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.

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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.

Abbreviations: DAEC, diffusely adherent *E. coli*; EAEC, enteroaggregative *E. coli*; EAF, EPEC adherence factor; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin-producing *E. coli*.

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 diarrhoea who were 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 laboratory at Universidade Federal de São Paulo, São Paulo, Brazil. Strains were cultivated in commercial test systems (PROBAC do Brazil, São Paulo) for biochemical confirmation of the species or genus. All *E. coli* colonies were submitted to slide agglutination with polyvalent and monovalent antisera (PROBAC do Brazil) against the O antigens of EPEC serogroups and an O157 EHEC strain. All *E. coli* strains were maintained on nutrient agar slants at room temperature.

DNA hybridization. All *E. coli* isolates were screened 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 *Bam*HI–*Sal*I fragment from plasmid pMAR2; *eae* (encoding intimin, an outer-membrane protein involved in the attaching and effacing lesions promoted by EPEC and EHEC strains), a 1 kb *Sal*I–*Kpn*I fragment of pCVD434; AA (aggregative adherence plasmid), a 1 kb *Eco*RI–*Pst*I fragment of pCVD432; *daaC* (diffuse adhesin), a 390 bp *Pst*I fragment of pSLM852; LT, a 1.3 kb *Bam*HI fragment of pCVD403; STh, a 216 bp *Eco*RI fragment of pCVD427; STp, a 216 bp *Eco*RI fragment of pCVD427; Inv (EIEC invasion plasmid), a 2.5 kb *Hind*III fragment of pPS55; *Ehly*, a 3.4 kb *Hind*III fragment of pCVD443; Stx1, a 1.1 kb *Bam*HI fragment of pJN37-19; and Stx2, a 842 bp *Sma*I–*Pst*I fragment of pNN110-18 (Nataro & Kaper, 1998). The DNA probes were labelled with 50 µCi (185 MBq) [α -³²P]dCTP by using a random primer extension kit (Rediprime DNA labelling system; Amersham). Colony blots were hybridized at 65 °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.

Statistical analysis. Data derived from children with diarrhoea and from control subjects were compared by a chi-squared or Fisher's exact test.

RESULTS AND DISCUSSION

Subjects

A total of 304 stool specimens from 218 children with diarrhoea and 86 children without diarrhoea (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 diarrhoea 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 diarrhoea and 230 from 86 children without diarrhoea, 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 diarrhoea, 20 (23.2 %) were from controls, and 6 were found with another bacterial pathogen. Five children with diarrhoea 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). 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.

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

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

Pathogen	No. (%) of children		P value*	Odds ratio
	Patients (n=218)	Controls (n=86)		
Rotavirus	74 (33.9)	0	<0.01	35.66
<i>Shigella</i> spp.	8 (3.7)	0	—	
<i>Salmonella</i> spp.	6 (2.7)	0	—	
DAEC	40 (18.3)	7 (8.1)	<0.05	4.92
aEPEC	12 (5.5)	6 (6.9)	NS	
tEPEC	2 (0.9)	0	—	
EAEC	10 (4.6)	6 (6.9)	NS	
ETEC	7 (3.2)	1 (1.2)	—	
EIEC	1 (0.4)	0	—	
DAEC + <i>Salmonella</i> spp.	1	0	—	
EAEC + <i>Salmonella</i> spp.	2	0	—	
aEPEC + <i>Shigella</i> spp.	1	0	—	
ETEC + <i>Salmonella</i> spp.	1	0	—	
ETEC + <i>Shigella</i> spp.	1	0	—	

*NS, Not significant ($P>0.05$).

detected in children aged 7–12 months. Five diarrhoea-genic *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 *et al.*, 1993; Jallat *et al.*, 1993; Germani *et al.*, 1996). This is the second case-control study that we have conducted in Brazil showing an association of DAEC with diarrhoea

Table 2. Prevalence and characteristics of diarrhoeagenic *E. coli* among patients and controls, by age

<i>E. coli</i> type	Reactivity with DNA probe	EPEC serogroup	No. (%) of positive children by age group					
			0–6 months		7–12 months		13–24 months	
			Patients (n=69)	Controls (n=30)	Patients (n=80)	Controls (n=15)	Patients (n=69)	Controls (n=41)
DAEC	<i>daaC</i>	O128	12 (17.4)	3 (10.0)	12 (15.0)	2 (13.3)	16 (23.2)	2 (4.9)*
EPEC								
Typical	<i>eae</i> , EAF	O55	0	0	2 (2.5)	0	0	0
Atypical	<i>eae</i>	O26, O119, O142	5 (7.2)	3 (10)	5 (6.2)	1 (6.7)	2 (2.9)	2 (4.9)
EAEC	AA	—	6 (8.7)	3 (10.0)	4 (5.0)	1 (6.7)	0	2 (4.9)
ETEC	LT	—	0	0	0	0	6 (8.7)	1 (2.4)
	LT, STh	—	0	0	0	0	1	0
EIEC	Inv	—	0	0	1 (1.2)	0	0	0

* $P=0.01$.

(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; Yatsuyanagi *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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