Human Parechovirus and Other Enteric Viruses in Childcare Attendees in the Era of Rotavirus Vaccines

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Received November 3, 2011; accepted January 2, 2012; electronically published May 3, 2012.

Objective. We studied the prevalence of enteric viruses, including rotavirus, enterovirus, norovirus, adenovirus, and human parechovirus (HPeV), in stool samples of childcare attendees. The prevalence of enteric viruses was described in children with and those without gastroenteritis.

Methods. Children aged 1-19 months were recruited from 2 childcare centers in Tacoma, Washington, from October 2008 through June 2009. Stool samples were obtained at enrollment and during diarrheal illnesses for enteric virus testing. A symptom diary was completed by parents.

Results. One hundred six children (mean age, 10 months) were followed for an average of 170 days. At enrollment, 78 asymptomatic children had stool samples available. Forty-eight illnesses with acute diarrhea (stool samples were available for 24 illnesses) occurred in 37 children. Rotavirus was not detected in samples from symptomatic or asymptomatic children. HPeV was present in 21% and adenovirus in 46% of symptomatic children. At least 1 virus was detected in 78% of samples from asymptomatic children, including HPeV in 27% and adenovirus in 55%. No differences were found in symptom prevalence between HPeV-positive and HPeV-negative diarrheal illnesses. Molecular analysis revealed a diversity of HPeV types.

Conclusions. Our study highlights the high level of HPeV circulation in childcare. The lack of rotavirus detected in this study supports the impact of rotavirus vaccine and emphasizes the need for a greater focus on the epidemiology of non-rotavirus etiologies of gastroenteritis.
The main objective of our study was to describe the prevalence of common enteric viruses in stool samples from healthy asymptomatic childcare attendees and those collected during subsequent diarrheal episodes in a setting where RV vaccine uptake was uniformly excellent. Secondary objectives of this study included identifying specific HPeV serotypes in children attending childcare and assessing the spread of HPeV within the childcare setting.

**PATIENTS AND METHODS**

**Study Design**

Children from 5 weeks through 24 months of age attending 1 of 2 large childcare centers on a military base in the Pacific Northwest were eligible for participation in a prospective study of enteric diseases. Children who attended at least 20 hours/week were eligible; those expected to leave the center in the next 3 months were excluded from participation. Parents provided informed consent, and the institutional review boards of Madigan Healthcare System (Seattle, WA) and Seattle Children’s Hospital (Seattle, WA) approved the study. Continuous recruitment began on 28 October 2008, and children enrolled in the study were followed until 30 June 2009. Stool samples were collected at the time of study enrollment at the childcare center by the study nurse whether symptoms were present or not. Baseline demographic characteristics, medical history, and immunization history were obtained from parent interviews and review of participant medical records. Throughout the study period, the study nurse was contacted by parents and/or the childcare provider when any child was ill [22, 23], and the nurse contacted the childcare center and/or parents weekly to identify unreported illness and follow illness progression. Diarrhea was defined as >3 loose or watery stools in a 24-hour period with or without emesis by parent report. Illness symptoms were confirmed with the parent by the study nurse, and a stool sample was collected either at the childcare center or directly from the parents. Parents completed a daily symptom diary for 14 days following illness onset. One of 3 study physicians documented medical visits related to the illnesses by use of a standardized form.

**Laboratory Analysis**

Stool samples were tested for RV by enzyme immunoassay (Rotazyme test, Abbott Laboratories, Abbott Park, IL) [24, 25]. Stool swabs were sent to the University of Washington Molecular Virology Laboratory (Seattle, WA) in 0.5 mL of lysis buffer for detection of HPeV and EVs by reverse-transcription polymerase chain reaction (RT-PCR) and AVs by PCR, as described elsewhere [12, 24]. The AV assay detected all genotypes of AV without differentiation. Norovirus was detected by an in-house-developed, quantitative real-time RT-PCR assay. The assay, which was developed for clinical use, used primers and a TaqMan probe targeting the conserved region between NV open reading frames 1 and 2. Total nucleic acids were extracted using the QIAamp DNA Mini kit (Qiaegen, Valencia, CA). An internal control made of jellyfish DNA transcripts was added to every sample as an internal positive control. A 1:10 dilution of the initial sample was tested if inhibition was seen in the original sample. The specific protocols for HPeV and EV RT-PCR have been described elsewhere [12, 26].

Samples positive for HPeV were subtyped using the same extraction protocol as that for RT-PCR and amplified using 2 sets of nested RT-PCR reactions targeting the VP1 and/or the VP3/VP1 junction regions [19, 27]. The HPeV type was determined by sequencing the
amplicons and blasting the sequences in GenBank. Samples with sequence matches of 80%-95% based on <100 base pairs are reported as “possible” types.

Statistical Methods
Comparisons of continuous measures of illness (eg, days of missed childcare) between viruses were made using nonparametric Mann-Whitney tests. Differences in symptom prevalence were compared using the Fisher exact test. Odds ratios and 95% confidence intervals comparing HPeV prevalence between asymptomatic and symptomatic periods were calculated using generalized estimating equations to account for correlation in events measured from the same children.

RESULTS
Patient Characteristics and Samples
One hundred six children were enrolled, including 54 boys (51%), at a mean age of 10 months (range, 1-19 months). Sixty children were enrolled from 7 different childcare rooms at one childcare center, and 47 children were enrolled from 7 different childcare rooms at a second childcare center. Racial background included Caucasian (n = 42), African American (n = 22), Hispanic (n = 9), Asian/Pacific Islander (n = 4), and mixed race (n = 26; race was unreported in 3 children). Children attended childcare an average of 43 hours/week (range 24-60 hours/wk). Children were followed for an average of 170 days (range, 27–238 days). All infants were vaccinated appropriately for their age at the time of enrollment, including with RV vaccine, with the exception of one 6-month-old infant who was up to date for vaccinations only through the age of 4 months. Seventy-eight children who were asymptomatic upon enrollment had stool samples obtained. During follow-up, 48 illnesses with acute diarrhea were captured in 37 children (8 children had 2 acute diarrheal illnesses, and 1 child had 4). Stool samples were available for viral detection in 24 (50%) of these illnesses from 21 children. (Three children had 2 illnesses each. Second illnesses occurred >3 weeks after resolution of the previous diarrheal illness, and no children with repeat illnesses had viruses in common between the first and second illnesses.) Illnesses with missing samples (n = 24) were compared with those with complete samples with regards to demographic characteristics of the child and symptoms, severity, and duration of the illness; no differences were found. The proportion of illnesses with samples that were missing was consistent throughout the study period.

Virus Detection in Stool Samples
At least 1 virus was detected in 61 (78%) of 78 asymptomatic stool samples (Table 1), including AV in 43 (55%), EV in 30 (38%), HPeV in 21 (27%), and NV in 5 (6.4%). At least 1 virus was detected in 17 (71%) stool samples obtained from 24 symptomatic children (Table 1), including AV in 11 (46%), HPeV in 5 (21%), NV in 5 (21%), and EV in 3 (13%). Rotavirus was not detected in any stool samples collected from childcare attendees during the study period, including samples from asymptomatic children and samples obtained during diarrheal illness. More than 1 virus was detected in 28 (36%) of the 78 asymptomatic samples and in 5 (21%) of the 24 symptomatic samples overall. Codetection of other viruses in HPeV-positive samples was more common in samples from asymptomatic children (15 of 21 samples) compared with samples from symptomatic children (1 of 5 samples). Notably, AV was detected in all coinfections identified in both asymptomatic and ill children. Comparison of the symptoms between children with HPeV-only and HPeV-negative acute gastroenteritis demonstrated no significant differences overall (Table 2).

Prevalence and Typing of HPeV
The prevalence of HPeV among asymptomatic children <12 months old was 17 of 53 (32%), whereas the

| Table 1. Viruses Detected in Asymptomatic and Symptomatic Children From Stool Samples |
|----------------------------------|-----------------|-----------------|
| Viruses                          | Asymptomatic Children (N = 78) | Symptomatic Children (N = 24) |
| AV only, No. (%)                 | 15 (19)          | 6 (25)          |
| EV only, No. (%)                 | 11 (14)          | 0 (0)           |
| HPeV only, No. (%)               | 6 (8)            | 4 (17)          |
| NV only, No. (%)                 | 1 (1)            | 2 (8)           |
| RV only, No. (%)                 | 0 (0)            | 0 (0)           |
| AV and EV, No. (%)               | 12 (15)          | 2 (8)           |
| AV and HPeV, No. (%)             | 6 (8)            | 0 (0)           |
| AV and NV, No. (%)               | 0 (0)            | 1 (4)           |
| AV, EV and HPeV, No. (%)         | 6 (8)            | 0 (0)           |
| AV, HPeV and NV, No. (%)         | 3 (4)            | 1 (4)           |
| AV, EV and NV, No. (%)           | 1 (1)            | 1 (4)           |
| Negative                         | 17 (22)          | 7 (29)          |

Abbreviations: AV, adenovirus; EV, enterovirus; HPeV, human parechovirus; NV, norovirus; RV, rotavirus.
prevalence among asymptomatic children 13–24 months old was 4 of 25 (16%; \( P = .18 \); Fisher exact test). The average age of asymptomatic patients demonstrating HPeV in their stool samples was 7.5 months. HPeV was detected in stool samples of 5 of the 78 asymptomatic children (6%).

The HPeV type was determined by sequencing in 12 of 21 HPeV-positive asymptomatic enrollment stool samples and in 3 of 5 HPeV-positive stool samples obtained from children with diarrhea. Four different HPeV types were identified from patients in our study population. HPeV-1 was detected in 6 asymptomatic samples and 1 symptomatic sample, possible HPeV-2 was detected in 5 asymptomatic samples, HPeV-3 was detected in 2 symptomatic samples, and HPeV-6 was detected in 1 asymptomatic sample (Figure 1). Clustering of types was noted within childcare rooms. For example, only HPeV-1 was found in stool samples obtained from children in room F1 and only HPeV-2 was found in samples from children in room G1. One sample with possibly \( >1 \) HPeV type was detected in a child attending childcare in room D1 with 2 other children from whom different HPeV types were found (HPeV-1 and HPeV-6, respectively). In addition, the 2 children with HPeV-3 detected during diarrheal illness had onset of symptoms within 1 week in the same childcare center, although not in the same room.

DISCUSSION

We describe the detection of viral enteric pathogens in asymptomatic healthy young children attending childcare and in the same children during periods of symptomatic gastroenteritis. We utilized molecular techniques to diagnose potential viral enteric pathogens including AV, NV, EV, and the newly described HPeV in our population that was well immunized against RV. We found high rates of asymptomatic shedding in the youngest children attending childcare and high rates of viral coinfection with AV, which was the most common enteric virus detected. Rates of detection of HPeV were higher than those of NV in asymptomatic children and surprisingly similar to those of NV in symptomatic children.

Overall, our prospective study detected at least 1 virus in 78% of stool samples from asymptomatic children and 71% of stool samples from symptomatic children. Notably, a previous emergency department–based study of diarrhea in our region found viruses in only 55% of stool samples and bacteria in only 4% of stool samples from patients aged <3 years [26]. Unfortunately, we were not able to perform cultures for bacterial or other viral gastrointestinal pathogens that may have contributed to the etiology of the 7 gastrointestinal illnesses for which no virus was identified. All 7 of these illnesses included fever (Table 2), suggesting that an alternate infectious etiology was present. Healthcare visits occurred for 5 of these 7 illnesses: 1 child was clinically diagnosed with gastroenteritis, 2 children were diagnosed with sinusitis, and 1 child was diagnosed with otitis.

HPeV was detected in stool samples collected from healthy children attending childcare at study enrollment and in stool samples obtained from subsequent episodes of diarrhea. Because our sample size was limited, we were unable to perform a crossover analysis to compare HPeV prevalence in asymptomatic versus symptomatic periods in individual children.

Table 2. Characteristics of 24 Diarrheal Illnesses With Stool Sample Collection

<table>
<thead>
<tr>
<th>Patient Characteristics and Symptoms</th>
<th>Any Virus Detected</th>
<th>HPeV Alone</th>
<th>HPeV With Another Virus</th>
<th>HPeV Not Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 &gt;1 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of illnesses</td>
<td>12 5 7 4</td>
<td>1 10 12.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, months</td>
<td>11.7 10.2 13.1</td>
<td>9.6 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean duration of diarrhea, days</td>
<td>1.8 3.8 3.7</td>
<td>2.8 3 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever, No. (%)</td>
<td>2 (17) 3 (60) 7 (100)</td>
<td>1 1 10 (53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheezing, No. (%)</td>
<td>4 (33) 0 3 (43)</td>
<td>1 0 6 (32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough, No. (%)</td>
<td>10 (83) 3 (60) 5 (71)</td>
<td>3 0 15 (79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhinorrhea, No. (%)</td>
<td>12 (100) 4 (80) 7 (100)</td>
<td>4 0 19 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting, No. (%)</td>
<td>5 (42) 4 (80) 5 (71)</td>
<td>2 1 11 (58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean time of childcare missed, days</td>
<td>1.9 2 3.4 2</td>
<td>4 1.7 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean time of parental work missed, days</td>
<td>1.9 1.8 1.9 2</td>
<td>4 2.4 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illness requiring healthcare visit, No. (%)</td>
<td>4 (33) 1 (20) 5 (71)</td>
<td>2 0 8 (42)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HPeV, human parechovirus.

*One data point was missing.
Nevertheless, the overall rate of HPeV detection in the stool samples of children who were confirmed by a nurse to be asymptomatic was surprisingly high (27%). It is possible that HPeV infection is more common in young infants <2–3 months of age, given that asymptomatic samples were only collected at enrollment, when study participants were youngest and more likely to be <3 months of age.

The association between HPeV and younger age has been suggested in other studies investigating serious HPeV infections [11, 13], including our recent hospital-based study assessing HPeV infection in hospitalized patients of all ages [12]. Several large retrospective studies have attributed gastrointestinal symptoms to HPeV infection [16–19], but only 1 other study has investigated this prospectively in a cohort of healthy children. Tapia et al [21] prospectively collected stool samples in Norwegian children to determine whether the presence of HPeV correlated with symptoms of infection. No correlation was detected between the presence of HPeV and coughing, fever, diarrhea, or vomiting. These authors concluded that infants frequently acquire HPeV at an early age without it causing disease. Our study also demonstrated an increased prevalence of HPeV positivity in younger infants. Another prospective study evaluated the presence of HPeV among children presenting with enteritis in Germany [15]. In that study, HPeV was detected more often in samples from children presenting for outpatient care than in samples from hospitalized children or those in enteritis outbreak cohorts. HPeV was rarely observed in secondary cases, leading the investigators to speculate that HPeV was probably not primarily responsible for viral enteritis epidemics. After eliminating all participants who were coinfected with AV, astrovirus, RV, NV, and EVs, the investigators determined that only 7 of 60 (12%) children <2 years of age with diarrhea were infected with HPeV alone.

We similarly found that only 17% of children with gastroenteritis symptoms were infected with HPeV alone, and we found that AV was commonly detected with HPeV. One of 5 (20%) children had codeletion of AV in their stool samples, and coinfection with AV was more common in stool samples from asymptomatic HPeV-positive participants, at 71%. Adenovirus has been associated with gastroenteritis symptoms in children [28], and asymptomatic detection could be due to an earlier episode of gastroenteritis. Furthermore, our high rates of AV shedding may not be limited to history of gastroenteritis illness, because extended shedding of AV is possible following respiratory illnesses as well.
Interestingly, HPeV types appeared to cluster by childcare room, suggesting that this virus is easily spread in a childcare setting. Tapia et al [21] evaluated the duration of shedding and estimated the median duration of infection to be 51 days. Such prolonged viral shedding could contribute to dissemination in a childcare setting. We found a diversity of HPeV types, including some that have not previously been reported from surveillance laboratories in the United States [9]. Similarly, diverse HPeV types were reported in a prospective German study [15]. As in our study, HPeV-1 was the most frequent type detected.

Remarkably, no RV was detected during our 9-month study period, which included the historical peak period of RV detection in our area. Although molecular methods of viral detection have expanded greatly since the late 1980s and 1990s, when early studies of RV epidemiology were conducted, the detection of RV in stool samples by enzyme-linked immunosorbent assay methods has been used in many previous studies as well as our study, and it is used in ongoing surveillance studies by the Centers for Disease Control and Prevention [3]. The RV test was performed in a clinical laboratory, and we were limited to the clinical assay in use. Although this assay may be less sensitive than other techniques, our findings reflect clinical determinations that would have been made had the children presented to their medical provider for care. Differences in laboratory methods or surveillance techniques used to detect RV are not likely to be related to this substantial decrease in RV disease. The differences in RV disease in our patient population appear to be directly related to the overall decrease in RV disease now documented in children throughout the United States [29]. The decrease of symptomatic RV infection from rates approaching 100% or more, if multiple infectious episodes are counted, to no cases in our study was unexpected and an encouraging marker for vaccine effectiveness in a real-world setting.

The presence of HPeV and other viruses in asymptomatic children highlights the need to consider the possible contribution of other pathogens when attributing a causal relationship between a stool pathogen and gastroenteritis symptoms. We did not collect past illness history at enrollment. Thus, isolates found in the stool samples of asymptomatic children at enrollment could be present due to prolonged shedding after a clinical illness. The collection of samples from asymptomatic children only at enrollment also might introduce bias if the child entered at a time when the virus was not circulating in the community. However, enrollment into the study was conducted from October through June, during which all of the enteric viruses surveyed were detected.

Our study was limited by its small sample size and by the 50% response rate of participants in contributing stool samples during gastrointestinal illness. However, these nonresponders were similar in patient and illness characteristics to those who contributed samples, and we do not expect that these missing samples biased our ability to detect RV, HPeV, or the other enteric viruses in this study. The lack of RV detected in this study emphasizes the need for a greater focus on the epidemiology of non-RV enteric viruses that are frequently detected in the stool samples of infants and young children attending childcare. The role of specific viruses, including NV, as individual causes of disease is also not well understood, and multiple viruses, including HPeV, were frequently found in stool samples obtained from healthy, asymptomatic childcare attendees. We found that HPeV is frequently detected in the stools of infants and young children attending childcare but may not necessarily be a cause of diarrheal illness. We found HPeV-3 only in samples from participants with diarrhea, suggesting the possibility that this type could potentially be a diarrheal pathogen. Further studies investigating how best to distinguish asymptomatic shedding of HPeV-3 and other enteric viruses from individual etiologies of diarrheal illness are needed.

Acknowledgments

We acknowledge Susan Chambers and Melinda Behrens, who assisted in the study overall as well as with data collection for this study.

Disclaimer. The views expressed are those of the authors and do not reflect the official policy of the Department of the Army, the Department of Defense, or the US Government. The investigators have adhered to the policies for protection of human subjects as prescribed in 45 CFR 46.

Financial support. This work was supported by an investigator-initiated grant from MedImmune.

Potential conflict of interest. J. E. has received research support from Novartis, ADMA, and Adams. E. M. has received research support from Vioguard.
All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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