Cosavirus Infection in Persons with and without Gastroenteritis, Brazil

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To determine possible cosavirus association with clinical disease, we used real-time reverse transcription PCR to test children and HIV-positive adults in Brazil with and without gastroenteritis. Thirteen (3.6%) of 359 children with gastroenteritis tested positive, as did 69 (33.8%) of 204 controls. Low prevalence, frequent viral co-infections, and low fecal cosavirus RNA concentrations argue against human pathogenicity.

The family Picornaviridae comprises 12 genera and includes several leading pathogens affecting human and animal health, e.g., foot-and-mouth-disease virus and polioviruses. With the advent of metagenomics, 3 novel human picornaviruses have been described since 2008: klsavivirus, salivirus, and cosavirus (1–3).

Besides being detected in raw sewage (4), cosaviruses have been detected in human fecal specimens and were tentatively associated with gastroenteritis in 2 patients from Australia and Scotland (3,5). Cosavirus pathogenicity in humans has remained unknown, however, because detection rates in patients and healthy controls were similar in the only available cohort studies of patients with acute flaccid paralysis in Southeast Asia (3) and with gastroenteritis in China (6). We investigated cosavirus prevalence in 464 children and HIV-infected adults with gastroenteritis and 253 controls without gastroenteritis in Brazil.

The Study

Fecal specimens from 6 clinical cohorts in Salvador, northeastern Brazil, were analyzed (Table 1). Adult cohorts comprised HIV-infected patients with gastroenteritis (105 persons) and without (49 persons) gastroenteritis. Child cohorts included 359 children with gastroenteritis and 204 healthy children from child-care centers. Nasal swab specimens were obtained from controls attending child-care centers and from children with gastroenteritis and concomitant respiratory symptoms. All specimens were stored at −30°C to −80°C until further processing.

Viral RNA was purified from ≥200 mg fecal specimen and 140 μL nasal swab specimen suspended in phosphate-buffered saline by using the Viral RNA Mini kit (QIAGEN, São Paulo, Brazil) as described (7). A nested reverse transcription PCR (3) detected cosavirus. After nucleotide sequencing of all PCR-positive specimens, we developed a specific real-time RT-PCR for quantifying viruses from Brazil (Table 2). Assay optimization and quantification relied on photometrically quantified cRNA in vitro transcripts, as described (8). After assay optimization, sensitivity was 6.8 copies per reaction.

In the adult cohorts, 1 of 105 HIV-infected persons with gastroenteritis had positive results for cosavirus. In the control group of 49 HIV-infected adults without gastroenteritis, none had positive cosavirus results (χ2 0.5; p = 0.49).

Considerably higher prevalence was detected among child cohorts. Of children with gastroenteritis, 13 (3.6%) of 359 patients were cosavirus positive. The proportion of cosavirus-positive controls without gastroenteritis, sampled in 2008, was significantly higher: 65 (49.2%) of 132 controls were cosavirus positive (χ2 149.1; p<0.0001). On resampling in 2011, 4 (6.5%) of 62 controls were cosavirus positive. Although the difference was not statistically significant (χ2 1.1; p = 0.3), this result was almost double that for patients. In another child-care center, none of 10 healthy children were cosavirus positive.

The higher prevalence detected in controls in 2008 could indicate a seasonal infection pattern because specimens were collected in a single weekend in a child-care center. The lower cosavirus-positive results in the same child-care center 3 years after the initial sampling support this idea. However, in sick children, cosavirus-positive specimens were obtained at similarly low rates throughout the year (Figure 1), which might argue against seasonal variation as a generic property of cosavirus infection.

To evaluate whether cosavirus causes disease in ill children, we analyzed co-infections with common viral pathogens causing diarrhea (astrovirus, norovirus, rotavirus, adenovirus) (Figure 2, panel A). In 10 (76.9%) of

1These authors contributed equally to this article.
13 cosavirus-positive patients, ≥1 of these pathogens could be detected.

Acute infection with an enteric virus is usually associated with high viral RNA shedding. Cosavirus concentrations were low in outpatient and control children associated with high viral RNA shedding. Cosavirus could be detected.

13 cosavirus-positive patients, >1 of these pathogens could be detected.

No controls sampled in 2008. None of the nasal specimens and 65 nasal swab specimens from cosavirus-positive controls sampled in 2008. None of the nasal specimens from controls with positive fecal specimens and only 1 nasal specimen from a patient unrelated to the cosavirus-positive fecal cases.

Figure 2, panel B).

Not differ significantly (analysis of variance $F2.0; p = 0.14$; Figure 2, panel B).

Low cosavirus concentrations in the enteric tract could result from swallowing viruses originating in the respiratory tract. Therefore, we tested for cosavirus in 96 nasal swab specimens from gastroenteritis patients, including 3 nasal swab specimens from persons with cosavirus-positive fecal specimens and 65 nasal swab specimens from cosavirus-positive controls sampled in 2008. None of the nasal specimens from controls with positive fecal specimens and only 1 nasal specimen from a patient unrelated to the 3 aforementioned persons was weakly positive (10$^3$ RNA copies/mL), which refutes this hypothesis.

To analyze whether a preceding point-source infection caused high cosavirus prevalence in the controls without gastroenteritis sampled in 2008, we determined the genomic sequence of the 5′ untranslated region PCR amplicons and phylogenetically analyzed the sequence (GenBank accession no. JN228118–JN228188). Cosaviruses from these controls were distributed across the phylogenetic tree (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1415-Techapp.pdf). Maximum nucleotide distance within these cosaviruses was up to 22.5% in the analyzed 398-nt fragment, making a recent point-source infection unlikely.

Conclusions

Human cosavirus infections were reported previously from a limited number of persons and geographic areas.

| Table 1. Clinical cohorts tested for cosavirus, Salvador, Brazil*

<table>
<thead>
<tr>
<th>Cohort no.</th>
<th>Cohort description†</th>
<th>Sampling site‡</th>
<th>Sampling time</th>
<th>Participant age, mo, mean (SD)</th>
<th>No. participants</th>
<th>No. (%) RT-PCR positive§</th>
<th>Virus concentration, log$_{10}$ RNA copies/g feces, mean (SD)¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HIV-infected adults with gastroenteritis</td>
<td>Infectious Diseases HIV</td>
<td>2007 Mar–2010 Mar</td>
<td>487.6 (114.4)</td>
<td>105</td>
<td>1 (1.0)</td>
<td>4.43</td>
</tr>
<tr>
<td>2</td>
<td>HIV-infected adults without gastroenteritis</td>
<td>Outpatient Department of Pediatrics</td>
<td>2007 Mar–2010 Mar</td>
<td>533.8 (115.9)</td>
<td>49</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Children with gastroenteritis</td>
<td>Department of Pediatrics</td>
<td>2006 Feb–2007 Sep</td>
<td>19.0 (15.6)</td>
<td>359</td>
<td>13 (3.6)</td>
<td>3.40 (0.93)</td>
</tr>
<tr>
<td>4</td>
<td>Control children without gastroenteritis</td>
<td>Community child-care center**</td>
<td>2008 Dec</td>
<td>29.6 (13.1)</td>
<td>132</td>
<td>65 (49.2)</td>
<td>2.97 (0.97)</td>
</tr>
<tr>
<td>5</td>
<td>Control children without gastroenteritis</td>
<td>Community child-care center**</td>
<td>2011 Nov–2011 Dec</td>
<td>14.3 (5.5)</td>
<td>62</td>
<td>4 (6.5)</td>
<td>3.41 (0.49)</td>
</tr>
<tr>
<td>6</td>
<td>Control children without gastroenteritis</td>
<td>University child-care center**</td>
<td>2011 Oct–2011 Nov</td>
<td>18.6 (4.2)</td>
<td>10</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>717</td>
<td>83 (11.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RT-PCR, reverse transcription PCR; §Samples only considered if positive in nested RT-PCR as in (3) and in strain-specific real-time RT-PCR.
†Gastroenteritis was defined as acute diarrhea with ≥3 watery stools in the previous 24 h and within 13 d before hospital admission.
‡All hospital units were located within the Hospital Professor Edgard Santos, Federal University of Bahia. All sites were located in Salvador, Bahia, in northeastern Brazil.
¶Measured by strain-specific RT-PCR.
**Written consent was obtained from adult family members.

### Table 2. Oligonucleotides used for detection and quantification of cosaviruses*

<table>
<thead>
<tr>
<th>Oligonucleotide identity</th>
<th>Sequence, 5′ → 3′</th>
<th>Genomic target region, RT-PCR type</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKV-N5U-F1</td>
<td>CGTCCTTTTACGCGTTTGA (+)</td>
<td>5′-UTR, nested RT-PCR 1st round</td>
<td>Cosavirus detection†</td>
<td>(3)</td>
</tr>
<tr>
<td>DKV-N5U-F2</td>
<td>GTACCTTTGAGGACATTTTG (−)</td>
<td>5′-UTR, nested RT-PCR 2nd round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKV-N5U-F2</td>
<td>ACGGTTTTTGACTCCACAC (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKV-N5U-R3</td>
<td>GTCCTTTTGGACAGGGCTT (−)</td>
<td>5′-UTR, real time</td>
<td>Brazilian cosavirus quantification†</td>
<td></td>
</tr>
<tr>
<td>HCosv-rtF351</td>
<td>TTAGGGATGCTGRTGTTGTG (−)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCosv-rtF783</td>
<td>FAM-AGCCTTACAGGCRACCCCTGC-3′ (+, Probe)</td>
<td>RT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCosv-rtR827-1</td>
<td>CCAYGTGATGGGCTTTC-5′ (−)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*RT-PCR, reverse transcription PCR; UTR, untranslated region; FAM, fluorescein; R, G/A; DDQ1, deep dark quencher 1; Y, C/T.
†RT-PCR reactions were carried out using the QIAGEN One-step RT-PCR kit as described by the manufacturer (QIAGEN, São Paulo, Brazil). 300 nmol/L of each primer, 200 nmol/L of the probe (real time RT-PCR assay), 1 μg bovine serum albumin, and 5 μL RNA extract. Second-round reactions used the Platinum Taq DNA Polymerase Kit as described by the manufacturer (Invitrogen, São Paulo, Brazil) with 2.5 mol/L MgCl$_2$ and 1 μL of first-round PCR product. Real time RT-PCR amplification involved 55°C for 15 min, 95°C for 15 min, and 45 cycles of 95°C for 15 s and 58°C for 30 s (fluorescence measured). Nested RT-PCR involved 30 min at 50°C; 15 min at 95°C; 10 cycles of 20 s at 94°C; 30 s starting at 60°C with a decrease of 1°C per cycle, and 50 s at 72°C; and 40 cycles of 20 s at 95°C, 30 s at 94°C, and 50 s at 72°C; and a final elongation step of 5 min at 72°C. Second-round reactions used 3 min at 94°C and thermal cycling as for the first round.

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In Brazil, the 3.6% detection rate in children with gastroenteritis was comparable to the 1.8% rate in a cohort study of gastroenteritis patients in China (6). Although the 6.5% detection rate in 1 control cohort in Brazil was compatible with the 1.7% rate in 60 healthy controls in China, the combined 33.8% prevalence detected in controls from 3 different samplings in Brazil was much higher. Nonetheless, the prevalence was comparable to the 43.9% detected in 41 healthy Southeast Asian children in the only other cohort study (3). Detecting cosavirus in 1 of 154 adults in Brazil was compatible with finding a single cosavirus-positive patient among 1,000 adults with gastroenteritis in Scotland, confirming that cosaviruses are rare and probably neither pathogenic nor commensal in adults (3).

The higher prevalence of cosavirus found in controls than in patients, the frequent co-infections with established pathogens, and the unusually low RNA virus concentrations give evidence against cosavirus involvement in human gastroenteritis. Viruses that replicate in the human gut generally reach concentrations 1,000- to 100,000-fold higher than those of cosavirus. This finding is exemplified by genetically related picornaviruses (Aichi viruses, parechoviruses, and cardioviruses) and established enteric pathogens (e.g., noroviruses and rotaviruses) (8–12). Notably, the aforementioned study on cardioviruses included the same specimens from Brazil, which indicates that poor sample quality was not a factor.

These low concentrations would be compatible with absence of replication in the enteric tract and passive virus ingestion, e.g., from nutritional sources, drinking water, or the respiratory tract. However, nutritional patterns of the tropical countries in which cosavirus have been detected certainly differ. Furthermore, in Brazil, adults are unlikely to have a completely different diet from infants and children. Moreover, the unprecedented detection of cosavirus in a respiratory tract specimen makes ingestion of viruses from nutritional sources alone unlikely, although a link to fluid droplets from drinking water in the respiratory tract is hypothetically possible.

Another explanation for low cosavirus RNA levels in fecal samples is that a cosavirus infection occurred early in the person’s life and produced partial mucosal immunity and limited subsequent cosavirus replication in the gut. This is exemplified for viruses transmitted by the fecal–oral

![Detection pattern of cosavirus in children with gastroenteritis throughout different seasons during 2006–2007, Brazil.](image1)

![Co-infections and fecal cosavirus (CosV) RNA concentrations. A) Co-infections with established viral causes of diarrhea in children with gastroenteritis who were positive for CosV. Viral RNA and DNA were detected by real-time PCR (methods available upon request) in the same eluates used for CosV detection. B) Boxplot generated with SPSS V19 (SPSS, Munich, Germany) of log_{10} CosV RNA concentrations per gram of feces in children with gastroenteritis and healthy control children from a child-care center in 2008 and 2011. Boxes show the medians and interquartile ranges (box length). The whiskers represent an extension of the 25th or 75th percentiles by 1.5× the interquartile range. Datum points beyond the whisker range are considered as outliers and marked as circles. GG/I/II, genogroups I and II.](image2)
route by up to 100-fold higher fecal shedding of vaccine rotavirus and poliovirus among seronegative persons than among seropositive or previously vaccinated persons (13,14). However, this explanation would be incompatible with the high prevalence of cosavirus in many control children, who were generally older than patients.

Prolonged low concentrations of picornavirus shedding has been demonstrated, e.g., by detectable hepatitis A virus RNA up to 3 months after acute infection (15). Nonetheless, this circumstance is unlikely to explain the low cosavirus concentrations, given the overall high number of persons with positive results.

Although our study extends the known geographic occurrence of cosavirus, whether it is a human pathogen at all remains to be determined. Future studies would be enhanced by serologic analyses and investigations focusing on nutrition and drinking water in tropical countries.

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References


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Technical Appendix

Technical Appendix Figure. Cosavirus (CosV) partial 5′ untranslated region phylogeny. The neighbor-joining phylogeny was generated by using MEGA5 (www.megasoftware.net), including a complete deletion option and a nucleotide percentage distance substitution model drawn to scale. Values at node points indicate support from 1,000 bootstrap reiterations (only values >70 are shown). The final dataset comprised 389 t corresponding to positions 512–909 in CosV A1 (GenBank accession no. FJ438902). All available CosV sequences covering the complete genomic region obtained in this study were included in the analysis and are given with strain name (when available), GenBank accession number and geographic origin. CosV 5′ untranslated region types (3) are indicated to the right of taxon names. Viruses from this study are color coded by clinical cohort: red, children with gastroenteritis; green, HIV-infected adults with gastroenteritis; blue, healthy control children from child-care centers. Scale bar indicates nucleotide substitutions per site.

Virology