Age-specific prevalence of diffusely adherent *Escherichia coli* in Brazilian children with acute diarrhoea

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In a prospective study between February 2003 and June 2004, stool specimens of children less than 2 years of age with diarrhoea (n=218) and without diarrhoea (n=86), living in Vitória, Espírito Santo, Brazil, were examined for the presence of diarrhoeagenic *Escherichia coli*. *E. coli* isolates were tested by colony blot hybridization with specific DNA probes designed to detect EPEC, ETEC, EIEC, EAEC, DAEC and EHEC/STEC. Diarrhoeagenic *E. coli* strains were detected as the sole pathogen in stools of 92 (30.3 %) children, including 72 (33.0 %) with diarrhoea and 20 (23.2 %) without diarrhoea. DAEC was the most frequent pathotype and was found significantly more often from patients (18.3 %) than from controls (8.1 %) (P<0.05), particularly among children more than 1 year of age (P<0.01). Atypical EPEC and EAEC isolates were isolated from both patients (5.5 % and 4.6 %, respectively) and controls (6.9 % and 6.9 %, respectively). ETEC was more frequently isolated from patients (3.2 %) than controls (1.2 %). Typical EPEC (0.9 %) and EIEC (0.4 %) isolates were detected only in children with diarrhoea. In conclusion, our data suggest that DAEC should be considered potential pathogens in the region of Brazil studied.

INTRODUCTION

Diarrhoeagenic *Escherichia coli* constitute an important group of pathogens associated with enteric diseases. Six *E. coli* pathotypes associated with diarrhoea are currently recognized: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). EPEC are divided into typical (tEPEC) and atypical (aEPEC) subgroups depending on the presence or absence of the EPEC adherence factor (EAF) plasmid, respectively (Nataro & Kaper, 1998).

Each type of diarrhoeagenic *E. coli* is defined on the basis of distinct virulence characteristics, and DNA probes for these characteristics have been developed to distinguish types of diarrhoeagenic *E. coli* from each other and from non-pathogenic *E. coli* strains of normal flora (Nataro & Kaper, 1998).

It has become clear that there are regional differences in the relative prevalence of the different types of diarrhoeagenic *E. coli* and such differences may affect the overall regional prevalence of diarrhoeal diseases. In Brazil, several studies have investigated the relative prevalence of diarrhoeagenic *E. coli* among children with and without diarrhoea. However, most of these studies have been performed predominantly in the cities of São Paulo and Rio de Janeiro, two large urban centres in south-eastern Brazil (Rosa et al., 1998; Gomes et al., 1991; Scaletsky et al., 1999, 2002b; Regua-Mangia et al., 2004; Araujo et al., 2007). The aim of the present study was to determine the relative prevalence and the role of the different *E. coli* pathotypes as cause of acute diarrhoea in the city of Vitória, Espírito Santo, another large urban centre in south-eastern Brazil, where childhood diarrhoea is endemic.
METHODS

Subjects. The study was conducted at the emergency room of Hospital de Pediatria in the city of Vitória, Espírito Santo, Brazil. From February 2003 to June 2004, all children less than 2 years of age with acute diarrhea who where brought to the hospital ambulatory clinic in the morning were enrolled in the study. Specimens were collected during Monday through Thursday of the following months: March, April, May, June, August, September, October, November and February. Demographic information such as age and gender, and clinical information, were obtained by means of a standard questionnaire administered by a physician. Clinical symptoms including vomiting, fever, abdominal pain and dehydration were recorded. A control group containing asymptomatic children was randomly selected from the well-child outpatient clinics of the same hospital. Children in the two groups were of low socioeconomic status and were matched by age and period of admission. The stool collection was performed with the consent of the children’s parent and the approval of the Hospital Committee on Ethics in Research.

Microbiological methods. Two rectal swab specimens were collected from each child, placed in Cary–Blair transport medium, and processed within 4 h. One swab specimen was processed by routine microbiological and biochemical tests to identify E. coli, Salmonella spp., Shigella spp., Campylobacter spp. and Yersinia enterocolitica (Ewing, 1986); the second swab specimen was stored in 2 ml phosphate-buffered saline (pH 7.4) at 4 °C until being tested for rotavirus and adenovirus by enzyme immunoassays (Flewett et al., 1989). E. coli were isolated on MacConkey agar plates. Five separate lactose-fermenting colonies and two non-lactose-fermenting colonies presumed to be E. coli by colony morphology were stored on nutrient agar slants and sent to the microbiological hybridization assay as described previously (Scaletsky et al., 2002). The O antigens of EPEC serogroups and an O157 EHEC strain. All colonies were submitted to slide agglutination with polyvalent and monovalent antisera (PROBAC do Brazil) against E. coli and processed within 4 h. One swab specimen was processed by colony DNA hybridization assay as described previously (Scaletsky et al., 2002c). The following specific DNA probes were used: EAF (EPEC adherence factor), a 1 kb BamHI–SalI fragment from plasmid pMAR2; cac (encoding intimin, an outer-membrane protein involved in the attaching and effacing lesions promoted by EPEC and EHEC strains), a 1 kb SalI–KpnI fragment of pCVD434; AA (aggregative adherence plasmid), a 1 kb EcoRI–PstI fragment of pCVD432; daaC (diffuse adhesin), a 390 bp PstI fragment of pSLM852; IT, a 1.3 kb BamHI fragment of pCVD403; StH, a 216 bp EcoRI fragment of pCVD427; Stp, a 216 bp EcoRI fragment of pCVD427; Inv (EIEC invasion plasmid), a 2.5 kb HindIII fragment of pPS55; Ehy, a 3.4 kb HindIII fragment of pCVD443; Stx1, a 1.1 kb BamHI fragment of pN37-19; and Stx2, a 842 bp SmaI–PstI fragment of pNN110-18 (Nataro & Kaper, 1998). The DNA probes were labelled with 50 μCi (185 MBq) [32P]dCTP by using a random primer extension kit (Rediprime DNA labelling system; Amersham). Colony blots were hybridized at 37 °C overnight, washed with 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS, and exposed to X-ray film overnight at −80 °C.

RESULTS AND DISCUSSION

Subjects

A total of 304 stool specimens from 218 children with diarrhea and 86 children without diarrhea (controls) were examined for enteric bacterial pathogens. Among the population studied, 99 (32.6%) children were aged less than 6 months; 95 (31.2%) and 110 (36.2%) were aged 7–12 months and 13–24 months, respectively. The overall sex distribution was 176 (57.9%) male and 128 (42.1%) female.

Prevalence of enteropathogens other than diarrhoeagenic E. coli

The prevalence of enteropathogens other than diarrhoeagenic E. coli identified in the stool cultures of children with diarrhea and asymptomatic controls is presented in Table 1. Rotavirus was the organism most commonly detected in diarrhoeal stools (33.9%; P<0.01), followed by Shigella spp. (3.7%) and Salmonella spp. (2.7%). Yersinia enterocolitica and Campylobacter spp. were not isolated in this study.

Prevalence of diarrhoeagenic E. coli

A total of 915 colonies of E. coli, 685 obtained from the 218 children with diarrhea and 230 from 86 children without diarrhea, were tested using DNA probes to classify them into the different pathotypes (Table 1). A total of 98 diarrhoeagenic E. coli strains were isolated from the 304 stool specimens: 72 (33.0%) were identified as the only pathogen in the stools of children with diarrhea, 20 (23.2%) were from controls, and 6 were found with another bacterial pathogen. Five children with diarrhea and one control were colonized with more than one diarrhoeagenic E. coli pathotype. DAEC was the most prevalent pathotype identified, followed by aEPEC, EAEC, ETEC, tEPEC and EIEC. No EHEC or STEC was isolated in this study.

The DAEC pathotype was isolated significantly more often from patients (18.3%) than from controls (8.1%) (P<0.05) (Table 1). Atypical EPEC and EAEC were isolated with similar frequencies from patients (5.5% and 4.6%, respectively) and controls (6.9% and 6.9%, respectively). ETIEC was also more frequently isolated from patients (3.2%) than controls (1.2%). Typical EPEC (0.9%) and EIEC (0.4%) isolates were detected only in children with diarrhea.

Considering the age groups, the DAEC pathotype was detected in all age groups, but a significantly higher prevalence was seen in children over 12 months of age (P=0.01) (Table 2). Atypical EPEC and EAEC were mainly detected among children less than 12 months of age, while ETEC was only detected in children over 12 months of age. The two tEPEC isolates and the one EIEC isolate were
detected in children aged 7–12 months. Five diarrhoeagenic \textit{E. coli} isolates from children with diarrhoea belonged to a classical EPEC serogroup; one DAEC belonged to the O128 serogroup, three aEPEC strains isolated from children with diarrhoea belonged to serogroups O26, O119 and O142, and one tEPEC belonged to the O55 serogroup. Of the eight ETEC strains isolated, seven produced the LT toxin only and one strain produced LT and ST toxins.

Clinical symptoms such as fever, vomiting, abdominal pain and dehydration of the children were recorded; their frequencies were similar in children with diarrhoea carrying DAEC, aEPEC, EAEC or ETEC isolates. The frequencies of fever and vomiting were 60\% and 90\%, respectively. Abdominal pain and dehydration were observed in 70\% and 30\% of the children. The two children carrying aEPEC isolates presented vomiting and one of them had fever and abdominal pain. The single EIEC isolate was recovered from a child presenting fever and vomiting.

Our results demonstrate the previously unrecognized importance of DAEC as a cause of childhood diarrhoea in the region of Brazil studied and support the evidence from prospective case-control studies showing an association of DAEC with age-dependent diarrhoea (Gunzburg \textit{et al.}, 1993; Jallat \textit{et al.}, 1993; Germani \textit{et al.}, 1996). This is the second case-control study that we have conducted in Brazil showing an association of DAEC with diarrhoea.

### Table 1. Frequency of isolation of bacterial pathogens among case patients and controls in Vitória, Espírito Santo, Brazil

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. (%) of children</th>
<th>Patients (n=218)</th>
<th>Controls (n=86)</th>
<th>P value*</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus</td>
<td>74 (33.9)</td>
<td>0</td>
<td>&lt;0.01</td>
<td>35.66</td>
<td></td>
</tr>
<tr>
<td>\textit{Shigella} spp.</td>
<td>8 (3.7)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{Salmonella} spp.</td>
<td>6 (2.7)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>DAEC</td>
<td>40 (18.3)</td>
<td>7 (8.1)</td>
<td>&lt;0.05</td>
<td>4.92</td>
<td></td>
</tr>
<tr>
<td>aEPEC</td>
<td>12 (5.5)</td>
<td>6 (6.9)</td>
<td>NS</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tEPEC</td>
<td>2 (0.9)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>EAEC</td>
<td>10 (4.6)</td>
<td>6 (6.9)</td>
<td>NS</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>7 (3.2)</td>
<td>1 (1.2)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>EIEC</td>
<td>1 (0.4)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>DAEC + \textit{Salmonella} spp.</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aEPEC + \textit{Shigella} spp.</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC + \textit{Salmonella} spp.</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC + \textit{Shigella} spp.</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NS, Not significant (P>0.05).

### Table 2. Prevalence and characteristics of diarrhoeagenic \textit{E. coli} among patients and controls, by age

<table>
<thead>
<tr>
<th>\textit{E. coli} type</th>
<th>Reactivity with DNA probe</th>
<th>EPEC serogroup</th>
<th>No. (%) of positive children by age group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0–6 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Patients (n=69)</td>
</tr>
<tr>
<td>DAEC EPEC</td>
<td>daaC</td>
<td>O128</td>
<td>12 (17.4)</td>
</tr>
<tr>
<td>Typical EPEC</td>
<td>cae, EAF</td>
<td>O55</td>
<td>0</td>
</tr>
<tr>
<td>Atypical EPEC</td>
<td>cae</td>
<td>O26, O119, O142</td>
<td>5 (7.2)</td>
</tr>
<tr>
<td>EAEC</td>
<td>AA</td>
<td>–</td>
<td>6 (8.7)</td>
</tr>
<tr>
<td>ETEC</td>
<td>LT</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>LT, STh</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>EIEC</td>
<td>Inv</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

*P=0.01.
(Scaletsky et al. 2002a). The first study was carried out in two urban centres in north-eastern Brazil, and DAEC were also significantly associated with diarrhoea in children over 12 months of age. Our study did not suggest a characteristic clinical presentation for DAEC infection. Whereas a high proportion of children infected with DAEC had fever, vomiting, and abdominal pain, the frequencies of these complaints were not different from those of children infected with aEPEC, EAEC or ETEC.

In our study, aEPEC strains were the second most frequent pathotype of E. coli; however, the isolation rate was similar among children with and without diarrhoea. aEPEC has been implicated as the causative agent in some outbreaks (Viljanen et al., 1990; Scotland et al., 1993; Hedberg et al., 1997; Yatsuynagai et al., 2002) and endemic diarrhoea (Bokete et al., 1995; Afset et al., 2003), whereas in other studies this pathotype has not been recovered more frequently from diarrhoeal cases than from controls (Echeverria et al., 1991). However, the role of aEPEC in diarrhoea has not been established conclusively. In Brazil, aEPEC has been increasingly reported (Vieira et al., 2001; Dulguer et al., 2003; Gomes et al., 2004) and was recently implicated as a cause of diarrhoea in São Paulo, another geographical region of Brazil (Araujo et al., 2007). Although geographical differences may exist, the prevalence of aEPEC in this and previous studies underscores the emergence of aEPEC strains in Brazil.

The very low rate of tEPEC in our study was not surprising. Recent studies in several regions of Brazil have shown a very low frequency of tEPEC and a relatively high frequency of aEPEC (Dulguer et al., 2003; Regua-Mangia et al., 2004; Gomes et al., 2004; Franzolin et al., 2005; Araujo et al., 2007). As demonstrated in this and other studies, aEPEC appears to have become a more frequent cause of diarrhoea than tEPEC among Brazilian children.

The low proportion of children with diarrhoea infected with EAEC was somewhat surprising. In recent epidemiological studies conducted in different urban centres of Brazil, EAEC strains were found to be dominant and associated with infantile diarrhoea (Fang et al., 1995; Scaletsky et al., 2002b; Regua-Mangia et al., 2004; Araujo et al., 2007). The low rate of EAEC in our study may be due to geographical variation; indeed, EAEC strains have been found to vary markedly by region in the proportion that hybridize with the AA probe (Baudry et al., 1990; Okeke et al., 2000; Scaletsky et al., 2002c). Since the identification of EAEC isolates in this study was based on DNA probes, and considering that the HEP-2 adherence assay is the ‘gold standard’ for detecting EAEC strains, it is possible that our findings may not reflect the true frequency of these organisms in the population studied.

The low frequencies of ETEC and EIEC strains in children over 12 months old in our study are, in general, in agreement with previous studies performed in other Brazilian locations (Gomes et al., 1991; Rosa et al., 1998; Regua-Mangia et al., 2004).

In conclusion, our data suggest that DAEC should be considered potential pathogens in the region of Brazil studied. Further studies are needed to investigate the pathogenic mechanisms of DAEC isolates from the present study.

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