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Introduction of a novel parechovirus RT-PCR clinical test in a regional medical center

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ABSTRACT

Background: Although data documenting the severity and frequency of human parechovirus (HPeV) infections have been published, detection of HPeV is not routinely performed in most clinical virology laboratories.
Objective: To describe diagnostic yield, epidemiology and clinical characteristics of patients infected with HPeV during the first year using a new HPeV reverse transcription (RT)-PCR.
Study design: We introduced an HPeV RT-PCR for the routine testing of cerebrospinal fluid (CSF) and blood samples submitted to our clinical laboratory for detection of human enteroviruses (HEV). Prospective testing of samples with retrospective analysis of medical charts was performed.
Results: Of the 499 clinical samples received between May, 2009 and May, 2010, 9.6% (46 patients) had HEV detected and 3.4% (15 patients) had HPeV detected. All patients infected by HPeV were <3 months old, hospitalized between June and October 2009, and all typed viruses were HPeV3. Clinical characteristics of HPeV and HEV infected infants were similar. However, patients infected with HPeV were more likely to have a normal leukocyte count in their CSF ($p < 0.001$). One HPeV3-infected infant developed encephalitis and another developed hepatitis.
Conclusion: In our institution, the HPeV RT-PCR was useful to diagnose a novel pathogen in infants with sepsis-like disease.

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1. Background

Although human parechoviruses (HPeV) are generally associated with mild gastrointestinal and respiratory symptoms, severe neonatal disease has been described.[1–3] Of 16 identified subtypes, HPeV type 3 is most commonly associated with sepsis-like disease, meningoencephalitis and hepatitis in infants.[4–6] Few data on the epidemiology of HPeV infections are available from North America; most studies are retrospective analyses of stored clinical specimens.[1,3,7,8] We designed an HPeV real-time reverse transcription (RT)-PCR for the routine prospective testing of cerebrospinal fluid (CSF) and blood samples submitted to our clinical virology laboratory for human enterovirus (HEV) detection.

2. Objective

We describe the diagnostic yield, epidemiology and clinical characteristics of patients infected with HPeV during the first year using a new HPeV RT-PCR.

3. Study design

3.1. HPeV RT-PCR design and evaluation

Primers and probes for a real time RT-PCR targeting HPeV were designed in the 5′ untranslated region (UTR) using 15 GenBank HPeV sequences representing subtypes 1–8. Prior to nucleic acid extraction (QIAamp RNA Mini Kit, Qiagen, Valencia, CA), jellyfish RNA transcripts (EXO) were added to clinical specimens to assess RNA extraction efficiency and PCR assay inhibition.[11] Two sets of HPeV primers and one Taqman probe were multiplexed with EXO primers and probe (Table 1, Supplemental data) in a reaction mix including UltraSense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA), HPeV primer mix (250 nM each), EXO primer mix (100, 200 nM), HPeV probe (100 nM), EXO probe (62.5 nM) and

Abbreviations: HPeV, human parechovirus; HEV, human enterovirus; RT-PCR, reverse transcription polymerase chain reaction; CSF, cerebrospinal fluid.

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Table 1
Results of RT-PCR testing of viral cultures.

Viral culture result	Numbers of positive results by RT-PCR		
	HPeV	HEV	HRV
HEV	6[a]	139[a]	NT[b]
HRV	2	3	98

[a] 3 cultures were strongly positive for HEV and weakly positive for HPeV.
[b] Not tested.

10 μl of extracted RNA. Reactions were amplified for 40 cycles using the cycling protocol recommended by the master mix manufacturer with an annealing temperature of 60 °C. The linear range of our HPeV RT-PCR, evaluated using a strain of HPeV1, was 5×10^8 viral copies/mL, the average R^2 value of the standard curve was 0.97 and the analytical limit of detection was 500 copies/mL.

Potential cross-reactivity of the HPeV RT-PCR for the 5′ UTR of HEV and rhinoviruses (HRV) was evaluated by testing 142 viral cultures, previously identified as HEV by cytopathic effect and immunofluorescence, with HPeV and HEV RT-PCR and 103 viral cultures, previously identified as HRV by cytopathic effect and acid lability testing, using HPeV, HEV, and HRV RT-PCR. HEV cultures included a wide range of strains identified as coxsackie A, coxsackie B3-5, echovirus 6, 9, 11, 30 and enterovirus 71. Residual clinical CSF samples ($n = 867$) collected between July, 2006 and December, 2008 that were previously submitted for HEV RT-PCR were retrospectively analyzed by HPeV. HEV RT- PCR, which included HEV specific primers and probe (Table 1, Supplemental data), was performed using the same extraction method, master mix and amplification protocol as the HPeV assay. Both RT-PCR assays were quantitative and included four point standard curves generated from quantified RNA transcripts. The HRV RT-PCR has been previously described.[9]

3.2. Prospective clinical testing and HPeV typing

CSF and blood samples (serum or plasma) from pediatric and adult patients submitted for HEV RT-PCR were simultaneously tested by HPeV RT-PCR beginning in May, 2009. Results of testing were reported to the patient's medical chart. Available HPeV positive samples were analyzed for subtype. Samples were extracted with QIAamp RNA Mini Kit (Qiagen) and amplified using two sets of nested RT-PCRs targeting the VP1 and the VP3/VP1 junction regions (Table 1, Supplemental data).[1,10] HPeV type was determined by sequencing the amplicons and blasting the sequences in GenBank.

3.3. Clinical data and statistical analysis

Review of medical records from HPeV and HEV positive infants was performed after receiving IRB approval from our institution. Two medical professionals independently reviewed all charts separately and completed a standardized data collection form. Clinical and laboratory characteristics of the HPeV infected infants were compared with those from patients <12 months of age infected with HEV who received care at the same hospital between 2007 and 2010. Two-tailed Wilcoxon rank sum test and Fisher's exact test were used to compare groups.

4. Results

4.1. HPeV RT-PCR analysis of cultures and retrospective samples

The results of RT-PCR testing of viral culture harvests previously identified as HEV or HRV by culture methods are shown in Table 1. HEV RT-PCR and HPeV RT-PCR showed a very small amount of cross-reactivity in high titer viral cultures identified as HEV based on the strong HEV RT-PCR results. Of 867 clinical CSF samples tested retrospectively by HEV and HPeV RT-PCR, 71 were positive for HEV (8.2%), 6 were positive for HPeV (0.7%) and 790 were negative for both viruses showing no cross-reactivity in clinical samples.

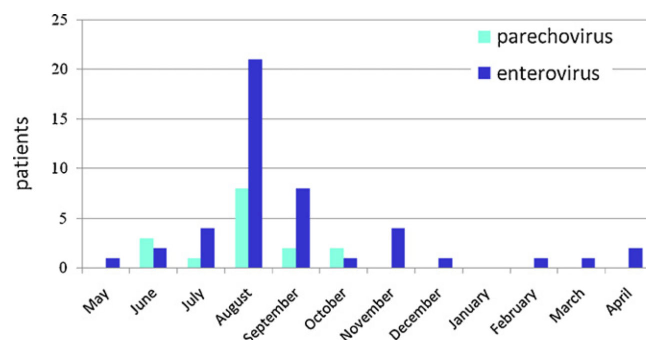

Fig. 1. Seasonal distribution of human parechovirus and human enterovirus in Seattle, WA, May 2009–May 2010.

4.2. Prospective clinical testing

We prospectively tested 499 clinical samples by HEV and HPeV RT-PCR between May, 2009 and May, 2010, including 409 CSF and 90 blood; 225 (45.1%) were from patients under 18 years of age (range: 1 day–88 years, median: 22 years, SD: 24.5 years). Forty-eight (9.6%) samples from 46 patients had HEV detected and 17 (3.4%) samples from 15 patients had HPeV detected. Introduction of this HPeV-specific RT-PCR added to the HEV PCR platform resulted in an increased rate of detection of CSF/blood viral pathogens of 35% during the first year the assay was used. The age of the HEV positive patients ranged from 5 days to 72 years (median: 17 years, SD: 17.8 years), while that of HPeV positive patients ranged from 7 days to 64 days (median: 31 days, SD: 19.3 days) ($p < 0.01$). Although HEV and HPeV were both more prevalent in August, HEV was detected throughout the year whereas HPeV was detected between June and October 2009 (Fig. 1).

4.3. HPeV typing

Of 15 patients infected with HPeV, 13 had samples available for analysis of HPeV subtype by sequencing, one had no sample available and one patient's sample had been sent to another laboratory for typing. Of 14 samples available, three did not amplify because of low viral load and 11 were type 3, including the sample evaluated at an outside laboratory.

4.4. Clinical characteristics

Clinical characteristics of 12 HPeV positive infants hospitalized in our medical center were compared to those of 26 infants <12 months of age hospitalized with HEV infection between May, 2007 and May, 2010 (Table 2). Infants infected with HEV and HPeV differed significantly in CSF laboratory values. HPeV-positive infants had significantly fewer white blood cells in their CSF than HEV-positive infants (2 vs. 161.5 cells/mm^3) ($p < 0.001$). One (10%) of 10 patients with HPeV had >10 cells/mm^3 in the CSF compared with 18 (81.8%) of 22 HEV patients ($p < 0.001$). Compared to HPeV-positive patients, the CSF protein level was significantly higher in the HEV group ($p = 0.03$), although the numbers of patients with elevated CSF protein were not different. Similarly, CSF glucose was significantly lower in the HEV group ($p = 0.003$), although no differences were observed in the number of patients with low CSF glucose concentrations. Peripheral leukocyte count was significantly lower in the HPeV group than the HEV group ($p = 0.008$), although both values were within the normal range for age. No deaths occurred

Table 2
Clinical characteristics of infants <12 month old infected with human parechoviruses (HPeVs) or human enteroviruses (HEVs).

	HPeV (*n*) 2009–2010	ENT (*n*) 2007–2010	*p* Value
Age (days)	27.5 (12)	39 (26)	0.198
Fever (days)	3.5 (12)	3 (26)	0.219
Diarrhea (%)	8.3 (12)	15.4 (26)	1.000
Vomiting (%)	8.3 (12)	26.9 (26)	0.393
Rash (%)	16.7 (12)	38.5 (26)	0.268
Respiratory symptoms (%)	16.7 (12)	26.9 (26)	0.689
Subjective irritability (%)	83.3 (12)	87 (26)	1.000
Hospitalization (days)	3 (12)	2 (26)	0.393
Antibiotics (days)	3 (12)	3 (26)	0.305
CSF viral load (copies/mL)	21,268 (10)	7682 (22)	0.339
CSF WBC (cells/mm^3)	2 (10)	161.5 (22)	<0.001
CSF WBC ≥ 10 cells/mm^3 (%)	10 (10)	81.8 (22)	<0.001
CSF protein (mg/dL)	54 (10)	86 (22)	0.03
CSF protein > 120 mg/dL (%)	10 (10)	22.7 (22)	0.637
CSF glucose (mg/dL)	45 (10)	40.5 (22)	0.003
CSF glucose < 40 mg/dL (%)	10 (10)	40 (22)	0.114
Peripheral WBC (10^3 cells/mm^3)	6.9 (12)	10.7 (25)	0.008

in either group. The mean viral loads of HEV and HPeV in the CSF of infected patients were not significantly different (7682 vs. 21,268 copies/ml, respectively).

Most infants infected with HEV or HPeV had a benign clinical course associated with viral meningitis or systemic infection and were discharged from the hospital shortly after the diagnosis of viral infection was made. However, two infants infected with HEV developed severe myocarditis and two infected with HPeV had serious clinical disease requiring prolonged hospitalization. One two-week-old infant with complicated HPeV infection presented with a fever of 38.7 °C, a petechial rash, seizures and respiratory distress. The patient was initially treated with phenobarbital and rapidly developed bradycardia and apnea requiring mechanical ventilation for 48 h. Magnetic resonance imaging (MRI) provided the diagnosis of encephalitis (Fig. 2). The infant was hospitalized for 11 days and discharged with levetiracetam. Anti-convulsive medication was discontinued at 8 months old and neurological development was evaluated as normal at that time. The second HPeV-infected child was admitted at five weeks old with fever of 40 °C, severe hepatitis (AST 3199 IU/L, GGT 288 IU/L, bilirubin conjugated 3.0 mg/dL), and mild coagulopathy. After 3 days of fever and progression of jaundice, the patient was transferred to our hospital for further evaluation of liver disease. The patient was discharged two days later with improving liver function and a diagnosis of viral hepatitis.

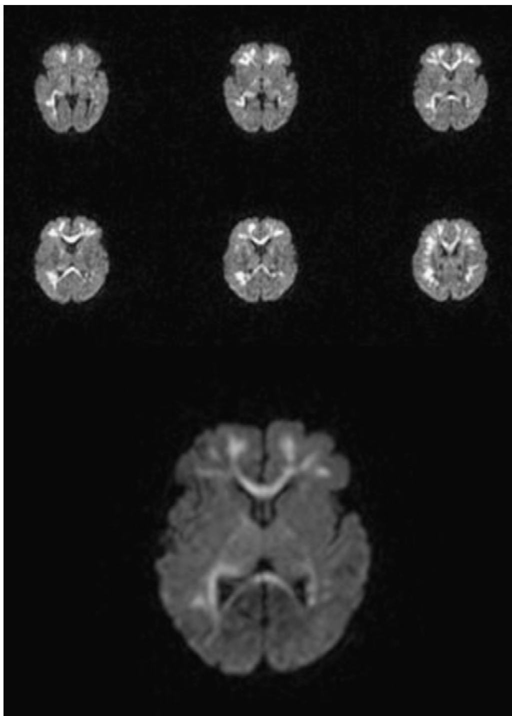

Fig. 2. Magnetic resonance imaging (MRI) compatible with encephalitis showing diffuse abnormal diffusion restriction throughout the cerebral white matter tracts in 2 week old infant infected with human parechovirus 3.

5. Discussion

This study represents the first prospective examination of the diagnostic utility of HPeV detection in a clinical virology laboratory servicing a large academic medical center. HPeV-specific RT-PCR added to the HEV platform resulted in an increased rate of detection of CSF/blood viral pathogens by 35% during the first year the new assay was used. This result is similar to the increased rate of detection of 32% found in a retrospective study.[8] However, all HPeV positive patients in our study were infants, questioning the clinical utility of systematic testing for HPeV in adult patients. HPeV type 3, which has previously been described only in a very young patient population,[5] was the most frequent viral pathogen observed in CSF from infants <3 months of age in our study. However, our study may have missed detection of HPeV in older children, who are less likely than very young children to have lumbar punctures performed.

This study confirms the epidemiology recently described in a retrospective study of HPeV infection in children hospitalized in Kansas City, MO.[11] The seasonality of our cases, which were detected between June and October, agrees with both the Kansas City and European data, in which HPeV was detected between April and October.[3,7,10] The prevalence of HPeV in CSF from children <5 years in The Netherlands varied by year, with rates of 8.2%, 0.4%, and 5.7% reported in 2004, 2005 and 2006, respectively.[8] A study in England reported an absence of HPeV in 2007 but an epidemic peak in 2008.[7] These reports suggest that HPeV occurs in Europe on a biannual basis.[5] Our positivity rate of 7.5% in children during 2009–2010, correlated with an epidemic period as reported in European studies. Preliminary data in Seattle from summer and fall 2010 show low rates of HPeV detection (data not shown), suggesting the possibility that biannual variability may be present in North America but with epidemics occurring in uneven years instead of even years.

Differences in symptoms between infants infected with HEV and HPeV were not observed. However, our data confirm previously published studies documenting differences in CSF laboratory parameters,[12] including a lower rate of pleocytosis in infants infected with HPeV compared to HEV. Nine of 10 infants with detection of HPeV in CSF had normal CSF leukocyte counts and concentrations of protein and glucose, providing evidence that normal CSF can occur with viral meningoencephalitis in infants. Although 1 of 12 infants (8.3%) with HPeV positive CSF had complicated neurological disease, further data are required to determine the true incidence of neurological complications related to this pathogen and to determine the potential for long-term sequelae. One patient developed severe hepatitis and mild coagulopathy as reported by other groups.[12,13] Although the rate of severe complication in our study was 16.6% for HPeV infections compared to 4% for HEV in infants <12 months old, these rates may depend on the types of HPeV and HEV circulating and the completeness of clinical evaluations of patients, including lumbar puncture.

Limitations of our study include the absence of extensive validation with a prototypes panel. Although our primers were designed to amplify HPeV subtypes 1–8 we did not have access to all pro-

totypes to confirm it. We identified only HPeV3 from CSF samples, but have sequenced types 1, 3, and 6 from other clinical specimens (data not shown). The use of standardized collection forms and data validation was utilized to minimize bias, although the retrospective design of the clinical data analysis could have been biased by this approach.

While our study was not designed to examine cost effectiveness, the additional of HPeV testing added minimal cost to the existing HEV RT-PCR platform. Results obtained on the 15 HPeV-positive patients allowed discontinuation of antibiotics, earlier discharge, and reassurance to the physicians and families that a pathogen likely to be responsible for the child's disease was detected. In the first year of our study, we estimate that the cost of testing 225 pediatric samples for HPeV and HEV was substantially less than the cost of hospitalization of 15 febrile infants for even one additional day.

In conclusion, our HPeV diagnostic assay was helpful in diagnosing infants with sepsis-like disease. Clinicians should not rely on pleocytosis to request HPeV RT-PCR testing since almost all our cases had normal CSF, but should be encouraged to consider this diagnosis in infants <3 months of age presenting with fever and other signs of sepsis during the summer and fall months.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.02.010.

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