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DIFFUSE AND ENTEROAGGREGATIVE PATTERNS OF ADHERENCE OF *ESCHERICHIA COLI* ISOLATED FROM STOOLS OF CHILDREN IN NORTHEASTERN BRAZIL

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ABSTRACT

Childhood diarrheal diseases remain highly endemic in northeastern Brazil. The attributable fraction of all diarrheal diseases among children less than 2 years of age due to *Escherichia coli* was examined in a 2-year prospective study in two large urban centers of Brazil. Between May 1997 and June 1999, fecal *E. coli* isolates from 237 children with diarrhea (217 acute and 20 persistent cases) and 231 children without diarrhea (controls) attending two hospitals in Northeast Brazil were tested for their pattern of adherence to HEp-2 cells and for colony hybridization with DNA probes specific for the six pathotypes of diarrheagenic *E. coli*. Enteroinvasive *E. coli*, enterotoxigenic *E. coli* and enterohemorrhagic *E. coli* were not isolated from any children. Diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) were the most frequent isolates with similar frequencies from children with or without diarrhea. Atypical EPEC (EAF-negative) strains were isolated with similiar frequency from both cases (5.5%) and controls (5.6%). Enteropathogenic *E. coli* (typical EPEC) strains, characterized by localized adherence pattern of adherence, hybridization with the EAF probe, and belonging to the classical O serogroups, were significantly associated with diarrhea ($P = 0.03$). These *E. coli* strains associated with diarrhea accounted for 9% of all children with diarrhea. Collectively, in Northeast Brazil, *E. coli* strains comprise a small proportion of severe diarrhea prevalence in children.

Key words: *Escherichia coli*, diffusely adherent *E. coli*, enteroaggregative *E. coli*, adherence patterns, childhood diarrhea.

INTRODUCTION

Diarrheagenic *Escherichia coli* comprise an important group of pathogens associated with enteric diseases. Six pathogenic types (pathotypes) of *E. coli* associated with diarrhea are currently recognized: enterotoxigenic (ETEC), typical enteropathogenic *E. coli* (typical EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse adherent *E. coli* (DAEC) (29). Recently, a new pathotype, named atypical EPEC was reported as a putative cause of diarrhea in infants in Brazil (defined below) (35). It has become clear that there are regional differences in the relative prevalence of the different diarrheagenic *E. coli* groups (1,4,6,7,8,14,17,21) and such a difference may affect the overall regional prevalence of diarrheal diseases.

Detection of diarrheagenic *E. coli* has been facilited by tissue culture assays as well as by DNA probes specific to each group of *E. coli*. The DNA fragments used as probes hybridize to either adhesin or toxin-encoding genes (5,19,26,30) or with genes linked with the presence of a phenotype (3,37).

EPEC, EAEC and DAEC are characterized by their distinct patterns of adherence to cultured epithelial cells *in vitro*. Typical

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EPEC strains bind to host cells in a pattern called localized adherence (LA), where microcolonies form on the surface of cells (HeLa or HEp-2) (32). EAEC bind in an aggregative adherence (AA) pattern, which is characterized by a stacked-brick-like arrangement on the surface of cells as well as of the glass or plastic (28). DAEC strains are defined by a diffuse pattern of adherence (DA), where the bacteria cover the entire surface of cells uniformly (32). Recently, Scaletsky *et al*. (34) described a new adherence pattern called localized adherence-like (LAL) pattern. This pattern is characterized by less compact microcolonies or clusters of bacteria on the surface of a few cells observed only after prolonged incubation periods (6 h). *E. coli* strains exhibiting such a pattern of adherence and also lacking the EAF plasmid were labeled atypical EPEC (35).

This study was designed to determine the relative prevalence and the role of the different *E. coli* pathotypes in acute and persistent diarrhea among infants in 2 large urban centers in northeastern Brazil known for their low socio economic status and high prevalence of diarrhea and malnutrition.

MATERIALS AND METHODS

Patients

The study was conducted at Hospital de Pediatria da Universidade Federal do Rio Grande do Norte (Natal, Rio Grande do Norte) and Hospital Universitário Materno-Infantil (São Luiz, Maranhão). From May 1997 to June 1999, all children less than 2 years of age with acute or persistent diarrhea who were brought to the hospital ambulatory clinics were enrolled in the study. Clinical information was collected by means of a standard questionnaire. The information requested included age, sex, clinical symptoms (fever, vomiting, and dehydration status), type and duration of diarrhea, and history of antibiotic therapy prior to the clinic visit. Diarrhea was defined as the excretion of three or more liquid stools during a 24-h period before admission. Acute diarrhea was defined as diarrhea of less than 14 days duration at the time of admission. Persistent diarrhea was defined as diarrhea of a presumably infectious etiology lasting more than 14 days. A control group containing asymptomatic children matched for age was randomly selected from the well-child outpatient clinic of the same hospitals, examined during the same study time period. Control infants had had no gastrointestinal symtoms for at least 30 days prior to inclusion in this study.

Microbiological studies

Two rectal swabs were collected from each child, placed in Cary-Blair transport medium, and processed within 4 h. One swab was processed by routine microbiological and biochemical tests to identify *E. coli*, *Salmonella* spp. *Shigella* spp.,*Campylobacter* spp., and*Yersinia enterocolitica* (11), while the second swab was stored in 2 ml of phosphate-buffered saline (pH 7.4) at 4ºC until tested for rotavirus by enzyme immunoassay (EIA) (13). Fecal samples and/or rectal swab specimens were obtained for detection of *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* spp (18). *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 distinct morphologic type were cultivated in commercial test systems (PROBAC do Brasil, São Paulo, Brazil) such as EPM (fermentation of sucrose, urea hydrolysis, and gas production) and MiLi (motility, indole, and lysine decarboxylase) (39) media for biochemical confirmation of species or genus. All *E. coli* colonies were tested for slide agglutination with commercial polyvalent and monovalent antisera (PROBAC do Brasil, São Paulo, Brazil) against the following serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128ab, O142, O157, and O158. When two or more colonies of identical serotypes were isolated from the same child, only one colony was kept. The *E. coli* strains were maintained in nutrient agar slants at room temperature.

Adhesion assay

All *E. coli* isolates were characterized by the pattern of adherence to HEp-2 cells in the presence of D-mannose according to the method described by Scaletsky et al. (32). Briefly, monolayers of 10^5 HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) using 24-well tissue culture plates (Falcon Becton Dickinson, New Jersey, EUA). Bacterial strains were grown statically in 3 ml of tryptic soy broth (Difco, Detroit, MI) for 16-18 h at 37ºC. The monolayers were infected with approximately 3×10^7 bacteria (40 µl of bacterial cultures added to 1 ml of DMEM) and incubated at 37ºC for 3 h. The infected monolayers were washed with sterile phosphate-buffered saline (PBS), fixed with methanol, stained with May-Grunwald-Giemsa stain, and examined under a light microscope. Strains that adhered to the monolayers were recorded as adhering in localized (LA), localized-like (LAL), diffuse (DA), or aggregative (AA) patterns.

DNA probe hybridization

The various DNA probes used for detection of diarrheagenic *E. coli* are shown in Table 1. DNA probes were prepared from recombinant plasmids containing the DNA probe fragments as inserts. Plasmids were prepared, purified, and digested with restriction endonucleases (Gibco-BRL, Grand Island, NY), and the appropriate restriction fragments were purified by gel extraction. The DNA fragments were labeled by random primer extension kit (Rediprime DNA Labelling System, Amersham) with 50 µCi of [a-^{32}P]dCTP (Amersham Life Science Products, Airlington Heights, Ill.). Colony blots were prepared on Whatman 541 filter papers which were then processed and hybridized under stringent conditions as described previously

(25). The positive control *E. coli* strains used for hybridization reactions included: EDL933 (for Shiga-like toxin I [stxI] and stxII), H10407 (for heat-labile [LT] and stable-labile [ST] enterotoxins); EDL1284 (for invasion); E2348/69 (for EPEC adherence factor [EAF], and attachment-effacement factor [AE]; 17-2 (for aggregative adherence); and C1845 (for diffuse adherence).

Statistical analysis

Frequency of isolation of pathogens from children with and without diarrhea was compared by a two-tailed chi-square or Fisher's exact test.

RESULTS

A total of 237 children (217 acute and 20 persistent) and 231 matched control children without diarrhea were studied. We identified potential diarrheagenic *E. coli* by assays of adhesion to HEp-2 cells and by hybridization with specific DNA probes.The isolation frequency of pathogens from children with diarrhea is shown in Table 2. Putative diarrheagenic *E. coli* was isolated from 51.9% (123 of 237) of children with diarrhea compared with 42.4% (98 of 231) of children without diarrhea ($P > 0.05$). In children with diarrhea, DAEC was the most prevalent *E. coli* group identified, followed by EAEC, typical EPEC and atypical EPEC. EIEC, ETEC, and EHEC were not detected in any children. Of children with diarrhea, 49 (20.7%) of 237 were infected with more than one of the potential diarrheal *E. coli* pathogens that were tested for, while 44 (18.7%) of 237 were colonized with both diarrheagenic *E. coli* and one of the other enteric pathogens, while no control children were colonized with more than one category of diarrheagenic *E. coli*. Rotavirus and *Shigella* spp were isolated from 51 (21,5%) and 38 (16%) children with diarrhea and from 13 (5.6%) and 5 (2.2%) controls, respectively ($P < 0.00$). Three children with persistent diarrhea were infected with *Shigella*, 4 children with EAEC, and 4 children with DAEC. Twenty infants were infected with DAEC and rotavirus, 4 infants with DAEC and *Shigella*, 14 infants were infected with EAEC and rotavirus, and one infant with atypical EPEC and rotavirus.

DAEC and EAEC strains were the most frequent isolates with similar frequencies from cases (20.7 - 16.9%) and controls (17.3 - 16.4%), respectively (Table 2). The detection frequency of DAEC and EAEC by specific DNA probes was different compared with the HEp-2 cell adherence pattern (Table 2). The *daaC* probe detected 58 (65.2%) of 89 isolates which yielded the DA pattern: 31 of 49 from cases and 27 of 40 from controls. Similarly, EAEC probe used to detect EAEC isolates reacted with 48 (61.5%) of 78 that yielded the AA pattern: 21 from cases and 27 from controls. One isolate with DA carrying *daaC* also hybridized with the *eaeA* probe. Three isolates with AA pattern hybridized with both EAEC and *daaC* probes.

From 89 DAEC strains producing DA on HEp-2 cells, only one from a child belonged to one of the classic serogroups, O128 (Table 3). Of the 78 EAEC isolates producing AA, 6 belonged to a classical serogroup. The serogroups detected were O125 (2 cases and 1 control), and O126 (1 case), and O127 (1 child each).

Typical EPEC was the only diarrheagenic category of *E. coli* significantly associated with diarrhea ($P = 0.03$) (Table 2). We characterized typical EPEC on the basis of its localized adherence (LA) pattern and positive reactions to EAF and *eaeA* probes. Strains with LA were more frequently isolated from the

Table 1. DNA probes used for detection of diarrheagenic *E. coli.*

E. coli pathotype	Pathogenic factor detecteda	Recombinant plasmid containing the probe	Probe obtained with restriction enzyme	Ref.
ETEC	Enterotoxin LT	pCVD403	1.3-kb *Bam*HI	26
	Enterotoxin STp	pCVD426	157-bp *Pst*I	
	Enterotoxin STh	pCVD427	216-bp *Eco*RI	
EIEC	Invasion	pPS55	2.5-kb *Hin*dIII	37
EHEC	Adherence	pCVD419	3.4-kb *Hin*dIII	30
	Shiga-toxin I	pJN37-19	1.142-kb *Bam*HI	
	Shiga-toxin II	pNN110-18	842-bp *Sma*I-*Pst*I	
EPEC	EAF	pJPN16	1-kb *Bam*HI-*Sal*I	27
	eaeA	pCVD434	1-kb *Sal*I-*Kpn*I	19
DAEC	*daaC*	pSLM852	390-bp *Pst*I	5
EAEC	AA	pCVD432	1-kb *Eco*RI-*Pst*I	3

[a] EAF, EPEC adherence plasmid; *eaeA*, encoding intimin, an outer membrane protein involved in the attaching-effacing lesions promoted by EPEC; *daaC*, associated with the biogenesis of F1845, a fimbrial adhesin involved in DA; AA, aggregative adherence plasmid.

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

Pathogen	No. of children (%) infected		*P* value
	Diarrheal	Control	
DAEC			
By HEp-2 assay (DA pattern)	49 (20.7)[b]	40 (17.3)	0.71
By *daaC* probe	31 (13.1)	27 (11.7)	0.75
EAEC			
By HEp-2 assay (AA pattern)	40 (16.9)	38 (16.4)	0.22
By EAEC probe	21 (8.9)[c]	27 (11.7)[c]	0.39
EPEC typical			
By HEp-2 assay (LA pattern)	21 (8.9)	7 (3.0)	0.03
By *eaeA*, EAF probes	21 (8.9)	7 (3.0)	0.03
EPEC atypical			
By HEp-2 assay (LAL pattern)	4 (1.7)	2 (0.9)	0.06
By *eaeA* probe	13 (5.5)	13 (5.6)	-
Rotavirus	51 (21.5)	13 (5.6)	0.00
Shigella spp.	38 (16.0)	5 (2.1)	0.00
Salmonella spp.	3 (1.3)	1 (0.4)	0.97
Entamoeba histolytica	2 (0.8)	1 (0.4)	0.33
Giardia lamblia	3 (1.3)	3 (1.3)	0.58

[a] 237 diarrheal children and 231 matched controls were studied during May 1997 to June 1999 in northeastern Brazil; [b]isolate also hybridize with the *eaeA* probe; [c]isolates also reacted with the *daaC* probe.

Table 3. Distribution of classical serogroups among EPEC, EAEC and DAEC strains in case and control infants.

Serogroup	DAEC Cases/ Controls (n = 49 / 40)	EAEC Cases/ Controls (n = 40 / 38)	Typical EPEC Cases/ Controls (n = 21 / 7)	Atypical EPEC Cases/ Controls (n = 13 / 13)
O26	0 / 0	0 / 0	0 / 0	1 / 0
O55	0 / 0	0 / 0	6 / 0	0 / 1
O111	0 / 0	0 / 0	4 / 0	0 / 1
O114	0 / 0	0 / 0	0 / 0	0 / 3
O119	0 / 0	0 / 0	7 / 2	0 / 1
O125	0 / 0	2 / 1	0 / 0	0 / 0
O126	0 / 0	1 / 0	0 / 0	2 / 0
O127	0 / 0	1 / 1	0 / 3	0 / 0
O128	0 / 1	0 / 0	0 / 0	0 / 0
O142	0 / 0	0 / 0	0 / 0	0 / 0
Total	0 / 1	4 / 2	17 / 5	3 / 6

diarrhea group than controls, 21 of 237 (8.9%) versus 7 of 231 (3.0%), respectively. The *eaeA* DNA probe, which is specific for both typical and atypical EPEC, detected 54 isolates (Table 2). Twenty-eight *eaeA* -positive isolates hybridized with the EAF probe and exhibited a LA phenotype, indicating that they belonged to the typical EPEC pathotype. Six out of the remaining 26 atypical EPEC (EAF- negative) showed an LAL pattern. Strains showing the LAL pattern were found both in children with diarrhea (1.7%) and controls (0.9%).

Twenty-two out of the 28 typical EPEC isolates from cases (17 children) and controls (5 children) belonged to a classical serogroup (Table 3): O55 (6 cases), O111 (4 cases), O119 (7 cases and 2 controls), and O127 (3 controls). Five typical EPEC isolates did not fall into any traditional EPEC serogroups and one detected in a case child belonged to serogroup O157. The 26 atypical EPEC isolated from cases and controls revealed classical serogroups in 9 isolates: O26 (1 case), O55 and O111 (1 control each), O114 (3 controls), O119 (1 control), and O126 (2 cases). Serogroups O26, O126, and O111 were found among atypical EPEC showing LAL.

DISCUSSION

Diarrhea remains an important public health problem for children in developing areas of Brazil's northeast. The importance of *E. coli* as a cause of diarrhea and its attributable fraction to the diarrhea prevalence in this region was unknown. The present study was performed to determine the prevalence of different *E. coli* pathotypes in children with diarrhea in two large urban centers in the northeast Brazil. While potential diarrheal *E. coli* strains were isolated from more than half (52%) of the children with diarrhea, only typical EPEC strains in children with diarrhea were clearly associated with diarrhea. These children accounted for 9% of all the cases of diarrhea. The study included children visiting hospital clinics with diarrhea and thus involved children with diarrhea severe enough to require medical attention. Thus, EPEC may represent just a small fraction of all those responsible for diarrheal diseases that occur in communities. After rotavirus and *Shigella*, these *E. coli* strains were responsible for more cases of diarrhea than any other pathogen.

In our study, all strains isolated from cases and controls showing LA hybridized with the EAF and *eaeA* probes. Gomes et al. (16) suggested that detection of EAF in a strain that belonged to the classical EPEC serogroup (20, 31) might be a reliable test for detecting EPEC associated with diarrhea. Although the number of strains detected in this study was too small to draw any significant conclusion, O55 and O111 serogroups were detected only in cases. These two serogroups have been prevalent in Brazil in the last 25 years (40, 41). The characteristic phenotype of LA known to correlate with the classic O serogroups (33) and diarrheal disease was confirmed in this study. Eighty-one percent of EPEC strains isolated from cases belonged to classic O serogroups. However, serogrouping DAEC and EAEC isolates, did not bring any additional information about their pathogenic potentials. O125 and O127 EAEC isolates were found in cases and controls.

The remaining *eaeA*-positive EAF-negative isolates in this study, considered atypical EPEC, could be either virulent or avirulent. These strains carry only the *eaeA* gene and do not possess the EAF plasmid. Indeed, volunteer-based studies performed by Levine *et al.* (22) have shown that JPN15 (a plasmid cured EPEC strain) caused diarrhea but it was less severe than that caused by the wild-type strain. Thus, it is possible that clinical isolates of EPEC lacking EAF have the ability to cause diarrhea. In the present study, atypical EPEC were detected both in cases and controls in similar frequency. Six of these 26 strains showed an LAL pattern, 4 in cases and 2 in controls. Although there were no statistically significant differences and the number of isolates was small, LAL pattern isolates were isolated twice as often from cases than from controls. In a previous study in São Paulo in southeast Brazil, we have demonstrated that atypical EPEC strains showing an LAL pattern were significantly associated with diarrhea (35). However, in this study, performed in another geographic region of Brazil, there was no significant correlation between *E. coli* exhibiting LAL and diarrhea.

Strains belonging to EAEC have been implicated as etiologic agents of infantile gastroenteritis in both developing (4, 9, 43) and developed countries (36), most predominantly among patients with diarrhea that persists longer than 14 days. Fang *et al*. (12) and Lima et al (24) working in Fortaleza, another large urban center in northeastern Brazil demonstrated a significant association between EAEC and persistent diarrhea. In the present study EAEC strains were equally detected in cases and controls without any statistical differences between the two groups. Conflicting results about the role of EAEC in persistent diarrhea have been published elsewhere (4,12,16, 21, 42). These differing results may reflect differences in the nutritional status of the populations examined. EAEC may be more important as a cause of persistent diarrhea in mal-nourished children.

These differences in results may also reflect differences in the detection tests used. In this study, EAEC strains were detected by the organism's HEp-2 adhesion pattern (78 strains) and by hybridization with the EAEC probe (48 strains). The relevance of the EAEC probe in epidemiological studies is uncertain. EAEC probe was highly sensitive and specific in an epidemiological study involving strains from Chile and India (3). However, in our study, only 61.5% of the EAEC strains exhibiting AA pattern of adherence were detect with this probe. Such discrepancies in the performance of this DNA probe were also reported in studies conducted in northeastern Brazil (12) and this prompted us to study new EAEC genotypes. Of the 78 EAEC strains, 4 hybridized with *daaC* probe. EAEC strains carrying *daaC* have been previously reported (15).

Most of *E. coli* strains isolated in this study belonged to the DAEC pathotype: 18% (89 of 468) isolates were defined phenotypically by their pattern of adherence to HEp-2 cells and 12.4% (58 of 468) were defined genotypically by hybridization with the *daaC* probe. There is still much debate over whether DAEC strains cause diarrhea, or whether DAEC strains comprise a distinct group of *E. coli*. Many field reports (2,10,15,16,17,23), as well as volunteer studies (38) report contradictory results. In the current study, there was no significant correlation between DAEC isolates and diarrhea. However, most of the children in the present study were under 1 year of age, and as suggested by other authors DAEC may be more important as a diarrheal pathogen in older populations or in specific age groups of children (17,21).

In conclusion, the majority of *E. coli* strains isolated in this prospective-study belonged to the DAEC and EAEC pathotypes. However, they were present in similar proportions in both children with or without diarrhea. On the other hand, EPEC strains were confirmed as important causes of enteric infections in children less than 2 years of age. They were the third most important group of pathogens associated with diarrhea. Further studies are needed to characterize the epidemiology of *E. coli* diarrhea in urban centers of Brazil.

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RESUMO

Isolamento dos padrões de adesão difusa e agregativa de *Escherichia coli* nas fezes de crianças da região nordeste do Brasil

Na região nordeste do Brasil a doença diarréica na infância continua sendo altamente endêmica. Com o objetivo de determinar a prevalência das diferentes categorias de *E. coli* diarreiogênica foi realizado um estudo prospectivo de dois anos em dois grandes centros urbanos dessa região. Entre maio de 1997 a junho de 1999, foram examinadas amostras de *Escherichia coli* isoladas de 237 fezes de crianças com diarréia (217 aguda e 20 persistente casos) e 231 de crianças sem diarréia (controles) atendidas em dois hospitais na região nordeste do Brasil quanto a adesão a células HEp-2 e hibridização com sondas genéticas específicas para as seis categorias de *E. coli* diarreiogênica. *E. coli* que adere difusamente (DAEC) e *E. coli* enteroagregativa (EAEC) foram as categorias mais frequentemente isoladas tanto em casos como em controles. *E. coli* enteropatogênica atípica (EPEC) foi isolada tanto em casos (5.5%) como em controles (5.6%). Amostras de *E. coli* enteropatogênica (EPEC típica), caracterizadas pelo padrão de adesão localizada, hibridização positiva com a sonda EAF e pertencentes aos sorogrupos clássicos foram mais frequentes em casos do que em controles,

significantemente relacionadas com diarréia ($P = 0.03$). Essas amostras constituíram o terceiro patógeno, depois de rotavirus e *Shigella* spp, com predomínio significantemente maior entre os casos. Concluindo, na região nordeste do Brasil as EPEC respondem por 9% dos casos de diarréia grave em crianças menores de dois anos.

Palavras-chave: *Escherichia coli*, *E. coli* que adere difusamente, *E. coli* enteroagregativa, padrões de adesão, diarréia infantil.

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