Diversity of Human Parechoviruses Isolated from Stool Samples Collected from Thai Children with Acute Gastroenteritis[†]

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A total of 82 fecal specimens which were known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus and which were collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand, from January to December 2005 were screened for human parechovirus (HPeV). HPeV was detected by reverse transcription-PCR with a primer pair that amplified the 5' untranslated region of its genome and was genotyped by sequencing of the VP1 region. HPeV was detected in 12 of 82 specimens tested, and the detection rate was found to be 14.6%. The capsid VP1 gene was successfully sequenced from nine of the HPeV strains detected. The HPeV strains studied clustered into four different genotypes, HPeV genotype 1 (HPeV1) to HPeV4, and the majority of the strains studied (five strains) belonged to HPeV1. This is the first finding of HPeV from children with acute gastroenteritis in Thailand. In addition, the diversity of the Thai HPeV strains was also noted.

Parechoviruses are small, nonenveloped, positive-sense single-stranded RNA (ssRNA) viruses and belong to the large family of *Picornaviridae*, which is a highly diverse family of important pathogens of humans and animals. The *Parechovirus* genus was defined in the early 1990s (15, 18). The genus is composed of two species: Ljungan virus, isolated from bank voles (27), and human parechovirus (HPeV), a frequent human pathogen. On the basis of serological characteristics, two HPeVs were characterized in early 2004. However, they were first classified as echovirus types 22 and 23 within the *Enterovirus* genus but were later reclassified as HPeV genotype 1 (HPeV1) and HPeV2, respectively, on the basis of their distinctive biological and molecular properties. The genome of HPeV has an average length of 7,300 nucleotides and is packaged into an icosahedral capsid made up of multiple copies of each of the capsid proteins VP0, VP3, and VP1 (15, 33).

Previous findings revealed the genetic variability of HPeVs, and the number of newly identified HPeV genotypes has been on the increase. On the basis of VP1 sequence comparisons, nine HPeV types have been described to date (1–9, 16, 24, 34). In addition, HPeVs have been classified into 14 genotypes, as described elsewhere (<http://www.picornaviridae.com/parechovirus/hpev/hpev.htm>); however, the nucleotide sequences of new HPeV strains from genotypes 9 to 13 and corresponding studies have so far not been published.

Acute gastroenteritis is among the diseases that are the primary cause of morbidity in Thailand, as documented in a Thai annual report of epidemiological surveillance ([\[moph.go.th\]\(http://moph.go.th\)\); and it is well established that rotaviruses, adenoviruses, astroviruses, and caliciviruses are the most important etiologic agents of acute gastroenteritis \(14, 19, 20, 25\). To date, however, there has been no report on HPeV infection in Thailand. The present study was aimed at screening stool samples collected from children with acute gastroenteritis in Chiang Mai, Thailand, for infection with HPeV, one of the less explored viral pathogens which has recently been reported to be associated with diarrhea, and characterizing the molecular properties of the HPeV strains detected.](http://epid</p></div><div data-bbox=)

MATERIALS AND METHODS

Clinical specimens. Eighty-two fecal specimens which had been shown to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus by reverse transcription (RT)-PCR and which were collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand, from January to December 2005 were subjected to screening for HPeV. All stool samples were stored without additives at -30°C for up to 3 years before analysis. The fecal specimens were diluted with distilled water to 10% suspensions and were clarified by centrifugation at $10,000 \times g$ for 10 min. The supernatants were collected and stored at -30°C until use for virus detection.

RNA extraction and RT. The RNA genome of HPeV was first extracted from 140 μl of a 10% fecal suspension by using a QIAamp viral RNA minikit (Qiagen, Inc., GmbH Hilden, Germany), according to the manufacturer's instructions. Then, for RT, 5 μl of the stored, extracted RNA was added to a reaction mixture consisting of $5\times$ first-strand buffer (Invitrogen, Carlsbad, CA), 10 mM deoxynucleoside triphosphates (dNTPs; Roche, Mannheim, Germany), 0.1 M dithiothreitol (Invitrogen, Carlsbad, CA), SuperScript reverse transcriptase III (200 U/ μl ; Invitrogen, Carlsbad, CA), random primer (1 $\mu\text{g}/\mu\text{l}$; hexa-deoxynucleotide mixture; Takara, Shiga, Japan), RNase inhibitor (33 U/ μl) (Toyobo, Osaka, Japan), and distilled water. The total volume of the reaction mixture was 15 μl . The RT reaction was carried out at 50°C for 1 h, followed by 95°C for 5 min, and the reaction mixture was then held at 4°C . The cDNA was stored at -30°C until it was used for the PCRs (29, 35).

PCR for detection of HPeV. After the addition of 2 μl of cDNA to 23 μl of the reagent mixture containing $5\times$ Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (10 mM), primers (20 μM), Taq DNA polymerase (5 U/ μl ; Promega), and distilled water, a screening PCR was conducted with primers ev22(+) and ev22(–) to amplify a 270-bp PCR product of the 5' untranslated region

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TABLE 1. Oligonucleotide sequences of the primers used in this study and their positions

Primer	Gene	Sequence (5' to 3') ^a	Sense	Position ^b	Amplicon	Reference or source
ev22(+)	5' UTR	CYCACACAGCCATCCTC	+	312–328	270	Joki-Korpela and Hyypia (17)
ev22(–)	5' UTR	TRCGGGTACCTTCTGGG	–	581–565		
VP1-parEchoF1	VP1	CCAAAATTCTRTGGGGTTC	+	2332–2349	760	Benschop et al. (6)
VP1-parEchoR1	VP1	AAACCYCTRTCTAAATAWGC	–	3090–3071		
Cap-parEcho-F	VP1	TCHACWTGGATGMGRAARAC	+	2162–2181	1076	This study
Cap-parEcho-R	VP1	TCYARYTCACAYTCYTCYTC	–	3237–3218		
3DparEcho-F	3D	GATTGGCACTTYATGATHAATG	+	6555–6576	383	This study
3DparEcho-R	3D	CAAATGAWTCTGCCATGAYAC	–	6937–6917		

^a Y, C or T; R, G or A; W, A or T; H, A, C, or T; M, C or A.

^b The sequence position is based on the full genome sequence of HPeV1 prototype strain Harris (GenBank accession number L02971).

(UTR) (17) (Table 1). The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and with a final extension at 72°C for 7 min.

Genotyping by VP1 sequencing and primer designation. At first, to amplify the VP1 segment, the primers developed by Benschop and colleagues and described previously (6) were used. However, because of the failure to obtain PCR products from most of the HPeV-positive samples except one, two new primers were designed for the first PCR. Then, the nested PCR was performed with the inner primer pair described by Benschop and colleagues (6).

For primer designation, to obtain the full length of 702 bases of the VP1 capsid gene, alignment of the full genome sequences of reference strains of eight known HPeV genotypes available in the GenBank database was performed with Clustal X software to find the conserved regions, and the two new primers were designed to be specific for sequences outside the VP1 region. The oligonucleotide sequences of the newly developed primers and their positions are described in Table 1.

The first PCR was done with the newly designed primers; and the thermal cycle program was as follows: 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The nested PCR was conducted with the known primer pair VP1-parEchoF1 and VP1-parEchoR1 (6) at an annealing temperature of 48°C to generate a 760-bp product (Table 1). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and the bands were visualized by staining with SYBR Safe (Invitrogen, Tokyo, Japan) under UV light. The HPeV-positive samples were retested with another PCR by using a newly designed primer pair, 3DparEcho-F and 3DparEcho-R, derived from the 3D gene of the HPeV genome (Table 1).

Sequencing and phylogenetic analysis. The PCR amplicons of the VP1 gene were purified and sequenced in both directions by using a BigDye Terminator cycle sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers used for amplification of the VP1 gene were used as sequencing primers. The sequence data were collected by an ABI Prism 310 genetic analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The sequences of the VP1 segments from the HPeV strains obtained in the present study and other reference HPeV strains of nine known genotypes available in the GenBank database were compared. The sequence data and the data from the phylogenetic analysis were analyzed by using BioEdit (version 7.0.5) software. A parsimony analysis was also conducted by using the MEGA (molecular evolutionary genetics analysis; version 3.1) program to determine the evolutionary relationship among the sequences studied (22). The method was performed by using a close-neighbor interchange with a random option and with 500 bootstrap repetitions.

Nucleotide sequence accession numbers. The nucleotide sequences of the following reference HPeV strains described in this study have been deposited in the GenBank database under the accession numbers indicated in parentheses: HPeV1 strains Harris (L02971), 652281 (FJ373120), BNI-R09/03 (EU024632), BNI-R32/03 (EU024636), BNI-R15/03 (EU024633), BNI-788St (EF051629), 677033 (FJ373136), 69960AE (AM933170), A229-05 (AB300968), A234-05 (AB300969), A708-99 (AB300935), BNI-R04/03 (EU024631), A65-05 (AB300963), A222-05 (AB300967), BNI-R21/03 (EU024634), 652780 (FJ373127), 650648 (FJ373108), A191-05 (AB300966), A527-99 (AB300928), BNI-90/03 (EU024630), and BNI-R30/03 (EU024635); HPeV2 strain Williamson (AJ005695); HPeV3 strains Can82853-01 (AJ889918), 677146 (FJ373162), A415-01 (AB300945), A308/99 (AB084913), and 651689 (FJ373153); HPeV4 strains Fuk2001-282

(AB433630), NII370-93 (AB434673), T75-4077 (AM235750), 653046 (FJ373170), and K251176-02 (DQ315670); HPeV5 strains CT86-6760 (AF055846), T92-15 (AM235749), and 676618 (FJ373175); HPeV6 strains 2005-823 (EU077518), NII561-2000 (AB252582), BNI-67/03 (EU024629), and 650045 (FJ373178); HPeV7 strain PAK5045 (EU556224); HPeV8 strain BR/217/2006 (EU716175); and HPeV14 strain 451564 (FJ373179). For the Thai strains studied, the accession numbers are FJ648755 to FJ648762 and GQ149453.

RESULTS

Of the 82 samples tested, 12 were positive for HPeV, and the rate of detection of HPeV was 14.6%. All 12 patients whose stool samples were positive for HPeV were children ages 6 to 24 months. Of these, six patients (50%) were from 6 to 18 months of age.

For genotyping, the full-length VP1 capsid sequences from only nine strains were successfully amplified and sequenced. The full length of the VP1 sequences of the nine strains studied was 702 bases.

Figure 1 shows the phylogenetic tree constructed from the 624-nucleotide sequences of the partial VP1 segments of the reference HPeV strains and nine Thai strains found in this study. On the basis of the specific clustering of the isolates with known HPeV types obtained from the GenBank database, the strains studied could be identified as HPeV genotypes 1 to 4. The majority of the Thai strains (five strains) belonged to HPeV1, the largest cluster of HPeVs. One Thai strain clustered together with the Williamson strain into the HPeV2 cluster. The two other strains were HPeV3, the second largest cluster of HPeVs. The remaining strain was genotyped as HPeV4, and it clustered along with strain K251176, which was recently detected in The Netherlands.

For the HPeV1 strains studied, three strains were found to cluster closely together with prototype strain Harris, and the amino acid similarities between the three strains and strain Harris ranged from 92.7% to 93.7%, while the two remaining HPeV1 Thai strains were in the larger cluster consisting of recently detected HPeV1 strains, and the amino acid similarities between those two strains and strain Harris were less than 90% (86.4% and 89.6%, respectively).

In the case of the HPeV2 strains, the amino acid similarity between the Thai strains studied and strain Williamson was 95.2%, while the mean amino acid similarities between the

FIG. 1. Phylogenetic tree constructed from the 624-nucleotide sequences of the VP1 gene of the strains studied and reference HPeV strains with 500 bootstrap repetitions. Bootstrap values above 70% are shown at the branch nodes. The HPeV strains studied are in boldface type.

Thai strains and strains of the other genotypes ranged from 63.8% (with HPeV3) and 74.2% (with HPeV1).

The two Thai HPeV3 strains studied clustered closely together with Japanese strain A308/99, the prototype strain of HPeV3, and had 96.6% amino acid similarity to that strain.

Within the HPeV4 cluster, the amino acid similarity between the Thai strains studied and other strains ranged from 97.8% to 99.1%.

Alignment of the deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeV

genotypes 1 to 8 and 14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered critical for HPeV1 entry, was present in the HPeV1, HPeV2, and HPeV4 strains studied but not the HPeV3 strains studied (data not shown).

DISCUSSION

To date, a variable spectrum of symptoms caused by HPeVs has been described. The common symptoms are similar to those caused by some enteroviruses, including mostly enteritis with diarrhea and respiratory disease (3, 6, 17, 31, 32). Other symptoms and syndromes as a result of HPeV infection, such as meningoencephalitis, encephalomyelitis, flaccid transient paralysis, nosocomial infection, neonatal sepsis, myocarditis, myositis, lymphadenopathy, hand-and-mouth disease, rash, fever of unknown origin, influenza-like illness, Reye's syndrome, and hemolytic-uremic syndrome, have also been reported (7, 10–13, 16, 21, 23, 26, 28, 30, 34).

By screening fecal samples known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus for human parechovirus, this study provides one more piece of evidence that HPeV infection is associated with acute gastroenteritis. In addition, with a detection rate of 14.6% among the samples tested, the study demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in Thailand.

In this study, the full length of the 702-base VP1 gene of the Thai strains studied was successfully obtained. However, some of VP1 sequences of the reference strains, especially strain 451564 of new genotype HPeV14 (4), available in the GenBank database were partial VP1 sequences. Consequently, the phylogenetic tree was constructed on the basis of 624 bases. The results showed that four different HPeV genotypes, HPeV1 to HPeV4, were present among Thai infants and children with acute gastroenteritis, and more than a half of the strains detected belonged to HPeV1. That finding was in good agreement with the findings of previous studies, which reported that HPeV1 was predominant over other the HPeV genotypes found in patients with acute gastroenteritis (3, 4, 6, 9).

Among the well-known HPeV genotypes, HPeV2 appears to be a rare genotype. Interestingly, one HPeV2 strain was found in this study. This finding is in support of the statement that HPeV2 infections rarely occur and are mostly associated with gastrointestinal symptoms.

Two HPeV3 strains were isolated in the present study. According to previous reports, infection with HPeV3 is associated with younger age and more severe disease than infection with HPeV1 or HPeV2. Unfortunately, in this study, analysis and comparison of the clinical symptoms related to HPeV3 infection could not be performed due to the lack of availability of clinical data.

In this study, samples positive for HPeV were retested and were confirmed to be HPeV positive. However, of the 12 samples positive for HPeVs, the virus from only 9 samples could be genotyped. Therefore, the possibility that the three samples containing unidentifiable HPeV genotypes contained virus whose genotypes could not be detected by the assay used cannot be excluded. In addition, the possibility that these three samples contained low viral loads also cannot be excluded. Therefore,

a real-time PCR method is more appropriate for the screening of fecal specimens for HPeVs. The fact that this study did not employ real-time PCR is also a limitation of this study.

In conclusion, this is the first report of HPeV infections among infants and children with acute gastroenteritis in Thailand. The diversity of the Thai HPeVs was found by the identification of the four different genotypes of HPeV (HPeV1 to HPeV4) in the samples tested. Taken together with the findings from previous studies, it is suggested that HPeV should be included in the spectrum of viruses for which children with acute gastroenteritis are routinely screened.

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