Diarrhea Etiology in a Pediatric Emergency Department: A Case Control Study

Donna M. Denno,1,2 Nurmohammad Shaikh,3 Jenny R. Stapp,5 Xuan Qin,6,4 Carolyn M. Hutter,7 Valerie Hoffman,5 Jody C. Mooney,5 Kelly M. Wood,5 Harold J. Stevens,3 Robert Jones,8 Phillip I. Tarr,3,4,a and Eileen J. Klein1,5,a

1Department of Pediatrics, 2Global Health, University of Washington, Seattle; 3Division of Gastroenterology and Nutrition, Department of Pediatrics, and 4Department of Molecular Microbiology, Washington University School of Medicine, St Louis, Missouri; 5Seattle Children’s Hospital, 6Department of Laboratory Medicine, University of Washington, 7Department of Public Health Sciences, Division of Public Health Sciences, Fred Hutchinson Cancer Center, and 8Craic Computing LLC, Seattle, Washington

Background. The etiology of childhood diarrhea is frequently unknown.

Methods. We sought Aeromonas, Campylobacter, Escherichia coli O157:H7, Plesiomonas shigelloides, Salmonella, Shigella, Vibrio, and Yersinia (by culture), adenoviruses, astroviruses, noroviruses, rotavirus, and Shiga toxin-producing E. coli (STEC; by enzyme immunoassay), Clostridium difficile (by cytotoxicity), parasites (by microscopy), and enteroaggregative E. coli (EAEC; by polymerase chain reaction [PCR] analysis) in the stools of 254 children with diarrhea presenting to a pediatric emergency facility. Age- and geographic-matched community controls without diarrhea (n = 452) were similarly studied, except bacterial cultures of the stool were limited only to cases.

Results. Twenty-nine (11.4%) case stools contained 13 Salmonella, 10 STEC (6 O157:H7 and 4 non-O157:H7 serotypes), 5 Campylobacter, and 2 Shigella. PCR-defined EAEC were present more often in case (3.2%) specimens than in control (0.9%) specimens (adjusted odds ratio [OR], 3.9; 95% confidence interval [CI], 1.1–13.7), and their adherence phenotypes were variable. Rotavirus, astrovirus, and adenovirus were more common among controls, but both groups contained noroviruses and C. difficile at similar rates. PCR evidence of hypervirulent C. difficile was found in case and control stools; parasites were much more common in control specimens.

Conclusions. EAEC are associated with childhood diarrhea in Seattle, but the optimal way to identify these agents warrants determination. Children without diarrhea harbor diarrheagenic pathogens, including hypervirulent C. difficile. Our data support the importance of taking into account host susceptibility, microbial density, and organism virulence traits in future case-control studies, not merely categorizing candidate pathogens as being present or absent.

The study of acute childhood diarrhea presents 2 major challenges: this disorder often eludes etiologic determination [1–5], and many putatively diarrheagenic agents detected in stools are of undetermined causative significance. For example, in a recent Seattle Children’s Hospital (SCH) emergency department (SCHED) study, 6% of diarrheal stools contained Clostridium difficile cytotoxin [1], but the role of C. difficile in childhood diarrhea is a complex issue because the stools of many healthy infants and children contain this organism [6, 7]. Here we report a comprehensive case-control analysis of diarrhea etiology at the same venue to determine if candidate pathogens such as C. difficile and enteroaggregative Escherichia coli (EAEC) are, in fact, associated with diarrhea.

METHODS

Study Design and Enrollment

This prospective case-control study was approved by the SCH and Washington University Institutional...
Review Board. Case subjects were children presenting with diarrhea from November 2003 to November 2005 to the SCHED (a facility with >60,000 patient encounters during that interval), whose parents agreed to participate. Families provided demographic data, illness characteristics, medication, and travel history on standardized questionnaires, on which notice was provided that responding on the questionnaire signified participation consent.

Potential controls were recruited before and during case enrollment into a reserve cohort using 72 family and pediatric practices in King County (which includes Seattle and its suburbs) and southern Snohomish County, Washington, where circa 90% of SCHED patients reside. Multilingual recruitment materials describing the study [8] were displayed; brochures contained return mail enrollment forms for interested participants. For each case subject, a computer algorithm selected 2 community controls from the control reserve cohort, matched sequentially to the closest zip code of residence of the patient to reduce confounding by geography and demography, age (closest ± 50%, up to 19 years old), and finally sex.

One of 2 research assistants called families of each computer-selected control to explain the study, determine eligibility, obtain consent, and administer the same travel and medication questions used for cases. Controls could participate only once and were ineligible if they had diarrhea in the preceding 30 days or had a sibling chosen as a control for the same case. Qualifying controls were then sent a sterile specimen cup, collection “hat,” an insulated box, aqueous freezer bricks, and instructions to freeze the bricks. Control stools were placed into the cup and returned in the supplied box with the frozen bricks via courier to the SCH clinical microbiology laboratory. A small monetary acknowledgement was provided in appreciation for participation. US census data [9] and inter-zip code driving distances (Google Maps, http://maps.google.com/maps?hl=en&tab=wl on 12 April 2012) were used to approximate median household incomes and median distances between cases and corresponding controls, respectively.

**Laboratory Analysis**

Fresh case and control stools were evaluated for Shiga toxin (Stx)-producing E. coli (STEC) using an Stx enzyme immunoassay (EIA) on overnight broth cultures, parasites (by microscopy) and C. difficile (by cytotoxicity assay), and plated on MacConkey agar; case stools were cultured for bacteria generally considered to be diarrheagenic (Aeromonas species, Campylobacter species, STEC O157:H7, Pleisomonas shigelloides, Salmonella, Shigella, Vibrio, and Yersinia species) [10, 11]. Stools were then frozen at −80°C for subsequent batch-testing for adenoviruses, astroviruses, noroviruses 1 and 2, and rotavirus (IDEIA EIA kits K6021, K6042, K6043, K6020, respectively, Dako, Carpinteria, California). Non-O157:H7 STEC were recovered by isolating, and subsequently testing up to 20 lactose fermenting colonies by polymerase chain reaction (PCR) [10].

We identified EAEC by PCR testing 3 colonies per subject for aatA and aggR (conditions and primers in Supplementary Table 1), subject to colony availability (Supplementary Table 2). The stx1 and stx2 PCR was performed on pure cultures of single colonies of all O157:H7 and non-O157:H7 STEC, and 8 colony pools of EAEC.

We extracted DNA (QIAmp, Qiagen, Valencia, California) from frozen stools that tested positive in the C. difficile cytotoxicity assay and from their matched cytotoxin-negative controls. We then employed PCR with primers specific for the universal bacterial 16S ribosomal RNA (rRNA) gene (UB16S) and the C. difficile 16S rRNA gene (CD16S), and, if these were present, C. difficile toxins A (tcdA) and B (tcdB), binary toxin (cdtA and cdtB), and intact or deleted tcdC (tcdC, when absent (deleted) from C. difficile is associated with enhanced expression of toxins A and B). We also tested 22 cytotoxin-negative stools from controls that were matched to cytotoxin-positive cases for CD16S using the same methodology.

We examined individual EAEC to determine their adherence phenotypes. HeLa cells were grown to nearconfluence in Dulbecco modified eagle medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) in glass chamber slides (5% CO2, 37°C). Bacteria were grown (37°C, overnight) in Luria-Bertani (LB) broth with shaking under aerobic conditions, and 0.3 mL were added to 2.7 mL of fresh LB broth and grown under the same conditions for 6 hours, washed in DMEM, and suspended in adherence media (DMEM containing 5% FCS and 0.5% D-mannose), which was then added to the chamber slides after removing the media under which the HeLa cells were growing. After incubation (1 hour, 5% CO2, 37°C), we aspirated the adherence media and washed the monolayers 3 times with DMEM. After adding fresh adherence media and incubating (3 more hours) in the same conditions, we washed the cells 3 times (phosphate-buffered saline [PBS]), and fixed (100% cold methanol, 5 minutes) and stained (0.5% crystal violet) them before applying a coverslip. An observer unaware of the identity of the bacteria in each chamber scored the assays as nonadherent or adherent and noted the characteristics of the positives. Positive and negative controls were E. coli strains EAEC O42 and ORN172, respectively.

Only subjects whose specimens were tested completely for all organisms of interest (STEC and viruses by EIA, C. difficile by cytotoxicity, parasites by microscopy, and EAEC by PCR for cases and controls, classic bacterial pathogens by culture for cases) were included in this analysis.
Statistical Analysis
We modeled power with different potential sample and effect sizes; with sample sizes of 250 case and 450 control subjects, the project had 74%, 89%, and 95% chances of finding differences of 2%, 3%, and 4% frequency in the case group, compared with the control group. We used STATA version 11 (Statacorp, College Station, Texas) to measure associations between organism and case status, and conditional logistic regression analyses to calculate univariate matched odds ratios (ORs) and 95% confidence intervals (CIs). To address confounding by demographic and socioeconomic factors (including potential residual confounding by matching variable), we calculated adjusted ORs (aOR) and 95% CIs using multivariate conditional logistic models that included variables found to be associated with disease status in our data, or, if cells contained no or 1 subject, we calculated $P$ values using the exact version of McNemar test ($P_{exact}$).

RESULTS
We enrolled 254 cases and 452 matched controls (Table 1; mean, 1.8 matched controls per case; standard deviation, 0.5; range, 1–4). A median of 39 days elapsed between receipt of case stools and their corresponding controls’ stools (interquartile range, 40; range, 8–121). Cases were matched to controls living in the same (31%), contiguous (36%), or adjacent to a contiguous (14%), zip code; the median distance between zip codes of residence of cases and matched controls was 11.9 km. Controls’ zip codes had higher mean incomes than those of the cases, and there was a higher proportion of whites among controls and Hispanics among cases, but no other significant differences between groups. For these reasons, we adjusted for mean income by zip code, race and ethnicity in the multivariate regression analyses.

Twenty-nine (11.4%) case stools contained 30 bacterial pathogens: 13 Salmonella (5 Salmonella serovar Typhimurium, 2 Salmonella serovar Heidelberg, and 1 each Salmonella subgenus I Group B (4,512:i,-) Salmonella subspecies I serotype 4,12:i,-, Salmonella serovar Newport, Salmonella serovar Minnesota, Salmonella serovar Enteritidis, and Salmonella serovar Brandenburg), 10 STEC, 5 Campylobacter (including 1 non-jejunii), and 2 Shigella sonnei.

The single specimen that was negative on the STEC EIA but which contained E. coli O157:H7 on the corresponding sorbitol MacConkey agar plate possessed stx1 and stx2 genes (Supplementary Table 4); a pure broth culture of this isolate was positive in the EIA. The stools containing STEC O26:H11 and O111:nonmotile also contained rotavirus and S. serovar Brandenburg, respectively, whereas those that contained STEC O121:nonmotile, O177:nonmotile, and O157:H7 possessed no other organism of interest (Tables 2, 3 and Supplementary Table 4). The statistical significance of the differences in positivity between STEC among cases and controls varied from $P_{exact} = .13$ for non-O157:H7 overall, to $P_{exact} = .50$ for non-O157:H7 where no other organism of interest was identified, to $P_{exact} = .06$ for the 5 cases of STEC O157:H7 identified by Stx EIA. We necessarily excluded the sixth case identified only by SMAC agar screening from this particular analysis as controls were not similarly screened.

An average of 2.92 colonies per subject (median 3, range 0–3) were tested for the 2 EAEC loci. EAEC from 4 cases and 2 controls contained both loci, whereas EAEC from 4 cases and 2 controls contained one or the other locus (Supplementary Table 2). Adherence phenotypes of EAEC varied and included nonadherent, sparsely and densely adherent, and adherent in a stippled pattern over cells (Supplemental Figure 1). Adherence phenotypes correlated with neither case nor control subject status, or EAEC genotype, and varied even between EAEC from the same individuals. All EAEC were stx–negative.

C. difficile cytotoxin positivity was similar in cases and controls (aOR: 0.9; CI: 0.4–2). Point estimates did not meaningfully change when further adjusted for a history of antibiotic use (data not shown), but did increase in magnitude when restricting analysis to subject sets older than one and three years of age (aORs: 1.7 [CI: 0.5–5.4] and 4.2 [CI: 2.7–25.4], respectively).

Table 1. Demographic and Specimen Characteristics of Cases and Controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case (n = 254)</th>
<th>Control (n = 452)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>121 (47.6)</td>
<td>216 (48.1)$^a$</td>
<td>.95</td>
</tr>
<tr>
<td>Age, median months</td>
<td>20 (10–46)</td>
<td>24 (11–49)</td>
<td>.49</td>
</tr>
<tr>
<td>Race$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>142 (58.0)</td>
<td>345 (77.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>28 (11.4)</td>
<td>56 (12.5)</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>25 (9.8)</td>
<td>39 (8.6)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>10 (4.8)</td>
<td>8 (1.8)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>44 (17.3)</td>
<td>42 (9.3)</td>
<td></td>
</tr>
<tr>
<td>Don’t know</td>
<td>26 (10.2)</td>
<td>2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>9 (3.5)</td>
<td>4 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Hispanic ethnicity</td>
<td>69 (27.2)</td>
<td>70 (15.5)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Don’t know</td>
<td>13 (5.2)</td>
<td>2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>0 (0)</td>
<td>3 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Mean household income of zip code$^{c}$</td>
<td>$$54,099$</td>
<td>$$55,849$</td>
<td>.02</td>
</tr>
</tbody>
</table>

Data are expressed as No. (%), unless otherwise indicated.

$^a$ Sex not provided for 3 controls.

$^b$ Percentages exceed 100 because of multiple responses by some subjects.

$^c$ Based on US census data for 1999 on median household income of households in participants’ zip code of residence.

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Table 2. Case-Control Results for Organisms of Interest

<table>
<thead>
<tr>
<th>Established or Candidate Pathogens</th>
<th>No. of Positive Cases (%)</th>
<th>No. of Positive Controls (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC EIA positiveb</td>
<td>9 (3.5)</td>
<td>0</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>O157:H7c</td>
<td>5 (2.0)</td>
<td>0</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Non-O157:H7c</td>
<td>4 (1.6)</td>
<td>0</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>EAEC</td>
<td>11 (4.3)</td>
<td>6 (1.7)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>8 (3.2)</td>
<td>1 (0.2)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Norovirus 1</td>
<td>2 (0.7)</td>
<td>4 (1.2)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>15 (5.5)</td>
<td>27 (7.2)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Parasites (any)f</td>
<td>1 (0.4)</td>
<td>16 (4.3)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Assorted</td>
<td>11 (4.3)</td>
<td>6 (1.7)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>8 (3.2)</td>
<td>1 (0.2)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
<tr>
<td>Norovirus 2</td>
<td>2 (0.7)</td>
<td>4 (1.2)</td>
<td>0.9 (.2–2.6)</td>
<td>0.9 (.2–2.6)</td>
</tr>
</tbody>
</table>

Some of these subjects’ specimens contained >1 target organism. Details of dual infections, and of characteristics of the subjects and their illnesses, are provided here and in Supplemental Tables 2–4. Odds ratios vary depending on the specific numbers of positive cases and controls in matched and unmatched sets. Abbreviations: CI, confidence interval; EAEC, enteroaggregative E. coli; EIA, enzyme immunoassay; OR, odds ratio; STEC, Shiga toxin-producing E. coli.

For organisms where no or only 1 subject’s specimen tested positive, we present P values from an exact version of McNemar test (P_exact), rather than ORs and CIs.

a Adjusted for mean income by zip code, race, and ethnicity

b These are defined as subjects whose broth cultures tested positive for Shiga toxin (Stx) by EIA and from which a non-O157:H7 or an O157:H7 STEC was recovered but does not include the additional case subject whose stool contained STEC O157:H7 as identified on sorbitol MacConkey agar, but whose toxin assay was negative.

c For organisms where no or only 1 subject’s specimen tested positive, we present P values from an exact version of McNemar test (P_exact), rather than ORs and CIs.
d These are defined as subjects whose broth cultures tested positive for Stx by EIA and from which an O157:H7 STEC was recovered but does not include the additional case subject whose stool contained STEC O157:H7 as identified on sorbitol MacConkey agar, but whose toxin assay was negative.

e These are defined as subjects whose broth cultures tested positive for Stx by EIA and from which a non-O157:H7 STEC was recovered.

f Cryptosporidia (1 case and 6 control subjects), Entamoeba coli (6 control subjects), Blastocystis hominis (1 control subject), Endolimax nana (2 control subjects), and Giardia lamblia and E. nana (1 control subject).

PCR evidence of C. difficile was lacking (ie, specimens were negative for CD16S) in one case and one control stool that were cytotoxicity assay positive. Each of 2 cytotoxin-negative specimens from the controls matched to cytotoxin-positive cases was negative for CD16S. Of the 12 cases and 27 controls whose cytotoxin-positive stools contained PCR confirmation of C. difficile presence, 3 (25%) and 2 (7.4%) contained a hypervirulent C. difficile (the tcdC deletion).

Only 1 case subject, a 4-year-old girl who was evaluated for 4 days of diarrhea following travel to Honduras, was infected with a parasite (Cryptosporidia), whereas 16 control subjects produced stools with parasites. Adenoviruses were identified in the same proportion of cases and controls as EAEC, and astrovirus were also associated with case status but noroviruses were not. Rotavirus was found overwhelmingly in case stools (Table 2).

One or more organisms of interest were found in the stools of 174 cases and 69 controls (aOR, 12.9; CI, 7.9–21.2). Seventeen cases and 6 controls had >1 such microbe in their stools (aOR, 6.0; CI, 2.4–15.1; Table 3). Only 1 stool (from a control) contained 3 organisms of interest (Cryptosporidia, adenovirus and EAEC).

DISCUSSION

The distribution of bona fide enteric pathogens (Campylobacter, STEC O157:H7, Salmonella, Shigella, and rotavirus) in this series resembles the etiologic distribution in this venue between 1998 and 2001. This constancy demonstrates small area (greater Seattle) stability of childhood diarrhea etiology, though both studies were conducted before widespread use of rotavirus vaccines. Our findings differ from recent North American and European studies of people presenting with acute diarrhea in the higher proportion of bacterial pathogens we have found, with the most pronounced difference in the higher recovery of STECs [2–4, 12–14]. It is possible that this high-acuity population, drawn entirely from a pediatric emergency facility, is responsible for this particular difference. Additionally, we identified a greater proportion of astrovirus in diarrhea than these other studies, though this is an agent that
has been reported in hospitalized North American children in frequencies similar to what we report [5].

The higher proportion of EAEC among cases than among controls corroborates findings from children in Cincinnati [3], New Haven, and Baltimore [4] and extends these findings by comparing community-acquired diarrhea to community-based age-matched controls. EAEC diarrhea is generally nonbloody and persistent, but the characteristics of the case subjects with these organisms in their stool in our study suggests a more acute picture, occasionally including bloody diarrhea, although these EAEC did not contain stx genes, unlike EAEC O104:H4, which caused a large and devastating epidemic in Europe in May–June 2011. We believe that future work should be directed to thoroughly define the scope of

<table>
<thead>
<tr>
<th>Established or Candidate Pathogens</th>
<th>No.</th>
<th>Mean Age (Months)</th>
<th>Fever†</th>
<th>Vomiting†</th>
<th>Abdominal Pain†</th>
<th>Stool Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC§</td>
<td>10</td>
<td>72</td>
<td>50</td>
<td>70</td>
<td>90</td>
<td>Blood† Mucus‡</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>5</td>
<td>85</td>
<td>60</td>
<td>60</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Salmonella</td>
<td>13</td>
<td>57</td>
<td>92</td>
<td>69</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Shigella</td>
<td>2</td>
<td>84</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Clostridium difficileh</td>
<td>14</td>
<td>54</td>
<td>57</td>
<td>71</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>EAECi</td>
<td>8</td>
<td>54</td>
<td>63</td>
<td>50</td>
<td>63</td>
<td>25</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>11</td>
<td>56</td>
<td>82</td>
<td>73</td>
<td>73</td>
<td>18</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>8</td>
<td>38</td>
<td>38</td>
<td>88</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>110</td>
<td>22</td>
<td>81</td>
<td>96</td>
<td>48</td>
<td>7</td>
</tr>
<tr>
<td>Norovirus 1</td>
<td>4</td>
<td>144</td>
<td>0</td>
<td>75</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>Norovirus 2</td>
<td>5</td>
<td>39</td>
<td>40</td>
<td>100</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Total No. of established or candidate pathogens</td>
<td>174</td>
<td>36</td>
<td>73</td>
<td>86</td>
<td>59</td>
<td>20</td>
</tr>
<tr>
<td>All patients with multiple established or candidate pathogens</td>
<td>17</td>
<td>40</td>
<td>65</td>
<td>88</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>All patients with no established or candidate pathogens</td>
<td>80</td>
<td>38</td>
<td>56</td>
<td>58</td>
<td>55</td>
<td>21</td>
</tr>
<tr>
<td>All patients</td>
<td>254</td>
<td>37</td>
<td>68</td>
<td>77</td>
<td>58</td>
<td>20</td>
</tr>
</tbody>
</table>

Symptoms and signs are family/self-reported on questionnaire.

Abbreviations: EAEC, enteraggregative E. coli; STEC, Shiga toxin-producing E. coli; WBC, white blood cells.

† History and symptoms were family/self-reported on questionnaire.

‡ Percentages calculated by dividing the number of subjects answering “yes” by total number of subjects in group (including those answering “don’t know” or leaving response blank) and multiplying by 100, and therefore represent minimal values.

§ Median No. of stools in 24-hour period before Seattle Children’s Hospital emergency department (SCHED) visit, among those providing a response other than “don’t know.”

‖ Median No. of days of diarrhea before SCHED visit, among those providing a response other than “don’t know.”

* Outside of the United States or Canada.

‡‡ Defined as white blood cells identified in the stools by Gram stain or wet mount.

h Includes patients infected with STEC O157:H7 (n = 6), O26:H11, O111:nonmotile, O121: nonmotile, and O177:nonmotile (n = 1 each). STEC-infected cases are those subjects whose broth cultures tested positive for Shiga toxin (Stx) by enzyme immunoassay (EIA) and from which a non-O157:H7 or an O157:H7 STEC was recovered, or whose sorbitol MacConkey agar plate culture yielded an O157:H7 STEC even though the Stx EIA was negative. The stx genotypes and associated illness characteristics are provided in Supplementary Table 4.

i Defined as patients whose stools were cytotoxin positive; characteristics of genotypes in individual positive specimens and corresponding subjects are provided in Supplementary Table 3.

j Defined as isolates positive for aatA and/or aggR loci by polymerase chain reaction; characteristics of genotypes of positive colonies and corresponding subjects are provided in Supplementary Table 2.

k 174 organisms were detected in 157 case patients.

l Case subjects had evidence of 2 organisms of interest in their stool: C. difficile toxin and rotavirus (n = 4), rotavirus and astrovirus (n = 2), rotavirus and EAEC (n = 2), C. difficile toxin and adenovirus, C. difficile toxin and norovirus 1, C. difficile toxin and norovirus 2, adenovirus and astrovirus, adenovirus and rotavirus, STEC O26:H11 and rotavirus, S. serovar Typhimurium and rotavirus, Shigella sonnei and EAEC, and STEC O111:nonmotile and S. serovar Brandenburg (n = 1 each pairing).
Categorical present/absent assignment to microbes might not adequately weight factors that would incriminate such organisms as pathogens when found in cases or exculpate them when found in controls. Factors not considered in present/absent results include host immunopropensity toward acquiring specific pathogens and then becoming ill; microbial density in analyte as has been noted with enteropathogenic E. coli [33]; presence and expression of genes encoding virulence factors by the putative pathogen in the host; and ambient flora that might alter expression of virulence. For EAEC, however, microbial enumeration is not so straightforward, because the defining loci are contained on plasmids of variable copy number, which hinder attempts to determine organism density per mass of stool. However, in this study, the mean numbers of positive colonies per EAEC-excreting cases (2.4) and controls (2.8) were similar.

We are also constrained by our diagnostic reliance on available reagents that target only “established” pathogens. Diagnostic and new technologic approaches to pathogen discovery and identification are needed to more thoroughly identify causes of diarrhea. One such example to illuminate diarrhea etiology is mass sequencing, which has identified several candidate diarrheagenic viruses in childhood stool [34–38]. Expanding databases of microbial sequences, developing enumeration tools that are more sophisticated than yes/no presence of organism, and factoring in host response to agent presence/absence will advance our abilities to confirm or refute virulence. Until these many case-control design and analysis issues can be resolved, it appears that outbreaks will remain the best way to establish pathogenicity of candidate diarrheagenic organisms.

Finally, the presence of diarrheagenic viruses, parasites, EAEC, and C. difficile (including potentially hypervirulent strains) in many control stools, sometimes in greater frequency than in case specimens, raises the possibility that future control strategies and modeling efforts might need to account for community reservoirs of these agents [39].

In summary, EAEC are statistically associated with diarrhea, but definition of this class of probable pathogens needs refinement, and optimal detection methodologies are lacking. Some community-acquired acute childhood diarrhea is probably caused by C. difficile, but we doubt that cytotoxin-producing C. difficile always warrants treatment when found in cases of acute diarrhea. This case-control study strengthens the case that some non-O157:H7 STEC are pathogenic but again portrays the predominance of the O157:H7 serotype in a high-acuity setting, and the superiority of SMAC agar screening for this pathogen. Finally, asymptomatic excretion of C. difficile, Cryptosporidium, and diarrheagenic viruses by community controls highlights the need to identify host and microbial factors and processes that differentiate asymptomatic carriage from pathogenicity and might have implications for transmission.
The high carriage rate also poses difficulties when using case-control studies to confirm or refute pathogenicity of candidate diarrheagenic agents.

**Supplementary Data**

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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