Diversity of human parechoviruses isolated in stool samples collected from
Thai children with acute gastroenteritis

Ngan Thi Kim Pham\textsuperscript{1}, Quang Duy Trinh\textsuperscript{1}, Pattara Khamrin\textsuperscript{2}, Niwat Maneekarn\textsuperscript{3}, Hidaaki Shimizu\textsuperscript{4}, Shoko Okitsu\textsuperscript{5}, Masashi Mizuguchi\textsuperscript{1}, and Hiroshi Ushijima\textsuperscript{2}\
*Correspondence to: Hiroshi Ushijima, M.D., Ph.D., Aino Health Science Center, Aino University, 2-17-3 Shibuya, Shibuya-ku, Tokyo 150-0002, Japan.

\textsuperscript{1}Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
\textsuperscript{2}Aino Health Science Center and Aino University, Tokyo, Japan
\textsuperscript{3}Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
\textsuperscript{4}Kawasaki City Institute of Health, Kanagawa, Japan
\textsuperscript{5}Aino Health Science Center and Aino College, Tokyo, Japan

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E-mail: ushijima-hiroshi@jcom.home.ne.jp, Phone and Fax: 81-3-3486-8481
ABSTRACT

A total of 82 fecal specimens, which had been known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus, collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand from January to December 2005, were subjected to screening for human parechovirus (HPeV). HPeV was detected by RT-PCR using a primer pair to amplify the 5’UTR 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%. For the detected HPeV, the capsid VP1 gene of nine strains was successfully sequenced. The studied HPeV strains clustered into 4 different genotypes from HPeV1-4, and the majority of the studied HPeV strains belonged to HPeV1. This is the first finding of HPeV from children with acute gastroenteritis in Thailand. In addition, the diversity of Thai HPeV was also noted.

Key words: human parechovirus, diversity, acute gastroenteritis, Thailand
INTRODUCTION

Parechoviruses are small, non-enveloped, 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 has been 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. Based on serological characteristics, two HPeVs were characterized in the early 2004. However, they were first classified as echovirus types 22 and 23, within the enterovirus genus, later were reclassified as HPeVs 1 and 2, respectively, based on distinctive biological and molecular properties. The genome of HPeVs 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).

The previous findings reveal the genetic variability of HPeVs and the number of newly identified HPeV genotypes has been on the increase. Based on VP1 sequence comparisons, there have been nine published HPeV types up to date (1-9, 16, 24, 34). In addition, according to the website of http://www.picornaviridae.com/parechovirus/hpev/hpev.htm, HPeVs have been classified into 14 genotypes, however, the nucleotide sequences of new HPeV strains from genotypes 9 to 13 and corresponding studies have not been published so far.

In Thailand, acute gastroenteritis is the primary cause of morbidity among diseases documented in the annual report of epidemiological surveillance [http://epid.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). Up to date, however, there has been no report on HPeV infection in Thailand. The present study was aimed to screen stool samples collected from children with acute gastroenteritis in Chiang Mai, Thailand for HPeV infection, one of less explored viral pathogens which has been reported to be associated with diarrhea recently; and to characterize the molecular properties of the detected HPeV strains.
MATERIALS AND METHODS

Clinical Specimens

Eighty two fecal specimens, which had been known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus by reverse transcription - polymerase chain reaction (RT-PCR), 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 three years before analysis. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 xg for 10 min. The supernatants were collected and stored at -30°C until use for the detection of virus.

RNA extraction and Reverse Transcription (RT)

The RNA genome of HPeV was first extracted from 140 µl of 10% fecal suspension using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany) according to manufacture’s instructions. Then, for reverse transcription, 5 µl of the stored, extracted RNA was added to a reagent mixture consisting of 5x First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 0.1M DTT (Invitrogen), SuperScript Reverse Transcriptase III (200 U/µl) (Invitrogen, Carlsbad, CA, USA), random primer (1 µg/µl) (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase Inhibitor (33 U/µl) (Toyobo, Osaka, Japan), and distilled water. The total volume of reaction mixture was 15 µl. RT reaction was carried out at 50°C for 1 hr, followed by 95°C for 5 min and then held at 4°C. The cDNA was stored at -30°C until using for PCR reactions (29, 35).

Polymerase Chain Reaction for detection of HPeV

After adding 2 µl of cDNA into 23 µl of the reagent mixture containing 5x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (10 mM), primers (20 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI, USA), and distilled water, screening PCR was conducted using primers ev22(+) and ev22(-) to amplify a 270-bp PCR product of 5’UTR region.
The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min.

Genotyping by VP1 sequencing and primer designation

At first, to amplify the VP1 segment, the previously described primers developed by Benschop and colleagues (6) were used. However, because of failure in obtaining PCR products of most HPeV-positive samples except for one, two new primers were designed for the first PCR. Then, for the nested PCR, it was performed using 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 full genome sequences of reference strains of 8 known HPeV genotypes available in GenBank databases was performed using Clustal X software to find the conserved regions and the two new primers were designed outside the VP1 region. Oligonucleotide sequences of the newly developed primers and their positions were described in Table 1.

The first PCR was done using the new 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, 72°C for 1 min, and a final extension at 72°C for 7 min. The nested PCR was conducted using the known primer pair: VP1-parEchoF1 and VP1-parEchoR1 (6) at 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 SYBR Safe (Invitrogen, Tokyo, Japan) staining under ultraviolet 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).

Sequence and Phylogenetic Analysis

The PCR amplicons of the VP1 gene were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers for amplification of 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 comparison analysis of the VP1 segment was conducted between the obtained HPeV strains and other reference HPeV strains of 9 known genotypes available in GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was also conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 to determine the evolutionary relationship among studied sequences (22). The method was performed using close-neighbor interchange with a random option and with 500 bootstrap repetitions.

The nucleotide sequences of the reference HPeV strains described in this study have been deposited in GenBank under accession numbers: HPeV1: 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), BNI-R30/03 (EU024635); HPeV2: Williamson (AJ005695); HPeV3: Can82853-01 (AJ889918), 677146 (FJ373162), A415-01 (AB300945), A308/99 (AB084913), 651689 (FJ373153); HPeV4: Fuk2001-282 (AB433630), NII370-93 (AB434673), T75-4077 (AM235750), 653046 (FJ373170), K251176-02 (DQ315670); HPeV5: CT86-6760 (AF055846), T92-15 (AM235749), 676618 (FJ373175); HPeV6: 2005-823 (EU077518), NII561-2000 (AB252582), BNI-67/03 (EU024629), 650045 (FJ373178); HPeV7: PAK5045 (EU556224); HPeV8: BR/217/2006 (EU716175); HPeV14: 451564 (FJ373179). For the studied Thai strains, they are FJ648755-FJ648762 and GQ149453.
RESULTS

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

For genotyping, only 9 strains were successfully amplified and sequenced of the full length VP1 capsid sequences. Full length of the VP1 sequences of the 9 studied strains was 702 bases.

Fig.1 shows the phylogenetic tree constructed from 624 nucleotide sequences of partial VP1 segment of reference HPeV strains and 9 Thai strains found in this study. Based on specific clustering of the isolates with known HPeV types obtained from GenBank, the studied strains could be identified as HPeV genotypes 1-4. The majority (5 strains) of the Thai strains belonged to HPeV1, the largest cluster of HPeVs. One Thai strain clustered together with the Williamson strain into 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 the K251176 strain, which has been recently detected in Netherlands.

For the studied HPeV1, 3 strains were found to cluster closely together with the prototype Harris strain and the amino acid similarities between the three strains and the Harris strain ranged from 92.7% to 93.7%; while the two others were in the larger cluster consisting of recently detected HPeV1 strains and the amino acid similarities between the two strains and the Harris strain were less than 90% (86.4% and 89.6%).

In the case of HPeV2 strains, the amino acid similarity between the Thai studied strain and the Williamson strain was 95.2%, while mean amino acid similarities between the Thai strain and other genotypes ranged from 63.8% (with HPeV3) and 74.2% (with HPeV1).

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

Within HPeV4 cluster, the amino acid similarity between the Thai studied strain 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 HPeVs genotypes 1-8 and 14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for HPeV1 entry, was present in the studied HPeV1, HPeV2, HPeV4 strains, except for the studied HPeV3 strains (data not shown).
DISCUSSION

To date, a variable spectrum of symptoms caused by HPeVs has been described. The common symptoms are similar to that caused by some enteroviruses, including mostly enteritis with diarrhea, and respiratory disease (3, 6, 17, 31, 32). Other symptoms and syndromes also have been reported 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, and Reye’s syndrome, haemolytic uremic syndrome (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 evidence of HPeV-associated acute gastroenteritis. In addition, with a detection rate of 14.6% of samples tested, the study demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in Thailand.

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

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

For HPeV3, there were 2 strains 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 on clinical symptoms related to HPeV3 infection could not be performed due to unavailable clinical data.

In this study, positive samples were retested and confirmed as positive. However, of the 12 samples positive for HPeVs, only 9 samples could be genotyped. Therefore, unidentified HPeV genotypes, which the assay used might not be able to pick up, contained in the three ungenotypable samples could not be excluded. In addition, a possibility of low viral load in the three ungenotypable samples could not be excluded as well. Therefore, a real-time PCR method is more appropriate in screening fecal specimens for HPeVs. Not employing real-time PCR is also a limitation of this study.

In conclusion, this is the first report on HPeVs among infants and children with acute gastroenteritis in Thailand. With the identification of the 4 different genotypes of HPeVs (HPeV1-4) in the samples tested, the diversity of Thai HPeVs was found. Taken together with the findings from previous studies, it is suggested that HPeV should be included into the spectrum of viruses which are routinely screened for among children with acute gastroenteritis.
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REFERENCES


TABLE AND FIGURE LEGENDS

Tab.1. Oligonucleotide sequences of the primers used in this study and their positions.
Y stands for C or T, R: G or A, W: A or T, H: A, C, or T, and M means C or A.
Sequence position (*) is based on the full genome sequence of the prototype HPeV1 strain, Harris strain, with the accession number of L02971.

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.
Percentage bootstrap values above 70% are shown at the branch nodes. The studied HPeV strains are in boldface type.
Table 1

<table>
<thead>
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<th>Primer</th>
<th>Gene</th>
<th>Sequence 5' to 3'</th>
<th>Sense</th>
<th>Position*</th>
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<th>Reference</th>
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<td>ev22(+)</td>
<td>5'UTR</td>
<td>CYCACACAGCCATCCTC</td>
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<td>312-328</td>
<td>270</td>
<td>Joki-Korpela and Hyypia, 1998</td>
</tr>
<tr>
<td>ev22(-)</td>
<td>5'UTR</td>
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<td>-</td>
<td>581-565</td>
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<tr>
<td>VP1-parEchoF1</td>
<td>VP1</td>
<td>CCAAAATTCRTGGGGTTC</td>
<td>+</td>
<td>2332-2349</td>
<td>760</td>
<td>Benschop et al., 2006</td>
</tr>
<tr>
<td>VP1-parEchoR1</td>
<td>VP1</td>
<td>TCYACAAACTCTCCACCGC</td>
<td>-</td>
<td>3090-3071</td>
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<td></td>
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<tr>
<td>Cap-parEcho-F</td>
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<td>2162-2181</td>
<td>1076</td>
<td>This study</td>
</tr>
<tr>
<td>Cap-parEcho-R</td>
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<td>-</td>
<td>3237-3218</td>
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<tr>
<td>3DparEcho-F</td>
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<td>GATTGGCACTTYATGATHAATG</td>
<td>+</td>
<td>6555-6576</td>
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<td>-</td>
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Figure 1