WHOLE GENOME SEQUENCES OF H5N1 INFLUENZA A VIRUS ISOLATED FROM A LITTLE GREBE IN THAILAND

Nikhom Naksupan[1, 4], Donruedee Sanguansermsri[1], Rosarin Wongvilairat[1], Pannika R Niumsup[1], Sutatip Pongcharoen[2], Pornchai Chamnanpood[3], Chanpen Chamnanpood[3] and Phanchana Sanguansermsri[1]

[1]Faculty of Medical Sciences, [2]Faculty of Medicine, Naresuan University, Phitsanulok; [3]Department of Livestock Development, Ministry of Agriculture and Cooperatives, Bangkok; [4]School of Medical Sciences, Naresuan University at Phayao, Phayao, Thailand

Abstract. This is the first report of the whole genome sequence of influenza A virus in an aquatic resident bird of Thailand. It was categorized into genotype Z according to its characteristics of a 20 amino acid deletion in neuraminidase and a five amino acid deletion in the non-structural protein. The indicator for a highly pathogenic trait of the virus is the presence of a polybasic amino acid sequence at the cleavage site of HA0. The feature of resistance to the antiviral drug amantadine is found at the 31st amino acid position of M2 (serine to asparagine). Phylogenic analyses revealed that virus A/little grebe/Thailand/Phichit-01/2004 (H5N1) is closely related to the chicken and human isolates recovered from Thailand. The high degrees of similarity among the sequences and phylogenic trees indicate there was no difference between the viruses isolated from poultry and aquatic birds in Thailand at the time of study. The results also suggest the source of H5N1 avian influenza virus in the little grebe and others in Thailand may have the same origin.

INTRODUCTION

Influenza A viruses are classified as members of the Orthomyxoviridae family. Their genomes consist of eight segments of linear negative-sense, single-strand ribonucleic acid (RNA). The eight segments of viral RNA encode for ten proteins (Horimoto and Kawaoka, 2005). Influenza A viruses of aquatic birds (wild birds) have been proposed as the ancestors of all influenza A virus subtypes (Horimoto and Kawaoka, 2001). Currently, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified (Fouchier *et al*, 2005). Avian influenza (AI) viruses preferentially infect cells lining the intestinal tract of birds and are excreted in high concentrations in their feces. While AI viruses are generally nonpathogenic in wild birds, they sometimes cause significant morbidity and mortality upon transmission to other species (Webster *et al*, 1992; Nicholson *et al*, 2003). It was found that AI viruses that caused outbreaks in poultry and humans in Thailand, Indonesia and Vietnam are genotype Z (Li *et al*, 2004). The H5N1 AI viruses isolated from chickens in Thailand (A/Chicken/Nakorn-Pathom/Thailand/CU-K2/2004) were closely related to the RNA segments of influenza A virus H5N1 isolated from humans in Vietnam (A/Vietnam/1196/2004). However, the Thai H5N1 AI viruses were not related to the early AI viruses during the outbreak in 1997, but remarkably resembled the AI viruses in the years 2000-2001(Viseshakul *et al*, 2004). There were several waves of AI outbreaks in several kinds of poultry in Thailand (Amonsin *et al*, 2006). The viruses caused deaths not

Correspondence: Phanchana Sanguansermsri, Faculty of Medical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
Tel: 66 (089) 7088384; Fax: 66 (055) 261197
E-mail: phanchana@hotmail.com

only in humans but in other mammalian species, including tigers, leopards, cats, and dogs (Keawcharoen *et al*, 2004; Songserm *et al*, 2006a,b; Witayathawornwong, 2006).

Tachybaptus ruficollis or the little grebe (common name) is classified into the Podicipedidae family. It is the smallest duck-like bird, with a thin, pointed bill and a truncated, tailless appearance. In Thailand, the little grebe is a common resident birds and can be found in marshes, ponds, lakes and canals (Lekagul and Round, 1991). After the start of the epidemic of bird flu, some little grebes in Phichit Province Thailand were infected with H5N1 AI virus resulting in death.

In this study, the entire genome sequences of the AI virus isolated from a little grebe in Thailand are described. The viral genome was analyzed and compared to the H5N1 influenza viruses isolated from humans and poultry during previous H5N1 outbreaks in Thailand to determine the likely evolutionary origin of these viruses as well as their relationships to each other.

MATERIALS AND METHODS

Virus

Influenza virus was isolated from a little grebe (*Tachybaptus ruficollis*) from Phichit Province, Thailand (kindly provided by the Northern Veterinary Research and Development Center, Phitsanulok). The virus was isolated by chicken egg embryo inoculation. Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays were used for subtype identification (OIE, 2004).

RNA extraction, PCR and DNA sequencing

Viral RNA was extracted from the allantoic fluid of an inoculated egg using RNeasy Mini-Kit as described by the manufacturer (Qiagen, Santa Clarita, Calif). Reverse transcription was performed with the Omniscritpt-Reverse Transcriptase kit (Qiagen, Santa Clarita, Calif) using uni12 primer as previously reported (Hoffmann *et al*, 2001). PCR amplification of the full length of the viral gene segment was performed employing specific primers (Hoffmann *et al*, 2001). The PCR products

Table 1
Sequencing primers.

Sequencing primers	Nucleotide (5′ - 3′) sequence
PB2-F578	CAATAACGAARGAGAAGAAAGAAGA
PB2-F1044	GAAGGAAGAGGAAGTGCTTACAGGC
PB2-F1455	AGGAGTAAGAGTTAGTAAARTGGGA
PB2-F1937	CTGTGAATGTGAGAGGCTCWGGAAT
PB2-R373	TGTAAACCTTTGGATAATGRACTGC
PB2-R1310	AATCTTTGGTTTGCTCTGTTTACGA
PB1-F426	TATGACTGGACATTGAAYAGAAACC
PB1-F861	GGAGGGAATGAGAAGAAGGCTAAAT
PB1-F1364	CCWATCCTCTGATGATTTCGCTCTC
PB1-F1876	ARTGGGARTTGATGGATGAAGATTA
PB1-R524	TCCYGAYTCATTGGCTGTTAGACCG
PB1-R1035	ATTCWGGYTGGTTCCTTGTGATGTA
PB1-R1633	TTTATCACTGTAACWCCAATGCTCA
PA-F426	AAGCYAACAAGATAAAATCCGAGAA
PA-F869	TTCTTGCTGATGGATGCYCTKAAAT
PA-F1344	GTATGAGAAGGAACTAYTTYACAGC
PA-F1748	ATGAAATGGGGSATGGAAATGAGGC
PA-R515	CAAGGGTGTAGTCMGCTTTRGTGGC
PA-R1098	TTTGTTTTTGGGATTTTMTCCTCAT
HA5-F228	GCTCCTCGGGAACCCAATGTGTGAC
HA5-F611	CAAGACTCTATCAAAACCCAACCAC
HA5-F931	CACCCTCTCACYATCGGRGAATGCC
HA5-F1387	CTTTACGACAAGGTCCGACTACAGC
HA5-F1645	GCTGGTCTATCTTTATGGATGTGCT
HA5-R-515	GCACTGTTCTTTKTGATAAGCCATA
HA5-R1298	AATCCGTCTTCCATCTTCTTGTTTA
NP-F450	TGCTGGTCTTACCCACCTGATGATA
NP-F876	TAAGTCCTGCTTRCCTGCTTGTGTG
NP-R556	GAGAGCACATCCTKGGGTCCATTCC
NP-R1115	ACTCTTGTCCCTCTGATGAAACTTG
NA-F407	CAAAGACAGAAGCCCTCACAGAACA
NA-F925	TCGGAGAYAATCCACGCCCCAATGA
NA-R413	GTCTTTGACAGTCCCRTTGGAGTGC
NA-R962	ACTACCKGTTCCATCATTGGGGCGT
M-F520	GGCRACTAYCACCAACCCACTAATC
M-R549	TGYCTGATTAGTGGGTTGGTGRTAG
NS-F503	AATCTCACCATTRCCTTCCCTTCCA
NS-R320	TGATGACTTTATCCATTATTGCCTG

Table 2
The length of the predicted amino acid sequences of A/little grebe/Thailand/Phichit-01/2004 (H5N1) obtained from the study.

Segment	Gene	Note	Length of amino acids	Accession number
1	PB2	Polymerase basic protein 2	760	DQ407243
2	PB1	Polymerase basic protein 1	758	DQ407244
3	PA	Polymerase acidic protein	717	DQ407245
4	HA	Hemagglutinin	569	AY553802
5	NP	Nucleoprotein	499	DQ407246
6	NA	Neuraminidase	450	AY553832
7[a]	M1	Matrix protein 1	253	DQ407247
	M2	Matrix protein 2	98	
8[b]	NS1	Nonstructural protein	226	DQ407248
	NS2 (NEP)	Nuclear export protein	122	

[a]RNA segment 7 encoded for two proteins: M1 and M2 ; [b]RNA segment 8 encoded for two protein: NS1 and NS2

were purified using the QIAquick PCR Purification Kit (Qiagen). The DNA sequences were determined by cycle sequencing (Big Dye Terminator V. 3.1 Cycle Sequencing Ready Reaction) using specific sequencing primers as shown in Table 1 on the ABI-Prism 310 Genetic analyzer (Perkin Elmer, Norwalk, CT).

Sequences analysis

The nucleotide sequences were compared with published sequences in GenBank by BioEdit v. 7.0.7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenic tree was drawn using PHYLIP v. 3.66 (http://evolution.genetics.washington.edu/phylip.html) and TreeView v. 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RESULTS

Genetic characterization

The AI virus isolated from the little grebe in this study was named A/little grebe/Thailand/Phichit-01/2004 (H5N1) according to its type, host, country of origin, specific number, the year of isolation, and subtype of hemagglutinin (HA: H5) and neuraminidase (NA: N1), respectively. The genome of the virus had eight RNA segments as follows: polymerase B2 (PB2), polymerase B1 (PB1), polymerase A (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS). All nucleotide sequences were deposited at the NCBI Database with the GenBank accession numbers: AY553802, AY553832, and DQ407243 to DQ407247. The length of the predicted amino acid sequences and accession numbers of the database are shown in Table 2. Sequence comparisons between A/little grebe/Thailand/Phichit-01/2004 (H5N1) and other H5N1 AI viruses in Thailand, such as chicken, human and cat, are presented in Table 3. Compared with previous records, all genes of the AI viruses revealed 99.1-100% similarity with both nucleotide and amino acid sequences.

Phylogenic trees analysis

Phylogenic analysis of HA and NA genes revealed that the A/little grebe/Thailand/Phichit-01/2004 (H5N1) was closely related to the chicken, tiger, and human isolates recovered in Thailand and Vietnam during 2004 and 2005 (Figs 4 and 5). The remaining gene segments (PB2, PB1, PA, NP, M, and NS) followed a similar pattern (data not shown).

Table 3
Percent nucleotide (amino acid) similarity between A/little grebe/Thailand/Phichit-01/2004 and some H5N1 AI viruses isolated in Thailand.

	Nontaburi: A/Ck/Th/ Non/CK-162/05[a]	Phichit: A/Ck/Th/ PC-168/06 [b]	Nakhon Phanom: A/Ck/ Th/NP-172/06[c]
PB2	99.2 (99.2; I260V, L512F, S629N)	99.1 (98.6; M11I, T106A, V338I, V560M, V649I)	97.5 (97.4; A105T, A108T, I147T, E208D, D254E, K339T, R368Q, D390N, I616V, V667I)
PB1	99.3 (98.9; R211K, K331E, L384I, T400A)	99.2 (98.4; M171I, E177D, K331E, I552V, V591I, V719M)	97.8 (98.9; K215R, K331E, V644I, K653R)
PA	99.5 (99.7; I690V)	99.3 (99.2; I330V, A369T, A404T)	93.5 (95.6; D27N, S58G, E101D, T129I, K204R, L261M, K339R, S388G, R391K, S400P, A404S, V554I, N614T, G631S, A669V, A712T)
HA	99.1 (98.3; V102A, T175I, R341K, T544A, S558F)	99 (97.9; K3R, K38R, Q154L, R513K, T554A, +569K, +570L)	96.7 (91.0; F8L, A9G, V102A, D110N, S140D, L145S, K156T, S157P, S171N, V190I, P197S, N209R, R228K, A230H, E243D, I248M, T279A, L285V, M298I, R326K, Q338L, I529T, K345-, V539A, T544A, +569K, +570L, +571E, +572S, +573E
NP	99.3 (99.2; S34G, I217M)	99.7 (99.4; A366S)	98.3 (98.8; D290N, F338L, V363I)
NA	99.7 (97.4; H36R, H44Q, V63A, A118S, N305T, D396N)	99.3 (99.0; T128S, S180N)	98.5 (91.0; K5Q, T17I, M19I, L29M, H39Q, K45Q, S50R, K58N, K64T, N75R, Y80H, S180N, H233Y, D250N, T269I, S320P, V326I, E362G, S366N, V369M, G434S)
M (M1)	99.7 (99.2; A83S)	99.4 (99.2; K27R)	98.5 (98.4; I14V, I205V)
NS (NS1)	99.3 (96.5; Q25R, R188Q, I193L, D204N)	98.9 (98.2; E71D, A81T)	98.2 (95.7; K75E, A81T, V122T, I124L, T190S)

[a]A/chicken/Thailand/Nontaburi/CK-162/2005 from Nonthaburi (accession number. DQ334773-80)
[b]A/chicken/Thailand/PC-168/2006 from Pichit (accession number. DQ999879-86)
[c]A/chicken/Thailand/NP-172/2006 from Nakhon Phanom (accession number. DQ999871-78)

DISCUSSON

To our knowledge, this is the first study of the whole genome sequence of H5N1 AI virus isolated from a wild aquatic bird in Thailand. A/little grebe/Thailand/Phichit-01/2004 contained an amino acids sequence RERRRKKR*GLF at the cleavage site in the HA0 precursor of HA. The series of basic amino acids which are cleaved by proteases

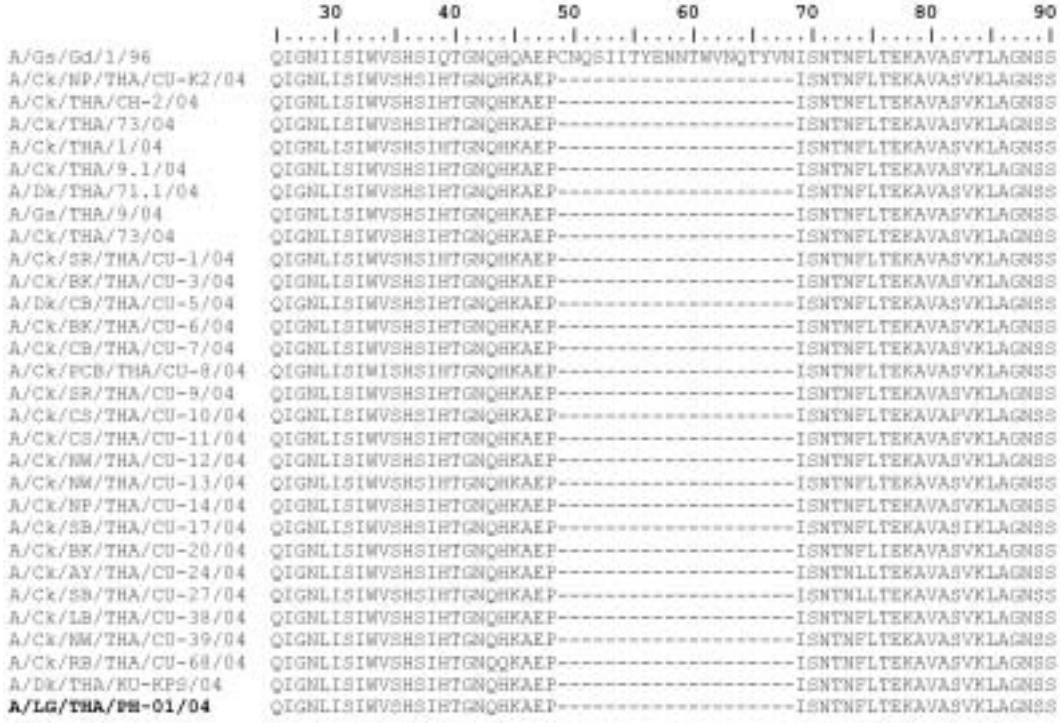

Fig 1–Alignment comparison of deduced amino acid sequences of the NA protein showed the Thai AI viruses exhibited a 20-amino acid deletion in the NA stalk region at the position 49-68.

Table 4
HA0 cleavage sequences of recent Thailand Influenza H5N1 viruses.

Viruses strain	HA0 cleavage site	Accession no.
A/LG/THA/PH-01/04[a]	RRRKKR*GLF	AY553802
A/Ck/NP/THA/CU-K2/04[b]	RRRKKR*GLF	AY590568
A/Ck/AY/THA/CU-24/04[c]	RRRKKR*GLF	DQ083569
A/Ck/KP-2-02/04[d]	KRRKKR*GLF	DQ017308
A/Ck/NW-2-07/04[e]	KRRKKR*GLF	DQ017307
A/Ck/THA/PL-02/04[f]	RRRKKR*GLF	AY553793
A/cat/THA/KU-02/04[g]	RRRKKR*GLF	DQ236077.1
A/tiger/SR/THA/Ti-1/04[h]	RRRKKR*GLF	AY646167.1
A/THA/1 (KAN-1)/04[i]	RRRKKR*GLF	AY555150.2
A/leopard/SR/THA/Leo-1/04[j]	RRRKKR*GLF	AY646175
A/open-billed Stork/THA/VSMU-15-ATG/05[k]	RRRKKR*GLF	EF206699.2
A/pigeon/THA/VSMU-25-BKK/05[l]	RRRKKR*GLF	EF206698.2
A/Ck/Th/Non/CK-162/05[m]	KRRKKR*GLF	DQ334776
A/Ck/Th/NP-172/06[n]	RRRKR*GLF	DQ999872

[a] A/little grebe/Thailand/Phichit-01/2004; [b] A/Chicken/Nakorn-Pathom/Thailand/CU-K2/2004; [c] A/Chicken/Ayutthaya/Thailand/CU-24/2004; [d] A/Chicken/Kumphangphet-2-02/2004; [e] A/Chicken/Nakornsawan-2-07/2004; [f] A/Chicken/Phitsanulok-02/2004; [g] A/cat/Thailand/KU-02/04; [h] A/tiger/Supanburi/Thailand/Ti-1/04; [i] A/Thailand/1 (KAN-1)/2004; [j] A/leopard/Suphanburi/Thialand/Leo-1/2004; [k] A/open-billed Stork/Thailand/VSMU-15-ATG/2005; [l] A/pigeon/Thailand/VSMU-25-BKK/2005; [m] A/chicken/Thailand/Nontaburi/CK-162/2005; [n]A/chicken/Thailand/Nakorn-Pathom-172/2006

Fig 2–Alignment comparison of the NS1 protein showed that Thai AI viruses exhibited a 5-amino acid deletion at the position 80-84.

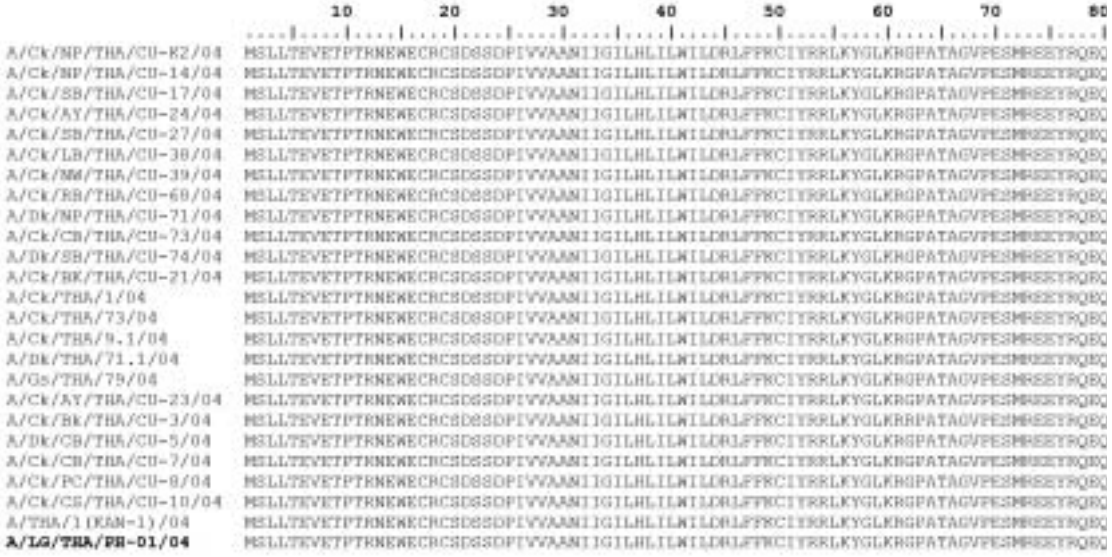

Fig 3–Alignment comparison of M2 protein revealed asparagine instead of serine at position 31.

found in a wide range of organs, such as furin and proprotein convertase 6 (Stieneke-Grober *et al*, 1992; Horimoto and Kawaoka, 2005) resulted in a lethal systemic infection, indicating the highly pathogenic nature of the influenza virus (HPAI) (Horimoto and Kawaoka, 1994). This cleavage sequence has been present in most AI viruses in Thailand found in both avian and mammalian species. (Table 4). The receptor binding region of the HA1 subunit of the HA for A/little grebe/Thailand/Phichit-01/2004 also possessed Q and G at position 222 and 224, respectively, similar to the H5N1 viruses recently isolated from humans and poultry in Thailand and Vietnam (Li *et al*, 2004; Matrosovich *et al*, 2004).

NA is a sialidase that prevents virion aggregation by removing cell and virion surface sialic acid (Horimoto and Kawaoka, 2001). The NA of A/little grebe/Thailand/Phichit-01/2004 revealed a 20 amino acid deletion at the stalk region (Fig 1) and a 5 amino acid deletion in the nonstructural 1 (NS1) protein (Fig 2), similar to previous studies (Li *et al*, 2004; Viseshakul *et al*, 2004). Deletion in the NA stalk region may be associated with adaptation of influenza viruses to land-based poultry (Matrosovich *et al*, 1999). In contrast, no deletion in NA was found in influenza viruses in 1996. The position of the deletion was similar

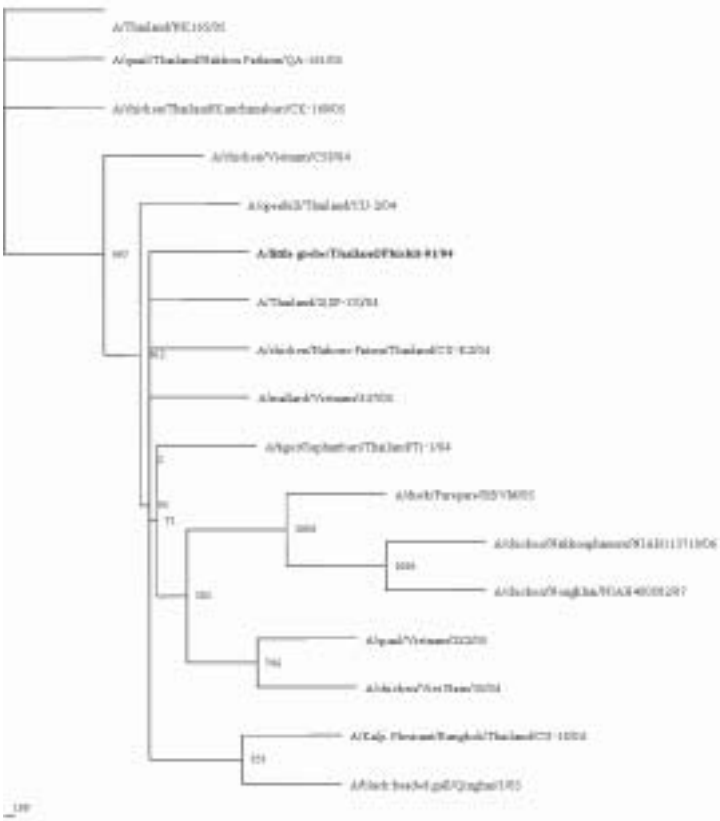

Fig 4–Phylogenic tree analysis of HA gene (nucleotide 5-1716) for H5N1 viruses. Nucleotide sequences were analyzed with a PHYLIP package. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join the nodes. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value.

but not identical to the 19 amino acid deletion found in the Hong Kong/1997 viruses (Puthavathana *et al*, 2005).

The NS-specific viral RNA segment of influenza A virus encoded for both NS1 and nuclear export protein (NEP). Unspliced NS-specific mRNA translates into the NS1 protein, while the spliced RNA directs the synthesis of the NEP (O'Neill *et al*, 1998). Previous studies have indicated that the NS1 gene functioned as an antagonist against the host interferon defense system in multiple ways (Garcia-Sastre *et al*, 1998). Additionally, glutamic acid at position 92 in the NS1 protein is associated with increased virulence in pigs (Seo *et al*, 2002). A/little grebe/Thailand/Phichit-01/2004 has aspartic acid at position 92 in the NS1 protein, similar to recent epidemic isolates (Li *et al*, 2004).

The amino acid comparison of matrix 1 (M1) and matrix 2 (M2) proteins showed a conserved sequence (more than 99.5-100% similarity) among the H5N1 AI in Thailand, including A/little grebe/ Thailand/ Phichit-01/2004 (Table 3). It has been demonstrated that amantadine inhibits influenza virus replication by blocking the activity of the M2 ion channel (Lubeck *et al*, 1978; Hay *et al*, 1985). Substitution mutations which confer resistance to amantadine have been mapped to amino acid 27 (V→A), 30 (A→T or V), and 31 (S→N) of the M2 gene segment (Suzuki *et al*, 2003). A/little grebe/Thailand/ Phichit-01/2004 revealed single amino acid substitutions at amino acid 31 (S→N) (Fig 3) in the transmembrane region of the M2 protein, which may confer amantadine resistance (Sweet *et al*, 1997; Scholtissek *et al*, 1998; Puthavathana *et al*, 2005). It is possible the use of amantadine on poultry farms in China since late 1990s is responsible for amantadine resistance to influenza viruses. Recent studies by WHO reference laboratories indicate amantadine resistance has been steadily increasing worldwide (Parry, 2005).

An E627K substitution mutation in the PB2 protein has been associated with increasing virulence in H5N1 viruses, resulting in a high mortality rate in mice (Shinya *et al*, 2004).

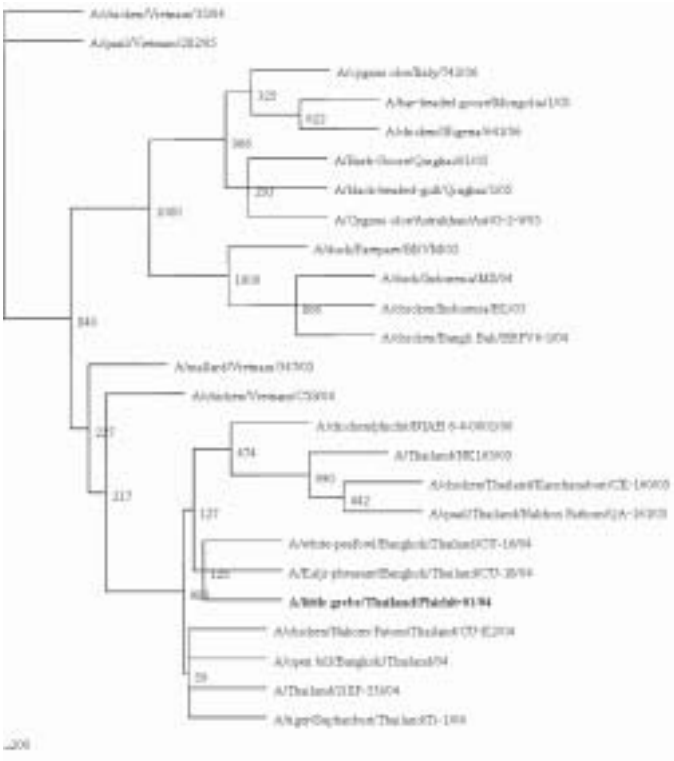

Fig 5–Phylogenic tree analysis of NA gene (nucleotide 31 to 1358) for H5N1 viruses. Nucleotide sequences were analyzed with a PHYLIP package. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join the nodes. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value.

A/little grebe/Thailand/Phichit-01/2004 has glutamic (E) acid (PFAAAPPEQSR) at position 627 in PB2, as found in other avirulent strains. PB2 is a determining of the RNA polymerase. Within PB2, residue 627 is always glutamic acid in avian isolates but lysine in viruses in humans (Subbarao *et al*, 1993). Previously, influenza viruses isolated from three of four humans in Vietnam had lysine at 627 in the PB2 protein (Li *et al*, 2004).

In conclusion, the amino acid sequence analysis of the A/little grebe/Thailand/Phichit-01/2004 (H5N1) revealed a polybasic amino acid at the HA0 cleavage site (RRRKKR*GLF). Amino acid sequences showed a 20 amino acid deletion in the stalk region and a 5 amino acid deletion in the NS1 protein, which is similar to the H5N1 viruses isolated in the same epidemic classified as genotype Z. Interestingly, the amino acid sequences of the M2 protein of A/little grebe/Thailand/Phichit-01/2004 indicate a substitution of a serine for asparagine at residue 31 which has been known to confer resistance to adamantanes drugs (amantadine and rimantadine). Phylogenic analysis showed that A/little grebe/Thailand/Phichit-01/2004 (H5N1) isolated from an aquatic bird in Thailand was similar to other H5N1 viruses in the same pandemic. Hence, the AI epidemic in Thailand can be found on the poultry farms and in other avian species, such as aquatic wild birds and resident birds. The high degree of similarity among the sequences and phylogenic trees indicates there is no difference between the viruses isolated from poultry and aquatic birds in Thailand at the time of study. The results also suggest that the sources of H5N1 AI viruses in a little grebe and in others avian species in Thailand may come from the same origin.

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