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**REVIEW ARTICLE**

Highly pathogenic avian influenza viruses with low virulence for chickens in *in vivo* tests.

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**REVIEW ARTICLE**

Highly pathogenic avian influenza viruses with low virulence for chickens in *in vivo* tests.

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**Summary**

Four avian influenza viruses have been recognised that have genetic coding for HPAI viruses, but do not show virulence for chickens. The two different mechanisms that prevent this potential being expressed have been determined for A/chicken/Pennsylvania/1/83 (H5N2) and A/goose/Guandong/2/96 (H5N1), but neither of these apply to A/turkey/England/87-92BFC/91 (H5N1) or A/chicken/Texas/298313/04 (H5N2).

## Introduction

The definition of highly pathogenic avian influenza (HPAI) virus adopted by the World Organisation for Animal Health (OIE) has both molecular and *in vivo* tests by which a virus can be considered to cause HPAI. In the definition any AI virus should be considered HPAI if inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus intravenously results in more than 75% mortality within 10 days, or if they have an intravenous pathogenicity index (IVPI) of greater than 1.2 (Alexander, 2005). But additionally, a virus of H5 or H7 subtype should be considered an HPAI virus if it has the presence of multiple basic amino acids at the cleavage site of the haemagglutinin precursor protein HA0 similar to those reported for known HPAI viruses (Alexander, 2005). The reason a molecular qualification was added to the purely *in vivo* definition recommended at the First International Symposium on Avian influenza (Bankowski, 1981) was that a virus of low virulence for chickens, A/chicken/Pennsylvania/1/83 (H5N2), but with multiple basic amino acids at the HA0 cleavage site, circulated for some time in poultry in the USA before virulent virus emerged, following a simple point mutation (Kawaoka *et al.*, 1984). It was considered that the risk of such viruses mutating to virulence was so much greater than those AI viruses of low virulence, termed low pathogenicity AI (LPAI) viruses, lacking multiple basic amino acids at the HA0 cleavage site that they should be included within the definition of HPAI.

**Molecular basis of virulence.** The haemagglutinin (HA) glycoprotein of influenza A viruses has two important functions that are required for the virus to be infectious. The HA first brings about attachment of a virus particle to a host cell and, after being taken in to an endosome, then brings about fusion between the host cell membrane

and the virus membrane so that the viral genetic material is introduced into the host cell. This glycoprotein is produced as a precursor, HA0, which requires post translational cleavage by host proteases before it is able to induce membrane fusion and virus particles become infectious (Rott, 1992).

Various studies have demonstrated that the amino acid motif at the cleavage site of the HA0 precursor protein is extremely important in facilitating its cleavage by different proteases and this is of paramount importance in the virulence of the virus. The HA0 precursor proteins of avian influenza viruses of low virulence for poultry have a single arginine at the cleavage site and another basic amino acid [arginine or lysine] at position -3 or -4. These viruses are limited to cleavage by extracellular host proteases such as trypsin-like enzymes and thus restricted to replication at sites in the host where such enzymes are found, i.e. the respiratory and intestinal tracts. In contrast HPAI viruses possess multiple basic amino acids at their HA0 cleavage sites either as a result of apparent insertion or apparent substitution (Vey *et al.*, 1992, Wood *et al.*, 1993, Senne *et al.*, 1996) and appear to be cleavable by a ubiquitous protease[s], probably one or more proprotein-processing subtilisin-related endoproteases of which furin is the leading candidate (Stieneke-Grober *et al.*, 1992). These viruses are able to replicate throughout the bird, damaging vital organs and tissues which results in disease and death (Rott, 1992).

To date only viruses of H5 and H7 subtypes have been shown to cause HPAI (with the exception of some nephropathogenic H10 subtype viruses that may cause high mortality when inoculated intravenously, but not by other routes (Swayne & Alexander 1994)). It appears that HPAI viruses arise by mutation after LPAI viruses have been introduced into poultry. The cleavage site motifs of the HPAI and H5 and H7 LPAI viruses isolated since 1959 are summarised in Table 1, earlier HPAI viruses

have also been shown to have multiple basic amino acids motifs. Vey *et al.*, (1992) suggested that the probable minimum requirement is RXR/KR\*GLF, but several very virulent virus do not entirely comply with this minimum. A/turkey/England/50-92/91 (H5N1) with a motif of PQRKRKTR\*GLF (Horimoto *et al.*, 1995) and A/chicken/Pennsylvania/1370/83 (H5N2), motif PQKKKR\*GLF, do not, nor do the Chile 2002 (Suarez *et al.*, 2004) and the Canada 2004 (Pasick *et al.*, 2005) H7N3 HPAI viruses as discussed below.

Several mechanisms may be responsible for the mutation resulting in the emergence of HPAI viruses. Most HPAI viruses appear to have arisen as result of spontaneous duplication of purine triplets which results in the insertion of basic amino acids at the HA0 cleavage site and that this occurs due to a transcription fault by the polymerase complex (Perdue *et al.*, 1998). However, as pointed out by Perdue *et al.*, (1998), this is clearly not the only mechanism by which HPAI viruses arise as some appear to result from nucleotide substitution rather than insertion, e.g. A/chicken/Scotland/59 (H5N1), which has the motif PQRKKR\*GLF, while others have insertions without repeating nucleotides. The Chile 2002 (Suarez *et al.*, 2004) and the Canada 2004 (Pasick *et al.*, 2005) H7N3 HPAI viruses show distinct and unusual cleavage site amino acid sequences of PEKPKTCSPLSRCRETR\*GLF and PENPKQAYRKRMTTR\*GLF respectively. These viruses appear to have arisen as a result of a recombination event between the HA gene and nucleoprotein gene and matrix gene respectively resulting in an insertion at the HA0 cleavage site of 11 amino acids for the Chile virus and 7 amino acids for the Canadian virus.

**HPAI qualifying viruses of low virulence.** Four viruses have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low virulence (IVPI <1.2) when inoculated into 6-week-old chickens intravenously.

*A/chicken/Pennsylvania/1/83 (H5N2).* During the epizootic in Pennsylvania beginning in 1983, an H5N2 virus of low virulence circulated for some months before it mutated to a virus with high virulence for chickens and other poultry. Sequencing studies showed that the original virus of low virulence, *A/chicken/Pennsylvania/1/83*, differed from highly virulent *A/chicken/Pennsylvania/1370/83* by two mutations, each of which resulted in an amino acid change in the HA protein. One amino acid change was also found to be present in other Pennsylvanian virus isolates of low virulence, but the other, at position 13 on the HA molecule (T to K), resulting in the loss of a glycosylation site at position 11 was exclusive to the high virulence isolates (Deshpande *et al.*, 1987; Ohuchi *et al.*, 1989). Glycosylation at this position, which although distant from the cleavage site linearly on the HA molecule, was close in the three dimensional structure. The loss of the glycosylation site at HA 11 is thought to allow the ubiquitous host protease access to the multiple basic amino acid cleavage site and released the potential virulence of the virus (Deshpande *et al.*, 1987; Ohuchi *et al.*, 1989).

*A/turkey/England/87-92BFC/91 (H5N1).* The second report concerned an isolate made during an investigation into a HPAI outbreak in Norfolk, England (Alexander *et al.*, 1993). One of a number of H5N1 viruses isolated from diseased turkeys caused no disease in inoculated chickens (IVPI 0.0), but had the same multiple basic amino acids



motif as the isolates that caused rapid mortality in inoculated chickens (IVPI 3.0) (Alexander *et al.*, 1994; Wood *et al.*, 1994).

*A/chicken/Texas/298313/04 (H5N2)*. The third report concerned the isolation of a virus of H5N2 subtype from chickens in Texas, USA in 2004. This virus failed to cause deaths or clinical signs in chickens infected intravenously but had a multiple basic amino acid motif at the HA0 cleavage site of PQRKKR\*GLF (Lee *et al.*, 2005), which was identical to the virulent virus *A/chicken/Scotland/59 (H5N1)*.

*A/goose/Guandong/2/96m(H5N1)*. Recently Li *et al.*, (2006) described the isolation of two H5N1 viruses from geese in China in 1996, both with the same multiple basic amino acid motifs at the HA0 cleavage site, but again showing the marked difference in virulence for chickens seen with the Norfolk and Texas viruses (Table 2).

Li *et al.*, (2006) sequenced the entire genome of the two goose viruses and reported only 5 amino acid differences between the two viruses (Table 2). By using reverse genetics to obtain four viruses containing one gene from the low virulence virus and seven genes from the virulent virus Li *et al.*, (2006) concluded that the change from A to V at position 149 of the NS1 protein was responsible for reducing the virulence of the virus *A/goose/Guandong/2/96*. The reason suggested was that the amino acid change at NS1 position 149 diminished the ability of the NS1 protein to antagonize interferon and thus reduced the virulence of the virus.

**Comparison of the four viruses.** For this review we compared the presence or absence of the amino acid differences reported by Li *et al.*, (2006) for their viruses in the other viruses with multiple basic amino acid HA0 cleavage site sequences, but low

virulence (Table 1). All had A at NS1 position 149 like the virulent A/goose/Guandong/1/96. All, like A/goose/Guandong/2/96, had M at position 179 in the M1 protein, but so did their virulent counterparts. These viruses also showed identity with the virulent Chinese goose isolate for the other three amino acids reported as different by Li *et al.*, (2006) (Table 1).

We also compared the potential glycosylation site at HA protein position 11 for each of the viruses. Lee *et al.*, (2005) had shown that removal of this glycosylation had no effect on the virulence for chickens of their Texas 2004 isolate. Similarly, glycosylation at HA 11 is unlikely to be significant in suppressing the virulence of A/turkey/England/87-92BFC/91 as the virulent counterpart virus also has this glycosylation site.

To date comparisons of the genomes of closely related viruses have revealed two distinct mechanisms by which the apparent genetic coding for high virulence for chickens has been prevented from being expressed phenotypically. Neither of these mechanisms appears to apply to A/turkey/England/87-92BFC/91 or A/chicken/Texas/298313/04, thus further emphasising the complex and multigenic nature of avian influenza virulence. Work continues to determine why these viruses do not show their apparent potential virulence. Sequencing of the whole genomes of the A/turkey/England/91 viruses has revealed 10 amino acid differences between the high and low virulence viruses (Londt *et al.*, 2007), but the significance of these differences is yet to be established.

## Discussion

Of the 4 low virulence HPAI viruses the one from Pennsylvania was isolated before the molecular definition was in place and those from China and England were isolated more or less simultaneously with high virulence HPAI viruses. Only the Texas virus caused questions to be asked of the OIE molecular definition of HPAI and in particular whether the full force of trade restrictions should have been imposed for a virus that caused little disease in poultry.

In the Terrestrial Animal Health Code 2005 the OIE revised the definition of notifiable avian influenza (OIE 2005). In the new definition notifiable avian influenza (NAI) was extended to include LPAI H5 and H7 subtype viruses (LPNAI) in addition to the HPAI viruses (HPNAI). Similarly, in the new European Union Directive (CEC 2006) control measures were extended to H5 and H7 subtype AI viruses that fulfilled neither molecular nor *in vivo* virulence criteria, although these are not as severe as control measures for HPAI viruses.

The two mechanisms by which known low virulence HPAI viruses have become highly virulent have required a single point mutation, whereas mutation from LPAI to HPAI involve multiple nucleotide changes. However, it seems the latter may occur as a single mutational event. There is an argument therefore that low virulence HPAI viruses should be treated as LPNAI viruses rather than HPNAI for trade and control purposes. However, it is worth noting that trade embargos and centralised control measures, including stamping out, for all LPNAI viruses have been practised by a number of countries in recent years (Capua & Alexander, 2003).

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**Table 1.** Amino acid motifs at the HA0 cleavage site of H5 and H7 subtype influenza A viruses<sup>a</sup>

Virus subtype	LPAI viruses	HPAI viruses <sup>b</sup>
H5	PQRETR*GLF	PQRKKR*GLF
		PQRRKKR*GLF
		PQKKKR*GLF
		PQRKRKKR*GLF
		PQRKRKTR*GLF
		PQRRRRKKR*GLF
		PQRRRKKR*GLF
		PQRERRRKKR*GLF
		PQRKKR*GLF
		PQREKRRKKR*GLF
H7	PEIPKGR*GLF PENPKGR*GLF	PETPKRRRR*GLF
		PEIPKKREKR*GLF
		PEIPKKKGR*GLF
		PEIPKRKGR*GLF
		PEIPKKRKR*GLF
		PEIPKKREKR*GLF
		PEIPKKKKR*GLF
		PEIPRKRKR*GLF
		PETPKRRKR*GLF
		PEIPRKRKR*GLF
		PEIPKGSRVRR*GLF
		PEIPKRRRR*GLF
		PEKPKTCSPLSRCRETR*GLF
		PENPKQAYRKRMR*GLF

\*Denotes cleavage site. <sup>a</sup>Some isolates from the same outbreak may show minor variations in the amino acids at the HA0 cleavage site that are not included in this Table. <sup>b</sup>Only the cleavage site motifs of HPAI viruses isolated since 1959 are listed.



**Table 2** Properties and amino acid differences in the viruses compared in this review<sup>a</sup>.

Virus	IVPI	HA0 cleavage site	HA 11,12,13	PA 95	NP 299	M1 112	M1 179	NS1 149
A/chicken/Pennsylvania/1370/83 (H5N2)	2.37	PQKKKR*GLF	NSK	C	V	A	M	A
A/chicken/Pennsylvania/1/83 (H5N2)	0.0	PQKKKR*GLF	NST	C	V	A	M	A
A/turkey/England/50-92/91 (H5N1)	3.0	PQRKRKTR*GLF	NST	C	V	A	M	A
A/turkey/England/87-92BFC/91 (H5N1)	0.0	PQRKRKTR*GLF	NST	C	V	A	M	A
A/chicken/Texas/298313/04 (H5N2)	0.0	PQKKKR*GLF	NST	C	V	A	M	A
A/goose/Guandong/1/96 (H5N1)	2.1	PQRRRRKKR*GLF	NST	C	V	A	I	A
A/goose/Guandong/2/96 (H5N1)	0.0	PQRRRRKKR*GLF	NST	S	I	T	M	V

<sup>a</sup>Data from Alexander *et al.*, (1993), Wood *et al.*, (1994), Lee *et al.*, (2005), Li *et al.*, (2006) and Banks (2001).