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Human Parechovirus Infections in Dutch Children and the Association between Serotype and Disease Severity

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Background. Human parechoviruses (HPeVs) are members of the family *Picornaviridae* and are classified into 3 known serotypes: HPeV1, HPeV2, and the recently identified HPeV3. HPeV1 and HPeV2 infections are most commonly associated with mild respiratory or gastrointestinal symptoms and occasionally with severe disease conditions, such as flaccid paralysis and encephalitis. HPeV3 infection has been associated with transient paralysis and neonatal infection and has until now only been reported in Japan and Canada.

Methods. Culture isolates considered to be enterovirus on the basis of cell culture but that were found to be enterovirus negative by 5'untranslated region reverse-transcriptase polymerase chain reaction (5'UTR RT-PCR) during the period December 2000 through January 2005 were selected. Isolates were tested by HPeV 5'UTR RT-PCR and were genotyped by sequencing the VP1 region. Phylogenetic analysis was performed, and the association with clinical symptoms was established.

Results. Thirty-seven (12%) of the 303 isolates that tested positive for enterovirus by cell culture were in fact HPeV. The majority of the HPeV-positive isolates ($n=27$) could be identified as HPeV1. The remaining 10 isolates, which were grown from samples obtained in 2001, 2002, and 2004, could be typed as the recently identified HPeV3. HPeV was exclusively detected in children aged <3 years. Children infected with HPeV3 were significantly younger than children infected with HPeV1, and sepsis-like illness and central nervous system involvement were more frequently reported in children infected with HPeV3.

Conclusions. We report HPeV infections in young children during the period of 2000–2005 and show an association between HPeV3 infection and sepsis-like illness and central nervous system involvement in neonates.

INTRODUCTION

Picornaviruses form a group of small, nonenveloped, positive single-stranded RNA viruses that are grouped into 9 genera, including enteroviruses and parechoviruses. The human parechoviruses (HPeVs) were previously known as enteroviruses echo22 and echo23, which were first isolated in 1956 [1, 2]. When molecular techniques were designed for

the detection of enterovirus, echo22 and echo23 could not be detected, and sequence analysis revealed that they were genetically distinct from all other picornaviruses. Therefore, they were renamed “HPeV1” and “HPeV2,” belonging to a new genus of parechoviruses within the *Picornaviridae* [3]. The HPeV genome is similar to that of other picornaviruses, except for the fact that the capsid protein VP0 is not cleaved for maturation into VP2 and VP4 [1, 2].

HPeV1 is considered to be a widely spread pathogen that mainly affects young children [1]. HPeV1 infections are most commonly associated with mild gastrointestinal or respiratory symptoms. Severe disease conditions, such as flaccid paralysis [4] and encephalitis [5] have also been reported, but they appear to occur less frequently in cases of HPeV1 infection than in cases of enterovirus infection [2]. HPeV2 infections rarely occur and are mostly associated with gastrointestinal symptoms [6].

Recently, a new HPeV serotype (HPeV3) was isolated from a 1-year-old patient with transient paralysis in Japan [7]. HPeV3 has also been found in 3 patients aged <1 month who had neonatal sepsis in Canada [8].

HPeV can grow on tertiary monkey kidney cells, human embryonic lung cells, and African green monkey kidney (Vero) cells, and it has the same cytopathogenic effect as enterovirus. With the development of molecular techniques, many diagnostic laboratories replaced cell culture with RT-PCR that is based on the highly conserved 5'untranslated region (5'UTR) to detect enterovirus in CSF specimens. However, PCR assays based on the 5'UTR region of enterovirus will not detect HPeV. RT-PCR assays for HPeV are available [9], but they are not routinely used to detect HPeV in clinical samples. Therefore, infections with HPeV are either missed or may be misdiagnosed as enterovirus infection.

Here, we studied whether enterovirus infections that were diagnosed by cell culture in our laboratory during the period of 2000–2005 could, in fact, have been HPeV infections. We also investigated whether the newly described HPeV3 serotype could be found in our clinical isolates by genotyping of the VP1 region. Classification of enteroviruses by VP1 genotyping closely relates to the classification by serotyping [10–14]. This has also been described for another picornavirus, the foot-and-mouth disease virus [15]. For HPeV, the variability within the VP1 region allows one to distinguish between the 3 serotypes of HPeV [7, 8]. Because it has been suggested that HPeV3 infection can lead to severe morbidity in young children, we studied the association between the different HPeV serotypes and various clinical symptoms.

METHODS

Clinical samples

Fecal samples and throat swab specimens that are sent to our laboratory for enterovirus diagnosis are routinely cultured on tertiary monkey kidney cells, Vero cells, and human embryonic lung cells. Viral culture is performed by cocultivation of patient material with tertiary monkey kidney cells, Vero cells, and human embryonic lung cells in tubes containing 1 mL of minimum essential medium Hanks 8% fetal cow serum. These viral cultures are examined twice weekly for the appearance of enterovirus-specific cytopathogenic effect. Preliminary identification of isolates is performed according to either the unstained cytopathogenic effect or immunofluorescence with a specific monoclonal antibody (DAKO-Enterovirus monoclonal antibody 5-D8/1; DAKO) [16]. Viral cultures are incubated for a maximum of 21 days before they are considered negative. All culture isolates that are determined by cell culture to be enterovirus are routinely sent to a reference laboratory (National Institute for Public Health and the Environment, Bilthoven, The Netherlands) for poliovirus surveillance.

From December 2000 through January 2005, a total of 284 enterovirus-positive culture isolates were sent to the National Institute for Public Health and the Environment. Thirty-five culture isolates that then tested enterovirus negative by the 5'UTR RT-PCR at the National Institute for Public Health and the Environment were selected for this study. Additionally, 19 samples that were found to be enterovirus positive in cell culture but were not yet sent to the National Institute for Public Health and the Environment were also included.

RNA extraction

RNA was extracted from culture isolates using the method described by Boom et al. [17]. Twenty-five μL of a 100-fold dilution in PBS of the culture isolates was added to 900 μL of lysis buffer and 20 μL of size-fractionated silica coarse particles. The extraction mixture was spiked with copies of armored enterovirus internal control RNA [16] when testing for the presence of HPeV or enterovirus by the 5'UTR RT-PCR, and it was not spiked when genotyping by sequencing of the VP1 region had to be performed. As positive controls, we included culture isolates that have previously been typed by the National Institute for Public Health and the Environment as echo22 (HPeV1) and echo23 (HPeV2). The negative control consisted of calf thymus DNA (20 ng/ μL ; Sigma).

5'UTR RT-PCR and detection

Forty μL of extracted RNA was used for reverse transcription. The final reverse-transcription mixture (50 μL) contained 1x reverse-transcriptase buffer CMB1 (10 mmol/L TRIS-HCl [pH, 8.3], 50 mmol/L KCl, 0.1% Triton-X100; Sigma), 5.0 mmol/L MgCl_2 (Sigma); 1.5 μg of random hexamers (Roche Diagnostics), 120 $\mu\text{mol/L}$ (each) dNTPs (Applied Biosystems), 280 ng/ μL α -caseine (lot number 17H9551; Sigma), 4 U of RNAsin (Promega), and 20 U of Superscript II (Invitrogen Life Technologies). The mixture was incubated for 30 min at 42°C, and 25 μL of the generated cDNA was used as input in the PCR. The PCR was performed in a 50- μL volume containing 1x PCR II buffer (Applied Biosystems); 200 $\mu\text{mol/L}$ of each dATP, dCTP, and dGTP (Applied Biosystems); 400 $\mu\text{mol/L}$ dUTP (Applied Biosystems); 0.1 $\mu\text{g}/\mu\text{L}$ bovine serum albumin (Roche Diagnostics); 400 ng/ μL α -caseine; 0.5 U of AmpErase (Uracil-N-glycolase; Applied Biosystems); and 2.5 U of Amplitaq Gold (Applied Biosystems). The final MgCl_2 concentration was 2.5 mmol/L [16].

For the 5'UTR enterovirus PCR, 200 ng of Entero-1 primer (5'-CCCTGAATGCGGCTAAT-3'; nt 452–468) and 200 ng of Bio-entero-2 primer (5'-ATTGTCACCATAAGCAGCC-3'; 5'biotinylated; nt 597–579) [16] were used. For the 5'UTR HPeV PCR, 200 ng of the K29 primer and 200 ng of the K30 primer [18] were used. Both 5'UTR PCRs were performed in an Applied Biosystems 9600 thermocycler, with the method described by Beld et al. [16].

The enterovirus 5'UTR amplicons were analyzed by hybridization and electrochemiluminescence measurement [16]. The HPeV 5'UTR amplicons were analyzed by gel electrophoresis.

VP1 one-step RT-PCR

To detect all 3 known HPeV serotypes, new primers were designed just outside the VP1 region, amplifying the complete VP1 region (760 bp). Primers were designed using the following reference strains: HPeV1 strain Harris (S45208), HPeV2 strain Williamson (AJ005695), and HPeV3 isolate A308-99 (AB084913) (table 1). The PCR was performed in a 50- μL volume containing 0.5 $\mu\text{mol/L}$ primer VP1-parEchoF1 and 0.5 $\mu\text{mol/L}$ primer VP1-parEchoR1 (table 1), 1x RT-PCR mix (67 mmol/L Tris [pH, 8.8], 17 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 6 $\mu\text{mol/L}$ EDTA, 2 mmol/L MgCl_2 , 1 mmol/L dithiothreitol; (Sigma), 200 $\mu\text{mol/L}$ (each) dNTPs, 400 ng/ μL α -caseine, 10 U of RNAsin, 3 U of AMV-RT (Roche), and 2.5 U of Taq DNA polymerase (Applied Biosystems). The RT-PCR was performed in an Applied Biosystems 9600 thermocycler by the method described by Oberste et al. [19].

Table 1. VP1 human parechovirus primers designed for genotyping of human parechoviruses, 2000-2005.

Primer	Sequence 5'-3'	Position ^a
VP1-parEchoF1	CCAAAATTCRTGGGG TTC	2332-2349
VP1-parEchoR1	AAACCYCTRTCTAAATAWGC	3090-3071

NOTE. Degeneracy: standard International Union of Biochemistry nucleotide ambiguity codes.

^a The reference strain was human parechovirus serotype 1 Harris (Genbank accession number S45208).

HPeV genotyping and phylogenetic analysis

The HPeV VP1 amplicons were gel purified and sequenced. The sequencing PCR was performed in a 20-mL volume containing 10 ng of either forward primer VP1-parEchoF1 or reverse primer VP1-parEchoR1 (table 1), 5 ng of (purified) amplicon, 1 mL of Big Dye Terminator ready reaction mix (Applied Biosystems), 7 mL of dilution buffer (400 mmol/L Tris HCl [pH, 8.0] and 5 mmol/L MgCl₂). The sequencing PCR is performed in an Applied Biosystems 9600 thermocycler, as follows: 1 min at 96°C, followed by 25 cycles that each consisted of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. The sequences were analyzed on an ABI 3730/3100 DNA analyzer (Applied Biosystems). Sequences were aligned using Clustal-W included in the Vector NTI suite 7 software package (InforMax). Sequences were edited using Genedoc software, version 2.6.02 [20]. Phylogenetic analyses were performed by the neighbor-joining method [21], as implemented in the Molecular Evolutionary Genetics Analysis software package, version 2.1 [22]. Jukes and Cantor distances [23] were estimated for the nucleotide sequences, and P-distances were used for amino acid sequences. One thousand bootstrap replicates were analyzed. The use of other methods for distance estimation did not influence the tree topology. As reference strains/isolates, we used HPeV1 strain Harris (S45208) and HPeV1 isolates A1086-99 (AB112485), A942-99 (AB112486), and A10987-00 (AB112487); and HPeV2 strain Williamson (AJ005695) and HPeV3 isolates A308-99 (AB084913), A317-99 (AB112482), A354-99 (AB112483), A628-99 (AB112484), and Can82853-01 (AJ889918). The previously typed echo22 (HPeV1) and echo23 (HPeV2) isolates from a National Institute for Public Health and the Environment panel were also included as a control that VP1 genotyping correlates with serotyping. HPeV genotype was assigned on the basis of phylogenetic clustering analysis. The nucleotide sequences of the VP1 region from the HPeV1 and HPeV3 isolates are deposited in GenBank under the accession numbers DQ172416-DQ172441 (HPeV1), and DQ172442-DQ172451 (HPeV3).

Clinical data and statistical analysis

Clinical data were collected from medical records and letters of discharge from the 37 patients infected with HPeV. The patient's age at time of virus isolation, sex, duration of hospitalization, underlying illnesses, and the ward of admission were documented. The patients were scored for the presence or absence of the following clinical symptoms: fever (temperature, $>38^{\circ}\text{C}$), sepsis-like illness (signs of circulatory or respiratory dysfunction), respiratory symptoms (rhinorrhea, cough, upper respiratory tract infections, or lower respiratory tract infections), gastrointestinal symptoms (diarrhea and/or vomiting alone or in combination with abdominal distension), and neurological symptoms (clinically suspected meningitis, lethargy, convulsions, or paralysis).

Statistical analysis was performed to compare the 2 groups of patients. To compare age distribution, the Mann-Whitney *U* test was used. Clinical symptoms were compared using Fisher's exact test.

RESULTS

Molecular identification of HPeV

From December 2000 to January 2005, a total of 303 clinical samples were determined to be positive for enterovirus by cell culture in our laboratory, and 284 of these culture isolates were sent to the reference laboratory for poliovirus surveillance. Of those, the reference center reported that 35 samples were found to be negative for enterovirus by 5'UTR RT-PCR. To identify whether these culture isolates could be HPeV, these 35 isolates and the additional 19 isolates that had not yet been sent to the reference laboratory (total number of isolates, 54) were tested by our 5'UTR RT-PCRs, which are specific for HPeV and enterovirus. All 37 isolates that had positive HPeV RT-PCR results had negative results of the enterovirus RT-PCR. The other 17 culture isolates tested positive for enterovirus and negative for HPeV by RT-PCR. Thus, all 54 culture isolates tested by RT-PCR could be identified as either HPeV or enterovirus, and no double infections were found in this subset. Remarkably, 5 isolates that had negative 5'UTR RT-PCR results at the reference center were found to be positive for enterovirus by our 5'UTR RT-PCR, indicating that the RT-PCR for enterovirus described by Beld et al. [16] is more sensitive. In conclusion, 37 (12%) of our 303 clinical isolates that were determined to be enterovirus on the basis of cell culture results were in fact HPeV.

Molecular typing of HPeV

Because genotyping based on VP1 sequencing has been shown to correlate with serotyping for both enterovirus and foot-and-mouth disease virus [10-15], we designed degenerate primers (table 1) to sequence the entire VP1 region, to distinguish between HPeV1, HPeV2, and HPeV3. All 37 HPeV culture isolates were amplified and sequenced. Culture isolates from a panel typed by the reference laboratory as echo22 (HPeV1) and echo23 (HPeV2) were also amplified and sequenced with these primers. The reference strains for HPeV1 (strain Harris), HPeV2 (strain Williamson), and HPeV3 (A308-99) and published isolates for HPeV1 and HPeV3 were included in the analysis. Figure 1 shows the phylogenetic tree based on amino acid

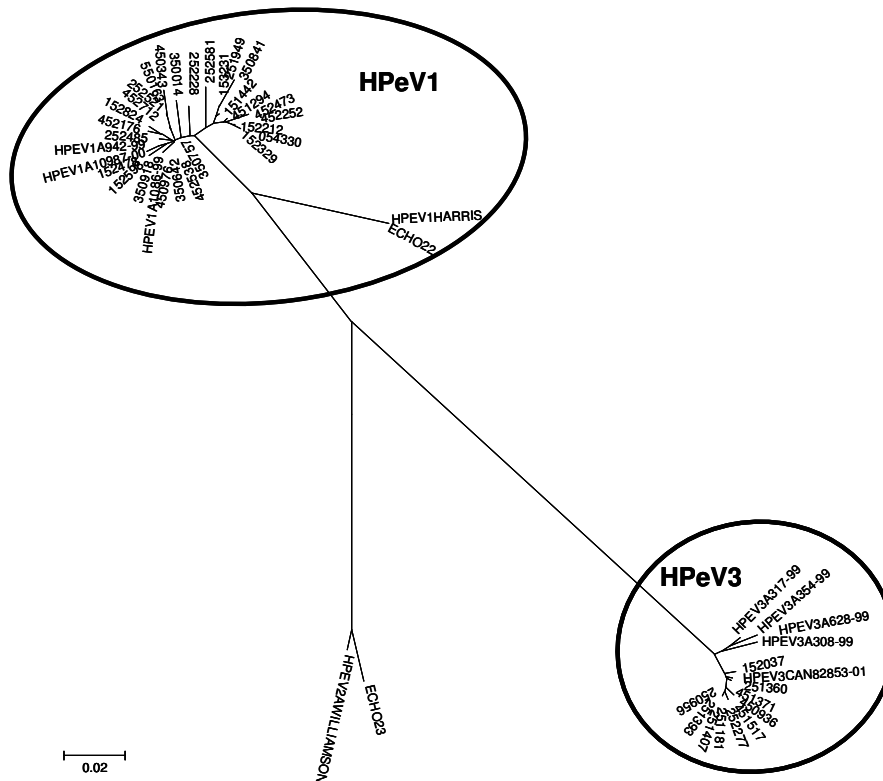


Figure 1. Unrooted phylogenetic tree showing the relationship between clinical isolates from January 2000-January 2005 and HPeV1 strain Harris (S45208) and isolates A1086-99 (AB112485), A942-99 (AB112486) and A10987-00 (AB112487); HPeV2 strain Williamson (AJ005695) and HPeV3 isolates A308-99 (AB084913), A317-99 (AB112482), A354-99 (AB112483), A628-99 (AB112484) and Can82853-01 (AJ889918) based on amino acid differences in the capsid protein VP1. The tree was constructed by using the neighbour-joining method. Numbers represent the frequency of occurrence of nodes in 1000 bootstrap replicas.

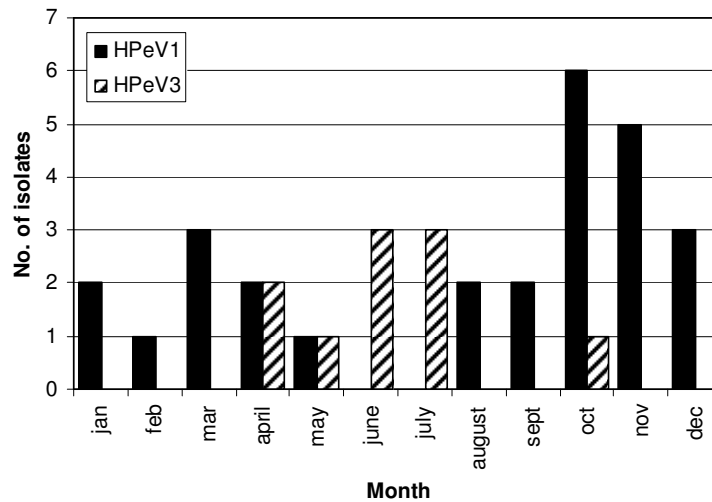


Figure 2. Seasonal distribution of culturing HPeV1 (white bars) and HPeV3 (hatched bars) isolates from patients over the period 2000-2005.

sequences. The tree based on nucleotide sequences revealed the same topology (data not shown). The previously typed echo22 and echo23 clustered with their respective reference HPeV genotype. The majority of the isolates ($n=27$) could be identified as HPeV1. In addition, 10 isolates could be typed as the recently identified HPeV3. The Dutch HPeV1 isolates ($n=27$) were only 91% homologous to the HPeV1 strain Harris. However, they clustered closely with the recently genotyped and serotyped Japanese HPeV1 sequences (98% homology) [7]. The Dutch HPeV3 isolates ($n=10$) were 97% homologous to the HPeV3 isolate A308-99 [7]. The Dutch HPeV3 isolates ($n=10$) clustered closely together (99% homology) and were more closely related to the Canadian isolate (CAN82853-01; 99% homology) than to the Japanese isolates (figure 1). Homology between the HPeV1 cluster and the HPeV3 cluster was 73%. Clustering of HPeV1 and HPeV3 isolates could not be related to year of isolation, hospital or ward of admission, or clinical features.

HPeV3 was already present in a patient in July 2001, whereas 6 isolates were cultured from patients in 2002, and the remaining 3 were cultured in 2004 (data not shown). For HPeV1, most isolates were cultured in the autumn-winter season, with the peak occurring in October, whereas none were cultured in June through July. For HPeV3, the opposite was observed (figure 2).

Table 2. Clinical symptoms in human parechovirus (HPeV) infection in Dutch children, 2000-2005.

characteristics	No. of patients with symptoms/ no. of patients with available data (%)		P
	HPeV serotype 1 (n= 27)	HPeV serotype 3 (n= 10)	
	Fever	16/25 (64)	
Sepsis-like-illness	2/26 (8)	7/10 (70)	<.001 ^a
Respiratory tract symptoms	13/27 (48)	3/10 (30)	.5
Gastrointestinal tract symptoms	21/26 (81)	7/10 (70)	.7
Central Nervous System symptoms	3/26 (12)	5/10 (50)	.02 ^a

NOTE. P values were determined by Fisher's exact test.

^a statistically significant.

Characterization of clinical symptoms

We identified 27 patients infected with HPeV1 and 10 infected with HPeV3. All patients were children aged <3 years. HPeV3 was exclusively present in young infants (median age, 1.3 months), whereas the median age of the children infected with HPeV1 was significantly higher (6.6 months; $P=.0039$). Remarkably, almost all HPeV3 infections were observed in boys (ratio of male to female children for HPeV1 and HPeV3 infections, 1.5:1 and 9:1, respectively).

In total, 23 children were admitted to the academic hospital, and 10 were admitted to a general hospital in the region. Most children were admitted to a general children's ward or a children's surgery ward, but 1 child was treated on an intensive care unit because of convulsions.

HPeV1 infections are generally associated with mild gastrointestinal or respiratory disease; however, HPeV3 has been found in children with more-severe clinical symptoms, such as paralysis [7] and neonatal sepsis [8]. Therefore, we compared the 2 groups of children for the presence or absence of fever, sepsis-like illness, respiratory tract symptoms, gastrointestinal tract symptoms, and neurological (CNS) symptoms (table 2). The majority of children in both groups were found to have fever for at least 1 day. In both groups, gastrointestinal tract symptoms were more frequent than respiratory tract symptoms. For the children infected with HPeV1, clinical symptoms were mainly gastrointestinal tract and/or respiratory tract symptoms, whereas sepsis-like illness and CNS symptoms were reported in only a minority of patients. However, in significantly more children infected with HPeV3, sepsis-like illness and CNS symptoms were reported. All children with sepsis-like illness were treated with antibiotics, which was usually stopped after 48–72 h because no bacterial agent could be cultured

and because the children improved clinically. There was no mortality among the children with sepsis-like illness or CNS symptoms. None of the children with CNS symptoms developed paralysis.

DISCUSSION

The newly identified HPeV3 was first isolated in 2004 and has since then only been reported in 4 children from Japan [7] and 3 children from Canada [8]. Until now, HPeV3 has not been reported in Europe. Seroepidemiological studies by Ito et al. [7] showed that HPeV3 existed long before its first isolation in 2004, because 87% of Japanese adults aged >40 years have antibodies against HPeV3. Here, we describe 10 children from The Netherlands with HPeV3 infection during the period of 2000–2005.

Typing of our clinical isolates was done by sequencing the entire VP1 region. The majority of the samples ($n=27$) clustered in a subgroup of HPeV1 isolates that was more closely related to the HPeV1 isolates identified in Japan in 2004 [7] than to the reference strain Harris, which was sequenced in 1992 [3] and isolated in 1956. The remaining 10 isolates clustered with the Japanese and Canadian HPeV3 isolates. The amino acid similarity of the VP1 region between the HPeV1 cluster and the HPeV3 cluster was 73%, which is in accordance with that found by Ito et al. [7]. The high amino acid homology of the Dutch HPeV3 isolates to the Canadian HPeV3 isolate suggests that HPeV3 circulating in The Netherlands is more closely related to HPeV3 circulating in Canada than in Japan. However, more data on geographical spread of HPeV3 are needed. When clustering of HPeV1 or HPeV3 strains was observed, it could not be related to the year of isolation, admittance to the same hospital (ward), or specific clinical features.

The previously described HPeV3 infections in children suggested a more severe clinical spectrum than occurs with HPeV1 infection, including transient paralysis [7] and neonatal sepsis [8]. This is the first report that directly compares the clinical symptoms in a group of children infected with HPeV3 with the symptoms in children infected with HPeV1. Although the groups are small, our analysis shows that the children infected with HPeV3 were significantly younger and that sepsis-like illness and CNS symptoms were observed more often in HPeV3-infected children than in HPeV1-infected children. It remains unclear why HPeV3 would infect younger children. This could be related to the presence of maternal antibodies for HPeV1 but not for HPeV3, although the reason for this is also unclear. In addition, it cannot be excluded that the severity of symptoms is related to the young age at which the infection tends to occur.

The seasonal distribution of our HPeV1 isolates is in agreement with data from a Swedish study, which showed that HPeV1 occurs in late summer to autumn and in winter to early spring [24]. The Dutch HPeV3 isolates were isolated in spring and the early summer. Originally, HPeV3 was found in a child admitted to the hospital in Japan in August. Two Canadian HPeV3 isolates were isolated from children admitted in September and from 1 child admitted in December. Our study indicates a different pattern of seasonal circulation for HPeV1 and HPeV3; however, more data are needed to confirm this trend.

We showed that 12% of our clinical isolates that were determined to be enterovirus by cell culture were, in fact, HPeV. All HPeV isolates were recovered from young children (mean age, 5.7 months). We confirm that HPeV mainly infects children before the age of 3 years, with the majority of children infected before the first year of age [9], whereas enterovirus infections occur during a much broader age range (mean age for the 17 children with enterovirus infection, 20 months; data not shown). Symptoms vary from mild gastrointestinal tract and respiratory tract symptoms to sepsis-like illness and meningitis. This indicates that HPeV is a relevant pathogen in young children. However, molecular techniques for diagnosis of enterovirus infection do not include HPeV. Thus, when only PCR for enterovirus is performed, HPeV infections will not be detected; therefore, we suggest the implementation of an RT-PCR for the detection of HPeV in clinical specimens obtained from children aged <3 years.

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