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Adherence Patterns and Adherence-Related DNA Sequences in *Escherichia coli* Isolates from Children with and without Diarrhea in São Paulo City, Brazil

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The correlation between various adherence patterns and adherence-related DNA sequences in *Escherichia coli* isolates from 1- to 4-year-old children with and without diarrhea in São Paulo, Brazil, was evaluated. A total of 1,801 isolates obtained from 200 patients and 200 age-matched controls were studied. The adherence patterns found were classified as diffuse, aggregative, aggregative in a 6-h assay, aggregative predominantly in coverslips, localized, localized-like, and noncharacteristic. In general, the DNA sequences used as probes showed excellent specificities (>93%), but their sensitivities varied. Thus, the results of bioassays and assays with DNA probes normally used to search for adherent *E. coli* did not correlate well, and the best method for the identification of these organisms in the clinical research setting remains controversial. Isolates presenting diffuse adherence or hybridizing with the related *daaC* probe, or both, were by far the most frequent in patients (31.5, 26.0, and 23.0%, respectively), followed by isolates presenting aggregative adherence or hybridizing with the related EAEC probe, or both (21.5, 13.0, and 10.5%, respectively). None of the different combinations of adherence patterns and adherence-related DNA sequences found were associated with acute diarrhea.

The first step in the establishment of the diarrheal diseases caused by the various categories of diarrheagenic *Escherichia coli* is adherence to epithelial cells of the intestinal mucosa. In vitro assays with eukaryotic cell lines (HeLa and HEp-2 cells) have identified three distinct adherence patterns among fecal isolates of *E. coli*: localized, diffuse, and aggregative (37, 38, 41). Localized adherence (LA) is characterized by formation of bacterial microcolonies on a restricted area(s) of the cell surface, while diffuse adherence (DA) is the scattered attachment of bacteria over the whole surface of the cell (41). The pattern of aggregative adherence (AA) consists of bacterial attachment to the cells and the intervening cell growth surface in a stacked brick-like lattice (37).

The LA pattern was first detected in strains classified as enteropathogenic *E. coli* (EPEC) among serogroups associated with outbreaks of infantile diarrhea (41). Although *E. coli* strains exhibiting DA (DAEC) have been isolated at similar frequencies from feces of infants and young children with acute diarrhea and nondiarrheic controls in some populations (3, 10, 11, 14, 18), they were significantly associated with diarrhea in other settings (1, 17, 24, 29, 33). *E. coli* strains showing AA, termed enteroaggregative *E. coli* (EAEC), have been linked to sporadic persistent diarrhea (3, 4, 7, 10, 13, 26, 27, 44) and to outbreaks of diarrhea in both developing and developed countries (8, 12, 28, 43). However, the role of EAEC in acute diarrhea is still controversial: some studies have shown a correlation (7, 23, 25, 27, 34, 37), but others (1, 3, 6, 10, 11, 13–15, 17, 18, 24, 26, 29, 33, 44) have not.

DNA probes derived from adherence-related sequences have been constructed (2, 5, 16, 31, 36) and used in hybridization assays for the detection of the different established and putative categories of diarrheagenic *E. coli* in many epidemiological studies.

We evaluated the relationship between the LA, DA, and AA patterns and hybridization with adherence-related DNA sequences and tested children 1 to 4 years old with and without acute diarrhea for the presence of adherent *E. coli* strains.

MATERIALS AND METHODS

Patients. Rectal swab specimens were collected from 200 children with acute (≤7 days) diarrhea (patients) and 200 children who had not had any gastrointestinal signs or symptoms during the 30 days prior to collection (controls). These children were selected by a computer-generated random-number table from among 505 patient-control pairs obtained for a study on the etiology of acute diarrheal diseases (19). The population analyzed consisted of children visiting the emergency room of the Hospital Infantil Menino Jesus, São Paulo, Brazil, for medical attention between April 1989 and March 1990; most children were of low socioeconomic status. Controls were matched with case patients in the following age groups: 12 to 23, 24 to 35, and 36 to 59 months.

Microbiologic methods. Adenovirus, *Aeromonas* spp., thermophilic *Campylobacter*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, and rotavirus were searched for by standard methods (20, 30). Five lactose-fermenting and any non-lactose-fermenting colonies typical of *E. coli* were selected from the isolation plates. Biochemically confirmed *E. coli* isolates were stored in 15.0% glycerol at −70°C. All *E. coli* isolates were tested with specific DNA probes designed to detect enterotoxigenic *E. coli* (ETEC) (LT-I, LT-II, ST-Ip, ST-Ih, and ST-II probes), enteroinvasive *E. coli* (EIEC) (Inv probe), and Shiga-toxin-producing *E. coli* (STEC) (Stx1 and Stx2 probes), as described previously (22).

HeLa cell adherence assays. The HeLa cell adherence assays were performed as described by Cravioto et al. (9) with HeLa cells grown to 60% confluence and a single infection period of 3 h. Weakly adherent and nonadherent isolates were retested by the same method with an additional incubation period of 3 h (6-h assay) (9). The following *E. coli* strains were included as controls: E2348/69 (LA)

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TABLE 1. Relationship between hybridization with DNA probes for adherence-related sequences and patterns of adherence to HeLa cells of *E. coli* isolated from children with and without diarrhea

Adherence pattern[a]	No. of isolates	No. (%) of isolates that hybridized with the following DNA probe(s):							
		daaC only	EAEC only	*daaC* and EAEC	*daaC* and *eaeA*	*eaeA* only	*eaeA* and *bfpA*	*eaeA*, EAF, and *bfpA*	None
DA	321	197 (61.4)	0	0	5 (1.5)	0	0	0	119 (37.1)
AA (typical)	256	0	134 (52.3)	56 (21.9)	0	0	0	0	66 (25.8)
AA6h	27	0	0	0	0	0	0	0	27 (100)
AAcs	132	3 (2.3)	5 (3.8)	0	0	3 (2.3)	0	0	121 (91.6)
LA (typical)	22	0	0	0	0	0	0	22 (100)	0
LAL	14	0	0	0	0	9 (64.3)	5 (35.7)	0	0
NC	183	2 (1.1)	2 (1.1)	0	7 (3.8)	41 (22.4)	2 (1.1)	0	128 (70.5)
NA	846	30 (3.6)	26 (3.1)	1 (0.1)	0	2 (0.2)	0	0	788 (93.0)
Total	1,801	232	167	57	12	55	7	22	1,249

(36), C1845 (DA) (5), and 0431-4/85 (AA) (18). Strains were tested in duplicate and were examined blindly by immersion microscopy.

Search for adherence-related DNA sequences in *E. coli*. The following specific DNA probes were used: *daaC* (associated with the biogenesis of F1845, a fimbrial adhesin involved in DA), a 350-bp *Pst*I fragment of pSLM852 (5); EAEC (EAEC adherence plasmid), a 1-kb *Eco*RI-*Pst*I fragment of pCVD432 (2); EAF (EPEC adherence factor), a 1-kb *Bam*HI-*Sal*I fragment derived from plasmid pMAR2 (36); *bfpA* (encoding the subunits of a fimbrial adhesin of EPEC strains, termed the bundle-forming pilus), an 852-bp *Eco*RI fragment of pMSD207 (16); and *eaeA* (encoding intimin, an outer membrane protein involved in the attaching-effacing lesions promoted by EPEC and some STEC strains), a 1-kb *Sal*I-*Kpn*I fragment from plasmid from pCVD434 (31). The fragment probes were labeled with [α-^{32}P]dATP and used in colony hybridization assays performed under stringent conditions as described by Maas (35).

Statistical analysis. Analysis of patients and controls maintained the matched pairs and used the Pike-Morrow adaptation of the Mantel-Haenszel test to calculate *P* values (42). Epi-Info 6.02 was used to perform the calculations.

RESULTS

***E. coli* adherence to HeLa cells.** The adherence patterns of 273 isolates could not be determined because they promoted complete detachment of the HeLa cell monolayers in the 3-h assays. Among the remaining 1,801 *E. coli* isolates tested (879 from patients and 922 from controls), 47.0% were nonadherent (NA; i.e., sparse bacteria adhering to <1% of the cells in the 6-h assay). The adherence patterns of the other isolates are presented in Table 1.

The degree and frequency of DA varied, but this pattern could always be detected in the 3-h assay. Regarding the AA pattern, 3 types were detected: typical AA, AA that could be clearly discerned only in the 6-h assay (AA6h), and AA predominantly in coverslips (AAcs). The typical LA adherence pattern was characterized by one to three tight bacterial clusters per cell on 75 to 100% of the cells. An LA-like (LAL) pattern of adherence (40), characterized by loose and compact clusters of bacteria on 1 to 45% of the cells, was detected in the 6-h assay for some isolates that presented an undefined pattern in the 3-h assay. Moreover, some isolates presented adherence patterns distinct from those described in the literature; the adherence patterns of these isolates were classified as noncharacteristic (NC).

Correlation between adherence patterns and hybridization with adherence-related DNA probes. The relationship between the distinct adherence patterns detected and hybridization with specific DNA probes found in this study is presented in Table 1. The *daaC* probe reacted with 202 of 321 isolates with DA (sensitivity, 62.9%) and with 99 isolates that were NA or that presented adherence patterns distinct from DA (specificity, 93.3%). The EAEC probe detected 190 of the 256 isolates with AA and 5 of the 132 isolates with AAcs (sensitivity, 47.0%) and reacted with only 29 isolates showing none of the different AA types (specificity, 97.9%). The *eaeA* probe reacted with all isolates with LA and LAL (sensitivity, 100.0%) and with 60 non-LA- and non-LAL-producing isolates (specificity for the detection of LA- and LAL-producing *E. coli*, 96%). All isolates with LA hybridized with the EAF and *bfpA* probes, whereas none of the isolates with LAL reacted with the EAF probe, and only five (35.7%) isolates reacted with the *bfpA* probe. Most (60.3%) of the 273 cytodetaching isolates did not hybridize with any of the probes used.

Prevalence of adherence patterns and adherence-related DNA sequences in patients and control children. The distribution of the *E. coli* isolates showing different patterns of adherence or related DNA sequences in patients and controls is presented in Table 2. Approximately 50% of the children studied carried more than one type of isolate. The statistical analysis of the association with diarrhea was performed for those children in whose stools none of the other pathogens were identified (adenovirus, *Aeromonas*, *Campylobacter*, EIEC, ETEC, rotavirus, *Salmonella*, *Shigella*, and STEC).

None of the adherence patterns detected was associated with acute diarrhea. DA was the most frequent pattern among isolates from both patients and controls (31.5 versus 28.5%), followed by AA (21.5 versus 19.0%) and AAcs (12.5 versus 15.0%). Likewise, none of the adherence-related DNA sequences were associated with acute diarrhea. Isolates carrying *daaC* were the most frequent among the patients and controls (26.0 versus 21.0%). Similarly, none of the different combinations of adherence patterns and related DNA sequences were associated with diarrhea (Table 3). Isolates carrying *daaC* and producing DA (23.0%) and EAEC-positive isolates producing AA (10.5%) were the most frequent in patients.

DISCUSSION

In this study we analyzed the correlation between different adherence patterns and the presence of adherence-related DNA sequences in *E. coli* isolates from diarrheic and control children. The adherence-related DNA probes used showed excellent specificities (>93.0%), but their sensitivities varied. The low sensitivities of the *daaC* and EAEC probes found in this study (62.9 and 47.0%, respectively) are in accordance with those found in other field studies and confirms the heterogeneous nature of the DAEC and EAEC categories (2, 11, 13, 17, 29). It is interesting that none of the isolates that showed AA6h reacted with the EAEC probe; whether these isolates comprise a homogeneous population remains to be established. Furthermore, all 56 isolates that were both EAEC and *daaC* positive produced AA but not DA, and the significance of *daaC* in these isolates is under investigation. All isolates with LA carried *eaeA*, *bfpA*, and EAF, whereas all isolates with LAL car-

TABLE 2. Association between the different patterns of adherence and the adherence-related DNA sequences in *E. coli* and acute diarrhea in 200 patients and 200 age-matched controls

E. coli property	No. (%) of children				Association with acute diarrhea	
	Patients		Controls			
	Total	Single pathogen[a]	Total	Single pathogen	Odds ratio[b]	*P* value
Pattern of adherence[c]						
DA	63 (31.5)	17 (8.5)	57 (28.5)	23 (11.5)	0.73	0.45
AA	43 (21.5)	16 (8.0)	38 (19.0)	18 (9.0)	0.89	0.89
AA6h	4 (2.0)	2 (1.0)	7 (3.5)	5 (2.5)	0.40	0.45
AAcs	25 (12.5)	13 (6.5)	30 (15.0)	15 (7.5)	0.86	0.86
LA	5 (2.5)	1 (0.5)	2 (1.0)	1 (0.5)	1.00	0.48
LAL	3 (1.5)	1 (0.5)	1 (0.5)	1 (0.5)	1.00	0.48
Hybridization with the following DNA probe:						
daaC	52 (26.0)	12 (6.0)	42 (21.0)	7 (3.5)	1.71	0.37
EAEC	26 (13.0)	9 (4.5)	34 (17.0)	17 (8.5)	0.53	0.19
daaC + EAEC	10 (5.0)	3 (1.5)	11 (5.5)	2 (1.0)	1.50	1.00
daaC + *eaeA*	5 (2.5)	1 (0.5)	0	0	1/0	1.00
eaeA	16 (8.0)	3 (1.5)	10 (5.0)	5 (2.5)	0.60	0.72
eaeA + *bfpA*	0	0	3 (1.5)	2 (1.0)	0.00	0.48
eaeA + EAF + *bfpA*	5 (2.5)	1 (0.5)	2 (1.0)	1 (0.5)	1.00	0.48

[a] Single pathogen, any pair in which none of the other pathogens identified, i.e., adenovirus, *Aeromonas*, *Campylobacter*, EIEC, ETEC, rotavirus, *Salmonella*, *Shigella*, and STEC, were found.
[b] Number of pairs with a single putative pathogen in which the adherence factor was reported in the case patient but not in the control divided by number of pairs in which the adherence factor was reported in the control but not the case patient.
[c] Cell-detaching *E. coli* strains and *E. coli* strains with an NC pattern of adherence were excluded from this analysis.

ried *eaeA* (100.0%) and occasionally *bfpA* (35.7%). So far, the distribution of *bfpA* has not been extensively analyzed, but this probe is considered to be more sensitive than the EAF probe in detecting LA-producing *E. coli* (16). However, our data suggest that both probes are equally sensitive in detecting LA.

The different combinations of adherence patterns and adherence-related sequences found in this study demonstrated that the results obtained with the DNA probes and by the bioassays normally used to search for DAEC and EAEC do not correlate well. Moreover, although adherence to HEp-2

TABLE 3. Prevalence of different patterns of adherence combined with adherence-related DNA sequences in *E. coli* isolated from 200 children with acute diarrhea and 200 age-matched controls[a]

Pattern of adherence	DNA probe	No. (%) of children				Association with acute diarrhea	
		Patients		Controls			
		Total	Single pathogen[b]	Total	Single pathogen	Odds ratio[c]	*P* value
DA	*daaC*	46 (23.0)	10 (5.0)	38 (19.0)	7 (3.5)	1.43	0.64
	daaC + *eaeA*	2 (1.0)	0	0	0		
AA	EAEC	21 (10.5)	8 (4.0)	26 (13.0)	13 (6.5)	0.62	0.40
	daaC + EAEC	10 (5.0)	3 (1.5)	10 (5.0)	2 (1.0)	1.50	1.00
AAcs	*daaC*	0	0	2 (1.0)	0		
	EAEC	1 (0.5)	0	3 (1.5)	1 (0.5)	0.00	1.00
	eaeA	0	0	2 (1.0)	1 (0.5)	0.00	1.00
LA	*eaeA* + EAF + *bfpA*	5 (2.5)	1 (0.5)	2 (1.0)	1 (0.5)	1.00	0.48
LAL	*eaeA*	3 (1.5)	1 (0.5)	0	0	1/0	1.00
	eaeA + *bfpA*	0	0	1 (0.5)	1 (0.5)	0.00	1.00
NC	*daaC*	1 (0.5)	0	1 (0.5)	0		
	EAEC	2 (1.0)	0	0	0		
	eaeA	13 (6.5)	2 (1.0)	6 (3.0)	4 (2.0)	0.50	0.68
	daaC + eaeA	3 (1.5)	1 (0.5)	0	0	1/0	1.00
	eaeA + *bfpA*	0	0	2 (1.0)	1 (0.5)	0.00	1.00

[a] Cell-detaching and nonadherent *E. coli* strains were excluded from this analysis.
[b] Single pathogen, any pair in which none of the other pathogens identified, i.e., adenovirus, *Aeromonas*, *Campylobacter*, EIEC, ETEC, rotavirus, *Salmonella*, *Shigella*, and STEC, were found.
[c] Number of pairs with a single putative pathogen in which the adherence factors were reported in the case patient but not in the control divided by number of pairs in which the adherence factors were reported in the control but not the case patient.

and HeLa cells has been used as the "gold standard" assay for the detection of these categories of isolates, variations in the AA (29, 32) and LA (22, 40) patterns are occasionally reported, and thus, the best method of identifying these organisms in the clinical research setting remains controversial.

None of the different adherence patterns or adherence-related DNA sequences, or a combination of both, were associated with acute diarrhea in children 1 to 4 years old in São Paulo. This lack of association was observed even when the data for any of the three age groups studied (12 to 23, 24 to 35, and 36–59 months) were analyzed (data not shown).

The children studied here were selected from a larger population in which *Shigella* and rotavirus were the most frequent pathogens found (19). Although DAEC and EAEC were very frequently found in these children, they were not associated with acute diarrhea, suggesting that both categories are heterogeneous and each comprises pathogenic and nonpathogenic strains or that the high level of asymptomatic carriage of DAEC and EAEC, often observed with other well-established enteropathogens (20), masks an association with the disease in our population. The low incidence of *E. coli* with LA found in this study is probably due to differences in the risk factors that these children have (39), since LA is mainly detected among strains of EPEC serotypes, which prevail in the first year of life (21).

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