Clinical and molecular insights into human parechovirus infection

Benschop, K.S.M.

5

High Prevalence of Human Parechovirus (HPeV) Genotypes in the Amsterdam Region and Identification of Specific HPeV Variants by Direct Genotyping of Stool Samples

K. Benschop, X. Thomas, C. Serpenti, R. Molenkamp, and K. Wolthers

Journal of Clinical Microbiology 2008; 46 (12):3965-3970

Lab. of Clinical Virology, Dept. of Medical Microbiology, Academic Medical Center, Amsterdam.

High Prevalence of Human Parechovirus (HPeV) Genotypes in the Amsterdam Region and Identification of Specific HPeV Variants by Direct Genotyping of Stool Samples

Human parechoviruses (HPeV) are widespread pathogens belonging to the *Picornavirus* family. Six genotypes, which have predominantly been isolated from children, are known. Data on prevalence of HPeV genotypes are generally based on cell culture, which may underestimate the prevalence of certain HPeV strains that are difficult to grow. We studied 1,824 stool samples from 1,379 children (<5 years old) sent to the Academic Medical Center, Amsterdam, The Netherlands, between 2004 and 2006. Samples were screened using specific human enterovirus (HEV) and HPeV real-time PCRs based on the 5'untranslated region. A high percentage of HPeV infections (16.3%), comparable to the percentage of HEV infections (18.4%), were found by PCR in stool samples. HPeV-positive stool samples were directly genotyped based on the VP1 region for the first time to avoid a culture bias. HPeV1 was found to be the most prevalent type. The majority of the HPeV1 strains clustered separately from the prototype strain, Harris, which has not been reported to circulate lately. However, we could identify three strains as HPeV1 Harris. HPeV3 was identified as the second most predominant type during 2004 and 2006 but was not found in 2005. HPeV4 to -6 were found in smaller numbers. One strain could not be associated with a known HPeV type (VP1 gene nucleotide similarity: 71%), possibly indicating a new genotype. Also, we report the first identification of three HPeV5 strains and one HPeV1 strain with a different motif at the C-terminal end of VP1, where the arginine-glycine-aspartic acid (RGD) motif is normally located.

INTRODUCTION

Human parechovirus 1 (HPeV1) and HPeV2 were first identified in the mid-1960s as human enteroviruses (HEVs) and were classified as echovirus 22 and 23 (30). Sequence analyses showed that these viruses were genetically distinct from the entire *Enterovirus* genus, and they were reclassified as the new genus *Parechovirus* in the family *Picornaviridae* (24). There are now six known HPeV genotypes (2, 8, 16, 29), which have been isolated mainly from young children. Infections with HPeVs have been shown to be widespread (17, 18, 25), but different HPeV genotypes were found to differ epidemiologically and clinically from each other (1, 6, 29).

Epidemiological data on HPeV prevalence are predominantly based on cell culture isolation of different HPeV strains. As HPeVs, in particular HPeV3, are found to be difficult to culture in standard diagnostic cell lines and their isolation is largely determined by the cell lines used (1, 29, 30), the exact HPeV prevalence might be underestimated.

Recent studies show that HPeV1 is still the most frequently isolated (1, 3, 6, 13, 29). However, genotyping showed the recently circulating HPeV1 strains to be phylogenetically distinct from the prototype strain, designated Harris, first isolated in 1956 (1, 6, 12, 29). Both the Harris strain and the recently isolated HPeV1 strains can be serotyped using antisera directed against the original Harris strain (1). Since the older studies depended on serotyping methods, while the more recent studies are based on genotyping, it is difficult to establish the time frame in which the prototype strain ceased circulation. The benefits of molecular typing instead of classical serotyping methods to type different HPeVs have already been proven by the reclassification of some HEVs as HPeVs (17, 26). In addition, genotyping has led to the reclassification of the second HPeV2 serotype, represented by the CT86-6760 strain (20), as the HPeV5 genotype (2), following the identification of HPeV3 and -4 (2, 8, 16).

Infections with HPeV3 have been associated with more severe symptoms than other HPeV infections, and HPeV3 has been reported to be either the second most predominant strain (1, 6, 16) or equal to HPeV1 in prevalence (13, 29). As HPeV3 was found to display different cell tropisms in different cell culture assays (1, 29) and as these studies were based on cell culture isolation of HPeVs, the use of different cell lines within different studies might account for these discrepancies.

Direct screening and typing of clinical samples would exclude such cell culture bias and therefore would provide a better estimation of the prevalences of different HPeV types. In addition, by screening over 700 cerebrospinal fluid (CSF) samples by real-time PCRs, HPeVs were identified in almost 5% of the children under the age of 5 years, in comparison to 14% for HEVs (5, 31). Since higher prevalences were found in 2004 and 2006 than in 2005, a 2-year cycle was suggested for HPeVs with neuroinvasive characteristics. Unfortunately, due to low HPeV viral load these CSF samples could not be genotyped. In order to gain insight into the circulation of different HPeV types within the Amsterdam region in those years, we retrospectively screened and directly typed stool samples from children under the age of 5 years from 2004 to 2006 by realtime PCR.

METHODS

Clinical samples

Stool samples from children under the age of 5 years obtained between 2004 and 2006 from the Amsterdam region were collected. Stool samples were suspended in 2% broth (Oxoid, Drongen, Belgium) and stored at -20°C. In total, 1,824 stool samples from 1,379 children were available for testing (808 boys and 571 girls).

RNA extraction

Broth-suspended stool samples (50 µl) were incubated for 10 min in lysis buffer L6 (10) and centrifuged for 2 min to remove any stool debris. The supernatant was further extracted using 20 µl of size-fractionated silica particles in combination with 900 µl of lysis buffer L6 as previously described by Boom et al. (9, 10). When testing for the presence of an HPeV and/or HEV infection, samples were coextracted with 6,250 copies of armored RNA of an internal control (IC), corresponding to 500 copies of IC cDNA in PCR (4). The IC was omitted from extraction when genotyping by sequencing of the VP1 region had to be performed (6). During the study period we changed from manual extraction to automatic extraction using the total nucleic acid isolation kit with the MagnaPure LC instrument (Roche Diagnostics, Almere, The Netherlands). Retesting of samples showed 100% recovery compared to the manual extraction. RNA was eluted in 50 µl of Tris-EDTA buffer in both extraction methods.

Detection of HPeV and HEV by real-time reverse transcription-PCR (RT-PCR)

Forty microliters of extracted RNA was used for reverse transcription using random hexamers (Applied Biosystems, Niewerkerk a/d IJssel, The Netherlands) as previously described (4). Five microliters of cDNA was used for both the HEV-specific duplex assay and the HPeV-specific single-target assay (5). The HEV-specific duplex assay was performed in a 25 µl volume containing 900 nM of each primer (entero-1-TM and entero-2-TM [5]), 200 nM of the EV-WT-MGB probe and IC-MGB probe (5), 400 ng/µl of bovine α-casein (lot number 17H9551; Sigma, Zwijndrecht, The Netherlands), and 1x TaqMan universal PCR master mix (Applied Biosystems). The HPeV-specific single-target assay was performed in a 25-µl volume containing 900 nM of each primer (ParechoF31 [5] and K30 [20]), 200 nM of the HPeV-WT-MGB probe (5), 400 ng/µl of bovine α-casein (Sigma), and 1x TaqMan universal PCR master mix (Applied Biosystems). The primers and probes were

obtained from Biolegio (Nijmegen, The Netherlands) and Applied Biosystems, respectively. The real-time PCRs were performed in an Applied Biosystems 7000 sequence analyzer as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles, each consisting of 15 s at 95°C and 1 min at 60°C.

Genotyping by VP1 sequencing

The previously described one-step VP1 RT-PCR (6) was adapted into a two-step assay. As the previously described primer set might not detect HPeV5 (13), a second primer set was designed to include degeneracy to all known HPeV types (VP1-parEchoF12 [5'-CCA RAA YTC ITG GGG YTC-3'] and VP1-parEchoR12 [5'-AAI CCY CTR TCY ARR TAW GC-3']). Two hundred sixty-one HPeV-positive samples from 216 children could be retrieved for typing, of which 168 samples from 130 children could be successfully genotyped using both primers sets. Forty microliters of newly extracted RNA (omission of IC) was used for reverse transcription as previously described using random hexamers (4). Twenty-five microliters of cDNA was used for the VP1 PCR. The PCR was performed in a 50-µl volume containing 1x PCR II buffer (Applied Biosystems); 200 µmol/liter each of dATP, dCTP, and dGTP (Applied Biosystems); 400 µmol/liter dUTP (Applied Biosystems); 0.1 µg/µl bovine serum albumin (Roche Diagnostics); 400 ng/µl α-casein; 1 µM VP1-parEchoF1/VP1-parEchoF12 and 1 µM VP1-parEchoR1/VP1-parEchoR12 (6); and 2.5 U of Amplitaq Gold (Applied Biosystems). The final $MgCl_2$ concentration was 2.5 mmol/liter. The HPeV VP1 amplicons were gel purified and sequenced as previously described (6).

Phylogenetic and statistical analysis

The sequences were analyzed on an ABI 3730/3100 DNA analyzer (Applied Biosystems). Sequences were aligned using Clustal-W (28), included in the VectorNTI suite 10 software package (Invitrogen) and edited using Simmonics v1.62 (http://www2.warwick.ac.uk/fac/sci/bio/research/devans/bioinformatics/simmonics, 23). Phylogenetic analyses were performed by the neighbor-joining method (22), as implemented in the Molecular Evolutionary Genetics Analysis software package, version 3.1 (19). P-distances were estimated for amino acid sequences. One thousand bootstrap replicates were analyzed. The HPeV genotype was assigned on the basis of phylogenetic clustering. HPeVs from nine children were previously typed from culture isolates obtained between 2000 and 2005 (6). The HPeV type was confirmed by genotyping the original stool samples.

Statistical analysis was performed using SPSS 12.1 for Windows based on the number of children. Children with more than one sample were identified as follow-up subjects if samples were less than 11 weeks apart (11). In addition, infections of children from whom multiple samples containing identical sequences were obtained were characterized as single HPeV infections. To compare age distributions, we used the Wilcoxon-Mann-Whitney test. To compare HPeV and HEV prevalences over the years, we used the chi-square distribution with 95% confidence intervals (CI).

Nucleotide sequence accession numbers

The nucleotide sequences of the VP1 gene region are deposited in GenBank under accession numbers FJ373059 to FJ373179.

RESULTS

HPeV in stool samples from 2004 to 2006

Stool samples (n = 1,824) from 1,379 children under the age of 5 years were obtained from 2004 to 2006 and screened retrospectively using HPeV- and HEV-specific real-time PCRs. HPeV infections were detected in 270 samples obtained from 225 (16.3%) children, and HEV infections were detected in 265 samples from 253 (18.4%) children (Table 1). Forty-one children showed a double infection with HPeV and HEV (Table 1).

The yearly prevalences for both HPeV and HEV varied during the 3 years studied. HPeV was detected in 17.5% (80/456), 13.2% (59/447), and 18.2% (86/476) of the children in 2004, 2005, and 2006, respectively (Table 1). HEV was detected in 20% (91/456), 14.2% (65/447), and 20.5% (97/476) of the children, respectively, in these 3 years (Table 1). The lower prevalence observed in 2005 was not significant for HPeV (P=0.09) and was just significant for HEV (P=0.04).

Table 1. Human Parechovirus (HPeV) and Human Enterovirus (HEV) infections detected in stool samples.

Virus(es)	Number (%) of virus-positive patients in:			
	2004 (n=456)	2005 (n=447)	2006 (n=476)	Total (n=1379)
HPeV	80 (17.5)	59 (13.2)	86 (18.2)	225 (16.3)
HEV	91 (20)	65 (14.5)	97 (20.6)	253 (18.4)
HPeV and HEV	12 (2.6)	8 (1.8)	21 (4.4)	41 (2.9)

Table 2. Number of neonates (< 28 days) found positive for Human Parechovirus (HPeV) and Human Enterovirus (HEV).

Virus(es)	No. of virus-positive neonates/no. tested (%) in:			
	2004	2005	2006	total
HPeV	9/80 (11.3)	2/59 (3.4)	7/86 (8.1)	18/225 (8.0)
HEV	16/91 (17.6)	8/65 (12.3)	12/97 (12.4)	36/253 (14.2)

The majority of the children infected with HPeV (84%, 189/225) were <2 years old, and 8% (18/225) were neonates (<28 days old). Of the children infected with HEV, 77% (196/253) were <2 years old and 14% (36/253) were neonates. As shown in Table 2, the number of neonates infected with HPeV was much lower in 2005 than in 2004 and 2006.

We found more boys infected with either HPeV or HEV than girls. However, taking into account that the ratio of boys to girls tested (1.4:1) was similar to the ratios of HPeV- and HEV-positive boys to girls (1.4:1 and 1.3:1, respectively), the relative risk (RR) for infection of boys with either virus was not higher than that for girls (RR_{HEV} = 1.1, 95% CI, 0.9 to 1.4; RR_{HPeV} = 1.0, 95% CI, 0.8 to 1.3).

Prevalence of HPeV types

In total, 168 samples from 130 children were successfully genotyped directly from stool samples as either HPeV1, -3, -4, -5, or -6 (Fig. 1). HPeV1 was the predominant type identified in all 3 years; 64.6% (84/130) of the children were found to be infected with strains of this type (Fig. 2). During 2004 and 2006, HPeV3 was identified as the second most predominant type (22.3%, 29/130). However, HPeV3 was not identified in 2005, nor was HPeV4 (Fig. 2). In total, HPeV4 was found in 9 children (7%). HPeV5 and -6 were identified at least once a year in the 3 years studied.

HPeVs could be isolated during the entire year. However, differences in seasonal distribution between the genotypes could be observed. HPeV1, -4, -5, and -6 were mostly identified during autumn and winter. However, HPeV3 was mainly found in summer. Interestingly, all HPeV types were less prevalent in spring.

In comparison to HPeV1 infections, more HPeV3 infections were found at the younger ages. For HPeV3, 66% (19/29) of the infected children were younger than 6 months, of which 24% (n = 7) were less than 28 days old. In contrast, for HPeV1, only 40% (34/84) of the infected children were younger than 6 months, of which 2.3% (n = 2) were neonates. Of the 261 samples available for typing, we were unable to type 93 samples from 86 children. Fifty samples from 49 children were found to have a low viral load (cycle

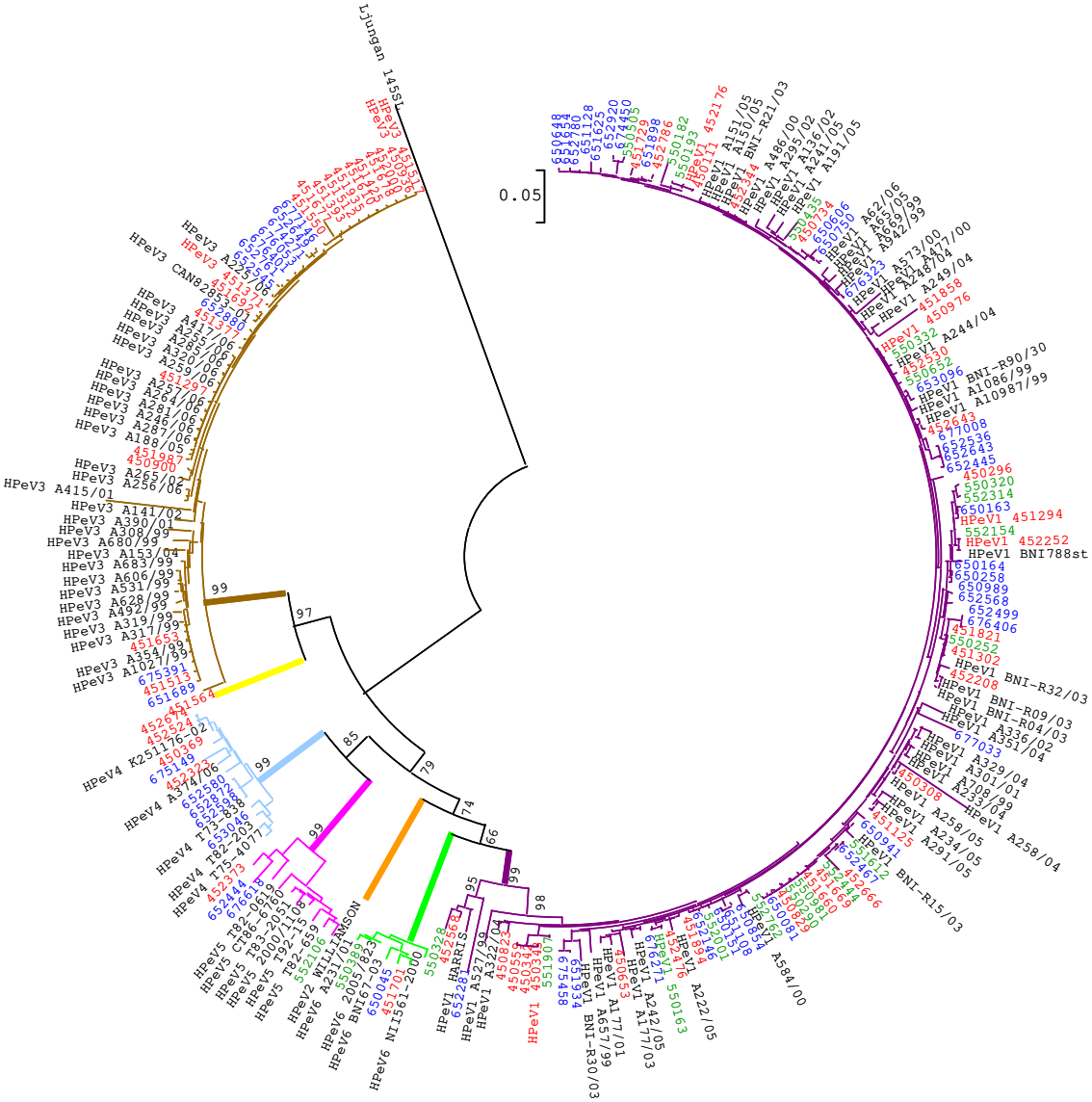

Figure 1. Rooted phylogenetic tree based on amino acid differences in the capsid protein VP1 (236 aa). The tree was constructed by using the neighbor–joining method. Numbers represent the frequency of occurrence of nodes in 1000 bootstrap replicas. The Dutch strains sequenced within this study contain a 6 digit numbering, and have been color-coded by year of isolation (2004: red, 2005: green, and 2006: blue). The different HPeV genotype clusters have been color-coded within the tree: HPeV1 (purple), HPeV2 (orange), HPeV3 (light brown), HPeV4 (light blue), HPeV5 (pink), HPeV6 (light green). As an outgroup, Ljungan Virus 145 SL (AF327922) was used. Reference strains and isolates were obtained from GenBank: HPeV1 Harris (S45208), BNI788st (EF051629), 450343 (DQ172430), 550163 (DQ172425), 450976 (DQ172417), 451294 (DQ172440), 452176 (DQ172431), 4522252 (DQ172435), A317/99, A354/99, A628/99, A1086/99, A10987/00 (AB112482-AB112487), A301/01 (AB300943)

A177/01, A584/00, A573/00, A486/00, A477/00 (AB300937-AB300941), A708/99 (AB300935), A669/99 (AB300932), A657/99 (AB300931), A527/99 (AB300928), A233/04, A244/04, A248/04, A249/04, A258/04, A322/04, A329/04, A351/04, A62/05, A65/05, A150/05, A151/05 (AB300954-AB300965), A295/02, A336/02, A177/03 (AB300949-AB300951), A136/02 (AB300946), A347/06 (AB300985), A191/05, A222/05, A229/05, A234/05, A241/05, A242/05, A258/05 (AB300966-AB300972), BNI-R90/03, BNI-R04/03, BNI-R09/03, BNI-R15/03, BNI-R21/03, BNI-R30/03, BNI-R32/03 (EU024630-EU024636); HPeV2 Williamson (AF055846); HPeV3 A308/99 (AB084913), CAN82853-01 (AJ889918), 451371 (DQ172449), 451517 (DQ172447), 450936 (DQ172446), A390/01 (AB300944), A415/01 (AB300945), A1027/99 (AB300936), A683/99 (AB300934), A680/99 (AB300933), A606/99 (AB300930), A531/99 (AB300929), A492/99 (AB300927), A319/99 (AB300926), A153/04 (AB300952), A141/02, (AB300947) A265/02 (AB300948), A471/06 (AB300986), A188/05, A225/06, A246/06, A255/06, A257/06, A259/06, A264/06, A265/06, A281/06, A285/06, A287/06, A320/06 (AB300973-AB300984); HPeV4 K251176-02 (DQ315670), T75-4077 (AM235750), T82-203 (AM234727), T73-838 (AM234725); HPeV5 CT86-6760 (AJ005695), T92-15 (AM235749), T820169 (AM234728), T82-659 (AM234726), T83-2051 (AM234724); HPeV6 NII561-2000 (AB252582), BNI67-03 (EU024629), 2005/823 (EU077518), A231/01 (AB300942).

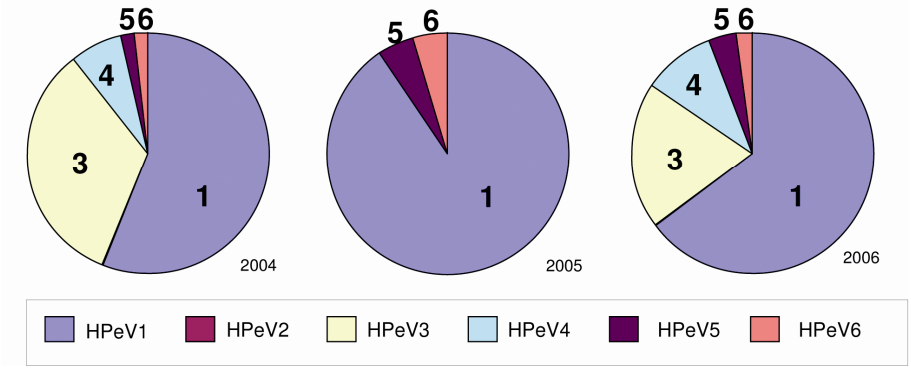

Figure 2. The prevalence, of the known Human Parechovirus (HPeV) types in 2004 (HPeV1, *n*=30; HPeV3, *n*=19; HPeV4, *n*=4; HPeV5, *n*=1 and HPeV6, *n*=1), 2005 (HPeV1, *n*=19; HPeV5, *n*=1 and HPeV6, *n*=1) and 2006 (HPeV1, *n*=35; HPeV3, *n*=10; HPeV4, *n*=5; HPeV5, *n*=2; HPeV6, *n*=1) identified in the Amsterdam region. The different types have been numerically numbered according to the HPeV genotype.

threshold [*CT*] value by real-time PCR > 38) and could therefore not be typed. Forty-three samples from 37 children were found to have a high enough viral load for genotyping based on the *CT* value by real-time PCR. As stool samples can be very incongruent, these samples were tested multiple times. However, after several tests, signs of degradation could be observed, as the *CT* values increased considerably after freeze-thawing steps (mean increase of *CT* value, 9; range, 7 to 11).

Phylogenetic characterization of HPeV types

Based on specific clustering of the isolates with known HPeV types obtained from GenBank, we could identify HPeV genotypes 1, 3, 4, 5, and 6 (Fig. 1). HPeV2 was not found. HPeV1 comprised the largest cluster. The majority of the HPeV1 strains (n = 81) were found to cluster closely together with strains identified in Japan (strains having five-digit numbers prefixed with “A”) (Fig. 1) and Germany (strains having three-digit numbers prefixed with “BNI-R”) (Fig. 1) and formed a separate cluster from the prototype strain, identified in 1956 (Harris). The amino acid similarity between the recently isolated HPeV1 strains, including those identified in Germany and Japan, and the prototype strain, Harris, was only 89.2%. Three strains were found to cluster closely with the Harris strain (amino acid similarity, 93.7%). We found no specific geographical or temporal separation between the different HPeV1 strains (Fig. 1). HPeV3 comprised the second-largest cluster, containing 29 Dutch stains. As seen within the HPeV1 cluster, two separate lineages, which previously had not been found, could be seen within the HPeV3 cluster based on the VP1 gene. The majority of the Dutch strains were found to form a tight cluster with the Japanese strains (amino acid similarity, 96.2%). However, one strain (651689; 2006) clustered outside the larger HPeV3 group and had 96.2% amino acid similarity to this group. In contrast to findings for HPeV1, we could identify geographical or temporal separation between the different HPeV3 strains (Fig. 1); however, due to the close similarities, more diverse sequences are needed for confirmation.

Based on cluster analyses, strains were further identified as either HPeV4, -5, or -6. The isolate from one positive HPeV stool obtained in 2004 could not be assigned to a specific HPeV cluster. Strain 451564 had the best nucleotide identity, 71.2% (79.8% amino acid identity), to the prototype strain, HPeV3 A308-99, identified in 2004. The second-best match was found to be less than 70% (66% identity to HPeV1 Harris). Based on previous proposed criteria to assign HPeV types (21), further characterization is needed. Strain 451564 was also found to lack the

arginine-glycine-aspartic acid (RGD) motif (Fig. 3), as previously seen for other HPeV3 genotypes.
Interestingly, the RGD motif was not found in three HPeV5 strains (452373 [2004], 652444 [2006], and 676618 [2006]) and one HPeV1 strain (652281 [2006]), which was most closely related to Harris. Instead of the deletion seen in HPeV3 and the unidentified strain 451564, all four strains contained a specific sequence at the C-terminal end of VP1, preceded by the well-conserved domain proline-alanine-proline-lysine (PAPK) and followed by the conserved sequences predicted to be part of the cleavage site seen in all known HPeV genotypes, (D/N)-(E/Q)^(S)-(P/L) (Fig. 3).

DISCUSSION

In the 3-year study period, we found a high percentage (16%) of HPeV-positive children by direct PCR screening from stool samples. Previous studies on the prevalence of HPeV were primarily based on culture isolates, which can bias the data due to the inability of virus variants to replicate in certain cell lines (1, 29). Only one other study also directly screened from stool samples (3). In that study a much lower prevalence of HPeV was found (1.3%). However, that study involved both children and adults, while our study was performed on stool samples obtained exclusively from children. When the prevalence of HPeV is calculated solely on the basis of samples from children less than 2 years old, the study by Baumgarte et al. (3) and the

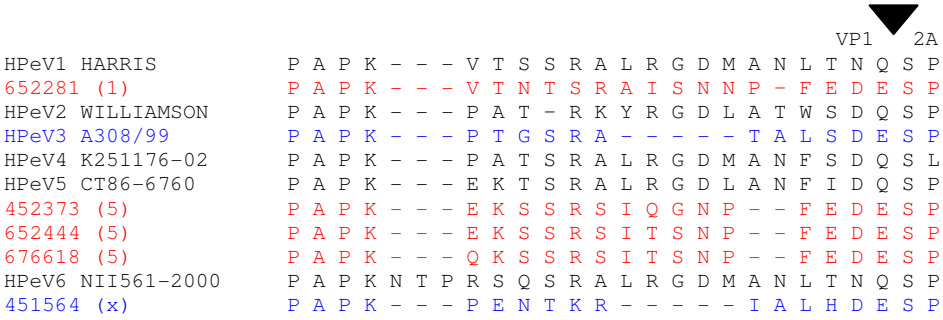

Figure 3. Alignment of the VP1 region flanking the RGD motif. The strains containing the RGD motif are shown in black. The strains lacking the RGD motif are shown in blue. The three HPeV5 strains and one HPeV1 strain, containing the different motif at the C terminal end of the VP1 gene are shown in red. The arrowhead marks the cleavage site of the VP1-2A junction.

study presented here show similar prevalences (11.6% and 13.4%, respectively).

In concordance with seroepidemiological data on HPeV1 (18, 25, 27) and HPeV3 (16), HPeV1 was the most common genotype found. The HPeV1 strains could be grouped in two lineages, of which one lineage comprises the prototype HPeV1 strain, designated Harris (30). HPeV1 Harris has not been identified in recent years (1, 2, 6, 29). The fact that we identified three strains as Harris shows that the “old” strain is either still circulating or recirculating, albeit at low frequency.

The study by Baumgarte et al. (3) found a third HPeV1 lineage intermediate between the recently circulating HPeV1 strains and the old Harris strain and suggested that this strain formed a transition group between the recent and old HPeV1 lineages (3). On the basis of the VP1 regions that we analyzed, we could observe several of these “transition” strains. Whether the recently circulating strains have gradually evolved from the old strain or are the result of several recombination events (7, 12) needs to be investigated further.

In contrast to the yearly circulation of HPeV1, HPeV3 was not observed in 2005. Remarkably, in that same year the HPeV prevalence in CSF was low (0.4%) (31). During the same study period, HPeV was found in almost 5% of CSF samples, and it was suggested that HPeV was the second most frequent viral cause of sepsis-like illness and meningitis after HEV, which was found in 14% of the children. However, screening of stool samples showed HPeV infections to be as prevalent as HEV infections, which is related to the fact that both viruses are transmitted through the fecal-oral route. In addition, double infections were found; these were not found within CSF. Although data on type-specific prevalence in CSF are lacking, the low prevalence in CSF and the absence of HPeV3 seen in stool samples in 2005 strongly suggest that HPeV3 might be the predominant genotype infecting the central nervous system. In addition, the number of HPeV-infected neonates was also lowest in 2005 (3%), again underlining the association between HPeV3 and infection at a younger age (6, 7, 29).

Although seroprevalence data are lacking for HPeV4 to -6, we speculate that these recent types circulate to a lesser extent than HPeV1 and -3, both in the Amsterdam region and globally.

To directly perform genotyping on stool samples, we optimized our VP1 RT-PCR (6) and a second primer pair was designed to be able to amplify all known HPeV genotypes. However, we were not able to type all available samples due to low virus titers or degradation. Thus, the prevalences of certain HPeV genotypes (indicated in Fig. 2 legend) might be slightly underestimated. We cannot exclude the possibility that these samples

contained unidentified HPeV genotypes which our assay might not be able to pick up. However, a majority of these samples (21/26) could still be typed using a nested approach based on the VP3/VP1 region as belonging to one of the known HPeV genotypes 1, 4, and 5 (15). Therefore, we speculate that both the incongruity of stool samples and the sensitivity of our assay caused the limitations of our assay. Despite these limitations, the use of this direct genotyping method comprising the entire VP1 gene has resulted in the identification of four interesting strains lacking the RGD motif. The RGD motif has been identified in all known HPeV genotypes, with the exception of HPeV3. This is the first report describing two HPeV genotypes whose VP1 regions have consistently been found to contain the RGD motif in different isolates and yet to also contain a different specific consensus sequence at the C-terminal end. The identification of two different variants of the same genotype is not uncommon within the family *Picornaviridae*. The echovirus 9 strain designated Barty (14) was shown to contain different consensus sequences, including the RGD motif, in comparison to the echovirus 9 strain designated Hill (14).

It has been proposed that the RGD motif is a key factor in defining cell tropism of the different HPeV genotypes (7, 16). Its absence in HPeV3 suggests an RGD-independent entry pathway.

The insertion of the specific sequence found in the four strains identified could indicate a second RGD-independent pathway. Preliminary culture data already showed these strains to be difficult to culture in standard diagnostic cell lines such as African green monkey kidney (Vero), human colon carcinoma (HT-29), and human lung carcinoma (A549) (1, 29; our unpublished data) cells. This was also observed for the unidentified HPeV strain 451564, which also lacked the RGD motif. In order to identify what specific cell entry pathways the different HPeV types use and what effect these different pathways have on their clinical outcome, more research is needed. In summary, this is the first study where HPeVs were directly typed from stool samples without being isolated first by cell culture. This resulted in the identification of an unidentified HPeV genotype and “RGD-absent” HPeV5 and HPeV1 strains. In addition direct screening from stool samples showed HPeV1 to be the most prevalent type, followed by HPeV3. As HPeVs are transmitted via the fecal-oral route, analysis of stool samples provided an unbiased analysis of different HPeV types.

Acknowledgements

We thank Hetty van Eijck for providing culture data on the new HPeV variants. This work was supported by the Department of Medical Microbiology, Academic Medical Center, Amsterdam, The Netherlands.

REFERENCES

1. **Abed, Y., and G. Boivin.** 2006. Human parechovirus infections in Canada. Emerg. Infect. Dis. **12:**969–975.
2. **Al-Sunaidi, M., C. H. Williams, P. J. Hughes, D. P. Schnurr, and G. Stanway.** 2007. Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. J. Virol. **81:**1013–1021.
3. **Baumgarte, S., L. K. de Souza Luna, K. Grywna, M. Panning, J. F. Drexler, C. Karsten, H. I. Huppertz, and C. Drosten.** 2008. Prevalence, types, and RNA concentrations of human parechoviruses, including a sixth Parechovirus type, in stool samples from patients with acute enteritis. J. Clin. Microbiol. **46:**242–248.
4. **Beld, M., R. Minnaar, J. Weel, C. Sol, M. Damen, H. van der Avoort, P. M. Wertheim-Van Dillen, A. van Breda, and R. Boom.** 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J. Clin. Microbiol. **42:** 3059–3064.
5. **Benschop, K., R. Molenkamp, A. van der Ham, K. Wolthers, and M. Beld.** 2008. Rapid detection of human parechoviruses in clinical samples by realtime PCR. J. Clin. Virol. **41:**69–74.
6. **Benschop, K. S., J. Schinkel, R. P. Minnaar, D. Pajkrt, L. Spanjerberg, H. C. Kraakman, B. Berkhout, H. L. Zaaijer, M. G. Beld, and K. C. Wolthers.** 2006. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin. Infect. Dis. **42:**204–210.
7. **Benschop, K. S., C. H. Williams, K. C. Wolthers, G. Stanway, and P. Simmonds.** 2008. Widespread recombination within human parechoviruses: analysis of temporal dynamics and constraints. J. Gen. Virol. **89:**1030–1035.
8. **Benschop, K. S. M., J. Schinkel, M. E. Luken, P. J. M. van den Broek, M. F. C. Beersma, N. Menelik, H. W. M. van Eijk, H. L. Zaaijer, C. M. J. E. VandenBroucke-Grauls, M. G. H. M. Beld, and K.**

C. Wolthers. 2006. Fourth human parechovirus serotype. Emerg. Infect. Dis. **12:**1572–1575.

9. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1–14.
10. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-Van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
11. **Chung, P. W., Y. C. Huang, L. Y. Chang, T. Y. Lin, and H. C. Ning.** 2001. Duration of enterovirus shedding in stool. J. Microbiol. Immunol. Infect. **34:**167–170.
12. **de Souza Luna, L. K., S. Baumgarte, K. Grywna, M. Panning, J. F. Drexler, and C. Drosten.** 2008. Identification of a contemporary human parechovirus type 1 by VIDISCA and characterisation of its full genome. Virol. J. **5:**26.
13. **de Vries, M., K. Pyrc, R. Berkhout, W. Vermeulen-Oost, R. Dijkman, M. F. Jebbink, S. Bruisten, B. Berkhout, and L. van der Hoek.** 2008. Human parechovirus type 1, 3, 4, 5, and 6 detection in picornavirus cultures. J. Clin. Microbiol. **46:**759–762.
14. **Eggers, H. J., and A. B. Sabin.** 1959. Factors determining pathogenicity of variants of ECHO 9 virus for newborn mice. J. Exp. Med. **110:**951–967.
15. **Harvala, H., I. Robertson, E. C. McWilliam Leitch, K. Benschop, K. C. Wolthers, K. Templeton, and P. Simmonds.** 2008. Epidemiology and clinical associations of human parechovirus respiratory infections. J. Clin. Microbiol. **46:**3446–3453.
16. **Ito, M., T. Yamashita, H. Tsuzuki, N. Takeda, and K. Sakae.** 2004. Isolation and identification of a novel human parechovirus. J. Gen. Virol. **85:**391–398.
17. **Joki-Korpela, P., and T. Hyypia.** 2001. Parechoviruses, a novel group of human picornaviruses. Ann. Med. **33:**466–471.
18. **Joki-Korpela, P., and T. Hyypia.** 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin. Infect. Dis. **27:**129–136.
19. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
20. **Oberste, M. S., K. Maher, and M. Pallansch.** 1998. Complete sequence of echovirus 23 and its relationship to echovirus 22 and other human enteroviruses. Virus Res. **56:**217–223.

21. **Oberste, M. S., S. M. Michele, K. Maher, D. Schnurr, D. Cisterna, N. Junttila, M. Uddin, J. J. Chomel, C. S. Lau, W. Ridha, S. al-Busaidy, H. Norder, L. O. Magnius, and M. A. Pallansch.** 2004. Molecular identification and characterization of two proposed new enterovirus serotypes, EV74 and EV75. J. Gen. Virol. **85:**3205–3212.
22. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
23. **Simmonds, P., and D. B. Smith.** 1999. Structural constraints on RNA virus evolution. J. Virol. **73:**5787–5794.
24. **Stanway, G., F. Brown, P. Christian, T. Hovi, T. Hyypia¨, A. M. Q. King, N. J. Knowles, S. M. Lemon, P. D. Minor, M. A. Pallansch, A. C. Palmenberg, and T. Skern.** 2005. Picornaviridae, p. 757–778. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy: classification and nomenclature of viruses. Eighth report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, United Kingdom.
25. **Stanway, G., P. Joki-Korpela, and T. Hyypia.** 2000. Human parechoviruses— biology and clinical significance. Rev. Med. Virol. **10:**57–69.
26. **Stanway, G., N. Kalkkinen, M. Roivainen, F. Ghazi, M. Khan, M. Smyth, O. Meurman, and T. Hyypia.** 1994. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J. Virol. **68:**8232– 8238.
27. **Takao, S., Y. Shimazu, S. Fukuda, M. Noda, and K. Miyazaki.** 2001. Seroepidemiological study of human parechovirus 1. Jpn. J. Infect. Dis. **54:**85–87.
28. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
29. **Watanabe, K., M. Oie, M. Higuchi, M. Nishikawa, and M. Fujii.** 2007. Isolation and characterization of novel human parechovirus from clinical samples. Emerg. Infect. Dis. **13:**889–895.
30. **Wigand, R., and A. B. Sabin.** 1961. Properties of ECHO types 22, 23 and 24 viruses. Arch. Gesamte Virusforsch. **11:**224–247.
31. **Wolthers, K. C., K. S. M. Benschop, J. Schinkel, R. Molenkamp, R. M. Bergevoet, I. Spijkerman, H. C. Kraakman, and D. Pajkrt.** 2008. Human parechoviruses as important viral cause of sepsislike illness and meninigitis in young children. Clin. Infect. Dis. **47:**358–363.