CHARACTERIZATION OF THE HEMAGGLUTININ AND NEURAMINIDASE GENES OF RECENT INFLUENZA VIRUS ISOLATES FROM DIFFERENT AVIAN SPECIES IN THAILAND

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Summary. – The hemagglutinin (HA) and neuraminidase (NA) genes of eight influenza A virus (H5N1) isolates obtained from various avian species in Thailand in 2003–2004 have been characterized in comparison with the Thai isolate A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04(H5N1). Phylogenetic analyses of both genes revealed that all the eight avian isolates were closely related to the A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04(H5N1). The amino acid sequence of the HA cleavage site revealed a common characteristic of a highly pathogenic virus strain. Moreover, a deletion of 20 amino acids in the NA stalk region was detected in all Thai isolates in contrast to the H5N1 strain that had caused outbreaks in eastern Asia in 1996–1997 and 2000–2001.

Key words: avian influenza; avian species; hemagglutinin gene; influenza A virus; neuraminidase gene; H5N1 subtype

Introduction

Avian influenza (AI) is an infectious disease of birds caused by influenza A virus (the species *Influenza A virus*, the genus *Influenzavirus A*). Most birds are susceptible to this infection, though some species are more resistant than others. AI is highly contagious, leads to severe epidemics; it causes a wide spectrum of symptoms in birds, ranging from mild illness to rapid fatal disease. Some migratory and water birds are thought to be the natural reservoirs of AI viruses, while domestic poultry including chickens and turkeys are considered particularly susceptible hosts (Horimoto and Kawaoka, 2001). To date, antigenic and genetic analyses of AI virus strains have identified 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. Of these, only H5 and H7 strains can become highly pathogenic upon transmission from wild birds to poultry. Recent outbreaks of severe AI were caused by a H7N7 subtype in the Netherlands in 2003 (Fouchier *et al*., 2004), H5N2 and H7N3 subtypes in northern Italy in 1997–1998 (Donatelli *et al*., 2001) and a H5N1 subtype in Hong Kong in 1997 and 2003 (Tam, 2002).

Owing to the lack of a proof reading mechanism, influenza viruses are thought to have a high error rate during the transcription of their genome (Suarez, 2000). As a result, the virus is thought to adapt rapidly to new host species. It is believed that a species crossover may result in genetic changes in HA and NA that alter the specificity of the receptor binding, the membrane fusion glycoprotein in cell entry (HA) and the receptor-destroying enzyme in virus release (NA). These genetic changes could result in new pandemic strains (Matrosovich *et al*., 1999; Suarez, 2000; Banks *et al*., 2001; Horimoto and Kawaoka, 2001).

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Abbreviations: HA = hemagglutinin; NA = neuraminidase; AI = avian influenza; MMLV = Moloney murine leukemia virus

The recent outbreak of AI, caused by highly pathogenic H5N1 strains in more than eight countries in Asia from December 2003 to September 2004, led to heavy economic losses in the poultry industry. Moreover, at least 29 deaths from a total of 42 human cases, caused by direct transmission of the virus from birds to humans, were reported in Thailand and Vietnam on September 23, 2004 (ProMED, 2004). The result of antigenic analysis and genome sequencing of the virus isolates from the infected persons proved that they were related to the strain responsible for the 1997 outbreak in Hong Kong (Subbarao *et al*., 1998; CDC, 2004). Moreover, the HA and NA genes of the isolates from human cases in Thailand were highly similar to those obtained from birds during the same epidemic (Viseshakul *et al*., 2004). Besides poultry suffering from an extremely high mortality, various other species of birds were infected and succumbed in this widespread epidemic.

In this study, we present the result of molecular characterization of the HA and NA genes of nine H5N1 influenza A virus isolates obtained from infected chickens, duck and other wild birds during the outbreak in Thailand in 2004 .

Materials and Methods

Viruses. Tissue specimens were collected during the outbreak of AI in Thailand in 2004 from following poultry and wild birds: four chickens (*Gallus domesticus*), a duck (*Anas domesticus*), a crow (*Corvus splendens*), a white peacock (*Brassica oleracea*), a Kalij pheasant (*Lophura leucomelanos*), and a Asian open bill (*Anastomus oscitans*). Suspensions (10%) of the specimens were inoculated in the allantoic cavity of 11-day-old SPF embryonated chicken eggs. HA titers were determined by using chicken erythrocytes.

Viral RNA was extracted from virus-infected allantoic fluids with the QIAamp[å] Viral RNA Mini Kit (Qiagen).

RT-PCR. cDNAs were synthesized from viral RNAs by using a universal primer specific for influenza A virus (5'-AGCAAA AGCAGG-3'), M-MLV Reverse Transcriptase ((Promega) and other components according to the manufacturer's (Promega) recommendations.

In the PCR, the following primer pairs specific for HA gene were employed: H5-F5' (5'-AGCAAAAGCAGGGGTCTGA TCTG-3', nt 1–23) and H5-R1 (5'-GCTCCTCTTTATTGTTGG GTATG-3', nt 565–543), H5-F2 (5'-TGAGAAAATTCAGATCAT CCCC-3', nt 409–430) and H5-R2 (5'-CAACGGCCTC AA ACTGAGTGT-3', nt 1265–1245), H5-F3 (5'-ACTCCAATGGGG GCGATAAAC-3', nt 914–934) and H5-R3' (5'-AGTAGAAAC AAGGGTGTTTTTAACTAC-3', nt 1778–1754).

NA gene-specific primer pairs were as follows:N1-F5' (5'-AGCAAAAGCAGGAG TTTAAAATG-3', nt 1–23) and N1-R1 (5'-TGATAGTGTCTGTTATTATGCC-3', nt 669–648), N1-F2 (5'-GTTTGAGTCTGTTGCTTGGTC-3', nt 539–559) and N1-R3' (5'-AGTAGAAACAAGGAGTTTTTTGAAC-3', nt 1458–1434). The reaction mixture (25 µl) consisted of 1 µl of cDNA, 10 µl of 2.5× Eppendorf MasterMix (Eppendorf, Germany), 0.5 µmol/l forward and reverse primer each and sterile water. After an initial denaturation step (94°C/2 mins), 40 cycles of amplification were performed, each including denaturation (94°C/30 secs), annealing (55°C/30 secs) and 1.30 min extension (72°C/90 secs), followed by a final extension (72°C/10 mins). The PCR was carried out in a Mastercycler Personal from Eppendorf (USA).

Nucleotide sequencing and phylogenetic analysis. The PCR-amplified DNAs were sequenced by using the Big Dye Terminator Sequencing Kit, version 3.0 (Amersham Biosciences) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were deposited at the GenBank under Acc. Nos. Y590563, AY590564, AY590567 – AY590570, AY590572, AY590576, AY590577, AY660554 – AY660558, and AY779048-51.

The HA and NA gene sequences obtained from the eight isolates were compared with those of A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04(H5N1) (Viseshakul *et al*., 2004) and other AI isolates from 1996–2004 available at the GenBank by using the BIOEDIT 5.0.9 program. Maximum likelihood trees were generated by using the DNASTAR-5.x program. Finally, a phylogenetic analysis of the compared isolates was performed.

Results and Discussion

As regards the sequences of HA ORF (nt 14–1,696), the ten AI virus isolates shared 99.64–99.94% identity with A/Chicken/Nakorn-Patom/Thailand/CU-K2/04(H5N1). The HA cleavage sites of all were indicative of highly pathogenic phenotype; minor differences were observed between the HA cleavage sites of the isolates from crow, white peacock, Kalij pheasant, and Asian open bill (Fig. 1). The HA cleavage site of these isolates consisted of multiple basic amino acid insertions, characteristic for highly pathogenic H5N1 subtype (Horimoto and Kawaoka, 2001; Tam, 2002). In addition, the viruses were isolated from tissues with specific AI lesions. Phylogenetic analysis of the HA gene of the isolates (Fig 2A) revealed a close relationship among the isolates from humans and poultry in Thailand, Vietnam, Indonesia and China during the initial outbreak in the 2003–2004 epidemic. All the ten isolates formed a separate cluster including goose isolates from Guangdong in 1996–1997 and were closely related to A/Duck/China/E319.2/03(H5N1).

As regards the sequences of NA ORF (nt 22–1,350), the isolates from poultry showed the highest identity (99.55–99.93%) with A/chicken/Nakorn-Patom/Thailand/CU-K2/04 (H5N1) In phylogenetic tree, the isolates from 1996–2001 formed a cluster separated from that of the isolates from the recent outbreak in eastern Asia (Fig. 2B). Analysis of the deduced amino acid sequences revealed that all the ten isolates had a deletion of 20 amino acids at positions 49–68 in the NA stalk region and were closely related to A/Duck/China/E319.2/03(H5N1).

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                          310        320        330        340        350        360
Ck/TH/04(CU-K2)           IHPLTIGECP KYVKSNRLVL ATGLRNSPQR ERRRKKRGLF GAIAGFIEGG WQGMVDGWYG
H/TH/04(KAN-1)            .......... .......... .......... .......... .......... ..........
H/TH/04(SP-33)            .......... .......... .......... .......... .......... ..........
Ck/TH/04(CU-K1)           .......... .......... .......... .......... .......... ..........
Ck/TH/04(CU-K3)           .......... .......... .......... .......... .......... ..........
Ck/TH/04(CU-21)           .......... .......... .......... .......... .......... ..........
Dk/TH/04(CU-2)            .......... .......... .......... .......... .......... ..........
OB/TH/04(Openbill)        .......... .......... .......... ....K..... .......... ..........
WP/TH/04(White peacock)   .......... .......... .......... ....K..... .......... ..........
KP/TH/04(Kajil pheasant)  .......... .......... .......... ....K..... .......... ..........
Cr/TH/04(Crow)            .......... .......... .......... ....K..... .......... ..........
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Fig. 1

Amino acid sequences of HA at the cleavage site

The sequences of the eight Thai islates are compared with that of A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04(H5N1) and human isolates. Amino acid sequences at the HA0 cleavage sites of H5 influenza viruses in this study.

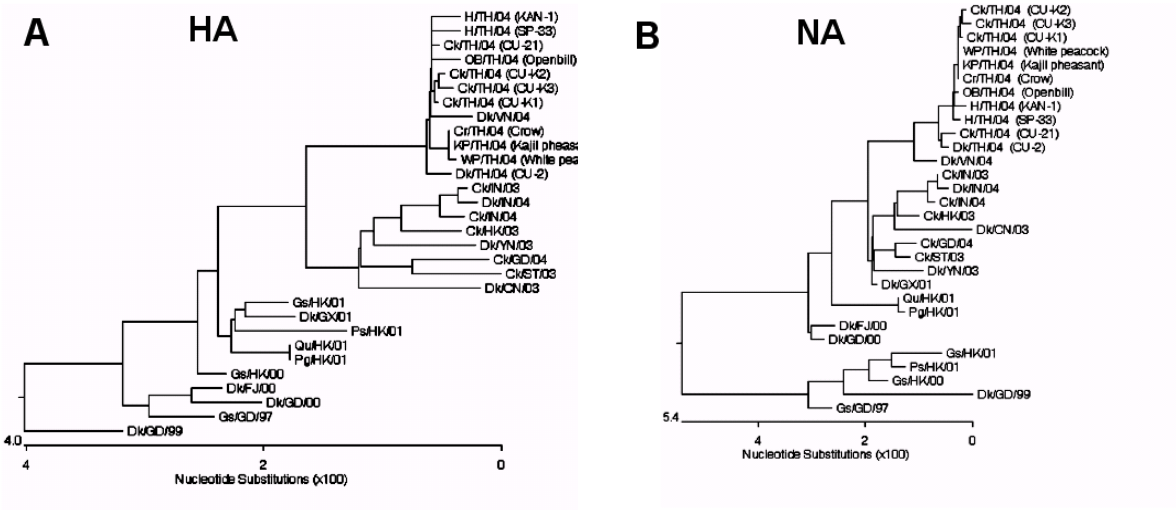

Fig. 2

Phylogenetic tree of HA (A) and NA (B) genes of various influenza virus isolates

Ck = chicken, Cr = crow, Dk = duck, Ps = pheasant, Pg = pigeon, Qu = quail, Gs = goose, OB = Asian open bill, KP = kalij pheasant, WP = white peacock, H = human, GD = Guangdong, HK = Hong Kong, VN = Vietnam, IN = Indonesia, CN = China, ST = Shantou, FJ = Fujian, YN = Yunnan, ZJ = Zhejiang, GX = Guangxi.

This study demonstrated that highly pathogenic influenza A virus (H5N1) isolates were obtained from a variety of infected avian species during the 2003–2004 epidemics in Thailand and eastern Asia. Phylogenetic analyses of these isolates based on HA and NA genes confirmed their close relationship to each other. A series of basic amino acids at the HA cleavage site, known to be associated with high pathogenicity, was found to display some variation among the isolates. Moreover, a glutamine at position 222 and glycine at position 224 of HA, indicative for avian cell surface receptor usage, were found in the isolates obtained from humans and poultry in Thailand and Vietnam (Li *et al*., 2004) and in isolates obtained from a tiger and a leopard in Thailand (Keawcharoen *et al*., 2004). NA stalk deletions were observed in all the isolates. This molecular feature has been described for isolates from domestic poultry (Campitelli *et al*., 2004) and suggested to affect the NA activity (Banks *et al*., 2001; Spackman *et al*., 2003;

Campitelli *et al*., 2004). A recent study of lethality of H5N1 strains to ferrets revealed that multiple molecular differences in other genes were important for a high level of virulence (Govorkova *et al*., 2005). Our study thus shows that wild and domestic birds investigated by us have been infected with a closely related virus strain during this outbreak. This has important implications for wild bird conservation and influenza virus epidemiology.

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