HUMAN ADENOVIRUS IN PATIENTS WITH INFLUENZA-LIKE ILLNESS AND/OR ACUTE GASTROENTERITIS IN THAILAND, 2016

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Abstract. Human adenovirus (HAdV) causes a variety of clinical manifestations, such as respiratory tract infections and gastrointestinal diseases. The study determined the prevalence of HAdV infection and HAdV types in Thailand (Bangkok and Khon Kaen Province) in 2016. Nasopharyngeal (NP) and fecal samples were collected from patients with influenza-like illness (n=3,451) or acute gastroenteritis (n=948). Gene amplification and direct sequencing detected HAdV in 3.6% and 14.1% of NP and fecal specimens, respectively, with HAdV-B3(48%),HAdV-B7 (3%), HAdV-B11 (2%),HAdV-C1 (17%), HAdV-C2(18%), HAdV-C5(10%), HAdV-C6(1%) and HAdV-E4 (1%) in NP specimens, and HAdV-C2(22%), HAdV-D13(2%), HAdV-D17(1%), HAdV-D56(1%), and HAdV-F41 (31%) in fecal samples. HAdV-B3, HAdV-C1 and HAdV-C2 were commonly associated with respiratory tract infection, and HAdV-F41 with acute gastroenteritis. The results will be of use in understanding prevalence and distribution of HAdV infection in Thailand, and in developing more effective management and prevention measures together with provision of baseline data for future studies on prevalence and epidemiology of HAdV types in Thailand.

Keywords: acute gastroenteritis, adenovirus, influenza-like illness, Thailand

INTRODUCTION

Human adenoviruses (HAdVs)can cause various diseases, such gastrointestinal disease in infants and young children, respiratory tract infection (RTIs), ophthalmic infection, genitourinary illnesses, and hemorrhagic cystitis (Echavarria, 2009). HAdV is a non-enveloped, double-stranded linear DNA virus belonging to family Adenoviridae and genus *Mastadenovirus* (Rowe *et al,* 1953). Characterization of HAdV types is based on immunotypic and molecular methods (Heim *et al, 2003*). The latest HAdV genotype number 86 was assigned in November, 2017 (Human Adenovirus Working Group, 2017).

Of the seven adenovirus species (A - G), species A, B, C, and E are associated with symptomatic RTIs, with species B type 7 linked high rates of morbidity and mortality (World, 2007; Ampuero *et al*, 2012; Tsou *et al*, 2012; Tórtora *et al*, 2015). About 5-7% of RTIs in children are related to HAdV infection (Echavarria,

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2009), with mild but endemic and most commonly seen in young children to HAdV-C (Lee, 2010). HAdV-D56 is a major causative agent of epidemic keratoconjunctivitis (EKC) and urethritis, indicating a possibility of an increase in HAdV-D56 infection associated with genital infection rate in the future (Hiroi *et al*, 2012). HAdV-E (adenovirus 4) was reported as the cause of an epidemic acute respiratory disease in US military recruits (Jacobs *et al*, 2004). Types F40 and F41 are associated with gastroenteritis (causing diarrhea and vomiting) are the third most common cause of viral gastroenteritis in children, with a prevalence of 4-12% (Echavarria, 2009).

In vitro antiviral drug responses vary according to HAdV type (Morfin *et al*, 2005). However, several new viral RTIs and gastroenteritis diseases with epidemic potential to threaten global health security have emerged in the past 15 years (Al-Tawfiq *et al*, 2014). Furthermore, epidemiological surveillance of viral RTIs and viral gastroenteritis for early identification of etiological agents, especially at the start of outbreaks, and optimal and timely management of large numbers of samples are necessary to prevent the spread of pathogens (Al-Tawfiq *et al*, 2014).

Herein, we report the discovery of HAdV infections types and determine their prevalence, and also that of influenza virus, respiratory syncytial virus (HRSV), human rotavirus (HRV), human norovirus (HNoV), human sapovirus(HSaV), human cosavirus (HCoV), human astrovirus (HAstV), human safford virus(HSFV), human bocavirus (HBoV), human parechovirus (HPeV), human rhinovirus (HRV), human poliovirus (HPV), human echovirus (EcoV),and human coxsackie virus (HCV) in Thailand in 2016 to provide basic data towards an understanding of the infection of these various groups of viruses in the etiology of respiratory tract infection and gastroenteritis. Nasopharyngeal (NP) swabs or aspirate samples were collected from patients with RTI and fecal samples from patients with acute diarrhea. The resulting data would provide molecular prevalence and seasonal distribution of HAdV infection in the country. In addition, information obtained can be used to develop more effective management and prevention programs and provide benchmarks for future implementations.

MATERIALS AND METHODS

Study population

The study was carried out at the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB 286/58).

There were 3,451 NP samples, 2,756 and 695 from Bangkok and Chum Phae Hospital, Khon Kaen Province, northeastern Thailand, respectively. Mean ± SD age of subjects was 4.6 ± 3.7 years (range, 17days -15 years), of whom 1,896 were males and 1,555 females. NP swabs collected as part of a routine influenza surveillance of in- and out-patients with influenza-like illness, were placed into viral transport media and subjected to routine screening for influenza virus by quantitative and RT-PCR assays (Tewawong *et al*, 2017), and then analyzed for HAdV types as described below.

Of the 948 fecal samples, 868 and 80 were from Bangkok and Khon Kaen Province, respectively. Mean ± SD age of the subjects was 27.1 ± 26.5 years (range, 3 days -101 years), of whom 449 were males and 499 females. Inclusion criteria

were symptoms of watery diarrhea (≥3 episodes within 24 hours) with vomiting and/or fever. These fecal samples were subjected to routine screening for EcoV, HAdV, HAstV, HBoV, HCoV, HCV, HNoV, HPeV, HPV, HRV, HSaV, and HSFV, and by gene amplification (Chieochansin *et al*, 2015), and then analyzed for HAdV type as described below.

HAdV typing

DNA was extracted using RibospinvRD II Extraction Kit (GeneAll, Seoul, Korea) and stored at -20°C until used. Nested PCR was used to detect HAdV hexon using two set of primer pairs: first pair, ADV_ F1 5′-AYGCYAMCTTYTTYCCCATGG C-3′ and ADV_ R1 5′-GTRGCGTTRCCGGCNGAGAA-3′; second pair, ADV_F2 5′-TTYCCCATGGCNCACAACAC-3′ and ADV R2 5′-GYYTCRATGAYGCCGCGGTG-3′ (where M is A or C, Nis A or C or G or T, R is A or G, and Y is C or T (Lu and Erdman, 2006). For the first round PCR, reaction mixture consisted of 2μl of DNA, 7.5 μl of 1X PCR Perfect AmpMaster (GeneAll, Seoul, Korea), 10 mM first primer pair, and distilled water to make a final volume of 25 μl. Thermocycling was conducted in Mastercycler personal (Eppendorf, Hamburg, Germany**)** as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and a final step of 72°C for 10 minutes. Then, 1.5 μl aliquot of first round PCR mixture was used for the second round PCR as described above but using the second round (inner) primer pair. Amplicons (956 bp) were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide dye, visualized using a UV-transilluminator, gel-purified using Hi Yield TM Fragment Extraction Kit (GeneAll), and sequenced (First Base Lab, Selangor Darul Ehsan, Malaysia).

Data and sequence analysis

HAdV nucleotide sequences were analyzed using BioEdit version 7.2.5.0, Chromaslite version 2.1.1.0, NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), and Clustal X version 2.1.0.0 softwares. MEGA 6.06 was employed for phylogenetic analysis and phylogenetic trees were constructed using neighbor-joining (NJ) algorithm, tested by applying bootstrap tests with 1,000 replications using MEGA6; Molecular Evolutionary Genetics Analysis Version 6.0 (Tamura *et al*, 2013). Sequences were deposited at GenBank, accession nos. KY704350-KY704467 (NP samples) and KY968993-KY969126 (fecal samples).

RESULTS

The 3,451NP samples were predominantly from children<3 years of age, with male:female ratio of 1.2:1(Table1). Specimens tested positive for HAdV were 114/3,451 (3.3%), influenza A H1N1 144/3,451 (4.2%), influenza A H3N2 170/3,451 (4.9%), influenza B 144/3,451 (4.2%), RSV-A 63/1,729 (3.6%), and RSV-B 72/1,729 (4.2%). There were two cases (<3 years of age) with co-infection of HAdV and RSV.The majority of HAdV-infected patients were 0-<3 years of age, followed by children 3-<6 years of age (Table1). HAdV in NP specimens was detected throughout the year, with two broad peaks one early and one later in the year 2016 (Fig 1A). Of the 114 HAdV-positive samples HAdV-B3 constituted nearly 50%, with the remaining being HAdV-B7, HAdV-B11, HAdV-C1, HAdV-C2, HAdV-C5, HAdV-C6, and HAdV-E4 (Fig 1C).

The 948 fecal samples were predominantly from individuals >15years of age (Table1). Children 0<3 years of age provided the majority of fecal samples with viral infection, followed by children 3-<6

Table 1
Characteristics of nasopharyngeal (NP) and fecal specimens collected from Bangkok and Khon Kaen province, Thailand in 2016.

Characteristic	NP specimen[a] (*n*=3,451)		Fecal specimen[b] (*n*= 948)	
	Number (%)	HAdV positive (%)	Number (%)	HAdV positive (%)
Gender				
Male	1,896 (54.9)	73 (64.0)	449 (47.4)	71 (53.0)
Female	1,555 (45.1)	41 (36.0)	499 (52.6)	63 (47.0)
Age (year)				
Mean (SD)		4.6 (3.7)		27.1 (26.5)
Age group (year)				
0-<3	1,261 (36.5)	69 (60.5)	269 (28.4)	54 (40.4)
3-<6	1,058 (30.7)	34 (29.8)	76 (8.0)	19 (14.2)
6-≤15	151,132 (32.8)	11 (9.7)	97 (10.2)	14 (10.4)
>15	None	None	506 (53.4)	45 (33.6)
No age data	None	None	None	2 (1.5)
Locality				
Bangkok	2,756 (79.9)	100 (3.6)	868 (91.6)	113 (13.0)
Khon Kaen	695 (20.1)	14 (2.0)	80 (8.4)	21 (26)

[a]Collected as part of a routine influenzasurveillance of in- and out-patients with influenza-like illness. [b]Collected from patients with symptoms of watery diarrhea (≥3 episodes within 24 hours) with vomiting and/or fever. HAdV, human adenovirus.

years of age (Table1).The numbers of samples tested positive for EcoV were 14 (1.5%),HAdV 134 (14.1%), HAstV 22 (2.3%),HBoV 15 (1.6%),HCoV 22 (2.3%),HCV 2 (0.2%), HNoV 133 (14.0%), HPeV 7 (0.7%), HPV 8 (0.8%), HRV 12 (1.3%), HSaV 11 (1.2%), and HSFV 1 (0.1%). The numbers of cases of HAdV co-infection with EcoV were 3, HAstV 3, HBoV 1, HCoV 3, HNoV 20, HPV 1, HRV 12, and HSaV 2. Three peaks of adenovirus prevalence were discerned: a broad peak early in the year, followed by a peak in July and a predominant one in December (19%) (Fig1B). HAdV-F41and -C2 were nearly equally the two most prevalent, the remaining being -B3, -B7,-C1,-C5,-C6,-D13, - D17,-D56, and -F40 (Fig 1D).

Phylogenetic analysis of HAdV*hexon* fragments from NP and fecal samples showed HAdV strains from the two specimen sources formed different clusters (Fig 2). The most common genotypes from NP samples formed cluster HAdV-B3. For fecal samples, the predominant genotypes were clustered in HAdV-F41.

DISCUSSION

HAdV specimens for typing were collected anonymously, and thus it was unclear which samples originated from

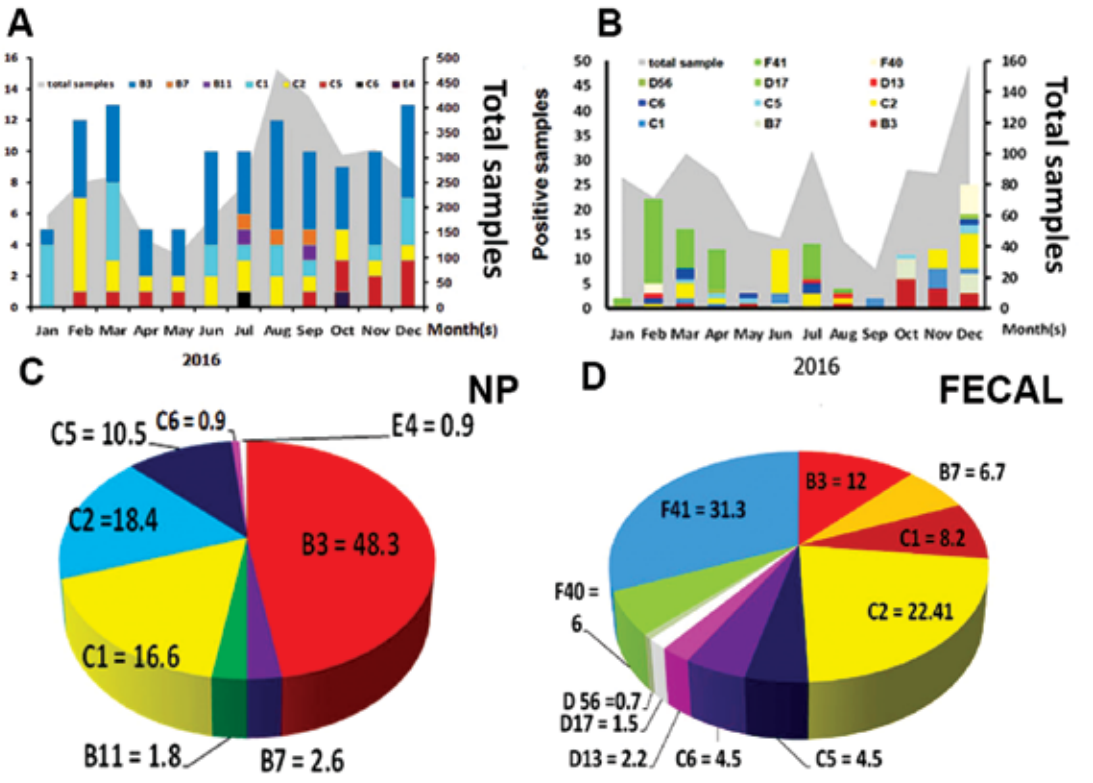
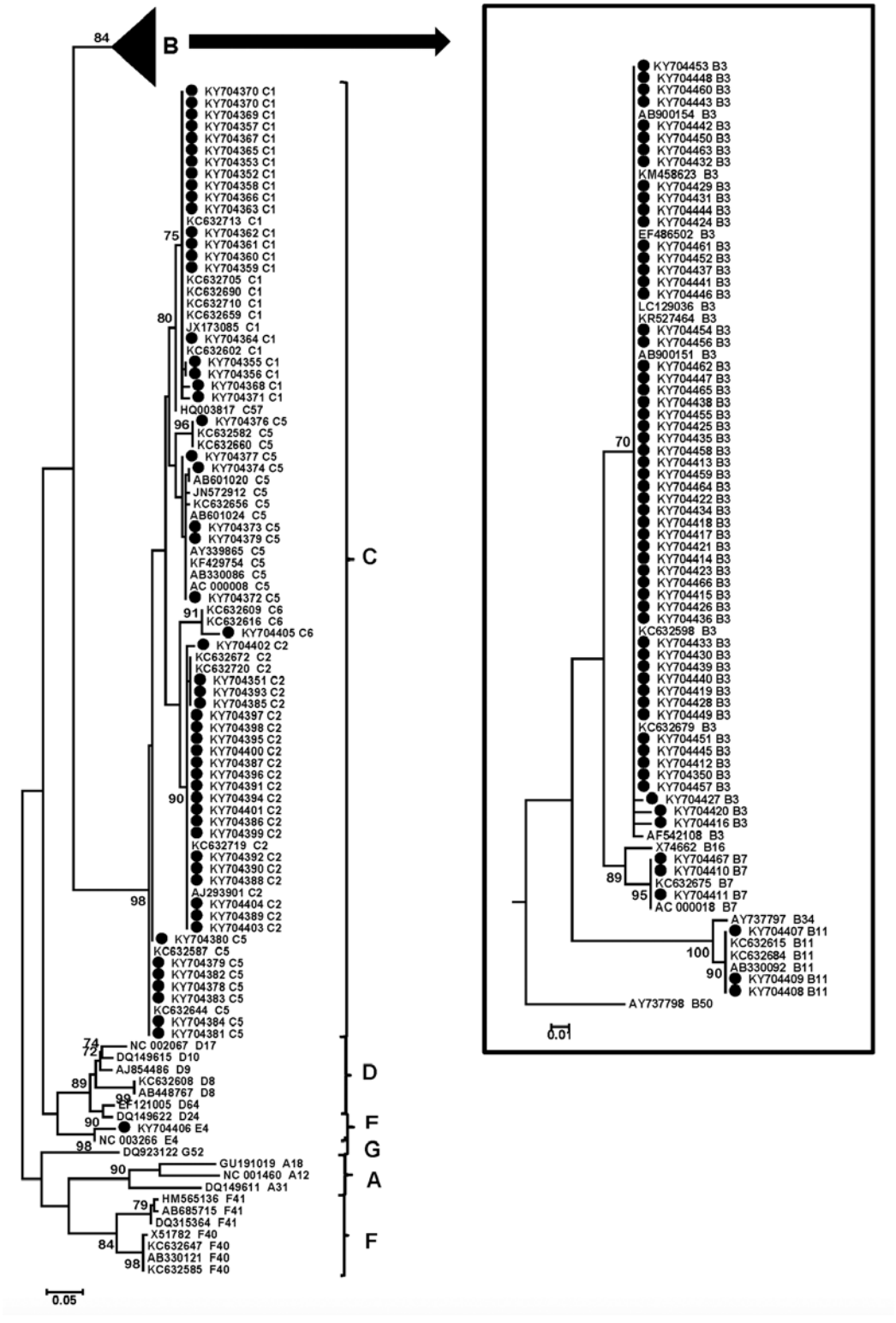


Fig 1-Numbers of nasopharyngeal (NP) (A) and fecal (B) positive samples per month (by type), and overall human adenovirus HAdV types distribution for NP (C) and fecal (D) samples collected in Bangkok and Khon Kaen Province, Thailand 2016. HAdV type was determined from sequence of HAdV *hexon* fragment. NP samples (n = 3,451) were collected as part of a routine influenza surveillance of in- and out-patients with influenza-like illness and fecal samples (n = 948) from patients with symptoms of watery diarrhea (≥3 episodes within 24 hours) with vomiting and/or fever. Colored bar indicates HAdV type.

in- or out-patients. However, the majority of the samples were known to be from out-patients. The prevalence of HAdV infection from NP specimens of 3.3% is higher than that (1.04%) in a previous study conducted in Thailand 2009–2012 (Sriwanna *et al*, 2013), and was nearly the same as that (4.8%) of a study in Japan 2009 (Kittigul *et al*, 2009). On the other hand, a multi-country study in 2017 (involving 17 centers in eight countries, namely, Australia, Latin America and South East Asia), patients with influenza-like illness reported a HAdV-positive prevalence of 9.8%(Taylor *et al*, 2017). A surveillance study in 2013 of patients with pneumonia on Thailand-Myanmar border obtained a HAdV-positive prevalence of 18.8% (Turner *et al*, 2013), and Li *et al* (2015) in China in 2015 observed a HAdV-positive prevalence of 13.8%. The HAdV infection prevalence in the present study was lower than that of Argentina (7.3%)(Marcone *et al*, 2015), Brazil (6.4%) (Ampuero *et al*, 2012), China (12%) (Chen *et al*, 2016), and Italy (19.9%) (Esposito *et al*, 2016). These differences could be partly explained by differences in the study areas, sporadic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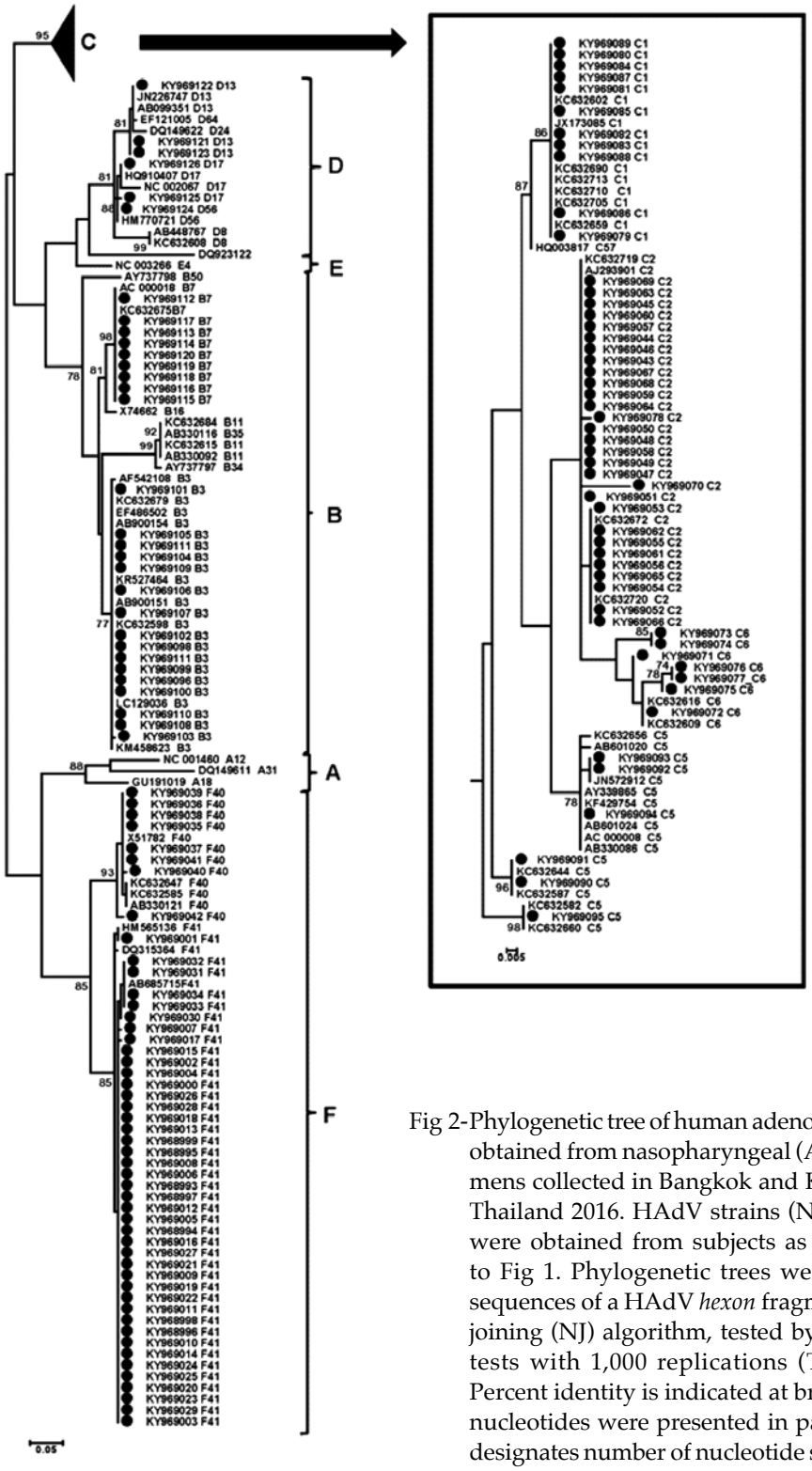

Fig 2-Phylogenetic tree of human adenovirus (HAdV) strains obtained from nasopharyngeal (A) and fecal (B) specimens collected in Bangkok and Khon Kaen Province, Thailand 2016. HAdV strains (NP = 118, fecal = 134) were obtained from subjects as described in legend to Fig 1. Phylogenetic trees were constructed from sequences of a HAdV *hexon* fragment using neighbor-joining (NJ) algorithm, tested by applying bootstrap tests with 1,000 replications (Tamura *et al*, 2013). Percent identity is indicated at branch node. Identical nucleotides were presented in parentheses. Scale bar designates number of nucleotide substitutions per site.

or outbreak infection, differences in immunity to the prevalent virus types, and characteristics of patients.

HAdV infection among those with influenza-like illness mostly affected children <3 years of age, compatible with previous studies of HAdV infection worldwide (Abd-Jamil *et al*, 2010; Selvaraju *et al*, 2011; Khor *et al*, 2012; Tsou *et al*, 2012; Dey *et al*, 2013; Chen *et al*, 2016; Esposito *et al*, 2016). In our study, specimens positive for influenza virus were mostly detected in older age groups, while HAdV-positive samples virus were present in the younger group. These observations should be of benefit in helping to guide diagnosis of HAdV and influenza infections.

HAdV prevalence lacked clear seasonality, similar to other reports (Ampuero *et al*, 2012; Qurei *et al*, 2012; Tsou *et al*, 2012). Our finding of HAdV-B3 was most common in NP samples is consistent with reports from Canada, China, Malaysia, Taiwan, and USA (Yeung *et al*, 2009; Abd-Jamil *et al*, 2010; Selvaraju *et al*, 2011; Khor *et al*, 2012; Tsou *et al*, 2012; Chen *et al*, 2016). On the other hand, HAdV-C was found to be predominant in Brazil (Tórtora *et al*, 2015), Canada (Yeung *et al*, 2009), Italy (Esposito *et al*, 2016), and Korea (Hong *et al*, 2001). Our survey in Thailand found only three HAdV types (B3, C1 and C2) in samples from Khon Kaen Province, whereas nine types (A7, B3, B7, B11, C1, C2, C5, C6 and E4) were detected in Bangkok samples. In Thailand in 2009 HAdV-C1 was reported to account for >50% of infections, followed by HAdV-B3; then in 2010, HAdV-C1 prevalence has decreased and in 2011 HAdV-B3 has increased to became the predominant type (Sriwanna *et al*, 2013). This was also the situation in 2016 based on assay of NP samples. Similarly, Mandelboim *et al* (2011) noted HAdV-C1 is predominant in Israel and one year later HAdV-B3 has become predominant in Palestine (Qurei *et al*, 2012). These inter- and intra-nation variations in HAdV types may be due to differences in geographical factors, immunity, population size, and HAdVtypes involved in outbreaks.

The possibility that immunity and or its lack might drive the prevalence of HAdV types circulating during an outbreak in any particular year is supported by observations on influenza B virus lineages circulation in Japan where the prevalence of HAdV-B7 is only 2.6% (Nakagawa *et al*, 2000), and in USA where HAdV-B7 has not been reported as a significant cause of severe respiratory disease (Scott *et al*, 2016).HAdV-E4 is a minor co-circulating HAdV type that causes conjunctivitis in North Africa (Fedaoui *et al*, 2017), with only one such sample detected in our study.

HAdV prevalence in fecal samples of 14.1% was higher than that (5.8%) reported in 2009 - 2012 (Sriwanna *et al*, 2013) but similar to those reported in other regions, *eg* Africa (9.6-13.3%)(Liu *et al*, 2016a), China (10.3%)(Liu *et al*, 2016b), India (11%) (Banerjee *et al*, 2017), Japan (8.2%) (Nakamura *et al*, 2016), and USA (7%) (Stockmann *et al*, 2017). The number of HAdV-positive fecal samples peaked in December, but elsewhere, such as China, HAdV is detected throughout the year, with no clear seasonal pattern (Liu *et al*, 2016a). This could be attributed to differences in such factors as population and geography. There were two notable viruses in fecal co-infection:12 cases of HAdV/HRVand 20 cases of HAdV/HNoV co-infections. The associations between co-infectious viruses need further clarification.

The relative frequencies of HAdV types in fecal samples (F41 being predominant, followed by C, B, and D) are similar to those previously reported in

Thailand in 2009 (Kittigul *et al*, 2009) and in 2009-2012 (Sriwanna *et al*, 2013), and also in Japan (Dey *et al*, 2013. These reports support the notion of continuing genetic selection over time to favor a particular antigenic type.

Although HAdV-F40 is an enteric virus, it is less common than HAdV-F41 in Thailand (Sriwanna *et al*, 2013). HAdV-F40 was present in 2010 and 2011 but not in 2012 (Sriwanna *et al*, 2013).The present study also identified HAdV-D13, HAdV-D17 and HadV-D56 in the fecal samples. As HAdV can cause genital ulcers, cervicitis and pharyngitis through vaginal or oral sexual intercourse, these possible routes for HAdV transmission need further investigation.

Systematic surveillance of pathogens that cause gastroenteritis is important to safeguard public health. In fecal samples, the rare HAdV-D13 was detected in one sample and HAdV-D17 in two samples from a hospital in Bangkok, but due to the anonymous sampling protocol, the origins of the samples could not be obtained.

Genotyping plays a key role in epidemiological investigations of virus infections, especially in closed settings where groups of individuals are at increased risk of gastroenteritis due to exposure or impaired immunity. Information on epidemiological agents of viral gastroenteritis in Thailand is useful for formulating policies for prevention and control of diarrheal disease. Detection and surveillance of HAdV emergence can lead to early identification of new virus types and variants of known types, which should be of assistance in explaining appearance clusters of cases and of sporadic cases of severe illness that are possibly related to changes to an existing predominant HAdV species. Long-term surveillance of virus types infecting a particular population can help to determine the molecular basis of antigenic variations in the population, which will facilitate in discerning the evolutionary molecular trajectories of circulating HAdVs.

In conclusion, the data gathered in the present study of HAdV infection in Thailand provide information on this virus that is currently sparse in the Southeast Asian region. The report updates HAdV prevalence in nasopharyngeal and fecal specimens collected in Thailand (Bangkok and Khon Kaen Province) in 2016. HAdV-B3, HAdV-C1, and HAdV-C2 were the most types present in nasopharyngeal specimens, whereas HAdV-F41 was most common in fecal specimens. Close observation and implementation of preventive measures against HAdV infection in young children are recommended. Further HAdV typing studies should be increased to supply more epidemiologic information for improvement in the treatment, prevention and control of HAdV-associated diseases in Thailand.

ACKNOWLEDGEMENTS

The work was supported by a Research Chair Grant from NSTDA (P-15-50004), a Dutsadi Piphat Scholarship, the Center of Excellence in Clinical Virology, Chulalongkorn University (GCE 59-00930-005), and MK Restaurant Company Ltd, Thailand. The authors thank staff of the Center of Excellence in Clinical Virology, Chulalongkorn University and of King Chulalongkorn Memorial Hospital for their kind assistance.

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