Human Caliciviruses as a Cause of Severe Gastroenteritis in Peruvian Children

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To define the role of human caliciviruses (HuCVs) in severe childhood gastroenteritis, fecal and paired serum samples from 233 Peruvian children hospitalized with gastroenteritis (case patients) and fecal samples from 248 control subjects were evaluated. Overall, 128 case patients (55%) demonstrated HuCV infection by either fecal (n = 81 [35%]) or serological (n = 96 [41%]) testing. HuCVs were more prevalent in fecal samples from case patients than those from control subjects (35% vs. 13%; P < .001). HuCV infection was more prevalent among case patients without another pathogen than in those who had a coinfected pathogen (77% [40/52] vs. 49% [88/181]; P < .001). HuCVs appear to be an important cause of gastroenteritis in Peruvian children.

Human caliciviruses (HuCVs) are genetically and antigenically diverse single-stranded RNA viruses that belong to 2 genera in the family Caliciviridae—the noroviruses and sapoviruses [1]. HuCVs, particularly noroviruses, have been firmly established as a leading cause of outbreaks of gastroenteritis worldwide [2]. Their role as causative agents of sporadic gastroenteritis in children is less clear, primarily because of the lack of simple and sensitive detection assays. The results of early studies indicated that the acquisition of antibodies to HuCVs occurs early in life, particularly among children in developing countries [3]. However, HuCVs have been infrequently identified by electron microscopic and EIA examination of fecal samples from patients with gastroenteritis [4]; therefore, it remained unclear whether they cause illness or only asymptomatic infections. The development and application of reverse-transcription polymerase chain reaction (RT-PCR) assays dramatically changed this situation—Pang et al. [5] found HuCVs in 29% of all episodes and 11% of severe episodes of gastroenteritis among Finnish children. These observations have been supported by the results of other recent studies in which HuCVs were detected in 8%–14% of children with moderate to severe gastroenteritis that required a clinic visit or hospitalization [6–9].

Previous experience has suggested that the true prevalence of HuCV infection may be underestimated unless RT-PCR and serological assays are used together. HuCV strains have many genetic clusters, so current RT-PCR assays may not efficiently detect all HuCV strains [10], and inhibitors of RT-PCR can also decrease the detection of enteric viruses in fecal samples. Serological assays alone may also miss some HuCV infections, because of the great antigenic diversity of HuCVs and the lack of broad cross-reactivity among strains [11]. To further clarify the role of HuCVs in the etiology of severe childhood gastroenteritis, we conducted a rigorous evaluation of children seeking treatment for gastroenteritis at a hospital in Lima, Peru. The present study is unique in that we used a variety of molecular assays to detect HuCVs in fecal samples and correlated the results with serological evidence of recent infection. Our data provide new insights into the importance of HuCV infections as a cause of severe gastroenteritis among children in a setting with great disease burden.

Patients, materials, and methods. The present study was conducted as an extension of a survey of the etiologic agents of diarrhea among children who were hospitalized at the Instituto Nacional de Salud del Nino, a hospital that serves children from a poor section of urban Lima, Peru. Details of the study design have been published elsewhere [12]. In brief, during a 2-year period from 15 February 1995 to 14 February 1997, all children <5 years old seeking medical attention for watery diarrhea (≥3 liquid or semisolid stools during a 24-h period) at the hospital were evaluated for inclusion in the study. A pediatrician or nurse assessed the child’s hydration status, and we included as case patients those children with diarrhea who had at least 2 of the following 3 features of dehydration: poor skin turgor, increased thirst, and altered sensorium. Informed consent was obtained from the parents or guardians of the patients for participation in the study, and human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of clinical research.
At or shortly after admission, fecal and acute-phase serum samples were obtained from the case patients, and, 26–30 days later, a convalescent-phase serum sample was obtained during a follow-up visit. Fecal samples from these patients were evaluated for rotavirus, astrovirus, adenovirus, enterotoxigenic Escherichia coli (ETEC), Campylobacter jejuni, Shigella species, Vibrio cholerae 01, Cryptosporidium parvum, Cyclospora cayetanensis, and Giardia species by use of methods described elsewhere [12].

To examine the association between HuCV infection and gastroenteritis, we compared the rates of detection of HuCVs in fecal samples from case patients with those in samples from children without diarrhea (control subjects). Control samples were obtained from children enrolled in a concurrent cohort study in an urban slum in Lima [13]. In that study, which began in February 1995 and ended in December 1998, 238 children were followed from birth for a period of 3 years. Diarrhea surveillance was conducted 5 days/week, and fecal samples were obtained from each child at 1–2-week intervals. Samples from control subjects were frequency-matched to those from case patients with regard to age and month and year of collection.

IgG antibody titers against HuCVs were measured by EIA in acute- and convalescent-phase serum samples by use of methods described and validated elsewhere [14]. Seven recombinant norovirus capsid proteins, expressed by a baculovirus vector and representing 5 genetic clusters, were used as antigens: GI/1 (Norwalk virus [rNV]), GI/1 (Hawaii virus), GI/3 (Mexico virus), GI/3 (Toronto virus), GI/4 (Lordsdale virus), GI/4 (Burberry Landing virus [rBLV]), and GI/6 (Florida virus [rFV]). In brief, micro-EIA plates (Dynatech) were coated overnight with 75 μL of 1.0 μg/mL of purified norovirus viral capsid protein or no-antigen control (diluent alone) in 0.06 mol carbonate buffer/L (pH 9.6) at 4°C. The plates were blocked with 5% nonfat milk in 0.01 mol PBS/L. Peroxidase-conjugated goat anti–human IgG was used as the detector antibody (Kirkegaard & Perry Laboratories). All test serum samples and conjugates were diluted 1:1000 in PBS–1% nonfat milk. Total IgG units were calculated by use of a standard curve generated from serial dilutions of a reference serum included on each plate. Seroconversion was defined as a ≥4-fold increase in IgG units between acute- and convalescent-phase serum samples.

Fecal samples were tested for HuCVs by RT-PCR by use of methods described elsewhere [8]. In brief, RNA was extracted from 100 μL of a 10% stool suspension, concentrated by ethanol precipitation, and resuspended in a final volume of 10 μL of water. Region B primers (Mon431, Mon432, Mon433, and Mon434) generated a 213-bp product of noroviruses, whereas region 5 primers (p289 and p290) generated 319- and 331-bp products of noroviruses and sapoviruses, respectively. Both RT-PCR assays were performed in 50 μL of reaction mixture. For RT-PCR with region B primer sets, the reaction mixture contained 25 μL of MasterAmp 2X PCR Premix G (Epicentre Technologies); 1 mmol dithiothreitol/L; 0.2% Triton X-100; 2 mmol 2-mercaptoethanol/L; 0.6 μmol Mon431, Mon432, Mon433, and Mon434/L; 20 U of RNAse Inhibitor (Roche Molecular Biochemicals); 2.7 U of avian myeloblastosis virus super reverse transcriptase (Molecular Genetic Resources); and 1.25 U of AmpliTaq DNA polymerase (PerkinElmer Biosystems). For RT-PCR with primer pair 289/290, the reaction mixture was similar, except that 25 μL of the PCR and 0.6 mol p289 and p290/L were used. The thermalcycler program used in both RT-PCR assays was as follows: 1 cycle of reverse transcription for 10 min at 42°C, 1 cycle of denaturation for 3 min at 94°C, 40 amplification cycles of denaturation for 30 s at 94°C, annealing for 1 min 30 s at 50°C, extension for 30 s at 60°C, and a final cycle of incubation for 7 min at 72°C. The amplification products were analyzed by electrophoresis in 3% agarose gel and visualized under UV illumination after staining with 0.5 μg/mL ethidium bromide for 30 min.

Each amplified RT-PCR product with region B primers was characterized by liquid hybridization assay (LHA) by use of 12 different genogroup- and genetic cluster–specific digoxigenin-labeled oligonucleotide probes [8]. RT-PCR products were hybridized with each probe simultaneously in a separate reaction. In brief, 2 μL of PCR product was mixed with 8 μL of hybridization solution; the final concentration was 1× standard saline citrate (SSC; 0.15 mol NaCl/L and 0.015 mol sodium citrate/L [pH 7.0]), 0.05% bromophenol blue, 0.05% xylene cyanol, 6% glycerol, and 0.01 μmol digoxigenin-labeled probe/L. These reaction mixtures were incubated for 2 min at 94°C, hybridized for 20 min at either 54°C (for GI) or 58°C (for GII), and then placed immediately on ice water. The products in the 3% agarose gel were then transferred overnight to a positively charged nylon membrane (Roche Molecular Biochemicals) with 20× SSC buffer and immobilized by UV cross-linking. For the reaction of the hybridized digoxigenin-labeled probe with the anti–digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche), the membrane was pretreated with blocking solution (100 mmol Tris-HCl/L, 150 mmol NaCl/L, and 2% blocking reagent [pH 7.5]) for 30 min and incubated in the block solution that contained the conjugate diluted 10,000-fold (75 μL/mL) for 1 h. After 3 washes with wash buffer 1 (100 mmol Tris-HCl/L [pH 7.5] and 150 mmol NaCl/L) and 1 wash with wash buffer 2 (100 mmol Tris-HCl/L [pH 9.5], 100 mmol NaCl/L, and 50 mmol MgCl2/L) for 5 min each, the ready-to-use chemiluminescent substrate CSPD (Roche Molecular Biochemicals) was applied to the membrane, and it was exposed to x-ray film after a 10-min incubation. All incubation and wash steps were done at room temperature with agitation.

To confirm the clustering of strains typed by LHA and to characterize the RT-PCR products with primer pair 289/290, all strongly positive RT-PCR products were excised from the gel, extracted, and purified by use of the Qiaex gel-extraction...
Table 1. Seroconversions to human caliciviruses (HuCVs) among 233 gastroenteritis case-patients, as detected by EIA testing of acute- and convalescent-phase serum samples, using recombinant norovirus antigens, Lima, Peru, 1995–1997.

<table>
<thead>
<tr>
<th>Antigen, genogroup/cluster</th>
<th>Norwalk GI/1</th>
<th>Hawaii GI/1</th>
<th>Mexico GI/3</th>
<th>Toronto GI/2</th>
<th>Burwash GI/4</th>
<th>Lordsdale GI/4</th>
<th>Florida GI/6</th>
<th>Any</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norwalk</td>
<td>8 (3)</td>
<td>4 (2)</td>
<td>4 (2)</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>2 (1)</td>
<td>...</td>
</tr>
<tr>
<td>Hawaii</td>
<td>...</td>
<td>44 (19)</td>
<td>28 (12)</td>
<td>23 (10)</td>
<td>29 (12)</td>
<td>28 (12)</td>
<td>16 (7)</td>
<td>...</td>
</tr>
<tr>
<td>Mexico</td>
<td>...</td>
<td>...</td>
<td>38 (16)</td>
<td>21 (9)</td>
<td>23 (10)</td>
<td>24 (10)</td>
<td>14 (6)</td>
<td>...</td>
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<tr>
<td>Toronto</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>36 (16)</td>
<td>51 (22)</td>
<td>43 (18)</td>
<td>14 (6)</td>
<td>...</td>
</tr>
<tr>
<td>Burwash</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>55 (24)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Lordsdale</td>
<td>...</td>
<td>...</td>
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<td>Florida</td>
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<td>...</td>
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<td>...</td>
<td>...</td>
<td>96 (41)</td>
</tr>
</tbody>
</table>

Table 2. Human calicivirus reverse-transcription polymerase chain reaction (RT-PCR) and liquid hybridization assay (LHA) testing of fecal samples from 233 case patients with gastroenteritis and 248 control subjects, Lima, Peru, 1995–1997.

<table>
<thead>
<tr>
<th>Test, result</th>
<th>Case patients (n = 233)</th>
<th>Control subjects (n = 248)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Positive</td>
<td>30 (13)</td>
<td>12 (5)</td>
</tr>
<tr>
<td>LHA-GI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Positive</td>
<td>13 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>RT-PCR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Positive</td>
<td>17 (7)</td>
<td>16 (6)</td>
</tr>
<tr>
<td>Any RT-PCR or LHA</td>
<td>Positive</td>
<td>81 (35)</td>
<td>32 (13)</td>
</tr>
</tbody>
</table>

-a Region B primers sets, including Mon431, Mon432, Mon433, and Mon434, for the detection of noroviruses.
-b LHA with GI-specific probes for GI/1, GI/2, and GI/3 clusters.
-c LHA with GII-specific probes for GII/1, GII/4, GII/2, GII/3, GII/5, and GII/6 clusters.
-d Primer pair 289/290 was for the detection of noroviruses and sapoviruses.

Overall, we detected evidence of HuCV in 128 (55%) of 233 case patients by use of either assay—a percentage that was comparable to the prevalence of rotavirus in fecal testing alone. The prevalence of HuCV infection was greater among the 52 case patients without another pathogen than among those with another pathogen (77% [40/52] vs. 49% [88/181]; P < .001). Of the 233 case patients, 40 (17%) were infected with HuCVs alone, including 20 (9%) who had both serological and RT-PCR evidence of infection, 13 (6%) who only had serological evidence of infection, and 7 (3%) who had only RT-PCR evidence of infection.

The examination of seasonal patterns for the most frequently
detected pathogens among the case patients demonstrated that HuCV and ETEC infections were more common during the summer months (January–March), whereas rotavirus infections were observed throughout the year, with a slight decline during the spring (November–December) months.

**Discussion.** To our knowledge, this is the first study to have combined both serological and RT-PCR assays to obtain a full measurement of the prevalence of HuCV infections among children hospitalized with gastroenteritis. Our data indicate that HuCV infections are common in Peruvian children, and 2 key findings point to their etiological role in severe gastroenteritis. First, the prevalence of HuCVs was significantly greater in fecal samples from case patients with severe diarrhea than in those from control subjects. Second, HuCV infection was significantly more common among case patients who did not have a coinfecting pathogen than among those with another pathogen. Because of the high rate of mixed infections in this setting, it is difficult to estimate the proportion of gastroenteritis episodes that was attributable to HuCVs, but it might be reasonable to estimate that HuCVs likely caused gastroenteritis in the 17% of all case patients in whom no other coinfecting pathogen was identified.

The prevalence of HuCV infections in the present study is slightly greater than that reported in previous studies [5–10], which is likely a function of both the poor sanitation and hygiene in the community served by the study hospital and the combined use of serological and fecal assays to provide a full estimate of infection. Although the RT-PCR assays might have detected the fecal shedding of HuCVs that was not related to clinical illness in patients, seroconversion provides evidence of a temporal association of infection with disease. The observed high rates of seroconversion are comparable to rates of rotavirus infection seen among Mexican children [15], where a fourth and fifth rotavirus infection was observed by 2 years of age in 20% and 13%, respectively, of a cohort of 200 prospectively monitored infants. Similar to the rotavirus study in Mexico, the identification of HuCV infection on the basis of serological response and the fecal excretion of virus was complementary.

The observed discordance between the serological and fecal assay results could be explained by several factors. Among children with seroconversion, the failure to detect HuCV in fecal samples might be attributable to an improperly obtained or stored fecal sample, the inability of the RT-PCR primers to detect the infecting HuCV strain, or the presence of RT-PCR inhibitors in the fecal sample. Among children with HuCVs detected in fecal samples, the failure to document seroconversion might be attributable to a high preexisting antibody titer that might preclude a 4-fold increase in antibody levels, the lack of an appropriate homologous recombinant antigen for the EIA, colonization of the gut with HuCVs in the absence of true infection, or the relative inability of the serum immune response to reflect local intestinal immune responses.

Some limitations of the study should be considered in the interpretation of the findings. First, because the original hospital-based study did not include a comparison group of children without diarrhea, we selected our control subjects from a concurrent prospective cohort study. We believe that this choice of control subjects, although not ideal, is scientifically valid because they were from a similar population as the case patients and were matched to the case patients for age and time of enrollment. Second, we evaluated HuCV infection among the case patients by use of both serological and fecal assays, but we were not able to conduct serological testing for control subjects to compare detection rates in the 2 groups. Third, we did not conduct serological testing for other pathogens among the case patients. As a result, we may have underestimated the number of infections caused by pathogens other than HuCVs, although, for most of these organisms, including rotavirus, fecal assays have high sensitivity and specificity, compared with serological assays.

In conclusion, our data indicate that HuCVs may be important etiological agents of severe gastroenteritis in young children. This high prevalence of viral agents and low rates of infection with *Shigella* species and *V. cholerae* support the recommendation that antibiotics are rarely required for the treatment of gastroenteritis in children. Currently available assays for HuCVs are cumbersome for routine use, but simpler EIAs for the detection of HuCVs in fecal samples may soon be available. The broader application of these assays will help in defining the disease burden of HuCVs in different settings and in evaluating the need for specific interventions.

**References**