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Brief Report

Genetic analysis of influenza A virus (H5N1) derived from domestic cat and dog in Thailand

A. Amonsin[1], T. Songserm[2], S. Chutinimitkul[3], R. Jam-on[2], N. Sae-Heng[2], N. Pariyothorn[1], S. Payungporn[3], A. Theamboonlers[3], Y. Poovorawan[3]

[1] Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok, Thailand
[2] Faculty of Veterinary Science, Kasetsart University, Kumphaengsaen Campus, Nakorn Pathom, Thailand
[3] Faculty of Medicine, Chulalongkorn University, Pathumwan, Bangkok, Thailand

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Summary

Complete genome sequences of H5N1 viruses derived from a domestic cat "A/Cat/Thailand/KU-02/04" and dog "A/Dog/Thailand/KU-08/04" were comprehensively analyzed and compared with H5N1 isolates obtained during the 2004 and 2005 outbreaks. Phylogenetic analysis of both cat and dog viruses revealed that they are closely related to the H5N1 viruses recovered from avian influenza outbreaks of the same period. Genetic analysis of 8 viral gene segments showed some evidence of virulence in mammalian species. In summary, the H5N1 viruses that infected a domestic cat and dog are highly pathogenic avian influenza viruses that are virulent in mammalian species, potentially indicating transmission of H5N1 viruses from domestic animals to humans.

Correspondence: Yong Poovorawan, Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Pathumwan, Bangkok 10330, Thailand
e-mail: Yong.P@chula.ac.th

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Highly pathogenic avian influenza (HPAI) (H5N1) causes fatal disease in many avian species and humans. In Thailand, AI outbreaks have occurred in the form of four major outbreaks between 2004 and 2006. In the course of these outbreaks, the infections affected many avian species and humans. Moreover, some mammalian species including a tiger, a leopard, a dog and a cat were also diagnosed with HPAI (H5N1) infection [1, 6, 16, 17].

HPAI infection in a domestic cat was first reported in early 2004, the first episode of AI outbreak in Thailand. The cat showed clinical signs of high fever, panting, depression and convulsions with sudden death. HPAI (H5N1) infection was confirmed by immunohistochemistry and virus isolation from animal tissues as well as multiplex RT-PCR to confirm the H5 and N1 serotype of the virus. The animal had a history of eating a pigeon carcass in the same area as the AI outbreak [16].

In October 2004, the second episode of AI outbreak, the first evidence of HPAI infection in domestic dogs in Thailand was reported. Based on owner information, the animal ate a duck car-

cass in the AI-affected area before developing the disease. HPAI infection in the domestic dog was confirmed by immunohistochemistry and multiplex RT-PCR [17].

The reports of HPAI (H5N1) infection in a domestic cat and dog have demonstrated that the H5N1 viruses circulating in Thailand are not only lethal to poultry, humans and tigers but also extend to some domestic animals including dogs and cats. This finding reinforces the necessity of H5N1 prevention and control of H5N1 infection in animals with close contact to humans.

In the present study, we have compared the complete genomes of two H5N1 viruses derived from a cat "A/Cat/Thailand/KU-02/04 (Cat-KU-02)" and a dog "A/Dog/Thailand/KU-08/04 (Dog-KU-08)". The viruses were comprehensively characterized and compared with H5N1 isolates obtained from chicken, duck, tiger, leopard and human in Thailand during the 2004 and 2005 outbreaks. The viruses were also compared to H5N1 isolates from several countries in Asia, Europe and Africa including China, Indonesia, Vietnam, Malaysia, Mongolia, Japan, Russia, Nigeria, Italy and Germany to determine genetic relatedness, genetic differences and possible virulence determinants among H5N1 isolates.

Both H5N1 viruses (Cat-KU-02 and Dog-KU-08) were obtained from cat and dog carcasses. The cat

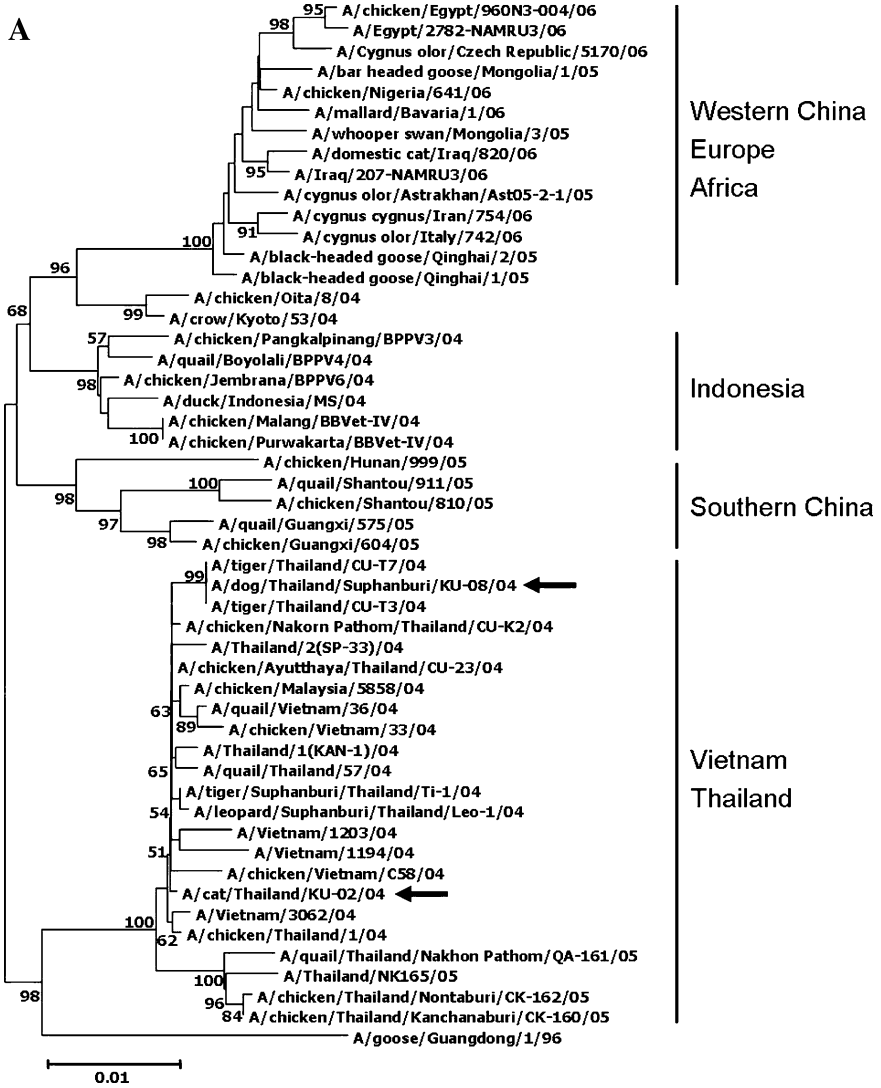

Fig. 1. Phylogenetic analysis of **A**: hemagglutinin (HA) and **B**: neuroaminidase (NA) gene of A/Cat/Thailand/KU-02/04 and A/Dog/Thailand/KU-08/04 viruses

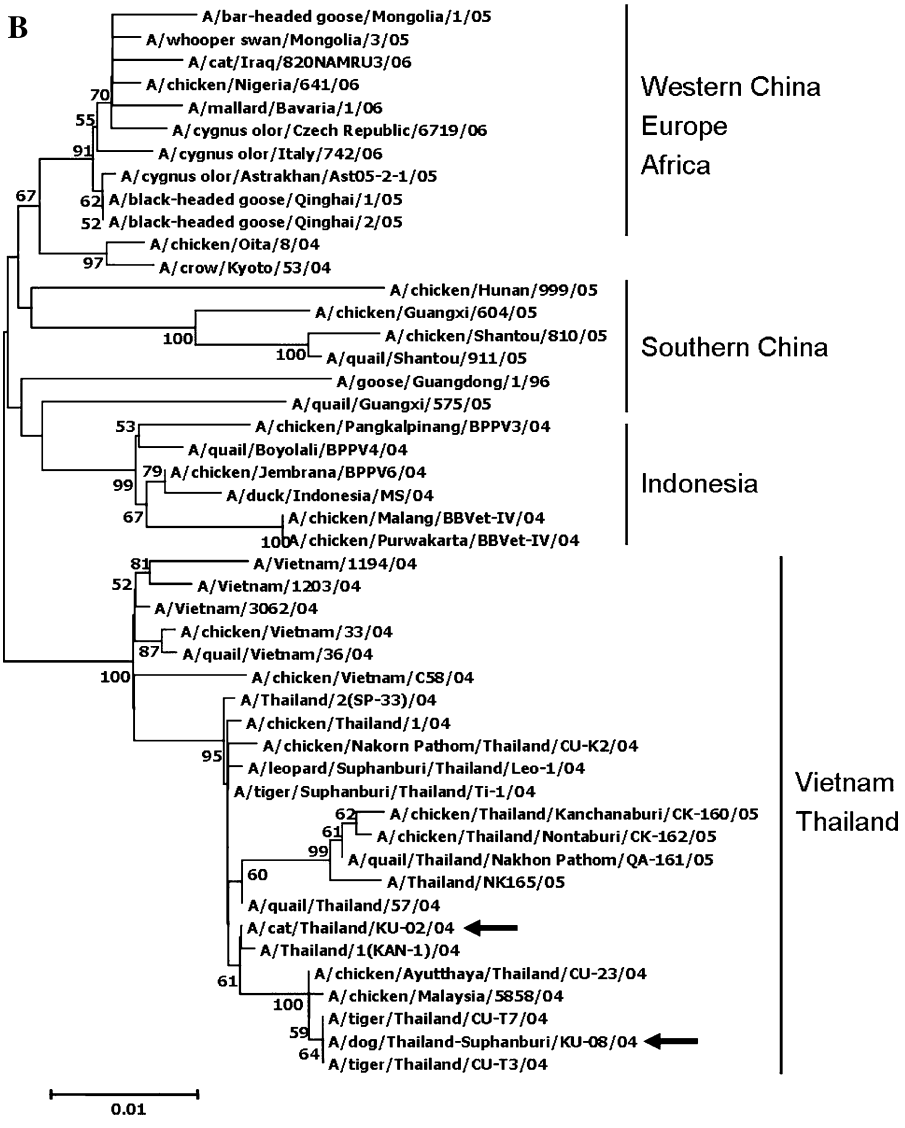

Fig. 1 (*continued*)

H5N1 isolate (Cat-KU-02) was obtained from the lung of a dead domestic cat in Suphanburi province, Thailand. The dog H5N1 isolate (Dog-KU-08) was isolated from the lung of a mixed-breed dog in the same province. The viruses were isolated by embryonated egg inoculation [11]. Viral RNA was extracted from H5N1-infected allantoic fluid using the RNeasy mini kit (Qiagen, GmbH, Germany). Reverse transcription was performed with a universal primer (Promega, Madison, WI) to generate cDNA. H5N1 virus identification was accomplished by multiplex RT-PCR aimed at the matrix (M), hemagglutinin (HA) and neuraminidase (NA) genes [12]. Both cat and dog isolates were characterized as H5N1 subtypes by multiplex RT-PCR, which yielded 276-, 189- and 131-bp PCR products of the M, H5 and N1 genes, respectively.

The complete genomes of the viruses were sequenced as previously described [19]. In brief, PCR products of each gene segment were generated by RT-PCR with primers specific for each gene. The PCR products were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg, Germany). DNA sequencing was carried out using a Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction (ABI, Foster City, CA) with an

Table 1. Genetic analysis of the amino acid sequences of the deduced proteins of A/Cat/Thailand/KU-02/04 and A/Dog/Thailand/KU-08/04 viruses

Virus	Gene															
	HA											NA				
	Connecting peptide sequences	Receptor binding site		N-linked glycosylation site	Antigenic site E		Antigenic site A			Receptor binding		NA stalk region	Amino acid			
	323–330	222	224	154–156	83	86	138	140	141	129	175		74	79	274*	332
A/Goose/Guangdong/1/96	RERRRKKR/G	Q	G	(NSA)	A	A	H	R	S	S	L	No deletion	F	A	H	T
A/Cat/Thailand/KU-02/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Dog/Thailand/KU-08/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	T	H	A
A/Leopard/Thailand/Leo-1/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Tiger/Thailand/Ti-1/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Tiger/Thailand/CU-T3/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	T	H	A
A/Tiger/Thailand/CU-T7/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	T	H	A
A//Thailand/2(SP-33)/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Chicken/Nakorn-Pathom/ Thailand/CU-K2/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Chicken/Ayutthaya/ Thailand/CU-23/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	A	H	A
A/Chicken/Kanchanaburi/ Thailand/CK-160/05	REKRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Thailand/NK-165/05	REKRRKKR/G	Q	G	NST	P	A	Q	K	S	V	L	20 aa deletion	F	A	H	T
A/Vietnam/3062/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Chicken/Vietnam/C58/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	F	A	H	T
A/Chicken/Malaysia/5858/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	A	H	A
A/Quail/Malaysia/6309/04	RERRRKKR/G	Q	G	NST	A	V	Q	K	S	L	L	20 aa deletion	L	A	H	A
A/Duck/Indonesia/MS/04	RERRRKKR/G	Q	G	(NSA)	A	A	Q	K	S	S	L	20 aa deletion	P	A	H	T
A/Chicken/Indonesia/ BBVet-IV/04	RERRRKKR/G	Q	G	(NSA)	A	A	Q	K	S	S	L	20 aa deletion	P	A	H	T
A/Quail/China/Guangxi/ 575/05	RERRRKKR/G	Q	G	(DNA)	A	A	Q	S	S	L	L	20 aa deletion	F	A	H	T
A/Chicken/China/Guangxi/ 604/05	RERRRKKR/G	Q	G	(DNA)	A	A	Q	S	S	L	L	20 aa deletion	F	A	H	T
A/Black-headed goose/ Qinghai/1/05	GERRRKKR/G	Q	G	(NNA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Cygnus olor/Italy/742/06	GERRRKKR/G	Q	G	(NDA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Chicken/Nigeria/641/06	GERRRKKR/G	Q	G	(DNA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Cygnus olor/Astrakhan/ Ast 05-2-1/05	GERRRKKR/G	Q	G	(DNA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Whooper swan/ Mongolia/3/05	GERRRKKR/G	Q	G	(DNA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Domestic Cat/Iraq/820/06	GERRRKKR/G	Q	G	(DNA)	I	A	Q	R	S	S	L	20 aa deletion	F	A	H	T
A/Iraq/207-NAMRU3/06	GERRRKKR/G	Q	G	(DNA)	I	A	Q	R	S	S	L	–	–	–	–	–

Virus	Gene																
	M2									NS1			PB2			PA	NP
	Amantadine-resistant amino acids						Human/ avian-like characteristic			5-amino-acid deletion	Virulence determinant		Virulence determinant				
	26	27	30	31	64	66	16	28	55	80–84	92	c-terminal	627	661	702	409	136
A/Goose/Guangdong/1/96	L	V	A	S	S	E	E	V	L	no	D	ESEV	E	A	K	S	M
A/Cat/Thailand/KU-02/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Dog/Thailand/KU-08/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Leopard/Thailand/Leo-1/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Tiger/Thailand/Ti-1/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Tiger/Thailand/CU-T3/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Tiger/Thailand/CU-T7/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Thailand/2(SP-33)/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Chicken/Nakorn-Pathom/ Thailand/CU-K2/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Chicken/Ayutthaya/ Thailand/CU-23/04	I	V	A	N	A	A	E	V	L	yes	N	ESEV	E	A	K	S	L
A/Chicken/Kanchanaburi/ Thailand/CK-160/05	I	V	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Thailand/NK-165/05	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Vietnam/3062/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Chicken/Vietnam/C58/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Chicken/Malaysia/5858/04	I	A	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Quail/Malaysia/6309/04	I	V	A	N	A	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Duck/Indonesia/MS/04	L	V	A	S	S	A	E	V	L	yes	D	ESEI	E	A	K	S	L
A/Chicken/Indonesia/BbVet-IV/04	L	V	A	S	S	A	E	V	L	yes	D	ESEI	E	A	K	S	L
A/Quail/China/Guangxi/575/05	L	V	A	S	S	A	E	V	L	yes	D	ESEV	E	A	K	S	L
A/Chicken/China/Guangxi/604/05	L	I	A	S	S	E	E	V	L	yes	D	ESKV	E	A	K	S	L
A/Black-headed goose/Qinghai/1/05	L	V	A	S	S	A	E	V	L	yes	D	ESEV	K	A	K	S	L
A/Cygnus olor/Italy/742/06	L	V	A	S	S	E	E	V	L	yes	D	ESKV	K	A	K	S	L
A/Chicken/Nigeria/641/06	L	V	A	S	S	E	E	V	L	yes	D	ESKV	K	A	K	S	L
A/Cygnus olor/Astrakhan/ Ast 05-2-1/05	L	V	A	S	S	E	E	V	L	yes	D	EPKV	K	A	K	S	L
A/Whooper swan/Mongolia/3/05	L	V	A	S	S	E	E	V	L	yes	D	ESKV	K	A	K	S	L

* Position 274 was indicated by N2 numbering order.

ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT). The sequences were edited and assembled using Bioedit 5.0.9 (Ibis Therapeutics, Carlsbad, CA). Phylogenetic analyses and genetic comparison were performed by the Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 program. The nucleotide sequences of 8 gene segments of both cat and dog viruses were submitted to GenBank under accession numbers Cat-KU-02 (DQ236077-DQ236084) and Dog-KU-08 (DQ530170-DQ530177).

Phylogenetic analysis of all genes of H5N1 viruses from cat and dog showed that both viruses clustered into the Thailand and Vietnam lineage. This lineage includes the H5N1 isolates from 2004 to 2005 from Thailand, Vietnam, and Malaysia. On the other hand, the H5N1 viruses from Indonesia (2003–2004) and China (2003–2005) as well as the H5N1 viruses from Europe and Africa (2005–2006) were grouped into a separate lineage of H5N1 genotype Z. Phylogenetic analysis of the HA gene (Fig. 1A) showed that the H5N1 virus from the cat (KU-02) was part of the early 2004 sub-lineage. In addition, the dog H5N1 isolate (KU-08) was closely related to the tiger H5N1 viruses (CU-T3 and CU-T7) obtained during the same period of AI outbreak in October 2004. It is noteworthy that the 2005 viruses isolated from chickens (CK-160, CK-162), quail (QA-161) and human (NK-165) belonged to a separate sub-lineage, indicating the viruses' continuous evolution in Thailand. The H5N1 isolates from Indonesia and South China (Shantou, Guangxi, Hunan, and Fujian) were grouped into separate lineages, Indonesia and South China, respectively. The recent 2005–2006 H5N1 isolates from West China (Qinghai), East Asia (Iran, Iraq and Mongolia), Europe (Russia, Italy, Germany and Czech Republic) and Africa (Egypt and Nigeria) clustered into yet another separate group (West China, Europe and Africa). It is noteworthy that H5N1 from a domestic cat in Iraq was also part of this lineage, which represents a group separate from that of H5N1 isolated from the cat in Thailand. Phylogenetic analysis of the HA gene led us to conclude that the HA gene of the viruses in Asia, Europe and Africa originated from one common ancestor, the ''A/goose/Guangdong/96-lineage''. This conclusion has been documented in additional publications [2].

Phylogenetic analysis of the NA gene (Fig. 1B) produced a result similar to the HA gene analysis in that the cat (KU-02) and dog (KU-08) viruses clustered into the Vietnam lineage. In addition, the 2005 H5N1 viruses from Thailand also clustered into the Vietnam sub-lineage, indicating genetic drift of the viruses. The H5N1 viruses from Indonesia, West China, Europe and Africa also clustered into separate lineages, clusters identical to those detected by HA gene analysis. Phylogenetic analyses of the M, NP, NS, PA, PB1, and PB2 genes of both cat and dog isolates also yielded results similar to those arrived at by HA and NA gene analysis (data not shown). In general, phylogenetic analysis of all gene segments indicated that the cat and dog viruses were closely related to tiger and chicken H5N1 viruses isolated during the same period of AI outbreaks. Moreover, it also demonstrated that cat and dog viruses were of avian origin and thus produced additional evidence for viruses of avian origin infecting several mammalian species, e.g. tiger, leopard, dog, cat and human.

The results obtained from genetic analysis of all 8 gene segments of the cat and dog viruses in comparison with other H5N1 viruses are shown in Table 1. The deduced amino acid sequences of the HA gene were aligned and compared in order to monitor genetic differences between the viruses. The HA connective peptide sequences (position 323–330) of cat and dog viruses contain multiple basic amino acids (RERRRKKR/G), similar to the 2004 H5N1 isolates from tigers, chickens, ducks and humans in Thailand, Vietnam, Malaysia and Indonesia. However, the 2005 H5N1 isolates from Thailand (CK-160, QA-161, CK-162, NK-165) harbor different basic amino acids (Arginine (R) to Lysine (K)) at position −6 of the HA cleavage site (REKRRKKR/G). The H5N1 isolates from West China (Qinghai), Europe and Africa contain different basic amino acids at position −8 (GERRRKKR/G), whereas most of the H5N1 isolates from South-East China contain multiple basic amino acids similar to the 2004 Thailand isolates (RERRRKKR/G) (Table 1). It is noteworthy that the cat and human H5N1 isolates from Iraq (A/Domestic cat/Iraq/820/06 and A/Iraq/207-

NAMRU3/06) displayed the same amino acids at the connecting peptide sequences as the West China (Qinghai), Europe and Africa groups, (GERRRKKR/G). By definition, the presence of multiple basic amino acids is characteristic of HPAI. These multiple basic amino acids located adjacent to the HA cleavage site are known to be related to high virulence of the H5N1 viruses due to efficient cleavage by intracellular proteases [5].

In the present study, the receptor-binding site at amino acid positions 222 and 224 of the HA gene of cat and dog isolates as well as all other H5N1 isolates displayed glutamine (Q-222) and glycine (G-224), indicating that the receptor-binding site is specific for the avian receptor, SA-α-2, 3-Gal. A previous study demonstrated that H5N1 virus preferably binds to the lower respiratory tract of human and cat, which is abundant in type II pneumocytes [18]. Among the more than 100 H5N1 isolates our laboratory has been monitoring, there is no evidence of genetic mutation at the receptor-binding site (data not shown). It is important to monitor this receptor-binding site, since amino acid residue changes at the receptor-binding pocket may indicate increased virulence of H5N1 viruses for mammalian species.

At least seven potential glycosylation sites were identified in the HA1 protein of both cat (KU-02) and dog (KU-08) viruses, similar to the glycosylation sites found in tiger and human viruses. The glycosylation sites are located at positions 10–12, 11–13, 22–24, 154–156, 165–167, 193–195, and 286–288 (data not shown). In our study, the most variable glycosylation site is located at positions 154–156 (Table 1). All H5N1 viruses from Thailand, Vietnam and Malaysia contained amino acid residues suitable for glycosylation (NST). This glycosylation site (154–156) was also found in a few isolates from Indonesia. Interestingly, most isolates from China, Europe and Africa do not contain a glycosylation site at this position. Some studies reported that a glycosylation site adjacent to the receptor binding sites may help augment viral infectivity in mammalian cells [9]. Absence of glycosylation at position 154–156 of H5N1 isolates from West China, Europe and Africa might be one of the virulence factors pertaining to low incidence of human cases in those particular regions.

We further investigated 7 amino acid residues in HA which are under positive selection pressure (five residues at the antigenic sites A and E (positions 83, 86, 138, 140, 141) and two residues at the receptor-binding site (positions 129, 175) [15]. Our result showed that the cat and dog isolates contained 7 amino acid residues similar to H5N1 isolates from tigers, chickens and humans in Thailand and Vietnam (Table 1). Only one human H5N1 isolate (NK-165) contained amino acid residues at positions 83 (P), 86 (A) and 129 (V) that were distinct from those of other Thai H5N1 viruses.

Genetic analysis of the NA gene of the cat and dog viruses showed a 20-amino-acid deletion at positions 49–68 of the NA stalk. This finding had been reported for the NA stalk region of all H5N1 isolates during 2003–2006. However, this deletion was not observed in an H5N1 isolate from China in 1996 (A/Goose/Guangdong/1/96). The deletion in the NA stalk region has been thought to correlate with an adaptation of H5N1 viruses aimed at efficiently infecting land-based avian species [9]. It is noteworthy that 3 amino acids at the variable positions 74, 79 and 332 of the NA gene of the cat isolates (early 2004 outbreak) were different from those of dog, tiger (CU-T3 and CU-T7) and chicken (CU-23) viruses isolated during the late 2004 outbreak. In the present study, we also analyzed oseltamivir resistance due to a single amino acid substitution from histidine (H) to tyrosine (Y) at position 274 of the neuraminidase active site [8]. Both H5N1 viruses isolated from cat (KU-02) and dog (KU-08) contained histidine at position 274, which implied that these viruses were sensitive to oseltamivir treatment.

Amino acid alignment of the non-structural (NS) protein of cat and dog H5N1 viruses showed five amino acid deletions at positions 80–84 of the NS1 protein. This deletion within the NS1 protein had previously been reported in tigers, humans and avian species during the 2004–2006 AI outbreaks but had not been found in H5N1 viruses recovered prior to 2000 [20]. Moreover, the amino acid at position 92 of the NS1 protein of H5N1 viruses from cat and dog was aspartic acid (D), which had been previously identified in all 2003–2006 H5N1 viruses, except for one chicken isolate (CU-23) (Table 1). The mutation of D92 to E92 is related

to H5N1 virulence in mammalian species [13]. However, in this study, we did not find this virulence determinant (E92) in the NS1 protein of cat and dog H5N1 viruses and neither had we found it in tiger and human viruses from previous studies. Another virulence determinant for H5N1 virus in mammals is the carboxy-terminus of the NS1 protein [7, 10]. In this study, the cat and dog viruses contained the PDZ binding sequence motif (ESEV) (Glu-Ser-Glu-Val) at the C-terminal of the NS1 protein similar to the majority of H5N1 isolates from avian species. A previous study reported that the presence of a functional carboxy-terminal PDZ-binding domain (ESEV/EPEV) may correlate with H5N1 virulence in mammals [10].

We aligned the deduced amino acids of the M2 protein in order to identify amantadine-resistant amino acids and characterize the viruses as avian- or human-related. The cat and dog H5N1 viruses displayed an amino acid mutation from serine to asparagine at position 31 (Ser31Asn) of the M2 protein, indicating amantadine resistance. This mutation was predominantly observed in H5N1 viruses from Thailand, Vietnam and Malaysia, whereas some isolates from Indonesia, China and Europe contain serine (S), indicating amantadine sensitivity. In addition, we screened for amino acid substitutions at positions Leu26Ile, Val27Ala/Val27Ile, Ala30Ser, and Ser31Asn, which are related to amantadine resistance [3]. We found that the cat and dog isolates as well as all Thailand and Vietnam isolates contained two amino acid substitutions at Leu26Ile and Ser31Asn, whereas Val27Ala and Ala30Ser were not detected. Thus, our study has supported the previous finding of a high frequency of dual amantadine resistance mutations at positions 26 and 31 (Leu26Ile- Ser31Asn) in H5N1 isolates from Thailand and Vietnam [3]. Furthermore, the M2 protein of cat and dog viruses harbors one human- (Val28) and two avian-related (Glu16 and Leu55) amino acids, indicating a mixed human-/avian-relatedness of the viruses.

Three polymerase genes encode the polymerase proteins (PA, PB1 and PB2), which are associated with viral replication by copying genomic RNAs into viral mRNAs. Genetic analysis of the PB2 protein revealed that the cat and dog viruses as well as those isolated from tigers and humans in Thailand contained lysine (K) at position 627. In contrast, H5N1 viruses isolated from avian species in Thailand contain glutamic acid (E). Those H5N1 viruses derived from western China, Europe and Africa contained Lys627. The presence of lysine at position 627 of the PB2 protein has been associated with an adaptation of H5N1 viruses facilitating virulence in mammalian species [14].

H5N1 infection of a domestic cat and dog investigated in this study indicated that the H5N1 of avian origin can cause fatal infection in mammals with close contact to humans. Even though H5N1 infections were rare in domestic animals, regular monitoring of H5N1infection in domestic animals should be implemented in order to prevent transmission to humans [4]. Phylogenetic analysis of both cat and dog viruses revealed that the viruses are closely related to H5N1 recovered from AI outbreaks during the same period. Genetic analysis of 8 virus gene segments showed some evidence of virulence in mammalian species as previously discovered in H5N1 isolates from a tiger, leopard and human. In conclusion, the H5N1 viruses infecting domestic cats and dogs are highly pathogenic avian influenza viruses with properties that render them virulent in mammalian species, indicating potential H5N1 transmission from domestic animals to humans.

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References

1. Amonsin A, Payungporn S, Theamboonlers A, Thanawongnuwech R, Suradhat S, Pariyothorn N, Tantilertcharoen R, Damrongwantanapokin S, Buranathai C, Chaisingh A, Songserm T, Poovorawan Y (2006) Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology 344: 480–491
2. Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung

CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y (2006) Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci USA 103: 2845–2850

3. Cheung CL, Rayner JM, Smith GJ, Wang P, Naipospos TS, Zhang J, Yuen KY, Webster RG, Peiris JS, Guan Y, Chen H (2006) Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J Infect Dis 193: 1626–1629
4. Cleaveland S, Meslin FX, Breiman R (2006) Dogs can play useful role as sentinel hosts for disease. Nature 440: 605
5. Hatta M, Gao P, Halfmann P, Kawaoka Y (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293: 1840–1842
6. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theambooniers A, Tantilertcharoen R, Pattanarangsan R, Arya N, Ratanakorn P, Osterhaus DM, Poovorawan Y (2004) Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis 10: 2189–2191
7. Krug RM (2006) Virology. Clues to the virulence of H5N1 viruses in humans. Science 311: 1562–1563
8. Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y (2005) Avian flu: isolation of drug-resistant H5N1 virus. Nature 437: 1108
9. 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
10. Obenauer JC, Denson J, Mehta PK, Su X, Mukatira S, Finkelstein DB, Xu X, Wang J, Ma J, Fan Y, Rakestraw KM, Webster RG, Hoffmann E, Krauss S, Zheng J, Zhang Z, Naeve CW (2006) Large-scale sequence analysis of avian influenza isolates. Science 311: 1576–1580
11. Office International des Epizooties O (2005) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.7.12 Avian Influenza. [Updated May 2005]
12. Payungporn S, Phakdeewirot P, Chutinimitkul S, Theamboonlers A, Keawcharoen J, Oraveerakul K, Amonsin A, Poovorawan Y (2004) Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol 17: 588–593
13. Seo SH, Hoffmann E, Webster RG (2004) The NS1 gene of H5N1 influenza viruses circumvents the host antiviral cytokine responses. Virus Res 103: 107–113
14. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y (2004) PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320: 258–266
15. Smith GJ, Naipospos TS, Nguyen TD, de Jong MD, Vijaykrishna D, Usman TB, Hassan SS, Nguyen TV, Dao TV, Bui NA, Leung YH, Cheung CL, Rayner JM, Zhang JX, Zhang LJ, Poon LL, Li KS, Nguyen VC, Hien TT, Farrar J, Webster RG, Chen H, Peiris JS, Guan Y (2006) Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology 350: 258–268
16. Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, Poovorawan Y (2006) Avian influenza H5N1 in naturally infected domestic cat. Emerg Infect Dis 12: 681–683
17. Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R, Poovorawan Y (2006) Fatal avian influenza A H5N1 in a dog. Emerg Infect Dis 12: 1744–1747
18. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T (2006) H5N1 virus attachment to lower respiratory tract. Science 312: 399
19. Viseshakul N, Thanawongnuwech R, Amonsin A, Suradhat S, Payungporn S, Keawchareon J, Oraveerakul K, Wongyanin P, Plitkul S, Theamboonlers A, Poovorawan Y (2004) The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. Virology 328: 169–176
20. Zhou H, Jin M, Chen H, Huag Q, Yu Z (2006) Genome-sequence analysis of the pathogenic H5N1 avian influenza A virus isolated in China in 2004. Virus Genes 32: 85–95