Detection of Human Parechoviruses from clinical stool samples in Aichi, Japan

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Abstract

Between April 1999 and March 2008, a total of 4,976 stool specimens collected from patients with suspected viral infection through the infectious agents surveillance in Aichi, Japan, were tested for the presence of human parechoviruses (HPeVs). We detected HPeVs in 110 samples by either cell culture or reverse transcriptase polymerase chain reaction (RT-PCR), or both. Serotyping either by neutralization test or by nucleotide sequence determination and phylogenetic analysis of the VP1 and 5’-UTR regions revealed that 63 were HPeV-1, followed by 44 HPeV-3, 2 HPeV-4, and 1 HPeV-6 strain. The high nucleotide and amino acid sequence identity of the Japanese HPeV-3 isolates in 2006 to the strains previously reported from Canada and Netherlands confirmed the worldwide prevalence of HPeV-3 infection. Ninety-seven % of the HPeV-positive patients were younger than 3 years, 86.2 % younger than 12 months. The clinical diagnoses of HPeV-positive patients were gastroenteritis, respiratory illness, febrile illness, exanthema, ‘hand, foot and mouth disease’, aseptic meningitis, and herpangina. Among 49 HPeV-positive patients with gastroenteritis, 35 were positive with HPeV-1, 12 with HPeV-3, and out of 25 with respiratory illness, 11 with HPeV-1 and 14 with HPeV-3, respectively. HPeV-3 seemed to be an important etiological agent of respiratory infection of children. While HPeV-1 was detected predominantly during fall-winter, the majority of the HPeV-3 was detected during summer and fall. A different pattern of clinical manifestations as well as seasonality suggested that there are different mechanisms of pathogenesis between HPeV-1 and HPeV-3 infection.
Introduction

Human parechoviruses (HPeVs) are members of the family *picornaviridae* and classified into 14 known genotypes (3, 4, 6, 11, 15, 17, 18, 25, 39). HPeV-1 and -2, previously known as echovirus 22 (E-22) and 23 (E-23), were first isolated in 1956. While HPeV-2 infection appears to be rare (2, 13, 20, 29), HPeV-1 is prevalent worldwide. HPeV-3 was recently isolated from a stool specimen of a young child with transient paralysis in our laboratory (18) and has been also reported to be detected from critically ill children who reported to have viremia, neonatal sepsis and sudden infant death syndrome (1, 2, 5, 33, 38). HPeV-4 was first reported in 2006 from stool specimen of a 6-day-old infant with a 2-day history of high fever and poor feeding (4). CT86-8760 (connecticut/86), formerly typed as E-23 (HPeV-2) by neutralization test (29), was recently reclassified as the fifth type of HPeV (HPeV-5) based on the phylogenetic analysis of the VP1 region (3). Recently, the sixth type of HPeV (HPeV-6) was isolated from cerebrospinal fluid of a patient with Reye syndrome (38). The seventh type of HPeV (HPeV-7) was found in stool sample from a healthy boy who had close contact with a person who had nonpolio acute flaccid paralysis (AFP) in Pakistan (25). The eight type of HPeV (HPeV-8) was detected by RT-PCR in stool sample from a child with enteritis in Brazil (11). The 14th type of HPeV (HPeV-14) was found in stool sample from a child in Netherlands (7). The nucleotide sequences of new HPeV strains, i.e., genotypes 9 to 13 have not been published so far.

Several former studies established and improved the sensitivity of HPeV detection from stool specimens (10, 24, 30), but the full spectrum of clinical manifestations and the epidemiology of HPeV infection, as well as the prevalence and pathogenesis of each HPeV type, still remain undetermined. In an attempt to characterize the epidemiologic and pathogenic aspects of each HPeV serotype, we here report the incidence and clinical
features of HPeV infection in children based on the analyses of epidemiological
information and stool specimens collected from approximately 5,000 patients visiting
pediatric clinics during recent 10 years as a routine surveillance system to identify the
prevalence of viral pathogens.

**Materials and Methods.**

*Clinical specimen collection.*

Specimens were collected as part of the National Epidemiological Surveillance of
Infectious Diseases in which 29 medical institutions in the Aichi Prefecture (except for
the Nagoya City area) were enrolled as the sentinels for infectious agent surveillance.
Between April 1999 and March 2008, a total of 4,976 stool specimens were collected
from patients with suspected viral infection (Table 1). All specimens were collected
after the parents of the enrolled children had given informed consent. Demographic
and clinical information were extracted from the patient record provided by the
attending physician to each specimen. The clinical diagnoses of these patients were
shown in Table 2.

*Virus isolation from stool specimens and serotyping*

Feces were diluted with veal infusion broth to 10% (w/v) suspensions, and centrifuged
at 10,000 x g for 20 min. The supernatant was inoculated onto HeLa and Vero cells, and
observed for up to 2 weeks, followed by subpassage to fresh cells for an additional
2-week observation for the presence of cytopathic effects (CPE). Following the
emergence of CPE, serotyping of each virus isolate was performed by neutralizing test
(26) using type-specific antisera, i.e., anti-HPeV-1 (Harris strain) distributed from the
National Institute of Infectious Diseases, Japan, and anti-HPeV-3 (A308/99 strain)
prepared in this laboratory as pooled guinea pig immune serum.
**RT-PCR and sequencing.**

Nucleic acid was extracted directly from the sample stool suspension prepared above and also from the supernatant of inoculated cell cultures using either the QIAamp Viral RNA (DNA) mini kit (QIAGEN) or High Pure Viral RNA kit (Roche) according to each manufacturer’s instructions.

RT-PCR was performed using the One-step RT-PCR kit (Invitrogen) as described by the manufacturer. For amplification of the part of 5’UTR region, HPeV PCR ev22 (+) and (-) primer pair (19) was used. In addition, the amplification of the nucleotides in VP1 region was also performed using the following primers designed by comparison of the known 6 HPeV serotypes (Fig. 1): A forward primer with a sequence of 5’-GGD ARR MTK GGD VAW GAY GC-3’ (HPeV-VP1S) and a reverse primer with a sequence of 5’-CCA TAR TGY TTR TAR AAA CC-3’ (HPeV-VP1AS) and nested-PCR reverse primer with a sequence of 5’-TCY ARY TGR TAY ACA YKS TCT CC-3’ (HPeV-VP1AS2). Cycling conditions were as follows: 50 °C for 30 min; 95 °C for 1 min; 35 cycles consisting of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 90 sec; followed by a final extension at 72 °C for 10 min. Amplicons were detected by 0.5 % TAE agarose gel electrophoresis and staining with ethidium bromide.

All samples from gastroenteritis patients were tested for the detection of Norovirus (22), Rotavirus (16), and Adenovirus (34) using PCR, which were performed according to previously published protocols.

Selected PCR-amplified products were purified and introduced into a pGEM-T vector (Promega). The DNA sequence was determined by an automated DNA sequencer (Model-4000, Li-cor Lincoln, Nebr., USA). Each serotype was determined by the comparison of the nucleotide sequence to the available HPeV sequences from GenBank.
using the Genetyx program (Genetix, New Milton, Hampshire, UK).

**Phylogenetic tree analysis.**

Nucleotide and deduced amino acid sequences of amplified VP1 regions were aligned with representative HPeV strains by using the Genetyx and Neighbor software package of the Phylip program in the Genetics Computer Group (GCG) package. Phylogenetic trees were constructed using UPGMA (unweighted pair group method with averages) in the same package. The following nucleotide sequences were obtained from GenBank:

- HPeV-1 (Harris strain) L02971; HPeV-1 (BNI-788st strain) EF051629; HPeV-2 (Williamson strain) AJ005695; HPeV-3 (A308-99 strain) AB084913; HPeV-3 (Can82853-01 strain) AJ889918; HPeV-4 (K251176-02 strain) DQ315670; HPeV-4 (T75-4077 strain) AM235750; HPeV-5 (Connecticut/86-6760 strain) AF055846; HPeV-5 (T92-15 strain) AM235749; HPeV-6 (NII561-2000 strain) AB252582; HPeV-6 (BNI-67-03 strain) EU024629; HPeV-7 (PAK5045 strain) EU556224; HPeV-8 (BR217 2006 strain) EU716175; HPeV-14 (451564 NL,2004 strain) FJ373179; Ljungan virus (AF327920); and published isolates for HPeV-1 and HPeV-3.

**[Nucleotide sequence accession numbers.]** The nucleotide sequences of HPeV strains reported in this paper were submitted to GenBank, under accession numbers AB300926 to AB300986 and AB469777 to AB469782.

**Results**

**Detection of HPeVs in clinical specimens.**

Out of 4,976 samples, 110 (2.2%) were positive for HPeVs either by RT-PCR using primers for 5’UTR region alone or by both virus isolation and RT-PCR (Table 1). HPeVs were isolated from 74 of 4,976 (1.5%) samples. All culture-positive samples
were also positive by RT-PCR as described below. Out of 110 samples that were
present for 5’UTR amplification, 62 were also positive for VP1 amplification and were
typed as described in the next section. Among 38 samples that were negative for VP1
amplification, five of them were positive for HPeV isolation, i.e., 1 HPeV-1 and 4
HPeV-3. The remaining 33 were identified as 23 HPeV-1 or 10 HPeV-3 using 5’UTR
sequences with 100% bootstrap support for standard strain and isolations identified by
neutralizing test. In addition, 5’UTR amplicons from the 5 VP1-negative specimens
mentioned above were also typed accordingly.

Of 74 isolates, 71 (95.9%) could be typed by the neutralization test using the antisera
against HPeV-1 and HPeV-3, 37 (50%) typed as HPeV-1, 34 (45.9%) as HPeV-3.
Serotypes of the remaining 3 isolates were determined as 2 HPeV-4 and 1 HPeV-6 by
molecular characterization. HPeV nucleotide sequences were detected in 110 of 4,976
samples, 63 (57.2%) were typed as HPeV-1, 44 (40%) were HPeV-3, two were HPeV-4 and one was HPeV-6.

Copathogens were also found in 8 (10.8%) of 74 samples positive for HPeV isolation.
Out of these 8 samples, 2 were positive for Norovirus genotype II, and the remaining 6
samples were positive for one of the following viruses: Enterovirus 71, Norovirus
genotype I, Group A Rotavirus, Adenovirus 2, Adenovirus 41, Coxsackievirus A8 (Cox.
A8). Enterovirus 71 was isolated from a patient with hand, foot and mouth disease,
and Cox. A8 was from one with herpangina, and remaining 6 were from those with
gastroenteritis.

**Genome sequence determination and phylogenetic analysis of VP1 region.**

Out of 4,976 samples, amplification of VP1 region by RT-PCR were successful for 72
(1.4%) samples, all of which were also positive for 5’UTR amplification. The
nucleotide sequences of these amplicons were determined and utilized for serotyping as
Serotypes were determined based on the nucleotide sequence of the entire VP1 region. A phylogenetic tree was constructed with these data and also with the partial nucleotide sequence of the published strains (Fig. 2). Our HPeV-1 isolates (n=39) were only 73.4 to 78.6% similar to the prototype Harris strain and formed several clusters, and related to those recent clinical isolates reported from the Netherlands and Germany. Nucleotide sequences of HPeV-1 isolates comprising different clusters showed 16.5% difference each other. Clustering was independent from chronological and geographical distribution or seasonality of sample collection or clinical manifestations.

All 29 HPeV-3 sequences determined in this study were related not only to the prototype A308-99 but also to the isolates reported from Canada and Netherlands. These strains showed 91.4 to 100% identity each other and formed the same branch of the VP1 tree, indicating a common ancestor prevalent in the world.

The intratypic nucleotide (and amino acid) similarity in the VP1 region of the HPeV-1, HPeV-3, HPeV-4, and HPeV-6 were 64-72% (62-81%), 64-70% (62-77%), 67-71% (65-78%), and 65-71% (67-81%), respectively.

Dermographic features of HPeV-positive patients.

The age at the specimen sampling was known for 106 of entire 107 HPeV-positive patients, mean age being 12.9 months. All 63 children positive for HPeV-1 were younger than 5 years (except for the one unknown), 55 (87.3%) less than 1 year, with an average 13 months; similarly, all 44 who are positive for HPeV-3 were younger than 6 years of age, 38 (86.3%) aged less than 1 year, with an average of 12.1 months.

The male-to-female ratio was 2.4 to 1 (44 vs 18) for patients with HPeV-1, and 1.32 to 1 (25 vs 19) for HPeV-3, respectively. The male to female ratio for the all stool samples was 1.3 to 1 (2,818 vs 2,144 except 14 unknown).
Clinical manifestations of HPeV-positive patients.

From stool specimens collected from pediatric outpatients, HPeV was isolated every year from 1999 to 2007, most frequently in 1999 (22) and the least in 2007 (6) (Table 1). Clinical diagnoses of these 110 HPeV-positive patients are summarized in Table 2. The most common diagnosis was gastroenteritis, followed by respiratory illness, unspecified febrile illness, exanthema, ‘hand, foot and mouth disease’, aseptic meningitis, and herpangina. Among the 63 patients who were positive for HPeV-1, 35 (55.6%) were diagnosed as gastroenteritis, 11 (17.5%) as respiratory illness. In contrast, of 44 HPeV-3-positive patients, 14 (31.8%) were with respiratory illness, followed by 12 (27.3%) with gastroenteritis.

Overall, both HPeV-1 and -3 were detected almost throughout the year but certain distinct seasonality was observed for both serotypes. Fifty-four (85.7%) out of 63 HPeV-1-positive samples were collected from August to January, and 33 of them (33/54, 61%) associated with gastrointestinal symptoms. In comparison, HPeV-3 appeared a couple of months earlier than HPeV-1, and 38 (86.4%) of 44 HPeV-3-positive samples were collected during June to November (Fig 3). In particular, 11 (91.7%) of 12 HPeV-3-positive specimens associated with gastrointestinal symptoms were collected during July and November.

The two HPeV-4 strains were detected in August and November, respectively, both from samples derived from patients with gastroenteritis. HPeV-6 was detected from one sample which had been collected in June from a patient with herpangina and also positive for Cox. A8.
Discussion

HPeVs have been classified into 6 serotypes based on neutralization test or molecular identification, and at least 3 new serotypes (HPeV-7, 8, 14) being identified (3, 4, 6, 7, 11, 15, 17, 18, 25, 39). Clinical manifestations that have been associated with HPeV infections include gastroenteritis, respiratory diseases, aseptic meningitis, encephalomyelitis, AFP, lymphadenopathy, myocarditis, haemolytic uraemic syndrome, neonatal sepsis-like syndromes with necrotizing enterocolitis, sudden infant death syndrome (SIDS), notably in young children (1-15, 18, 19, 23-25, 27, 28, 32, 33, 35, 39). We therefore tested stool specimens collected from children visiting pediatric clinics with suspected viral infections between year 1999 and 2008 for HPeV-3 as well as the other HPeVs, and detected HPeV in 110 (2.2%) of 4,976 samples by RT-PCR. To our knowledge, this is the first description of routine surveillance findings in which a new and improved method directed specifically for detection of all 6 types of HPeVs has been applied.

To determine the efficiency of current molecular diagnostic methods using HPeV VP1 and/or 5’-UTR sequences obtained by RT-PCR (2, 5, 18, 19, 30), we performed RT-PCR which detect HPeV-1, 3, 4, and 6 simultaneously, in parallel with the conventional virus isolation using Vero and HeLa cell culture. We evaluated the incidence over a full year period by RT-PCR testing and cell culture. In conclusion, RT-PCR method as well as cell culture is recommended as the primary diagnostic tool for HPeVs infections, if confirmation using cell culture for isolation of copathogens and new strains that are untypable by the sequence of RT-PCR products.

In addition, we designed a new RT-PCR primer that amplify VP1 region of all six known serotypes of HPeV. Phylogenetic analysis using VP1 region was possible for 72 of 110 (65.5%) HPeV RNA-positive samples. There were 33 samples from which
VP1 sequence could not be amplified by the current RT-PCR protocol which were typed comparing 5’UTR sequences to the standard strain/isolate with 100% bootstrap support. As 5’UTR sequences tend to be highly conserved and therefore not entirely suitable for serotyping (31, 38), development of a more sensitive RT-PCR protocol for VP1 region is required. Still, molecular typing methods have established to circumvent practical problems associated with traditional serum neutralization and provide an indispensable supporting data for serotyping. In this study, two HPeV-4 and one HPeV-6 that were not neutralized with anti HPeV-1,-2 and -3 sera were typed solely by molecular methods described above. Viral genomic sequences should also be useful for the identification of previously unreported genotypes. They also provide an essential information for the determination of specific molecular target sequences for the virus identification.

By phylogenetic analysis of HPeV-1, each of our HPeV-1 isolates belonged to several clusters, with no specific temporal or geographic patterns. In contrast to HPeV-1, we could identify close similarity between HPeV3 strains in (Fig 2)

Of the 110 children positive for HPeVs, HPeV-3 was second most important pathogen following HPeV-1. As reported previously from Europe (5) and North America (2), we confirmed that HPeVs are associated not only with gastroenteritis but also with respiratory illnesses of young children. While HPeV-1 was most prevalent in patients with gastroenteritis, representing almost 55.6%, HPeV-3 seems to be responsible not only for gastroenteritis but also, or more notably, for respiratory symptoms.

Whereas HPeV-1 did not seem to be involved in central nervous system (CNS) infections, HPeV-3 has been reported to be associated with sepsis-like illness or CNS involvement or cases of SIDS (1, 33). While we could not determine the involvement of HPeV-3 in these serious clinical situations, it should be noted that HPeV-3 was detected from 9 samples from unrelated patients, 6 with febrile illness and 3 with
aseptic meningitis. Although HPeV was detected throughout the year, we noticed a
different pattern of seasonality between HPeV-1 and HPeV-3, suggesting different
pathogenesis between these two serotypes.

All HPeV-positive samples obtained in this study were from children who were 6 years
or younger, majority of those younger than 1 year of age (96/109; 88.1%). Previous
seroepidemiology revealed that the proportion of seropositive individuals increases
rapidly with age, reaching 75 to 100% by 6 years of age in this area for both HPeV-1
and HPeV-3 (18). These results suggested that both HPeV-1 and -3 are endemic and
most HPeV infections occur in pediatric population. More HPeV infections were
observed in male than in female patients. In particular, HPeV-1 was detected in male
2.5 times more than female, inspiring a need to further investigate if there is any
different susceptibility between sexes.

HPeV-4 was recently isolated from a stool specimen of a 6-day-old patient with fever
and poor feeding and no history of specific gastrointestinal or respiratory symptoms in
Netherlands. In our study, the two HPeV-4 strains, A374-06 and A386-07 were both
isolated from patients with gastroenteritis, aged 2 month and one year, respectively.
Adenovirus 2 was also detected from the stool sample with A386-07. HPeV-6 was
first reported from a cerebrospinal fluid of patient with Reye syndrome in Niigata,
Japan (NII561-2000 in Fig. 2) (39). In this study, the HPeV-6 isolate (A231-01) was
isolated from the stool of 11 month old patient with herpangina, whose throat swab was
positive for Cox.A8. Since we detected only two and one strain in this 9-year
surveillance, both HPeV-4 and 6 are either less related to disease manifestations or less
prevalent, or both, in Aichi area than HPeV-1 and 3. Further researches, especially on
both case- and sentinel-based surveillances, are needed to clarify the prevalence and
etiological importance of each serotype of HPeV in the general population.
References


17. Hyypia, T., Horsnell, C., Maaronen, M., Khan, M., Kalkkinen, N., Auvinen, P.,


Table 1 Detection of HPeVs from 4,976 fecal samples obtained between April 1999 and March 2008 in Aichi Prefecture, Japan.

<table>
<thead>
<tr>
<th>Year</th>
<th>No of samples</th>
<th>No of positive samples</th>
<th>HPeV-1</th>
<th>HPeV-3</th>
<th>HPeV-4</th>
<th>HPeV-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999 (Apr. to Dec.)</td>
<td>748</td>
<td>22(16)</td>
<td>9(5)</td>
<td>13(11)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2000</td>
<td>739</td>
<td>14(5)</td>
<td>11(5)</td>
<td>3(3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2001</td>
<td>263</td>
<td>7(4)</td>
<td>4(1)</td>
<td>2(2)</td>
<td>0</td>
<td>1(1)</td>
</tr>
<tr>
<td>2002</td>
<td>274</td>
<td>10(8)</td>
<td>5(3)</td>
<td>5(5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>327</td>
<td>10(1)</td>
<td>6(1)</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2004</td>
<td>462</td>
<td>13(10)</td>
<td>10(8)</td>
<td>3(2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2005</td>
<td>529</td>
<td>12(8)</td>
<td>12(6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2006</td>
<td>837</td>
<td>16(16)</td>
<td>1(1)</td>
<td>14(14)</td>
<td>1(1)</td>
<td>0</td>
</tr>
<tr>
<td>2007</td>
<td>674</td>
<td>6(6)</td>
<td>5(3)</td>
<td>0</td>
<td>1(1)</td>
<td>0</td>
</tr>
<tr>
<td>2008 (Jan. to Mar.)</td>
<td>105</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4976</td>
<td>1(1074)</td>
<td>6(337)</td>
<td>44(34)</td>
<td>2(2)</td>
<td>1(1)</td>
</tr>
</tbody>
</table>

A parenthesis Number of cell culture positive
Table 2 Detection of HPeVs from fecal samples with various clinical manifestations.

<table>
<thead>
<tr>
<th>Clinical symptoms</th>
<th>No of patients with HPeV (% of total)</th>
<th>No of patients with HPeV-1 (% of total)</th>
<th>HPeV-3 (% of total)</th>
<th>HPeV-4</th>
<th>HPeV-6</th>
<th>No of patients tested (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastroenteritis</td>
<td>49 (44.5)*</td>
<td>35 (55.6)</td>
<td>12 (27.3)</td>
<td>2</td>
<td>0</td>
<td>2204 (44.3)</td>
</tr>
<tr>
<td>Respiratory illness</td>
<td>25 (22.7)</td>
<td>11 (44.2)</td>
<td>14 (54.5)</td>
<td>0</td>
<td>0</td>
<td>735 (14.8)</td>
</tr>
<tr>
<td>Undifferentiated febrile illness</td>
<td>14 (12.7)</td>
<td>8 (57.1)</td>
<td>6 (42.9)</td>
<td>0</td>
<td>0</td>
<td>190 (3.8)</td>
</tr>
<tr>
<td>Exanthema</td>
<td>10 (9.1)</td>
<td>6 (60.0)</td>
<td>4 (40.0)</td>
<td>0</td>
<td>0</td>
<td>106 (2.1)</td>
</tr>
<tr>
<td>Hand-foot-mouth disease</td>
<td>7 (6.4)</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
<td>0</td>
<td>0</td>
<td>274 (5.5)</td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>4 (3.6)</td>
<td>1 (25.0)</td>
<td>3 (75.0)</td>
<td>0</td>
<td>0</td>
<td>249 (5)</td>
</tr>
<tr>
<td>Herpangina</td>
<td>1 (0.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>193 (3.9)</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1025 (20.6)</td>
</tr>
<tr>
<td>Total</td>
<td>110 (100)</td>
<td>63 (100)</td>
<td>44 (100)</td>
<td>2</td>
<td>1</td>
<td>4976 (100)</td>
</tr>
</tbody>
</table>

* % of total
Fig. 1

Ambiguity codes: D, A or T or G; K, T or G; M, A or T; R, A or G; S, C or G; V, A or C or G; W, A or T; and Y, C or T.

Positions are relative to the genome of HPeV-1-Harris (GenBank accession No. L02971).
Monthly distribution of HPeVs.
Figure legend

**Fig1.** Schematic representation of the parechovirus genome and locations of the primer sites.

**Fig2.** Phylogenetic analysis of HPeV isolates and prototype based on nucleotide sequences of the VP1 region. The corresponding gene sequences of previously reported Canadian, Dutch, and German isolates were also included. The dendrograms were generated by evolutionary distances, as computed by UPGMA. The isolates reported in this study are indicated by the isolate number and the year (e.g., A322-04). The Canadian isolate is indicated by Can82853-01. The Germany isolate is indicated by BNI-788St. The Dutch isolates are indicated by GenBank accession numbers DQ172419-DQ172450.

**Fig3.** Histogram of monthly distribution of RT-PCR positive clinical cases for HPeV between year 1999 and 2008.