HUMAN ASTROVIRUS AMONG PATIENTS WITH ACUTE GASTROENTERITIS IN THAILAND, 2009-2017

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Abstract. Human astrovirus (HAstV) is associated with10% of non-bacterial acute gastroenteritis worldwide and in 0.5-15% of acute diarrhea outbreaks in children. HAstV is transmitted through a fecal-oral route and outbreaks are attributed to virus-contaminated food. Etiology of many sporadic incidences of diarrhea often remains undetermined, especially that due to HAstV infection among patients with diarrhea in Thailand. In this study, the prevalence and genotype of HAstV in 4,117 stool samples of patients with acute gastroenteritis in Thailand between 2009 and 2017 were determined using RT-PCR targeting open reading frame 2 region of the virus. Nucleotide sequence and phylogenetic analysis revealed a 3.1% prevalence, with the majority (1.6%) being HAstV genotype 1 (HAstV-1), followed by HAstV-2 (0.5%), HAstV-4 (0.1%), and HAstV-5 (0.9%). This study provides a baseline of the prevalence and diversity of HAstV associated with acute gastroenteritis in Thailand.

Keywords: diarrhea, HAstV genotype, human astrovirus, Thailand

INTRODUCTION

Human astrovirus (HAstV) is increasingly recognized as a significant cause of non-bacterial acute gastroenteritis, infecting individuals of all age especially very young children (Olortegui *et al*, 2018), with an average prevalence of 11% worldwide (Bosch *et al*, 2014). Symptoms may include diarrhea, abdominal pain, vomiting, and fever, which typically last 2-3 days and generally resolve spontaneously (Herrmann *et al*, 1991). HAstV is transmitted through a fecal-oral route, and outbreaks have been associated with virus-contaminated food (Iritani *et al*, 2014). Virus infection is generally restricted to the gastrointestinal tract (De Benedictis *et al*, 2011).

HAstV, belonging to family Astroviridae and genus *Mamastrovirus*, is a non-enveloped positive-sense single-stranded RNA virus with a genome of ~7 kb in length, encoding three overlapping open reading frames (ORFs) (Bosch *et al*, 2011; Bosch *et al*, 2014). ORF1a codes for a non-structural protein, ORF1b an RNA-dependent RNA polymerase, and ORF2 a shell and spike protein (a region often used for typing). There are several divergent HAstV species, of which the classical

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HAstV species comprises eight genotypes (HAstV-1 to -8). HAstV-1 accounts for the majority of infection worldwide, followed by genotypes 2-5 and occasionally genotype 8 depending on the region, while HAstV-6 and -7 are least frequently reported (Méndez *et al*, 2013). Other species of HAstV, HAstV-MLB (strains MLB1 to MLB3) and HAstV-VA/HMO (≥4 distinct strains), have been identified in recent years (Bosch *et al*, 2014). Thus, the increasing diversity of HAstV warrants molecular surveillance in acute diarrhea.

HAstV genome mutation rate is generally similar to other single-stranded positive sense RNA viruses such as the picornaviruses. Genetic variability in HAstV ORF2 is higher than ORF1a and 1b especially in porcine, ovine, mink and turkey when compare to humans, cat and chicken, with variation rates ~$3.7x10^{-3}$ nucleotide substitution/site/year and ~$2.8x10^{-3}$ synonymous change/site/year (Lukashov and Goudsmit, 2002; van Hemert *et al*, 2007).

In this study, the prevalence of HAstV in archived stool samples obtained between 2009 and 2017 from Thai patients of all ages presented with acute gastroenteritis were examined to provide baseline data for future epidemiology and diagnostic studies.

MATERIALS AND METHODS

Study samples

For eight consecutive years (January 2009 - December 2017) in Thailand, archived stool samples from patients (Bangkok, n = 2,383 and Khon Kaen, n = 1,734) with gastroenteritis admitted for acute diarrhea were analyzed. Samples were from 2,252 children <5 years of age (infant and pre-school children), 271 children 5 to <15 years of age (school children), and 1,594 individuals ≥15 years of age (adult) (mean age of 18.6 years, median age of 2.8 years).

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 286/58). Prior written informed consents were obtained from adult participants or legal guardians in the case of children.

RT-PCR and molecular typing

Virus RNA was isolated using RiboSpinvRD kit (GeneAll, Seoul, South Korea) and cDNA synthesized using random hexamer primers and ImProm-II Reverse Transcription System (Promega, Madison, WI). A semi-nested PCR to amplify HAstV ORF2 region was performed initially using primers HAstV-F4286 (5′-GGACTGCWAAGCAGCTTCGTG-3′) and HAstV-R5164 (5′-GCAGCATADCCDGTRAARCACCA-3′) for 1st round, followed by primers HAstV-F4586 (5′-AARCAACTCAGGAAACARGGTGT-3′) and HAstV-R5164 for 2nd round [where D = (A or G or T), R = (A or G) and W = (A or T)] (Najafabadi *et al*, 2008). In the 1st round, reaction mixture contained 2 μl of cDNA, 0.5 μM each primer, 10 μl of 2xAccustart II PCR SuperMix (Quantabio, Beverly, MA), and nuclease-free water to a final volume of 25 μl. In the 2nd round, reaction mixture contained 1 μl of PCR product from 1st round, 0.5 μM each primer, 10 μl of 2xAccustart II PCR SuperMix (Quantabio, Beverly, MA), and nuclease-free water to a final volume of 25 μl. Thermocycling of both rounds was conducted using Mastercycler nexus X2 (Eppendorf, Hamburg, Germany) under the following conditions: 95˚C for 5 minutes; 40 cycles of 95˚C for 30 seconds, 55˚C for 45 seconds and 72˚C for 90 seconds; and a final step of 72˚C for10 minutes. PCR amplicons (578 bp) were separated

by 2% agarose gel-electrophoresis, stained with fluorescent dye (SMOBIO Technology, Hsinchu, Taiwan) and visualized by UV transilluminator (Vilber Lourmat, Collegien, France), purified and sequenced (FirstBASE Lab, Seri Kembangan, Malaysia). Nucleotide sequences were subjected to BLAST analysis and deposited at GenBank database, accession nos. MG970001-MG970130.

Phylogenetic analysis

A phylogenetic tree was constructed from HAstV ORF2 sequences from different parts of the world available from GenBank database together with those of HAstV-1 to -8, -MLB and -VA/HMO reference strains using MEGA6 (Tamura *et al*, 2011) with maximum likelihood method to estimate genetic distances using Kimura two-parameter method (Kimura, 1980). Reliability of the phylogenetic tree was assessed by bootstrap analysis of 1,000 pseudo-replicates and bootstrap values >70% are considered significant.

RESULTS

HAstV characterization

HAstV was detected in stool samples from 2009 to 2017 with an overall prevalence of 3.1% and 2.5% from Bangkok and Khon Kaen samples, respectively. Annual prevalence varied from 1.2% in 2013/2014 to 5.4% in 2011 (Table 1). The most common genotype was HAstV-1 and no samples containing HAstV-3, -6, -7 and -8 were found during the study period. Between 2009 and 2011, HAstV-1 and HAstV-2 were the most frequently detected genotypes. There was an absence of HAstV in samples of 2012; in 2015, HAstV-1 became predominant, while in 2016 and 2017 there was an equal predominance of HAstV-1 and -5. The seasonal pattern of HAstV infection seemed

Table 1
HAstV and genotypes in fecal samples from diarrheal patients in Thailand, 2009 - 2017.

Year	Number of samples	Number of HAstV positive samples (%)	Genotype (%)			
			HAstV-1	HAstV-2	HAstV-4	HAstV-5
2009	347	17 (5)	13 (4)	2 (1)	-	2 (1)
2010	268	5 (2)	3 (1)	2 (1)	-	-
2011	315	17 (5)	2 (1)	13 (4)	-	2 (1)
2012	403	0	-	-	-	-
2013	161	2 (1)	-	-	-	2 (1)
2014	157	3 (2)	-	-	2 (1)	1 (1)
2015	435	18 (4)	16 (4)	1 (<1)	-	1 (<1)
2016	943	22 (2.3)	7 (0.7)	3 (0.3)	-	12 (1.3)
2017	1,088	44 (4.0)	23 (2.1)	1 (0.1)	3 (0.3)	17 (1.6)
Total	4,117	128 (3.1)	64 (1.6)	22 (0.5)	5 (0.1)	37 (0.9)

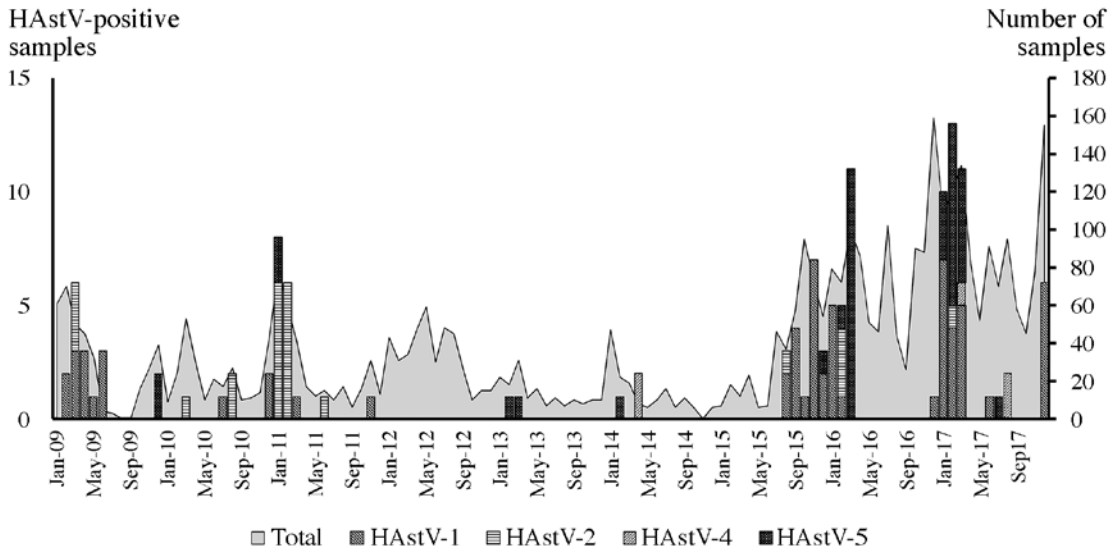

Fig 1-Trimonthly prevalence of human arbovirus (HAstV) genotypes in diarrheal patients, Thailand, 2009 - 2017. Fecal samples (n = 4,117) were examined for HAstV genotypes using RT-PCR and direct sequencing of a 578-bp ORF2 fragment. Line graph indicates trimonthly number (right y-axis) of samples. Bar graph indicates trimonthly number (left, y-axis) of HAstV genotype. Jan, January - April; May, May - August; Sep, September - December.

to have a higher prevalence in the winter season (December-February) (Fig 1).

Of the 128 HAstV-positive samples, 70 (55%) were from pre-school children, 10 (8%) from school children and 48 (37%) from adults (data not shown).

Phylogenetic analysis

Phylogenetic tree of HAstV ORF2 578-bp sequences demonstrated clustering of HAstV strains of the study with the reference strains (Fig 2). While HAstV-5 strains were all tightly grouped with the reference strain Goiania5/GO/12/94/Brazil, HAstV-1 and -2 strains formed two closely related but slightly divergent clusters.

DISCUSSION

HAstV is associated with 0.5-15% of acute diarrhea outbreaks in children and up to 20% of sporadic non-bacterial diarrhea, with 2% of children shedding virus without clinical symptoms (Moser and Schultz-Cherry, 2005; Kumthip *et al*, 2018). Several outbreaks were reported as being associated with consumption of contaminated food (Oishi *et al*, 1994; Mitchell *et al*, 1995); however, their prevalence is dependent on region and test setting. A number of epidemiological studies suggested the highest detection rate is among children between 2-4 years of age and in the winter season (De Benedictis *et al*, 2011; De Grazia *et al*, 2011). One of the earliest studies on the prevalence of HAstV in Thailand utilized monoclonal antibody-based enzyme immunoassay, which showed HAstV is present in 8.6% of children <5 years of age with gastroenteritis compared to 2.1% of age-matched children without the condition, with 95% of HAstV-positive children ≤2 years old

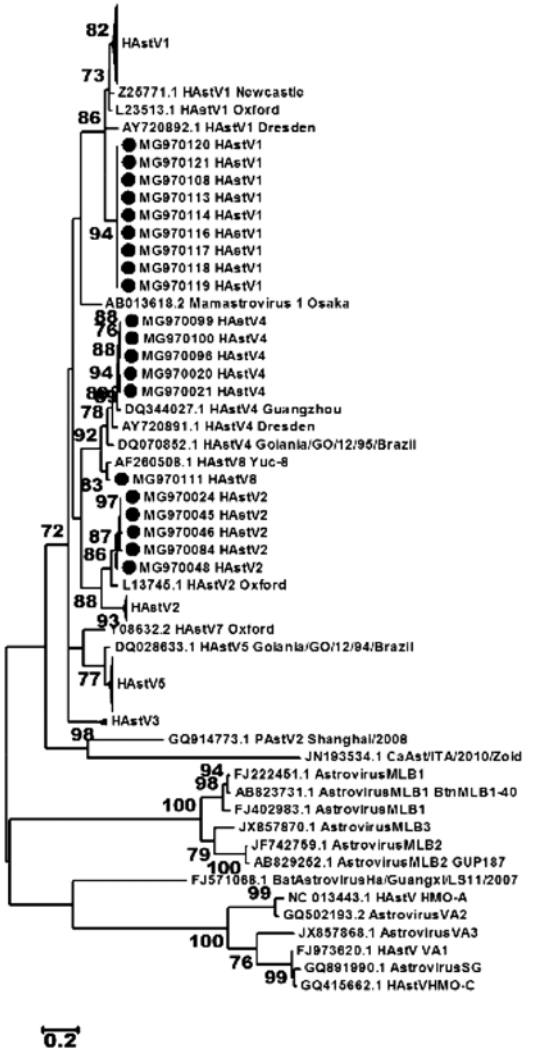

Fig 2-Phylogenetic tree of human arbovirus (HAstV). Tree was constructed using the maximum likelihood method implemented in MEGA6 from comparisons of a 578-bp fragment of ORF2 region. Nucleotide sequences were from representative strains of various geographical regions, reference genotype strains and those from the study (all with GenBank accessions numbers). Bootstrap value >70% is shown at branch node. Scale bar represents percent nucleotide substitution.

(Herrmann *et al*, 1991). A larger study involving RT-PCR-based screening of HAstV in children ≤5 years old with gastroenteritis in northern Thailand reported a prevalence <1.4% in the past decade (Malasao *et al*, 2012). The current study of stool samples from diarrheal children ≤5 years old in central and northeastern regions of the country from 2009 to 2017 detected a higher prevalence (1.7%). It is worth noting overall HAstV prevalence during the study period was higher in central than northeastern Thailand.

The classic HAstV-1, -2, -3, and -5 have been reported to be circulating worldwide at low frequency (0.5-15%) (Vu *et al*, 2017). This study reveals these HAstV genotypes were circulating twice as frequently in Thailand, albeit still at very low prevalence compared to the more common enteric viruses, such as adenovirus, norovirus, rotavirus, and even enterovirus (Chansaenroj *et al*, 2017). As expected, there was a predominance of HAstV-1, similar to other studies (Guo *et al*, 2010; De Grazia *et al*, 2011; Nakamura *et al*, 2016) and shared more than 90% nucleotide sequence identity. HAstV-4 was identified for the first time in Thailand, thereby increasing the overall diversity of the classic HAstV genotypes known to circulate in the country. Although HAstV-MLB-1 and -MLB-2 were previously identified in children stool samples (Linsuwanon *et al*, 2015) but were absent in all samples in this study. The absence of HAstV-3, -6, -7, and -8 in Thailand may indicate geographical restrictions of these genotypes, or possible (but unlikely) failure of the RT-PCR assay used in genotyping. The earlier relatively high frequency of detection based on immunoassay compared to current RT-PCR methods may be due to false positive detection of viruses antigenically related to HAstV by antibodies employed at that

time, or might reflect improvement in sanitary practices. However, to date, there is no information on correlation between symptoms and HAstV genotype.

The prevalence of HAstV-associated gastroenteritis in other Southeast Asian countries is lacking. Within the Pacific Rim countries, a prevalence comparable to our findings has been reported in Korea (1%) (Ham *et al*, 2014), Japan (2.3-2.8%) (Thongprachum *et al*, 2015) and China (significantly higher 7.8-10.0%) (Liu *et al*, 2007; Guo *et al*, 2010). The majority of these studies focused on the pediatric population, while prevalence in adults is unclear. Our study included individuals of all ages, but age distribution was skewed towards the very young children. Although HAstV infection is resolved without requiring hospitalization (Vu *et al*, 2017), morbidity may be severe in this age group, as well as in the elderly and the immunocompromised patients.

Because symptoms of HAstV infection is virtually indistinguishable from that of rotavirus and norovirus infections (Vu *et al*, 2017), there is a lack of awareness of HAstV among healthcare workers in resource-limited healthcare settings. Testing for HAstV is rarely sought in patients with acute gastroenteritis. It is unclear how HAstV maintains its circulation in the population, but HAstV nucleic acid was detected in environmental water sources within Bangkok (Ng *et al*, 2012).

Although HAstV can circulate year-round, higher incidence has been reported in cooler months such as in Spain and Uruguay (Bosch *et al*, 2014; Lopez *et al*, 2017). Our study demonstrated higher prevalence in the winter season (December-February). Annual circulation of HAstV could be explained by the emergence and re-emergence of different lineages over time due to accumulated mutations, which might be accelerated by viral genome recombination, a property intrinsic to positive strand RNA viruses (De Benedictis *et al*, 2011). This study supports the notion that even though HAstV is less common than rotavirus among children with diarrhea, it is a potentially important virus to consider in investigations of the etiology of acute gastroenteritis in the absence of rotavirus and norovirus laboratory detection.

In summary, this consecutive 9-year study is the most recent for circulating human astrovirus associated with acute gastroenteritis in Thailand. Although it had a relatively low detection frequency compared to other more common enteric viruses, the contribution of human astrovirus to disease severity, especially in young children (≤5 years of age) should not be underestimated. Periodic surveillance and determination of baseline prevalence of circulating human astrovirus (including genotypes) are important drivers in the continuing assessment of this viral infection in the Southeast Asian region.

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