

Phylogenetic characterization of avian influenza H5N1 subtype viruses isolated in Thailand during 2006–2008

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Abstract

Highly pathogenic avian influenza A H5N1 subtype emerged in Thailand during 2006 to early 2008. All the viral genes were of avian influenza origin, which indicates the absence of reassortment with human influenza viruses. The phylogenetic analysis of HA gene demonstrated that all of the studied viruses belonged to Clade1 (A/ck/Phichit//NIAH1/06, A/ck/Angthong/NIAH101204/07, A/ck/Nakhonsawan/NIAH600567/08 and A/ck/Phichit/NIAH600674/08) and Clade 2.3.4 (A/ck/Nakhonphanom/NIAH113718/06, A/ck/Nongkhai/NIAH400802/07 and A/c/kMukdahan/NIAH40390/07). Four isolates (A/ck/Nakhonsawan/NIAH600567/08, A/ck/Phichit/NIAH600674/08, A/ck/Angthong/NIAH101204/07, A/ck/Phichit/NIAH1/06) may be resistant to the amantadine drugs but sensitive to neuraminidase inhibitors (Oseltamivir carboxylate). The A/ck/Nakhonphanom/NIAH113718/06, A/ck/Nongkhai/NIAH400802/07 and A/ck/Mukdahan/NIAH40390/07 isolates showed an evidence of the antigenic shift of RNA polymerase acid (PA) gene and lack of one Lys(K) of the polybasic cleavage site (PLRERRRK_R/GLF) of the haemagglutinin (HA) gene, but in all isolates, there was deletion of 20 amino acid at the position 49 - 68 of neuraminidase (NA) gene and 5 amino acid at the position 80 - 84 of nonstructure (NS1) gene.

Key words: Highly Pathogenic Avian Influenza virus, H5N1, phylogenetic analysis, sequence analysis

Introduction

Highly Pathogenic Avian Influenza (HPAI) virus subtype H5N1 is an emerging issue for world health; it has caused numerous disease outbreaks in domestic poultry and wild bird populations, and threatened human health. Between 1997 and early 2008, a total of 385 cases of human infection were found. The H5N1 avian influenza viruses continue to be a threat to public health. Since late 2003, highly pathogenic H5N1 viruses have continued to cause outbreaks in countries in South-East Asia, China, central Asia, the Middle East and Africa as they were reported to the World Health Organization, resulting in 243 (63%) deaths (WHO, 2008). There has been an increasing concern to the public that that H5N1 could become the next pandemic influenza strain (Alexander, 2000).

HPAI virus belonging to the family *Orthomyxoviridae* is divided into type A, B and C based on morphology. The pleomorphic (80 – 120 nm) virion is made up of a lipid bilayer envelope derived from host cell, proteins, an envelope, a single stranded and negative sense RNA genome of eight segments, including PB2, PB1, PA, HA, NP, NA, M, and NS (ICTV, 2005). Influenza virus type A is divided further into subtypes based on the antigenic relationships in the surface glycoproteins, haemagglutinin (HA), and neuraminidase (NA). Currently, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) that potentially allow 144 various combinations have been recognized (Fouchier et al., 2005). Types B and C virus are not divided into subtypes. Genetic analyses have shown that most H5N1 influenza viruses from poultry and humans in 2004 to 2008 were first recognized in poultry from southern China in 2002 (Guan et al., 2004; Li et al., 2004). However, genetic characterization of H5N1 viruses from Southeast Asia and southern China showed that amino acid residues in the HA protein are distinct from the viruses isolated from other regions, and that these changes are correlated to the antigenic and receptor-binding sites (WHO, 2005). In addition, aquatic birds are thought to be the reservoir for genetic diversity and the source for transmission of HPAI viruses to other animal species such as domestic poultry, pigs, horses and humans (Webster et al., 1992).

Thailand has had 6 major outbreaks, 1) 23 January 2004 to 24 May 2004, 2) 3 July 2004 to 12 April 2005, 3) 1 July 2005 to 9 November 2005, 4) 11 January to 7 November 2006, 5) 15 January to 18 June 2007 and 6) 23 January to 25 April 2008 (OIE, 2008). The aim of this study is to collect 7 strains of HPAI H5N1 subtype viruses that were isolated from outbreaks in different regions of Thailand during 2006 and early 2008 and to conduct a sequence analysis by comparing with previously published isolates.

Materials and methods

Virus

A total of 7 Thai HPAI isolates (Table 3) were submitted to National Institute of Animal Health, Regional Veterinary Research and Development center, Phitsanulok and Khonkaen. All isolates were propagated in 9-day-old embryonated eggs by using standard procedures and identified with the standard hemagglutination (HA) test and hemagglutination inhibition (HI) test according to Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2004). Infective allantoic fluids from different isolates were harvested and used as a stock at -70°C for sequence analysis.

Reverse transcription-polymerase chain reaction

Viral RNA was extracted from infective allantoic fluids using the Qiagen RNA viral kit (Qiagen, Germany). The viral RNA was resuspended in DEPC-treated water and stored at -70°C. The RNA samples were reverse transcribed and amplified in a one-step RT-PCR assay with the following primers of the full genomes of eight segments described previously (Li et al., 2007; Hoffmann et al., 2001). The sequences of primers are indicated in Table 1. Briefly, 5 µL of RNA were added in a 50 µL RT-PCR reaction containing 25 µL of 2x reaction buffer (Invitrogen), 1 µL each (10 µM) of forward and reverse primers, 1 µL of SuperScript III/Platinum Taq High Fidelity enzyme mix (Invitrogen) and 17 µL of DEPC-treated water. Reactions were incubated at 50°C for 30 min, 94°C for 2 min, and 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.5 min, followed by 72°C for 7 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide (EtBr), and visualized under ultraviolet transillumination.

Sequencing and phylogenetic analysis

The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia Calif.) and the concentration of transcripts was determined using an ND-1000 NanoDrop spectrophotometer (NanoDrop® Technologies, USA) at 260 nm, ligated to a pGEM®-T vector system (Promega®, USA), and transferred into *DH5α Escherichia coli* by using Gene Pulser (Bio-RAD, USA). The clones were determined using gene specific primers and Bigdye® Terminator version 3.1 chemistry (Applied Biosystems, USA), according to the manufacturer's

instructions. Reactions were run on a 3100 DNA analyser (Applied Biosystems, USA). Sequencing was performed from both directions of M13 primers (pGEM[®]-T vector) in the PCR amplification, assembly of sequences, removal of low quality sequence data by using SeqScape[®] software version 2.5 (Applied Biosystems, USA), nucleotide sequence translation into protein sequence, additional multiple sequence alignments and processing were performed with the Bioedit software version 7.0.4.1 with an engine based on the Clustal W algorithm (Thompson, 1994). The phylogenetic analysis of the partial genome nucleotide and the deduced amino acid sequences were constructed with Molecular Evolutionary Genetics Analysis (MEGA, version 4) (Tamura et al., 2007) using neighbor joining tree inference analysis with the Tamura-Nei model, with 1000 bootstrap replications.

Table 1 Primers used in this study.

Name	Sequences (5 to 3)	Target gene	Reference
Bm-PB2-1	TATTGGTCTCAGGGAGCGAAAGCAGGTC	PB2-fragment 1	(Li et al., 2007)
PB2-1250R	TCYTCYTGTGARAAYACCAT		
PB2-1105F	TAYGARGARTTCACAATGGT		
Bm-PB2-2341R	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	PB2-fragment 2	(Li et al., 2007)
Bm-PB1-1	TATTCGTCTCAGGGAGCGAAAGCAGGCA		
PB1-1262R	TTRAACATGCCCATCATCAT		
PB1-1124F	ARATACCNGCAGARATGCT	PB1-fragment 2	(Li et al., 2007)
Bm-PB1-2341R	ATATCGTCTCGTATTAGTAGAAACAAGGCATT		
Bm-PA-10	TATTCGTCTCAGGGAGCGAAAGCAGGTAC		
PA-1498R	TNGTYCTRCAYTTGCTTATCAT	PA-fragment 1	(Li et al., 2007)
PA-747F	CATTGAGGGCAAGCTTTC		
Bm-PA-2233R	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT		
Bm-HA-1	TATTCGTCTCAGGGAGCAAAAGCAGGGG	HA-fragment	(Hoffmann et al., 2001)
Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT		
Bm-NP-1	TATTCGTCTCAGGGAGCAAAAGCAGGGTA		
Bm-NP-1565R	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT	NP-fragment	(Hoffmann et al., 2001)
Bm-NA-1	TATTCGTCTCAGGGAGCAAAAGCAGGAGT		
Bm-NA-1413R	ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT		
Bm-M-1	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	M-fragment	(Hoffmann et al., 2001)
Bm-M-1027R	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT		
Bm-NS-1	TATTCGTCTCAGGGAGCAAAAGCAGGGTG		
Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	NS-fragment	(Hoffmann et al., 2001)

Results

Analysis and comparison of inferred amino acid sequences

The HA gene of the 7 isolates of HPAI-H5N1 viruses sequenced in this study have the receptor binding pocket of HA1 retained amino acid residues Q222 and G224 (positions 226 and 228 in H3 numbering, positions 238 and 240 when numbered from the H5 ATG; this numbering is used throughout). In addition, all isolates have the multiple basic amino acids (PQRERRRRKKR/GLF, PQREKRRKKR/GLF and PLRERRRRK_R/GLF showed in Table 3) at the connecting peptide between HA1 and HA2 genes. The NA gene of these 7 strains had 20 amino acids deletion in the stalk region (positions aa49 to aa68) and Amino acid position of NA are specific H275. The NS1 gene of 7 Thai isolates showed D92 of amino acid residues and has a conserved a 5 amino acid deletion (position aa80–aa84). The PB2 gene of these 7 Thai isolates showed E627 of amino acid residues. The M2 gene of four isolates (A/ck/Nakhonsawan/NIAH600567/08, A/ck/Phichit/NIAH600674/08, A/ck/Angthong/NIAH101204/07 and A/ck/Phichit/NIAH11/06) had 2 amino acid changes (from I to L at aa26 and N to S at aa31). The PA gene of three isolates (A/ck/Nakhonphanom/NIAH113718/06, A/ck/Nongkhai/NIAH400802/07 and A/ck/Mukdahan/NIAH40390/07) had 7.1%(100-92.9) of maximum nucleotide divergence from A/bird/Thailand/3.1/2004 (nt33-1553).

Table 2 Analysis and comparison of specific amino acid sequences of different gene segments of Thai HPAI viruses

H5N1 virus	Amino acid (aa) at indicated position									
	HA		NA		M2		NS		PB2	PA
	238(226 ^a) (Q)	240(228 ^a) (G)	stalk deletion (49 to 68)	274 (H)	26 (L)	31 (S)	deletion (80 to 84)	92 (D)	627 (E)	% homology with A/bird/Thailand/3.1/2004
1. A/ck/Phichit/NIAH1/06	Q	G	Yes	H	I	N	Yes	D	E	99.1
2. A/ck/Nakhonphanom/NIAH113718/06	Q	G	Yes	H	L	S	Yes	D	E	93
3. A/ck/Nongkhai/NIAH400802/07	Q	G	Yes	H	L	S	Yes	D	E	92.7
4. A/ck/Mukdahan/NIAH40390/07	Q	G	Yes	H	L	S	Yes	D	E	93
5. A/ck/Angthong/NIAH101204/07	Q	G	Yes	H	I	N	Yes	D	E	96.3
6. A/ck/Nakhonsawan/NIAH600567/08	Q	G	Yes	H	I	N	Yes	D	E	98.4
7. A/ck/Phichit/NIAH600674/08	Q	G	Yes	H	I	N	Yes	D	E	98.4
8. A/ck/Sukhothai/NIAH114843/08	Q	G	000	0	I	N	000	0	0	000

^aH3 numbering

A/bird/Thailand/3.1/2004 Accession number AY651607 (nt33-1553)

Phylogenetic analysis

Phylogenetic analysis of Thai H5N1 isolates and study of their phylogenetic relationships with other representative H5N1 isolates in GenBank was performed based on nucleotide sequence of the HA and PA gene. According to phylogenetic results of HA segments mentioned Figure 1, the seven Thai H5N1 viruses isolates in Thailand in 2006-2008 belonged to Clad 1 and Clade 2.3.4 (Table 3). These studies have identified to ten major Clades of H5N1 HPAIV of world origin (WHO/FAO/OIE H5N1 Evolution Working Group, 2007) (Figure 1). And the phylogenetic results of PA gene mentioned Figure 2, the first Cluster (I) consisting four Thai strains (in this study), one Thai strain (reference strain) and eighteen Chinese strains, second Cluster (II) consisting of four Chinese strains including A/goose/Guangdong/1996, the third Cluster (III) consisting of three Thai strains, one Laos strain, one Vietnamese strain, one Malaysian strain and one Japanese strain, the fourth Cluster (IV) consisting of three Chinese strains.

Table 3 GenBank accession number, Clad and HA connecting peptide sequence of viruses isolated in Thailand.

Isolate	Accession number	Clade	Cleavage site
1. A/ck/Phichit/NIAH1/06	EU919137	1	PQRERRRKKR/GLF
2. A/ck/Nakhonphanom/NIAH113718/06	EF419242	2.3.4	PLRERRRK_R/GLF
3. A/ck/Nongkhai/NIAH400802/07	EF419243	2.3.4	PLRERRRK_R/GLF
4. A/ck/Mukdahan/NIAH40390/07	EU919136	2.3.4	PLRERRRK_R/GLF
5. A/ck/Angthong/NIAH101204/07	EU 919140	1	PQREKRRKKR/GLF
6. A/ck/Nakhonsawan/NIAH600567/08	EU919139	1	PQRERRRKKR/GLF
7. A/ck/Phichit/NIAH600674/08	EU919138	1	PQRERRRKKR/GLF
8. A/ck/Sukhothai/NIAH114843/08	00000000	1	PQRERRRKKR/GLF

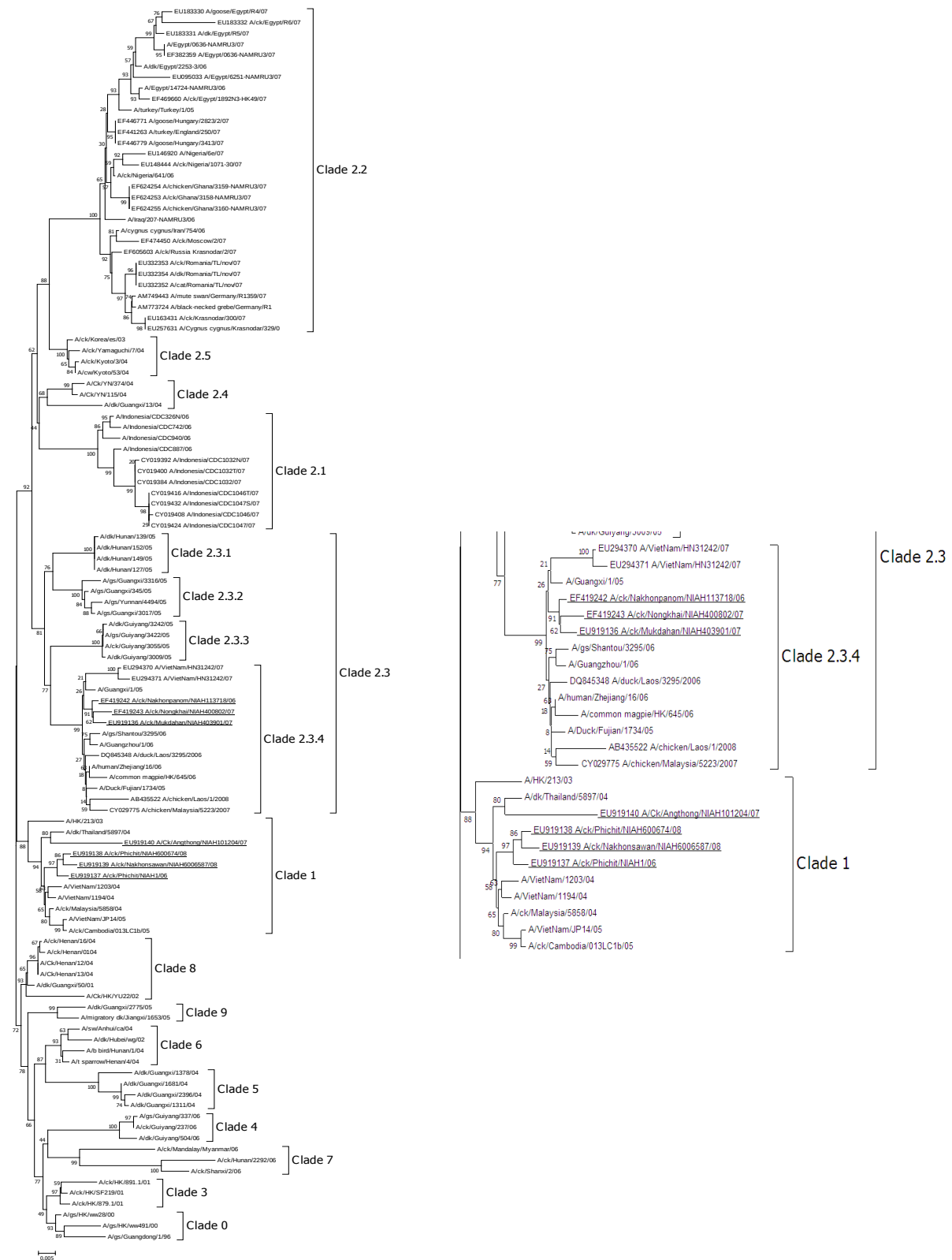


Figure 1 Phylogenetic relationships of the HA protein of representative H5N1 viruses isolated in Thailand and compared with other H5N1 sequences in GenBank. Tree was generated by using MEGA 4 program (neighbor-joining analysis method and with Tamura -Nei model). Number below branches indicates bootstrap values from 1000 replicates. Analysis was based on nucleotides 49 to 1570 (nt 1521 bp).

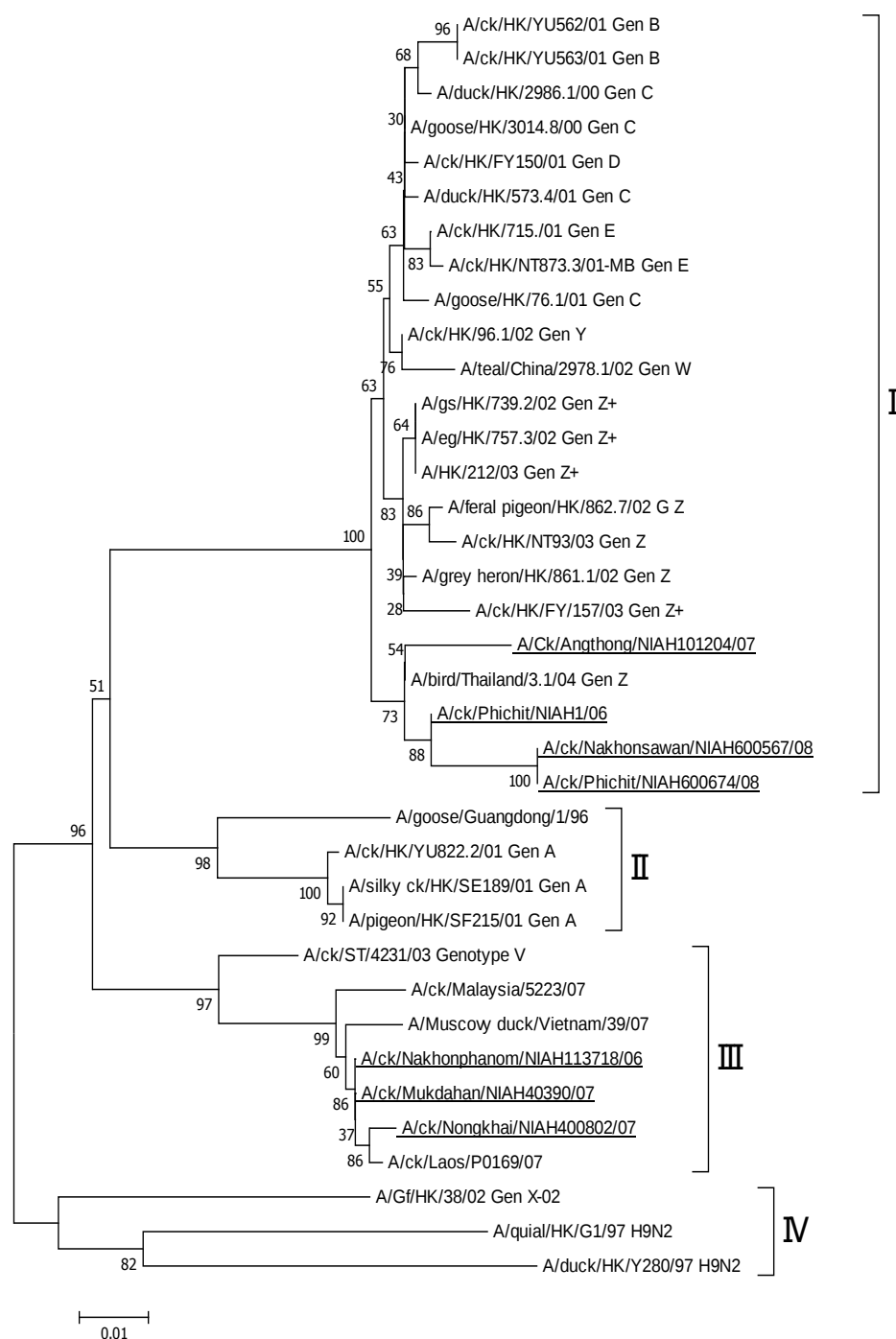


Figure 2 Phylogenetic relationships of the PA protein of representative H5N1 viruses isolated in Thailand and compared with other H5N1 sequences in GenBank. Tree was generated by using MEGA 4 program (neighbor-joining analysis method and with Tamura-Nei model). Number below branches indicates bootstrap values from 1000 replicates. Analysis was based on nucleotides 1430 to 1972 (nt 542 bp).

Discussion

The HA gene of the 7 isolates of HPAI-H5N1 viruses sequenced in this study has had receptor binding pocket of HA1 retained amino acid residues Q222 and G224 that preferentially bind to 2,3-NeuAc - Gal linkages of avian cell-surface receptors. In addition, all isolates had the multiple basic amino acids at the connecting peptide between HA1 and HA2 genes (PQRERRRKRR/GLF, PQREKRRKRR/GLF and PLRERRRK_R/GLF showed in Table 1). This showed that H5N1 viruses had continued to evolve since early 2004 with minor changes but remained unchanged in their pathogenicity. However, it is known to be one of the most important pathogenicity markers of H5N1 influenza A viruses. Because of this cleavage, the virus becomes infectious (Weis et al., 1988; Ha et al., 2001; Li et al., 2004; Smith et al., 2006a). According to phylogenetic results mentioned above, the seven viruses belonged to the 2 Clades (Table 1). The first Clade 1 viruses fell into the same Clade as viruses found in Cambodia, Malaysia, Laos and Vietnam in 2003 to 2004 (Fig1). These viruses were mainly found in the central area of Thailand such as provinces around Bueng Boraphet Lake and agriculture areas such as Phitsanulok, Phichit and Nakhonsawan provinces. And the second of Clade 2.3.4 circulated in late 2006 to early 2007 contains only in the Northeast of Thailand such as Nakhonphanom, Nongkhai, Mukdahan provinces and clustered together with Fujian-like viruses, which have been isolated in south of China since 2005 include some isolate from Vietnam, Malaysia and Laos in 2006 to 2007. The NA genes of these seven strains have a similar 20 amino acid deletion in the stalk region (positions 49aa to 68aa) and Amino acid NA 275 specified H for all HPAI isolates from 2006 to 2008, thus predicting full susceptibility to oseltamivir (Smith et al., 2006b). Analysis of amino acid residues shows that D92 in NS1 was maintained in Thailand viruses isolate, and all strains have a conserved deletion (position 80aa–84aa) in the middle of peptide (Subbarao et al., 1998). The position 627 of PB2 in all Thai H5N1 strains are glutamic acid (E627), which is the same as the avirulent strains of Hong Kong H5N1/97. However, some H5N1 viruses isolated from Human in Vietnam were shown with lysine (K627) (Mukhtar et al., 2007). Previous research demonstrated that the point mutation of K627 increased the replicate efficiency in mice, which limited the virus replication only in respiratory organs (Subbarao et al., 1993; Rimmelzwaan et al., 2006). We identified the two substitutions in the transmembrane region of the M2 protein (I26L and N31S) that seemed to be unique to

viruses isolated from 4 Thailand isolates become may resistant to amantadine and rimantadine (Crumpacker et al., 2001). The PA protein found genetic shift in 3 Thai isolates (A/ck/Nakhonphanom/NIAH113718/06, A/ck/Nongkhai/NIAH400802/07 and A/ck/Mukdahan/NIAH40390/07) by using the maximum nucleotide divergence was at 7.1% and supported the phylogenetic analysis, the these Thai H5N1 strains formed a separate group (third Cluster (III)) different from four Thai isolates (first Cluster (I)) and may have originated from the same ancestor with A/ck/ST/4231/03 strain. These results indicated that these isolates had a major change in PA protein, but remained unchanged in their pathogenicity. The PA tree also revealed that the 3 Thai isolates were significantly closer to Fujian - like viruses isolated such as Laos, Vietnam and Malaysia (Fig 2). In conclusions, the results of sequence and phylogenetic analysis data help to understand the dynamics of HPAI H5N1 subtype viruses evolution and support epidemiological observations.

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การศึกษาคุณสมบัติทางด้านโมเลกุลของเชื้อไข้หวัดนก H5N1 subtype ในประเทศไทย ระหว่างปี พ.ศ. 2549-2551

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บทคัดย่อ

การศึกษาทางชีวโมเลกุลของเชื้อไวรัสไข้หวัดนก สายพันธุ์ H5N1 จำนวน 7 สายพันธุ์ ที่ระบาดในประเทศไทย ระหว่างปี พ.ศ. 2549 ถึง 2551 ในระดับโมเลกุล โดยศึกษาการเรียงลำดับเบสของรหัสพันธุกรรม (DNA sequencing) และ การวิเคราะห์ทาง Phylogenetic ของ โปรตีน Hemagglutinin (HA) พบว่า ยีนทั้ง 7 สายพันธุ์ยังคงเป็นไวรัสที่มีแหล่งที่มา จากสัตว์ปีกยังไม่เกิดการ reassortment ของไวรัสที่มาจากคน และเชื้อที่แยกได้ อยู่ใน Clade 1 จำนวน 4 สายพันธุ์ ประกอบด้วย A/ck/Phichit/NIAH1/06 A/ck/Angthong/NIAH101204/07 A/ck/Nakhonsawan/NIAH600567/08 และ A/ck/Phichit/NIAH600674/08 และ อยู่ใน Clade 2.3.4 จำนวน 3 สายพันธุ์ ประกอบด้วย A/ck/Nakhonphanom/NIAH113718/06 A/ck/Nongkhai/NIAH400802/07 และ A/ck/Mukdahan/NIAH40390/07 และพบว่ามี 4 สายพันธุ์ A/ck/Phichit/NIAH1/06 A/ck/Angthong/NIAH101204/07 A/ck/Nakhonsawan/NIAH600567/08 และ A/ck/Phichit/NIAH600674/08 อาจจะมีดื้อต่อยา amantadine แต่ยังไม่พบการดื้อยา neuraminidase inhibitors (Oseltamivir carboxylate) และ พบว่ายีน PA ของ 3 สายพันธุ์ A/ck/Nakhonphanom/NIAH113718/06 A/ck/Nongkhai/NIAH400802/07 และ A/ck/Mukdahan/NIAH40390/07 มีการเปลี่ยนแปลง Major change ของเชื้อไข้หวัดนกทั้งสามสายพันธุ์ นอกจากนี้ พบว่ามี deletion 1 amino acid ของ ยีน Haemagglutinin (HA) ที่ตำแหน่ง polybasic cleavage site (PLRERRRK_R/GLF), มี deletion จำนวน 20 amino acid ที่ตำแหน่ง 49-68 ของยีน Neuraminidase (NA) และ มี deletion จำนวน 5 amino acid ที่ตำแหน่ง 80-84 ของยีน Nonstructure (NS1)

คำสำคัญ เชื้อไวรัสไข้หวัดนก H5N1 phylogenetic analysis sequence analysis