GENETIC CHARACTERIZATION OF NONSTRUCTURAL GENES OF H5N1 AVIAN INFLUENZA VIRUSES ISOLATED IN THAILAND IN 2004-2005

Narutchala Suwannakhon[1], Sarutsawadee Pookorn[2], Donruedee Sanguansermsri[1], Chanpen Chamnanpood[1, 3], Pornchai Chamnanpood[3], Rosarin Wongvilairat[1], Sutatip Pongcharoen[4], Pannika R Niumsup[1], Duangkamol Kunthalert[1] and Phanchana Sanguansermsri[1]

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

Abstract. The outbreak of highly pathogenic avian influenza (HPAI) viruses has severely disrupted poultry production and trade. Humans have been infected with HPAI H5N1 viruses and many have died. The nonstructural (NS) proteins of the virus are a factor that determines virulence. In this report, 80 NS genes of H5N1 HPAI viruses isolated from Thailand were completely sequenced and phylogenically analyzed. The percentages of identity and variable site NS1 genes were similar to NS2/nuclear export protein (NEP) genes. All NS1 genes from the samples were located in allelic group A. The NS1 and NS2/NEP proteins possess 225 and 121 amino acids, respectively. All NS1 protein samples had five amino acid deletions typical of avian influenza viruses isolated since 2002. An amino acid substitution at position 92 (G92E) of the NS1 protein, known to promote the inhibition of host immune responses, was also found in the samples.

INTRODUCTION

The H5N1 avian influenza virus epidemic is a significant problem for the poultry industry and human health and has worldwide distribution (Webster and Hulse, 2004; Smith *et al*, 2006). In Thailand and neighboring countries, more than 150 million poultry and more than 184 people have been killed by the H5N1 avian influenza virus. The H5N1 avian influenza virus is also expected to cause the next influenza pandemic (Viseshakul *et al*, 2004; Smith *et al*, 2006; Palese and Shaw, 2007). It is not clear why the H5N1 avian influenza virus is virulent. Hemagglutinin has been identified as one of the virulence factors. The nonstructural 1 protein also contributes to the virulence of the influenza virus (Nicholson *et al*, 2003; Barclay and Zambon, 2004; Seo *et al*, 2004; Govorkova *et al*, 2005; Li *et al*, 2006b; Palese and Shaw, 2007).

The H5N1 avian influenza virus belongs to the family Orthomyxoviridae. Its genome contains eight separate segments of single-stranded negative sense RNA named polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS), which encode ten to eleven viral proteins (Chen *et al*, 2004; Palese and Shaw, 2007). The smallest segment of RNA, the NS, encodes two proteins, the nonstructural 1 protein (NS1) and

Correspondence: Phanchana Sanguansermsri, Faculty of Medical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
Tel: 66 (0) 89 708 8384; Fax: 66 (055) 261 197
E-mail: phanchana@hotmail.com

the nuclear export protein (NS2/NEP). The NS1 protein is translated directly from the mRNA (Porter *et al*, 1980). It consists of 124-237 amino acids, depending on the virus strain (Suarez and Perdue, 1998; Wang *et al*, 2005). The NS1 protein is the only virus protein not present in the virus particle. It accumulates in the nucleus of infected cells (Li *et al*, 1998; Ozaki and Kida, 2007; Satterly *et al*, 2007). The NS1 protein is a multifunctional protein that plays important roles in viral replication and the anti-immune response of the host cell. The NS1 protein structure contains an RNA binding domain and an effector domain (Wang *et al*, 1999; Hayman *et al*, 2007; Lin *et al*, 2007). The RNA binding domain is located at the 1 to 73 amino-terminus, and the effector domain extends from 74 to the end C-terminus. The RNA binding domain can interact with double stranded RNA (dsRNA), viral RNA (vRNA), poly (A) binding protein, U6 snRNA, protein kinase R (PKR), 2′5′ oligoadenylate synthetase (2′5′ OAS)/RNase L pathway, and eukaryotic initiation factor (eIF4GI) (Hatada and Fukuda, 1992; Qian *et al*, 1995; Bergmann *et al*, 2000; Burgui *et al*, 2003; Min and Krug, 2006; Lin *et al*, 2007; Min *et al*, 2007).

NS1 protein binds to cellular multifunctional protein nucleolin and NS1-binding protein (NS1-BP) (Wolff *et al*, 1998; Murayama *et al*, 2007). The effector domain contains the binding site of 30 kDa cleavage and polyadenylation specificity factor (CPSF), poly (A) binding protein II (PABII) (Chen *et al*, 1999; Noah *et al*, 2003). The NS2/NEP protein is translated from the NS2/NEP mRNA which is produced by NS mRNA splicing in the nucleus of the host cell. The greatest length of the NS2/NEP protein is 121 amino acids (Porter *et al*, 1980; Palese and Shaw, 2007). The NS2/NEP protein may promote the formation of a stable export complex of new viral RNP (Neumann *et al*, 2000). In this report, we demonstrate the genetic analysis of eighty segments of NS genes of H5N1 avian influenza virus isolated in Thailand during 2004-2005.

MATERIALS AND METHODS

Viruses

Eighty H5N1 influenza viruses used in this study were isolated from diseased or dead poultry in Thailand during 2004-2005 (Table 1). The samples were obtained from the Northern Veterinary Research and Development Center. The viruses were isolated and identified according to the standard method describe in the OIE Terrestrial Manual (OIE, 2004). Briefly, the tracheal or cloacal swab samples were inoculated into 10-day-old-specific-pathogen-free (SPF) embryonated chicken eggs. These eggs were then incubated at 37°C for up to 4 days. Dead eggs and all remaining eggs at the end of the incubation period for collecting allantoic fluid were tested for hemagglutination (HA) activity. The virus subtypes were identified by hemagglutination inhibition (HI) and real-time RT-PCR. Samples yielding positive results were kept at -80°C until used.

RNA extraction, PCR, and DNA sequencing

Viral RNA was extracted from allantoic fluid using the RNeasy Mini kit (Qiagen) as described by the manufacturer. Reverse transcription was performed with oligonucleotide influenza universal primer Uni 12 (Hoffmann *et al*, 2001) using the InpromII kit (Promaga) as described by the manufacturer. The cDNAs were amplified with primers Bm-NS-1 and Bm-NS-890-R as previously reported (Hoffmann *et al*, 2001). The amplification program was started at 94°C for 40 seconds followed by 35 cycles at 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 1 minute, and a 10 minute final extension at 72°C. To confirm proper amplification, PCR products were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining under UV illumination.

PCR products were purified with the QIAquick PCR purification kit (Qiagen) as de-

Table 1

H5N1 avian influenza viruses in this study and their GenBank accession numbers.

No.	Viruses	Accession number
01	A/Partridge/Thailand/Sukhothai-01/2004(H5N1)	EU153264
02	A/Chicken/Thailand/Tak-01/2004(H5N1)	EU153265
03	A/Chicken/Thailand/Nakornsawan-01/2004(H5N1)	EU153266
04	A/Chicken/Thailand/Phitsanulok-01/2004(H5N1)	EU153267
05	A/Chicken/Thailand/Sukhothai-01/2004(H5N1)	EU153268
06	A/Duck/Thailand/Phichit-01/2004(H5N1)	EU153269
07	A/Chicken/Thailand/Phitsanulok-02/2004(H5N1)	EU153270
08	A/Chicken/Thailand/Kamphaenphet-01/2004(H5N1)	EU153271
09	A/Chicken/Thailand/Nakornsawan-02/2004(H5N1)	EU153272
10	A/Chicken/Thailand/Phetchabun-01/2004(H5N1)	EU153273
11	A/Chicken/Thailand/Phetchabun-02/2004(H5N1)	EU153274
12	A/Chicken/Thailand/Sukhothai-02/2004(H5N1)	EU153275
13	A/Chicken/Thailand/Pathumthani-01/2004(H5N1)	EU153276
14	A/Chicken/Thailand/Udonthani-01/2004(H5N1)	EU153277
15	A/Chicken/Thailand/Udonthani-02/2004(H5N1)	EU153278
16	A/Chicken/Thailand/Udonthani-03/2004(H5N1)	EU153279
17	A/Chicken/Thailand/Mahasarakham-01/2004(H5N1)	EU153280
18	A/Quail/Thailand/Phitsanulok-01/2004(H5N1)	EU153281
19	A/Quail/Thailand/Phichit-01/2004(H5N1)	EU153282
20	A/Open-bill ibis/Thailand/Nakornsawan-01/2004(H5N1)	EU153283
21	A/Pigeon/Thailand/Uttaradit-01/2004(H5N1)	EU153284
22	A/Pigeon/Thailand-01/2004(H5N1)	EU153285
23	A/Sparrow/Thailand/Phitsanulok-01/2004(H5N1)	EU153286
24	A/Bird/Thailand/Nakornsawan-01/2004(H5N1)	EU153287
25	A/Pigeon/Thailand/Phitsanulok01/2004(H5N1)	EU153288
26	A/Duck/Kamphaenphet/NIAH6-2-0045/2004(H5N1)	EU153289
27	A/Chicken/Kamphaenphet/NIAH6-2-0046/2004(H5N1)	EU153290
28	A/Chicken/Sukhothai-2-01/2004(H5N1)	EU153291
29	A/Chicken/Uttaradit-2-01/2004(H5N1)	EU153292
30	A/Chicken/Uttaradit-2-02/2004(H5N1)	EU153293
31	A/Chicken/Kamphaengphet-2-01/2004(H5N1)	EU153294
32	A/Chicken/Kamphaengphet-2-02/2004(H5N1)	EU153295
33	A/Chicken/Kamphaengphet-2-03/2004(H5N1)	EU153296
34	A/Chicken/Kamphaengphet-2-04/2004(H5N1)	EU153297
35	A/Chicken/Uthaithani-2-01/2004(H5N1)	EU153298
36	A/Chicken/Kamphaengphet-2-06/2004(H5N1)	EU153299
37	A/Chicken/Nakornsawan-2-01/2004(H5N1)	EU153300
38	A/Chicken/Nakornsawan-2-04/2004(H5N1)	EU153301
39	A/Chicken/Nakornsawan-2-05/2004(H5N1)	EU153302
40	A/Chicken/Nakornsawan-2-06/2004(H5N1)	EU153303
41	A/Chicken/Nakornsawan-2-07/2004(H5N1)	EU153304
42	A/Duck/Nakornsawan-2-02/2004(H5N1)	EU153305
43	A/Chicken/Uthaithani-2-02/2004(H5N1)	EU153306

Table 1 (continued).

No.	Viruses	Accession number
44	A/Chicken/Uthaithani-2-03/2004(H5N1)	EU153307
45	A/Chicken/Uthaithani-2-05/2004(H5N1)	EU153308
46	A/Duck/Uthaithani-2-02/2004(H5N1)	EU153309
47	A/Chicken/Phitsanulok-2-01/2004(H5N1)	EU153310
48	A/Duck/Phitsanulok-2-01/2004(H5N1)	EU153311
49	A/Duck/Phitsanulok-2-02/2004(H5N1)	EU153312
50	A/Chicken/Phitsanulok-2-03/2004(H5N1)	EU153313
51	A/Chicken/Phitsanulok-2-04/2004(H5N1)	EU153314
52	A/Duck/Phichit-2-01/2004(H5N1)	EU153315
53	A/Duck/Phichit-2-02/2004(H5N1)	EU153316
54	A/Little-cuckoo-dove/Tak-2-01/2004(H5N1)	EU153317
55	A/Chicken/Phetchabun-2-01/2004(H5N1)	EU153318
56	A/Chicken/Phetchabun-2-02/2004(H5N1)	EU153319
57	A/Chicken/Nakornsawan/NIAH6-3-0014/2005(H5N1)	EU153320
58	A/Chicken/Sukhothai/NIAH6-3-0005/2005(H5N1)	EU153321
59	A/Duck/Phitsanulok/NIAH6-3-0015/2005(H5N1)	EU153322
60	A/Chicken/Nakornsawan/NIAH6-3-0004/2005(H5N1)	EU153323
61	A/Chicken/Phitsanulok/NIAH6-3-0016/2005(H5N1)	EU153324
62	A/Duck/Uthaithani/NIAH6-3-0003/2005(H5N1)	EU153325
63	A/Chicken/Phitsanulok/NIAH6-3-0017/2005(H5N1)	EU153326
64	A/Chicken/Phitsanulok/NIAH6-3-0011/2005(H5N1)	EU153327
65	A/Duck/Phichit/NIAH6-3-0018/2005(H5N1)	EU153328
66	A/Chicken/Uthaithani/NIAH6-3-0007/2005(H5N1)	EU153329
67	A/Chicken/Thailand/Kamphaengphet-3-04/2005(H5N1)	EU153330
68	A/Chicken/Kamphaengphet/NIAH6-3-0028/2005(H5N1)	EU153331
69	A/Chicken/Thailand/Kamphaengphet-3-07/2005(H5N1)	EU153332
70	A/Chicken/Thailand/Kamphaengphet-3-02/2005(H5N1)	EU153333
71	A/Chicken/Kamphaengphet//NIAH6-3-0019/2005(H5N1)	EU153334
72	A/Chicken/Kamphaengphet/NIAH6-3-0020/2005(H5N1)	EU153335
73	A/Chicken/Sukhothai/NIAH6-3-0013/2005(H5N1)	EU153336
74	A/Chicken/Uthaithani/NIAH6-3-0021/2005(H5N1)	EU153337
75	A/Chicken/Phitsanulok/NIAH6-3-0022/2005(H5N1)	EU153338
76	A/Chicken/Nakornsawan/NIAH6-3-0023/2005(H5N1)	EU153339
77	A/Chicken/Phitsanulok/NIAH6-3-0024/2005(H5N1)	EU153340
78	A/Chicken/Nakornsawan/NIAH6-3-0025/2005(H5N1)	EU153341
79	A/Chicken/Phitsanulok/NIAH6-3-0026/2005(H5N1)	EU153342
80	A/Chicken/Kamphaengphet/NIAH6-3-0027/2005(H5N1)	EU153343

scribed by the manufacturer. The purified PCR products were sequenced by the sequencing primers Bm-NS-1, Bm-NS-890R, NS-F503, and NS-R320 as previously reported (Hoffmann *et al*, 2001) using the BigDye Terminator Sequencing kit™ (Applied Biosystems). The sequencing products were precipitated by sodium-acetate ethanol precipitation and then analyzed with an automated sequencer (Genetic Analyzer 310 Applied Biosystems).

Table 2
Related sequences of the NS gene of H5N1 avian influenza virus from the GenBank database for genetic analysis.

Viruses	Accession number
A/Tiger/Thailand/VSMU-1-SPB/2004(H5N1)	EF178527
A/cat/Thailand/KU-02/04(H5N1)	DQ236083
A/chicken/Bangkok/Thailand/CU-21/04(H5N1)	DQ083659
A/Gs/Thailand/79/2004(H5N1)	AY651548
A/little grebe/Thailand/Phichit-01/2004(H5N1)	DQ407248
A/mallard/Vietnam/3/2003(H5N1)	DQ493215
A/dog/Thailand-Suphanburi/KU-08/04(H5N1)	DQ530177
A/Goose/Guangdong/178/04(H5N1)	AY028445
A/Tuekey/England/50-92/91(H5N1)	U85447
A/Chicken/NY/13142-5/94(H5N1)	AF001409
A/Duck/Michigan/1980(H5N1)	U85381

Sequence analysis

Assembly of the sequences, translation of the nucleotide sequences in protein sequences, pair wise alignment, and multiple alignment were performed using the BioEdit program version 7.0.7 (Hall, 1999). Calculation of variable site and non-synonymous/synonymous ratios were performed with DnaSP version 4.10.9 (Rozas *et al*, 2003) and K-estimator version 6.0 (Comeron, 1995, 1999).

The sequence samples in this study and additional data of other related sequences of the NS gene H5N1 avian influenza virus from the GenBank database (Table 2) were used for phylogenetic analysis. The multiple alignments were performed with clustalX, version 1.83, before the phylogenetic tree was constructed by PHYLIP (Phylogeny inference package) software, version 3.66.

RESULTS

Genetic characterization

The nucleotide sequences of NS genes of 80 isolates of H5N1 avian influenza viruses were completely identified. The data from this study are available from GenBank under the accession number EU153264 to EU153343.

In this study, the NS1 protein of all 80 isolates of H5N1 was found to have five amino acids at positions 80-84. The length of this NS1 protein was 225 amino acids. The NS2 protein in all isolates contained 121 amino acids residues. All NS1 and NS2/NEP genes shared the first 30 nucleotides of the coding region. All NS1 genes were 678 nucleotides in length (including stop codon). The NS2/NEP genes had 457 nucleotides in their one intron, making the second exon reside in a different reading frame than the gene NS1. The lengths of the NS1 and NS2/NEP proteins were 225 and 121 amino acid residues, respectively. The NS2/NEP genes of all H5N1 samples also had the same length. The lengths of all NS coding sequence, non-overlapping and overlapping regions of the gene NS1 and NS2/NEP, percent identity, and numbers of variable sites in each region are summarized in Table 3.

In regard to genetic variation, analysis of the coding region, the non-synonymous/synonymous ratio for the NS2/NEP region (0.394805) was higher than the NS1 region (0.255446) (data not shown). There were 20 groups of amino acid substitutions in the NS1

Table 3
The percentages of identity and the numbers of variable site comparisons of the NS1 and NS2/NEP coding sequences and deduced amino acid sequences.

	Lengths	% Identity			Variable site	
		Max	Min	Average	Number	%
All coding sequence	823	100	98.5	99.46	55	6.68
Non-overlapping	602	100	98.3	99.46	46	7.64
Overlapping	221	100	98.6	99.51	9	4.07
NS1 gene	678	100	98.5	99.48	46	7.09
NS2/NEP gene	366	100	98.0	99.49	18	4.91
Amino acids in NS1	225	100	97.3	99.57	22	9.77
Amino acids in NS2/NEP	121	100	96.7	98.81	13	10.74

protein and 14 groups of amino acid substitutions in the NS2/NEP protein as shown in Tables 4 and 5, respectively.

Phylogenetic tree analysis

The coding region of the NS1 gene from 35 different H5N1 influenza virus samples from this study and another 11 NS1 genes of H5N1 influenza viruses obtained from the GenBank database were used for phylogenetic analysis (Fig 1). The results reveal there were 35 NS1 genes located on allele A. The NS1 gene for H5N1 viruses isolated in Thailand could be divided into two different subgroups.

DISCUSSION

Since the outbreak of the H5N1 highly pathogenic avian influenza (HPAI) viruse epidemic in Thailand in 2004, these viruses have spread rapidly to many provinces resulting in the death of more than 62 million birds. In humans, at least 14 deaths and 22 cases of H5N1 HPAI infection have been reported (Songserm *et al*, 2006; Maton *et al*, 2007). There have been few studies on the NS genes of H5N1 viruses in Thailand. In the present study, the NS coding sequences of H5N1 avian influenza were completely analyzed. The NS1 gene of influenza A viruses is generally divided into alleles A and B (Suarez and Perdue, 1998). Phylogenetic analysis in this study indicates that all the isolates of NS1 genes of H5N1 influenza viruses belonged to allele A and are subdivided into two subgroups. The subgroups are divided by nucleotide substitutions. Analysis of sequence identity and variable sites of the NS coding sequence, NS1 gene, and NS2/NEP gene indicate the genes are highly conserved (identity in more than 99%). The NS1 proteins were more conserved than the NS2/NEP proteins. This is further supported by calculations of non-synonymous/synonymous ratios of the two genes. The value for the NS1 gene is lower than the NS2/NEP gene (NS1 0.25546 and NS2/NEP 0.394805). Our results are different from the study by Suarez and Perdue (1998), but are similar to the study done in Israel (Wang *et al*, 2005; Banet-Noach *et al*, 2007). In addition, the numbers of variable sites in non-overlapping regions (7.64%) were higher than those in the overlapping regions (4.07%).

The NS2/NEP protein function depends on the nuclear export signal (NES) motif in its N-terminus region. The amino acid sequence in this region is highly conserved. The amino acid sequence of NES and the NS2/NEP sequence in the A/WSN/33 viruses has previously been

Table 4
Amino acid substitution in the NS1 protein.

Groups	Amino acid substitution	Sample number (as describe in Table 1)
1	-	01, 02, 06, 07, 10, 12, 13, 15, 16, 17, 20, 21, 23, 24, 25, 26, 28, 31, 32, 33, 34, 35, 36, 37, 38, 42, 43, 47, 49, 51, 52, 55, 56, 57, 58, 59, 65, 66, 67, 70, 71, 72, 75, 76, 77, 78, 80
2	D53G	04, 40, 46, 62, 73
3	E91K	05
4	V65M	08
5	L27M	09
6	D53N	11
7	R95G	14
8	L90P	18
9	R67W	19
10	S3P	22
11	M79L	48
12	E71K	50, 53, 61
13	A81T, R95K, I123V, E191K	68
14	D134G	03
15	N171I	27, 29, 30, 45, 54, 64, 79
16	A197V	39, 44
17	V122T, A127G	63
18	I140M	74
19	I221T	41, 60
20	P80S	69

Table 5
Amino acid substitution in the NS2/NEP protein.

Groups	Amino acid substitution	Sample number (as described in Table 1)
1	-	01, 02, 03, 04, 05, 07, 08, 09, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 32, 34, 40, 42, 46, 53, 62, 63, 68, 73
2	A22E	06
3	R86K	15
4	I89V	41, 60
5	S3P	22
6	L45F, R61K	39, 44
7	K39R, R61K	48
8	R42K, R61K	67, 69, 70, 71, 72, 80
9	R61K, E82G	56
10	R61K	26, 31, 33, 35, 36, 37, 38, 43, 47, 49, 51, 55, 66, 74, 75, 76, 77, 78
11	I80V, A115T	57
12	R61K, A115T	65
13	M19L, I80V	27, 29, 30, 45, 55, 64, 79
14	I80V	23, 24, 25, 28, 50, 52, 58, 59, 61

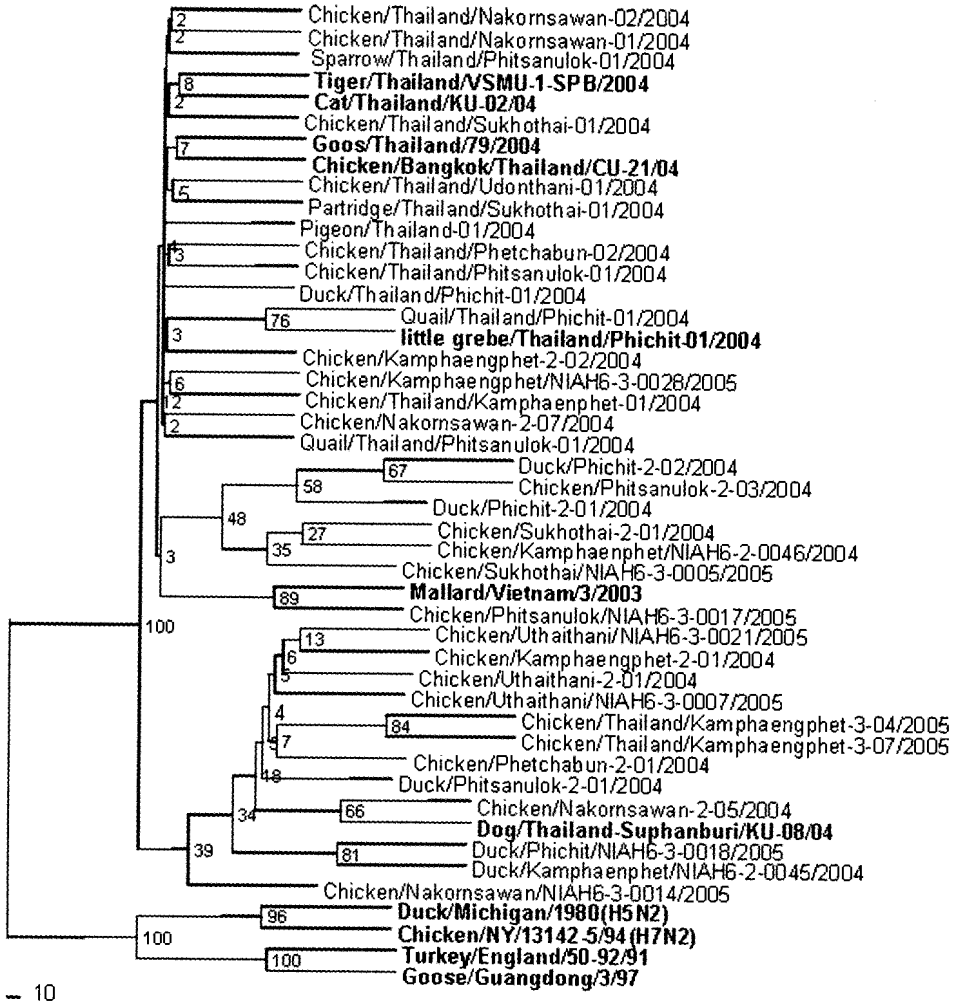

Fig 1–Phylogenetic relationships in influenza A virus based on the nucleotide sequences of the nonstructural genes generated using the DNA maximum likehood method. Samples with bold type face were taken from the GenBank as indicated in Table 2.

identified to be $_{12}$ILMRMSKMQL$_{21}$ (Iwatsuki-Horimoto *et al*, 2004). The NES sequence of the NS2/NEP H5N1 samples in this present is located at $_{12}$ILVRMSKMQL$_{21}$.

NS1 proteins have been found only in infected cells. These proteins regulate many cell functions in infection (Lin *et al*, 2007). The NS1 protein has two nuclear localization signals (NLS1 and NLS2) (Greenspan *et al*, 1988; Qian *et al*, 1994). NLS1 is conserved in all avian influenza viruses that contain a stretch of basic amino acids at positions 34 to 38 (DRLRR). NSL2 is found between amino acids 223 and 237 at the C-terminus. The basic amino acid residues at positions 231 and 232 are also part of the nucleolar localization signal (NoLS). The nuclear accumulation of NS1 protein may contribute to efficient viral replication in vero cells (Greenspan *et al*, 1988; Melen *et al*, 2007; Ozaki and Kida, 2007). In our samples, no NS1 proteins had NSL2 at their C-terminus. NS1 function is important to viral replication and the host immune response. The NS1 protein can act as antagonist to the human alpha/beta

interferon response and prevent the activation of NF-kappa B. Some molecular changes affect the virulence of H5N1 influenza viruses. H5N1 viruses contain amino acid deletions at 80 to 84 leading to an amino acid change at position 92 (D92E). These viruses induce an increased cytokine response (Wang *et al*, 2000; Seo *et al*, 2004; Hayman *et al*, 2007). NS1 protein in this study contained an amino acid substitution at 92. Another molecular mutation in NS1 protein is an amino acid substitution at the 149 amino acid residue (E149V). NS1 protein from H5N1 samples did not vary at this position (Li *et al*, 2006b). The RNA binding domains of NS1 protein in our H5N1 samples were at R38 and K41. These amino acids are important for the functioning of this domain (Wang *et al*, 1999; Lin *et al*, 2007). The effector domain of the NS1 protein binds to the 30 kDa CPSF (binding site at 144 to 186 amino acid residues) and for the PAB II at the amino acid region 223 to 237 (Nemeroff *et al*, 1998; Chen *et al*, 1999; Twu *et al*, 2006). The effector domain at amino acid residues 123 to 127 interacts with the PKR to inhibit the function of PKR (Bergmann *et al*, 2000; Li *et al*, 2006a; Min *et al*, 2007). The binding site of PKR in our NS1 protein was conserved among H5N1 Thailand isolates. These isolates were not the PBA II binding site in the NS1 protein because the NS1 protein of all H5N1 Thailand isolates was 225 amino acid residues long. The 3′ end C-terminus sequence motifs of some H5N1 influenza viruses were predicted to mediate binding to a PDZ domain. This domain plays an important role in many key signaling pathways. NS1 proteins in all isolates were contained in the EPEV amino acid residues at the NS1 3′ end sequence (Obenauer *et al*, 2006).

Other roles of the NS1 proteins include its association with anti-apoptosis or the programed cell death of host cells. A recent report in Israel showed that influenza A viruses containing K70, E76, G86, A143, and T171 have high apoptosis inducing activity (Schultz-Cherry *et al*, 2001; Ito *et al*, 2002; Banet-Noach *et al*, 2007). The NS1 protein in our H5N1 samples showed the amino acid sequences of E70, A76, T86, R143, and N or I 171.

ACKNOWLEDGEMENTS

A part of this work was supported by Agriculture Research Development Agency (Public Organization). The authors would like to thank Miss Purinthra Pensuwan for her technical assistant and the Northern Veterinary Research and Development Center for the viral specimens and Naresuan University Hospital for the use of the automated DNA sequencer.

REFFERENCES

Banet-Noach C, Panshin A, Golender N, *et al.* Genetic analysis of nonstructural genes (NS1 and NS2) of H9N2 and H5N1 viruses recently isolated in Israel. *Virus Genes* 2007; 34: 157-68.

Barclay WS, Zambon M. Pandemic risks from bird flu. *BMJ* 2004; 328: 238-9.

Bergmann M, Garcia-Sastre A, Carnero E, *et al.* Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* 2000; 74: 6203-6.

Burgui I, Aragon T, Ortin J, Nieto A. PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. *J Gen Virol* 2003; 84: 3263-74.

Chen GW, Yang CC, Tsao KC, *et al.* Influenza A virus PB1-F2 gene in recent Taiwanese isolates. *Emerg Infect Dis* 2004; 10: 630-6.

Chen Z, Li Y, Krug RM. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. *Embo J* 1999; 18: 2273-83.

Comeron JM. K-Estimator: calculation of the number of nucleotide substitutions per site and the confidence intervals. *Bioinformatics* 1999; 15: 763-4.

Comeron JM. A method for estimating the numbers of synonymous and nonsynonymous substitu-

tions per site. *J Mol Evol* 1995; 41: 1152-9.

Govorkova EA, Rehg JE, Krauss S, *et al.* Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J Virol* 2005; 79: 2191-8.

Greenspan D, Palese P, Krystal M. Two nuclear location signals in the influenza virus NS1 nonstructural protein. *J Virol* 1988; 62: 3020-6.

Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999; 41: 95-8.

Hatada E, Fukuda R. Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol* 1992; 73 (Pt 12): 3325-9.

Hayman A, Comely S, Lackenby A, *et al.* NS1 proteins of avian influenza A viruses can act as antagonists of the human alpha/beta interferon response. *J Virol* 2007; 81: 2318-27.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 2001; 146: 2275-89.

Ito T, Kobayashi Y, Morita T, Horimoto T, Kawaoka Y. Virulent influenza A viruses induce apoptosis in chickens. *Virus Res* 2002; 84: 27-35.

Iwatsuki-Horimoto K, Horimoto T, Fujii Y, Kawaoka Y. Generation of influenza A virus NS2 (NEP) mutants with an altered nuclear export signal sequence. *J Virol* 2004; 78: 10149-55.

Li KS, Guan Y, Wang J, *et al.* Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 2004; 430: 209-13.

Li S, Min JY, Krug RM, Sen GC. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 2006a; 349: 13-21.

Li Z, Jiang Y, Jiao P, *et al.* The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. *J Virol* 2006b; 80: 11115-23.

Li Y, Yamakita Y, Krug RM. Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein. *Proc Natl Acad Sci USA* 1998; 95: 4864-9.

Lin D, Lan J, Zhang Z. Structure and function of the NS1 protein of influenza A virus. *Acta Biochim Biophys Sin (Shanghai)* 2007; 39: 155-62.

Maton T, Butraporn P, Kaewkangwal J, Fungladda W. Avian influenza protection knowledge, awareness, and behaviors in a high-risk population in Suphan Buri Province, Thailand. *Southeast Asian J Trop Med Public Health* 2007; 38: 560-8.

Melen K, Kinnunen L, Fagerlund R, *et al.* Nuclear and nucleolar targeting of influenza A virus NS1 protein: striking differences between different virus subtypes. *J Virol* 2007; 81: 5995-6006.

Min JY, Krug RM. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc Natl Acad Sci USA* 2006; 103: 7100-5.

Min JY, Li S, Sen GC, Krug RM. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology* 2007; 363: 236-43.

Murayama R, Harada Y, Shibata T, *et al.* Influenza A virus non-structural protein 1 (NS1) interacts with cellular multifunctional protein nucleolin during infection. *Biochem Biophys Res Commun* 2007; 362: 880-5.

Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. *Mol Cell* 1998; 1: 991-1000.

Neumann G, Hughes MT, Kawaoka Y. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *Embo J* 2000; 19: 6751-8.

Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet* 2003; 362: 1733-45.

Noah DL, Twu KY, Krug RM. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAS. *Virology* 2003; 307: 386-95.

Obenauer JC, Denson J, Mehta PK, *et al.* Large-scale sequence analysis of avian influenza isolates. *Science* 2006; 311: 1576-80.

OIE. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: World Organization for Animal Health (OIE), 2004.

Ozaki H, Kida H. Extensive accumulation of influenza virus NS1 protein in the nuclei causes effective viral growth in vero cells. *Microbiol Immunol* 2007; 51: 577-80.

Palese P, Shaw M. Orthomyxoviridae: the viruses and their replication. In: Knipe D, Griffin D, Lamb R, *et al*, eds. Fields virology. Vol 2. Philadelphia: Lippincott Williams & Wilkins, 2007: 1647-89.

Porter AG, Smith JC, Emtage JS. Nucleotide sequence of influenza virus RNA segment 8 indicates that coding regions for NS1 and NS2 proteins overlap. *Proc Natl Acad Sci USA* 1980; 77: 5074-8.

Qian XY, Alonso-Caplen F, Krug RM. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J Virol* 1994; 68: 2433-41.

Qian XY, Chien CY, Lu Y, Montelione GT, Krug RM. An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. *Rna* 1995; 1: 948-56.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 2003; 19: 2496-7.

Satterly N, Tsai PL, van Deursen J, *et al.* Influenza virus targets the mRNA export machinery and the nuclear pore complex. *Proc Natl Acad Sci USA* 2007; 104: 1853-8.

Schultz-Cherry S, Dybdahl-Sissoko N, Neumann G, Kawaoka Y, Hinshaw VS. Influenza virus NS1 protein induces apoptosis in cultured cells. *J Virol* 2001; 75: 7875-81.

Seo SH, Hoffmann E, Webster RG. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. *Virus Res* 2004; 103: 107-13.

Smith GJ, Naipospos TS, Nguyen TD, *et al.* Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. *Virology* 2006; 350: 258-68.

Songserm T, Jam-on R, Sae-Heng N, *et al.* Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerg Infect Dis* 2006; 12: 575-81.

Suarez DL, Perdue ML. Multiple alignment comparison of the non-structural genes of influenza A viruses. *Virus Res* 1998; 54: 59-69.

Twu KY, Noah DL, Rao P, Kuo RL, Krug RM. The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. *J Virol* 2006; 80: 3957-65.

Viseshakul N, Thanawongnuwech R, Amonsin A, *et al.* The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. *Virology* 2004; 328: 169-76.

Wang S, Shi WM, Mweene A, Wei HL, Bai GR, Liu JH. Genetic analysis of the nonstructural (NS) genes of H9N2 chicken influenza viruses isolated in China during 1998-2002. *Virus Genes* 2005; 31: 329-35.

Wang W, Riedel K, Lynch P, Chien CY, Montelione GT, Krug RM. RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. *Rna* 1999; 5: 195-205.

Wang X, Li M, Zheng H, *et al.* Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol* 2000; 74: 11566-73.

Webster RG, Hulse DJ. Microbial adaptation and change: avian influenza. *Rev Sci Tech* 2004; 23: 453-65.

Wolff T, O'Neill RE, Palese P. NS1-Binding protein (NS1-BP): a novel human protein that interacts with the influenza A virus nonstructural NS1 protein is relocalized in the nuclei of infected cells. *J Virol* 1998; 72: 7170-80.

EPIDEMIOLOGY OF INTUSSUSCEPTION IN MALAYSIA: A THREE-YEAR REVIEW

Chan Lee Giak[1], HSS Amar Singh[2], Revathy Nallusamy[3], Teoh Yee Leong[4], Timothy L Ng[4] and Hans L Bock[4]

[1]Pediatric Department, Sarawak General Hospital, Sarawak; [2]Pediatric Department, Hospital Ipoh, Perak; [3]Pediatric Department, Hospital Pulau Penang, Penang, Malaysia; [4]GlaxoSmithKline Biologicals, Rixensart, Belgium

Abstract. This study aimed to document the baseline incidence and epidemiology of intussusception (IS) in Malaysia. This retrospective surveillance examined hospital discharge data from three hospitals in Malaysia to identify IS cases over a 3-year period (2000-2003) in children <5 years of age. Identification of definite cases of IS was done through a search of computerized hospital discharge records (ICD-9-CM code 560.0) followed by confirmation of diagnosis through medical record review. The definition of IS was based on the clinical guidelines from the IS Brighton Collaboration Working Group, version 2002. During the 3-year study period, there were 62 cases hospitalized due to IS, of which 74.2% were <1 year of age. The incidences for hospitalization due to IS in children <1 year old and <5 years old averaged 17.8 and 4.8 per 100,000 person-years, respectively. No IS-associated deaths were recorded and all IS cases had a favorable outcome. No distinct seasonality with IS occurrence was observed.

INTRODUCTION

Rotavirus (RV) has been identified as the most common cause of severe gastroenteritis (GE) in infants and young children throughout the world (Salinas *et al*, 2004), leading to more than 600,000 deaths per year in the developing countries (Parashar *et al*, 2003, 2006). Of these deaths, 90% occur in the poorest countries of Africa and Asia (Parashar *et al*, 2003; Bresee *et al*, 2005; Glass, *et al*, 2005).

In Southeast Asia, RV is estimated to cause death in 1 out of every 111-203 Bangladeshi children (Unicomb *et al*, 1997) and up to a staggering 100,000 deaths in India every year (Jain *et al*, 2001). A recent study conducted in Malaysia showed that RV disease is the cause of one-half of all hospitalizations for acute GE. Approximately, 23,000 inpatient and outpatient visits for acute GE were caused by RV annually. These data show the significant burden of RV disease in Malaysia (Hsu *et al*, 2005).

In the United States severe RV disease is associated with 500,000 physician visits per year, with 60,000 hospitalizations, 20-40 deaths, and annual costs exceeding $1 billion (Glass *et al*, 2005).

These alarming figures led the World Health Organization (WHO), the Institute of Medicine, and the Global Alliance for Vaccines and Immunization (GAVI), to identify RV vaccines as a priority for development (Glass *et al*, 2006). However, the first licensed rotavirus vaccine, *RotaShield*™ (Wyeth-Lederle), which was highly efficacious in preventing severe GE and hospitalizations due to RV infection, was suspended 9 months after introduction due to reports of a temporal association between

Correspondence: Dr Chan Lee Gaik, 23 Pediatric Department, Sarawak General Hospital, Jalan Tun Ahmad Zaidi Adruce, 93586 Kuching, Sarawak, Malaysia.
Tel: +60-82-276666; Fax: +60-82-242087
E-mail: dr_chanlg@hotmail.com; hsclgc@tm.net.my