HUMAN PARAINFLUENZA VIRUS INFECTION IN THAI CHILDREN WITH LOWER RESPIRATORY TRACT INFECTION FROM 2010 TO 2013

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Abstract. Human parainfluenza virus (HPIV) is a common cause of upper and lower respiratory illness in infants and young children. In order to classify the HPIV isolates circulating in the central part of Thailand, 650 samples obtained from the lower respiratory tract of patients from two hospital pediatric wards during 2010 to 2013, were analyzed for the presence and types of HPIVs by multiplex semi-nested PCR of hemagglutinin-neuraminidase (HN) gene. The results showed that 4.8% of the samples were positive for HPIV, among which 0.5%, 2.5% and 1.5% were positive for HPIV-1, HPIV-3, and HPIV-4, respectively, and none were positive for HPIV-2. A phylogenetic tree constructed from 31 HPIV HN gene sequences compared to those in GenBank showed greater than 80% identity to other reference strains. Prevalence of HPIV infection and phylogenetic characteristics of the circulating HPIVs may help explain the impact of HPIVs infection in Thai children.

Keywords: human parainfluenza virus (HPIV), classification, epidemiology, hemagglutinin-neuraminidase gene, Thailand

INTRODUCTION

Respiratory tract infections (RTIs) affecting the sinus, throat, airway or lung are usually caused by viruses or bacteria. Bacterial pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b, are estimated to account for 36% and 16%, respectively, of the global pneumonia mortality in children under 5 years of age in 2000 (O'Brien *et al*, 2009; Watt *et al*, 2009). Major viral contributors to childhood RTIs are respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), human metapneumovirus (HMPV), and influenza virus (Murphy, 1988; Forster *et al*, 2004; Mullins *et al*, 2004).

First discovered in 1959, HPIV is a common virus that causes acute RTI worldwide including rhinitis, otitis, laryngotracheobronchitis, bronchitis, and pneumonia (Billaud *et al*, 2005). Asymptomatic infections are also suspected from infections with HPIV (Laurichesse *et al*, 1999). HPIV is an enveloped single

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strand negative sense RNA virus belonging to Paramyxoviridae family. This virus can be divided into two groups, namely, *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4). HPIV-4 is further divided into 4A and 4B subtypes by hemagglutination inhibition and neutralizing tests (Lau *et al*, 2009). Its genome encodes at least six structural common proteins: nucleoprotein (NP), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large protein (L) (Vainionpaa and Hyypia,1994). HPIV envelope contains two major surface glycoproteins, HN and F (Vainionpaa and Hyypia,1994). HN glycoprotein plays dual biological functions, that of a hemagglutinin and a neuraminidase, and also plays an essential role in promoting fusion by the F protein, and thus regulates the interaction between virus and host cells (Mao *et al*, 2012). In addition, HN glycoprotein possesses the largest antigenic and genetic differences among HPIV types and strains within one type (van Wyke Coelingh *et al*, 1987; Klippmark *et al*, 1990; Henrickson, 1991; Palermo *et al*, 2009). Therefore, HN glycoprotein, the target for protective humoral immunity, has been used widely for typing HPIV in molecular epidemiological investigations (Henrickson,1991; Zambon *et al*, 1998; Jalal *et al*, 2007; Lau *et al*, 2009).

HPIV is commonly responsible for many respiratory tract symptoms, which can be found in both children and adults. Risk factors that increase the incidence and severity of lower respiratory infections in patients living in developing countries include large family size, crowded living condition, low birth weight, malnutrition, vitamin A deficiency, lack of breast feeding, pollution, and young age (Berman, 1991). In infants and young children, symptoms of HPIV infection of the upper respiratory tract include fever, runny nose, and cough. HPIV can cause a more severe lower respiratory illness, such as croup or pneumonia, especially in young children (Henrickson, 2003).

Although HPIV causes many illnesses at low rates, patients often have severe symptoms (Rubin *et al*, 1993) especially those undergoing bone marrow transplant (Zambon *et al*, 1998). HPIV circulates at low levels throughout the year (Billaud *et al*, 2005). In temperate countries, an association between the HPIV types and seasonal incidence of respiratory viruses has been reported (Laurichesse *et al*,1999). In tropical countries, however, correlation between respiratory viral incidences and seasons are not so well defined, which may suggest that more complex interactions are involved (Olsen *et al*, 2010).

Surveys of HPIV prevalence in neighboring countries of Thailand found 3.74% in China and 3.48% in Malaysia (Fe *et al*, 2008; Mao *et al*, 2012). Previous studies found that HPIV-1 and HPIV-3 are common in China, Japan, Malaysia, and Brazil (Fe *et al*, 2008; Mizuta *et al*, 2011; Khor *et al*, 2012; Mao *et al*, 2012), while HPIV-2 and HPIV-4 predominate in North America and Canada (Henrickson, 2003; Vachon *et al*, 2006). In the United States, the most common HPIV types associated with respiratory illness are HPIV-1 and HPIV-3, whereas HPIV-2 and HPIV-4 are less frequently detected (Fry *et al*, 2006). HPIV-3 circulates annually with seasonal peaks in the spring and summer months, while HPIV-1 peaks biannually in the fall during odd-numbered years (Fry *et al*, 2006). In tropical countries, a seasonal pattern is observed whereby the proportion of HPIV-positive cases tend to be highest between January and April, but not always limited to a single type. Studies of HPIV infection in Southeast Asia also

found seasonal peaks: a 2-year study in Thailand found a February-March peak, while a 4-year study in Singapore found a February-May peak (Suwanjutha *et al*, 1990; Chew *et al*, 1998).

Thus far, there is little information regarding the disease burden or prevalence of HPIVs in Thailand. The aim of this study was to investigate the prevalence of HPIVs in pediatric patients under 5 years of age from central Thailand with lower respiratory tract illness during the years 2010 to 2013. In addition, classification of HPIV types was determined based on the genetic variations within HN gene. Knowledge gained from this study will be useful for the surveillance and prevention of this respiratory virus infection in the future.

MATERIALS AND METHODS

Sample collection

Respiratory specimens (nasopharyngeal aspirate, endotracheal tube suction, and tracheal suction or bronchoalveolar lavage) were collected during July 2010 to November 2013 from patients under five years of age with acute lower respiratory tract infection admitted to two pediatric wards, King Chulalongkorn Memorial Hospital, Bangkok and Chon Buri Hospital, Chon Buri Province, both located in central Thailand. Inclusion criteria included fever over 37.8ºC and a tachypnea with the following respiratory conditions: respiratory rate ≥ 60, ≥ 50, ≥ 40, and ≥ 30 beats/min for patients < 2 months, between 2 months and 1 year, between 1 to 5 years, and > 5 years, respectively. All specimens were kept in viral transport media, which contains minimum essential medium Hanks salts 10.7 g/l, lactalbumin 0.25% w/v, glycerol 1% v/v, penicillin 4 unit/ml, streptomycin 4 mg/ml, polymy-

cin B sulfate 1 unit/ml, nystacin 0.25 unit/ml, gentamicin 1.25 mg/ml. The RNA extraction was performed upon arrival at Center of Excellence in Clinical Virology. Subsequently, RNA was frozen at -70ºC immediately until used.

The study protocol was approved by the institutional review board of the Faculty of Medicine, Chulalongkorn University (IRB378/56). The study was conducted on clinical specimens collected during routine examinations and stored as anonymous specimens. Patient's identifiers including personal information (name, address) and hospital identification number were removed from these samples to protect patient's confidentiality and did not appear in any part of the documents of the study. Permission for specimen utilization was granted by the Director of King Chulalongkorn Memorial Hospital.

Multiplex semi-nested RT-PCR assay

Total RNA was extracted from 200 l of each respiratory specimen using HiYield Viral Nucleic Acid Extraction kit (RBC Real Genomics, Taipei, Taiwan) following manufacturer's instructions. Total RNA was converted to cDNA using ImProm-II Reverse Transcription System (Promega, Madison, WI) with 10 M random hexamers and stored at -20ºC until used.

Nucleotide sequences of the HPIV HN gene were obtained from GenBank database (accession numbers AB367954, AB542810, AB543337, AB753465, AB753469, AB753478, AB753481-2, AB753484, D00865.1, EU326526.1, EU627591, EU814626.1, FJ455842.2, GU732171, HM460888, JX131646, KF483663, KF530212, KF687308, KF687311-14 and KF687328). Multiple sequence alignments were performed using Clustal W implemented in BioEdit (version 7.2.5). PCR primers were selected from the

Table 1
Primers used for multiplex semi-nested PCR assay for HPIV detection and typing.

Primer	Sequence (5′→3′)[a]	Application
F_PIV 1	<u>ATTTAG GTGCACTATAG</u>ACACCAGCAGGAAGGACACA	First and second round PCR
F_PIV 2	<u>ATTTAGGTGACACTATAG</u>AAATCAATTCCTAAAAGGACATGT	
F_PIV 3	<u>ATTTAGGTGACACTATAG</u>CTCACCAACAAGATAACATATAT	
F_PIV 4	<u>ATTTAGGTGACACTATAG</u>ATTCAATGGTGAAAAGAACATGG	
R1_PIV1&3	TAA**W**AC**Y**TGATATGA**Y**TTCCCTATA	First round PCR
R1_PIV2&4	ATTA**Y**ATTGTG**R**GT**R**TAACACCAAT	
R2_PIV 1	CCTACGGGACATCTCCAGA	Second round PCR
R2_PIV 2	TTAGGCGTCCCATTGTATGATT	
R2_PIV 3	CCAGACGTGCATCTCCAGA	
R2_PIV 4	GAGGAGTTGTAGCAGTTGGAA	

[a]Underline indicates SP6 promoter sequence used for nucleotide sequencing.
R = A or G, **W** = A or T, **Y** = C or T.

conserved regions of HN gene and the specificity of each primer was verified by BLAST analysis. The primers used are listed in Table 1.

The resulting cDNA was PCR amplified in the first round using four forward primers (F_PIV 1, F_PIV 2, F_PIV 3, and F_PIV 4) and two reverse primers (R1_PIV1&3 and R1_PIV2&4), and in the second round using the same forward primers and four other reverse primers (R2_PIV 1, R2_PIV 2, R2_PIV 3, and R2_PIV 4). PCR was carried out in a 25 μl reaction mixture containing 1 μl of cDNA template (for first round PCR) or PCR product (for second round PCR), 1X Perfect *Taq* Master Mix (5 PRIME, Darmstadt, Germany), 0.1 μM each primer, 1.5 mM MgCl$_2$ and nuclease-free water. Thermocycling (Mastercycler® Personal; Eppendorf AG, Hamburg, Germany) was as follows: 94°C for 4 minutes; 40 cycles of 94°C for 45 seconds, 53 °C (first round PCR) or 55°C (second round PCR) for 45 seconds, and 72°C for 45 seconds; and a final step at 72°C for 7 minutes.

Amplicons were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide, and visualized by UV transilluminator (Gel Doc 1000, Bio-Rad, Hercules, CA). The expected amplicons for HPIV-1, HPIV-2, HPIV-3 and HPIV-4 are 428, 428, 422 and 486 bp, respectively.

Nucleotide sequencing and analysis

Amplicons from the second round PCR were purified from agarose gel using Expin™ Gel SV kit (GeneAll, Seoul, Korea) and were sent to First BASE Laboratories (Salangor Darul Ehsan, Malaysia) for direct nucleotide sequencing using a universal primer specific to the SP6 promoter (5′ ATTTAGGTGACACTATAG 3′).

Sequences were analyzed by SeqMan program (DNASTAR version 6). Identification and typing of HPIVs were performed using BLAST analysis (www.ncbi.nlm.nih.gov/Blast). Sequences were submitted to the GenBank database (accession numbers KJ187002 - KJ187032.). The nucleotide sequences were also aligned with reference sequences (from Australia, Canada, China, Denmark, France, India,

Table 2
HPIV types and respiratory samples from which they were isolated.

HPIV type	NPA (%)	Tracheal suction (%)	ET suction (%)	Bronchoalveolar lavage (%)	Total (%)
HPIV-1	5/613 (0.8)	0/27 (0)	0/8 (0)	0/2 (0)	5/650 (0.8)
HPIV-2	0/613 (0)	0/27 (0)	0/8 (0)	0/2 (0)	0/650 (0)
HPIV-3	14/613 (2.3)	1/27 (4)	0/8 (0)	0/2 (0)	15/650 (2.3)
HPIV-4	9/613 (1.5)	1/27 (4)	1/8 (12.5)	0/2 (0)	11/650 (1.7)

ET, endotracheal suction; NPA, nasopharyngeal aspirate.

Japan, Mexico, Saudi Arabia, UK, and USA) and used for phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0.5 software (http://www.megasoftware.net/). The phylogenetic tree was generated using Neighbor-Joining (NJ) algorithm with bootstrap resampling of 1,000 replicates.

Statistical analysis

Statistical data were analyzed using SPSS software (version 17; Chicago, IL). Data comparisons were performed by the chi-square test, and results are considered statistically significant at $p < 0.05$.

RESULTS

Prevalence of HPIV during 2010 to 2013

Out of a total of 650 lower respiratory tract samples, 613 (94.3%) were obtained from nasopharyngeal aspirate (NPA), 27 (4.1%) from tracheal suction, 8 (1.2%) from endotracheal tube suction (ET suction), and 2 (0.3%) from brochoalvelolar lavage. All respiratory specimens were tested for HPIV by multiplex semi-nested PCR assay, which revealed that 31 (4.8%) samples were positive for HPIV, including 5 patients infected with HPIV-1, 15 with HPIV-3, 7 with HPIV-4A, and 4 with HPIV-4B. HPIV-2 was not detected in the samples (Table 2). The proportions of HPIV infection per year were 5% (2/39) in 2010, 2% (4/228) in 2011, 8% (19/237) in 2012, and 4% (6/146) in 2013.

Seasonal trends

In 2011 and 2013, very few cases of respiratory tract infections occurred during the first half of the year (Fig 1). Most types of HPIV were detected throughout 2012, but HPIV-2 was not found in any of the samples. HPIV-1 was found in February and March, which correspond to the summer season in Southeast Asia. HPIV-3 was present all year-round, and HPIV-4 can be found most often in July to September, the rainy season.

Characteristics of HPIV infection

HPIVs were identified in samples from pediatric patients with respiratory tract diseases, such as pneumonia, bronchiolitis and bronchitis. The majority of symptoms associated with HPIVs infection was pneumonia. When stratified according to age, HPIV was detected in all age groups, but the majority of HPIV infection was found in children 13-24 months of age (Table 3). These were primarily HPIV-3 and HPIV-4. Single infection of HPIV was detected in 26/31 (84%) positive cases. Three cases were co-infected with rhinovirus (HRV), one case was co-infected with metapneumovirus (MPV), and one with respiratory syncytial
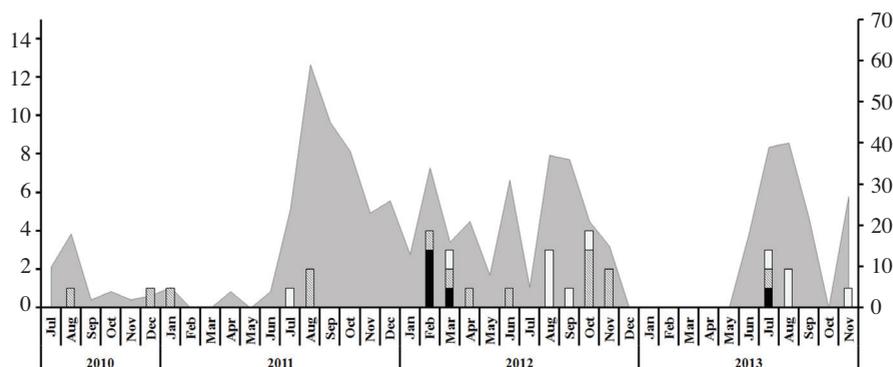
Fig 1-Seasonal variations of HPIVs from 2010 to 2013 among children < 5 years old with acute respiratory tract infection. HPIV detection and typing were conducted by multiplex semi-nested RT-PCR of hemagglutinin-neuraminidase gene. ■ , HPIV-1; ▨ , HPIV-3; ▢ , HPIV-4; ▨ , total specimens. * HPIV-2 not found in this study.

Table 3
Distribution of HPIV types isolated from patients of different age groups.

Age groups	HPIV-1	HPIV-2	HPIV-3	HPIV-4
0-3 months	0	0	0	1
4-6 months	2	0	1	1
7-12 months	1	0	1	2
13-24 months	2	0	9	7
3-5 years	0	0	4	0

virus (RSV) (Table 4). RSV and MPV infections generally are not seasonal, while HRV infection occurs most often during the rainy season. Thus, it is not surprising that we found more co-infections of HPIV with HRV. Clinical characteristics do not differ significantly between patients with single HPIV infection and co-infection.

Molecular classification of HPIVs and phylogenetic tree using HN gene

The genetic relatedness among the HPIV types identified in this study and those of the reference strains was analyzed by comparing the sequences of HN gene. Phylogenetic analysis showed that HPIV HN sequences are separated into two main groups, with each group being further classified into sub-branches that may be labeled as types (Fig 2). The average nucleotide identity of HPIV HN genes in this study, when compared with strains in other countries, was > 80%. Based on the phylogenetic tree constructed from HN nucleotide sequences, all 4 types of HPIVs were clearly separated with high bootstrap value of nearly 100. All HPIV-1 samples from this study were highly similar to the reference strains from Australia, France, Japan, South Africa and USA. Two of the 15 HPIV-3 positive samples were closely related to those from India (strain DEL/139/05) and Saudi Arabia (strain Riyadh 45/2008) with the nucleotide identity matrix of 99.5%. The other 13 samples were similar to strains from China, Japan and Mexico at 95.8%, 97.2% and 96.7% identity, respectively. HPIV-4 sequences identified from this study were clustered together with those from Japan (strains 1461-Yamagata-2010, 2266-Yamagata-2011, and 149-Yamagata-2012) with a bootstrap value of 99, but

Table 4
Single and co-infection of HPIV with other respiratory viruses.

Infection	HPIV-1	HPIV-2	HPIV-3	HPIV-4
Single infection	5	0	11	10
Co-infection with rhinovirus	0	0	3	0
Co-infection with metapneumovirus	0	0	1	0
Co-infection with respiratory syncytial virus	0	0	0	1
Co-infection with influenza virus	0	0	0	0

only one sample (KJ187024) has a base change T169C similar to the 222-Yamagata-2010 strain.

DISCUSSION

During 2010 to 2013, we identified HPIVs in 4.8% of the 650 respiratory specimens obtained from children with respiratory tract infection admitted to two hospital pediatric wards located in central Thailand. This prevalence of HPIV infection in Thailand is comparable to the previous studies in other countries, which ranged from 5% to 30% of hospitalized children with respiratory tract infection, depending on the year of study, case-definition, diagnostic technique used, type of specimen collected, and season (Downham, 1973; Templeton *et al*, 2004; Fe *et al*, 2008; Noh *et al*, 2013). The majority of pediatric patients in Thailand were infected with HPIV-3 (48%) and HPIV-4 (35%). For HPIV-3, the figure is different from the previous report of 3% in Sa Kaeo and Nakhon Phanom Provinces (Morgan *et al*, 2013). We found the higher rate of HPIV-4 (35%) than HPIV-1(17%), which is not unexpected because the total HPIV types appear to be very variable among different studies (Monto, 1973; Rubin *et al*, 1993; Laurichesse *et al*, 1999; Billaud *et al*, 2005). These variations in HPIV types reported may be attributed to the sensitivities of the detection methods employed

in different laboratories, particularly techniques used for HPIV culture and typing (Billaud *et al*, 2005). Other important factors that might affect the frequency of HPIV-4 are the duration of the study and the season and the year of observation (Billaud *et al*, 2005).

The absence of HPIV-2 in our study is not likely due to lack of sensitivity of the nested RT-PCR technique used because our method was able to directly detect other HPIV types in respiratory specimens, with a detection limit of 10 copies for HPIV (data not shown). Similar to other respiratory viruses in Thailand, HPIVs circulate at low levels throughout the year (Olsen *et al*, 2010). However, the seasonal trend of HPIV positive cases was similar to China (Liu *et al*, 2013), similar to other previous studies of HPIV infection in other Southeast Asian countries (Suwanjutha *et al*, 1990; Chew *et al*, 1998).

During the four years of observation, HPIV-3 was detected throughout the year, while HPIV-4 was observed in the rainy season. Biennial fall epidemics of HPIV-1 have been reported previously (Murphy *et al*, 1980; Marx *et al*, 1997; Carballal *et al*, 2001; Fry *et al*, 2006). HPIV-2 has also been reported to cause infections biennially together with HPIV-1, or in alternate year with HPIV-1, or causing yearly outbreaks (Downham, 1973; Murphy *et al*, 1980; Belshe *et al*, 1983). HPIV-3 has been
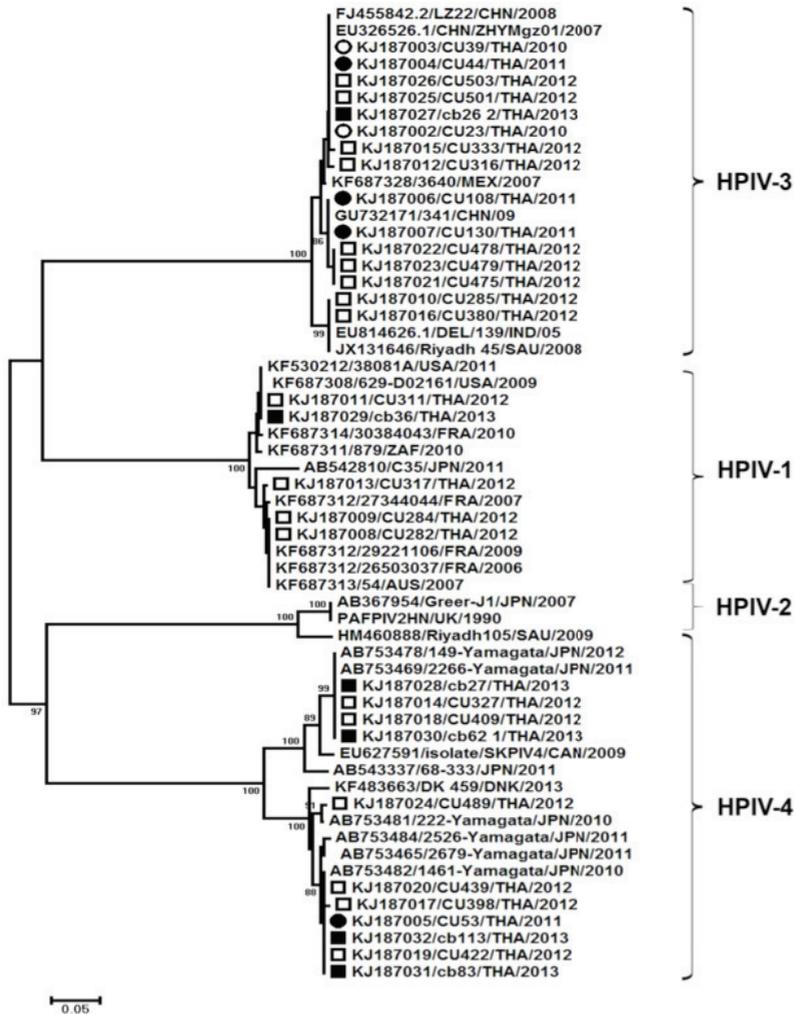
Fig 2–Phylogenetic analysis of HPIV hemagglutinin-neuraminidase gene from 31 clinical specimens. The scale bar represents the number of nucleotide substitutions per site. ○, 2010; ●, 2011; □, 2012; ■, 2013.

reported to occur annually during April to June in the United States (Fry *et al*, 2006). In temperate climate, the peak incidence of HPIV-4 infection is often in late fall and late winter months (Billaud *et al*, 2005; Lau *et al*, 2005; Vachon *et al*, 2006; Liu *et al*, 2013). However, other studies have detected peaks outside the usual seasons (Billaud *et al*, 2005; Ren *et al*, 2011), emphasizing a poorly understood epidemiology

and seasonality of HPIV infections (Fry *et al*, 2006).

As mentioned above, the lack of sufficient data and knowledge of the epidemiology of HPIV-4 have left many questions unanswered. The different geographic locations may result in different seasonal distributions of HPIVs (Liu *et al*, 2013). HPIV-1 and HPIV-2 are not isolated as frequently as HPIV-3 and HPIV-4. The
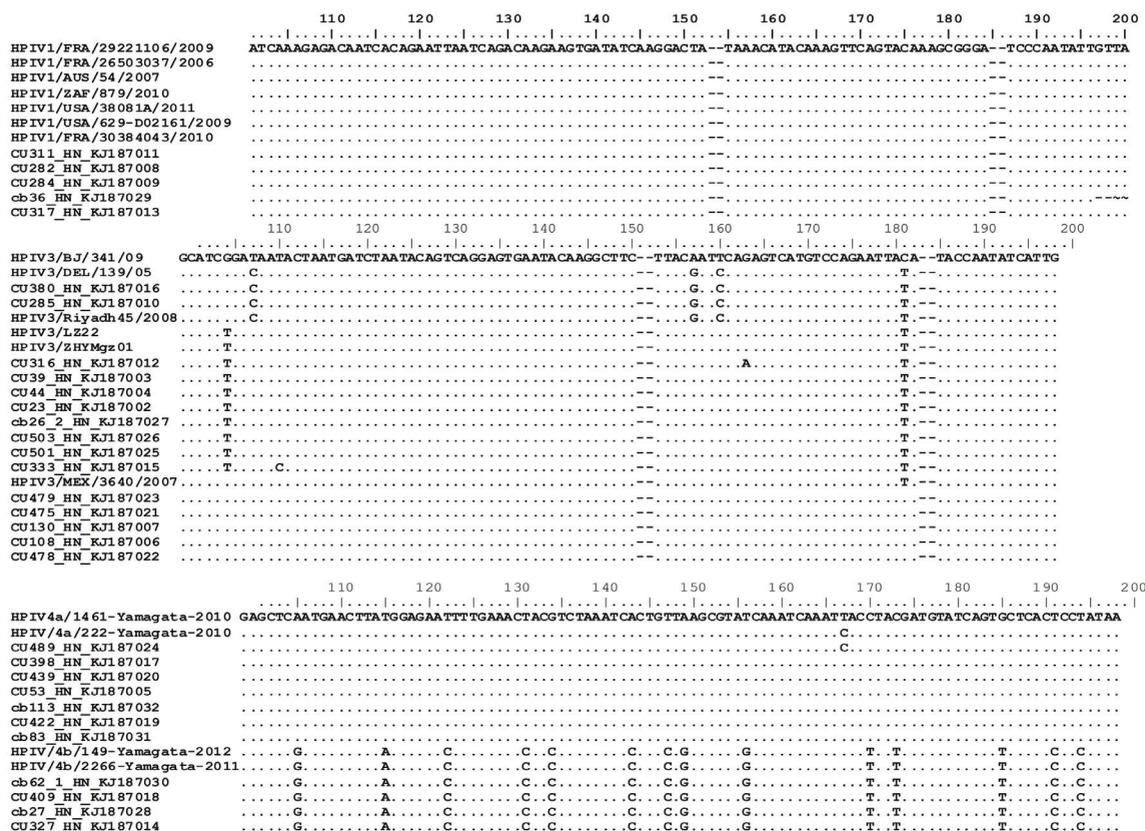
Fig 3–Nucleotide sequence alignment of hemagglutinin-neuraminidase gene of representative HPIV types and strains from other countries. Identical residues are indicated by dots.

frequencies of detection of HPIV-1 and HPIV-2 increase when detection of HPIV-3 and HPIV-1 decline.

Our study showed that HPIV infection was a major cause of lower respiratory tract infection, mostly in very young children (13-24 months of age). Due to the incomplete immunity to HPIVs, recurrence of infection is possible throughout life (Henrickson, 2003). HPIVs were detected in patients over a wide age distribution. However, many more children are infected than adults, with the vast majority of HPIV infections occurring in patients under 5 years of age (Liu *et al*,

2013). In this study, the four types of HPIVs varied among the age distribution of patients infected. HPIV-1 was mainly detected in patients less than 3 years, while HPIV-3 was isolated from a broader age distribution. There was no infant under one month of age who was HPIV positive. These results are in accordance with seroprevalence studies indicating that newborn infants have high levels of passive immunity to HPIV from the mother, and that these levels decrease substantially by 7 to 12 months of age (Henrickson, 2003).

Co-infection of HPIV with other

respiratory viruses was found for three viruses, namely, HRV, RSV, and MPV. Co-infections have been found in children, especially in patients under 5 years of age (Liu *et al,* 2013). This might indicate that immature immune systems of children leave them vulnerable to potential pathogens. However, no significant differences in clinical characteristics are seen between patients infected solely with HPIVs and patients co-infected with HPIVs and other respiratory viruses.

The phylogenetic analysis of HPIVs isolated showed an overall high level of nucleotide sequence identity (> 80%) of the HN region, suggesting that individual lineages of highly conserved HN HPIVs are widespread in the central region of Thailand. Sequence analysis of HN genes of HPIV-1 and HPIV-3 strains suggests that geographically defined genetic lineages might have developed (Henrickson and Savatski,1992; Hetherington *et al,* 1994). As shown by the phylogenetic tree, the isolates from Thailand and the reference sequences from different countries formed a unique branch, suggesting that variations within the HN gene of HPIV-3 could be correlated with the geographic origin of the strain (Mao *et al,* 2012). Most isolates of HPIV-4 from various countries in recent years were likely to be clustered into the same lineage (Henrickson and Savatski, 1992; Hetherington *et al,* 1994).

Although the prevalence of HPIV infections in the central part of Thailand was very low, it still poses a financial burden on the healthcare system. There is currently no vaccine or effective treatment available to such patients. The knowledge gained from this study should provide useful information for surveillance and prevention of the spread of this disease in the future.

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