

Sequence diversity of the haemagglutinin open reading frame of recent highly pathogenic avian influenza H5N1 isolates from Egypt

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Abstract The sequences encoding the haemagglutinin (HA) of twelve H5N1 isolates obtained in 2006 and 2007 from different avian species in backyard holdings and poultry farms in Egypt revealed amino acid variations across the polypeptide and also in the polybasic cleavage motif of three of the isolates from backyard poultry with one, so far, unique mutation in an isolate from a chicken. The HAs of two isolates (*A/goose/Egypt/R4/2007*, *A/chicken/Egypt/R3/2007*) collected on the same day in the same village from two neighbouring houses were found to differ from each other. Five out of the seven nucleotide exchanges in these two isolates were translationally silent, and two resulted in amino acid substitutions: one in the polybasic cleavage motif and the other in the signal peptide. Circulation of different H5N1 strains possessing considerable variations in backyard poultry, particularly domestic waterfowl, draws attention to the evolution of H5N1 subtypes in Egypt.

Introduction

Avian influenza viruses belong to type A viruses within the family Orthomyxoviridae. Further subtyping of influenza A viruses is based on antigenic differences between subtypes of the two surface glycoproteins haemagglutinin (H1–H16) and neuraminidase (N1–N9) [3]. H5N1 highly pathogenic avian influenza viruses (HPAIV) have emerged since 1997 in Southeast Asia and have undergone rapid evolution, resulting in multiple genotypes [4]. HPAIV H5N1 strains currently circulating in Europe and Africa belong to a single genotype “Z” and can be sub-grouped into numerous clades on the basis of their HA sequences. All HPAIV H5N1 outside Asia fall into clade 2.2; the progenitors of these viruses arose in 2005 during the Lake Qinghai outbreak in northwestern China. Meanwhile, sublineages of this clade have emerged and continue to evolve rapidly [7, 10, 12, 14].

HPAIV H5N1 is an important poultry pathogen and a major menace to the poultry industry. Furthermore, HPAIV H5N1 infections in poultry constitute a threat to humans. As of June 2, 2009, 433 human cases of H5N1 infection worldwide, with 262 deaths, were reported by the WHO. A total of 78 cases with 27 deaths have been recorded in Egypt, where 27 of the 38 cases confirmed so far in 2009 were observed (http://www.who.int/csr/disease/avian_influenza/country/en). Extensive surveillance and genetic studies have revealed that since 2003, H5N1 viruses had become endemic in poultry in many countries including Egypt [1]. Although the promoters of endemicity in Egypt have not yet been definitely clarified, clinically unapparent infected free-ranging ducks and geese as well as mixed-species backyard holdings are suspected to play a pivotal role [1]. Such domestic bird holdings are difficult to control. Poultry rearing according to cultural and social

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traditions as well as the predominance of live bird trading and economically motivated hesitant responses to public programs trying to raise awareness of the potential dangers are at the basis of the endemic status of HPAIV H5N1.

Long-term endemic influenza virus infections in poultry increase exposure risks to humans, and, in turn, create opportunities for the emergence of human-adapted strains with pandemic potential [8, 13]. Thus, sustained viral sequence comparisons and phylogenetic analyses of current HPAIV H5N1 are necessary to recognize newly emerging influenza variants and to monitor the global spread of these viruses [11]. In this study, we present sequences (GenBank accession numbers EU183321 to EU183332) and analyses of the HA genes of HPAIV H5N1 isolates from Egyptian backyard holdings as well as from poultry farms to contribute to the compilation of possible sequence variations.

Twelve isolates, six from backyard holdings from Beni-Suef Governorate and six from poultry farms from Beni-Suef, El-Minia, Al-Fayoum, and Qaluobia Governorates, were isolated in embryonated hen eggs from cloacal swab material (Table 1). The principal clinical sign in chickens and turkeys was sudden death. Some birds showed cyanosis of the head and shank as well as facial oedema. Mortality approached 100%. Postmortem findings included generalized congestion in all parenchymatous organs, congestion and petechial haemorrhages in the intestinal tract. In ducks and geese, mortality did not exceed 30%. Nervous manifestations were the common clinical features. Viral RNA was extracted from infected chorioallantoic membrane (CAM) homogenates of the first passage in hen eggs using TRIR (ABgene) as recommended by the supplier. All samples were tested by real-time reverse transcriptase polymerase chain reaction (RRT-PCR) according to the diagnostic cascade recommended by the Diagnostic Manual issued by the European Commission. All isolates were

found positive in RRT-PCR for M, H5 and N1 genes and pathotyped as HPAIV using the restriction enzyme cleavage pattern (RECP) assay as described recently [2]. One-step RT-PCR amplification of four overlapping regions of the H5 gene was performed with gene-specific primer sets using SuperScriptIII One-Step RT-PCR with Platinum[®] Taq DNA polymerase (Invitrogen) (sequences of primer sets are available upon request). RT-PCR amplicons were sequenced directly using an ABI Prism BigDye terminator cycle sequencing reaction kit (Applied Biosystems). Selected amplicons spanning the cleavage site of certain isolates were sequenced after cloning into plasmid vector pSP73 (Promega); five clones were sequenced per isolate. A BLAST analysis was initially performed to exclude sequence redundancy with existing GenBank entries. Comparative analyses were performed using MUSCLE alignments. These alignments (comprising an additional 105 full-length HA sequences from Egyptian H5N1 hp viruses that are publicly available in GenBank) were used to calculate phylogenies after selecting an appropriate nucleotide substitution model (SYM + G or HSK + I + G) using jModelTest [9]. Phylogenetic trees were constructed by maximum likelihood (PhyML [5]), maximum parsimony (TNT, <http://www.zmuc.dk/public/phylogeny/tnt>) and a Bayesian approach (BAMBE [6]). These analyses revealed the emergence of two separate sublineages, A and B, among Egyptian H5N1 hp viruses (Fig. 1). Most of the available sequences from 2008 belonged to the younger sublineage B, but some 2008 sequences still clustered in A (A2, A4, A5), indicating co-circulation of several distinct lineages of HPAIV H5N1 in Egypt. All sequences determined in this study belonged to different clusters of lineage A, and some were located near the root and may represent close relatives of the initially introduced virus(es). Egyptian avian and human H5N1

Table 1 Origin of HPAIV H5N1 analysed in this study

Isolate	Location	Species	Age	Vaccination	Collection Date
A/chicken/Egypt/R2/2007	BS	Chicken	Mixed (75–180 days)	1 month before infection	1 January 2007
A/chicken/Egypt/R1/2006	BS	Chicken	150 days	–	25 December 2006
A/chicken/Egypt/R3/2007	BS	Chicken	Mixed (1.5 and 2 years)	–	1 February 2007
A/duck/Egypt/R5/2007	BS	Duck	60 days	–	20 February 2007
A/goose/Egypt/R4/2007	BS	Goose	3 years	–	1 February 2007
A/chicken/Egypt/R6/2007	BS	Chicken	150 days	–	26 February 2007
A/duck/Egypt/F5/2006	Minia	Duck	30 days	–	25 December 2006
A/chicken/Egypt/F6/2007	BS	Chicken	42 days	Day old	6 March 2007
A/chicken/Egypt/F4/2006	BS	Chicken	130 days	5 day before infection	5 December 2006
A/turkey/Egypt/F2/2006	Qalu	Turkey	120 days	–	1 March 2006
A/turkey/Egypt/F1/2006	Minia	Turkey	150 days	–	25 February 2006
A/chicken/Egypt/F3/2006	Fayoum	Chicken	40 days	–	1 June 2006

BS Beni-Suef, *Qalu* Qaluobiya

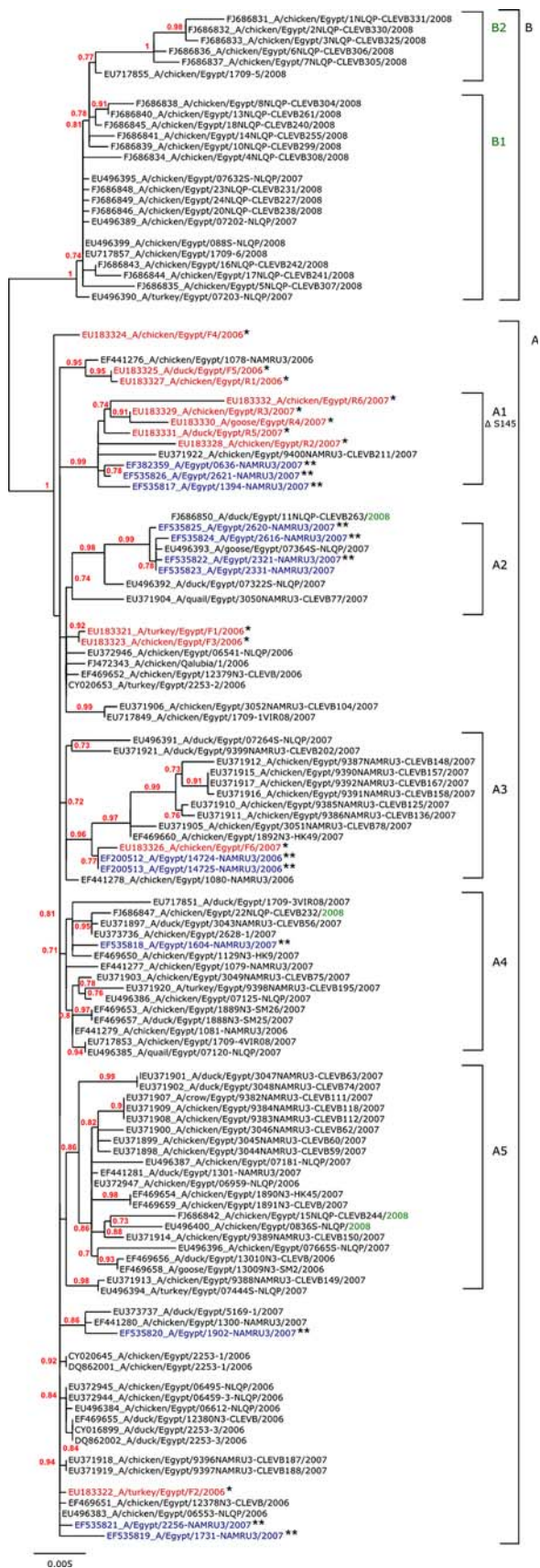


Fig. 1 Phylogenetic analysis of full-length HA genes of HPAIV H5N1 isolates from Egypt. The tree was generated using maximum-likelihood estimation (PhyML). An HSK model comprising a proportion of invariable sites and rate variation among sites with a number of rate categories was postulated (jModelTest). The bar represents genetic distance and is drawn to scale. *Single asterisks* indicate sequences determined in this study. All other sequences were obtained from GenBank in May 2009. *Double asterisks* indicate isolates of human origin. *Numbers* represent bootstrap values after 200 replications. Branches with support values <50 were collapsed. The topologies of trees established by maximum parsimony (TNT) or Bayesian methods (BAMBE) were very similar (not shown)

isolates were interspersed in our phylogenetic trees, indicating that no specific “humanized” lineage had evolved.

The deduced amino acid exchanges are distributed across the HA protein and are found in the signal peptide of three isolates as well as in the HA1 and HA2 subunits (Fig. S1) and in the polybasic cleavage motif (Fig. S1), whose consensus sequence is GERRRKKR*GLF for clade 2.2 viruses. This motif was present in nine of the isolates, whereas A/chicken/Egypt/R2/2007 contained an R at position K345, an exchange that had not been found before in H5N1 isolates. A/goose/Egypt/R4/2007 and A/chicken/Egypt/R6/2007 both showed an exchange from E340 to K but differed in the amino acid (aa) composition within the signal peptide (aa13), HA1 (aas 27, 39, 41, 61, 63, 87, 112, and 118) and HA2 (aa 479). All variations within the polybasic cleavage motif probably do not affect cleavage of the HA precursor molecule, since the RX(K/R)R consensus motif for cleavage by furin or subtilisin-like protein convertases [15] remains conserved. By contrast, amino acid differences at positions 27, 39 and 41 might result in a loss of one or two N-glycosylation sites in the HA of isolate A/chicken/Egypt/R6/2007 in comparison to the HA molecules of the other Egyptian isolates, which contain a total of seven potential N-glycosylation signals (Fig. S1). In addition, five of the six isolates from 2007 lack aa S145. This deletion is also present in all other viruses grouped into 2.2 sublineage A1, which also include sequences from human H5N1 isolates (Fig. 1). The significance of this deletion is unknown, but it should be noted that this position is close to a domain modulating receptor interaction. Interestingly, strains with this deletion appear to evolve towards a receptor usage that is similar to that of seasonal human H1N1 [11].

The isolates A/goose/Egypt/R4/2007 and A/chicken/Egypt/R3/2007 were collected on the same day from neighbouring houses and found to be different from each other. Five of the seven nucleotide exchanges were translationally silent, and two resulted in amino acid substitutions: one in the polybasic cleavage motif and the other in the signal peptide (Figs. S1, S2).

Field observations regarding the pathogenicity of HPAIV H5N1 in Egypt revealed considerable variation among backyard holdings, where ducks and geese seemed to be significantly less affected, showing fewer overt clinical signs. It is not yet clear whether amino acid substitutions in the HA that potentially result in altered glycosylation patterns, as also observed in the isolates analysed here, contribute to decreased pathogenicity in these species [8] or whether host-related factors are solely responsible.

In summary, our results are in accordance with the notion that several lineages of HA of HPAIV H5N1 strains are endemically co-circulating in Egypt. It remains to be determined whether lineage-A viruses which apparently arose in 2007, about a year after launching of a nationwide poultry vaccination campaign, represent rapidly evolving variants that have escaped vaccine-induced immunity.

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References

1. Aly MM, Arafa A, Hassan MK (2008) Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. *Avian Dis* 52:269–277
2. Fereidouni SR, Harder T, Starick E (2008) Rapid pathotyping of recent H5N1 highly pathogenic avian influenza viruses and of H5 viruses with low pathogenicity by RT-PCR and restriction enzyme cleavage pattern (RECP). *J Virol Methods* 154:14–19
3. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 79:2814–2822
4. Guan Y, Peiris JS, Lipatov AS, Ellis TM, Dyrting KC, Krauss S, Zhang LJ, Webster RG, Shortridge KF (2002) Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc Natl Acad Sci USA* 99:8950–8955
5. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
6. Larget B, Simon DL, Kadane JB (2002) Bayesian phylogenetic inference from animal mitochondrial genome rearrangements. *J Roy Stat Soc* 64:681–693
7. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepongstie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby J, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430:209–213
8. Matrosovich M, Zhou N, Kawaoka Y, Webster R (1999) The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J Virol* 73:1146–1155
9. Posada D (2008) jModelTest: phylogenetic model averaging. *Mol Biol Evol* 25:1253–1256
10. Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Aly MM, Brown IH, Couacy-Hymann E, De Mía GM, Dung DH, Guercio A, Joannis T, Ali ASM, Osmani A, Padalino I, Saad MD, Savić V, Sengamalay NA, Yingst S, Zaborsky J, Zorman-Rojs O, Ghedin E, Capua I (2007) Genome analysis linking recent European and African influenza (H5N1) Viruses. *Emerg Infect Dis* 13:713–718
11. Veljkovic V, Veljkovic N, Muller CP, Müller S, Glisic S, Perovic V, Köhler H (2009) Characterization of conserved properties of hemagglutinin of H5N1 and human influenza viruses: possible consequences for therapy and infection control. *BMC Struct Biol* 9:21
12. Weber S, Harder T, Starick E, Beer M, Werner O, Hoffmann B, Mettenleiter TC, Mundt E (2007) Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. *J Gen Virol* 88:554–558
13. Webster RG, Wright SM, Castrucci MR, Bean WJ, Kawaoka Y (1993) Influenza-a model of an emerging virus disease. *Intervirology* 35:16–25
14. WHO/OIE/FAO H5N1 Evolution Working Group (2009) Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. *Influenza Other Respi Viruses* 3:59–62
15. Zhou A, Webb G, Zhu X, Steiner DF (1999) Proteolytic processing in the secretory pathway. *J Biol Chem* 274:20745–20748