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Diffusely Adherent *Escherichia coli* as a Cause of Acute Diarrhea in Young Children in Northeast Brazil: a Case-Control Study

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In a prospective study carried out in two urban centers in northeastern Brazil, 195 HEp-2-adherent *Escherichia coli* strains were isolated; 110 were identified as the only pathogen in stools of children with diarrhea, and 85 were from controls. Enteropathogenic *E. coli* isolates were identified in 21 children with diarrhea (8.9%) and 7 children without diarrhea (3.0%), and they were significantly associated with diarrhea ($P < 0.01$). Enteroaggregative *E. coli* strains were isolated from 40 children with diarrhea (16.9%) and 38 children without diarrhea (16.4%) and showed no correlation with diarrhea ($P > 0.5$). In 49 children with diarrhea (20.7%) and 40 children without diarrhea (17.3%), diffusely adherent *E. coli* (DAEC) isolates were detected and were not found to be associated with diarrhea ($P = 0.41$). However, after stratification, for children older than 12 months of age a significant correlation between DAEC infection and diarrhea was detected ($P = 0.01$). These results suggest that DAEC isolates should be considered potential pathogens in northeastern Brazil and also confirm the association of DAEC with age-dependent diarrhea.

Diarrhea remains an important public health problem for children in developing areas of northeastern Brazil. The bacterial pathogen most commonly associated with endemic forms of childhood diarrhea is *Escherichia coli*. At least five categories of diarrheagenic *E. coli* strains are recognized on the basis of distinct epidemiological and clinical features, specific virulence determinants, and an association with certain serotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC) (13). Recently, diffusely adherent *E. coli* (DAEC) strains have been recognized as the sixth class of diarrheagenic *E. coli* and appear as a heterogeneous group (13). EPEC, EAEC, and DAEC isolates are characterized by their distinct patterns of adherence to cultured epithelial cells in vitro. EPEC strains bind to host cells in a pattern called localized adherence (LA), in which microcolonies form on the surfaces of the cells (15). EAEC isolates bind in an aggregative adherence (AA) pattern, which is characterized by a stacked brick-like arrangement on the surfaces of the cells as well as those of glass or plastic containers (13). DAEC strains are defined by a pattern of diffuse adherence (DA), in which the bacteria uniformly cover the entire cell surface (15). The implication of DAEC strains in diarrhea remains controversial, since some studies have reported that these strains are found similarly in children with and without diarrhea (6, 8). Tacket et al. (18) were unable to conclusively induce diarrhea with DAEC in adult volunteers but suggested that DAEC may cause disease in immunologically naive or malnourished children. Discrepancies among epidemiological studies could be explained by age-dependent susceptibility to diarrhea or by the use of an inappropriate detection method such as DNA probing (10). The current prospective case-control study was done to determine the role of DAEC strains as a cause of acute diarrhea in northeastern Brazil, where childhood diarrhea is endemic.

The study was conducted at the Hospital de Pediatria da Universidade Federal do Rio Grande do Norte (Natal, Rio Grande do Norte) and the Hospital Universitário Materno-Infantil (São Luiz, Maranhão). All children less than 2 years of age with acute diarrhea who were brought to the hospital ambulatory clinics from May 1998 to June 1999 were enrolled in the study. Clinical information was collected by means of a standard questionnaire. A control group of children who were examined during the same time period contained asymptomatic children who were matched with children in the study group by age and who were randomly selected from the well-child outpatient clinics of the same hospitals.

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*, while the second swab specimen was stored in 2 ml of phosphate-buffered saline (pH 7.4) at 4°C until it was tested for rotavirus by enzyme immunoassay. Fecal samples and/or rectal swab specimens were obtained for detection of *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* spp. *E. coli* strains were isolated on MacConkey agar plates. Four separate lactose-fermenting colonies, presumed to be *E. coli* by colony morphology, and two non-lactose-fermenting colonies of each

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TABLE 1. DNA probes and control strains used for DNA hybridization assays

E. coli category	DNA probe specific for[a]:	Fragment used as a probe [restriction endonuclease(s)]	Control strain(s)[b]	Reference
ETEC	Enterotoxin LT	pCVD403 (1.3-kb *Bam*HI fragment)	H10407	11
	Enterotoxin STp	pCVD426 (157-bp *Pst*I fragment)		
	Enterotoxin STh	pCVD427 (216-bp *Eco*RI fragment)		
EIEC	Invasion	pPS55 (2.5-kb *Hin*dIII fragment)	EDL1284	17
EHEC	Adherence	pCVD419 (3.4-kb *Hin*dIII fragment)	EDL933	14
	Shiga toxin 1	pJN37-19 (1.142-kb *Bam*HI fragment)		
	Shiga toxin 2	pNN110-18 (842-bp *Sma*I-*Pst*I fragment)		
EPEC	EAF	pJPN16 (1-kb *Bam*HI-*Sal*I fragment)	E2348/69	12
	eaeA	pCVD434 (1-kb *Sal*I-*Kpn*I fragment)		9
DAEC	*daaC*	pSLM852 (390-bp *Pst*I fragment)	C1845	3
	AIDA-I	pIB6 (450-bp *Eco*RI fragment)	2787	2
EAEC	AA	pCVD432 (1-kb *Eco*RI-*Pst*I fragment)	042	1

[a] EAF, EPEC adherence factor plasmid; *eae*, the gene encoding intimin, an outer membrane protein involved in the attaching-effacing lesions promoted by EPEC; *daaC*, a gene associated with the biogenesis of F1845, a fimbrial adhesin involved in DA, AIDA-I, a protein associated with the DA phenotype; AA, with aggregative adherence plasmid pattern; LT, heat-labile enterotoxin; STp, porcine heat-stable enterotoxin; STh, human heat-stable enterotoxin.
[b] The negative control was *E. coli* K-12 strain C600.

distinct morphologic type were cultivated in commercial test systems (PROBAC do Brasil, São Paulo, Brazil) for biochemical confirmation of the species or genus. All *E. coli* colonies were submitted to slide agglutination with polyvalent and monovalent antisera (PROBAC do Brasil) against the O antigens of EPEC serogroups and EHEC strains. All *E. coli* strains were maintained in nutrient agar slants at room temperature.

All *E. coli* isolates were screened by colony DNA hybridization for detection of the diarrheagenic *E. coli* isolates listed in Table 1. The DNA probes were labeled with 50 μCi of [α-^{32}P]dCTP by use of 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)–0.1% sodium dodecyl sulfate solution, and exposed to X-ray film overnight at −80°C.

All *E. coli* isolates were individually tested for specific patterns of LA, DA, and AA to HEp-2 cells as described by Scaletsky et al. (15).

Data derived from children with diarrhea and from controls were compared by two-tailed chi-square and Fisher's exact tests.

In total, 468 fecal samples (of which 237 samples were from symptomatic children) were examined for enteric pathogens (Table 2). Rotavirus and *Shigella* spp. were isolated from 51 (21.5%) and 38 (16%) children with diarrhea, respectively, and from 13 (5.6%) and 5 (2.2%) controls, respectively (for both groups, $P < 0.01$). *Salmonella* spp. were isolated from three children with diarrhea and one control, *G. lamblia* was isolated from three children with diarrhea and three controls, and *E. histolytica* was isolated from two children with diarrhea and one control ($P > 0.05$).

E. coli isolates were tested with DNA probes to classify them into the different categories, and the same *E. coli* strains were also tested by the HEp-2 cell adhesion assay to identify the different adherence patterns (Table 2). A total of 221 diarrheagenic *E. coli* strains were isolated from the 468 fecal specimens: 26 nonadherent EPEC strains (atypical EPEC) and 195 HEp-2-adherent *E. coli* strains. Among the latter isolates, 110 were identified as the only pathogen in the stools of children with diarrhea, and 85 were from controls.

Twenty-one children with diarrhea (8.9%) and seven children without diarrhea (3.0%) carried typical EPEC strains that

TABLE 2. Isolation of pathogens from the stools of children with diarrhea[a]

Pathogen and test	No. (%) of children infected		*P* value[b]
	Diarrhea group	Control group	
Rotavirus	51 (21.5)	13 (5.6)	**<0.01**
Shigella spp.	38 (16.0)	5 (2.1)	**<0.01**
Salmonella spp.	3 (1.3)	1 (0.4)	0.97
E. histolytica	2 (0.8)	1 (0.4)	0.33
G. lamblia	3 (1.3)	3 (1.3)	0.58
Rotavirus + EAEC	2 (0.8)	0	
Rotavirus + DAEC	3 (1.3)	0	
Shigella spp + DAEC	2 (0.8)	0	
DAEC			
With *daaC* probe	31 (13.1)	27 (11.7)	0.75
By HEp-2 assay (DA pattern)	49 (20.7)	40 (17.3)	0.41
EAEC			
With AA probe	21 (8.9)	27 (11.7)	0.39
By HEp-2 assay (AA pattern)	40 (16.9)	38 (16.4)	1.00
EPEC typical			
With *eaeA* and EAF probes	21 (8.9)	7 (3.0)	**<0.01**
By HEp-2 assay (LA pattern)	21 (8.9)	7 (3.0)	**<0.01**
EPEC atypical, by *eaeA* probe	13 (5.5)	13 (5.6)	0.89

[a] A total of 237 children with diarrhea less than 2 years of age and 231 matched controls were studied.
[b] *P* values were determined by chi-square or Fisher's exact, test. Boldface data indicate a significant difference.

TABLE 3. Diarrheagenic *E. coli* detected among patients and controls by age group[a]

Organism	Age group[b] (mo)	No. (%) of infected children		*P* value[f]
		Diarrhea group	Control	
DAEC	0–5	8 (13.8)[c]	9 (16.1)[c]	0.93
	6–12	27 (21.6)[c]	28 (22.6)[c]	0.97
	13–24	14 (25.9)	3 (5.9)	**0.01**
	Total	49 (20.7)	40 (17.3)	0.41
EAEC	0–5	13 (22.4)[c]	7 (12.5)[c]	0.25
	6–12	21 (16.8)[c]	24 (19.3)[c]	0.13
	13–24	6 (11.1)[c]	7 (13.7)[c]	0.88
	Total	40 (16.9)	38 (16.4)	1.00
EPEC,[d] typical	0–5	14 (24.1)	2 (3.6)	0.003
	6–12	7 (5.6)	5 (4.0)	0.77
	13–24	0 (0.0)	0 (0.0)	
	Total	21 (8.9)	7 (3.0)	**<0.01**
EPEC,[e] atypical	0–5	5 (8.6)	1 (1.8)	0.11
	6–12	6 (4.8)	9 (7.2)	0.58
	13–24	2 (3.7)	3 (5.9)	0.47
	Total	13 (5.5)	13 (5.6)	0.89

[a] A total of 237 children with diarrhea less than 2 years of age and 231 matched controls were studied.
[b] For children with diarrhea there were 58, 125, and 54 children in each of the three age groups, respectively. For the controls, there were 56, 124, and 51 children in each of the three age groups, respectively.
[c] The isolates hybridized with the specific DNA probe.
[d] Typical isolates were EAF positive.
[e] Atypical isolates were EAF negative, and nonadherent.
[f] *P* values were determined by chi-square or Fisher's exact test. Boldface data indicate a significant difference.

were significantly associated with diarrhea ($P < 0.01$), particularly among children <6 months old (Table 3). EPEC has been shown to be an important cause of diarrhea in the region studied as well as in other regions of Brazil (16).

Forty children with diarrhea (16.9%) and 38 children without diarrhea (16.4%) were infected with EAEC; thus, there was no correlation between EAEC carriage and diarrhea ($P = 1.00$). Similarly, there was no age-related association of diarrhea with the presence of EAEC in feces (Table 3). However, in the group aged 0 to 5 months, the frequency of EAEC isolation was twofold higher for children with diarrhea. A number of EAEC isolates failed to hybridize with the EAEC probe. Studies in Fortaleza, another large urban center in northeastern Brazil, have associated EAEC with persistent diarrhea (4). However, in the present study, all the episodes identified were acute diarrhea.

DAEC was recovered from the stools of 49 children with diarrhea (20.7%) and 40 children without diarrhea (17.3%) (Table 2). Thus, overall, DAEC was not significantly associated with diarrhea ($P = 0.41$). In children <12 months of age, the presence of a DAEC isolate was not significantly associated with diarrhea ($P = 0.95$). In contrast, DAEC was significantly associated with diarrhea in children >12 months of age ($P = 0.01$) (Table 3); the symptoms found in these children were not distinct from those observed in children <12 months of age. None of the DAEC strains were positive for the classic serogroups tested, and none of them reacted with the AIDA-I probe. Thirty-one (13.1%) DAEC strains from children with diarrhea and 27 (11.7%) DAEC strains from the control group hybridized with the *daaC* probe; all the DAEC isolates from children >12 months of age were negative for hybridization with this probe. In a study conducted in New Caledonia, France, DAEC probe-positive strains were significantly associated with diarrhea only in children upon 2 years of age (5).

Our results demonstrate the previously unrecognized importance of DAEC as a cause of childhood diarrhea in the region of Brazil studied and support the evidence from prospective case-control studies showing an association of DAEC with age-dependent diarrhea (7). In the present study, DAEC was associated with diarrhea in children >12 months of age; in contrast, the presence of DAEC in younger children was not associated with diarrhea. Further characterization of the virulence factors of DAEC strains, including identification of the adhesins of *daaC*-negative DAEC isolates from the present study, is under investigation.

In conclusion, our findings support the association of DAEC with diarrheal disease in children >12 months of age. Statistical analysis of *E. coli* strains isolated from diarrheal stool specimens and controls indicated that diffusely adherent *E. coli* strains should be considered potential pathogens in northeastern Brazil.

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