Detection of Human Parechoviruses from Clinical Stool Samples in Aichi, Japan

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Between April 1999 and March 2008, a total of 4,976 stool specimens collected from patients with suspected viral infection through infectious agent surveillance in Aichi, Japan, were tested for the presence of human parechoviruses (HPeVs). We detected HPeVs in 110 samples by either cell culture, reverse transcriptase PCR (RT-PCR), or both. Serotyping either by neutralization test or by nucleotide sequence determination and 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 a stool sample from a child in Netherlands (7). The 14th type of HPeV (HPeV-14) was found in a 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 previous 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 a recent 10-year period 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 was extracted from the patient record provided by the attending physician for each specimen. The clinical diagnoses of these patients are shown in Table 2.

**Virus isolation from stool specimens and serotyping.** Feces were diluted with veal infusion broth to 10% (vol/vol) suspensions and centrifuged at 10,000 × 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 period. The clinical diagnoses of these patients are shown in Table 2.
observation for the presence of cytopathic effects (CPE). Following the emergence of CPE, serotyping of each virus isolate was performed by neutralization test (26) using type-specific antisera, i.e., anti-HPeV-1 (Harris strain) distributed by the Genex and Neighbor software package of the Phylogeny Program (Genetix, New Milton, Hampshire, United Kingdom).

**Phylogenetic tree analysis.** Nucleotide and deduced amino acid sequences of amplified VP1 regions were aligned with those of representative HPeV strains by using the Genetyx program (Genetix, New Milton, Hampshire, United Kingdom).

**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 the 5′UTR region alone or by both virus isolation and RT-PCR (Table 1).

HPeVs were isolated from 74 of 4,976 samples (1.5%). All isolated samples were also positive by RT-PCR as described below. Out of 110 samples that were positive 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, 5 of them were positive for HPeV isolation, i.e., 1 HPeV-1 strain and 4 HPeV-3 strains. The remaining 33 were identified as 23 HPeV-1 strains and 10 HPeV-3 strains using 5′UTR sequences with 100% bootstrap support for the standard strain and isolates identified by neutralizing test. In addition, 5′UTR amplicons from the five 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 and 34 (45.9%) as HPeV-3. The serotypes of the remaining three isolates were determined as HPeV-4 (two isolates) and HPeV-6 (one isolate) by molecular

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

<table>
<thead>
<tr>
<th>Yr (April-December)</th>
<th>No. of samples*</th>
<th>HPeV-1</th>
<th>HPeV-3</th>
<th>HPeV-4</th>
<th>HPeV-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>748</td>
<td>22 (16)</td>
<td>9 (5)</td>
<td>13 (11)</td>
<td>0</td>
</tr>
<tr>
<td>2000</td>
<td>738</td>
<td>14 (5)</td>
<td>11 (5)</td>
<td>3 (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>
</tr>
<tr>
<td>2002</td>
<td>274</td>
<td>10 (8)</td>
<td>5 (3)</td>
<td>5 (5)</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>327</td>
<td>10 (1)</td>
<td>6 (1)</td>
<td>4 (0)</td>
<td>0</td>
</tr>
<tr>
<td>2004</td>
<td>482</td>
<td>13 (10)</td>
<td>10 (8)</td>
<td>3 (2)</td>
<td>0</td>
</tr>
<tr>
<td>2005</td>
<td>528</td>
<td>12 (8)</td>
<td>12 (8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2006</td>
<td>537</td>
<td>16 (16)</td>
<td>1 (1)</td>
<td>14 (14)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>2007</td>
<td>674</td>
<td>6 (6)</td>
<td>5 (5)</td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>2008</td>
<td>105</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis indicate the number of cell culture-positive samples.

**TABLE 2. Detection of HPeVs in fecal samples from patients with various clinical manifestations**

<table>
<thead>
<tr>
<th>Clinical symptom</th>
<th>No. (%) of patients with:</th>
<th>No. (%) of patients tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastroenteritis</td>
<td>49 (44.5)</td>
<td>110 (100)</td>
</tr>
<tr>
<td>Respiratory illness</td>
<td>25 (22.7)</td>
<td>63 (100)</td>
</tr>
<tr>
<td>Undifferentiated febrile illness</td>
<td>14 (12.7)</td>
<td>44 (100)</td>
</tr>
<tr>
<td>Exanthema</td>
<td>10 (9.1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Hand-foot-mouth disease</td>
<td>7 (6.4)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>4 (3.6)</td>
<td>0</td>
</tr>
<tr>
<td>Herpangina</td>
<td>1 (0.9)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Total 110 (100)
characterization. HPeV nucleotide sequences were detected in 110 of 4,976 samples (2.2%) by RT-PCR. Of 110 amplicons, 63 (57.2%) were typed as HPeV-1, 44 (40%) were HPeV-3, 2 were HPeV-4, and 1 was HPeV-6.

Copathogens were also found in 8 (10.8%) of 74 samples positive for HPeV isolation. Out of these eight samples, two were positive for norovirus genotype II, and the remaining six samples were positive for one of the following viruses: enterovirus 71, norovirus genotype I, group A rotavirus, adenovirus 2, adenovirus 41, or coxsackievirus A8 (Cox.A8). Enterovirus 71 was isolated from a patient with hand, foot, and mouth disease; Cox.A8 was from a patient with herpangina; and the remaining six were from patients with gastroenteritis.

Genome sequence determination and phylogenetic analysis of the VP1 region. Out of 4,976 samples, amplification of the VP1 region by RT-PCR was successful for 72 samples (1.4%), all of which were also positive for 5′UTR amplification. The nucleotide sequences of these amplicons were determined and utilized for serotyping as described below.

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 sequences 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 they were related to those recent clinical isolates reported from Netherlands and Germany. Nucleotide sequences of HPeV-1 isolates comprising different clusters showed a 16.5% difference from each other. Clustering was independent from chronological and geographical distribution, 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 to each other and formed the same branch of the VP1 tree, indicating a common ancestor prevalent in the world.

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

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

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

Clinical manifestations in 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 least frequently in 2007 (6) (Table 1). The 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 having gastroenteritis and 11 (17.5%) as having respiratory illness. In contrast, all 44 HPeV-3-positive patients, 14 (31.8%) had respiratory illness, followed by 12 (27.3%) with gastroenteritis.

Overall, both HPeV-1 and -3 were detected almost throughout the year, but a 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%) were associated with gastrointestinal symptoms. In contrast, 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 asso-
FIG. 2. 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 are 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 to DQ172450.
HPEVs have been classified into six serotypes based on neutralization test or molecular identification, and at least three new serotypes (HPEV-7, -8, and -14) have been 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, hemolytic uremic syndrome, neonatal sepsis-like syndromes with necrotizing enterocolitis, and 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 1999 and 2008 for HPEV-3 as well as the other HPEVs, and we 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 six 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 detects HPEV-1, -3, -4, and -6 simultaneously, in parallel with conventional virus isolation using Vero and HeLa cell culture. We evaluated the incidence over a full year by RT-PCR testing and cell culture. We conclude that the RT-PCR method as well as cell culture can be recommended as the primary diagnostic tool for HPEV infections, using confirmation by cell culture for isolation of copathogens and new strains that are untypeable by using the sequences of RT-PCR products.

In addition, we designed a new RT-PCR primer that amplifies the VP1 regions of all six known serotypes of HPEV. Phylogenetic analysis using the VP1 region was possible for 72 of 110 HPEV RNA-positive samples (65.5%). There were 33 samples from which the VP1 sequence could not be amplified by the current RT-PCR protocol, and these were typed by comparing 5’UTR sequences to those of 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 the VP1 region is required. Still, molecular typing methods have been established to circumvent practical problems associated with traditional serum neutralization and provide indispensable supporting data for serotyping. In this study, two HPEV-4 isolates and one HPEV-6 isolate that were not neutralized with anti HPEV-1, -2, and -3 sera were typed solely by the molecular methods described above. Viral genomic sequences should also be useful for the identification of previously unreported genotypes. They also provide essential information for the determination of specific molecular target sequences for 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 the case for HPEV-1, we could identify close similarity between HPEV-3 strains (Fig. 2).

Of the 110 children positive for HPEVs, HPEV-3 was second most important pathogen, following HPEV-1. As reported previously for samples from Europe (5) and North America (2), we confirmed that HPEVs are associated not only with gastroenteritis but also with respiratory illness in young children. While HPEV-1 was most prevalent in patients with gastroenteritis, representing almost 55.6% of the cases, 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, 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 in nine samples from unrelated patients, six with febrile illness and three 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 pathogeneses of these two serotypes.

All HPEV-positive samples obtained in this study were from children who were 6 years or younger, with the majority (96/109, 88.1%) being younger than 1 year of age. 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 that most HPEV infections occur in the pediatric population. More HPEV infections were observed in male than in female patients. In particular, HPEV-1 was detected in males 2.5 times more than in females, indicating a need to further investigate if there is any different susceptibility between the 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...
REFERENCES


