Phenotypic and Genotypic Characteristics of *Escherichia coli* Strains of Non–Enteropathogenic *E. coli* (EPEC) Serogroups that Carry *eae* and Lack the EPEC Adherence Factor and Shiga Toxin DNA Probe Sequences

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This study was conducted to characterize the virulence potential of 59 *Escherichia coli* strains carrying *eae* and lacking the enteropathogenic *E. coli* adherence factor and Shiga toxin probe sequences. In hybridization studies, all strains carried the locus of enterocyte effacement (LEE)–associated DNA sequences. Of the other 15 virulence DNA sequences tested, *hly* was the most frequent (44.1%); 17 combinations of these sequences were found, but strains carrying *eae* only (*eae* profile) were the most frequent (35.6%). Except for 1 cytodetaching strain, all others adhered to HeLa and Caco-2 cells, most of which (~75.0%) showed variations of the localized adherence pattern. Actin accumulation was detected in 75.9% of the nondetaching strains. Most strains had LEE, probably inserted in *pheU* (49.2%), and presented a nontypeable intimin (83.1%). Translocated intimin receptor–derived DNA sequences correlated with enteropathogenic and enterohemorrhagic *E. coli* in 61.0% and 32.0% of the strains, respectively. Thirty-five different serotypes were found. Only strains with the *eae* profile were associated with diarrhea ($P = .039$).

Enteropathogenic *Escherichia coli* (EPEC) is responsible for a large number of cases of infantile diarrhea in several developing countries [1]. These strains belong to specific serotypes within 12 different *E. coli* serogroups (O groups) [2] and produce a characteristic adherence pattern in tissue culture cells called localized adherence (LA) [3]. In the LA pattern, bacteria bind to localized areas of the cell surface, forming compact microcolonies (bacterial clusters) that are visualized after 3 h of contact of bacteria with the cells [3]. This phenomenon is associated with the presence of the large EPEC adherence factor (EAF) plasmid, which carries the EAF probe sequence [4]. Also present in the EAF plasmid is the cluster of genes that encode bundle-forming pili (BFP) that seem to interconnect bacteria within microcolonies and thus promote their stabilization [5].

The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E) that is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at the sites of bacterial attachment [1]. The fluorescent actin–staining (FAS) test allows for the identification of strains that produce A/E lesions, because of the detection of aggregated actin filaments beneath the attached bacteria [6]. The ability to produce A/E lesions also has been detected in strains of enterohemorrhagic *E. coli* (EHEC), Shiga toxin–producing *E. coli* (STEC), and other bacterial species [1].

The EAF plasmid is not essential for the formation of A/E lesions, although its presence enhances the efficiency with which they occur, probably because of the influence of a cluster of regulatory genes *(per A, perB,* and *perC)* [7]. The determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), which is a pathogenicity island that contains the genes encoding intimin (an outer membrane adhesion molecule), a type III secretion system, a number of secreted (Esp) proteins, and the translocated intimin receptor (Tir) [8]. Two different LEE insertion sites on the *E. coli* chromosome have been described, and a third unidentified insertion site has been reported [9].

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Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene, is responsible for the intimate adherence between bacteria and enterocyte membranes [8]. Studies on antigenic variations in the 280–amino acid residues of the C-terminal portion of intimin (the receptor-binding domain of the protein) and the use of polymerase chain reactions (PCRs) allowed for the classification of ⩾5 distinct intimin types or subtypes among EPEC and EHEC/STEC strains [10, 11]. The Esp molecules (EspA, EspB, and EspD) are involved in the formation of a translocon that delivers effector molecules to the host cell and disrupts the cytoskeleton, which subverts the host cell functions [8]. Tir is one of the EPEC translocated proteins; it is inserted into the host cell membrane, where it acts as a receptor to intimin [12].

E. coli strains carrying *eae* but lacking the EAF plasmid probe sequence and the Shiga toxins genes *(stx)* have been detected in different studies [1]. Many of these strains belong to EPEC serogroups (but not serotypes) [13]. However, in a study carried out in São Paulo, Brazil, on the epidemiology of acute infantile diarrhea, we found various *E. coli* strains with these characteristics that did not belong to the EPEC serogroups [14]. In the present study, these strains were characterized further at the genotypic and phenotypic levels, with the aim of better understanding their potential as putative enteropathogens. We determined the presence and some characteristics of the LEE region and searched for the occurrence of virulence associated markers within the *E. coli* species. Furthermore, we also determined their adherence patterns, ability to promote actin accumulation in vitro, and serotypes.

Materials and Methods

Origin and characteristics of the bacterial strains studied. The strains analyzed in this work were isolated during a study on the epidemiology of acute diarrhea in children (1–4 years old) who presented to the emergency room of Hospital Infantil Menino Jesus (São Paulo, Brazil) for medical attention (April 1989–March 1990) [14]. That study comprised rectal swab specimens obtained from 505 children with bloody and nonbloody acute diarrhea (case patients) and 505 children without any gastrointestinal signs or symptoms during the 30 days before collection (control patients). Except for 2 case patients and 5 control patients, none of the children had received antibiotics in the 14 days before fecal specimens were collected.

In the study mentioned [14], each fecal specimen was examined by standard methods for the presence of the following enteropathogenic bacteria: *Shigella* species, *Salmonella* species, *Yersinia enterocolitica, Aeromonas* species, and *Campylobacter* species. Adenovirus and rotavirus were tested by using an ELISA kit. Five lactose-fermenting and any non–lactose-fermenting colonies of each morphological type 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 in colony blots with radioactive-specific DNA probes for enterotoxigenic *E. coli* (ETEC; LT-I, LT-II, ST-Ip, ST-Ih, and ST-II probes), enteroinvasive *E. coli* (EIEC; Inv probe), EHEC/STEC (*stx1* and *stx2* probes), and EPEC (*eae* and EAF). Of the 1010 fecal specimens analyzed, 59 (32 from nonbloody diarrhea from 505 case patients and 27 from 505 nonmatched control patients) carried *E. coli* isolates bearing only the *eae* sequence; none of these isolates belonged to any of the established EPEC-bearing serogroups (O26, O55, O111, O114, O119, O125, O126, O127, O128, O142, and O158), as tested by slide agglutination with O-specific antisera. The number of isolates with these characteristics in each specimen varied from 1 to 5. For the present study, only 1 isolate from each of these 59 specimens was selected; thus, each isolate was regarded as 1 strain. Only 16 of the 59 strains (11 from case patients and 5 from control patients) were associated with 1 or 2 of the following enteropathogens: enteroaggregative *E. coli* (EAEC), rotavirus, *Aeromonas caviae, Shigella sonnei,* EIEC, and ETEC (LT-I producers). Rotavirus was the most frequently associated pathogen (found in 6 case patients), followed by enteroaggregative *E. coli* (found in 4 case patients and 1 control patient).

Search for virulence DNA sequences of the different categories of pathogenic E. coli. Hybridization assays were performed with specific DNA fragments amplified from prototype strains or fragments derived from cloned DNA probes (table 1). These fragments were labeled with [α-d-^{32}P]-dATP, and colony hybridization assays were performed as described elsewhere [28].

Hemolysin production. Two microliters of overnight bacterial cultures were inoculated onto Blood Agar Base (Difco) supplemented with 10 m*M* of calcium chloride and 5% of defibrinated sheep erythrocytes washed 3 times with PBS [29, 30]. The plates were incubated at 37°C and were examined after 3 h and 24 h for the presence of zones of hemolysis around bacterial growth.

Adherence pattern. The assay described by Cravioto et al. [31] was used with some adaptations, to determine the adherence pattern of the strains. HeLa cells at 60% confluence and polarized Caco-2 cells were cultivated in 24-well tissue culture plates containing MEM and Dulbecco's modified eagle medium (DMEM), respectively, supplemented with 5% (MEM) or 10% (DMEM) of fetal calf serum. After 2 washes with PBS, 1.0 mL of fresh media (MEM or DMEM supplemented with 2% D-mannose) was dispensed on the cell monolayers. *E. coli* strains were grown overnight in Luria broth without shaking and were diluted 1:50 in the media contained in the microplates. After an incubation period of 3 h at 37°C, monolayers were washed 10 times with PBS, fresh media was added to the wells, and an additional incubation period of 3 h proceeded (6 h assay). After 5 washes with PBS, the preparations were fixed with methanol, were stained with May Grünwald-Giemsa, and were examined blindly by light microscopy. *E. coli* HB101 was used as a negative control. *E. coli* strains E2348/69 [32], C1845 [25], and 0431-4 [33] were used as controls of the localized, diffuse, and aggregative adherence patterns, respectively. Each strain was tested at least twice, and 3 different examiners read the slides.

Detection of the ability to aggregate actin. The FAS assay described by Knutton et al. [6] was used. In brief, at the end of the adherence assay, performed as described above, monolayers were fixed in 3% formalin, were washed three times with PBS, and were treated with 0.1% Triton X-100. After 3 washes, monolayers were incubated with 5 mg/mL of fluorescein isothiocyanate–labeled phaloidin (Sigma) for 30 min and were washed 3 times (30-min incubation period each). Preparations then were examined by fluorescence microscopy. *E. coli* HB101 and *E. coli* E2348/69 were used as negative and positive controls, respectively.

Table 1. Genetic probes for virulence markers associated with different categories of pathogenic *Escherichia coli*.

Genetic probe	Associated property	*E. coli* category	Fragment	Reference
LEE A	Right extremity of LEE	EPEC	2870-bp fragment of pCVD453	[15]
LEE B	Part of *escV* and *escN*	EPEC	2946-bp fragment of pCVD461	[15]
LEE C	Part of *eae*	EPEC	1050-bp fragment of pCVD443	[15]
LEE D	Part of *espA* and *espB*	EPEC	2300-bp fragment of pCVD460	[15]
inv	Invasion plasmid of EPEC	EPEC	3800-bp fragment of pLV527	[16]
bfpA	Bundle-forming pilus	EPEC	852-bp fragment of pMSD207	[17]
perA	Plasmid-encoded regulator	EPEC	3500 bp fragment of pCVD450	[7]
EHEC-*hly*	EHEC hemolysin	EHEC	3400-bp fragment of pCVD419	[18]
hly	α-Hemolysin	UPEC, others	6400-bp fragment of pSF4000	[19]
afa	Afimbrial adhesin of the Dr family	UPEC, others	750-bp amplified fragment	[20]
pap	P fimbriae	UPEC	328-bp amplified fragment	[20]
sfa	S fimbriae	UPEC	410-bp amplified fragment	[20]
aggA	Subunit of aggregative adherence fimbriae I	EAEC	450-bp amplified fragment	[21]
aafA	Subunit of aggregative adherence fimbriae II	EAEC	550-bp amplified fragment	[22]
astA	Heat-stable toxin of EAEC (EAST-1)	EAEC, others	111-bp amplified fragment	[23]
EAEC	Plasmid sequence associated with aggregative adherence	EAEC	1000-bp fragment of pCVD432	[24]
daaC	Usher of the F1845 adhesin	DAEC, others	350-bp fragment of pSLM852	[25]
cdt	Cytolethal distending toxin	UPEC, others	1357-bp fragment of pCVD448	[26]
cnf	Cytotoxic necrotizing factor	UPEC, others	335-bp fragment of pEOSW1	[27]

NOTE. DAEC, diffusely adhering *E. coli;* EAEC, enteroaggregative *E. coli;* EAST-1, enteroaggregative *E. coli* heat-stable enterotoxin 1; EHEC, enterohemorrhagic *E. coli;* EPEC, enteropathogenic *E. coli;* LEE, locus of enterocyte effacement; UPEC, uropathogenic *E. coli.*

Detection of intimin expression. To verify the expression of intimin by strains that lacked the ability to aggregate actin (FAS-negative strains), bacterial strains were grown in Luria broth to the stationary phase, were diluted 1:100 in DMEM (an OD_{A600} of 0.05), and were incubated at 37°C for 3 h [10]. Bacterial whole-cell lysates were electrophoresed, as described by Laemmli [34], in 8% SDS-PAGE, were transferred onto nitrocellulose filter membranes (Hybond-C; Amersham Pharmacia Biotech) at 80 V for 90 min, and were blotted according to the method of Towbin et al. [35]. After blocking overnight in 3% bovine serum albumin, membranes were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and were incubated with universal anti-intimin–diluted sera (1:500) [36] for 2 h. Membranes then were washed with PBST and were developed with horseradish peroxidase-conjugated goat anti-rabbit serum (1:2000; Sigma) for 2 h and were developed with hydrogen peroxide and 3′3′-diaminobenzidine (Sigma).

Insertion sites of the LEE region. To verify whether LEE was inserted downstream of the *selC* locus, PCR reactions that amplify the junctions of this locus with the *E. coli* chromosome were performed [15]. For the reactions, 10 μL of template DNA (from a boiled suspension in distilled water of bacteria grown in MacConkey agar), 1.5 mM of $MgCl_2$, 200 μM of dNTPs, 0.5 μM of each primer, and 1 unit of Taq DNA polymerase were mixed. PCR reactions also were conducted to verify whether *pheU* (another possible locus for LEE insertion) was intact [9]. The amplification conditions and oligonucleotide sequences used are described in table 2.

Intimin typing. To identify the intimin type of the strains, PCR reactions 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 of an O157:H7 strain (Int γ). A conserved reverse primer (Int Ru) was used in all reactions [10]. The reaction conditions used were 10 μL of template DNA (obtained as described above), 200 μM of dNTPs, 1 unit of *Taq* DNA polymerase, 1.5 mM of $MgCl_2$, and 25 pmol of the primers described above. The amplification conditions and primer sequences used are indicated in table 2.

Tir typing. To detect the variant forms of the Tir protein, PCR reactions that amplify a region corresponding to the specific Tir sequences of EPEC and EHEC were done with primers Tir F1 (5′-GGAATTCATGCCTATTGGTAACCT) and Tir R4 (5′-CGGGATCCTTAAACGAAACGTACTGGTCC) and with primers Tir F3 (5′-GGAATTCATGCCTATTGGTAA) and Tir R3 (5′-CGGGATCCTTAGACGAAACGATGGGATCC), respectively. These primers were designed on the basis of the *tir* sequences of EPEC strain E2348/69 (GenBank accession no. AF013122) [12] and STEC strain 95SF2 (EH; GenBank AF070067) [37]. Both pairs of primers amplified the nucleotide sequences corresponding to amino acids 1–573 of the Tir proteins, which generated a DNA fragment of 1700 bp. The reaction conditions used were the same as those described for intimin typing, and the amplification cycles used are described in table 2.

Serotyping. Identification of somatic (O) and flagellar (H) antigens was performed by standard agglutination methods [38], with specific antisera O1 to O173 and H1 to H52 kindly provided by the Centers for Disease Control and Prevention (CDC; Atlanta). Thirty-five strains also were tested in the Enteric Section of Instituto Adolfo Lutz (São Paulo), using antisera prepared with type strains.

Statistical methods. Differences between 2 groups of data were tested by using the χ^2 test to calculate P values. Epi-Info version 6.02 (CDC) was used to perform calculations. $P < .05$ was considered to be significant.

Table 2. Primer sequences and amplification cycles used to verify some LEE characteristics in strains of *Escherichia coli* carrying the *eae* gene and lacking the EAF and Stx probe sequences.

Characteristic	Primer	Amplification cycle	Control strain		Reference
			Positive	Negative	
Right junction of LEE in *selC*	K255 and K260	94°C, 2 min; 50°C, 2 min; 72°C, 3 min	E2348/69	0311-1	[15]
Left junction of LEE in *selC*	K296 and K295	94°C, 1 min; 52°C, 1 min; 72°C, 3 min	E2348/69	0311-1	[15]
selC intact	K261 and K260	94°C, 1 min; 52°C, 1 min; 72°C, 3 min	0311-1	E2348/69	[15]
pheU intact	K913 and K914	94°C, 1 min; 52°C, 1 min; 72°C, 2 min	E2348/69	0311-1	[9]
Intimin α	Int-α and Int-Ru	95°C, 20 s; 45°C, 1 min; 74°C, 1 min	E2348/69	HB 101	[10]
Intimin β	Int-β and Int-Ru	95°C, 20 s; 45°C, 1 min; 74°C, 1 min	0311-1	HB 101	[10]
Intimin γ	Int-γ and Int-Ru	95°C, 20 s; 55°C, 1 min; 74°C, 1 min	EDL933	HB 101	[10]
Intimin δ	Int-δ and Int-R	95°C, 20 s; 45°C, 1 min; 74°C, 1 min	O86:H34	HB 101	[10]
Tir-EP	Tir-F1 and Tir-Ru4	94°C, 1 min; 50°C, 1 min; 72°C, 1 min 42 s	E2348/69	EDL933	
Tir-EH	Tir-F3 and Tir-R3	94°C, 1 min; 50°C, 1 min; 72°C, 1 min 42 s	EDL933	E2348/69	

NOTE. EAF, enteropathogenic *Escherichia coli* adherence factor; LEE, locus of enterocyte effacement; Stx, Shiga toxin; Tir-EH, enterohemorrhagic *E. coli* translocated intimin receptor; Tir-EP, enteropathogenic *E. coli* translocated intimin receptor.

Results

A collection of 59 *E. coli* strains of non-EPEC serogroups, presenting the *eae* gene and lacking the EAF and Stx probe sequences, was tested for a number of genotypic and phenotypic characteristics associated with potential *E. coli* virulence factors. The frequency of the strains with distinct characteristics regarding their association with diarrhea was further analyzed.

Presence of DNA sequences related to virulence in EPEC, EHEC, and other pathogenic categories of E. coli. All 59 strains 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). Of the remaining probe sequences tested, *hly* was the most frequently found (26 strains [44.1%]). Only 9 (34.6%) of the 26 strains bearing *hly* produced the clear halo of hemolysis after 3 h of incubation, which is characteristic of strains secreting Hly [29]. Most of the remaining strains carrying *hly* and 3 strains lacking this sequence produced a turbid halo of hemolysis after 24 h of incubation in blood agar.

Seventeen different combinations of specific virulence DNA sequences (genetic profiles) were detected, but 14 (82.4%) of these profiles were found in only $\leq$3 strains each (table 3). Strains with the *eae* profile (i.e., carrying only the *eae* sequence) were the most frequent (21 [35.6%]).

Adherence patterns. HeLa and Caco-2 cells were used, because the LA pattern characteristic of typical EPEC strains was originally described in the former lineage, whereas the latter represents a system that is closer to the human intestinal cells. One strain promoted total detachment (D) of both types of cells tested after a 3-h incubation period and was not characterized further. All the remaining strains adhered to both HeLa and Caco-2 cells, but, in the 3-h assays, they showed an indefinite pattern of adherence. In prolonged assays (6 h), 6 distinct adherence patterns were observed, 3 of which apparently consisted of variations of the typical LA pattern. These variant LA patterns were provisionally termed LC (for loose clusters of bacteria), with bacteria loosely organized in groups of various sizes over the cells' surfaces, coexisting with dispersed individual bacteria (figure 1*A*); LCC (for loose and compact bacterial clusters), which differs from the former LC pattern by the presence of compact bacterial clusters (figure 1*B*) and LA6, which is identical to the typical LA, except for the time required to be discerned (6 h instead of 3 h; figure 1*C*). A fourth pattern, IS (for isolated bacteria), was also seen. In this pattern, bacterial clusters were absent, but few individual bacteria were consistently found dispersed on the cell monolayers (figure1*D*). Strains presenting diffuse adherence (DA) (7 strains) or aggregative adherence (AA; 2 strains) [1] also were detected in the HeLa cells system. Of the strains tested, 91.4% presented the same adherence pattern in both cell lines used, but the AA pattern was not detected in Caco-2 cells. LC was the most frequent pattern on both cell lines, followed by the LCC and LA6 patterns (24, 11, and 9 strains, respectively, in HeLa cells).

Ability to aggregate actin. Forty-three (74.1%) and 45 (77.6%) of the 58 nondetaching strains were able to aggregate actin in Hela and Caco-2 cells, respectively, as verified by the FAS test. Four strains were FAS positive only in Caco-2 cells, whereas 2 strains were FAS positive only in HeLa cells. Table 3 presents the adherence patterns of the strains showing different genetic profiles, as well as the results of the FAS assays. About 64.0% of the strains presented both variations of the LA pattern (i.e., LC, LCC, or LA6) and the ability to aggregate actin. Western blot experiments with universal intimin antiserum revealed a lack of reactivity in 10 of the 15 FAS-negative strains tested (figure 2).

LEE insertion sites. Table 3 presents the LEE insertion sites of the strains studied, according to the different genetic profiles found. In 24 (40.7%) strains, LEE was inserted in *selC,* but, in 2 of these strains, *pheU* was also disrupted. Conversely, 29 (49.2%) strains had *selC* intact, but their *pheU* loci were disrupted, which suggests that the latter was an alternative insertion site for the LEE region. Of the remaining 6 strains, 5 had

Table 3. Characteristics of 59 *Escherichia coli* strains presenting the *eae* gene and carrying different putative virulence DNA sequences.

Genetic profile, adherence patterns[a]	Actin aggregation[b]	Intimin expression[c]	LEE in *selC*	*pheU* disrupted	Intimin type	Tir subtype	Serotype	No. of strains (origin)[d]
eae (21)								
LA6	+	ND	No	Yes	NT	EH	NT:H19	1 (p)
LA6	+	ND	No	Yes	β	EP	O153:H7	1 (c)
LA6	+	ND	No	Yes	NT	EH	NT:H−	1 (p)
LA6	+	ND	No	Yes	NT	NT	O70:H2	1 (p)
LA6	+	ND	No	Yes	NT	EP	NT:H2	1 (p)
LC	+	ND	No	No	γ	EP	O51:H40	1 (p)
LC (IS)	+	ND	No	Yes	NT	EP	NT:H40	1 (p)
LC	+	ND	No	Yes	NT	EH	O51:H−	1 (p)
LC	+	ND	Yes	No	NT	EH	O63:H6	1 (p)
LC	− (+)	−	No	Yes	β	EP	O11:H16	1 (p)
LC	−	−	No	No	NT	EP	O51:H40	1 (c)
LC	−	+	Yes	No	NT	EP	NT:H40	1 (p)
LC	−	−	Yes	No	NT	EP	NT:H19	1 (c)
LCC	+	ND	No	Yes	NT	EH	NT:H−; NT:H2	2 (p)
LCC	+	ND	No	Yes	β	EP	NT:H−	1 (c)
LCC	+	ND	ND[e]	Yes[e]	NT	EH	O104:H−	1 (p)
IS	+ (−)	ND	No	Yes	NT	EP	NT:H40,43	1 (c)
IS	−	−	No	No	NT	EP	O51:H40	2 (p/c)
DA (LC)	−	+	No	No	NT	EP	O51:H40	1 (p)
eae hly (9)								
LC	+	ND	Yes	No	NT	EP	O2ab:H45	1 (c)
LC (LCC)	+	ND	No	Yes	NT	EH	O162:H33	1 (p)
LC	+	ND	Yes	No	NT	EP	R:H28	1 (p)
LC	+	ND	Yes	No	γ	EP	NT:H−	1 (p)
LC	+	ND	Yes	No	NT	EP	NT:H8	1 (c)
LC	− (+)	−	No	Yes	NT	EP	NT:H40,43	1 (c)
LA6	+	ND	No	Yes	NT	EH	R:H11,21	1 (c)
DA	+ (−)	ND	No	Yes	NT	EP	NT:H−	1 (p)
LCC	+	ND	No	Yes	β	NT	NT:H−	1 (p)
eae hly astA (5)								
LC	+	ND	Yes	No	NT	EP	NT:H29,31	1 (c)
LCC	+	ND	No	Yes	NT	EH	NT:H−	1 (p)
LA6	+	ND	No	Yes	NT	EH	NT:H11	1 (c)
IS	+	ND	Yes	No	NT	EP	O13:H11	1 (c)
AA (LA6)	+	ND	Yes	No	NT	EP	O154:H9	1 (p)
eae hly daaC afa astA (3)								
DA	+	ND	Yes	No	NT	EP	NT:H−	1 (p)
DA	−	−	Yes	No	NT	EP	NT:H−	2 (c)
eae hly astA inv (3)								
LC	+	ND	Yes	No	NT	EH	O101:H33	1 (c)
LC	+	ND	Yes	No	NT	EP	NT:H−	1 (p)
LCC	+	ND	No	Yes	NT	EH	R:H11,21,41	1 (c)
eae astA (3)								
LCC	+	ND	Yes	No	NT	EH	O63:H6	1 (p)
LA6	+	ND	No	Yes	β	EP	NT:H−	1 (c)
AA (LC)	+	ND	No	Yes	NT	EH	O104:H12	1 (p)
eae bfpA perA astA (2)								
LC	+	ND	Yes	No	NT	EP	NT:H33	1 (c)
LCC	+	ND	No	Yes	γ	EP	NT:H8	1 (c)
eae hly daaC afa (2)								
DA	+	ND	Yes	No	NT	EP	O2:NT	1 (c)
DA	+	ND	No	Yes	NT	EH	O162:H−	1 (c)
eae hly inv (2)								
LC	+	ND	Yes	No	NT	EP	O109:H9	1 (c)
LC	−	+	Yes	Yes[e]	NT	NT	NT:H33	1 (p)
eae inv (2)								
LCC	+	ND	Yes	Yes	NT	EH	NT:H34	1 (p)
LA6	+	ND	No	Yes	NT	EP	O41:H−	1 (p)
eae hly daaC afa sfa pap (1)								
D	ND	ND	No	Yes	NT	EP	O4:H1	1 (p)
eae hly perA inv (1)								
LCC	−(+)	+	Yes	No	NT	EP	NT:NT	1 (c)
eae bfpA E-hly (1)								
LC	+	ND	Yes	No	γ	EP	O98:H8	1 (c)

(continued)

Table 3. *(Continued.)*

Genetic profile, adherence patterns[a]	Actin aggregation[b]	Intimin expression[c]	LEE in *selC*	*pheU* disrupted	Intimin type	Tir subtype	Serotype	No. of strains (origin)[d]
eae astA E-hly (1)								
IS	− (+)	+	No	Yes	NT	NT	NT:H−	1 (p)
eae astA inv (1)								
LC	−	−	Yes	No	NT	EH	NT:H−	1 (c)
eae E-hly (1)								
LCC	+	ND	No	Yes	β	EH	O16:H−	1 (p)
eae bfpA (1)								
LC	−	−	No	Yes	NT	EP	NT:H7	1 (c)

NOTE. AA, aggregative adherence; DA, diffuse adherence; EAEC, enteroaggregative *E. coli;* EH, Tir sequence of enterohemorrhagic *E. coli* strain 95SF2; EP, Tir sequence of enteropathogenic *E. coli* strain E2348/69; IS, isolated bacteria; LA6, localized adherence (time required for discernment, 6 h); LC, loose clusters of bacteria; LCC, loose and compact clusters of bacteria; LEE, locus of enterocyte effacement; ND, not determined; NT, nontypeable; Tir, translocated intimin receptor; +, positive; −, negative.

[a] All strains carried DNA sequences homologous to LEE A, B, C, and D, but none carried the *cdt, cnf, aggA, aafA,* and EAEC sequences. Adherence patterns were obtained in HeLa and Caco-2 cells. Parentheses indicate when different results were obtained in Caco-2 cells.

[b] As detected by fluorescent actin staining test in HeLa and Caco-2 cells. Parentheses indicate different results obtained in Caco-2 cells.

[c] As detected by using Western blot with universal intimin antiserum.

[d] Origin indicates if the strain was isolated from a case patient (p) or a control patient (c).

[e] Polymerase chain reaction amplification reactions yielded band fragments of larger-than-expected sizes.

both the *selC* and *pheU* loci intact. The remaining strain had both genes disrupted, but no amplification with the primers for the left junction of LEE in *selC* was detected, and amplification of the right junction resulted in a larger-than-expected fragment (table 3). In PCR experiments with primers that identify intact *pheU,* this latter strain and 1 of the 2 strains that had LEE inserted in *selC* and *pheU* yielded larger-than-expected fragments. Seventeen (81.0%) of the 21 strains with the *eae* profile had LEE not inserted in *selC.*

Intimin typing. On the basis of PCR reactions, most of the strains studied produced a nontypeable intimin, whereas strains producing β and γ intimins occurred infrequently (6 and 4 strains, respectively), and α and δ intimins were not detected.

Tir typing. Sequences similar to that amplified with the primers designed on the basis of the Tir sequence of EPEC strain E2348/69 (Tir type EP) were found in 36 (61.0%) of the strains studied, whereas the primers based on the Tir sequence of EHEC strain 95SF2 (Tir type EH) amplified a fragment with the expected size in 19 (32.2%) strains. Neither Tir sequence was found in 4 of the strains tested (table 2). The same number of strains (18 strains) presented Tir type EP combined with LEE inserted and not inserted in *selC.* On the other hand, 12 (63.2%) of the 19 strains with Tir type EH had LEE not inserted in *selC.*

Serotypes. The strains studied belonged to 16 distinct serogroups (table 3), of which O51 was the most frequent (4 from children with diarrhea and 2 from children without diarrhea). Only 25 (42.4%) strains were O typeable, and 3 were autoagglutinable. Thirty-five different serotypes were found, NT:H− being the most frequent followed by O51:H40. It is interesting that the NT:H− strains seemed to represent different serogroups, because the Tir subtype and LEE insertion sites varied among these strains. On the other hand, all 5 strains of serotype O51:H40 belonged to the *eae* profile, had LEE inserted in neither *selC* nor *pheU,* and had Tir type EP; however, their adherence patterns were distinct, and only 1 promoted actin aggregation and had intimin type γ. No correlation between a specific serotype and an adherence pattern and/or ability to aggregate actin was observed.

E. coli *of non-EPEC serogroups carrying* eae *and lacking the EAF and Stx probe sequences and diarrhea.* A case-control analysis was performed regarding the frequency of strains with the different genetic profiles. Only those strains carrying only *eae* (*eae* profile) were associated with diarrhea (48.4% vs. 22.2%; $P = .039$; table 4). This difference became more statistically significant when only the FAS-positive strains within the *eae* profile were considered (11 [35.5%] with diarrhea vs. 3 [11.1%] without diarrhea; $P = .031$; table 4).

None of the different patterns of adherence or serotypes found was significantly more prevalent in case patients than in control patients. Although FAS-positive strains were more frequently found in case patients than in control patients (80.6% vs. 66.6%, in HeLa cells, and 81.3% vs. 70.4%, in Caco-2 cells), this difference was not statistically significant, unless they were analyzed with strains of the *eae* profile.

Discussion

The 59 *E. coli* strains analyzed in the present study were isolated from children with and without diarrhea during an epidemiological study conducted in São Paulo. Despite the fact that 29 of the fecal specimens from which these strains were originally derived contained other isolates with the same characteristics (i.e., presented the *eae* gene and lacked the EAF and Stx probe sequences), only 1 isolate of each fecal sample was selected for the present study. This single isolate probably, but not definitely, represents the flora not analyzed.

According to the hybridization studies, all 59 strains tested

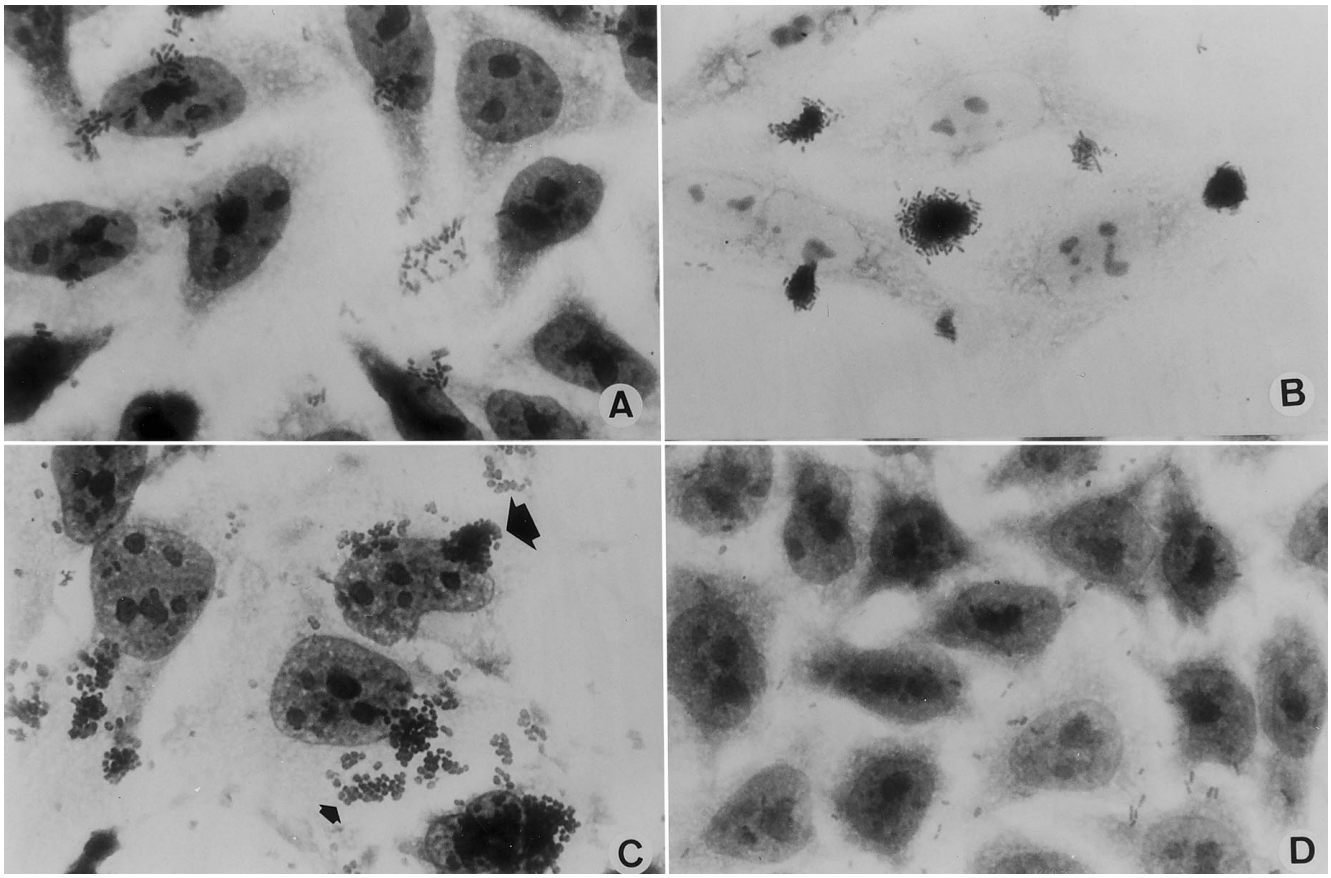

Figure 1. Adherence patterns in HeLa cells presented by *Escherichia coli* strains of nonenteropathogenic *E. coli* (EPEC) serogroups carrying *eae* and lacking the EPEC adherence factor and Shiga toxin probe sequences. *A,* LC pattern (loose clusters of bacteria), with bacteria loosely organized in groups of various sizes over the cells' surfaces, coexisting with dispersed individual bacteria. *B,* LA6 pattern (localized adherence), compact bacterial clusters discerned only in the 6-h assay. *C,* LCC pattern (loose [*small arrow*] and compact [*large arrow*] bacterial clusters), which differs from the LC pattern by the presence of compact bacterial clusters. *D,* IS pattern (few isolated individual bacteria over the cells). Original magnification, ×1000.

carried a conserved LEE region, as suggested by the presence of DNA sequences similar to the LEE probes; however, 15 (25.9%) of the 58 nondetaching strains tested were unable to promote actin accumulation in Hela cells, as detected by the FAS assay. In 5 of these 15 FAS-negative strains, intimin was expressed in normal levels, as detected by using Western blot with universal intimin antiserum (figure 2), which suggests that other factors prevented actin accumulation in these strains. The lack of expression of ⩾1 of the genes encoding the type III secretion system proteins and/or the secreted LEE proteins could explain the inability of these 5 strains to aggregate actin. It is also possible that these FAS-negative strains present some deficiency in events after the intimate adherence that prevented the recruitment of cytoskeleton components. In the remaining 10 strains that were FAS negative in HeLa cells, the lack of actin accumulation was probably due to the lack of or reduced intimin expression. Four of these 10 strains, however, were FAS positive in Caco-2 cells (table 3). It is interesting that 2 of these latter strains lacked reactivity with the universal intimin antiserum; production of an intimin whose epitopes were not recognized by the antiserum used could be an explanation for this discrepancy, but the reason why such a variant intimin was inactive in HeLa cells is not known.

The enteropathogenic potential of the FAS-negative strains found in this study should not be underestimated. Recently, in an epidemiologic study on strains isolated in the United Kingdom, it has been found that a large proportion of *eae*-positive isolates that were FAS negative on HEp-2 cells induced A/E lesions on human biopsies. This may imply that contact with an organized mucosal surface induces expression of some virulence factors involved in the A/E lesion formation (e.g., intimin; S. Knutton and A. Phillips, personal communication). In addition, Elliot at al. [39] reported that the LEE region of EHEC strain 85-170 (unlike that of EPEC strain E2348/69) is unable to confer the A/E phenotype when cloned into *E. coli* K-12, which suggests that the expression of the LEE genes may not

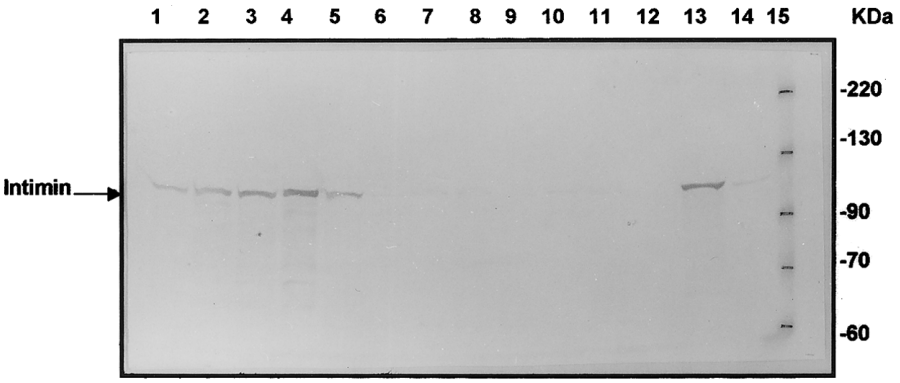

Figure 2. Western blot analysis with universal intimin antiserum against crude extracts of representative strains of *Escherichia coli* presenting the *eae* gene and lacking the enteropathogenic *E. coli* adherence factor and Shiga toxin probe sequences. Reactivity shown by 4 fluorescent actin–staining (FAS)–positive strains *(lanes 1–4)* and 1 FAS-negative strain *(lane 5). Lanes 6–11,* Weak or no reactivity with FAS-negative strains; *lane 12, E. coli* HB101; *lane 13, E. coli* E2348/69 (serotype O127:H6); *lane 14, E. coli* EDL933 (O157:H7); *lane 15,* molecular weight markers (Life Technologies).

be sufficient to provide some *E. coli* strains with the ability to produce A/E lesions.

Regarding the other DNA sequences for which we searched, *hly* was the most frequent (44.1%). Because Hly production is infrequent among EPEC and EHEC/STEC strains, the role of this protein in the putative pathogenesis of some of the *E. coli* strains studied is not known. Moreover, most of the strains carrying *hly* in this study produced a turbid halo of hemolysis within 24 h, instead of the typical clear halo of hemolysis detected within 3 h, of incubation in blood agar base [29]. These latter strains could both lack *hly* expression and produce another type of hemolysin or, alternatively, possess a defective mechanism of Hly secretion. Furthermore, only 3 strains presented similarity with the EHEC-*hly* probe that is associated with the hemolysin found in EHEC strains [18].

Because genes encoding virulence factors are located on transmissible plasmids, pathogenicity islands, transposons, or bacteriophages, the emergence of different combinations of virulence gene sequences (genetic profiles) found in the *E. coli* strains studied is not surprising. These sequences might have been transferred horizontally in the intestine and/or environment to the *E. coli* strains studied. An interesting combination of virulence markers was found in some of the strains studied; 1 strain of serogroup O4, for example, carried *eae, hly, daaC, afa, sfa,* and *pap,* which suggests that this strain is a potential uropathogenic *E. coli* (UPEC). The significance of the presence of the LEE region in such a strain, however, remains to be studied.

Despite the fact that Caco-2 cells are derived from human intestinal cells, whereas HeLa cells are not, the adherence patterns produced by the *E. coli* strains studied were similar in both cell lines (91.4% agreement). The pattern of adherence to cell lines cultivated in vitro has been used as a means of differentiating EPEC strains (that present LA in the 3-h assay) from diffusely adherent *E. coli* (DAEC) and EAEC, which characteristically produce DA and AA, respectively [1]. In this study, neither of the 2 strains producing AA carried the EAEC sequence; however, 4 of the 6 strains producing DA carried *daaC* and *afa,* which comprise sequences associated with the Dr family of adhesins [40]. Beinke et al. [41] reported that some *E. coli* strains producing DA might secrete homologues of Esp proteins that could contribute to the virulence of these strains.

Variations of the typical LA pattern were detected in most of the strains studied (~71.0%) in both cell lineages used, but none corresponded to the recently described LA-like (LAL) pattern [42]. LAL is characterized by the presence of fewer compact clusters of bacteria in a few cells, detected only in the 6-h assay [42]. The LC and LCC patterns identified in our strains were

Table 4. Frequency of *Escherichia coli* strains carrying *eae* only or presenting different genetic profiles, and ability to promote actin aggregation in case patients and control patients.

Genetic profile, actin aggregation[a]	No. (%) of strains in children		
	Case patients (n = 31)[b]	Control patients (n = 27)	Total (n = 58)
eae			
+	11 (35.5)[c]	3 (11.1)[c]	14 (24.1)
−	4 (12.9)	3 (11.1)	7 (12.1)
Total	15 (48.4)[d]	6 (22.2)[d]	21 (36.2)
Others			
+	14 (45.2)	15 (55.6)	29 (50.0)
−	2 (6.5)	6 (22.2)	8 (13.8)
Total	16 (51.6)[e]	21 (77.8)[e]	37 (63.8)

NOTE. The χ^2 test and Epi-Info 6.02 were used to perform calculations. $P < .05$ was considered to be significant.

[a] All strains carried DNA sequences related to locus of enterocyte effacement (LEE) A, B, C, and D, but none carried the *cdt, cnf, aggA, aafA,* and enteroaggregative *E. coli* sequences. Actin aggregation is as detected by fluorescent actin staining test.

[b] One strain presented cell-detaching activity and was not tested.

[c] $P = .031$, χ^2; 1 *df,* 4.28.

[d] $P = .039$, χ^2; 1 *df,* 4.68.

[e] $P = .039$, χ^2; 1 *df,* 4.28.

fairly different from LAL, because, in addition to the less compact clusters, they presented dispersed bacteria (LC pattern) or compact bacterial clusters (LCC pattern). No relationship among strains presenting any variant LA pattern and diarrhea, however, was found in the present study. Thus, although these variant patterns were very reproducible, they were described in the present study with the only purpose of stressing further differences among the strains studied rather than of proposing confusing new adherence pattern classifications.

It is probable that intimin plays a role, at least in part, in the adherence of the strains studied, as was suggested for atypical EPEC strains of serotypes O26:H−, O26:H11, O55:H7, O111ab:H−, O111ab:H9, and O119:H2 [13]. Furthermore, most strains lacked *perA,* which is a regulatory sequence involved in the expression of BFP and the A/E lesion; the lack of such an activity could explain the lower efficiency with which some of the strains adhered to the cell lines used.

LEE insertion sites vary according to the evolutionary lineage of the strains studied, but, in strains of the EPEC 1 and EHEC 1 clusters, it is generally inserted downstream of *selC* [43]. About 40.7% of the *E. coli* strains analyzed in our study had LEE inserted in *selC* and thus could be evolutionarily more akin to the EPEC 1 and EHEC 1 clones than to other clones. However, it has been reported that, in strains of serotypes O26 (H− and H11) and O111ac (H8 and H−), the LEE region is probably inserted in *pheU* [9, 44]. Similarly, half the strains in the present study probably had LEE inserted in *pheU.* This locus was also the probable insertion site of ~52.0% of all FAS-positive strains and of 57.1% of the strains with the *eae* profile.

Of note are the 5 strains of serotype O51:H40 (*eae* profile), which had LEE inserted in neither *pheU* nor *selC,* and a strain (*eae* profile) that had both *selC* and *pheU* disrupted and that yielded no DNA bands when amplified with primers for the left junction, whereas amplification with primers for the right junction resulted in a larger-than-expected fragment (table 3). Also interesting are the 2 strains (*eae* and *eae hly inv* profiles) that consistently presented faint bands above the expected size in PCR assays for the intact *pheU* locus. It has been reported that the LEE region carries large amounts of insertion sequences that favor the occurrence of different recombinational events, as well as the occurrence of LEE regions of different sizes [45]. We currently are investigating whether the strains mentioned above carry an additional copy of the LEE region or a second pathogenicity island.

Only 10 of the strains of the present study expressed β or γ intimins, and none expressed α or δ subtypes. According to Phillips and Frankel [46], different intimin types may possess different receptor-targeting specificities. Therefore, further studies are necessary to identify the intestinal target for colonization by most of the *E. coli* strains studied here.

The majority (61.0%) of the 59 strains tested presented Tir sequences similar to that of the prototype EPEC strain E2348/69 (Tir type EP). On the other hand, 32.2% of the strains presented Tir sequence similarity with that of EHEC strain 95SF2 (Tir type EH), and the Tir sequences of 4 strains were of neither type. By using a multiplex PCR specific for variants of the *eae, tir, espA,* and *espB* genes from human EPEC and EHEC and from rabbit EPEC strains, China et al. [47] showed that some bovine strains presented intimin derived from an EHEC strain of serotype O157:H7 *(eae*γ*),* as well as *tir* and *esp* genes of prototype EPEC strain E2348/69 *(tir*α *espA*α *espB*α*)*. Thus, recombination among at least some of the LEE genes seems to be a frequent event in *E. coli* strains. Most of the Tir protein of the strains in our study are probably binding an intimin type different from those of EPEC or EHEC/STEC, because only 10 of the strains carried any of the intimin types described thus far.

In a preliminary analysis, the *E. coli* strains of non-EPEC serogroups of the present study were classified as atypical EPEC strains (i.e., as strains capable of causing A/E lesions that do not produce Shiga toxins and lack the EAF plasmid [1]). After our extensive genotypic and phenotypic analysis, one may consider that these strains comprise a miscellaneous group composed by atypical EPEC, EAEC, DAEC, and UPEC strains that have acquired LEE by horizontal transfer. In addition, some of the strains could represent EPEC strains that lost the EAF plasmid (or part of it), EHEC/STEC strains that lost *stx* phage sequences, or even *E. coli* from the normal flora that had gained the LEE region.

The large variety of serotypes and the diversity of genetic virulence markers found in *E. coli* strains of non-EPEC serogroups carrying the LEE region poses a challenge in the diagnosis of these putative pathogens, because both serology and presence of the *eae* sequence are not sufficient to distinguish the truly pathogenic strains. Furthermore, none of the different combinations of adherence patterns, actin aggregation ability, and/or serotype was significantly associated with diarrhea, although the FAS-positive strains were more frequent in children with diarrhea (80.6%) than in children without diarrhea (66.6%); however, strains with the *eae* profile statistically were associated with diarrhea ($P = .039$), and 11 (73.3%) of the 15 strains from patients with this profile were isolated from children with diarrhea who carried no other well-established enteropathogen. Further studies in different intestinal systems are necessary to determine which of these strains are enteropathogenic and to establish the mechanisms that they might use to evoke diarrhea.

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