Prevalence of *Escherichia coli* Strains with Localized, Diffuse, and Aggregative Adherence to HeLa Cells in Infants with Diarrhea and Matched Controls

TANIA A. T. GOMES,^{1*} PAUL A. BLAKE,² AND LUIZ R. TRABULSI¹

Department of Microbiology, Immunology, and Parasitology, Escola Paulista de Medicina, Rua Botucatu 862, 04023 São Paulo, Brazil,¹ and Enteric Diseases Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333²

Received 17 August 1988/Accepted 18 October 1988

To determine the possible role of *Escherichia coli* strains with three different patterns of adherence to HeLa cells in causing diarrhea in infants in São Paulo, Brazil, we studied stool specimens from 100 infants up to 1 year of age with acute diarrheal illnesses and 100 age-matched control infants without recent diarrhea. *E. coli* with localized adherence to HeLa cells was much more common in patients (23%) than in controls (2%) ($P < 0.0001$) and was detected more frequently than rotavirus (19%) was in patients, even though the study was conducted during the coldest months of the year. Most (80%) of the *E. coli* colonies with localized adherence were of traditional enteropathogenic *E. coli* serotypes. Little difference was found between patients and controls in the rate of isolation of *E. coli* with diffuse adherence (31 and 32%, respectively) or aggregative adherence (10 and 8%, respectively). A genetic probe used to detect a plasmid-mediated adhesin which confers expression of localized adherence proved to be 100% sensitive and 99.9% specific in detecting *E. coli* with localized adherence to HeLa cells. Although *E. coli* strains with localized adherence have now been shown to be enteric pathogens in several parts of the world, the role of strains showing diffuse adherence and aggregative adherence is still uncertain.

Bacterial adherence to the intestinal mucosa is essential to the ability of many enteropathogenic *Escherichia coli* (EPEC) strains to cause diarrhea (2). Adhesion of enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) to the gut is mediated by diverse fimbrial structures (8, 10). So far, no adhesive structures have been described in enteroinvasive *E. coli* (EIEC).

Cravioto et al. (4) described in vitro mannose-resistant adhesiveness of *E. coli* strains to HEp-2 cells; most of those strains belonged to the classical EPEC serogroups. Subsequently, Scaletsky et al. (24) demonstrated that *E. coli* adhesiveness to HeLa cells could be of two distinctive types: localized adhesion (LA) and diffuse adhesion (DA). Further investigation showed that LA was mainly associated with certain EPEC bioserotypes, while DA was not associated with any particular group of *E. coli* (23). LA and DA could also be distinguished in HEp-2 cells (20). Baldini et al. (1) showed that LA expression is conferred by a plasmid-mediated adhesin named EPEC adherence factor (EAF), which was later demonstrated to be associated with enteropathogenicity in adult volunteers (11). A sensitive and specific genetic probe (EAF probe) has been constructed which permits identification of *E. coli* bearing EAF-coding genes (18).

Both tissue culture assays and the EAF probe have recently been employed to assess the frequency of isolation of LA- and DA-producing *E. coli* from diarrheal and nondiarrheal stools in diverse geographic regions (3, 19). In general, LA has been consistently associated with diarrhea, while the results for DA have been inconsistent (3, 14, 16, 19).

Recently, Nataro and co-workers have described a third *E. coli* adherence pattern in HEp-2 cells which was called

aggregative adhesion (AA) (19). In this form of adhesion, clumps of bacteria may be seen on the surfaces of HEp-2 cells as well as on the glass slide free from cells, assuming a characteristic stacked-brick pattern. *E. coli* presenting the AA pattern was shown to be associated with diarrhea in Chilean children (19). The primary purpose of the present study was to determine the prevalence of LA-, DA-, and AA-producing EPEC and non-EPEC strains among urban infants with acute diarrheal illness and age-matched controls in the largest city in Brazil, São Paulo. We also evaluated the sensitivity and specificity of the EAF probe in detecting LA-producing *E. coli* strains.

MATERIALS AND METHODS

At the emergency room of the Hospital Infantil Menino Jesus in São Paulo, fecal specimens were collected from urban infant patients (children <1 year old) with diarrhea lasting <8 days and from individually age-matched control infants who visited the hospital at the same time for other reasons and had not had diarrhea during the previous 30 days. Specimens were collected between May and August of 1985. Patient-control pairs were entered into the study until 100 pairs were accumulated in which both the patient and the control had *E. coli* detected in stools as described below; 11 other pairs were excluded because one of the subjects did not have *E. coli* detected in stools.

E. coli strains were isolated on MacConkey agar. Three to five separate lactose-fermenting colonies, presumed to be *E. coli* by colony morphology, and one non-lactose-fermenting colony of each distinct morphologic type were cultivated in EPM (26) and MILi (25) media for biochemical confirmation of species or genus. All *E. coli* colonies were submitted to slide agglutination with polyvalent and monovalent antisera (PROBAC do Brasil, São Paulo, Brazil) against O antigens of EPEC, EIEC, and EHEC (O157) serogroups. The EPEC

* Corresponding author.

TABLE 1. Sensitivity and specificity of the EAF probe in detecting *E. coli* producing LA to HeLa cells

Hybridization with EAF probe	No. of colonies showing the following adherence pattern to HeLa cells			
	LA	AA	DA	NA ^a
+	38	0	0	1
-	0	38	180	618

^a NA, Nonadherent.

serogroups considered were as follows: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128ab, O142, and O158. The EIEC serogroups sought were as follows: O28ac, O29, O112ac, O124, O136, O143, O144, O152, O164, and O167. Serogroups were confirmed by tube agglutination (6). H antigens were identified by standard procedures (6, 7) by using H1 to H50 antisera of the Centers for Disease Control, Atlanta, Ga. When two or more colonies of identical serotypes were isolated from the same child, only one colony was kept. All strains were maintained in nutrient agar slants at room temperature.

EPEC, EIEC, EHEC, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, and rotavirus were searched for as described elsewhere (17, 21, 28).

Mannose-resistant adherence to HeLa cells was assessed essentially as described by Scaletsky et al. (24). Only EPEC strains and *E. coli* strains that were neither invasive nor toxigenic and did not belong to EHEC serotype O157:H7 were tested. The laboratorian performing the assay did not know whether the strains being tested were from patients or controls.

To identify EAF-bearing (EAF⁺) colonies, we also used the 1-kilobase EAF fragment described by Nataro et al. (18) which was radiolabeled with [α -³²P]dATP by nick translation (22). Strains submitted to the adherence assay were also spotted onto MacConkey agar and incubated at 37°C overnight. Colonies were then transferred to Whatman 541 paper (Whatman, Inc., Clifton, N.J.), and the filters were processed and hybridized as described by Maas (13). Results were revealed by autoradiography.

The Fisher two-tailed exact test was used for statistical analysis.

RESULTS

A total of 875 *E. coli* colonies from the 100 patients (408 colonies) and 100 controls (467 colonies) were submitted to both HeLa cell adherence assay and hybridization with the EAF probe. No colony presented more than one adhesion type simultaneously. Results of HeLa cell and EAF probe assays are shown in Table 1. Thus, for detecting LA-

TABLE 2. Prevalence of adherent *E. coli* strains in infants with diarrhea and in age-matched controls in São Paulo

<i>E. coli</i> adhesion pattern	% of infants infected		P value
	Patients (n = 100)	Controls (n = 100)	
LA	23 ^a	2	<0.0001
DA	31	32	1.000
AA	10	8	0.806

^a Three children had LA⁺ *E. coli* of two different serotypes.

TABLE 3. Prevalence of enteropathogens among 100 infants with diarrhea and 100 age-matched controls in São Paulo from May through August of 1985

Enteropathogen	% with enteropathogen detected ^a		P value
	Patients (n = 100)	Controls (n = 100)	
LA ⁺ <i>E. coli</i>	23	2	<0.0001
Rotavirus	19	2	0.0001
<i>Salmonella</i> spp.	8	4	0.373
EPEC	4	4	1.000
<i>Shigella</i> spp.	3	1	0.621
<i>Campylobacter</i> spp.	1	0	1.000
EIEC	0	1	1.000

^a Subjects with multiple enteropathogens are listed once for each pathogen detected.

producing (LA⁺) *E. coli*, the EAF probe was 100% sensitive and 99.9% specific.

Of the 38 LA⁺ colonies, 30 (80%) belonged to traditional EPEC serotypes. The single probe-positive nonadherent *E. coli* belonged to EPEC serotype O111:H2. Only one DA-producing (DA⁺) *E. coli* strain was of an EPEC serogroup; it was serotype O119:H9. The remaining DA⁺ and AA-producing (AA⁺) strains did not belong to EPEC, EIEC, or O157 serogroups.

The frequencies with which LA⁺, DA⁺, and AA⁺ *E. coli* strains were isolated from patients and controls are shown in Table 2. The number of adherent colonies varied from one to five in each child studied. LA⁺ *E. coli* was much more common in patients than in controls ($P < 0.0001$). Three patients had LA⁺ *E. coli* colonies of two different serotypes. Of the 23 patients bearing LA⁺ *E. coli*, 20 (87%) had no other enteropathogens detected. DA⁺ *E. coli* strains were isolated with similar frequencies from patients and controls. When the analysis was confined to subjects with no enteropathogens detected (including *E. coli* of EPEC serogroups), DA⁺ *E. coli* was found in 12% of patients and 27% of controls. AA⁺ *E. coli* was not associated with infantile diarrhea; when subjects with known enteropathogens were excluded, 7% of patients and 6% of controls harbored AA⁺ *E. coli*.

The frequency with which enteropathogens were detected in patients and controls is shown in Table 3. LA⁺ *E. coli* were the organisms most commonly detected in diarrheal stools, followed by rotavirus and *Salmonella* spp. *Y. enterocolitica* was not found in the population studied.

DISCUSSION

In this study, we have shown that the EAF probe constructed by Nataro et al. (18) is highly sensitive and specific in identifying *E. coli* strains from São Paulo, Brazil, with LA to HeLa cells. A high sensitivity and specificity have been reported previously for the EAF probe by researchers studying strains from other geographic areas (3, 18, 20). The only probe-positive strain in our study that did not adhere to HeLa cells belonged to a traditional EPEC serotype (O111:H2) which has consistently produced LA in the past. Agarose gel electrophoresis revealed a 60-megadalton plasmid (i.e., similar in size to the EAF plasmid) which reacted with the EAF probe in a Southern blot analysis (data not shown). This suggests that this probe-positive nonadherent *E. coli* may carry EAF genes that were incorrectly expressed or not expressed at all. Nataro et al. (20) have observed one strain that adhered to HeLa but not to HEP-2 cells; it was probe

sensitive. Thus, it would be interesting to test our strain in HEp-2 cells and analyze the regulation and expression of the EAF genes.

In São Paulo, LA⁺ *E. coli* was detected much more frequently in infants with diarrhea than in their matched controls, and they were the most important enteric pathogen in these patients, exceeding the rate for rotavirus even though the cases occurred during the colder months. LA⁺ *E. coli* has been shown to be associated with infantile diarrhea in a paired patient-control study of children under 1 year of age in Thailand (5), and the importance of EAF expression for in vivo pathogenicity has been demonstrated in adult volunteers (11). Although enteropathogens like *Aeromonas* spp., *Plesiomonas* spp., *Clostridium difficile*, *Vibrio* spp. and viruses other than rotavirus were not searched for in our study, the fact that 20 (87%) of the 23 patients with LA⁺ *E. coli* in their stools had none of the other enteropathogens searched for in this study (ETEC, EIEC, EHEC, *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, *Campylobacter* spp., and rotavirus) supports their role as a cause of infantile diarrhea. It should be emphasized that the study was conducted during a 4-month period, May through August, and the relative frequency of specific enteropathogens may be different during other seasons. LA production was associated with EPEC serotypes, as described by other researchers (5, 9, 18, 19).

Mathewson et al. (14) showed that *E. coli* strains with mannose-resistant adherence to HEp-2 cells were twice as frequent in American adult students who had travelled to Mexico and acquired diarrhea as in their controls. According to Mathewson et al. (14), 61% of the adherent strains were LA⁺ and the rest were DA⁺. One LA⁺ and one DA⁺ *E. coli* strain were shown to cause diarrhea in adult volunteers (15). The LA⁺ strain from the volunteer study has subsequently been reported to be AA⁺ and EAF⁻, however, and to have H33 flagellar antigen, which is common in AA⁺ strains (27). Mathewson et al. (16) observed that DA⁺ *E. coli* strains were significantly associated with diarrhea affecting children 3 to 84 months of age in Guadalajara, Mexico. On the other hand, Cravioto et al. (A. Cravioto, M. Lince, J. Carrillo, and G. Cancino, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 47, 1986) found that DA⁺ *E. coli* strains were not associated with diarrhea in Mexican children. In São Paulo, we found DA to be equally common in infants with and without diarrhea. DA⁺ *E. coli* strains were not associated with diarrhea in children up to 5 years of age in Bangkok, Thailand (3) or in infants and young children in Chile (19).

AA *E. coli* have been reported to be associated with diarrheal disease in Chilean children (12, 19). However, although the association was statistically significant, the relative risk was only 1.57 (35.7% of patients and 22.7% of controls had AA⁺ *E. coli* in their stools). Our study of infants in Brazil found AA⁺ *E. coli* in a smaller proportion of patients (10%) and controls (8%); the relative risk was only 1.25 and was even lower when infants with recognized enteropathogens detected in their stools were excluded. Thus, more studies are needed both to establish the role of AA *E. coli* in the etiology of diarrhea and to determine the epidemiologic characteristics of the disease.

Clearly, LA⁺, DA⁺, and AA⁺ *E. coli* are very different in their mechanisms of cell association, and DA⁺ and AA⁺ *E. coli* may not be enteric pathogens or may have different epidemiologic characteristics. Knowledge about these organisms is now sufficient to conclude that it would be

incorrect to lump all types of adherent *E. coli* into one enteroadherence category as was proposed in 1985 (14).

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