Prevalence, Types, and RNA Concentrations of Human Parechoviruses, Including a Sixth Parechovirus Type, in Stool Samples from Patients with Acute Enteritis

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Parechovirus epidemiology and disease association are not fully understood. Real-time reverse transcriptase PCR (RT-PCR) for all human parechoviruses (HPeV) was applied on stool samples from two groups of patients. Both groups contained patients with acute enteritis of all age groups, seen during one full year. Patients with norovirus, adenovirus, enterovirus, astrovirus, or rotavirus infections were excluded. In 118 patients from outbreak and hospital settings, no HPeV was detected. In a prospective study group of 499 nonhospitalized patients, the detection rate was 1.6%. One virus-positive patient was detected from 39 control patients. Positive samples occurred only in summer and autumn. Only one patient had accompanying respiratory symptoms. An association with travel or animal contact was not found. All positive patients except one were <2 years of age, with a neutral gender ratio. In children <2 years of age, the detection rate was 11.6% (7 of 60 children). The range of viral loads was 3,170 to 503,377,290 copies per gram or milliliter of stool. One of the highest viral loads occurred in a control child without symptoms at the time of testing. Phylogenetic analysis showed mainly contemporary HPeV1 strains in our patients but also showed a separate new lineage of HPeV1 in evolutionary transition from the historical prototype strain. Moreover, a novel sixth HPeV type was identified. Full genome analysis of the two viruses revealed recombination between HPeV1 and -3 in one and HPeV6 and -1 in another. HPeV seems relevant in children <2 years and specific RT-PCR for HPeV should be included in enteritis screening.

The Picornaviridae are a highly diverse family of small nonenveloped RNA viruses, many of which cause disease in humans. Among the nine genera within the family, there are important pathogens such as the rhinoviruses, enteroviruses, and hepatoviruses. Another human pathogenic genus that was defined in the early 1990s is Parechovirus (14, 17). Within this genus, there are two species, Ljungan virus and parechovirus. Until their reclassification, human parechovirus types 1 and 2 (HPeV1 and -2) have been known as echovirus types 22 and 23 within the enterovirus genus (31). Reclassification was justified (HPeV1 and -2) have been known as echovirus types 22 and 23 within the enterovirus genus (31). Reclassification was justified not only upon genetic and serological observations but also for reasons relating to their different biological properties. One major difference, for example, is a lacking cleavage site between virus proteins 4 and 2 in the parechoviruses which is present and used in most of the other Picornaviridae (26).

The spectrum of symptoms caused by HPeV is similar to that caused by some enteroviruses, including mostly enteritis with diarrhea and respiratory disease (7, 25). Rarer conditions such as meningoencephalitis (18), flaccid/transient paralysis (11, 15), neonatal sepsis (8), and Reye’s syndrome (30) have been reported. In studies conducted in different countries worldwide, almost the whole adult population had anti-HPeV antibodies (16). A recent study on children in Finland showed that the median age of infection with HPeV1 was 18 months, with 20% being infected after the first year of life (28). In another study, 95% of newborn infants had maternal antibodies against HPeV1 (16).

Improved molecular diagnostic methods and better clinical surveillance have recently led to the identification of novel parechoviruses. HPeV3 was identified in Japan in 2004 by molecular and serological methods (15) and confirmed in North America and Europe shortly thereafter (2, 7, 8). Infections with HPeV3 seem to peak at an earlier age than with HPeV1, may be associated with male gender, and seem to involve more sepsis-like illness and central nervous symptoms (7). HPeV4 was detected in The Netherlands by serological and molecular methods (6) and confirmed in a phylogenetic study from stored isolated virus stocks, along with the characterization of HPeV5 (5). The latter two studies suggested using genetic data rather than seroreactivity for HPeV typing. One major argument in favor of molecular typing was the potential of parechoviruses to recombine, which was detected for the first time in these studies (5, 6).

In this study, a diagnostic real-time reverse transcriptase PCR (RT-PCR) assay covering all known parechoviruses was developed and used to test two large cohorts of patients with enteritis. Phylogenetic analysis of the obtained virus RNA sequences independently identified a novel parechovirus type 6 that was described very recently (30). In addition, we found an
FIG. 1. Nucleic acid alignment of the hybridization sites of diagnostic real-time RT-PCR oligonucleotides. Oligonucleotides are shown below the alignment panel. The base count in the top line is based on HPeV1 reference strain Harris, which also serves as the comparison sequence in the alignment. Dots represent identical bases in compared sequences; deviations are spelled out. A slash (/) represents a gap in the alignment. (rc) means that the reverse complementary sequence is shown for the antisense primer. Note that almost all nucleotide mismatches in this design are stable G:T non-Watson-Crick base pairs (23, 32). FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Patients and samples. Stool samples from patients with acute enteritis (n = 118) were obtained from the routine diagnostic laboratory of a municipal health service. Young children were hospitalized in municipal hospitals. All other patients in this subcohort were not hospitalized but were subjected to enteritis investigations in outbreaks (depending on age: kindergartens, catering kitchens, and retirement homes).

Another set of stool samples (n = 538) was collected from 1 January to 31 December 2004 in a prospective study on acute, community-acquired diarrhea. All patients were outpatients seen by general practitioners. Diarrhea in these patients was defined as excretion of at least two loose and malodorous stools during 24 h for breastfeeding infants and at least two loose stools in a 24-h period for all other patients. Patients were excluded if they had inflammatory bowel disease, celiac disease, cystic fibrosis, food intolerance, or a known malignant disease. The cohort included stool samples from 39 control patients of compatible age distribution with conditions other than enteritis. Written informed consent was obtained from all patients or parents. Study protocol and data handling were approved by the local ethics committee.

In both groups, patients with norovirus, adenovirus, enterovirus, astrovirus, or rotavirus infection were excluded. All stool samples were stored without additives at −20°C for up to two years before analysis.

Cell culture. African green monkey kidney cells (GMK-AH1), human rhabdomyosarcoma cells (RD-18S), human amnion cells (FL), primary human skin fibroblasts, fetal rhesus monkey kidney cells (FRHK-4), Crandle feline kidney cells (CRFK), and Vero cells were inoculated at 33°C with 10% stool suspensions purified from bacteria by ultrafiltration (0.2 μm) or chloroform treatment. Inoculation medium was exchanged after 24 h, and the cells were observed for 14 days for cytopathogenic effects. Supernatants were serotyped with reference serum pools A to H, from the Statens Seruminstitut, Copenhagen, Denmark (20), containing polyclonal sera against echovirus types 1 to 7; human enterovirus types 9 and 11 to 33; poliovirus types 1 to 3; and coxsackie virus types A7, A9, A16, and B1 to B6.

Preparation of stool samples for RT-PCR. RNA was extracted from stool samples stored at −20°C with a Qiagen DNA stool kit, according to the manufacturer’s instructions. The protocol used an input of about 200 mg of solid stool or 200 μl of liquid stool. Effectively, 1/10th of the input (~20 mg) was brought into an elution volume of 200 μl. It should be noted that the kit was specified for recovery of DNA, but in our hands viral RNA was copurified with good efficiency (9; our unpublished data). In a later phase of the study a Qiagen viral RNA kit was used instead, in order to save costs: it was noted that this kit extracted viral RNA with high efficiency from 100-mg or 100-μl stool samples prediluted 1:10 in phosphate-buffered saline. Suspensions were vortexed and centrifuged, and 100 μl of supernatant was extracted according to the manufacturer’s instructions. The elution volume was 10 μl. Effectively, RNA from 10 mg of stool was brought into an elution volume of 100 μl.

Materials and Methods

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Methods used for pretesting of stool samples. IDEIA rotavirus, adenovirus, and astrovirus antigen enzyme immunoassays (DakoCytomation, Cambridge, United Kingdom) were used as recommended by the manufacturer.

Nestled RT-PCR for norovirus was done as previously described (22). Nested RT-PCR for enteroviruses followed a very similar protocol, using outer primers CAACACCTCTCGTGTCCCCGG and ATTTGCACCATATAACGGCCA and inner primers TACTTCGAGAAACCCYAGTA and AACACGACAGCACCCAAA GTA.

Parvovirus broad-range real-time RT-PCR reaction. Reaction mixtures (25 μl) contained 1 μl of RNA extract, 1X reaction buffer from enzymes from a Qiagen OneStep RT-PCR kit, 400 μM of each primer HPS (GTGCCCTCTGGGGCCAAA AG), 400 μM of each primer HPA (TCAGATCCTAGTGTGGTGTAC), and 200 μM of probe HPP (FAM-CGAGAGTAGCCAAAAAGTGATCCCT-TAM RA). Cycling in an Applied Biosystems 7700 SDS instrument involved the following steps: 50°C for 30 min, 95°C for 15 min, and 45 cycles of 95°C for 5 s/58°C for 30 s. For the calculation of approximate virus RNA concentrations in stool samples, we assumed 100% efficiency of RNA recovery in the purification step. The projected equivalent amount of stool tested per PCR vial, receiving 3 μl of RNA eluate, was 0.3 μg or 0.3 μl (see description of nucleic acid extraction).

Synthetic RNA standard. A PCR fragment of the real-time RT-PCR screening assay was obtained from strain BNI-788t, a HPeV1 identified in our laboratory by cell culture and random amplification with the VIDISCA method (29; our unpublished observations). cDNA was ligated into a pCR 2.1 T-vector TA cloning kit (Invitrogen, Karlsruhe, Germany). Plasmids were purified, sequenced, and reamplified with plasmid-specific primers (M13F-20 and M13r, from the kit) to lower the plasmid background in subsequent in vitro transcription. Reamplification products were transcribed into RNA with a MegaScript T7 in vitro transcription kit (Ambion, Austin, TX). After DNase I digestion, RNA transcripts were purified with Qiagen RNeasy columns and quantified in a spectrophotometer. By RT-PCR with and without reverse transcriptase enzyme, the RNA/RNA ratio in the preparation was determined to be 100.

Sequencing. 5'-Noncoding region sequences were determined from PCR products. Sets of downstream primers in the 3' end of VP1 were designed from an alignment of GenBank sequences and used to amplify complete P1 fragments. These were analyzed by a primer walking technique. For strain BNI-67, a 2C to 3D protein sequence was obtained by amplifying the highly conserved distal segment of the 3D gene. The obtained fragment served as a template for the design of a specific reverse primer. This was combined with a set of candidate upstream primers as derived from an alignment of all prototype strains available in GenBank in November 2005. Long-range PCR was done with an Expand high-fidelity kit (Roche, Penzberg, Germany). The products obtained from successful long-range amplifications were cloned in pCR4 vectors (Invitrogen) and sequenced by a primer walking technique. Primers in VP1 and 2C to 3D fragments were used to amplify a VP1 to 2C protein fragment, which was also cloned and sequenced by primer walking. All primer sequences are available upon request.

In silico methods. Statistical analysis was done using Statgraphics 5.0 (Manugistics, Dresden, Germany). Phylogenetic analyses were conducted in MEGA4 (27). SimPloots were done with SimPlot software (19). Oligonucleotide
In order to appreciate the relevance of parechoviruses in patients with acute enteritis, a real-time RT-PCR assay was established in the conserved 5′-noncoding region of the parechovirus genome. Figure 1 shows an alignment of amplicon sequences with prototype strains of all six parechovirus types known at the time of writing. The sensitivity of the assay was estimated in vitro-transcribed RNA was then used as a quantification standard to determine virus RNA concentrations in stool samples.

In total, 656 stool samples from outpatients of all age groups were tested for HPeV with the broad-range real-time RT-PCR assay (Fig. 2). Samples were taken throughout the year. All samples had been pretested by antigen enzyme immunoassay for rotavirus, astrovirus, and adenovirus as well as by RT-PCR for enterovirus and norovirus. Samples from the first 141 patients were obtained from the diagnostic laboratory of a municipal health service (from S. Baumgarte). This cohort comprised hospitalized young children as well as older children and adults of all age groups who were tested in the context of enteritis outbreak investigations. No parechoviruses were found.

The second group of patients stemmed from a study on community-acquired enteritis. Only patients seen by general practitioners were tested. Again, adenovirus, astrovirus, rota-, noro-, and enteroviruses had tested negative in all patients. Nine parechoviruses were found in 499 patients with acute enteritis and in one of 39 control patients without enteritis. The detection rate in symptomatic patients was thus 1.6% (8/499; 95% confidence interval = 1 to 3%), but detection rates in patients and controls (1/39) were not significantly different at the 95% confidence level (two-tailed t test). All positive patients except one were below 2 years of age, and the gender ratio was neutral (the one patient >2 years was six years old). If the cohort would have been restricted to young children, i.e., children less than 2 years of age, the detection rate of parechoviruses would have been 11.6% (7 of 60).

More clinical information and viral load data on all HPeV-positive patients are shown in Table 1. In analogy with enteroviruses, positive samples occurred only in summer and autumn. There was no obvious association with travel or animal contact. Only one patient had accompanying respiratory symptoms. A very large range of viral loads was observed (3,170 to 503,377,290 copies/ml), and one of the highest viral loads occurred in a control patient without symptoms.

All positive stool samples were subjected to cell culture. Isolates were obtained only in those two samples that had the highest virus RNA concentrations (refer to Table 1). In both cases virus was culturable on RD, GMK, CRFK, and Vero cells but not on primary human skin fibroblasts, FL, and FRHK cells.

Analysis of parechoviruses. In order to appreciate the types of parechoviruses present in our patients, the whole P1 protein (VP0, VP3, and VP1) was sequenced in all samples positive for parechovirus. Only sample BNI-R17, which showed the lowest virus concentration, could not be sequenced. As shown in Fig. 3, most viruses clustered with a group of contemporary HPeV1 strains. In concordance with earlier observations (5), the prototype HPeV1 strain Harris had only basal relationship with the contemporary group. Amino acid identity with strain Harris was around 92%. One virus from our cohort, BNI-R30, segregated as a separate lineage between the historical type 1 Harris strain and the contemporary group. Another virus, BNI-
67, could not be associated with any type. It had basal relationship with types 1 and 2.

As proposed recently for parechovirus type determination (5), a 207-amino-acid fragment from the VP1 protein was compared in strain BNI-67 with established reference genotypes. As shown in Table 2, the amino acid distance between BNI-67 and the most similar established HPeV type, HPeV2, was 0.22. This value exceeded the distance between the two most similar established HPeV types (HPeV1 and -2, 0.21). It was thus assumed that BNI-67 constituted a novel HPeV type.

During the preparation of our manuscript, a sixth parechovirus type was identified by Watanabe and colleagues (30). When this strain was included in the P1 phylogenesis, it clustered with BNI-67 with high bootstrap support. VP1 amino acid distance was 0.04. It was concluded that BNI-67 was an HPeV type 6.

As BNI-67 could not be isolated, its nearly full genome sequence was determined from the original stool sample (GenBank accession number EU022171). Similarity plot analysis was conducted and recombination was analyzed by Bootscan analysis, as shown in Fig. 4. BNI-67 was a recombinant of mainly HPeV6, -5, and -1. Major recombination breakpoints existed between the 2A/2B, 2B/2C, and 3B/3C protein portions, respectively.

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<td>BNI-67</td>
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*The following reference strains were used to define types: type 1, Harris (14) and 1086-99 (5); type 2, Williamson (13); type 3, A308-99 (115) and Can82853-01 (2); type 4, K251176-02 (6) and T75-4077 (5); type 5, CT86-6760 (formerly classified as type 2 [21]) (5) and T92-15 (5). Calculation was based on a 207-amino-acid fragment of the VP1 gene as described in reference 5, using the p-distance model in MEGA4. Distance values for types represented by more than one reference strain are based on average distances.

FIG. 3. Phylogenetic analysis of P1 protein regions (amino acids 76 to 773; isolate Harris), analyzed using the p-distance substitution model. VP1-based HPeV types are shown next to clades on the right margin. Analysis was conducted in MEGA4 (27). The evolutionary histories were inferred using the neighbor-joining method (24). Relevant bootstrap values from 500 replicate trees are shown next to the branches (10). The scale shows the evolutionary distance from each root (trees are drawn to scale). GenBank accession numbers: BNI-67, EU022171; BNI-R90, EU024630; BNI-R4, EU024631; BNI-R9, EU024632; BNI-R15, EU024633; BNI-R21, EU024634; BNI-R30, EU024635; and BNI-R32, EU024636. Strain BNI-788st (EF051629) is not described in this study and will be described in more detail separately.

FIG. 4. Recombination analysis carried out with SimPlot software (19), using a 600-bp sliding window. Window’s middle position is given on the x axis. Prototype strains used for comparison are shown in the inserts in each window. (A) Similarity plot analysis. The y axis shows percent nucleic acid identities in the sliding analysis window. (B) Bootscan analysis. Bootstrapped phylogenesis is performed in a sliding window. The y axis shows for each of the prototype strains the percentage of permuted trees in which the prototype strain clusters with strain BNI-67.
DISCUSSION

Enteritis is one of those clinical conditions which is caused by a spectrum of viruses that is most likely not fully appreciated. When testing stool samples on cell culture, virus isolates are sometimes obtained which cannot be typed by current methods. We developed the first broad-range real-time RT-PCR assay for parechoviruses, determined the detection frequency in two different cohorts of patients with acute enteritis, and identified independently a novel parechovirus type.

The assay presented in this article facilitates broad-range testing with high sensitivity and with all advantages of real-time RT-PCR, compatible with modern clinical laboratory settings. Broad applicability is demonstrated by the fact that HPeV6 was readily identified in a stool sample even though this type was not known when the assay was designed. Good concordance with all known HPeV types is demonstrated by nucleic acid alignment of the oligonucleotide target regions. Of note, mismatches observed in the alignment are almost exclusively G:T base pairs, which are thermodynamically nearly as stable as regular Watson-Crick base pairs (23). We have proven the concept of this primer design strategy recently on another RNA virus (32). Importantly, most HPeVs in this study were detectable by RT-PCR only. Virus could be cultured only from two samples that had extraordinarily high virus concentrations. It can thus be emphasized that specific, broad-range RT-PCR assays for the Pareovirus genus should be applied in the clinical laboratory to ensure complete detection (1, 7).

Due to the fact that agents of enteritis especially in outpatients are often not tested for, we applied the novel assay in this type of patient. In concordance with other studies HPeV were mostly restricted to young children <2 years of age (1, 7, 28). Interestingly, HPeV seemed to be detectable at higher frequencies in outpatient children seen by practitioners than in hospitalized children and those involved in outbreaks of enteritis (P < 0.05, comparing detection rates in children in the first and second cohorts tested). Moreover, secondary cases in contact persons were only rarely observed. One could therefore speculate that HPeVs may not be primarily responsible for epidemics of viral enteritis. They would rather be observed on a sporadic, endemic basis in children who normally remain undiagnosed. Associations with travel or animal exposure could not be identified in our study.

The detection frequency in outpatient children <2 years of age, at 11.6%, should lead to a consideration of HPeV in the differential diagnosis of enteritis. The true population prevalence of HPeV is probably somewhat lower because patients with other common enteric infections were eliminated from the study group. However, such elimination minimizes the rate of patients with double infections and emphasizes those in whom symptoms are caused exclusively by HPeV. On the basis of our data it would be advisable to include RT-PCR for HPeV in routine viral enteritis testing, at least as a second-line screening assay.

On the other hand, it must not be overlooked that an HPeV was also found in a healthy control child. Importantly, the child showed one of the highest viral loads of all patients. As this study is the first to determine virus concentrations in HPeV patients, it is difficult to derive conclusions regarding the relevance of HPeV from this finding. It should be noted that there were no clinical records about the immediate course of this patient. It cannot be excluded that this patient may have developed clinical signs shortly after the sample was taken. Future studies should put emphasis on the inclusion of proper control groups and possibly also determine viral loads in larger numbers of patients, correlating these with clinical courses. Some recent targeted virus discovery approaches yielded novel viruses of still-unclear disease association (3, 4, 12), underlining the importance of clinical studies with appropriate control groups which need to be conducted in a timely manner along with the identification of a potential agent. As with other diseases, the availability of viral load data from real-time RT-PCR will add an additional dimension to the appreciation of disease association.

The independent identification of a sixth parechovirus type, the characterization of another recombinant parechovirus, as well as the confirmation of a predicted (5) transition group between the historical and contemporary type 1 HPeVs underlines the lack of information currently available on the ecology of these agents. Recombination seems to be an important issue in HPeV evolution, and the current methods of HPeV typing, using VP1 sequence typing or serotyping, are probably not sufficient to describe the true diversity and composition of HPeV populations present in humans (5, 6). More-detailed genotyping is probably necessary to understand transmission patterns and strain-specific disease associations.

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