Arch Virol (2007) 152: 585–593
DOI 10.1007/s00705-006-0860-6
Printed in The Netherlands

Archives of Virology

Genome characterisation of the newly discovered avian influenza A H5N7 virus subtype combination

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Received April 3, 2006; accepted August 18, 2006; published online November 20, 2006

Summary

In Denmark, in 2003, a previously unknown subtype combination of avian influenza A virus, H5N7 (A/Mallard/Denmark/64650/03), was isolated from a flock of 12,000 mallards. The H5N7 subtype combination might be a reassortant between recent European avian influenza A H5, H7, and a third subtype, possibly an H6. The haemagglutinin and the acidic polymerase genes of the virus were closely related to a low-pathogenic Danish H5N2 virus A/Duck/Denmark/65041/04 (H5N2). The neuraminidase gene and the non-structural gene were most similar to the highly pathogenic A/Chicken/Netherlands/1/03 (H7N7) and the human-fatal A/Netherlands/219/03 (H7N7), respectively. The basic polymerase 1 and 2 genes were phylogenetically equidistant to both A/Duck/Denmark/65047/04 (H5N2) and A/Chicken/Netherlands/1/03 (H7N7). The nucleoprotein and matrix gene had highest nucleotide sequence similarity to the H6 subtypes A/Duck/Hong Kong/3096/99 (H6N2) and A/WDk/ST/1737/2000 (H6N8), respectively. All genes of the H5N7 strain were of avian origin, and no further evidence of pathogenicity to humans has been found.

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Introduction

Influenza A viruses are ubiquitous in aquatic birds and ducks, and in general, these infections cause no clinical disease, but the virus is excreted in large numbers with the faeces. It is believed that all 16 subtypes of haemagglutinin (HA) are perpetuated in the aquatic bird population and reassort with each other with high frequency [9, 45]. Avian influenza viruses (AIV) in the aquatic bird reservoir may spill over to swine, horses, domestic poultry, sea mammals, and in rare cases also to humans, and cause infections. Influenza A viruses are continuously changing their antigenicity through antigenetic drift, i.e. site mutations in the surface glycoproteins HA and neuraminidase (NA). In addition, reassortment might occur by two viruses exchanging segments through infection of the same cell. The emergence of new viral strains by reassortment has led to the influenza A pandemics of the last century, e.g. Asian/57 (H2N2) and Hong Kong/68 (H3N2), possibly with the exception of the 1918 'Spanish Flu', which is believed to be merely of avian origin

adapted to humans [32, 40, 45]. AIVs are divided into viruses of low pathogenicity (LPAIV) and high pathogenicity (HPAIV) based on their virulence in chickens (fowl). All HPAIV have been of either H5 or H7 subtypes, although many viruses of these subtypes are of low pathogenicity (LP). It is believed that the HPAIV H5N1 virus that infected 18 humans and killed 6 in Hong Kong in 1997 was a reassortant between A/Goose/Guangdong/1/96 (H5N1)-like viruses, A/Teal/Hong Kong/W312/97 (H6N1)-like viruses, and A/Quail/Hong Kong/G1/97 (H9N2)-like viruses [12, 14, 19]. Since 1997 there have been increasing numbers of cases of AIV infections in humans, directly transmitted by close contact with infected birds. The H5N1 AIV has caused direct human infections with lethal outcome in Asia on several occasions [5, 17, 31]. Also, avian-derived H9N2 and H7N7 subtypes have proved capable of infecting humans [10, 29]. In addition, the H7N7 from the Netherlands in 2003 was capable of human-to-human transmission [10, 22]. Antiviral therapy plays an important role in prevention of influenza illness and especially in the case of a pandemic when time is short and a vaccine containing the appropriate strain takes time to manufacture. Amantadine, which acts on the matrix 2 ion channel protein of influenza A viruses, has been used in some countries for almost 25 years, and natural amantadine-resistant strains are quite prevalent [16, 20, 33]. The neuraminidase inhibitors zanamivir and oseltamivir were first used in clinical practice between 1999 and 2002. Mutations in NA might affect the binding to each of these drugs due to differences in the chemical structures of the inhibitors. It is of great concern that recent human infectious H5N1 virus isolates have shown resistance to oseltamivir [6, 24], the drug that has been stockpiled in case of a new pandemic.

The presence of multiple basic amino acids at the HA0 cleavage site is indicative of high pathogenicity (HP) [28, 30], and many HPAIVs possess an additional glycosylation site at the globular head of HA [2]. Amino acid substitution E627K in the basic polymerase 2 (PB2) protein and a D92E in the non-structural 1 (NS1) protein have been shown to increase replicative efficiency in mice and virulence in pigs, respectively [34, 36].

In the present study, we have genetically characterised a newly discovered AIV subtype combination, H5N7, A/Mallard/Denmark/65112/03 (H5N7), isolated from a stock of 12,000 mallards in Denmark with an increased mortality rate [3]. Interestingly, the virus did not possess any known sequence characteristics of HP in the HA protein, although the virus was isolated from multiple organs, including the brain [3]. We have compared the genome of the H5N7 strain with is closest HA and NA sequence homologues, the LP A/Duck/Denmark/65047/04 (H5N2) and the HP A/Chicken/Netherlands/1/2003 (H7N7), respectively, in relation to representatives of known genetic lineages of influenza A virus in the GenBank. We examined host-specific amino acids and amino acids at known inhibitory drug resistance sites, the extent of possible reassortment, and further genetic characteristics associated with increased virulence.

Methods

Samples from game ducks, mallard (Anas platyrhynchos)

In September 2003, AIV of subtype H5N7 was isolated from an outdoor-bred commercial flock of 12,000 mallards in Denmark, produced for restocking of game. More detailed information on the sampling and diagnostic procedures was described previously [3].

RNA extraction and full-length one-step RT-PCR

Viral RNA isolation by QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and RT-PCR by OneStep® RT-PCR Kit (QIAGEN, Hilden, Germany) was performed directly on tissue homogenate as described previously [3]. The primers were segment-specific but subtype-universal, targeting the highly conserved noncoding RNA region of 13 and 12 nucleotides at the 5′- and 3′-end of each segment, respectively [18]. Amplification of the internal genes PB2, PB1, and PA was performed with an annealing temperature of 63 °C and an elongation time of 7 minutes due to the larger size of these fragments. The PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, USA) prior to sequencing.

Sequencing and phylogenetic analyses

Purified PCR products were sequenced directly. Internal sequencing primers are available upon request. Sequencing was performed using a ABI PRISM® BigDye™ Terminators

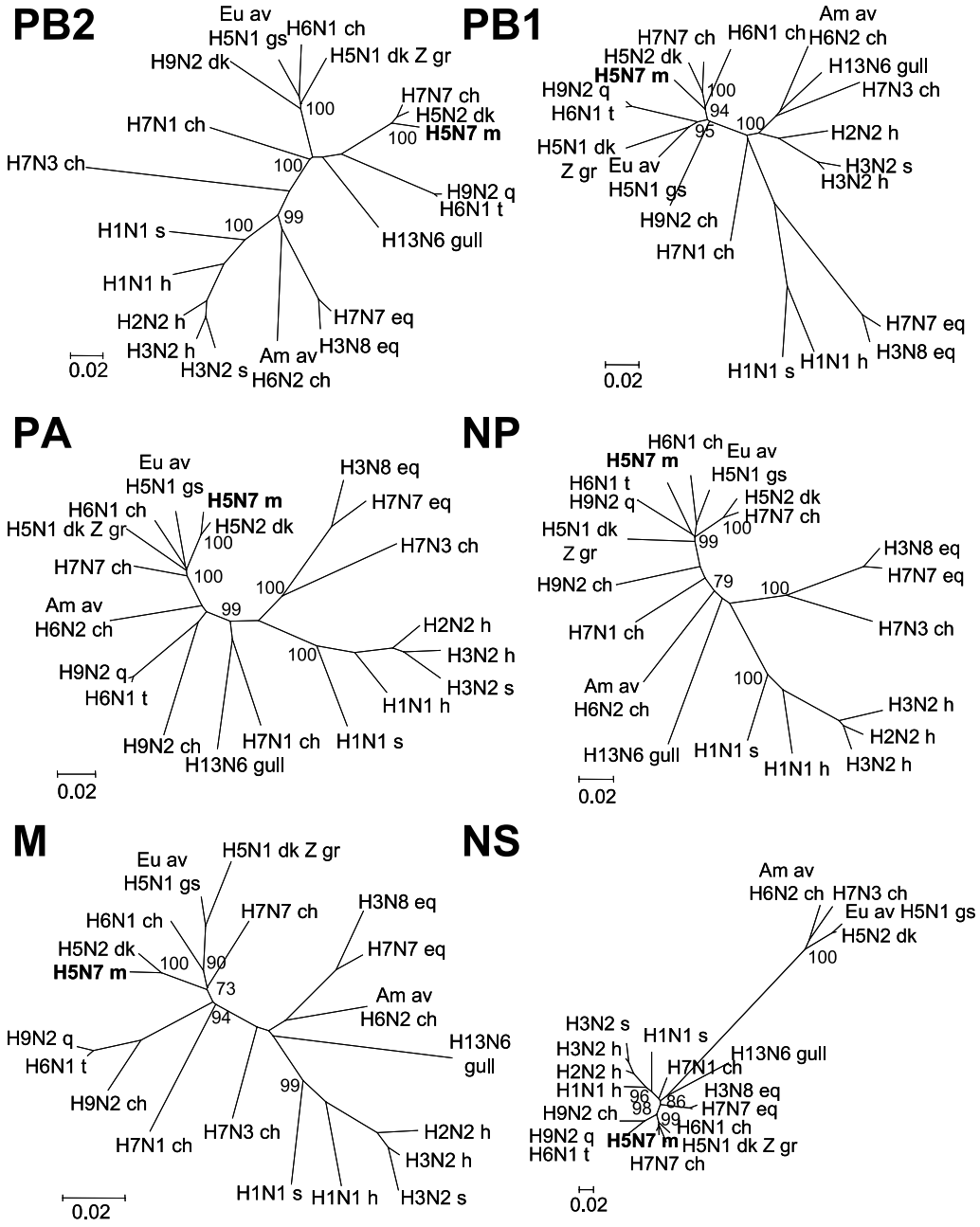

Fig. 1. Unrooted phylogenetic trees of the A/Mallard/Denmark/64650/03 (H5N7) genome (in bold typeface) in comparison to different reference sequences. The phylogenetic trees of the genes coding for the internal proteins represent the genome of A/Mallard/Denmark/64650/03 (H5N7 m) in comparison to A/Duck/Denmark/65047/04 (H5N2 dk) and A/Chicken/Netherlands/1/03 (H7N7 ch). Included in the trees are representatives of the known genetic lineages of influenza A virus: Am av H6N2 ch: A/Chicken/California/431/00, Eu av H5N1 gs: A/Goose/Guangdong/1/96, H13N6 gull: A/Gull/Maryland/704/77 (A/Gull/Maryland/1824/78 (M)), H3N8 eq.: A/Equine/Kentucky/2/86 (A/Equine/Tennessee/5/86 (PB1)), H7N7 eq: A/Equine/London/1416/73 (A/Equine/Newmarket/1/77 (M), A/Equine/London/1417/73 (NS)), H3N2 s.:A/Swine/Colorado/1/77, H1N1 s.:A/Swine/Iowa/1976/31, H1N1 h.: A/Puerto Rico/8/34, H2N2 h: A/Leningrad/134/17/57, H3N2 h.: A/Hong Kong/1/58. Also included is H7N3 ch: A/Chicken/Chile/4957/02, H7N1 ch: A/Chicken/FPV/Rostock/34, H9N2 q: A/Quail/Hong Kong/G1/97, H9N2 dk: A/Duck/Hong Kong/y280/97, H6N1 ch: A/Chicken/Taiwan/7-5/99, H6N1 t: A/Teal/Hong Kong/w312/97 and H5N1 dk Z gr: A/Duck/China/E319-2/03. All trees were constructed based on nucleotides in the open reading frame of each gene. The scale bars represent nucleotide distance units

v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) as described previously [3].

The development of the sequences was performed on an automatic ABI PRISM® 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries. Consensus sequences were generated using SeqScape® software v2.5 (Applied Biosystems), and assembly and alignment trimming were performed with the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison, WI). ClustalX [41] was used for multiple sequence alignments, and alignments were edited and translated using GeneDoc, version 2.6.002 (www.psc.edu/biomed/genedoc). Comparisons with published sequences were performed by searches in both the NCBI BLAST database [1] and in the Influenza Sequence Database Los Alamos [26]. Phylogenetic trees were generated using the PHYLIP package, version 3.57c [8]. Genetic distance matrices were calculated with the DNADIST program, using the Kimura 2-parameters model with a transition-to-translation ratio of 2.0. Neighbor-joining trees were generated with the Neighbor program. Trees were visualised in MEGA (www.megasoftware.net). The nucleotide sequences representing the known genetic lineages of influenza A viruses used as references in the phylogenetic trees of the genes coding for the internal proteins are given elsewhere [10] (see also legend, Fig. 1). Sequence representatives for common H7, H9, and H6 subtypes, the H6 (A/Teal/Hong Kong/W31/97 (H6N1)) and H9 (A/Quail/Hong Kong/G1/97 (H9N2) subtypes believed to contribute to the internal genes of the 1997 HPAIV H5N1 viruses, and a representative of the H5N1 Z-genotype (A/Duck/China/E319-2/03) were also included (see legend, Fig. 1). Nucleotide sequences obtained from this study are available under accession numbers DQ251441–DQ251454.

Results

Phylogenetic analysis and sequence comparisons

Genome characterisation of the A/Mallard/Denmark/64650/03 (H5N7) strain was obtained by full-length sequencing of all eight segments. Based on sequence nucleotide distances and BLAST searches, we found that the H5N7 strain possessed a HA gene closely related to a recent Danish LPAIV A/Duck/Denmark/65047/04 (H5N2) isolate with an HA nucleotide sequence identity of 98.9% (Table 1). The NA gene of the H5N7 virus was closely related to the HPAIV H7N7 causing infections in humans and lethal influenza A outbreaks in chickens in the Netherlands in 2003 (A/Chicken/Netherlands/1/03 and A/Netherlands/33/03 (H7N7)) with a nucleotide sequence identity of 96.8% (Table 1) [3]. The basic poly-

Table 1. Comparisons of A/Mallard/Denmark/64650/03 (H5N7) with isolates in GenBank of highest nucleotide and amino acid identity (%) including A/Duck/Denmark/65047/04 (H5N2)

Gene	Nucleotide sequence identity				Amino acid sequence identity			
	Most similar isolate	Subtype	No. different/no. compared	Identity (%)	Most similar isolate	Subtype	No. different/no. compared	Identity (%)
HA	A/Duck/Denmark/65047/04	H5N2	18/1702	98.9	A/Duck/Denmark/65047/04	H5N2	7/564	98.8
NA	A/Chicken/Netherlands/1/03	H7N7	46/1416	96.8	A/Chicken/Netherlands/1/03	H7N7	14/471	97.0
PB2	A/Duck/Denmark/65047/04/A/Chicken/Netherlands/1/03	H5N2 H7N7	66/2341 66/2341	97.2 97.2	A/Duck/Zhejiang/11/2000	H5N1	7/759	99.1
PB1	A/Duck/Denmark/65047/04/A/Chicken/Netherlands/1/03	H5N2 H7N7	115/2317 118/2324	95.0 94.9	A/Chicken/Netherlands/1/03	H7N7	4/757	99.5
PA	A/Duck/Denmark/65047/04	H5N2	42/2151	98.0	A/Duck/Denmark/65047/04	H5N2	5/716	99.3
NP	A/Duck/Hong Kong/3096/99	H6N2	46/1497	96.9	A/Duck/Denmark/65047/04	H5N2	4/498	99.2
M	A/WDk/ST/1737/2000	H6N8	14/984	98.6	A/Chicken/Netherlands/1/03[a]	H7N7	0/252	100
NS	A/Netherlands/219/03	H7N7	14/890	98.4	A/WDk/ST/1737/2000[b]	H6N8	0/230	100

[a] Other strains with 100% M1 amino acid identity comprise subtypes H1–H7, H9, H11, and H13.
[b] Other strains with 100% NS1 amino acid identity comprise subtypes H4 and H11.

merase gene (PB2) was common to both H7N7 (A/Chicken/Netherlands/1/03 (H7N7)) and H5N2 (A/Duck/Denmark/65047/04 (H5N2)), with a nucleotide identity of 97.2% (Table 1, Fig. 1). The PB1 gene was about equidistant, ∼95% nucleotide identity (Table 1), to both of its closest neighbors A/Chicken/Netherlands/1/03 (H7N7) and A/Duck/Denmark/65047/04 (H5N2) (Fig. 1). The acidic polymerase gene (PA) was most similar to the PA gene from the A/Duck/Denmark/65047/04 (H5N2) strain (98.0%). The nucleoprotein (NP) gene of H5N7 shares a common ancestor with the A/Teal/Hong Kong/W31/97 (H6N1) and A/Quail/Hong Kong/G1/97 (H9N2) viruses, which are believed contributed to the internal genes of the 1997 HP H5N1 strains from Hong Kong (Fig. 1). A BLAST search showed that the H5N7 NP gene had highest nucleotide sequence identity to an H6 virus (e.g. A/Duck/Hong Kong/3096/99 (H6N2)) (96.9%), whereas the closest isolate in amino acid sequence identity was A/Duck/Denmark/65047/04 (H5N2) (99.2%) (Table 1). The matrix gene (M) has a nucleotide sequence identity of 98.1% with the A/Duck/Denmark/65047/04 (H5N2) strain (Fig. 1). However, the closest isolate in GenBank is A/WDK/ST/1737/2000 (H6N8) (98.6%), and some virus strains of subtypes H1-H7, H9, H11, and H13 have 100% amino acid sequence identity to A/Mallard/Denmark/64650/03 (H5N7) (Table 1). The non-structural gene (NS) has greatest nucleotide sequence similarity to the human-fatal isolate A/Netherlands/219/03 (H7N7) (98.4%). However, virus strains with 100% amino acid sequence identity comprise subtypes H6, H4, and H11 (Table 1). The NS gene of the H5N7 isolate belongs to NS subgroup A as does the NS gene of the H7N7 from the Netherlands, whilst the NS gene of the H5N2 from Denmark belongs to NS subgroup B.

Sequence analysis

As described previously [3], the HA gene of A/Mallard/Denmark/64650/03 (H5N7) showed a phylogenetic relationship with the HPAIV H5N2 strains from Italy (e.g. A/Chicken/Italy/312/97 (H5N2)) that caused eight outbreaks of H5N2 infection in chickens in 1997–98 [7]. However, the H5N7 strain did not possess any of the known characteristics of the HP-like multibasic HA0 cleavage site sequence nor an additional glycosylation site in the globular head, and the chicken intravenous pathogenicity index was 0.00 [3]. Other indications of HP are the substitution K627 in the PB2 protein, which has been associated with increased virulence of H5N1 viruses in mice and H7N7 viruses in humans, and the presence of E92 in the NS1 protein, which has been shown to increase virulence in pigs [34, 36]. However, none of these mutations could be found in the H5N7 strain. The strain A/Netherlands/219/03 (H7N7), isolated from the fatal human case of acute respiratory distress syndrome in the Netherlands in 2003, differed by 14 substitutions in the HA, NA, PB2, PA, and NS genes compared to the other human and avian infectious H7N7 viruses at that time [10]. Some of these substitutions were also found in the gene segments of the H5N7 genome, namely PB2 V297I, PA F666L, NA P458S, and NS V137I.

Resistance

Surveillance of influenza susceptibility to rimantadine and amantadine and to neuraminidase inhibitors like zanamivir and oseltamivir is of great importance in order to be prepared for a new pandemic. The most common NA inhibitor-resistance mutations found clinically are E119V and R292K in N2 and H274Y in N1 [42, 44]. The H5N7 strain does not possess any of the typical substitutions for oseltamivir, zanamivir, or amantadine resistance (L26F, V27A, A30T/S, S31N, and G34E) [4, 15, 38].

Host specificity

All 32 amino acid residues in the genes M1, M2, NP, PA, and PB2 described as host-specific residues [35] were avian-like in the H5N7 strain. The HA of the H5N7 virus possesses a Q226 (H3 numbering, 238 from the H5N7 start) and a G228 amino acid (240 from the H5N7 start), indicating that the virus preferentially binds to the avian-like NeuAca2,3-Gal receptor linkage.

Discussion

AIV of subtype H5 was discovered for the first time in Denmark in 2003 in an outdoor-bred commercial flock of 12,000 mallard ducks that had shown increased mortality for some time [3]. The strain, A/Mallard/Denmark/64650/03, possessed a previously undescribed influenza A subtype combination, H5N7 [3]. It was promptly suggested that the new subtype combination was of low pathogenicity due to the lack of multibasic amino acids in the HA0 cleavage site, the lack of an additional glycosylation site in the globular head of HA, and a negative chicken intravenous pathogenicity index [3]. The HA protein of the H5N7 strain possessed an LP HA0 cleavage site (K-E-T-R) rendering it dependent on trypsin-like host cell proteases in order to become infectious, an indicator of localised infection. However, the H5N7 virus was isolated from pools of brain and of intestine, besides the pools of lung, kidney and spleen, often a characteristic of HPAIV. It was therefore necessary to genetically characterise the full genome of the H5N7 virus to trace any characteristics of HP and possible drug resistance and to determine the degree of reassortment of the H5N7 genome. Experience with the 1918 H1N1 ''Spanish flu'' pandemic virus has show that pathogenicity to humans is not completely dependent upon a multibasic HA0 cleavage site or multiorgan tropism, but the combination of genes, especially the HA and the polymerase genes, were essential for optimal virulence of the 1918 H1N1 strain [43]. The ''Spanish flu'' virus did not possess any of the known HA and NA characteristics of HP, but was still responsible for the deaths of up to 50 million people worldwide.

Since the first incidence of AIV transmission to humans [23], there has been considerable reassortment in the genes coding for the internal proteins of H5 subtypes with other AIVs. We found that the A/Mallard/Denmark/64650/03 (H5N7) strain possesses genes of both H5 and H7 subtype origin and possibly a third H6 subtype; however, it can not be excluded that the prevalence of the H5N7 subtype has been low and that the virus has existed in the wild bird population, giving rise to other reassortants. The closest isolates based on nucleotide sequence comparisons of HA and NA were an LP Danish H5 isolate A/Duck/Denmark/65047/04 (H5N2) and HP A/Chicken/Netherlands/1/03 (H7N7) [3], respectively. The phylogenetic analyses revealed that the H5N7 strain was composed of a PB2 gene similar to both A/Duck/Denmark/65047/04 (H5N2) and A/Chicken/Netherlands/1/03 (H7N7). The PB1 gene was equidistant in nucleotide sequence with its two closest isolates A/Duck/Denmark/65047/04 (H5N2) and A/Chicken/Netherlands/1/03 (H7N7). The NP gene was most closely related to viruses of H6 subtype origin as was the M gene. The PA gene clustered with A/Duck/Denmark/65047/04 (H5N2). The NS gene belonged to NS subgroup A, with no deletions in the coding sequence, while the A/Goose/Guangdong/1/96 (H5N1) – like viruses and the second Danish H5 isolate A/Duck/Denmark/65047/04 (H5N2) belong to subgroup B. The H5N7 NS gene had highest nucleotide sequence with the NS gene from the human-fatal isolate A/Netherlands/219/03 (H7N7). This H5N7 strain possessed one amino acid substitution, V137I, in the NS1 protein compared to the non-fatal chicken isolate A/Chicken/Netherlands/1/03 (H7N7) [10], which was also found in the NS protein of our H5N7 strain, but also in the NS1 gene of several other subtypes. The importance of this substitution in the NS1 protein is not clear, but the NS gene plays a key role in the pathogenesis of the influenza A virus by circumventing the host antiviral cytokine responses and acts as an antagonist on interferon and tumor necrosis factor alpha [34, 39]. Other substitutions common with the human-fatal case H7N7 strain were found in the PB2, PA and NA proteins of the H5N7 strain. It is not known if any of these substitutions are important for host infection or virulence. Further studies might explain if any of these substitutions contributed to the multiorgan tissue tropism seen for the H5N7 strain.

An amino acid substitution, E627K, in the PB2 molecule has been proposed to be an indication of systemic spreading and neurovirulence in H5N1-infected mice [13] and important for mammalian adaptation [37]. This substitution was found in the H7N7 virus from the human-lethal case in the Netherlands in 2003 and also in the ''Spanish flu''

virus from 1918 as well as in several HP H5N1 strains [10, 13, 27, 40]. However, the PB2 molecule of H5N7 possessed a glutamic acid at this position as did the H7N7 chicken isolate from the Netherlands in 2003. It has been shown that this substitution is not essential for high lethality in ferrets [11] and is not present in some human and avian HP H5N1 isolates [25]. A glutamic acid in position 92 of the NS protein is associated with increased virulence in pigs [34], but an aspartic acid was found in this position in the H5N7 NS protein. The NS1 E92 substitution was present in several Japanese HP H5N1 isolates from 2003 to 04 [27]; however, many recent HP H5N1 viruses do not [25], nor did the ''Spanish flu'' virus [43].

HA possessing L226 has been shown to bind to the human NeuAca2,6-Gal receptor linkage, whereas HA possessing Q226 has been shown to bind to the avian NeuAca2,3-Gal receptor linkage. Host-specific amino acid residues in the genome of the H5N7 virus were all of the avian origin, and the HA protein possessed the amino acids Q226 and G228, indicating that the virus preferentially binds to the avian-like receptor. Our results suggest that the H5N7 strain is of low virulence and not adapted to a mammalian host. The presence of virus in the brain and the pool from internal organs may be explained by weakness due to primary systemic bacterial infection of the sick ducks. There are no genetic indications of resistance to matrix- or neuraminidase-inhibitory drugs.

The new subtype combination H5N7 possesses genes closely related to HP avian influenza viruses. If such a virus is allowed to circulate in poultry or even wild-bird-breeding colonies, mutations may merge, and the low-pathogenic virus can become pathogenic, as LPAIV strains previously have been shown to be precursors of HPAIV strains [21, 22]. Because none of the genetic indicators for HP in the HA protein are a certainty of a HP phenotype, it is also necessary to characterise the genes coding for the internal proteins. Our results emphasize the need for increased surveillance of occurrences of AIV and LP and well as HP in order to keep the information on the circulating gene pool up-dated, thus optimizing preparedness of outbreaks of HPAIV and the risk of a derived pandemic in humans.

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