Characterization of Enteroinvasive *Escherichia coli* Strains Isolated from Children with Diarrhea in Chile

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Analysis of stool samples from 912 cases of diarrhea among Chilean infants and 1,112 controls resulted in the isolation of 17 enteroinvasive *Escherichia coli* (EIEC) strains from diarrhea cases (1.9%) and 3 EIEC from the asymptomatic controls (0.3%). Biochemical analysis of the 20 isolates showed variability among them. However, the majority were lysine decarboxylase negative and nonmotile and utilized sodium acetate. The strains belonged to the O groups 28ac, 124, 143, or 144 or were untypable with the antisera used. Most of them had conjugative plasmids which mediated multiple antibiotic resistance. There was a strong correlation in this group of strains between a positive Sereny test, the presence of a plasmid of 120 megadaltons, and hybridization with the invasiveness probe, an *Hind*III fragment derived from the plasmid pPS15A. The isolates had a wide range of plasmid profiles. Bioassays and colony and Southern hybridization tests with iron uptake DNA probes indicated that 80% of the EIEC strains produced aerobactin and expressed its receptor, the genes for which are known to be chromosomally located.

Enteroinvasive *Escherichia coli* (EIEC) share important features with *Shigella* spp. Both are able to produce the dysenteric syndrome, which is characterized by severe abdominal pain and frequent stools containing blood and mucus. Both pathogens have the ability to invade epithelial cells, which is dependent, in EIEC as in *Shigella* spp., on the presence of a 120- to 140-megadalton (MDa) plasmid (12). EIEC can be detected by biochemical and serological tests, but the diagnosis must be confirmed by biological assays (21). An alternative procedure to identify potentially invasive *E. coli* isolates is the hybridizations assays with DNA probes derived from the large invasiveness plasmid (23).

Aerobactin is a hydroxamate-type chelator produced by many species of the *Enterobacteriaceae* family (20). This siderophore confers a selective advantage to pathogens in extraintestinal infections (30, 32), but its role in the pathogenesis of diarrhea and enteric infections is still under study. A potential role for aerobactin in the virulence of some enteroinvasive strains has been suggested recently (11, 19, 31). We decided to investigate the presence of this siderophore and its receptor in EIEC strains by biological assays and DNA-DNA hybridization.

Notwithstanding the progress in health care and treatment of the dehydration produced by diarrhea in infants, infectious diarrhea continues to be a problem in developing countries (24). In Chile, a nontropical country, infant diarrhea is still an important cause of morbidity and mortality among infants, being a common cause of medical consultation for children of that age, especially in the warm season (2, 3).

Current studies on the ecology of diarrhea in Chile provided the opportunity to study the role of EIEC as a cause of diarrhea among infants living in a temperate climate and to define some biochemical, genetic, and molecular characteristics of these strains. This information may be useful in improving our understanding of the pathogenesis of EIEC infections and of the epidemiology of EIEC in Chile and other nontropical countries.

MATERIALS AND METHODS

Strains. This study included 20 *E. coli* strains isolated from 912 cases of diarrhea and 1,112 stool samples from asymptomatic individuals, all of them infants dwelling in the marginal slums of Santiago, Chile. The isolates were collected between December 1982 and December 1986 and stored in nutrient broth plus 30% glycerol at -20°C. Stool samples were obtained by using a sterile swab and transported in Cary-Blair transport medium. The bacterial enteropathogens enteropathogenic *E. coli*, enterotoxigenic *E. coli*, EIEC, *Shigella*, *Salmonella*, and *Campylobacter* spp. were identified by standard biochemical and serological procedures (10, 17). In addition, lysine decarboxylase-negative strains were regularly assayed by agglutination with O antisera corresponding to the following EIEC serogroups: O28ac, O112ac, O124, O136, O143, O144, O152, and O164. The agglutination test of the positive strains was repeated with cell suspensions heated at 100°C. *E. coli* HB101 (pPS15A) containing a 2.5-kilobase (kb) *Hind*III DNA fragment from the invasiveness plasmid of an EIEC was obtained from P. Small and S. Falkow, Stanford University, Stanford, Calif. (23). *E. coli* K-12 LG1522 (*fepA* ColV, K-30 *iuc*), used as the indicator strain in bioassays to detect aerobactin production, was obtained from P. H. Williams, University of Leicester, Leicester, U.K. (8).

Bioassay for invasiveness. The Sereny test (21) was used to demonstrate the invasiveness of all the strains with an enteroinvasive biotype.

Aerobactin production and susceptibility to cloacin DF13. The production of aerobactin was determined by cross-feeding *E. coli* LG1522 (8). Briefly, a portion of the supernatant of an overnight culture of each strain tested, grown in minimal M9 medium with 200 µM α,α'-dipyridyl, was added to paper disks on the surface of M9 agar plates containing 200 µM dipyridyl previously covered with soft agar containing *E. coli* LG1522. A halo of growth around the disks indicated production of aerobactin. The presence of the aerobactin receptor was detected by the cloacin DF13 sensitivity test, as the colicin and iron siderophore complex have the same receptor (5, 29). The production of hemolysin

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was determined by growing the strains in 5% sheep blood agar plates.

Isolation of plasmid and chromosomal DNA. Plasmid DNA was extracted by the method of Birnboim and Doly and analyzed by agarose gel electrophoresis in parallel with DNA of plasmids of known molecular weight (7). Chromosomal DNA was obtained essentially as described by Manning et al. with minor modifications (16).

Colony and Southern hybridizations. All the Sereny-positive strains were also tested in colony hybridization assays with an enteroinvasive probe, a 2.5-kb *Hind*III DNA fragment from plasmid pPS15A (23); with an aerobactin synthesis gene probe, a 2-kb *Ava*I DNA fragment of pABN-5 (6); and with a 2.3-kb *Pvu*II DNA fragment of pABN-1 as the aerobactin receptor probe (6). DNA probes were labeled with [α - 32 P]deoxynucleotides by nick translation (15). Bacteria were grown overnight on nitrocellulose filters placed on MacConkey plates, and the filters were prepared and hybridized as described by Moseley et al. (18). Simultaneously, the same strains were assayed by Southern hybridization. In this assay, plasmid DNA was extracted by the method of Birnboim and Doly (7), electrophoresed in 1% agarose slab gels, transferred to nitrocellulose filters, and hybridized with the 32 P-labeled probes as described previously (14, 25). Filters were exposed at -70°C to Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.).

Detection of antibiotic resistance. The antibiotic susceptibility of the strains was determined by the disk diffusion method of Bauer et al. (4). Production of colicin V was detected as described previously (9).

PAGE of outer membrane proteins. Outer membrane proteins were extracted and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (1). The gels were run at 120 V for 10 h. After SDS-PAGE, they were stained with silver (33).

RESULTS

Frequency of EIEC. EIEC were isolated from 17 of 912 (1.9%) diarrhea cases and from 3 of 1,112 (0.3%) stool samples from asymptomatic control individuals. The *E. coli* strains suspected of being enteroinvasive by their biotype or serogroup were tested for invasiveness by the Sereny test. Only 20 of them gave a positive result. To confirm the presence of genes responsible for invasiveness, we performed colony and Southern hybridization with a 32 P-labeled *Hind*III probe from pPS15A (23). Of the 20 initially Sereny-positive strains, 17 hybridized with the probe (Table 1). Analysis of the plasmid DNA from the enteroinvasive strains showed that they harbored a variety of plasmids. Southern hybridization experiments indicated that the plasmids which hybridized with the probe were always of high molecular weight, ranging between 120 and 140 MDa (Fig. 1). There was a positive correlation between the loss of this large plasmid, lack of invasiveness in the Sereny test, and absence of hybridization with the probe (Table 1).

Detection of the aerobactin system and location of the aerobactin genes. Of 20 strains, 16 were able to support the growth of the indicator strain *E. coli* LG1522 and were sensitive to cloacin DF13 (Table 1); thus, they expressed the complete aerobactin uptake system. Colony and Southern DNA-DNA hybridization experiments were performed to locate the aerobactin system genes, with both an aerobactin probe (the *Ava*I fragment of ABN-5), and a receptor probe (the *Pvu*II fragment of ABN-1). The colony hybridizations were positive in all of the aerobactin-producing strains, whereas no hybridization with plasmids was detected in the Southern assays. These results indicated that the aerobactin system genes must be located on the chromosome in this group of EIEC. Moreover, the hybridization assays of chromosomal DNA digested with different restriction en-

TABLE 1. Properties of EIEC strains isolated in Chile

EIEC strain	Source ^a	Sereny test ^b	Hybridization with invasive probe		Aerobactin bioassay	Hybridization with aerobactin synthesis probe		Aerobactin receptor bioassay	Hybridization with aerobactin receptor probe	
			Colony	Southern		Colony	Southern		Colony	Southern
1	D	+	+	+	+	+	—	+	+	—
2	D	+	+	+	+	+	—	+	+	—
3	D	+	+	+	+	+	—	+	+	—
4	D	+	+	+	+	+	—	+	+	—
5	D	+	+	+	+	+	—	+	+	—
6	D	+	+	+	—	—	—	—	—	—
7	D	(+)	—	—	+	+	—	+	+	—
8	D	+	+	+	+	+	—	+	+	—
9	D	+	+	+	+	+	—	+	+	—
10	D	+	+	+	+	+	—	+	+	—
11	D	+	+	+	+	+	—	+	+	—
12	D	(+)	—	—	+	+	—	+	+	—
13	D	+	+	+	+	+	—	+	+	—
14	D	(+)	—	—	—	—	—	—	—	—
15	D	+	+	+	+	+	—	+	+	—
16	D	+	+	+	+	+	—	+	+	—
17	D	+	+	+	—	—	—	—	—	—
18	C	+	+	+	—	—	—	—	—	—
19	C	+	+	+	+	+	—	+	+	—
20	C	+	+	+	+	+	—	+	+	—

^a D, Diarrhea; C, healthy control.

^b Signs in parentheses correspond to strains that gave a positive Sereny test immediately after being isolated. The strains which become Sereny negative upon storage did not hybridize with the probe.

FIG. 1. Plasmid content and hybridization with the invasiveness DNA probe of selected EIEC. (A) Plasmid profiles; (B) Southern hybridization. Lanes: 1, EI 1 (O124); 2, EI 3 (O124); 3, EI 13 (O124); 4, EI 2 (O28); 5, EI 8 (O28); 6, EI 16 (O28); 7, EI 10 (O143); 8, EI 19 (O143); 9, EI 18 (O144); 10, EI 9 (untypable); 11, EI 12 (untypable; negative control). The arrows indicate the plasmid that hybridized with the invasiveness probe.

zymes and hybridized with the aerobactin synthesis and aerobactin receptor probes produced specific patterns related to the strain serogroup. For example, strains belonging to serogroup O28ac (Fig. 2, lanes 1 to 3) had patterns of

FIG. 2. Hybridization of chromosomal DNA of different EIEC with iron uptake probes. (A) Chromosomal DNA digested with *Bam*HI and hybridized with the aerobactin synthesis DNA probe. (B) Chromosomal DNA digested with *Eco*RI and hybridized with the receptor DNA probe. Lanes: 1, EI 16 (O28); 2, EI 4 (O28); 3, EI 2 (O28); 4, EI 1 (O124); 5, EI 3 (O124); 6, EI 11 (O143); 7, EI 15 (O143); 8, EI 19 (O143); 9, EI 9 (untypable); 10, phage λ DNA; 11, *Shigella flexneri* chromosomal DNA.

hybridization similar to those of the O124 strains (Fig. 2, lanes 4 and 5) when assayed with the aerobactin synthesis and aerobactin receptor DNA probes. These patterns were different from the hybridization patterns produced by the strains belonging to the O143 serogroup (Fig. 2, lanes 6, 7, and 8).

Serogroup, biotype, antibiotic resistance, and outer membrane proteins. The majority of the strains studied belonged to serogroups and biotypes already described for EIEC strains (22, 27). Fifteen of the 20 strains included in this study belonged to the O groups O28ac, O124, O143, and O144, and 5 were untypable. The biochemical characteristics of the 20 EIEC strains grouped in accordance with serotype are presented in Table 2. All of the strains except one were lysine decarboxylase negative, and only two of them were motile. All of the strains were able to utilize sodium acetate. The strains belonging to the O143 serogroup presented the same pattern of biochemical reactions and a common pattern of antibiotic resistance. The majority of the strains expressed different patterns of multiple antibiotic resistance (Table 2), which could be easily transmitted by conjugation (data not shown).

There was also a relationship between the pattern of outer membrane proteins and some serogroups (Fig. 3). All the O28 strains expressed in their outer membranes a protein of 34 kDa, while the O143 strains expressed a protein of 35.5 kDa. There were other less drastic differences in the pattern of the outer membrane protein of these two serogroups.

None of the strains were able to produce hemolysin or colicin V.

DISCUSSION

EIEC are an infrequent cause of diarrhea in Chile. The epidemiology of these pathogens in Chile is therefore rather

TABLE 2. Biochemical reactions and antibiotic resistance of EIEC strains according to the O serogroup

Test or antibiotic	No. of positive or resistant strains				
	O28ac (n = 5)	O124 (n = 5)	O143 (n = 4)	O144 (n = 1)	Non- typable (n = 5)
Biochemical test					
Lysine decarboxylase	1	0	0	0	0
Motility	1	2	0	0	0
Indol	2	5	4	1	5
Ornithine decarboxylase	1	0	0	1	2
Sodium acetate	5	5	4	1	5
Lactose	4	2	4	1	2
Arabinose	5	4	4	1	5
D-Mannitol	5	5	4	1	5
Rhamnose	4	3	4	0	4
Sorbitol	5	5	4	0	4
Xylose	5	5	4	1	4
Antibiotic tested					
Ampicillin	1	3	4	1	3
Carbenicillin	1	3	4	1	4
Cephalothin	0	0	0	0	0
Chloramphenicol	1	0	0	1	1
Gentamicin	0	0	0	0	0
Nalidixic acid	0	0	0	0	0
Kanamycin	0	0	0	0	0
Streptomycin	1	2	4	1	4
Tetracycline	0	1	4	0	3

FIG. 3. SDS-PAGE profiles of outer membrane proteins from EIEC strains. Lane 1, Molecular weight standards; lanes 2 to 4, strains belonging to the O28 serogroup; lanes 5 to 7, strains belonging to the O143 serogroup.

similar to that in tropical countries (26, 27). This suggests that deficient sanitary conditions are more important than climate in explaining the impact of these enteric pathogens in different geographical areas. The significantly different frequency ($P < 0.003$) with which these strains are isolated from diseased and healthy children supports a pathogenic role for them in diarrhea. The low pathogenicity of EIEC in conditions in which other enteric pathogens are transmitted freely may be due either to their limited virulence or to environmental or host conditions which make infection and disease caused by these pathogens uncommon. The increase in infections by shigellae and other enteric pathogens in the past few years in Chile, in the absence of an increase in EIEC infections, suggests that the low degree of virulence of the pathogen is at least partly responsible for this behavior (C. S. Heignere, Ph.D. thesis, University of Denver, Denver, Colo., 1983).

As previously found in other geographical areas, the group of EIEC from Chile correspond to serogroups which have already been characterized as enteroinvasive (22, 27). Analyses of sugar fermentation patterns, antibiotic resistance, and plasmid content indicate a great degree of variability among the strains. The use of any of these characteristics as epidemiological markers is therefore limited. However, it is interesting that all the strains belonging to the O143 serogroup had common patterns of sugar fermentation and antibiotic resistance and a very similar but not identical SDS-PAGE pattern of plasmid DNA. We confirmed that the lysine decarboxylase and motility assays are useful as screening tests to detect these strains in stools. The utilization of sodium acetate could be useful in distinguishing EIEC from *Shigella* spp. A positive Sereny test was always correlated with the presence of the large plasmid and hybridization with the enteroinvasive probe. The strains which lost the large plasmid upon storage became Sereny negative and unable to hybridize with the probe, confirming the role of this plasmid in invasiveness.

The iron transport system mediated by the hydroxamic siderophore aerobactin is encoded by chromosomal genes or by ColV plasmids in *E. coli* (28), whereas in *Shigella* spp. it has been always found to be encoded by chromosomal genes (13). Our results demonstrate the presence of the aerobactin system in the chromosome DNA in 80% of the EIEC studied by colony and Southern hybridization, suggesting a possible pathogenic role for these genes. Moreover, the hybridization

of chromosomal DNA with aerobactin system probes indicates that the patterns of hybridization are different but conserved among some serogroups, suggesting its usefulness as an epidemiological marker. SDS-PAGE of outer membrane proteins could also be used with a similar purpose.

The expression of one or more antibiotic resistances by the majority of the EIEC strains (80%) raises the potential for problems when treating diarrhea produced by this pathogen.

More studies will be needed to dissect the role played by the aerobactin iron uptake system in the pathogenesis of EIEC disease and to answer the question regarding the different frequency of infections produced by this pathogen and *Shigella* spp. in Chile.

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