Virulence determinants of high-pathogenic avian influenza viruses in gallinaceous poultry

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Review
Future Virology

High-pathogenic avian influenza viruses (HPAIV) cause devastating outbreaks in domestic poultry worldwide. Moreover, they repeatedly lead to severe, even fatal disease in humans, raising concerns about their pandemic potential. HPAIV have evolved from circulating low-pathogenic precursors in several independent events by spontaneous acquisition of a polybasic cleavage site in the hemagglutinin (HA) envelope protein. Remarkably, in nature, HPAIV are confined to the HA serotypes H5 and H7 from the 16 HA serotypes known in birds. However, experimental introduction of a polybasic cleavage site into non-H5/H7 HA may result in a highly pathogenic phenotype, indicating that emergence of HPAIV with novel serotypes is conceivable, but requires further adaptation to the chicken host.

High-pathogenic avian influenza viruses: a scourge of poultry & a lurking pandemic threat

High-pathogenic avian influenza viruses (HPAIV), formerly known as fowl plague viruses, cause devastating losses in domestic poultry worldwide. The infection of HPAIV in gallinaceous birds results in a systemic disease affecting several organs including the lungs, heart, digestive tract and CNS. Affected birds may die within 2–7 days after infection and the lethality within affected flocks may range from 70 to 100% [1–4]. The gold standard for assessing virulence is the determination of the intravenous pathogenicity index in chickens, yielding values of 1.20 and higher for HPAIV [5], although there are a few strains with high virulence after intravenous but not oculonasal administration [6]. Since no vaccine is available that disrupts infection chains with certainty [7–10], in most countries the control measures in case of an HPAIV outbreak are based on rigorous depopulation and the establishment of quarantine zones around affected holdings. Within the last 135 years, more than 40 outbreaks of HPAIV have been reported worldwide (TABLE 1) [11,101]. Moreover, repeated HPAIV infections in humans, some associated with high lethality [12–17], have raised concerns about further HPAIV adaptation to the human host sparking a novel pandemic.

HPAIV differ from low-pathogenic avian strains by their polybasic hemagglutinin cleavage site & their restriction to the hemagglutinin serotypes H5 & H7

In 1878, the well-known fowl plague was first recognized as a specific poultry disease and in

1955 its causative agents were demonstrated to be influenza A viruses [18,19]. Later, those highly virulent virus strains were renamed HPAIV. Influenza A viruses belong to the family *Orthomyxoviridae*, which also comprise the genera *influenzavirus B*, *influenzavirus C*, *Isavirus* and *Thogotovirus* [20]. All orthomyxoviruses carry a segmented ssRNA genome of negative polarity with different numbers of gene segments, and are mainly discriminated by different antigenicity of their nucleoproteins [21–23]. The genome of influenza A viruses consists of eight segments that are organized in ribonucleoprotein complexes (RNPs). Each RNP contains the viral RNA encased by nucleoproteins and one bound viral polymerase complex composed of three subunits, the PB2, PB1 and PA proteins. Within the virion, the eight RNPs are enclosed by the matrix protein M1 and a lipid envelope in which the three surface proteins hemagglutinin (HA), neuraminidase (NA) and the proton channel protein M2 are inserted [24].

All influenza A viruses are dependent on the cleavage of the HA precursor HA0 into the HA1 and HA2 fragments for infectivity [25,26]. Only after proteolytic activation is the trimeric HA able to alter its conformation at the acidic pH in the endosome, resulting in exposure of the N termini of the HA2 fragments, which contain the hydrophobic fusion peptides necessary to merge the virion envelope with the endosomal membrane during viral endocytotic entry [27–29]. This proteolytic activation depends on several proteases that are provided by the host and recognize different cleavage motifs. All human, mammalian and low-pathogenic avian

Keywords

- cleavage site ■ fowl plague
- H5N1 ■ hemagglutinin
- high-pathogenic avian influenza virus ■ pathogenesis
- proteolytic activation
- virulence determinants

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Table 1. High-pathogenic avian influenza virus outbreaks reported from the late 19th century to January 2013.

Year	Subtype	Country
1878–1935	H7?	Italy – enzootic
1890–1930	H7?	Germany – enzootic
1922	H7?	UK
1923–1945	H7?	Egypt – enzootic
1924–1925	H7?	USA
1927	H7?	Indonesia
1929	H7?	UK
1929	H7?	USA
1959	H5N1	Scotland
1961	H5N3	South Africa
1963	H7N3	UK
1966	H5N9	Canada
1976	H7N7	Australia
1979	H7N7	UK
1979	H7N7	Germany
1983/1984	H5N2	USA
1983/1984	H5N8	Ireland
1985	H7N7	Australia
1991	H5N1	UK
1992	H7N3	Australia
1994/1995	H5N2	Mexico
1994	H7N3	Australia
1994	H7N3	Pakistan
1996 (ongoing) [†]	H5N1	Asia, Europe and Africa
1997	H7N4	Australia
1997	H5N2	Italy
1999	H7N1	Italy
2002	H7N3	Chile
2003	H7N7	Netherlands
2004	H7N3	Canada
2004	H5N2	USA
2004	H5N2	South Africa
2004	H7N3	Pakistan
2004	H5N2	Chinese Taipei
2005	H7N7	North Korea
2005/2006	H5N2	Zimbabwe
2007/2008	H7N3	Canada
2008	H7N7	UK
2009/2010	H7N7	Spain

[†]Since the emergence of the H5N1 high-pathogenic avian influenza virus in southern China and Hong Kong in 1996, an unprecedented multitude of outbreaks has been reported. Moreover, in Bangladesh, China, Egypt, India, Indonesia and Vietnam, the H5N1 high-pathogenic avian influenza viruses have become enzootic [101]. The reported high-pathogenic avian influenza virus outbreaks were compiled from [11,101].

Table 1. High-pathogenic avian influenza virus outbreaks reported from the late 19th century to January 2013 (cont.).

Year	Subtype	Country
2011/2012	H5N2	South Africa
2012	H7N7	Australia
2012	H5N2	Chinese Taipei
2012	H7N3	Mexico
2012	H7N1	South Africa
2012 (ongoing)	H7N3	Mexico

**Since the emergence of the H5N1 high-pathogenic avian influenza virus in southern China and Hong Kong in 1996, an unprecedented multitude of outbreaks has been reported. Moreover, in Bangladesh, China, Egypt, India, Indonesia and Vietnam, the H5N1 high-pathogenic avian influenza viruses have become enzootic [101]. Reported high-pathogenic avian influenza virus outbreaks were compiled from [11,101].*

influenza viruses (LPAIV) carry an HA cleavage site (HACS) with a motif of one basic amino acid, almost always consisting of an arginine. By stark contrast, all HPAIV carry a polybasic HACS. This difference in cleavage motifs determines which host protease is capable of cleaving HA0. Accordingly, the monobasic HACS motif is cleaved by tissue-restricted proteases such as TMPRSS2, HAT, matriptase, other trypsin-like proteases [30–35], and perhaps yet unidentified proteases, whereas the polybasic HACS is recognized by the ubiquitous proprotein convertase furin and related subtilases [36,37]. Such different tissue restriction of the host proteases entails (but is not sufficient for) distinct viral tissue tropisms. Thus, LPAIV are mostly confined to the digestive or respiratory tracts [38–40], whereas HPAIV exhibit a very broad tissue tropism afflicting many organs, including the CNS, often considered the prime cause of death in infected birds (FIGURE 1) [1–4].

Beside the polybasic HACS, HPAIV differ from LPAIV in that they are restricted to the HA serotypes H5 and H7, two of 16 HA serotypes known in avian influenza viruses [1,41,42]. While all 16 HA serotypes are prevalent in LPAIV with some preferences for certain avian species [43], only H5 and H7 are found both in low- and high-pathogenic strains (FIGURE 2).

Origin & evolution of HPAIV

All HPAIV have evolved from low-pathogenic precursors that circulate in the global population of wild aquatic birds, due to an extension of the HACS by an insertion mutation coding for a stretch of basic amino acids. LPAIV closely related to the subsequent HPAIV were detected in domestic poultry weeks or months prior to HPAIV outbreaks, such as in Pennsylvania 1983 [44,45], Italy 1999 [46,47], The Netherlands 2003 [15] or Chile 2004 (FIGURE 1) [48]. Therefore,

it appears that, at least in those instances, the evolution from LPAIV to HPAIV took place in gallinaceous birds.

Remarkably, the HACS of HPAIV differ in their amino acid composition, proving independent mutation events (FIGURE 1) [44,48–50]. Furthermore, phylogenetic analyses indicated that the HA of HPAIV do not form unique lineages [51]. For a few HPAIV, the inserted nucleotide sequence encoding multiple basic amino acids was found to originate from the viral nucleoprotein or matrix protein genes (FIGURE 1) [48,49] or host ribosomal 28S RNA [52], indicating RNA recombination. On the other hand, the creation of the polybasic cleavage site was hypothesized to be due to polymerase slippage during genome replication at purine-rich regions preceding the HACS coding sequence [53]. It should be emphasized, however, that in most HPAIV the mechanism for the insertion mutations is still elusive.

The polybasic HA cleavage site is the prime virulence determinant of HPAIV

The relevance of the polybasic HACS for virulence of HPAIV in mammals and chickens was addressed by experimental conversion into a monobasic motif. In mice and ferrets, HPAIV-derived mutants with a monobasic cleavage site display a considerably reduced level of virulence, demonstrated by less severe symptoms and confinement of viral replication to the respiratory tract [54–56]. In chickens, the experimental removal of the polybasic HACS from HPAIV led to an even more pronounced reduction of virulence, resulting in a seemingly avirulent phenotype of the HACS mutants with a monobasic cleavage motif [57,58]. Thus, the polybasic HACS is the dominant virulence determinant in HPAIV with regard to induction of disease in chickens. In HPAIV-derived monobasic HACS mutants, other still partially

Figure 1. Evolution of high-pathogenic avian influenza virus from low-pathogenic avian influenza virus. In low-pathogenic strains, the monobasic HACS (containing either arginine or, rarely, lysine in position 1 of the HACS) is associated with low virulence in chickens (blue). Highly pathogenic strains originate from low-pathogenic precursors by acquisition of an elongated polybasic HACS due to an insertion mutation, leading to high virulence (red). The amino acid residues in the precursor hemagglutinin flanking the insertion are printed in bold. Whereas in most cases the mechanism of this mutational insertion has remained uncertain, some HPAIV have acquired their polybasic HACS by recombination with host or viral RNA. Accordingly, the Chile and Canada strains carry elongated HACS regions with fragments originating from the NP or M genes [48,49]. HACS: Hemagglutinin cleavage site; HPAIV: High-pathogenic avian influenza virus; LPAIV: Low-pathogenic avian influenza virus.

unknown virulence determinants that remained after mutagenesis of only the HACS region, are dormant. Thus, at the present level of knowledge, any low-pathogenic strain of the H5 or H7 serotype has to be considered a potential HPAIV precursor. In summary, those studies showed that the polybasic HACS is the key virulence determinant of HPAIV.

Introduction of a polybasic HA cleavage site into LPAIV does not inevitably result in a highly pathogenic phenotype

In nature the emergence of HPAIV is restricted to serotypes H5 and H7. However, the molecular basis for this restriction is unclear. To distinguish between the compatibility of a polybasic

cleavage site with H5/H7 HA only and/or a unique predisposition of those two serotypes for insertion mutations, we introduced a polybasic HACS into the HA of non-H5/H7 serotypes. Integration of a polybasic HACS into the HA of several LPAIV, namely a duck H3, duck H4 or chicken H9 strain [59–61], resulted in the immediate transformation to an *in vitro* phenotype resembling HPAIV. These viruses replicated in cell culture independently of the presence of the protease trypsin, exogenously added to activate monobasic HA0 of LPAIV, and also produced plaques without the need for an exogenous protease. Thus, in these parameters they were indistinguishable from natural HPAIV. However, none of those viruses exhibited a highly

pathogenic phenotype in chickens, separating the *in vitro* traits from the *in vivo* phenotype. Remarkably, an engineered polybasic HACS mutant of a low-pathogenic H5N1 isolated from teal, which as an LPAIV of H5 serotype has to be considered a potential HPAIV precursor, did not display high virulence either [62]. By contrast, insertion of a polybasic HACS into an H6 LPAIV isolated from mallards resulted in a virulent phenotype [63], although this was not as highly pathogenic as natural H5 or H7 HPAIV. Interestingly, four naturally occurring

avian strains have been described that carry a polybasic HACS, but demonstrate low virulence in chickens [64]. Thus, insertion of a polybasic HACS, although it represents the dominant virulence marker in natural HPAIV, is not sufficient to confer a highly pathogenic phenotype on any LPAIV, although it may suffice in certain viral backgrounds. Moreover, the low-pathogenic precursor of the H5N2 HPAIV leading to the fowl plague outbreak in 1983 in Pennsylvania carried a polybasic HACS that was prevented from furin cleavage by nearby

Figure 2. Hemagglutinin serotype prevalence in different avian reservoirs. LPAIV (blue) with serotypes H1–H16 are maintained in aquatic wild birds (left panel) and domestic poultry (middle panel) comprising *Anseriformes* and *Galliformes* [76,77], in which they might undergo prior host adaptation, facilitating later evolution into HPAIV. Natural HPAIV (right panel) were found predominantly in gallinaceous domestic birds (and occasionally in wild birds), originate from low-pathogenic precursors and are restricted to the hemagglutinin (HA) serotypes H5 and H7 (red). However, experimental HPAIV (orange) carrying a non-H5/H7 HA with an engineered polybasic HA cleavage site exhibit a lethal phenotype in chickens, as well [60–63]. H14 and H16 have not been detected in domestic poultry to date. HPAIV: High-pathogenic avian influenza virus; LPAIV: Low-pathogenic avian influenza virus.

site-specific glycosylation. The subsequent loss of that glycosylation site by mutation resulted in a high-pathogenic virus [65]. On the other hand, prolonged passaging in cell culture in the absence of trypsin of an avian-like H7N7 virus from seal led to the spontaneous acquisition of a polybasic HACS accompanied by high virulence, especially in chickens [66]. These findings indicate that even LPAIV not isolated from gallinaceous poultry may be able to transform into an HPAIV upon acquisition of a polybasic HACS, whereas not every H5 or H7 LPAIV may serve as a HPAIV precursor without further adaptation.

Reassortment studies reveal the existence of additional virulence determinants in HPAIV

Earlier reassortment studies revealed that the immediate precursor of the Pennsylvania outbreak in 1983 carried virulence determinants in all viral proteins aside from the HA already including a polybasic HACS shielded by a carbohydrate attached to the stalk [45,67]. Remarkably, reassortment of two H5N1 HPAIV may result in decreased virulence [68]. Furthermore, the *NS* gene was shown to contribute to high pathogenicity in chickens [69]. Moreover, reassortants carrying the *HA* gene from an H5 HPAIV with the other seven genes originating from either an H9 or H5 LPAIV showed transient, nonlethal disease in chickens, whereas reassortants with the reciprocal gene constellation (i.e., the LPAIV H9 or H5 HA with artificial polybasic HACS and the genetic background from an HPAIV), were lethal for chickens [61,62]. Therefore, virulence determinants in HPAIV exist beyond the essential polybasic HACS and reside both within the *HA* gene and in the other gene segments. In addition, there might be several independent combinations of virulence determinants in different HPAIV strains. This trait renders any assessment of the risk of circulating H5 or H7 LPAIV becoming transformed into HPAIV very difficult.

HA of serotypes other than H5 or H7 can support high virulence

Experimental introduction of a polybasic HACS into an H6 LPAIV and generation of an HPAIV reassortant carrying an H9 HA with polybasic HACS resulted in a strikingly increased virulence for chickens [61,63]. However, with IPVI values of 1.40 and 1.23, respectively, the level of virulence was still lower than those of natural H5 or H7 HPAIV, which specify IPVI values of

above 2. To investigate whether HPAIV of other serotypes was also capable of supporting a highly pathogenic phenotype, a polybasic cleavage site was engineered into the HA of LPAIV with serotypes H1, H2, H3, H4, H6, H8, H10, H11, H14 or H15. Respective HA reassortants were generated by cotransfection with the genes from either a low-pathogenic H9N2 or a high-pathogenic H5N1 avian strain. Those recombinants that contained the engineered H2, H4, H8 or H14 genes in the HPAIV background were lethal in chickens and exhibited intravenous pathogenicity index values of 2.79, 2.37, 2.85 and 2.61, respectively, equivalent to naturally occurring H5 or H7 HPAIV. Thus, in the presence of a polybasic HACS, non-H5/H7 HA can support a high-pathogenic phenotype in the appropriate viral background, indicating the need for further adaptation. Hence, the observed restriction of natural HPAIV to serotypes H5 and H7 is likely due to their unique predisposition for acquisition of a polybasic HACS [60], but may not be absolute.

Conclusion

In light of our results that non-H5/H7 HPAIV can be generated experimentally [60,61], it is conceivable that HPAIV with novel serotypes could emerge in the future. Until the late 1950s, known fowl plague viruses were invariably H7, while HPAIV with the serotype H5 appeared thereafter. Today, both H7 and H5 HPAIV occur independently all over the world (TABLE 1). However, the molecular basis for the emergence of an HPAIV is still not fully understood. Previous observations suggest that the evolution of an LPAIV into HPAIV takes place in gallinaceous poultry prior to an HPAIV outbreak. Accordingly, prolonged circulation for weeks, or even months, of low-pathogenic precursor viruses was observed before emergence of an HPAIV. Since the acquisition of a polybasic HACS is due to an insertion mutation and, thus, occurs at considerable lower probabilities than any sequence of point mutations, virulence or host range determinants have to enable survival of those LPAIV in the chicken host. Such determinants, in addition to those acquired after the formation of a polybasic HACS, may comprise a lowered optimal pH to trigger fusion of the HA [70,71] and additional modifications [59,62,68,69,72], such as a deletion within the NA stalk region [73–75]. Furthermore, in nature, a polybasic HACS has so far only been found in HA of the H5 or H7 serotypes. This HA serotype restriction

of natural HPAIV points to a predisposition of the H5/H7 HA for acquisition of a polybasic HACS. However, if additional virulence determinants in the HA and other genes are dormant in the absence of a polybasic HACS, the experimental introduction of a polybasic HACS can yield highly pathogenic viruses with HA serotypes other than H5 or H7. Thus, the emergence of HPAIV with novel serotypes is conceivable.

Future perspective

Previous experiments have revealed that beside the polybasic HACS, HPAIV carry virulence determinants within the HA and other genes. For an evidence-based risk assessment of any given LPAIV, in particular those of the H5 and H7 serotypes, to transform into HPAIV, future studies should map those sites by generating single-gene reassortants between

well-characterized LPAIV and HPAIV with and without polybasic HACS. In a subsequent step, the virulence determinants may be narrowed to single amino acid exchanges by investigating viruses with chimeric gene segments. Likely candidates for virulence determinants include changes affecting the pH stability of the HA [71] or the NA stalk deletion [73–75]. Above all, it should be considered that different combinations of virulence determinants within one viral genome might yield a highly pathogenic phenotype. Therefore, those studies will have to be performed with a multitude of HPAIV/LPAIV pairs. So far, most of the experimental work on HPAIV/LPAIV has centered on H5 serotypes. To obtain a comprehensive picture and, hopefully, deduce congruent evolutionary parameters, similar extensive studies also need to be performed with H7 viruses and with strains specifying other HA serotypes.

Executive summary

High-pathogenic avian influenza viruses are a unique group among the influenza A viruses

- High-pathogenic avian influenza viruses (HPAIV) differ from low-pathogenic avian influenza viruses (LPAIV) by the presence of a polybasic hemagglutinin (HA) cleavage site (HACS).
- While there are 16 HA serotypes known in avian influenza virus strains, HPAIV are confined to the serotypes H5 and H7.

Origin & evolution of HPAIV

- Circulating H5 or H7 LPAIV may evolve into HPAIV.
- The heterogeneous HACS motifs found in HPAIV indicate independent mutational events and suggest different mechanisms of acquisition of a polybasic HACS.

A polybasic HA cleavage site is the prime virulence determinant

- Experimental removal of the polybasic HACS from HPAIV invariably leads to a low-pathogenic phenotype in chicken.
- Unknown virulence determinants may be dormant in circulating LPAIV.

Artificial introduction of a polybasic HACS into LPAIV

- Experimental introduction of a polybasic HACS into LPAIV may not necessarily result in a highly pathogenic phenotype.
- In addition, avian strains with polybasic HACS but low virulence in chicken underline the requirement for further adaptation.
- LPAIV isolated from bird species other than chicken may be able to evolve into HPAIV.

Reassortment studies with HPAIV

- Circulating H5 or H7 LPAIV may immediately transform into HPAIV upon acquisition of a polybasic HACS.
- Reassortment studies revealed that besides the polybasic HACS, there are further virulence determinants in the HA and the other genes, and that several combinations of virulence determinants in different viral strains may exist.

HA of serotypes other than H5 or H7 can support high virulence

- HA of serotypes other than H5 or H7 can support high virulence in the genetic background of an HPAIV. Thus, provided further adaptation, novel HPAIV with serotypes other than H5 or H7 could emerge in the future.
- The generation of functional non-H5/H7 HA with polybasic HACS suggests that the natural restriction of HPAIV to serotypes H5 and H7 may be due to a predisposition of the H5/H7 HA for an insertion mutation coding for a polybasic HACS.

Future perspective

- Identification of other virulence determinants residing in the HA or other genes, such as the neuraminidase, polymerase, nucleoprotein or NS genes should be carried out.
- Several HPAIV/LPAIV pairs should be studied.
- Future studies should elucidate the virulence potential of H7 LPAIV and the virulence determinants in H7 HPAIV.
- Evidence-based analysis of the risk for circulating LPAIV transforming into HPAIV should be performed.

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