Specific Association of Human Parechovirus Type 3 with Sepsis and Fever in Young Infants, as Identified by Direct Typing of Cerebrospinal Fluid Samples

H. Harvala, I. Robertson, T. Chieochansin, E. C. McWilliam Leitch, K. Templeton, and P. Simmonds

1Specialist Virology Centre, Royal Infirmary of Edinburgh, and 2Centre for Infectious Diseases, University of Edinburgh, Summerhall, Edinburgh, United Kingdom; 3Biomedical Science Centre of Excellence in Clinical Virology, Chulalongkorn University and Hospital, Bangkok, Thailand

Background. Human parechoviruses (HPeVs), along with human enteroviruses (HEVs), are associated with neonatal sepsis and meningitis. We determined the relative importance of these viruses and the specific HPeV types involved in the development of central nervous system–associated disease.

Methods. A total of 1575 cerebrospinal fluid (CSF) samples obtained during 2006–2008 were screened for HPeV by means of nested polymerase chain reaction. All samples for which results were positive were typed by sequencing of viral protein (VP) 3/VP1. Screening for HEV was performed in parallel, as was detection of HPeV in respiratory and fecal surveillance samples, to identify virus types circulating in the general population.

Results. HPeV was detected in 14 CSF samples obtained exclusively from young infants (age, <3 months) with sepsis or pyrexia. The frequency of detection of HPeVs varied greatly by year, with the highest frequency (7.2%) noted in 2008 exceeding that of HEVs. Direct typing of CSF samples revealed that all infections were caused by HPeV type 3, a finding that is in contrast to the predominant circulation of HPeV1 in contemporary respiratory and fecal surveillance samples.

Conclusion. HPeV was a significant cause of severe sepsis and fever with central nervous system involvement in young infants, rivaling enteroviruses. The specific targeting of young infants by HPeV type 3 may reflect a difference in tissue tropism between virus types or a lack of protection of young infants by maternal antibody consequent to the recent emergence of HPeV.

Human parechoviruses (HPeVs) are small, nonenveloped, single-stranded, positive-sense RNA viruses within the Parechovirus genus of the Picornaviridae family. To date, HPeVs have been classified into 6 different types. HPeV type 1 and HPeV type 2 (previously known as echoviruses 22 and 23, respectively) were first isolated in 1956 [1] and have been associated with gastrointestinal and respiratory tract symptoms, as well as with occasional cases of encephalitis and flaccid paralysis [2–5]. HPeV type 3 (HPeV3) was first described in 2004; the strain was isolated from a stool sample obtained from a 1-year-old female Japanese infant who had transient paralysis with fever and diarrhea [6], as well as from stool samples obtained from neonates with sepsis [7], leading many investigators to propose an association between HPeV3 and sepsis-like illness in young children [7–10]. The role of HPeV type 4, HPeV type 5, and HPeV type 6 (HPeV6) as human pathogens is still poorly understood; HPeV type 4 and HPeV6 have been implicated as a cause of diarrhea in children [11–13].

Most HPeVs have been identified by typing of cell-cultured virus isolated from fecal or nasopharyngeal aspirate (NPA) specimens. Reflecting the much lower viral loads noted in cerebrospinal fluid (CSF) samples, there are only 2 descriptions of propagation of HPeV from these samples [14]. One isolate was HPeV3, which is associated with fever, and the other was HPeV6, which was recovered from an individual with Reye syndrome. The inability to directly type HPeV in clinical samples has complicated previous attempts to associate certain variants with different disease presentations, such as...
demonstrations of associations between HPeV3 infections and central nervous system (CNS)-related disease [9, 10, 15].

To address this issue, and to assess the importance of HPeV and human enteroviruses (HEVs) in childhood sepsis and meningitis, we screened for HPeV in diagnostic CSF samples collected between 2006 and 2008, by use of highly sensitive nested polymerase chain reaction (PCR) analysis. We also compared the frequencies of detection of HPeV with those of HEV. Other neuropathogenic viruses (e.g., herpes simplex virus types 1 and 2 [HSV1 and HSV2, respectively] and varicella-zoster virus [VZV]) were also the subject of routine screening tests. HPeV-positive samples were typed by amplification and sequencing of the viral protein (VP) 3/VP1 region [16]. We observed a specific association of HPeV3 infections with CNS-related disease in very young children (age, <3 months); this finding contrasted with the widespread circulation of HPeV1, HPeV3, and HPeV6 in contemporary respiratory and fecal surveillance samples.

MATERIALS AND METHODS

Test specimens. CSF samples referred to the Specialist Virology Centre, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom, during the period from 3 January 2006 through 18 August 2008 (representing >90% of all CSF samples obtained during the period) were anonymized and deposited in the Specialist Virology Centre CSF sample archive. Although the samples were anonymized before testing, epidemiologic and demographic information was retained while patient confidentiality was strictly protected (in accordance with a protocol approved by the Lothian Regional Ethics Committee [protocol 2002/4/36]) [16, 17]. Data comprised the age group (with the youngest group [age, 0–3 months] subsequently referred to as “young infants”), partial postcode, recorded symptoms or clinical information, source of referral, month of sample collection, and results of other virologic testing of the sample.

A total of 2295 anonymized respiratory samples that were collected from 1525 different individuals (792 males and 733 females) between January 2008 and June 2008 were included in the study, screened in pools of 10, and resolved by splitting, as described elsewhere [16]. Screening tests were also performed on pooled fecal surveillance samples referred for bacteriologic screening and collected in March, June, and September 2008 in Edinburgh, although identification of individual positive samples was precluded.

HPeV detection and typing. RNA was extracted from 200 μL of pooled/individual specimens (i.e., CSF and NPA samples and clarified fecal supernatant) and placed into 40 μL of Tris-ethylenediaminetetraacetic acid buffer, as described elsewhere [16]. Samples were screened for HPeV RNA sequences in pools of 3 (for CSF samples) or 10 (for NPA samples and fecal supernatant), by means of reverse-transcription (RT) nested PCR performed with 5′ untranslated region (5′ UTR) primers, as described elsewhere [16]. Positive pools were split into individual components and were retested to identify positive samples. Routine PCR testing for HSV1, HSV2, VZV, and enteroviruses was performed on individual CSF samples, by use of an assay described elsewhere [18].

Positive samples were amplified in the VP3/VP1 junction region and sequenced between nucleotide positions 2182 and 2437 to identify HPeV types, as described elsewhere [16]. Nucleotide sequences from the VP3/VP1 region were assigned GenBank accession numbers FJ847944–FJ848013.

RESULTS

Study group. A total of 1575 CSF samples obtained from 1480 different individuals (770 males and 710 females) were predominantly referred from very young children (age, <3 months) (30% of samples), middle-aged adults (age, 37–65 years) (25% of samples), and older patients (age, >65 years) (25% of samples). Routine screening detected HSV1 in 14 samples, HSV2 in 7 samples, VZV in 5 samples, and enteroviruses in 93 samples (overall frequency of virus detection, 7%).

Frequency of HPeV and enterovirus infections. The sensitivity of the screening RT-PCR using 5′ UTR primers for the detection of HPeV RNA has been established elsewhere [16]. Screening of the 1575 CSF samples detected HPeV RNA in a total of 14 individual specimens obtained from 14 different study subjects: 3 (0.7%) of 440 samples were obtained in 2006, 0 in 2007, and 11 (7.2%) of 153 in 2008. In comparison, enteroviruses were detected in a total of 93 specimens: 23 (4.6%) of 502 in 2006, 0 in 2007, and 21 (3.8%) of 551 in 2008. All HPeV-positive samples (14 samples obtained from 14 study subjects) identified using 5′ UTR screening primers could be amplified in the VP3/VP1 region (table 1). All sequences clustered closely with HPeV3 (accession numbers AJ889918 and AB084913) (figure 1).

Epidemiologic and clinical associations between HPeV and enterovirus infections. All children infected with HPeV were young infants (age, <3 months); the frequency of detection of HPeV in this subject group was 2.9% (14 of 480), whereas enterovirus infections were widely distributed among most age groups (figure 2). Although the frequency of enterovirus infections was also high in young infants (37 [7.1%] of 518 study subjects), it was lower than that found in adults (26 [11.8%] of 219 study subjects 21–36 years of age). The highest frequency of VZV and HSV1 CNS-associated infections was seen in study subjects >65 years of age, whereas HSV2 infections were predominantly noted in the younger adult age group (21–36 years; data not shown). Although a higher proportion of the HPeV-infected study subjects were male (8 [57.1%] of 14 subjects), they were not significantly overrepresented in the whole study group (770 [52.0%] of 1480; P = .59, by Fisher’s exact test). Similarly,
more HEV infections were seen in males (51 [55%] of 93 study subjects).

There were marked temporal changes in the incidence of HPeV over the study period (figure 2). HPeV was detected in 3 (2.0%) of 120 and 11 (7.2%) of 153 young infants during 2006 and 2008, respectively, but it was not detected in 2007. This variability was much greater than that noted for enterovirus infections of the CNS in the same age group, which had relatively constant annual detection frequencies (11 [7.0%] of 158 in 2006, 15 [7.7%] of 193 in 2007, and 11 [6.6%] of 167 in 2008) (figure 2). However, these figures conceal major changes in the different age groups infected between years, as well as marked changes in incidence in different quarters.

Clinical presentation of HPeV infection. Referral information, including the source of the samples and the reported symptoms, for subjects with HPeV-positive samples was compared with that for subjects with samples positive for enteroviruses, VZV, HSV1, and HSV2. HPeV3 infection was most commonly associated with sepsis (in 80% of cases), with the remaining cases referred with unspecific fever (figure 3 and table 1). Although the presentation of enterovirus infections in young infants was similar to that of HPeV infections (with sepsis in 30% of cases and with fever in 40% of cases), several cases of meningitis were also recorded. Furthermore, enterovirus and HSV1 infections in study subjects >1 year of age were associated with meningitis and headache. In our study group, encephalitis was associated only with HSV1 or VZV infections, and it was not documented for any subjects from whom HPeV or enterovirus isolates were recovered.

Comparison of HPeV types detected in the CSF with circulating virus populations. To determine whether the HPeV3 variants detected in CSF samples were typical of virus circulating in the general population over the study period, we compared frequencies of detection, age distributions of infection, and phylogenetic relationships of HPeV types recovered from CSF with types recovered from contemporary respiratory diagnostic specimens in 2007 [16] and 2008 and from those recovered from fecal surveillance samples in 2008 (figures 1 and 4).

The exclusive detection of HPeV3 in CSF samples was not reflected in other sample types. For example, in 2008, HPeV3 accounted for only 36%-40% of total HPeV infection variants detected in respiratory and fecal samples, in which HPeV1 predominated (P < .001 and P < .001, respectively) (figure 4A). However, consistent with the absence of any diagnosed cases of parechovirus-associated sepsis in 2007 and reflecting substantial variability in the circulation of this type between years, HPeV3 was completely absent from respiratory samples in 2007 (during which time HPeV1 and HPeV6 were detected).

The targeting of very young children by HPeV3 with sepsis (figure 2) was reflected in respiratory samples, for which detection of HPeV3 was again almost entirely confined to samples obtained from the 0- to 3-month age group (figure 4B). In contrast, infections with HPeV1 and HPeV6 were detected in older age groups, although infections due to these types were invariably detected in subjects <5 years of age in both study years. Of the 4 subjects with HPeV3-positive CSF samples who had a respiratory sample collected at the same time, 3 had an HPeV-positive result for both samples, consistent with the systemic

Table 1. Patient demographic and clinical characteristics and parechovirus typing results.

<table>
<thead>
<tr>
<th>Year of collection, specimen</th>
<th>Age, months</th>
<th>Sex</th>
<th>Month</th>
<th>Diagnosis</th>
<th>Subject location</th>
<th>Virus type</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF-721/06</td>
<td>&lt;3</td>
<td>F</td>
<td>Apr</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-874/06</td>
<td>&lt;3</td>
<td>M</td>
<td>July</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-1112/06</td>
<td>&lt;3</td>
<td>F</td>
<td>Nov</td>
<td>Fever</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF-2147/08</td>
<td>&lt;3</td>
<td>F</td>
<td>Apr</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2162/08</td>
<td>&lt;3</td>
<td>M</td>
<td>Apr</td>
<td>Neonatal sepsis</td>
<td>PHDU</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2169/08</td>
<td>&lt;3</td>
<td>M</td>
<td>Apr</td>
<td>Neonatal fever</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2273/08</td>
<td>&lt;3</td>
<td>M</td>
<td>June</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2288/08</td>
<td>&lt;3</td>
<td>M</td>
<td>June</td>
<td>Neonatal sepsis</td>
<td>PITU</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2307/08</td>
<td>&lt;3</td>
<td>F</td>
<td>June</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2327/08</td>
<td>&lt;3</td>
<td>M</td>
<td>June</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2369/08</td>
<td>&lt;3</td>
<td>M</td>
<td>July</td>
<td>Fever</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2387/08</td>
<td>&lt;3</td>
<td>M</td>
<td>July</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2389/08</td>
<td>&lt;3</td>
<td>F</td>
<td>July</td>
<td>Sepsis</td>
<td>GCW</td>
<td>HPeV3</td>
</tr>
<tr>
<td>CSF-2426/08</td>
<td>&lt;3</td>
<td>F</td>
<td>Aug</td>
<td>Neonatal sepsis</td>
<td>PITU</td>
<td>HPeV3</td>
</tr>
</tbody>
</table>

NOTE. CSF, cerebrospinal fluid; F, female; GCW, general children ward; HPeV3, human parechovirus type 3; M, male; NNU, neonatal unit; PHDU, pediatric high-dependency unit; PITU, pediatric intensive care unit.
Figure 1. Phylogenetic comparisons of the viral protein (VP) 3/VP1 region sequences amplified from human parechovirus (HPeV)–positive specimens (circles) with all available nonidentical published sequences (labeled according to GenBank accession number). Trees were constructed by neighbor-joining of maximum composite likelihood (nucleotide) pairwise distances, with bootstrap resampling done to demonstrate the robustness of groupings; values ≥70% are shown. Multiple positive (respiratory [Resp]) samples obtained from the same individual were invariably identical, and only the first sample collected is shown. Paired arrows denote human parechovirus variants from the 3 subjects with paired positive cerebrospinal fluid (CSF) and respiratory samples. Fec, fecal; HPeV1–HPeV6, HPeV types 1--6.
disseminated parechovirus infections associated with sepsis in young infants. Phylogenetic analysis confirmed that HPeV3 variants in paired samples were identical or close to identical to each other (figure 1).

Phylogenetically, HPeV1 and HPeV3 variants recovered from different sample types were interspersed (figure 1), with there being no evidence for HPeV variants specifically associated with respiratory, enteric, or CNS infections. These observations argue against the existence of a specifically pathogenic (clonal) strain that might account for the development of sepsis in neonates infected with HPeV3.

HPeV3 variants recovered in 2006 and 2008 showed substantially less genetic diversity than did HPeV1 variants recovered in those years. Mean pairwise Jukes-Cantor–corrected distances of 0.024 between HPeV3 sequences were less than one-third of the distances between HPeV1 sequences (0.086), and they indicate a

Figure 2. Age distributions of human parechovirus (HPeV)–infected (A) and human enterovirus (HEV)–infected (B) study subjects, subdivided by referral year (2006, 2007, and 2008). The numbers above the bars denote total numbers of subjects with positive specimens. Dotted lines denote mean frequencies of detection of HPeV and HEV for the whole study group.
Over the study period, screening detected enteroviruses, HSV1, VZV, and HSV2, and VZV in 6.8% of samples obtained from children <5 years of age; the addition of screening for HPeV increased our rate of detection by more than one-quarter, to 8.6%.

Although several RT-PCR–based methods for the detection of HPeV in CSF samples have been described elsewhere [15, 19–22], to our knowledge, this is the first systematic study to apply direct HPeV type identification to CSF specimens. As a result, we were able to show an absolute association of HPeV type 3 with CNS-related infection in young infants, an association that could only be indirectly inferred from epidemiologic data and reliance on other sample types in previous studies [9, 10, 15]. Given the marked differences between HPeV types in terms of epidemiologic profile and disease associations, typing has important value in the diagnosis and clinical assessment of childhood sepsis and CNS-related diseases in neonates and infants.

**HPeV epidemiologic profile and disease associations.** There were striking differences in seasonal distribution, age distribution, and clinical outcomes between HPeV types. In common with findings from previous studies [12, 16, 23], HPeV1 was the most common type detected in non-CSF samples, with similar incidences noted in 2007 and 2008, reflecting the likely continuous circulation of this HPeV type throughout the 2000s [9, 12–14, 16, 23, 24]. In contrast, on the basis of our data from CSF screening tests, HPeV3 circulated only in 2006 and 2008, a biannual cycle consistent with previous reports of much higher frequencies of HPeV3 infections occurring in even-numbered years between 2000 and 2006, and was virtually absent in intervening years [10, 20]. The factors underlying the periodicity of HPeV3 transmission are unclear, although distinct epidemic patterns of circulation have been described for several enteroviruses. Regular sharp increases in reported cases are observed every 3–5 years, in association with the emergence of new genetic lineages [25–27]. Whether this phenomenon underlies the biannual cycle of HPeV3 is unclear. In the short VP3/VP1 sequence obtained in the current study, there was no evidence for a systematic difference between variants recovered from different years; most HPeV3 variants could be classified into 2 lineages, both of which circulated equally in 2008 and one of which contained variants from 2007.

**DISCUSSION**

**Detection and typing of HPeV.** Although enteroviruses are a well-known cause of aseptic meningitis and severe neonatal sepsis, the incidence and clinical associations of infections are less well established. Our observation that, in the first half of 2008, the frequency of HPeV infections (7.2%; all of which were due to HPeV3) was actually greater than that of enterovirus infections (6.6%; caused by 8 different serotypes) directly demonstrates that severe HPeV infections can at least episodically represent a significant cause of sepsis and CNS-related disease in young infants. These findings, in agreement with those of recently performed studies [7, 9, 10, 15], strongly support the introduction of routine HPeV screening of CSF specimens. The introduction of such screening would indeed significantly increase the rate of positive results of screening CSF specimens. Over the study period, screening detected enteroviruses, HSV1, HSV2, and VZV in 6.8% of samples obtained from children <5 years of age; the addition of screening for HPeV increased our rate of detection by more than one-quarter, to 8.6%.

Although several RT-PCR–based methods for the detection of HPeV in CSF samples have been described elsewhere [15, 19–22], to our knowledge, this is the first systematic study to apply direct HPeV type identification to CSF specimens. As a result, we were able to show an absolute association of HPeV type 3 with CNS-related infection in young infants, an association that could only be indirectly inferred from epidemiologic data and reliance on other sample types in previous studies [9, 10, 15]. Given the marked differences between HPeV types in terms of epidemiologic profile and disease associations, typing has important value in the diagnosis and clinical assessment of childhood sepsis and CNS-related diseases in neonates and infants.

**Figure 3.** Comparison of recorded symptoms of and/or diagnoses for study subjects with human parechovirus (HPeV)–positive samples with those found in association with infection due to other viruses detected during screening of cerebrospinal fluid (CSF) specimens. HSV1, herpes simplex virus type 1; HSV2, herpes simplex virus type 2; VZV, varicella-zoster virus; <3 months, subjects aged <3 months; ≥3 months of age, subjects ≥3 months of age.

More recent origin for HPeV3 variants circulating in Edinburgh over the study period. These observations are consistent with the phylogenetic position of HPeV3 sequences as direct descendants of the original A308 isolate recovered in 1999 (accession number AB084913) (figure 1).
that were referred (all 3 of which were found to be HPeV3 positive), an observation that underlines the high morbidity associated with these infections in this age group. However, in contrast to the findings of earlier studies [28, 29], no HPeV-infected young infants in the present study presented with encephalitis.

Mechanisms underlying HPeV type–associated differences. Two different possible explanations could account for the marked HPeV3-associated differences in the epidemiologic profile and morbidity [6, 9, 10]. HPeV3 differs from other types by not encoding an RGD (arginine-glycine-aspartic acid) motif in VP1 [6, 8], which has been suggested to enable HPeV1 and presumably other HPeV types to use integrins as virus receptors [30, 31]. The use of different receptors may thus confer upon HPeV3 a different cellular tropism, thereby accounting for its greater pathogenicity and neurotropism. However, this hypothesis awaits direct experimental confirmation.

Interestingly, neither hypothesis accounts for the observation that HPeV3 infections were almost entirely confined to young infants. Neither putative differences in cellular tropism nor a lack of protection by maternal antibody early in life would preclude transmission between older children, as observed for other HPeV types. A possible epidemiologic clue is the observation in the current study of a highly restricted variability of HPeV3 compared with HPeV1 variants characterized in the present study and in isolates recovered in Canada [7] and with other HPeV types based on available published sequences (figure 1). All

Figure 4. A, Comparison of human parechovirus (HPeV) types in different samples, shown as proportions of total samples. Differences in distributions were assessed using 3 × 2 exact contingency tables, with significant differences (P < .05) underlined. B, Age distributions of HPeV type 1 (HPeV1), HPeV type 3 (HPeV3), and HPeV type 6 (HPeV6) infections. Data for respiratory samples obtained during 2007 were derived from [18]. CSF, cerebrospinal fluid.
though the rate of substitution for the analyzed region of VP3/VP1 of HPeV has not been determined, rates between $4 \times 10^{-3}$ and $14 \times 10^{-3}$ substitutions per site per year typify structural gene regions of other picornaviruses (e.g., enteroviruses and aphthoviruses, as reviewed in [34]). If these reflect HPeV, a conservative (minimum) estimate of the substitution rate of $5 \times 10^{-3}$ substitutions per site per year predicts only 9 years of divergent sequence drift between Edinburgh variants and the originally described HPeV3 isolate (A308/99) from Japan from 1999, an estimate that closely fits the difference in sample dates. These dates support the hypothesis for extremely recent global spread of HPeV3 into naive human populations and may therefore account for the lack of maternal protection of neonates specific to this HPeV type. Population structures and evolutionary histories of different HPeV types can be reconstructed by coalescent methods [35] and can allow the association between emergence and pathogenicity to be further explored.

Acknowledgments

We thank Peter McCullough, Julie White, Mary Notman, Eleanor Leslie, and Carol Thomson for providing samples, data, and other virus testing results from the respiratory sample archive. We also thank Elly Gaunt for help with sample extraction, pooling, and archive storage of respiratory specimens.

References