Prevalence of Enteraggregative *Escherichia coli* among Children with and without Diarrhea in Switzerland

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In a prospective study between July 1999 and September 2000, stool specimens of children below the age of 16 years with (*n* = 187) and without (*n* = 137) diarrhea were tested for the presence of enterovirulent bacteria by standard culture methods and by PCR. Targets for the PCR were the plasmid pCVD432 for enteroggregative *Escherichia coli* (EAEC), the verotoxin 1 and verotoxin 2 genes for enterohemorrhagic *E. coli*, *ipaH* for enteroinvasive *E. coli* (EIEC) and *Shigella* spp., genes coding for heat-stable and heat-labile toxins for enterotoxigenic *E. coli* (ETEC), and the eaeA gene for enteropathogenic *E. coli*. The following bacteria could be associated with diarrhea: *Salmonella enterica* (*P* = 0.001), *Campylobacter* spp. (*P* = 0.036), ETEC (*P* = 0.012), and EAEC (*P* = 0.006). The detection of EAEC, ETEC, and *S. enterica* was strongly associated with a history of recent travel outside of Switzerland. EAEC isolates were found in the specimens of 19 (10.2%) of 187 children with diarrhea and in those of 3 (2.2%) of 137 children without diarrhea (*P* = 0.006) and were the most frequently detected bacteria associated with diarrhea. Among the children below the age of 5 years, the specimens of 18 (11.9%) of 151 with diarrhea were positive for EAEC, while this agent was found in the specimens of 2 (2.2%) of 91 controls (*P* = 0.007). Enteropathogenic *E. coli* isolates were found in the specimens of 30 (16.4%) of the patients and in those of 15 (10.9%) of the controls, with similar frequencies in all age groups (*P* > 0.05). We conclude that EAEC bacteria are involved in a significant proportion of diarrhea cases among children. Children younger than 5 years of age are more often affected by EAEC than older children.

Six different types of diarrheagenic *Escherichia coli* have been identified. These include enteraggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAD). The importance of EAEC bacteria as diarrheagenic agents in children remains controversial. Although the association of EAEC with diarrhea in children has been documented in different studies in industrially developing and developed countries (4, 5, 7, 13, 15, 19, 23), other studies did not associate EAEC with diarrhea (2, 8, 11). This lack of association may be partially explained by the significant strain-to-strain heterogeneity due to the presence of various presumed virulence factors that are not conserved among all EAEC bacteria or by the subjective interpretation of the standard laboratory test, i.e., the observation of an aggregative adherence pattern in a HEp-2 cell assay. Moreover, the importance of EAEC appears to vary geographically.

The aim of the present prospective study was to determine the importance of EAEC among children with diarrhea in Switzerland by using the PCR targeting plasmid pCVD432.

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MATERIALS AND METHODS

**Clinical samples.** Stool specimens were collected prospectively from children with acute or chronic diarrhea who were seen at the Children’s University Hospital of Zurich and at the Ostschweizer Children’s Hospital between August 1999 and September 2000. Diarrhea was defined as at least two loose stools within 24 h. An episode was considered chronic if it lasted for more than 14 days. Patients who had developed diarrhea only after admission to the hospital and those who had received antimicrobials in the previous 14 days were excluded. Clinical symptoms, including vomiting, a fever of >38.5°C, and dehydration, as well as any history of recent travel outside of Switzerland, were recorded. Dehydration was defined by clinical characteristics, e.g., tacky mucous membranes, sunken eyes or fontanel, and poor skin turgor.

Stool specimens from children seen at the two hospitals during the same time period for treatment of other diseases, i.e., children who had not experienced diarrhea, vomiting, or abdominal pain in the preceding 2 weeks prior to admission and who had not received antimicrobials in the previous 2 weeks, served as controls. Informed consent to collect a stool specimen from each of the children was gathered from their parents or guardians. The study was approved by the institutional ethics committee.

Stool specimens were either inoculated onto culture media within 6 h or stored in Cary-Blair transport medium for up to 72 h.

**Bacterial cultures.** The bacterial pathogens *Salmonella enterica*, *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., and *Aeromonas* spp. were sought by standard methods (17).

**Detection of enterovirulent *E. coli* by PCR.** The oligonucleotide primers used in this study are listed in Table 1. Stool specimens were inoculated onto MacConkey agar plates and incubated aerobically at 37°C for 24 h. Bacteria from the area of confluent growth were suspended in 5 ml of a 0.85% NaCl solution until a density equivalent to McFarland standard 6 was reached and were stored at −20°C until use. After thawing, 0.5 ml of the bacterial suspension was centrifuged and the supernatant was discarded. The pelleted cells were resuspended in 200 μl of 4% Chelex (Bio-Rad Laboratories AG, Reinach, Switzerland), and the DNA was extracted at 95°C for 15 min. After centrifugation, PCR was performed directly from the supernatant. The PCR mixtures with a final volume of 50 μl consisted of 5 μl of bacterial lysate (supernatant with template DNA); 0.2 mM each dGTP, dATP, dTTP, and dCTP (Roche Diagnostics GmbH, Mannheim, Germany); a 0.2 μM (0.25 μM in assays for pCVD432, genes coding for heat-
TABLE 1. Oligonucleotide primer pairs used in the study

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence size (bp)</th>
<th>Reference</th>
<th>Primer pair</th>
</tr>
</thead>
<tbody>
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<td>22</td>
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<td></td>
<td></td>
<td></td>
<td>5′-CAATGTATAGAATACCCGTGTT-3′</td>
</tr>
<tr>
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<td>752</td>
<td>10</td>
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<td>VT 2 geneb</td>
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<td></td>
<td></td>
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<td>ST gene</td>
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<td></td>
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<td></td>
<td>5′-TTATAGACCGCGTACAAGC-3′</td>
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</table>

a Specimens positive for pCVD432 were interpreted as harboring EAEC.
b Specimens positive for the VT 1 or VT 2 gene with a positive result for eaeA (a gene on the locus of enterocyte effacement) were interpreted as harboring EHEC. VT 1 gene- or VT 2 gene-positive specimens without a positive eaeA result were interpreted as harboring VTEC.
c Specimens positive only for eaeA represent EPEC.
d Since ipaH is present in both EIEC and Shigella spp., specimens positive for ipaH with cultural detection of Shigella spp. were considered to contain Shigella spp., whereas specimens positive for ipaH with negative culture results were interpreted as EIEC.
e ST and LT are virulence factors of ETEC.

labile toxin [LT] and heat-stable toxin [ST], and ipaH concentration each of the reverse and forward primers (Microsynth, Balgach, Switzerland); 10 mM Tris-HCl; 1.5 mM MgCl2; 50 mM KCl; 0.5 μM EDTA; 25 μM dithiothreitol; 1.25 U of Taq DNA polymerase; stabilizers; and 0.25% glycerol (Amersham Biosciences AB, Uppsala, Sweden). PCR was performed with the GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif.). The reaction was started with a 5-min denaturation step at 94°C. In the PCRs for the verotoxin 1 (VT 1) gene, the VT 2 gene, and eaeA, the temperature cycles consisted of 15 s at 94°C, followed by 15 s at 65°C and 75 s at 72°C. In the PCRs for pCVD432, the LT gene, the ST gene, and ipaH, the temperature cycles consisted of 30 s at 95°C, followed by 30 s at 55°C and 30 s at 72°C. Each cycle was repeated 35 times, and the final cycle was followed by incubation of the reaction mixture for 5 min at 72°C. The amplified DNA was separated by submarine gel electrophoresis on 1% agarose, stained with ethidium bromide, and visualized under UV transillumination.

**Detection of rotavirus and adenovirus.** Rotavirus and adenovirus were detected by commercially available antigen detection assays (Dialex; Orion Diagnostica, Espoo, Finland) in patients with diarrhea only.

**Statistical analysis.** Data were compared by a two-tailed chi-square test and Fisher’s exact test. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Demographics.** During the 15-month study period, stool specimens were investigated from a total of 187 children 1 to 189 months of age (mean, 38 months; median, 21 months) with diarrhea and from a total of 137 randomly selected children 1 to 174 months of age (mean, 50 months; median, 27 months) without diarrhea seen at two children’s hospitals in Switzerland. Among the children with diarrhea, 79 (42.2%) were girls, and among those without diarrhea, 58 (42.3%) were girls.

**Isolation of enteric pathogens.** One or more potential enteric pathogens were found in the specimens of 94 (50.3%) of the 187 patients with diarrhea and in 20 (14.6%) of the 137
Among the 94 patients with diarrhea, 63 were found to have both viral and bacterial pathogens in their stool specimens. Multiple pathogens were detected in 17 patients with diarrhea, whereas more than one pathogen was never isolated from a control child. Controls had clinical signs of dehydration and needed intravenous rehydration, and 2 patients (18%) had a fever of more than 38.5°C.

Isolation of bacterial enteropathogens other than E. coli. Table 2 lists the identified bacterial enteropathogens other than E. coli. Shigella spp. and 12 and 6 times, respectively, more often than isolates of EAEC, EHEC or VTEC, 1.1% for EIEC, and 4.3% for ETEC.

Detection of enterovirulent E. coli. The results of the PCR assays for enterovirulent E. coli are presented in Table 3. Among the children with diarrhea, EPEC isolates were detected in similar frequencies in children ≤5 years and children >5 years. Among the children with diarrhea, EPEC isolates were found to have both viral and bacterial pathogens in their stool specimens. Multiple pathogens were detected in 17 patients with diarrhea, whereas more than one pathogen was never isolated from a control child.
Travel history. Thirty-one (16.6%) of the patients with diarrhea, but none of those without diarrhea, had a history of recent travel outside of Switzerland. Among patients whose stool specimens tested positive for pathogens, the frequencies of their having a history of travel abroad were as follows: for patients with *S. enterica*, 50%; for patients with *Shigella* spp., 50%; for patients with *Campylobacter* spp., 17%; for patients with EAEC, 47%; for patients with EPEC, 37%; and for patients with ETEC, 75%. Patients with a history of travel abroad were found more often to be positive for *S. enterica* (19 versus 4%; *P* = 0.006), EAEC (29 versus 6%; *P* = 0.001), EPEC (36 versus 12%; *P* = 0.003), and ETEC (19 versus 1%; *P* < 0.0001) than were patients with no history of travel abroad. The most common travel destinations of the patients with diarrhea were Turkey (19%), the Balkan Peninsula (19%), and northern Africa (13%). Of note, five of the eight patients with ETEC had returned from these three destinations.

**DISCUSSION**

This prospective study demonstrates that EAEC bacteria are involved in a significant proportion of cases of diarrhea among children from an industrialized country, as are *S. enterica*, *Campylobacter* spp., and ETEC. EPEC isolates were detected in the specimens of children with diarrhea and in those of children without diarrhea with similar frequencies. The detection of EAEC, ETEC, and *S. enterica* was strongly associated with a history of recent travel outside of Switzerland, and EAEC isolates were detected more often in the specimens of children younger than 5 years of age than in those of older children.

The identification of EAEC in this study was based on PCR with primers complementary for pCVD432 (22), a test representing one of the reliable means for detecting EAEC (21). The HEp-2 adherence test is regarded as the “gold standard” for the identification of EAEC (18), but it is conducted only in reference laboratories, as it requires specialized facilities (21). Moreover, since other enteric bacteria also exhibit aggregative phenotypes, confirmation of *E. coli* speciation is mandatory to unequivocally identify EAEC (21). The PCR assay, based on a previously described DNA probe (3), identified 86% of reference EAEC strains found to be positive by the HEp-2 cell adhesion assay and identified <1% of other diarrheagenic *E. coli* strains (22). In a study conducted in Calcutta, India, the same PCR assay performed with similar sensitivity and specificity (7). In a Swiss study investigating the pathogenic role of EAEC among human immunodeficiency virus-infected persons, the pCVD432 PCR assay was demonstrated to better correlate with clinical findings than the cell adherence assay (6). Other studies, however, have provided evidence that pCVD432-probe-negative EAEC strains may also be associated with diarrheal illness (15, 20). An extreme example is represented by an outbreak of EAEC in Japan, where all isolates were negative by the pCVD432 PCR assay (14). Thus, in the present study, we may have underestimated the frequency of EAEC, but this would apply both to patients with diarrhea and to asymptomatic children.

A substantial proportion of the diarrheic children had a history of travel outside of Switzerland. Although the percentage of patients suffering from diarrhea who had a history of travel was higher at one enrolling site (Zurich, 18%) than at the other (St. Gallen, 6%), this difference was not statistically significant (*P* = 0.32). Almost half of the patients with diarrhea positive for EAEC, but none of the control children with EAEC, had a history of recent travel outside of Switzerland. For comparison, a study conducted by Hupperetz et al. in Germany found that approximately one-third of the children with diarrhea and detection of EAEC had traveled abroad (13). However, no information on the travel history was provided for other patients with diarrhea or for asymptomatic children. In adults, EAEC and ETEC were shown to be the major etiologic agents of traveler’s diarrhea (1). In our study, ETEC was detected only in children with diarrhea, and most of these children (75%) had a history of recent travel abroad. Thus, our data suggest that EAEC and ETEC may play a considerable role in traveler’s diarrhea during childhood, too.

The proportion of children with diarrhea who were demonstrated to be infected with EAEC was unexpectedly high (10%). The frequency of EAEC detection in control children was 2%. In the large German study by Hupperetz et al. (13), the frequency of EAEC detection in children was 2% and that in asymptomatic controls was 0%. Studies of EAEC conducted in less industrially developed countries revealed a prevalence of 39% in children with diarrhea and 27.7% in children without diarrhea in Nigeria (20) and a prevalence of 26.9% in children with diarrhea and 15% in controls in Venezuela (12). Thus, in developing countries, the prevalence of EAEC is considerably higher than in industrially developed countries. These observations may explain at least in part the high proportion of patients with diarrhea and EAEC associated with recent travel abroad.

An observation of considerable interest was that children younger than 5 years of age were significantly more often...
affected by EAEC than older children. Other studies also noted differences in prevalence of EAEC in various age groups. In the Venezuelan study, the difference in EAEC prevalence between children with diarrhea and asymptomatic controls was significant for the group 0 to 2 months of age but not for older infants (12). In the study conducted in Nigeria, however, EAEC was significantly more often isolated from children with diarrhea than from healthy control subjects in children >6 months of age but not in those <6 months of age (20). Our data suggest that the prevalence and significance of EAEC infections depend on age. Discordant results from other studies may be explained by the strain-to-strain heterogeneity in other geographical areas or by the existence of asymptomatic carriers. ETEC bacteria, however, show similar frequencies in children 0 to 5 years of age and in children 5 to 16 years of age.

Eight (42%) of the 19 children with diarrhea and EAEC were found to harbor one or two additional potentially enterovirulent agents, EPEC being the most frequently detected (Table 4). The detection of multiple pathogens in diarrheic children with EAEC has also been reported from other studies (7, 12, 13). Since EPEC in this study was found in similar frequencies in diarrheic children and in nondiarrheic children, the pathogenic role of E. coli harboring only the eaeA gene has to be questioned. Consequently, it seems justified to disregard EPEC as a cause of diarrhea in those children harboring EAEC, which would increase the number of diarrheic children with EAEC as the only identified pathogen from 11 to 14.

Only two-thirds of stool specimens from patients with diarrhea and none of the specimens of controls were analyzed for the presence of viruses, i.e., rotavirus and adenovirus, which may be regarded as a limitation of this study. Among diarrheic stools investigated for viruses, more than one-quarter of the specimens harbored either virus. Whereas rotavirus was found in combination with a bacterial pathogen in 2 of 25 cases only, adenovirus was detected together with a bacterial pathogen in 5 of 8 cases. Among the 69 diarrheic children with a potential bacterial enteric pathogen, 25 have not been screened for viruses. Of the eight diarrheic patients with EAEC who were tested for rotavirus and adenovirus, one patient was positive for adenovirus. In the study conducted by Huppertz et al. in Germany, 81% of the EAEC-positive patients were screened for the presence of rotavirus but not for adenovirus (13). All EAEC-positive specimens investigated in our study were negative for rotavirus.

Finally, the additional clinical signs in diarrheic patients infected with EAEC included vomiting in more than half of the patients and dehydration requiring intravenous fluid replacement in almost half of the patients. Furthermore, in about one-quarter of the patients, diarrhea became chronic, i.e., it lasted for more than 14 days, according to our definition. These observations are consistent with the so-far limited available data (13, 21). In children younger than 5 years of age with continuing diarrhea and a history of travel abroad, inclusion of tests for EAEC should be considered in the microbiological workup for diarrhea.

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