Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia

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In February 2004 a highly pathogenic avian influenza (HPAI) outbreak erupted in British Columbia. Investigations indicated that the responsible HPAI H7N3 virus emerged suddenly from a low pathogenic precursor. Analysis of the haemagglutinin (HA) genes of the low and high pathogenic viruses isolated from the index farm revealed the only difference to be a 21 nt insert at the HA cleavage site of the highly pathogenic avian influenza virus. It was deduced that this insert most probably arose as a result of non-homologous recombination between the HA and matrix genes of the same virus. Over the course of the outbreak, a total of 37 isolates with, and 3 isolates without inserts were characterized. The events described here appear very similar to those which occurred in Chile in 2002 where the virulence shift of another H7N3 virus was attributed to non-homologous recombination between the HA and nucleoprotein genes.

Avian influenza manifests itself in domestic poultry in two distinct ways, depending on the virulence of the strain involved. Low pathogenic avian influenza (LPAI) is a localized infection of the respiratory and digestive tracts characterized by mild clinical signs, unless exacerbated by other infections or environmental conditions. In contrast, highly pathogenic avian influenza (HPAI) is a systemic disease characterized by mortality rates that can reach 100% (for review see Swayne & Halverson, 2003).

Although virulence of avian influenza viruses, as with most other viruses, is multigenic in nature, the viral haemagglutinin (HA) is recognized as playing a major role (Rott et al., 1976; Scholtissek et al., 1977). While HPAI viruses appear to exclusively express H5 and H7 haemagglutinin subtypes, not all H5 and H7 viruses are highly pathogenic. The virulence of H5 and H7 viruses is largely controlled by the cleavability of the HA precursor, HA0, by host proteases (Bosch et al., 1979, 1981). Proteolytic cleavage of HA0 generates two disulfide-linked subunits, HA1 and HA2, which serves to expose a fusion peptide at the newly formed amino-terminal end of HA2. This fusion peptide is in turn responsible for the low pH induced fusion of the viral envelope with host endosomal membranes, necessary for influenza virus infectivity (Wiley & Skehel, 1987). The HA0 of LPAI viruses is processed by extracellular proteases that are secreted by cells that line the respiratory and digestive tracts, while the HA0 of HPAI viruses is processed by a family of intracellular proteases, which have a broader tissue distribution. This difference is believed to be the primary reason why LPAI viruses produce localized infections while HPAI viruses produce systemic infections.

HPAI H5 and H7 viruses are thought to emerge from low pathogenic precursors only after the latter have been introduced into domestic poultry. This hypothesis is supported by work which demonstrated that HPAI viruses appear not to form a separate phylogenetic lineage or lineages in waterfowl, which are the natural reservoirs for all type A influenza viruses (Banks et al., 2000). Low-pathogenicity H5 and H7 strains isolated from feral birds typically have only two basic amino acids at positions −1 and −3 from the cleavage site (Wood et al., 1993), while HPAI viruses bear the minimum sequence motif R-X-R/K-R (Vey et al., 1992). This motif is also found in the cleavage sites of many proproteins that require cleavage for their activation (Barr, 1991). Though a number of subtilisin-related endoproteases cleave proproteins containing this sequence motif, furin is recognized as the most likely candidate
involved in processing the HA0 precursor of HPAI viruses (Stieneke-Grober et al., 1992).

The emergence of HPAI from LPAI has been proposed to occur by a number of mechanisms. These include: (i) the insertion of basic amino acids at the HA cleavage site, possibly the result of duplication of purine triplets due to a transcription fault of the polymerase complex (Horimoto et al., 1995; Garcia et al., 1996), (ii) the progressive accumulation of basic amino acids at the cleavage site by a stepwise process involving amino acid substitutions (Horimoto et al., 1995; Spackman et al., 2003), and (iii) non-homologous recombination resulting in the insertion of a foreign nucleotide sequence adjacent to the HA cleavage site (Suarez et al., 2004).

In February 2004, an outbreak of highly pathogenic avian influenza arose in a chicken broiler breeder farm in British Columbia (BC). The index premise was comprised of two flocks 24 and 52 weeks of age. Birds in the older flock presented with a mild drop in egg production and feed consumption, along with a small increase in mortality that resolved after a few days. Necropsies performed on the affected birds revealed inflammation of the trachea and lungs from which an influenza A virus was isolated. The farm was placed under quarantine pending the results of virus characterization.

The isolate was subtyped by microtitre plate haemagglutinin-inhibition and neuraminidase-inhibition (Van Deusen et al., 1983) assays as an H7N3. H7-specific primers F, 5′-AGCAAAGCAGGGGATACAAATG-3′ and G, 5′-TCT-CCCTGTCATTTTGATGCC-3′ (Senne et al., 1996) were used to amplify and sequence a 1158 bp segment of the HA gene containing the cleavage site. The deduced amino acid sequence of the cleavage site (PENPKTR/GLF), along with an intravenous pathogenicity index (IVPI)=0, demonstrated that this isolate was of low pathogenicity. During the quarantine period, a pronounced shift in mortality, from <1 to nearly 20 %, was observed in the younger flock. Additional tissue specimens were collected from these birds and an H7N3 virus was once again isolated in chicken embryos. Total RNA was extracted from allantoic fluid and used to sequence the HA gene from which a PENPKTR/GLF cleavage site was deduced. However, these same allantoic fluid samples induced a cytopathic effect in QT (Quail fibrosarcoma)-35 cells in the absence of exogenously added trypsin. Furthermore, systemic disease with high mortality occurred within the first 24–48 h following intravenous inoculation of 4-week old specific-pathogen-free chickens, resulting in an IVPI=2.96. These birds showed signs of severe depression, laboured breathing, peri-oral oedema, cyanosis of the comb and wattles, and petechial haemorrhages on the scales covering the tarsus and metatarsus. (Supplementary material of the gross and microscopic lesions produced in experimentally infected chickens can be found in JGV Online.) An H7N3 virus was reisolated from the tissues of these birds and found to contain a 7 aa insert (PENPKQAYKRMTR/GLF) at its haemagglutinin cleavage site. A BLAST search of the nucleotide sequence of this insert indicated that it most probably originated from nt 737 to 757 of the matrix gene (M1). The M1 gene of the original LPAI virus isolate was cloned and sequenced using primers: 5′-BamHI-AGGAAAAGCAGGGTAGATATTGAAA-3′ and 5′-XbaI-AGTTGAAAACAAGG TAGTTTTTACTC-3′. Twenty of 21 nt of the inserted fragment were identical with that of the corresponding M1 gene sequence (GenBank accession #AY677732).

The contradictory results, involving the pathotype of the virus isolated from the younger birds on the index farm, which was initially LPAI based on sequencing, and subsequently determined to be HPAI based on growth in tissue culture and IVPI, can be best explained by the presence of a mixed viral population in which the low pathogenic form predominated. The existence of mixed influenza virus populations in a single virus isolate has been described by others (Perdue et al., 1992, 1994), and is consistent with the viral quasispecies concept (Domingo & Holland, 1997).

In early March, an H7N3 avian influenza virus was isolated from a second farm approximately 3 km west of the first. The only clinical sign observed with birds on this farm was a sudden increase in mortality. The HA of this isolate contained the protease cleavage site PENPKQAYKRMTR/GLF. In this case the insert was 100 % identical with the corresponding region of the M1 gene. Additional infected farms were identified by screening cloacal and oropharyngeal swab specimens for the presence of type A influenza virus RNA by using a real-time RT-PCR assay which targeted the M1 gene (Spackman et al., 2002). Specimens that gave positive results were further processed for virus isolation in chicken embryos. By the second week of May, when the last infected farm had been identified, a total of 40 isolates had been characterized, 37 of which contained an HA cleavage site bearing a 7 aa insert. Sequencing of HA1 for the majority of these isolates was accomplished by direct sequencing of RT-PCR amplicons using primers F and G (Senne et al., 1996). In some instances it was necessary to clone the RT-PCR amplicons using the pGEM-T-Easy vector system (Promega). The resulting clones were then characterized by cycle sequencing using T7/SP6 primers. Table 1 summarizes the nucleotide and deduced amino acid sequences of the various isolates, along with their intravenous pathogenicity indices.

Viruses with QAYKRM (GenBank accession #AY725855), QAYHKRM (AY730057), QAYRKRM (AY644402), QAHKRM (AY731820) and QAYQKRM (AY736323) inserts most probably evolved from viruses with the QAYQKRM (AY724684) insert. Three nucleotide changes were observed within the codon encoding glutamine at position 4 of the insert, which resulted in substitution with a basic amino acid at this position. The presence of the additional basic amino acid at this position did not appear to significantly alter the virulence of viruses bearing QARYKRM and QAYKKRM inserts (Table 1).
The two most recognized mechanisms responsible for genetic and phenotypic variation of influenza A viruses include high mutation rates which give rise to antigenic drift, and genetic reassortment among different viruses which gives rise to antigenic shift. RNA recombination has only recently been considered as a third mechanism by which influenza A viruses can undergo rapid evolutionary change. Non-homologous recombination involving influenza A viruses has been reported rarely in the literature (Fields & Winter, 1982; Bergman et al., 1992; Khatchikian et al., 1989; Orlich et al., 1990, 1994; Suarez et al., 2004). Two reports of non-homologous recombination involving the haemagglutinin gene are of particular interest with regards to the BC HPAI outbreak. The first involves a variant of the H7N3 A/turkey/Oregon/71 that arose following 12 serial passages in chicken embryo cells in the absence of extracellular proteases. This variant contained a 54 nt insert derived from the 28S rRNA gene at the haemagglutinin cleavage site, which enabled it to be cleaved by intracellular proteases (Khatchikian et al., 1989). This variant also demonstrated increased pathogenicity for chickens, inducing death 4–5 days following intramuscular inoculation (Orlich et al., 1990). The second report described a non-homologous recombinant involving the haemagglutinin and nucleoprotein genes of the H7N7 A/seal/Massachusetts/1/80 virus (Orlich et al., 1994). This variant arose following five serial passages of wild-type virus in chicken embryo cells in the absence of exogenously added trypsin. Molecular characterization revealed the presence of a 60 nt insert corresponding to nt 284–343 of the nucleoprotein gene (NP) in-frame with, and immediately adjacent to, the haemagglutinin cleavage site. This insertion mutant demonstrated a broadened host range with no requirement for exogenous trypsin, and an increased pathogenicity in White Leghorn chickens.

The above examples, which demonstrate the ability of influenza A viruses to utilize non-homologous recombination to acquire a virulence trait, had, until only recently, been documented under experimental conditions. The first report of RNA recombination being responsible for a shift in virulence in a natural outbreak of avian influenza involved an H7N3 A/turkey/Oregon/71 that arose following 12 serial passages in chicken embryo cells in the absence of extracellular proteases. This variant contained a 54 nt insert derived from the 28S rRNA gene at the haemagglutinin cleavage site, which enabled it to be cleaved by intracellular proteases (Khatchikian et al., 1989). This variant also demonstrated increased pathogenicity for chickens, inducing death 4–5 days following intramuscular inoculation (Orlich et al., 1990). The second report described a non-homologous recombinant involving the haemagglutinin and nucleoprotein genes of the H7N7 A/seal/Massachusetts/1/80 virus (Orlich et al., 1994). This variant arose following five serial passages of wild-type virus in chicken embryo cells in the absence of exogenously added trypsin. Molecular characterization revealed the presence of a 60 nt insert corresponding to nt 284–343 of the nucleoprotein gene (NP) in-frame with, and immediately adjacent to, the haemagglutinin cleavage site. This insertion mutant demonstrated a broadened host range with no requirement for exogenous trypsin, and an increased pathogenicity in White Leghorn chickens.

The outbreak described in this report is only the second time that natural emergence of a HPAI virus from a LPAI virus could be attributed to non-homologous...
Table 2. Comparison of the various inserts that have been introduced into the haemagglutinin cleavage site by non-homologous recombination

Inserts are indicated in bold.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>No. amino acids in insert</th>
<th>Origin of insert</th>
<th>Amino acid sequence of cleavage site</th>
<th>Pathotype*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Ck/BC/2004 H7N3</td>
<td>–</td>
<td>–</td>
<td>PENPKLTR/GLF</td>
<td>L</td>
<td>This study</td>
</tr>
<tr>
<td>A/Ck/Chile/2002 H7N3</td>
<td>7</td>
<td>M1</td>
<td>PENPKQAQYRKTR/GLF</td>
<td>H</td>
<td>Suarez et al. (2004)</td>
</tr>
<tr>
<td>A/Ty/Oregon/71 H7N3</td>
<td>10</td>
<td>NP</td>
<td>PENPKTSLSLPGRGTDLQVPTAR/GLF</td>
<td>H</td>
<td>Khatrckian et al. (1989)</td>
</tr>
<tr>
<td>A/Seal/Mass/1/80 H7N7</td>
<td>18</td>
<td>28S rRNA</td>
<td>PENPKTSLSLPGRGTDLQVPTAR/GLF</td>
<td>H</td>
<td>Orlich et al. (1994)</td>
</tr>
</tbody>
</table>

*Pathotype: H, high pathogenicity; L, low pathogenicity.

recombination. Interestingly, all intersegmental recombinants involving the haemagglutinin that have been described to date have involved the H7 subtype, and all insertions have occurred between positions −1 and −2 or −3 relative to the site of cleavage (Table 2). Additionally, all have occurred following passage of the virus in chickens or cells of chicken origin. The mechanism by which these inserts enhance cleavability by intracellular proteases is not known. X-ray crystallographic analysis of a soluble HA0 precursor that is resistant to tryptic cleavage revealed that the cleavage site forms a nearly circular loop structure, 19 aa in length, which projects perpendicularly from the long axis of the haemagglutinin (Chen et al., 1998). Furthermore, Lys-326 at critical position −4 relative to where cleavage occurs was not optimally situated for binding by the furin active site. The inserts described in this report and by others (Khatchikian et al., 1989; Orlich et al., 1994; Suarez et al., 2004) may also enhance cleavage by making the cleavage site more protease accessible.

In conclusion, a highly pathogenic H7N3 avian influenza virus appears to have emerged suddenly from a low pathogenic H7 influenza A virus in Mexico. The authors thank Stacey Halayko, Shanon Toback, Michelle French, Marlee Ritchie, Lisa Manning, Shelley Ganske and Margaret Krzywlewski for their excellent technical assistance. We also thank the British Columbia Ministry of Agriculture and Food for their collaboration during the outbreak.

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References


