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Virology 344 (2006) 480 – 491

VIROLOGY

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Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand

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Received 27 April 2005; returned to author for revision 14 July 2005; accepted 22 August 2005
Available online 27 September 2005

Abstract

The H5N1 avian influenza virus outbreak among zoo tigers in mid-October 2004, with 45 animals dead, indicated that the avian influenza virus could cause lethal infection in a large mammalian species apart from humans. In this outbreak investigation, six H5N1 isolates were identified and two isolates (A/Tiger/Thailand/CU-T3/04 and A/Tiger/Thailand/CU-T7/04) were selected for whole genome analysis. Phylogenetic analysis of the 8 gene segments showed that the viruses clustered within the lineage of H5N1 avian isolates from Thailand and Vietnam. The hemagglutinin (HA) gene of the viruses displayed polybasic amino acids at the cleavage site, identical to those of the 2004 H5N1 isolates, which by definition are highly pathogenic avian influenza (HPAI). In addition, sequence analyses revealed that the viruses isolated from tigers harbored few genetic changes compared with the viruses having infected chicken, humans, tigers and a leopard isolated from the early 2004 H5N1 outbreaks. Sequence analyses also showed that the tiger H5N1 isolated in October 2004 was more closely related to the chicken H5N1 isolated in July than that from January. Interestingly, all the 6 tiger H5N1 isolates contained a lysine substitution at position 627 of the PB2 protein similar to the human, but distinct from the original avian isolates.

Keywords: Avian influenza; H5N1 influenza A virus; Tigers; General characterization

Introduction

Since January 2004, H5N1 influenza A virus has caused a nationwide avian influenza (AI) outbreak in Thailand and another eight Asian countries including Cambodia, China, Indonesia, Japan, Laos, Malaysia, South Korea and Vietnam (OIE, 2005). The second wave of AI outbreak among poultry in Thailand was reported during July–October 2004. Moreover, the AI-H5N1 was reportedly transmitted to humans with a high mortality rate (Chotpitayasunondh et al., 2005; Ungchusak et al., 2005). In Thailand, as of April 2005, 17 confirmed human cases of avian influenza A (H5N1) infection have been reported, 12 of them fatal (WHO, 2004).

Apart from humans, H5N1 influenza A infections have been reported in several mammalian species such as monkeys (Kuiken et al., 2003), mice (Gao et al., 1999; Lu et al., 1999), pigs (Hatta et al., 2001b), ferrets (Zitzow et al., 2002), domestic cats (Kuiken et al., 2004) as well as tigers and leopards (Keawcharoen et al., 2004). Generally, it was quite unusual for H5N1 influenza viruses to cause disease in feline species such as tigers and leopards. The first outbreak of H5N1 influenza virus infection in tigers and leopards was reported in a zoo in Suphanburi province, central Thailand, in early 2004. During the outbreak, two tigers and two leopards died and H5N1 influenza viruses isolated from a tiger and a leopard were characterized by entire genome analysis. Those H5N1

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doi:10.1016/j.virol.2005.08.032

Table 1
List of H5N1influenza viruses isolated from zoo tigers

Virus	Source/pre–post-Oseltamivir	GenBank accession number
A/Tiger/Thailand/CU-T3/04	Nasal swab/ pre treatment	PB2: AY907672 PB1: AY972550 PA: AY972549 HA: AY842935 NP: AY972548 NA: AY842936 M: AY972547 NS: AY907673
A/Tiger/Thailand/CU-T7/04	Nasal swab/ post-treatment	PB2: AY907671 PB1: AY972554 PA: AY972553 HA: AY866475 NP: AY972552 NA: AY866476 M: AY972551 NS: AY907674
A/Tiger/Thailand/CU-T4/04	Pooled tissues/ pre-treatment	HA: AY972539 NA: AY972543 PB2: DQ017251
A/Tiger/Thailand/CU-T5/04	Pooled tissues/ pre-treatment	HA: AY972540 NA: AY972544 PB2: DQ017252
A/Tiger/Thailand/CU-T6/04	Kidney/ pre-treatment	HA: AY972541 NA: AY972545 PB2: DQ017253
A/Tiger/Thailand/CU-T8/04	Pooled tissues/ post-treatment	HA: AY972542 NA: AY972546 PB2: DQ017254

viruses (A/tiger/Thailand/Ti-1/04 and A/leopard/Thailand/Leo-1/04) are genetically similar to the viruses isolated from poultry during the same period (Keawcharoen et al., 2004).

The second outbreak, in mid-October, was reported in zoo tigers (*Panthera tigris*) in the Sriracha district, Chonburi province, Thailand, with a high number of infected animals, coinciding with the second poultry outbreaks in Thailand. The clinical signs included high fever, respiratory distress with bloody nasal discharge and sudden death. The outbreak resulted in 45 deaths with serious economic losses due to temporary closure of the zoo. Subsequent epidemiological investigation revealed that the tigers had been fed on contaminated fresh chicken carcasses (Thanawongnuwech et al., 2005).

The outbreak among tigers demonstrated that the H5N1/04-like viruses circulating in Thailand are not only lethal to poultry and humans but also to many mammalian species including tigers. During the October outbreak, Oseltamivir was given to all tigers in the same zone for disease treatment and prevention. However, the treatment was not successful possibly due to improper dosage and time of drug administration (data not shown).

In this study, we characterized the entire genome of H5N1 influenza viruses (A/Tiger/Thailand/CU-T3/04 and A/Tiger/Thailand/CU-T7/04) isolated from zoo tigers that had perished during the mid-October 2004 H5N1 influenza outbreak. These viruses had been isolated from animals both pre- and post-treated with Oseltamivir (75 mg/60 kg, twice per day) for 6 days. The viral genomes were analyzed and compared to the H5N1 influenza viruses isolated from tigers, leopards, humans and poultry during previous H5N1 outbreaks in Thailand as well as Indonesia, Vietnam and China to determine the genetic relatedness and possible genetic changes among the viruses.

Results

During the mid-October H5N1 avian influenza outbreak among zoo tigers, six H5N1influenza viruses were isolated from 4 dead tigers and from 2 nasal swabs taken from sick animals that subsequently perished (Table 1). All six isolates were identified as H5N1 subtypes by multiplex RT-PCR yielding accurate amplicons of 276, 189 and 131 bp size representing the M, H5 and N1 genes, respectively (Fig. 1). To study the relationship and genetic characteristics of the viruses isolated from tigers, two isolates, "A/Tiger/Thailand/CU-T3/04" (CU-T3) and "A/Tiger/Thailand/CU-T7/04" (CU-T7), were selected for sequence analysis of the entire genome. CU-T7 was isolated from a tiger treated with Oseltamivir at 75 mg/60 kg twice daily for 4 days, prior to specimen collection. In addition, the HA, NA genes and PB2 of all 6 tiger isolates were also sequenced and included in the phylogenetic and genetic analyses.

Phylogenetic analysis

Phylogenetic analysis of the hemagglutinin gene (HA) and neuraminidase gene (NA) showed that all the six tiger isolates were grouped into the lineage represented by the Thailand and Vietnam, 2004 isolates (Fig. 2). In contrast, the viruses isolated in Indonesia during 2003–2004 formed a separate lineage. The viruses isolated from poultry and swine in China during 2003–2004 grouped into a cluster that was closely related to the viruses collected in Hong Kong during 1996–2001. Topologically, the lineage of viruses from Thailand was continuously evolving. Based on chronological information available on H5N1 isolates, the recent tiger isolates from Thailand were closely related to each other and clustered with the chicken, tiger and leopard isolates previously characterized during the first (January) and second (July) H5N1 outbreaks in Thailand. This finding confirmed that the H5N1 viruses circulating among poultry and tigers in Thailand were closely related. Phylogenetic analyses of the other six gene segments of the tiger isolates collected in October 2004 including PA, PB1, PB2, NP, NS, and M, also showed high degrees of genetic

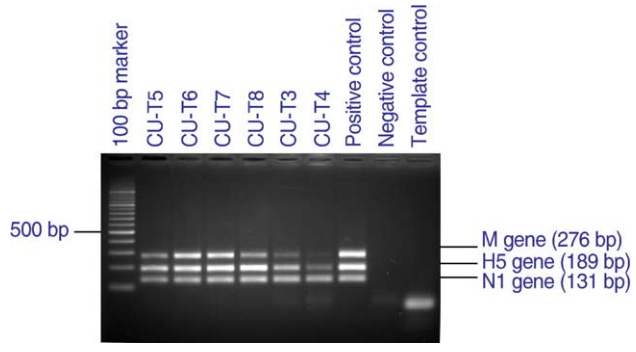

Fig. 1. Multiplex PCR for the Identification of Avian influenza (H5N1) isolated from tigers.

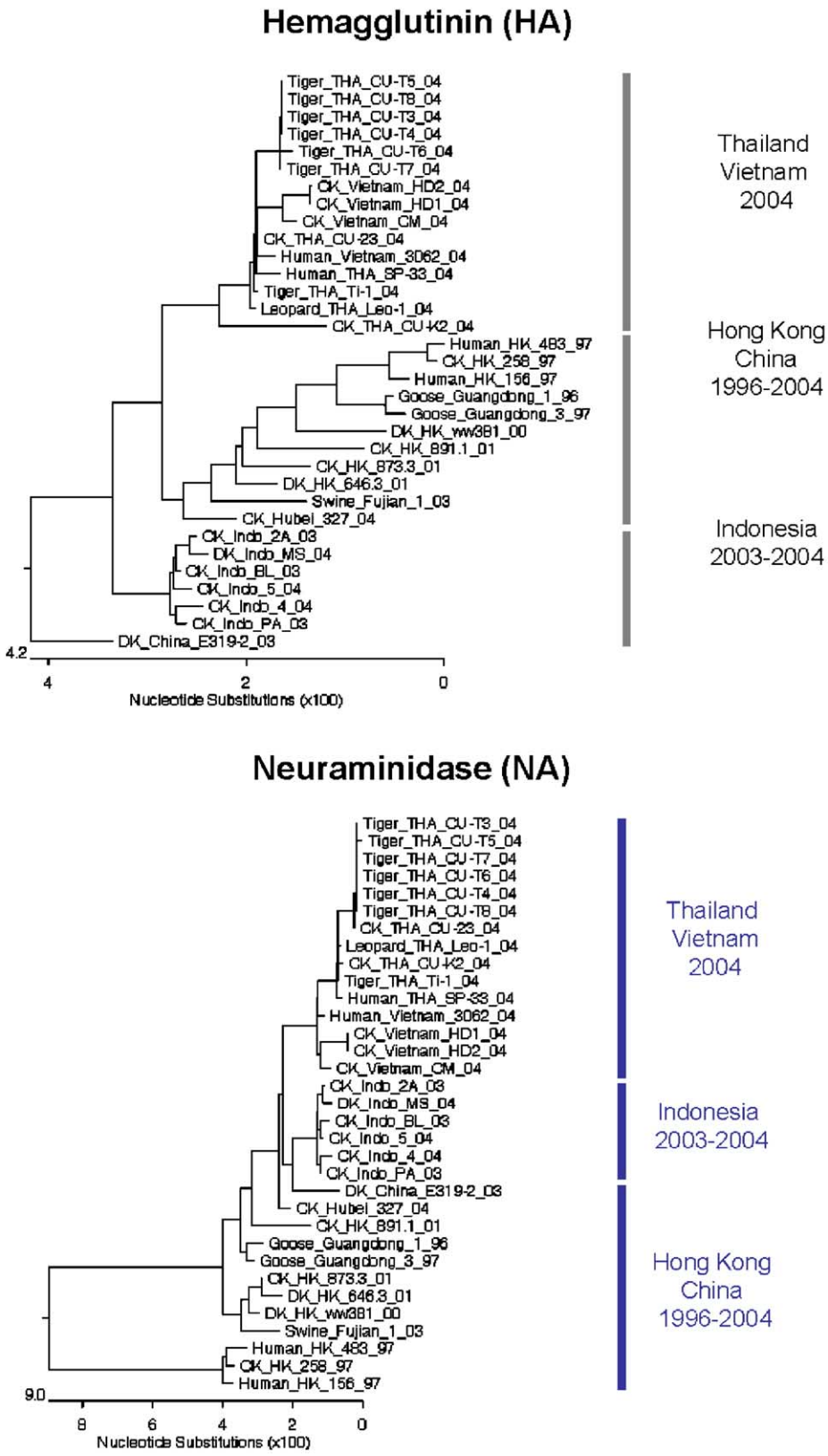

Fig. 2. Phylogenetic analysis of HA and NA gene sequences of H5N1 influenza viruses from 1996–2004.

relatedness to the chicken and tiger isolates collected earlier in the year (data not shown). In general, phylogenetic analysis of all eight gene segments indicated that the tiger viruses were of avian origin and exhibited the highest sequence similarity to those of chicken H5N1 isolated in July (A/Chicken/Thailand/CU-23/04) (Table 2).

Table 2
Comparison of the gene segments of A/Tiger/THA/CU-T3/04 to those of H5N1 isolates from Thailand

Gene	Region of comparison (bp)[a]	% Nucleotide identity					
		CU-T7	CU-23	CU-K2[b]	SP-33[c]	Leo-1[d]	Ti-1[d]
		October	July	January	January	January	January
		Tiger	Chicken	Chicken	Human	Leopard	Tiger
PB2	82–2220	99.9	99.7	99.3	99.3	99.3	99.3
PB1	63–2226	100	99.9	99.6	99.8	99.8	99.8
PA	26–2150	100	99.6	99.4	99.6	99.5	99.5
HA	45–1635	100	99.7	98.2	99.5	99.6	99.7
NP	58–1479	100	99.9	99.8	99.8	99.7	99.6
NA	25–1299	100	99.9	99.3	99.3	99.4	99.5
M	1–952	100	99.9	99.6	99.7	99.4	99.5
NS	48–826	100	99.4	99.0	99.2	99.2	99.4

[a] Position of nucleotides is based on A/Tiger/THA/CU-T3/04.
[b] CU-K2 (Viseshakul et al., 2004).
[c] SP-33 (Li et al., 2004).
[d] Leo-1/04 and Ti-1/04 (Keawcharoen et al., 2004).

Genetic analyses

Pair-wise sequence comparisons among the eight genes of the tiger isolates are presented in Table 2. Two tiger isolates were compared with five representative H5N1 viruses obtained in early 2004 that had been completely sequenced (Table 2). All genes of the 2 tiger isolates displayed the highest percentage of nucleotide identity. The percent homology between the eight gene segments of the two tiger isolates (CU-T3 and CU-T7) ranged between 99.9 and 100%. In addition, all gene segments of the CU-T3 isolate were closely related (>99% homology) to those of the isolates collected from humans, chicken, tigers and leopards. Interestingly, the genome of the tiger viruses, in particular the HA gene, was more closely related to the chicken H5N1 (A/Chicken/Thailand/CU-23/04) collected in July 2004 (second H5N1 outbreak), than that isolated in January 2004 (Table 2).

The results obtained from analyzing the eight gene segments of H5N1 isolates are presented in Table 2. The hemagglutinin (HA) genes of all the six tiger isolates were almost identical to each other and displayed profound nucleotide sequence identity (99.7%) with A/Chiken/Thailand/CU-23/04 and A/Tiger/Thailand/Ti-1/04. Only the HA gene of CU-T3 had the lowest homology (98.25%) with that of the chicken isolate (A/Chicken/Thailand/CU-K2/04). Based on the deduced amino acid sequence, the HA1–HA2 connecting peptides of the tiger isolates harbor multiple basic amino acids (SPQRERRRKKR), similar to the majority of H5N1 isolates circulating during 1996–2004 (Fig. 3). All 7 tiger isolates, including those from 1996–2004, had a glutamine at position 222 and a glycine at position 224, which are related to receptor binding sites specific for avian species (Fig. 3 and Table 3). At least seven glycosylation sites were found in the HA1 gene of tiger H5N1 viruses. Interestingly, a glycosylation site at positions 154–156 of the HA1 gene was found only in H5N1 viruses isolated from Thailand and Vietnam yet not in those from Hong Kong, Indonesia (A/Duck/Indonesia/MS/04) and China.

Sequence analyses of the NA gene of the H5N1 tiger viruses revealed that all six tiger isolates contained a 20 amino acid deletion in the NA stalk (position 49–68) that had previously been identified in the NA of other 2003–2004 isolates (Fig. 4) (Li et al., 2004; Viseshakul et al., 2004). On the other hand, a 19 amino acid deletion in a similar region of the NA protein (position 54–72) was observed in a 1997 human isolate (HK-156), whereas no amino acid deletion was detected in either the 1996–1997 goose isolates (Guangdong-1 and Guangdong-3) or the 2003 swine isolates (Fujian-1). The deletion in the stalk region had been reported as an adaptation of H5N1 viruses from wild aquatic birds to domestic chickens (Matrosovich et al., 1999). It should be noted that 3 amino acids at positions 74 (L; Leucine), 79 (T; Threonine) and 332 (A; Alanine) of six tiger isolates from the October outbreak were different from those of the tiger (Ti-1) and leopard (Leo-1) isolates previously analyzed during the January outbreak (Fig. 4).

Alignment of the M2 protein of H5N1 viruses is shown in Fig. 5. The matrix protein (M2) was identical in the tiger isolates from both the October and the January outbreaks. The tiger H5N1 viruses (CU-T3 and CU-T7) contained a mutation at position 31 (N; Asparagine) indicating amantadine resistance properties (Scholtissek et al., 1998). In addition, we found some polymorphisms at amino acid residues 26 (I; Isoleucine), 64 (A; Alanine) and 66 (A; Alanine) which had been observed in most of the 2003–2004 H5N1 isolates but not in the 1996–1997 H5N1 isolates (Fig. 5 and Table 3). The M2 gene of the 2004 H5N1 isolates, including CU-T3 and CU-T7, harbors one amino acid at position 28 (V; Valine) similar to human H5N1 and two amino acids at positions 16 (E; Glutamic acid) and 55 (L; Leucine) reminiscent of avian H5N1.

Genetic analysis of the PB2 protein is shown in Fig. 6. Two amino acids at positions 661(A; Alanine) and 702 (K; Lysine) similar to avian H5N1 were identified in PB2 of two tiger isolates from this outbreak, as had been found in tiger, leopard, chicken and human isolates from 2004. Interestingly, the nucleotide substitution at position 627 of PB2 to lysine (K) was observed in the tiger (CU-T3 and CU-T7), human (SP-33) and leopard (Leo-1), but not in the tiger (Ti-1) and chicken (CU-K2 and CU-23) isolates. Partial sequences of PB2 in four tiger isolates (CU-T3, CU-T5, CU-T6, CU-T8) were also analyzed.

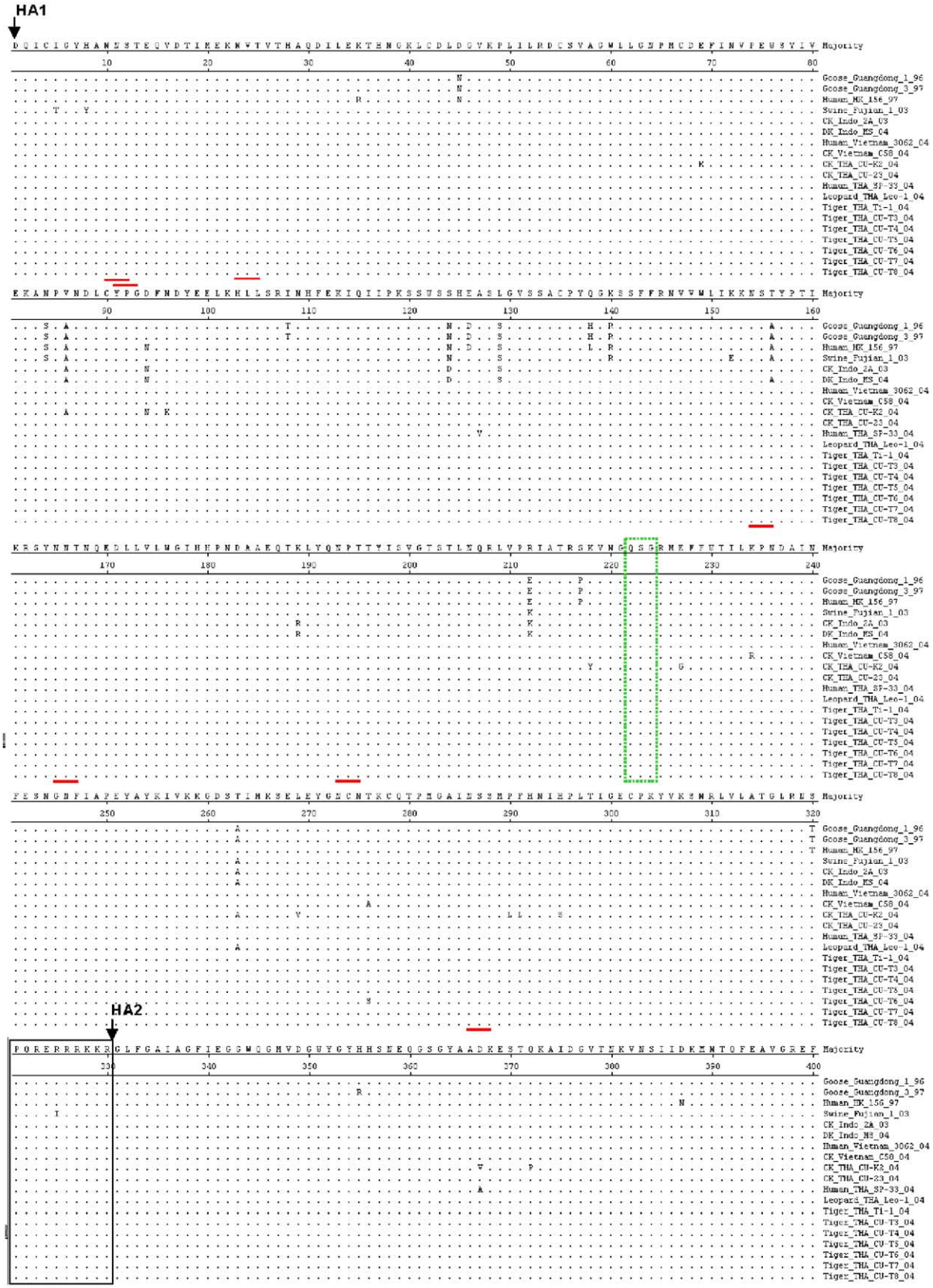

Fig. 3. Alignment of HA1 amino acid sequences of tiger isolates with those of H5N1 isolates from 1996–2004. The hemagglutinin cleavage site contained multiple base amino acid (SPQRERRRKKR) and is indicated by an open box. The receptor binding site at residues 222 (Q; Glutamine) and 224 (G; Glycine) are located by dotted-box. The potential glycosylation sites are indicated by underline.

Table 3
Analysis of the amino acid sequences of the proteins of A/Tiger/Thailand/CU-T3/04 and A/Tiger/Thailand/CU-T7/04

Virus	Gene			
	HA			
	Connecting peptide sequences	Receptor binding site		N-link glycosylation site
		222	224	154–156
Goose/Guangdong/1/96	TPQRERRRKKR	Q	G	– (NSA)
Goose/Guangdong/3/97	TPQRERRRKKR	Q	G	– (NSA)
Human/HK/156/97	TPQRERRRKKR	Q	G	– (NSA)
Swine/Fujian/1/03	SPQREIRRKKR	Q	G	– (NSA)
Chicken/Indonesia/2A/03	SPQRERRRKKR	Q	G	NST
Duck/Indonesia/MS/04	SPQRERRRKKR	Q	G	– (NSA)
Human/Vietnam/3062/04	SPQRERRRKKR	Q	G	NST
Chicken/Vietnam/C58/04	SPQRERRRKKR	Q	G	NST
Chicken/THA/CU-K2/04	SPQRERRRKKR	Q	G	NST
Chicken/THA/CU-23/04	SPQRERRRKKR	Q	G	NST
Human/THA/SP-33/04	SPQRERRRKKR	Q	G	NST
Leopard/THA/Leo-1/04	SPQRERRRKKR	Q	G	NST
Tiger/THA/Ti-1/04	SPQRERRRKKR	Q	G	NST
Tiger/THA/CU-T3/04	SPQRERRRKKR	Q	G	NST
Tiger/THA/CU-T7/04	SPQRERRRKKR	Q	G	NST

Virus	Gene			
	NA			
	NA stalk region	74	79	332
Goose/Guangdong/1/96	No deletion	F	A	T
Goose/Guangdong/3/97	No deletion	F	A	T
Human/HK/156/97	19 aa deletion (54–72)	F	A	T
Swine/Fujian/1/03	No deletion	F	T	T
Chicken/Indonesia/2A/03	20 aa deletion (49–68)	P	A	T
Duck/Indonesia/MS/04	20 aa deletion (49–68)	P	A	T
Human/Vietnam/3062/04	20 aa deletion (49–68)	F	A	T
Chicken/Vietnam/C58/04	20 aa deletion (49–68)	F	A	T
Chicken/THA/CU-K2/04	20 aa deletion (49–68)	F	A	T
Chicken/THA/CU-23/04	20 aa deletion (49–68)	L	A	A
Human/THA/SP-33/04	20 aa deletion (49–68)	F	A	T
Leopard/THA/Leo-1/04	20 aa deletion (49–68)	F	A	T
Tiger/THA/Ti-1/04	20 aa deletion (49–68)	F	A	T
Tiger/THA/CU-T3/04	20 aa deletion (49–68)	L	T	A
Tiger/THA/CU-T7/04	20 aa deletion (49–68)	L	T	A

Virus	M				NS		PB2			PA	NP
	26	31	64	66	Amino acid deletion (79–83)	92	627	661	702	409	136
Goose/Guangdong/1/96	L	S	S	E	No deletion	D	E	A	K	S	M
Goose/Guangdong/3/97	L	S	S	E	No deletion	D	E	A	K	S	L
Human/HK/156/97	L	S	S	E	No deletion	E	E	T	K	N	M
Swine/Fujian/1/03	L	S	S	E	5 aa deletion	D	E	A	K	S	L
Chicken/Indonesia/2A/03	L	N	S	A	5 aa deletion	D	E	A	K	S	L
Duck/Indonesia/MS/04	L	S	S	A	5 aa deletion	D	E	A	K	S	L
Human/Vietnam/3062/04	N	N	A	A	5 aa deletion	D	E	A	K	S	L
Chicken/Vietnam/CM/04	N	N	A	A	5 aa deletion	D	E	A	K	S	L
Chicken/THA/CU-K2/04	N	N	A	A	5 aa deletion	D	E	A	K	S	L
Chicken/THA/CU-23/04	N	N	A	A	5 aa deletion	N	E	A	K	S	L
Human/THA/SP-33/04	N	N	A	A	5 aa deletion	D	K	A	K	S	L
Leopard/THA/Leo-1/04	N	N	A	A	5 aa deletion	D	K	A	K	S	L
Tiger/THA/Ti-1/04	N	N	A	A	5 aa deletion	D	E	A	K	S	L
Tiger/THA/CU-T3/04	N	N	A	A	5 aa deletion	D	K	A	K	S	L
Tiger/THA/CU-T7/04	N	N	A	A	5 aa deletion	D	K	A	K	S	L

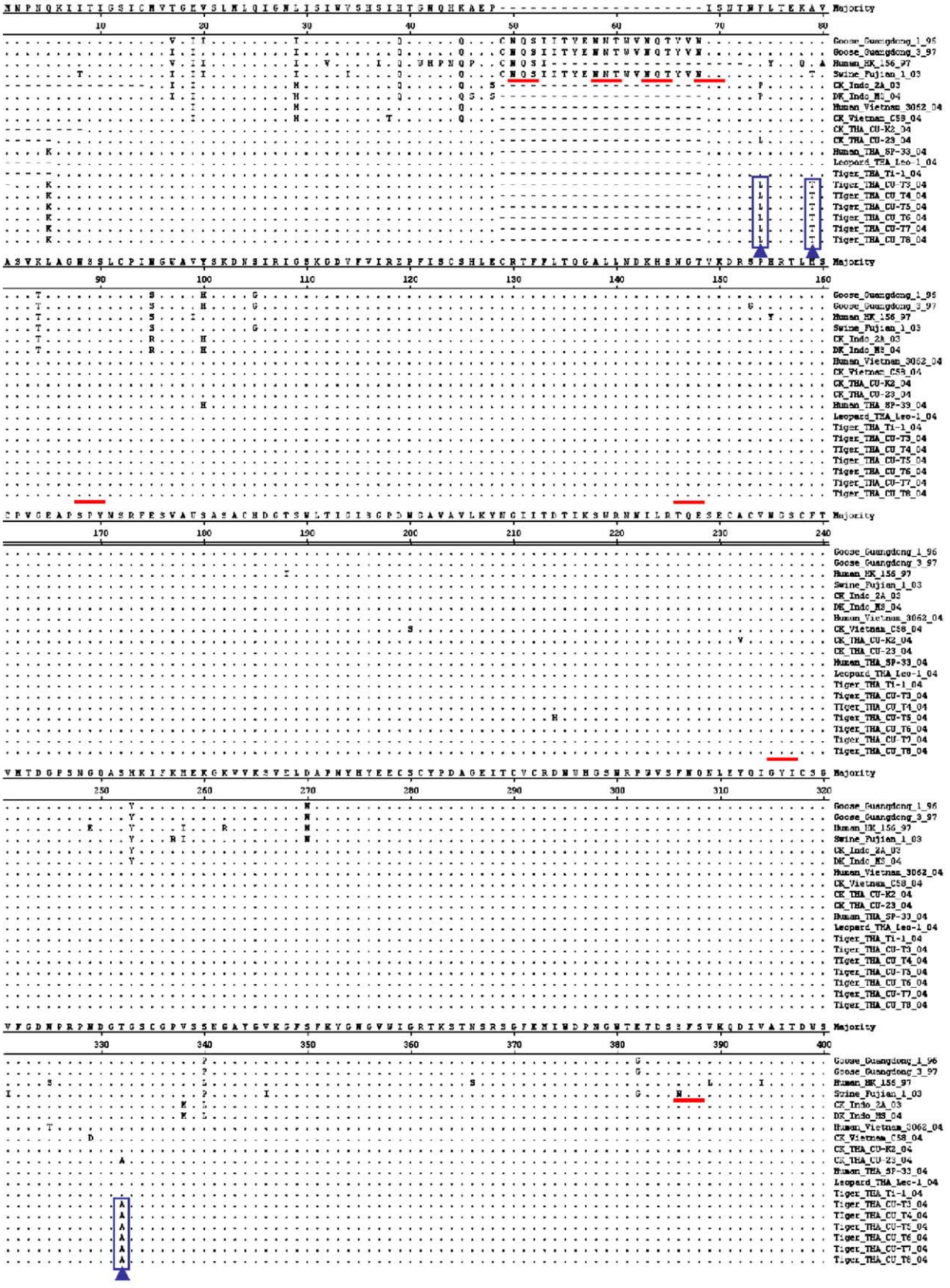

Fig. 4. Alignment of NA amino acid sequences of H5N1 influenza viruses from 1996–2004. Dashes represent amino acid deletions. Underlines represent potential glycosylation sites (N-T/S). Amino acid residues in the opened boxes represent amino acid differences resulting from comparison of tiger isolates with previously characterized tiger and leopard isolates.

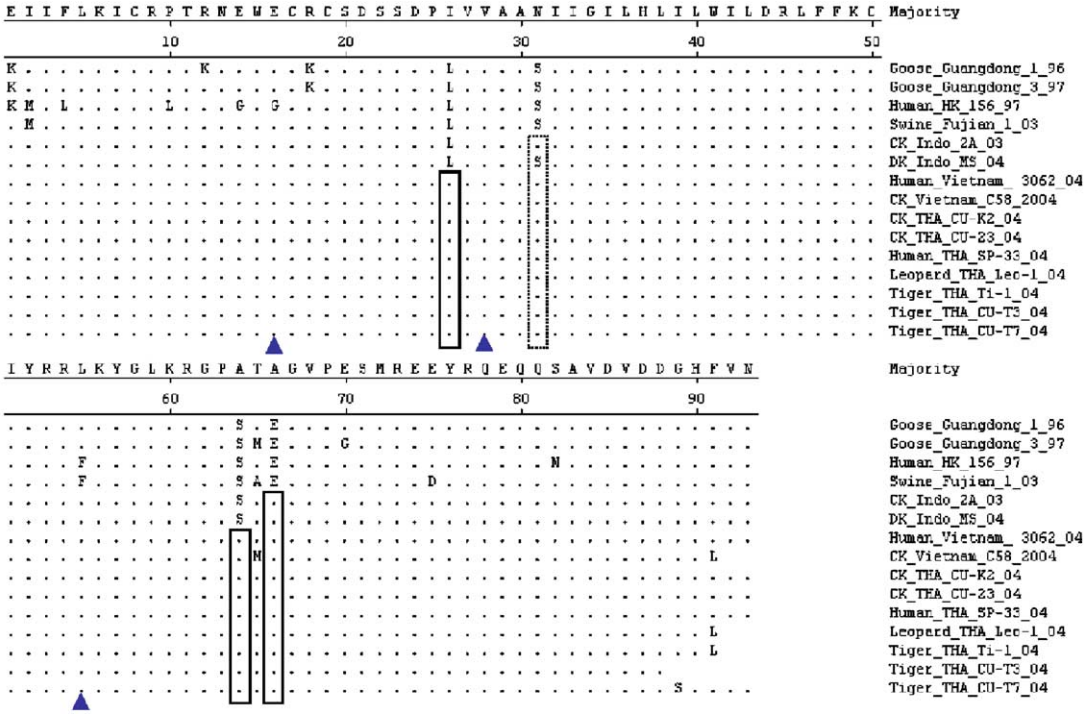

Fig. 5. Amino acid alignment of matrix protein of H5N1 influenza viruses from 1996–2004. Dots represent residues that match the consensus. Amino acid residues 31 (N; Asparagine) in dashed-open box indicate amantadines resistance site. Mutation of amino acids residues 26 (I; Isoleucine), 64 (A; Alanine) and 66 (A; Alanine) is present in open boxes. Arrows represent amino acids that have avian-like characteristic 16 (E; Glutamic acid), 55 (L; Leucine) and human-like characteristic 28 (V; Valine).

Lysine (K) at position 627 was observed in all tiger isolates from this outbreak. To confirm this finding, 189 H5N1 isolates from 1996–2004 were analyzed for the presence of lysine (K) at position 627 (Table 4). We found that only the H5N1 isolates found in tigers, leopard and humans, but not poultry, exhibited this K627 characteristic. The nucleotide substitution of glutamic acid (E) to lysine (K) at residue 627 of the PB2 protein has repeatedly been crucial for a high virulence of avian influenza virus in mice and other mammalian species (Hatta et al., 2001a). Based on the analysis of the non-structural protein gene (NS), the tiger isolates contained five-amino acid deletions at positions 79–83 of the NS1 protein, as has been previously reported in all H5N1 isolates from the 2003–2004 outbreaks (Table 3). It should be noted that most isolates from 2003–2004 contained aspartic acid (D) at amino acid position 92. Some studies reported a mutation of aspartic acid (D) to glutamic acid (E) at position 92 of the NS1 protein to be related to virulence of H5N1 in mammalian species (Seo et al., 2004). The specific amino acids in the NP, PB1, PB2 and PA proteins associated with host specificity have not been found in any tiger isolates in this study (Table 3).

Discussion

In this study, we identified H5N1 influenza in six samples using the previously established multiplex PCR method (Payungporn et al., 2004). The samples were collected from tigers that had been previously treated or post-treated with Oseltamivir. The characterized H5N1 influenza viruses displayed 99.9–100% nucleotide identity among all gene segments. During the outbreak, it was unlikely that Oseltamivir therapy could suppress the H5N1 virus infection. The result of Oseltamivir treatment was not promising possibly due to improper dosage and timing of the drug administration. Moreover, in all likelihood, the tigers were infected by horizontal transmission (Thanawongnuwech et al., 2005).

Phylogenetic analysis of all eight gene segments revealed that the H5N1 tiger viruses belonged to genotype Z, the same genotype as chicken and human isolates responsible for the regional outbreak in 2004 (Li et al., 2004). The hemagglutinin gene (HA) of most H5N1 isolates, including the tiger isolates in this study harbors multiple insertions of basic amino acids at the cleavage site, which by definition is characteristic for highly pathogenic avian influenza (HPAI) (Claas et al., 1998). During our ongoing investigation on H5N1 occurrence in Thailand, we also found two duck isolates with one basic amino acid deletion (KRKKR). The viruses were isolated from dead animals but were less pathogenic in chicken embryonated egg and cell cultures (data not shown). In addition, one basic amino acid deletion (KRKKR) had also been reported in H5N1 viruses from South Korea and one duck isolate from China (A/Duck/China/E319-2/03) (Lee et al., 2005). In this study, the tiger isolates shared the same amino acids (Q222 and G224) at positions 222–224 as chicken, swine, duck and human isolates (Table 3). The presence of Q222 and G224 at the receptor binding site is related to the preferential binding of α-2,3 linkage, typical for the avian but not the human virus (Connor et al., 1994). This finding demonstrated that the tiger isolates

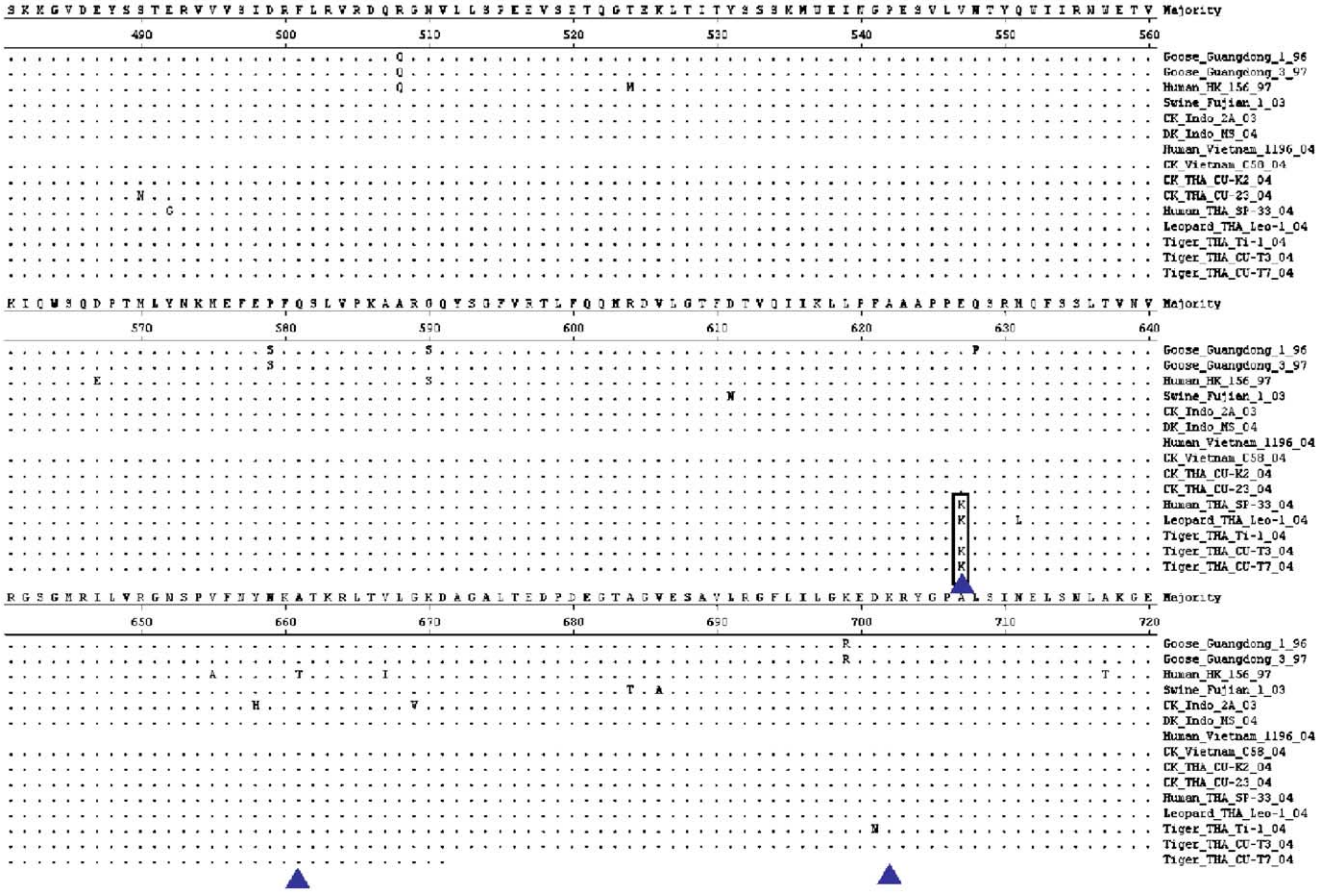

Fig. 6. Amino acid alignment of PB2 protein of H5N1 influenza viruses from 1996–2004. Dots represent residues that match the consensus. Arrows represent amino acids that have avian-like characteristic 627 (E, Glutamic acid), 661 (A; Alanine) and 702 (K; Lysine). Residue 627 with human-like amino acid (K, Lysine) is indicated by an open box.

with avian specific receptor binding properties can replicate and cause fatal infection in tigers, similar to what has been reported on human isolates (Matrosovich et al., 1999). In addition, the tiger H5N1 viruses proved to replicate very well in multiple organs of the animals, as virus could be isolated from various organs of the infected tigers (Data not shown). Our finding demonstrated that the H5N1 influenza virus could cross directly from poultry to tigers without requirement for an intermediary host/vector as had been previously reported in the case of humans (Osterhaus et al., 2002). The tiger viruses contain a glycosylation site at positions 154–156 of the HA gene, which presumably could impede the receptor binding site and therefore modify the receptor-binding properties. Many studies reported that glycosylation sites adjacent to receptor binding sites are not only related to the binding of carbohydrates on the HA globular head, and therefore, preventing the

Table 4
Analysis of amino acid at position 627 of PB2 from 189 H5N1 isolates

		Goose	Duck	Chicken	Human	Bird[a]	Swine	Leopard	Tiger	
China	1996–2004	2	31	10		1	2			46
Hong Kong	1997–2004	10	9	47	18	12				96
Thailand	2004	1	1	5	3(2)[b]	2		1(1)[c]	7(6)[d]	20
Vietnam	2004		1	8	4(3)[e]	1				14
Indonesia	2003–2004		1	5						6
Japan	2004			2		3				5
Korea	2003		1	1						2
Total		13	44	78	25	19	2	1	7	189

The number of isolates that contain lysine (K) at amino acid residue 627 is represented in ( ).

[a] Bird: Quail, Crow, Pheasant, Falcon.

[b] A/Thailand/5(KK-494)/2004 (AY627892), A/Thailand/2(SP-33)/2004 (AY627898).

[c] A/Leopard/Suphanburi/Thailand/Leo-1/04 (AY646182).

[d] A/Tiger/Chonburi/Thailand/CU-T3/04 (AY907672), A/Tiger/Chonburi/Thailand/CU-T7/04 (AY907671), CU-T4 (DQ017251), CU-T5 (DQ017252), CU-T6 (DQ017253), CU-T8 (DQ017254).

[e] A/Vietnam/1194/2004 (AY651718), A/Vietnam/3062/2004 (AY651721), A/Vietnam/1203/2004 (AY651719).

virus from binding to a non-specific target, but also compensate for the decreased enzymatic activities of NA (Matrosovich et al., 1999; Shortridge et al., 1998). Thus, the carbohydrate at the HA site of the tiger viruses might help improve the viral infectivity within mammalian hosts.

Phylogenetic analysis of the NA gene showed that the NA gene of the tiger isolates also belonged to the Thailand and Vietnam lineage (Fig. 2). Grouping of the NA phylogenetic tree was primarily based on the differences in the stalk region length, including a 20 amino acid deletion (2003–2004), a 19 amino acid deletion (1997–2001) and no deletion (1996–1997) in the stalk region (Viseshakul et al., 2004). The NA gene is often variable in both amino acid sequences and length. In this study, a 20-amino acid deletion at the NA stalk region was observed in all tiger isolates and all H5N1 isolates from Thailand, Vietnam and Indonesia. The shortening of the NA stalk was suggested as an adaptation of H5N1 viruses after circulating from wild aquatic birds to domestic poultry such as chickens and ducks (Matrosovich et al., 1999). The virus has somehow corrected the enzymatic activity of NA by deletion of its NA stalk region (Matrosovich et al., 1999). Oseltamivir, the neuraminidase inhibitor, has been proven effective for both prevention and treatment of acute influenza, but was not effective in this tiger H5N1 outbreak. However, the amino acid substitutions at the conserved residues in the NA active site related to Oseltamivir resistance were not observed in any H5N1 tiger isolates (Gubareva et al., 2000). It has been documented that resistance to Oseltamivir relates to changes of amino acid residues 119 (E to V), 293 (R to K) and 295 (N to S) (Kiso et al., 2004) and histidine (H) to tyrosine (Y) at position 274 in the N1 numbering system (McKimm-Breschkin et al., 2003). However, in this outbreak, there was no mutation of histidine to tyrosine at position 274 of the neuraminidase molecule after Oseltamivir treatment (CU-T7).

It has been known that amantadine binds to the ion channel region of the M2 protein and thus prevents the release of viral RNA into cells (Pinto et al., 1992). The changes of amino acids at positions 27 (Val), 30 (Ala) and 31 (Ser) can also lead to amantadine resistance (Scholtissek et al., 1998; Suzuki et al., 2003). All H5N1 viruses isolated in 2003–2004, except for the duck isolate from Indonesia (Indonesia-MS) displayed asparagine (N) at residue 31 of the M2 protein and therefore, the viruses were presumably resistant to amantadines.

A reverse genetics study reported that a single amino acid substitution at position 627 of the PB2 protein from Glu (E) to Lys (K) is responsible for the virulence of H5N1 in mammalian species (Hatta et al., 2001b; Shinya et al., 2004). The amino acid change at position 627 from glutamic acid (E) to lysine (K) affects the efficiency of virus replication (Shinya et al., 2004). Interestingly, the PB2 protein of six tiger isolates (CU-T3–CU-T8) in this outbreak as well as the leopard (Leo-1) and human (KK-494 and SP-33) isolates from the previous outbreak harbors lysine (K) at amino acid position 627 (Table 4). Conversely, most poultry isolates from previous outbreaks in Thailand and other countries display glutamic acid (E) at this position. Therefore, a change from glutamic acid (E) to lysine (K) at position 627 of PB2 could serve as a marker of the virulence phenotype of H5N1 viruses in tigers. The temperature at the site of infection in mammalian species (primarily, the respiratory tract) may be one of the factors influencing the infectivity of the H5N1 viruses. A previous study reported that residue 627 of PB2 is related to temperature sensitivity of the virus, which can affect efficacy of viral replication. The avian-like virus (E627) exhibited cold sensitivity (33 °C), whereas the human-like virus (K627) displayed higher polymerase complex activity during viral replication at a low temperature (Massin et al., 2001). Thus, more efficient virus replication due to Lysine (K) at position 627 of PB2 may explain the high virulence of the H5N1 viruses causing the high mortality rate among tigers in this outbreak.

An association between glutamic acid (E) at position 92 of the NS1 protein and the resistance of H5N1 virus to the antiviral effects of interferons and TNF-α has been previously reported (Seo et al., 2004). The mutation of aspartic acid (D) to glutamic acid (E) at position 92, which was required for the viruses' high virulence in mammalian species especially swine, was not observed in this study (Seo et al., 2004). Our investigation showed that all of the tiger isolates as well as poultry and human isolates from 1996–2004 harbor aspartic acid (D) at this position. This finding implicates the possibility that other factors or amino acid changes may be involved in the viruses' virulence in tigers.

Amino acids specific for the polymerase (PA, PB1, PB2), nucleoprotein (NP), nucleocapsid (NS) and matrix (M) genes associated with host range (Zhou et al., 1999) were not implicated in this study. Our analysis has shown that H5N1 influenza viruses isolated from tigers contained most of the amino acids specific for the avian virus. The PB2 of H5N1 tiger viruses contains two avian virus-like amino acids (alanine, A; 661 and lysine, K;702) (Fig. 6). These specific amino acids are located in the functional domain of the PB2 gene which is hypothetically responsible for interaction with other polymerase components (Perales et al., 1996). The NP protein of the H5N1 tiger viruses contains one avian virus-like amino acid (leucine, L; 136) which is located at the ion channel domain (Albo et al., 1995). Conversely, the tiger isolates contain two human virus-like amino acids at position 28 (valine; V) of M2, which is related to the ion channel domain (Holsinger et al., 1995; Zhou et al., 1999) and at position 627 (lysine; K) of PB2, which is related to viral replication (Shinya et al., 2004).

In this study, the outbreak of H5N1 in zoo tigers inflicting high mortality indicated that the H5N1 virus of avian origin could cause fatal infection in large mammalian species, apart from humans. One remaining question is why feline species are more susceptible to the H5N1 influenza A virus than other domestic animals. As the virus isolated from tigers displayed very few genetic changes compared to the virus found in poultry, this would suggest that the viruses have not yet fully adapted to infecting domestic mammals. One possible explanation might be that anatomical structure and eating habits could facilitate direct contact between the viruses and both nasal and oral cavities. Another possible explanation might be that the uncharacterized host receptor specificity of the feline species might play a major role in the virulence of H5N1

influenza. Further studies are required to elucidate the as yet unidentified host receptor of the feline species.

In summary, this study explored the genetic relatedness of the AI-H5N1 isolated from tigers in comparison with other H5N1 isolates from several species during the recent AI outbreak in Thailand. The sequence analyses revealed that the tiger H5N1 isolated in October was more closely related to the chicken H5N1 isolated in July than that from January. Interestingly, continuous genetic drift of the AI genes, in particular the HA gene, was observed chronologically. Moreover, the tiger H5N1 isolates contained a lysine substitution at position 627 of the PB2 protein similar to that of the human, yet distinct from the original avian isolates. This work highlights the significance of molecular epidemiology in studying the dynamics and evolution of the circulating AI viruses.

Materials and methods

Virus isolation

The H5N1 influenza viruses were obtained either from nasal swabs of sick animals or from tissues of dead tigers in the Sriracha district, Chonburi province, Thailand (Table 1). The viruses were isolated by embryonated chicken egg inoculation (OIE, 2000). The allantoic fluids containing virus were stored at −70 °C until further investigation.

Virus identification

Viral RNA was extracted from virus-containing allantoic fluid using the RNAeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. A random primer (Promega, Madison, WI, USA) was used for reverse transcription. Virus identification was performed by multiplex RT-PCR developed to identify the M, HA and NA genes applying specific primers for each gene (Payungporn et al., 2004). In brief, the 25 μl multiplex PCR amplification reaction mixture comprised 1× Master mix (Access Quick Master Mix; Eppendrof, Westbury, NY), 0.5 μM of each primer, 5 μl of cDNA from the previous reverse transcription reaction and RNase-free water at a final volume of 25 μl. The amplification reaction comprised an initial denaturation step at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, concluded by a final extension step at 72 °C for 7 min. A 7 μl volume of the PCR products was mixed with 2 μl of loading buffer (2% Orange G in 50% glycerol). The mixture was subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml) upon conclusion of electrophoresis. The gel was photographed under UV light with a gel documentation system (Vilber Lourmat, Lavalle Cedex, France). The expected sizes of the multiplex PCR products for the M, H5 and N1 genes were 276, 189 and 131 bp, respectively.

Genome sequencing and analysis

Viral genome sequencing and analysis were performed as previously described (Viseshakul et al., 2004). In brief, viral RNA was extracted and purified from infected allantoic fluid using the RNAeasy mini kit (Qiagen). Reverse transcription was performed using a universal primer to generate cDNA. PCR was performed employing primers specific for each gene (primer sequences are available upon request). The PCR products were purified using the Perfectprep Gel Cleanup Kit (Eppendrof). For the sequencing reaction, the Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction was used according to the manufacturer's protocol (ABI, Foster City, CA). The samples were analyzed by the ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT). The sequences were edited and assembled using Bioedit 5.0.9 (Ibis Therapeutics, Carlsbad, CA). Genetic and phylogenetic analyses were performed applying the Clustal V method, MegAlign Program (DNASTAR, Madison, WI).

Nucleotide sequence accession numbers

The nucleotide sequences obtained from this study can be found at GenBank under the accession numbers listed in Table 1.

Acknowledgments

This study was supported by a grant from the National Research Council of Thailand, the Thailand Research Fund, Senior Research Scholar and the emergency funding from Chulalongkorn University. We also would like to thank Venerable Dr. Mettanando Bhikkhu of The Foundation of King Rama IX, the Great and Ms. Petra Hirsch for reviewing the manuscript.

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