MAJOR ARTICLE

Atypical Enteropathogenic *Escherichia coli* Strains: Phenotypic and Genetic Profiling Reveals a Strong Association between Enteroaggregative *E. coli* Heat-Stable Enterotoxin and Diarrhea

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The virulence profiles of most atypical enteropathogenic *Escherichia coli* (EPEC) strains are unknown. A total of 118 typical and atypical strains of EPEC serotypes and non-EPEC serogroups isolated from children with or without acute diarrhea who were from different cities in Brazil were examined for virulence-associated markers and adherence to HEp-2 cells, and also had random amplified polymorphic DNA (RAPD) analysis performed. Atypical strains were identical to typical strains with regard to the virulence factors encoded on the locus of enterocyte effacement (LEE). In contrast with typical EPEC strains, none of the atypical strains reacted with the *bfpA* probe, and half of the strains hybridized with the *perA* probe. Most atypical strains presented Tir sequences that correlated with enteropathogenic or enterohemorrhagic *E. coli* (98%), had LEE inserted in either *selC* or *pheU* (88%), and presented a typeable intimin (52%). Eighteen new serotypes were found in the EPEC strains. Atypical and typical EPEC strains belonged to different RAPD clusters. Most atypical strains showed a localized-like adherence pattern (61.5%). Of the non–LEE-encoded virulence factors, enteroaggregative *E. coli* heat-stable enterotoxin was noted most frequently (45%) and was significantly associated with diarrhea ($P = .01$). Thus, this virulence marker may be used as an additional tool for the diagnosis of truly atypical pathogenic strains.

There currently are 6 groups of *Escherichia coli* that have been found, by case-control epidemiological studies, to be associated with gastrointestinal disease [1]. Enteropathogenic *E. coli* (EPEC) produces a characteristic histopathological lesion on the intestinal epithelium that is known as the "attaching and effacing lesion" (A/E lesion). Enterotoxigenic *E. coli* produces heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST) and ⩾1 intestinal colonization factors. Enteroinvasive *E. coli* invades the colonic epithelium. Enterohemorrhagic *E. coli* (EHEC) produces Shiga toxins, an α-hemolysin (E-hly), and, like EPEC, A/E lesions. Enteroaggregative *E. coli* (EAEC) adheres to HEp-2 cells in an aggregative adherence pattern and produces an ST-like toxin (EAST1), an LT toxin, and fimbrial colonization factors called "AAFs" (aggregative adherence fimbria). Diffusely adherent *E. coli* adheres to HEp-2 cells in a diffuse pattern and may carry the F1845 adhesin, which is related to the afimbrial adhesin AFA-I of uropathogenic *E. coli*. In addition to the aforementioned 6 classes of diarrheogenic *E. coli*, there are other potential classes that produce cytolethal distending toxin or cytotoxic necrotizing factor toxin, or that have the capacity to detach tissue culture cells.

EPEC strains that cause infantile diarrhea among individuals in developing countries [2, 3] adhere to epithelial cells in a characteristic pattern called "localized adherence" (LA) [4]. A similar adherence pattern,

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which is known as "localized-like adherence" (LAL), has been seen in HEp-2 cells infected with EPEC for 6 h [5].

The LA phenotype is dependent on the presence of the EPEC adherence factor (EAF) plasmid [6]. This plasmid harbors a 14-gene operon, which encodes a type IV pilus known as the "bundle-forming pilus" (BFP) [7, 8]. A subset of 3 genes also encoded on the EAF plasmid, known as the "plasmid-encoded regulator" *(perABC),* is involved in the transcriptional regulation of virulence genes, including *bfp* [9, 10].

The A/E lesion formation requires the products of several chromosomal genes encoded on a 35-kb pathogenicity island called the "locus of enterocyte effacement" (LEE) [11, 12]. The LEE encodes a type III secretion system [13], multiple secreted proteins, and a bacterial adhesin called "intimin" [14]. Two LEE insertion sites *(selC* and *pheU)* on the *E. coli* chromosome have been described, and a third unidentified insertion site has been reported [12, 15].

Intimin, a 94-kD outer membrane protein that is encoded by the gene *eae,* is responsible for the intimate adherence between bacteria and enterocyte plasmatic membranes. Studies of antigenic variations in the 280-aa residues of the C-terminal portion of intimin (the receptor-binding domain of the protein) and the use of polymerase chain reaction (PCR) analysis allow the classification of distinct intimin types (designated "intimin α," "intimin β," "intimin δ," and "intimin γ") and a nontypeable group [16]. Tir is one of the EPEC translocated proteins that is inserted into the host cell membrane, where it acts as a receptor to intimin [17].

Recently, EPEC was classified into 2 subcategories on the basis of hybridization results with the EAF probe: EPEC strains that hybridize with the EAF probe have been designated "typical EPEC," whereas EPEC strains that do not hybridize with the EAF probe have been designated "atypical EPEC" [1]. The most-studied EPEC strains belong to a series of O antigenic groups known as "EPEC O serogroups." Twelve EPEC serogroups (O26, O55, O86, O111, O114, O119, O125–O128, O142, and O158) were recognized by the World Health Organization in 1987. These serogroups include both typical and atypical EPEC strains.

Typical EPEC serogroups are the most frequently isolated bacterial diarrheal pathogens in developing countries, but diarrhea caused by atypical EPEC serogroups is now increasingly recognized in many countries [5, 18]. Several atypical strains of non-EPEC serogroups have also been identified in different epidemiological studies [19, 20]. However, these pathogens have not been thoroughly or well characterized for virulence genes and properties, as have typical EPEC strains.

Serotyping of atypical strains is insufficient to assess the pathogenic properties of such strains, because such organisms are quite variable in their repertoire of virulence determinants. We have applied genetic and phenotypic analysis to a collection of 118 typical and atypical strains of EPEC and non-EPEC serogroups, to identify common and unique virulence loci and traits in these organisms. We determined the presence of and some characteristics of the LEE region, and we searched for the occurrence of virulence-associated markers within the *E. coli* species. Furthermore, we also determined their adherence patterns and serotypes. These data can be used to detect atypical EPEC in clinical specimens and to elucidate the role of specific virulence factors.

MATERIALS AND METHODS

Bacterial strains. The strains examined in this report were isolated during an epidemiological study of acute diarrhea in children <2 years of age; the study was conducted in different regions of Brazil in 1997–1999 [3, 21]. The children were admitted to public hospitals for treatment in the following cities: São Paulo, Santa Catarina, Rio Grande do Norte, Goiania, and Maranhão. In the study, rectal swab specimens were obtained from 438 children with acute diarrhea (case patients) and from 422 children without any gastrointestinal symptoms (controls) for ⩾30 days before inclusion in the study.

In the aforementioned epidemiological study, each fecal specimen was examined, by use of standard methods, for the presence of *Shigella* species, *Salmonella* species, *Giardia lamblia, Yersinia enterocolitica, Campylobacter* species, *Cryptosporidium* species, and rotavirus. Four separate lactose-fermenting colonies and 2 non–lactose-fermenting colonies of each distinct morphological type were cultivated in commercial test systems (PROBAC do Brasil) for biochemical confirmation of species or genus. All *E. coli* isolates were tested with specific DNA probes designated to detect enterotoxigenic *E. coli* (LT and ST probes), enteroinvasive *E. coli* (Inv probe), Shiga toxin–producing *E. coli* (Stx1 and Stx2 probes), enteroaggregative *E. coli* (EAEC probe), diffusely adhering *E. coli* (*daaC* and AIDA-I probes), and EPEC (*eae* and EAF probes).

Serotyping. Identification of somatic (O) and flagella (H) antigens of typical and atypical strains of non-EPEC serogroups was done using standard agglutination methods [22], with specific antisera O1–O175 and H1–H56 acquired commercially (from the Universidad de Santiago de Compostela; Lugo, Spain). Ten strains also were tested in the Enteric Section of Instituto Adolfo Lutz (São Paulo, Brazil), with the use of H antisera prepared with type strains.

DNA hybridization. All strains were tested by colony blot hybridization with the probe fragments shown in table 1. Colony blots were prepared with Whatman 541 filter papers. The DNA probes were prepared by extracting plasmids by use of the method of Birnboim and Doly [34], digesting them with appropriate restriction endonucleases or amplifying them from prototype strains by use of PCR, and purifying fragments by

Table 1. Description of genetic probes for virulence markers used in colony blot hybridization of enteropathogenic *Escherichia coli* isolates.

Probe	Associated property	Description of probe	Reference
perA	Plasmid-encoded regulator	3500-bp fragment of pCVD450	[9]
LEE probe			
A	Right extremity of LEE	2870-bp fragment of pCVD453	[12]
B	Part of *escV* and *escN*	2940-bp fragment of pCVD461	[12]
C	Part of *eae*	1050-bp fragment of pCVD443	[12]
D	Part of *espA* and *espB*	2300-bp fragment of pCVD460	[12]
bfpA	Bundle-forming pilus	852-bp fragment of pMSD207	[23]
E-hly	EHEC hemolysin	3400-bp fragment of pCVD419	[24]
hly	α-Hemolysin	6400-bp fragment of pSF400	[25]
afa	Afimbrial adhesin of Dr family	750-bp amplified fragment	[26]
pap	P fimbriae	328-bp amplified fragment	[26]
sfa	S fimbriae	410-bp amplified fragment	[26]
aggA	AAF/I fimbrial subunit	450-bp amplified fragment	[27]
aafA	AAF/II fimbrial subunit	550-bp amplified fragment	[28]
aag3A	AAF/III fimbrial subunit	462-bp amplified fragment	[29]
astA	Heat-stable toxin (EAST1)	111-bp amplified fragment	[30]
cdt	Cytolethal distending toxin	1357-bp fragment from pCVD448	[31]
cnf	Cytotoxic necrotizing factor	335-bp fragment of pEOSW1	[32]
aer	Aerobactin	602-bp amplified fragment	[33]

NOTE. AAF, aggregative adhesion fimbria; E-hly, enterohemorrhagic *E. coli* hemolysin; EHEC, enterohemmorhagic *E. coli;* LEE, locus of enterocyte effacement.

gel extraction. The probes were labeled with [α-^{32}P]dCTP, and colony hybridization assays were performed as described elsewhere [35].

Insertion sites of the LEE region. To verify whether LEE was inserted downstream of the *selC* locus, PCRs that amplify the junctions of this locus with the *E. coli* chromosome were performed [12, 15]. For the reactions, 10 μL of template DNA (from a boiled suspension [in distilled water] of bacteria grown in MacConkey agar), 50 μmol/L each dNTP, 1 U of *Taq* DNA polymerase, 1.5 mmol/L $MgCl_2$, and 0.5 μmol/L each primer were mixed. PCRs also were performed to check whether *pheU* was intact [15]. The amplification conditions and primer sequences that were used are presented in table 2.

Intimin typing. To identify the intimin type of the strains, PCRs were performed with forward primers designed on the basis of the *eae* sequence of EPEC strains of serotypes O127:H6 (Int-α), O111:H− (Int-β), and O86:H34 (Int-δ), and an O157:H7 strain (Int-γ). A conserved primer (Int-Ru) was used in all reactions [16]. The amplification conditions and primer sequences that were used are presented in table 2.

Tir typing. To detect the 2 forms of the Tir protein, EPEC Tir and EHEC Tir, PCRs that amplified both coding regions of the EPEC and EHEC *tir* genes were performed using Tir004 and Tir005 primers (table 2). Tir004 hybridized to the ribosome binding site and to the 5′ end of both *tir* genes, whereas Tir005 hybridized to the 3′ end sequences that span the stop codons [36].

HEp-2 adherence 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. [4]. Monolayers were examined after incubation for 3 h. In brief, monolayers of 10^5 HEp-2 cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco-BRL) containing 10% fetal bovine serum, by use of 24-welled plates (Falcon Becton Dickinson). Bacterial strains were grown statically in 3 mL of tryptic soy broth (Difco) for 16–18 h at 37°C. Cell monolayers were infected with $\sim 3 \times 10^7$ bacteria (40 μL of bacterial cultures added to 1 mL of DMEM) and were incubated at 37°C for 3 h. The infected monolayers were washed with sterile PBS, fixed with methanol, stained with May-Grünwald and Giemsa stain, and examined under a light microscope. When the adherence pattern was weak or negative, a new preparation was made and examined after a 6-h incubation period.

Random amplified polymorphic DNA (RAPD) reaction. To investigate the genetic relationship between typical and atypical EPEC strains, we used RAPD analysis to study our collection of strains. Seventy-eight strains of EPEC serotypes and non-EPEC serogroups were typed by RAPD analysis. RAPD profiles were generated using 3 different primers with distinct G+C contents. These primers generated 105 polymorphisms

Table 2. Primer sequences and amplification cycles used to verify some locus of enterocyte effacement characteristics in enteropathogenic *Escherichia coli* strains.

Characteristic	Primer	Amplification cycle	Reference
selC Intact	K261 and K260	At 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min	[12]
Junction of LEE in *selC*			
Right	K255 and K260	At 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min	[15]
Left	K296 and K295	At 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min	[15]
pheU intact	K913 and K914	At 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min	[15]
Intimin			
α	Int-α and Int-R	At 95°C for 20 s, 45°C for 1 min, and 74°C for 1 min	[16]
β	Int-β and Int-R	At 95°C for 20 s, 45°C for 1 min, and 74°C for 1 min	[16]
γ	Int-γ and Int-Ru	At 95°C for 20 s, 55°C for 1 min, and 74°C for 1 min	[16]
δ	Int-δ and Int-Ru	At 95°C for 20 s, 45°C for 1 min, and 74°C for 1 min	[16]
Tir	Tir004 and Tir005	At 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min	[36]

that were used to construct a binary data matrix of the presence and absence of shared bands. The data from these comparisons were used to calculate the similarities between pairs of samples by use of the Jaccard coefficient. Genomic DNA was extracted and purified from bacterial cultures in Luria-Bertani broth by use of a kit (Easy-DNA Kit; Invitrogen), according to the manufacturer's instructions. PCR for the RAPD reaction was performed in 20-μL reaction volumes containing 10 ng of DNA, 20 mmol/L Tris-HCl (pH, 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 50 μmol/L each dNTP (Gibco BRL), 0.3 μL of random primers (OPE 16 [5′-GGTGACTGTG-3′], OPP-03 [5′-CTGATACGCC-3′], and OPJ18 [5′-TGGTCGCAGA-3′]; Operon Technologies), 1.3 U of *Taq* DNA polymerase (Gibco BRL) and overlaid with 30 μL mineral oil.

Amplification reactions were performed in a thermalcycler (Eppendorf Mastercycler Gradient) and included one previous step at 94°C for 4 min and 40 cycles, followed by denaturation at 94°C for 45 s, annealing at 35°C for 45 s, and extension at 72°C for 2 min. An additional extension step at 72°C for 7 min was included at the end of the PCR cycles. Amplified products were electrophoresed in 1.4% agarose gels, stained with ethidium bromide, and visualized using UV light. The 1-kb DNA ladder (Gibco BRL) was used as a molecular size marker in all gels.

Statistical analysis. Statistical analysis of the data was performed using the numerical taxonomy and multivariate analysis system software program (NTSYS), version 1.7 (Exeter Software). Data for children with diarrhea and data for controls were compared using a 2-tailed χ^2 test or Fisher's exact test.

RESULTS

A total of 438 children with diarrhea and 422 matched control children without diarrhea were studied. We identified potential diarrheogenic *E. coli* by use of assays of adhesion to HEp-2 cells and by hybridization with specific DNA probes. The frequency of isolation of pathogens from children with diarrhea is shown in table 3.

Of the 860 fecal specimens analyzed, 42 (4.9%; 36 from case patients and 6 from controls) were typical EPEC and 21 (2.4%; 14 from case patients and 7 from controls) were atypical EPEC; they belonged to any of the established EPEC serogroups (O26, O55, O111, O114, O119, O125–O128, O142, and O158). Eleven fecal specimens (1.3%; 9 from case patients and 2 from controls) contained typical non-EPEC serogroups and O nontypeable, and 44 (5.1%; 24 from case patients and 20 from controls) contained atypical non-EPEC serogroups and O nontypeable, as tested by slide agglutination with O-specific antisera. The number of isolates with these characteristics in each specimen varied from 1 to 3. For the present study, only 1 isolate from each of these 118 specimens was selected. Only 12 of the 118 strains (9 from case patients and 3 from controls) were associated with enteroaggregative *E. coli*, diffusely adherent

Table 3. Organisms isolated from stool specimens.

Organism	No. (%) of isolates recovered			P[a]
	From patients with diarrhea (n = 438)	From controls (n = 422)	Total (n = 860)	
Typical EPEC				
EPEC O serogroup	36 (8.2)	6 (1.4)	42 (4.9)	.000
Non-EPEC O serogroup	9 (2.1)	2 (0.5)	11 (1.3)	.036
Atypical EPEC				
EPEC O serogroup	14 (3.2)	7 (1.7)	21 (2.4)	.149
Non-EPEC O serogroup	24 (5.5)	20 (4.7)	44 (5.1)	.647
EAEC	75 (17.1)	49 (11.6)	124 (14.4)	.022
DAEC	81 (18.5)	65 (15.4)	146 (17)	.242
Shigella species	38 (8.7)	5 (1.2)	43 (5)	.000
Salmonella species	3 (0.7)	1 (0.2)	4 (0.5)	.624
Rotavirus	51 (11.7)	13 (3.1)	64 (7.4)	.000

NOTE. DAEC, diffusely adherent *Escherichia coli;* EAEC, enteroaggregative *E. coli;* EPEC, enteropathogenic *E. coli.*

[a] Calculated using the χ^2 test; $P < .05$ was considered to be significant.

E. coli, Shigella species, or rotavirus. Rotavirus was the most frequently associated pathogen (found in 6 case patients), followed by *Shigella* species (found in 3 case patients). In the present study, typical EPEC and non-EPEC strains were significantly associated with diarrhea, and, although not significantly associated with diarrhea, atypical EPEC and non-EPEC strains were recovered more frequently from children with diarrhea than from children without diarrhea (table 3).

In total, 53 typical EPEC ($n = 42$) and non-EPEC ($n = 11$) strains and 65 atypical EPEC ($n = 21$) and non-EPEC ($n = 44$) strains were characterized by virulence-associated markers, adherence to HEp-2 cells, and RAPD analysis (tables 4 and 5). The frequency of the atypical strains with distinct characteristics with regard to their association with diarrhea was further analyzed.

Characteristics of typical strains. Of the 53 typical strains, 42 (80%) belonged to 5 EPEC serogroups (37 strains were of serogroups O55, O111, and O119), 8 were classified as non-EPEC serotypes (O2:H2, O2:H45, O101:H33, O145:HNT, O157:HNT, O162:NM, and O162:H33), and 3 were nontypeable (table 4). All typical strains, but 2 EPEC strains (O119:H6), reacted with the *bfpA* and *perABC*. All 53 strains that were tested carried LEE-associated DNA sequences, as determined by hybridization with specific LEE-derived gene probes (LEE A, LEE B, LEE C, and LEE D [i.e., LEEA–D]). With regard to the presence of DNA sequences related to virulence in other pathogenic categories of *E. coli*, only 3 EPEC strains (O86:NM, O86:H34, and O111:H2) hybridized with the *cdt* (for "cytolethal distending toxin") probe.

Concerning the insertion site of the LEE as determined by

Table 4. Genotypic and phenotypic characterization of 53 typical enteropathogenic *Escherichia coli* (EPEC) and non-EPEC serogroups.

Serotype	Strain	Source(s)[a]	Target genes	LEE insertion	Intimin type	Adhesion (3 h)
EPEC O serogroup						
O55:NM	T3, T6, T16, T17, T22, T25, T27, T31, T42, T45, and T53	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	γ	LA
O86:NM	T33	Patient	*bfpA, perA,* LEEA–D, Tir, and *cdt*	*selC*	δ	LA
O86:H34	T1	Patient	*bfpA, perA,* LEEA–D, Tir, and *cdt*	*selC*	δ	LA
O111:NM	T7 and T20	Patient	*bfpA, perA,* LEEA–D, and Tir	ND	β	LA
	T9	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	β	LA
	T24 and T28	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	α	LA
O111:H2	T37	Patient	*bfpA, perA,* LEEA–D, Tir, and *cdt*	*selC*	β	LA
O119:NM	T11	Patient	*bfpA, perA,* LEEA–D, and Tir	ND	β	LA
	T13 and T30	Patient and control	*bfpA, perA,* LEEA–D, and Tir	*selC*	β	LA
O119:H6	T4, T5, T8, T10, T26, T32, and T46	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	β	LA
	T35, T38, T40, T49, T52, and T54	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
	T48	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
	T14	Patient	*bfpA, perA,* LEEA–D, and Tir	ND	β	LA
	T29	Control	*bfpA,* LEEA–D, and Tir	*selC*	β	LA
	T15	Patient	LEEA–D and Tir	*selC*	β	LA
O127:NM, H6	T41 and T43	Control	*bfpA, perA,* LEEA–D, and Tir	*selC*	α	LA
O127:H6	T44	Control	*bfpA, perA,* LEEA–D, and Tir	ND	γ	LA
Non-EPEC O serogroup						
O2:H2	T18	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
O2:H45	T19	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	δ	LA
O101:H33	T36	Control	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
O145:HNT	T23	Control	*bfpA, perA,* LEEA–D, and Tir	*selC*	γ	LA
O157:HNT	T47 and T34	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	α	LA
O162:NM	T12	Patient	*bfpA, perA,* LEEA–D, and Tir	*pheU*	NT	LA
O162:H33	T21	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
ONT:H45, HND	T2 and T51	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	NT	LA
ONT:HND	T50	Patient	*bfpA, perA,* LEEA–D, and Tir	*selC*	α	LA

NOTE. LA, localized adherence; LEEA–D, locus of enterocyte effacement probes A–D; ND, nondetermined; NM, nonmotile; NT, nontypeable.

[a] Patients were children with acute diarrhea, and controls were children without acute diarrhea.

Table 5. Genotypic and phenotypic characterization of 65 atypical enteropathogenic *Escherichia coli* (EPEC) and non-EPEC serogroups.

Serotype	Strain(s)	Source(s)[a]	Target genes	LEE insertion	Intimin type	Adhesion (6 h)
EPEC O serogroup						
O26:NM	A1 and A14[b]	Patient	*perA,* LEEA–D, Tir, and *astA*	*pheU*	NT, β	LAL
	A24	Patient	*perA,* LEEA–D, Tir, and *astA, afa*	*selC*	NT	LAL
	A27	Control	*perA,* LEEA–D, and Tir	*pheU*	β	LAL
O55:NM	A46	Control	*perA,* LEEA–D, Tir, and *astA*	*selC*	γ	LAL
O111:NM	A3	Patient	*perA,* LEEA–D, Tir, and *astA*	ND	α	LAL
	A4	Control	*perA,* LEEA–D, and Tir	ND	α	LAL
	A12	Control	LEEA–D and Tir	ND	NT	LAL
O114:NM	A45	Control	LEEA–D and Tir	*selC*	NT	LAL
O119:H2	A60, A62, A66,[c] and A67	Patient and control	*perA,* LEEA–D, Tir, and *astA*	*pheU*	β	LAL, DE
O126:NM	A13	Patient	*perA,* LEEA–D, and Tir	*pheU*	NT	LAL
O127:NM	A34	Control	LEEA–D and Tir	*pheU*	δ	LAL
O127:H40	A5[d] and A7	Patient	LEEA–D and Tir	ND	γ	LAL
O128:NM	A2	Patient	LEEA–D and Tir	ND	β	LAL
O142:NM	A11	Patient	*perA,* LEEA–D, Tir, and *astA*	*selC*	α	LAL
O142:H2	A44 and A55	Patient	LEEA–D and Tir	*selC*	α	LAL, DE
Non-EPEC serogroup						
O33:H6	A43	Control	*perA,* LEEA–D, and Tir	*pheU*	γ	LAL
O35:H19	A42	Control	LEEA–D and Tir	*selC*	NT	LAL
O85:H40	A15	Patient	*perA,* LEEA–D, and Tir	ND	NT	LAL
O101:NM	A21	Patient	*perA,* LEEA–D, and Tir	*pheU*	NT	LAL
O103:NM	A25 and A28	Control	*perA,* LEEA–D, Tir, and *astA*	*selC, pheU*	β, α	LAL
O105:H7	A17	Control	LEEA–D and Tir	*selC*	γ	LAL
O108:H31	A58	Control	LEEA–D and Tir	ND	γ	DE
O109:H54	A40	Control	LEEA–D and Tir	ND	β	LAL
O141:HNT	A47	Control	LEEA–D, Tir, and *astA*	*selC*	NT	LAL
O156:H16	A19	Patient	*perA,* LEEA–D, Tir, and *astA*	ND	α	LAL
O157:NM	A29 and A36	Control	LEEA–D and Tir	*selC*	δ	NA, NA
ONT:H18	A18	Patient	LEEA–D and *astA*	*pheU*	β	AA
ONT:NM	A23	Patient	*perA,* LEEA–D, Tir, *astA,* and *afa*	*selC*	NT	DE
	A26 and A32	Control	LEEA–D, Tir, and *astA*	*selC*	γ	DE, DA
ONT:NM, HND	A30, A38, A51–A53, A57, A59, A63, A64, and A68	Patient	*perA,* LEEA–D, Tir, and *astA*	v	v	v
	A16, A33, and A65	Control	*perA,* LEEA–D, and Tir	v	v	v
	A10, A20, A22, A31, A35, A37, A39, A41,[c] A48,[c] A49, A50, A54,[c] A56,[c] and A61[c,e]	Patient and control	LEEA–D and Tir	v	v	v

NOTE. AA, aggregative adherence; DA, diffuse adherence; DE, cell detaching; LAL, localized-like adherence; LEEA–D, locus of enterocyte effacement probes A–D; ND, not determined; NM, nonmotile; NT, nontypeable; v, variable.

[a] Patients were children with acute diarrhea, and controls were children without acute diarrhea.
[b] Positive for E-hly.
[c] Strains isolated from control.
[d] Positive for *astA* and *afa.*
[e] Negative for Tir.

PCR analysis, within a specific serotype, all typical strains gave the same result. Forty-seven (89%) of the strains had LEE inserted downstream in *selC,* 1 non-EPEC strain had LEE inserted in *pheU,* and 5 EPEC strains had an unidentified insertion site; those strains had both the *selC* and *pheU* loci intact, but no amplification with the primers for the left and right junction of LEE in *selC* was detected.

On the basis of PCRs, strains of serotypes O111:NM, O127:NM, O127:H6, and O157:HNT produced intimin α, most of the strains of serotypes O111:NM, O111:H2, O119:NM, and O119:H6 contained intimin β, and strains belonging to serotypes O55:NM, O127:H6, and O145:HNT contained intimin γ, whereas strains of serotypes O86:NM, O86:H34, and O2:

H45 were specifically amplified with the intimin δ primer. Seven EPEC and 6 non-EPEC strains produced a nontypeable intimin.

Sequences similar to that amplified with the primers designed on the basis of the Tir sequences of EPEC strain 2348/69 and EHEC strain 86/24 were found in all EPEC and non-EPEC strains. All typical strains showed only the LA pattern.

Characteristics of atypical strains. Twenty-one atypical strains belonged to EPEC serogroups (18 belonged to serogroups O26, O111, O119, O127, and O142), 13 represented non-EPEC serogroups (O33, O35, O85, O101, O103, O105, O108, O109, O141, O156, and O157), and 31 were nontypeable (table 5). According to the hybridization studies, none of the atypical strains hybridized with the *bfpA* probe, and 33 strains (51%; 13 belonging to EPEC serogroups and 20 belonging to non-EPEC serogroups) reacted with the *perABC* probe. All 65 strains hybridized with the LEEA–D probes. Twenty-nine atypical strains (45%) reacted with *astA* (for EAST1 toxin), 3 strains had the *afa* sequence (for afimbrial adhesin), and 1 carried *E-hly* (for EHEC hemolysin).

With regard to the LEE insertion sites, LEE was inserted downstream in *selC* in EPEC strains from serotypes O55:NM, O114:NM, O142:NM, and O142:H2, and it was inserted in *pheU* in strains from serotypes O26:NM, O119:H2, O126:NM, and O127:NM. In non-EPEC serogroups, LEE was inserted downstream in *selC* in 6 strains from serogroups O35, O103, O105, O141, and O157, and it was inserted in *pheU* in 3 strains from serogroups O33, O101, and O103. Of the remaining 35 non-EPEC strains, 17 had LEE inserted downstream in *selC,* 10 had LEE inserted in *pheU,* and 8 had an unidentified insertion site.

Regarding intimin types, most of the strains of serotypes O111:NM, O142:NM, O142:H2, O103:NM, and O156:H16 produced intimin α, strains of serotypes O26:NM, O119:H2, O128:NM, O103:NM, and O109:H54 contained intimin β, and strains belonging to serotypes O55:NM, O127:H40, O105:H7, and O108:H31 contained intimin γ, whereas strains from serotypes O33:H6, O127:NM, and O157:NM were specifically amplified with the intimin δ primer. Of the remaining 31 strains, 12 produced a nontypeable intimin, 8 produced intimin γ, and 6 produced intimin β, whereas strains that produced intimins α and δ (3 and 2 strains, respectively) occurred infrequently. Tir sequences correlated with EPEC strain 2348/69 and EHEC strain 86/24 were found in all atypical strains, with the exception of one atypical nontypeable strain. Forty atypical strains (61.5%) showed the LAL pattern in the 6-h assay, 3 strains showed the aggregative adherence pattern, 1 strain showed the diffuse adherence pattern, and 11 strains promoted cell detaching.

Distribution of virulence markers in children with or without diarrhea. The distribution of typical and atypical strains, showing the different virulence markers in case patients and controls, is presented in table 6. With rare exceptions, typical strains produced only the virulence factors encoded by the LEE region and the EAF plasmid. In contrast, atypical strains expressed EAST1 and other potential virulence factors not encoded in the LEE region. Only the atypical strains that carried the *astA* sequence were associated with diarrhea (22 [58%] of 38 strains vs. 7 [26%] of 27 strains; $P = .01$). Strains showing the LAL pattern were found both in children with diarrhea (61%) and in controls (62%).

RAPD analysis. The dendrogram presented in figure 1 shows 2 main groups. Group A includes typical and atypical strains (most of them had LEE inserted in *selC*), and group B encompasses 6 clusters. Clusters B1–B3 and B6 include, with the exception of one typical strain (O2:H2), most of the atypical strains with different virulence profiles. Clusters B4 and B5 contain both typical and atypical strains that are genetically distinct. The main clusters encompassed many small clusters corresponding to different serotypes. Different RAPD types were also found among strains from different Brazilian regions.

DISCUSSION

The 118 EPEC strains (typical and atypical) that we analyzed during an epidemiological study conducted in different regions of Brazil were isolated from children with or without diarrhea [3, 21]. Although typical EPEC and non-EPEC O serogroups were significantly associated with diarrhea (45 [10.3%] of 438 specimens from children with diarrhea; $P < .01$), atypical strains were found in 8.7% of children with diarrhea. Many of these strains belong to the EPEC serogroups, but, without comparison with a truly pathogenic and typical EPEC, we cannot assign

Table 6. Distribution of virulence markers among typical and atypical enteropathogenic *Escherichia coli* strains in patients with acute diarrhea and controls.

	No. (%) of typical strains		No. (%) of atypical strains	
Virulence marker	From patients ($n = 45$)	From controls ($n = 8$)	From patients ($n = 38$)	From controls ($n = 27$)
A–D	45 (100)	8 (100)[a]	38 (100)	27 (100)
Tir	45 (100)	8 (100)[a]	38 (100)	26 (96)
Bfp	44 (98)	8 (100)[a]	0	0
Per	44 (98)	7 (88)[a]	23 (61)	10 (37)
EAST1	0	0	22 (58)	7 (26)[a]
CDT	3 (67)	0	0	0
Afa	0	0	3 (8)	0
E-hly	0	0	1 (3)	0

NOTE. Afa, afimbrial adhesin of the Dr family; Bfp, bundle-forming pilus; CDT, cytolethal distending toxin; EAST1, enteroaggregative *Escherichia coli* heat-stable enterotoxin; E-hly; enterohemorrhagic *E. coli* hemolysin; LEE, locus of enterocyte effacement; Per, plasmid-encoded regulator; Tir, translocated intimin receptor.

[a] Significant at *P.*

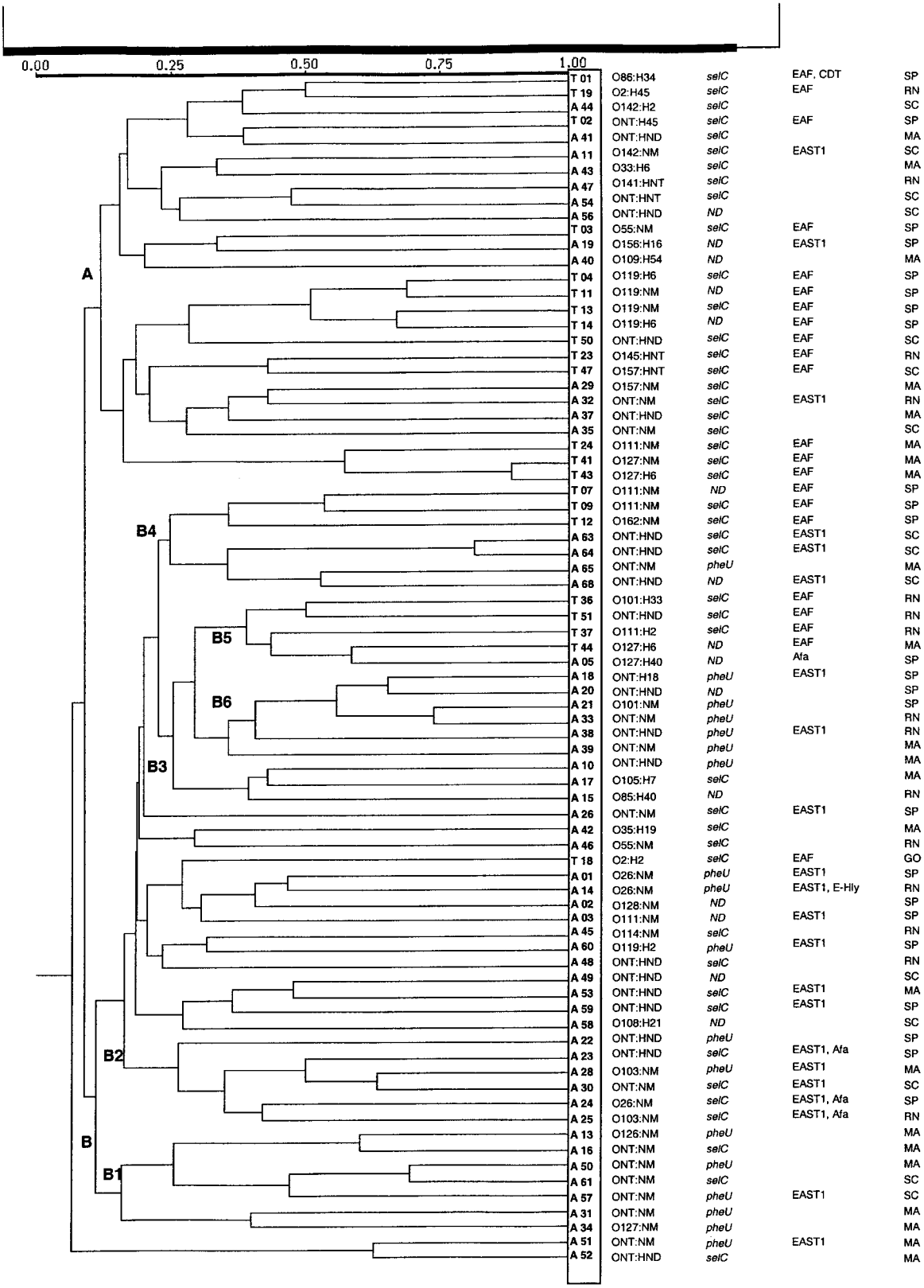

Figure 1. Dendrogram based on UPGMA (unweighted pair group method with averaging) cluster analysis of Jaccard coefficients. Shown are the no. of the strains *(column 1),* their serotypes *(column 2),* their locus of enterocyte effacement (LEE) insertion site *(column 3),* their non–LEE-encoded virulence factors *(column 4),* and the location of isolation in Brazil *(column 5).* GO, Goiania; MA, Maranhão; RN, Rio Grande do Norte; SC, Santa Catarina; SP, São Paulo.

a definite pathogenic role to the atypical EPEC strains identified by probing in our previous study.

The division of EPEC strains into typical and atypical groups is based on the presence of the EAF plasmid, as demonstrated by hybridization with a DNA probe, which is a region of 1-kb fragment, derived from the EAF plasmid. This plasmid encodes a BFP and a transcriptional activator *(perABC),* which is a regulatory sequence involved in the expression of BFP and the A/E lesion [8, 9]. Complete agreement between results obtained with *bfpA* or *perABC* probes and those obtained with the EAF probe has been observed in previous studies [19, 21]. In the present study, with the exception of 2 strains, all typical EPEC and non-EPEC strains reacted with the *bfpA* and *perABC* probes. In contrast, none of the atypical strains hybridized with the *bfpA* probe, and most atypical strains lacked *per.*

To fully characterize the strains, we examined the presence of the genes' LEE region by probe hybridization. All typical and atypical strains hybridized with LEEA–D. This result was expected, because all the strains were positive for the fluorescein-actin staining test (data not shown). The majority of the typical and atypical strains of EPEC and non-EPEC serogroups (74%) expressed intimins α, β, γ, or δ. All strains, with the exception of one atypical non-EPEC strain, presented Tir sequences correlated with enteropathogenic or enterohemorrhagic *E. coli.*

Our results, when combined with those of previous studies [37], indicate that typical and atypical EPEC represent collections of distinct serotypes and virulence properties. Typical and atypical EPEC strains belong to 2 different sets of serotypes. Most of the serotypes found may easily be classified as typical or atypical. However, some serotypes were not so readily classified; the most frequently noted of these serotypes were O111:NM, O55:NM, and O127:NM. In fact, these serotypes are derived from motile strains, and their O:H types could also be classified. We found new serogroups in the typical strains (O2, O145, O157, and O162); most of them had not yet been identified, and they may represent unrecognized EPEC serogroups [38–41].

Typical and atypical strains also differ in adherence patterns. All typical strains showed only the LA pattern mediated by the BFP fimbriae, whereas atypical strains showed the LAL pattern, the diffuse adherence pattern, the aggregative adherence pattern, or cell detaching.

Regarding virulence characteristics, typical strains are more homogeneous in their virulence characteristics than are atypical ones. All typical strains, with the exception of 3 EPEC strains that produced the cytolethal distending toxin, expressed only the virulence factors encoded by the LEE region and the EAF plasmid. In contrast, almost one-half of the atypical EPEC strains expressed EAST1 or other potential virulence factors not encoded in the LEE region. Accordingly, there were 2 kinds of atypical strains: those that express only the LEE-encoded virulence factors and those that express both LEE and the non–LEE-encoded virulence factors. The occurrence of atypical strains that express both LEE and the non–LEE-encoded virulence factors was significantly associated with diarrhea ($P = .01$).

It has been reported that the EAF plasmid may be lost during storage or even during infection [42]. There is also evidence suggesting that atypical EPEC strains may be ancestors to typical EPEC and EHEC bacteria [43]. The analysis of RAPD polymorphisms revealed that typical and atypical strains are genetically different, and that they also have more than a single clonal origin. These results confirm previous findings obtained by multilocus enzyme electrophoresis typing, and they are consistent with the concept that typical EPEC and atypical EPEC are distinct bacterial lineages.

In a previous study [19], atypical strains were considered to be a miscellaneous group composed of atypical EPEC, enteroaggregative *E. coli,* diffusely adherent *E. coli,* or uropathogenic *E. coli* strains that had acquired a LEE region by horizontal transfer. In the present study, with few exceptions, the atypical strains could represent 2 groups of strains: those that express only the LEE-encoded virulence factors and those that express both LEE and EAST1 toxin.

Although most of the genotypes and phenotypes examined were common in both atypical EPEC strains isolated from patients and controls, we found that the EAST1 toxin was detected much more frequently in atypical strains isolated from case patients than in those isolated from controls ($P = .01$). This virulence marker may be a useful tool for the diagnosis of truly atypical EPEC pathogenic strains.

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