

Heterogeneity among Strains of Diffusely Adherent *Escherichia coli* Isolated in Brazil

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Received 11 May 2004/Returned for modification 6 August 2004/Accepted 16 December 2004

One hundred twelve diffusely adherent *Escherichia coli* strains isolated from children in a case control study were evaluated for virulence-associated characteristics, serotyping, antibiotic resistance, and plasmid profiles. Half of the strains hybridized with the probes for *icuA* (aerobactin) and *fimH* (type 1 pili); *daaE* (F1845 fimbriae), *afa* (afimbrial Dr adhesin), *agg-3A* (aggregative adhesion fimbria type III fimbriae), *pap* (P fimbriae), *astA* (EAST1 toxin), and *shET1* (*Shigella* enterotoxin 1) sequences were present in <20% of the strains. The *shET1* gene was noted most frequently in strains isolated from patients. A minority (7%) of the strains produced hemolysin or colicin or showed cytotoxic effects on Vero cells. Forty-five different serotypes were found. The majority (70%) of the strains presented multiple antibiotic resistance. Antibiotic resistance and diffuse adherence were located on the same conjugative plasmids. These results suggest that the transfer of these potential virulence markers could be important in the epidemiology of diffusely adherent *E. coli*.

Diffusely adherent *Escherichia coli* strains that are identified by their diffuse adherence (DA) pattern on cultured epithelial cells in vitro (24) have been recognized as the sixth class of diarrheagenic *E. coli* (17).

Two putative adherence factors have been described for diffusely adherent *E. coli* strains. Bilge et al. (6) have characterized surface fimbriae (designated F1845) that are responsible for the DA phenotype in a prototype strain, C1845. The gene cluster that encodes this fimbriae can be found on either the bacterial chromosome or a plasmid. F1845 fimbriae are homologous at the nucleotide and amino acid levels with members of the Afa/Dr family of adhesins (Afa-I, Afa-III, Dr, Dr-II, and F1845) (18). Afa/Dr diffusely adherent *E. coli* strains are identified in epidemiological studies by hybridization to a specific probe, *daaC*, which is common to operons encoding Afa/Dr adhesins (6). A second putative adhesin associated with the DA phenotype, an outer membrane protein, designated AIDA-I, has been described by Benz and Schmidt (4).

In a number of epidemiological studies, diffusely adherent *E. coli* strains have been associated with diarrheal disease in different geographic areas (2, 11, 12, 13). However, the diffusely adherent *E. coli* virulence markers associated with diarrhea are yet to be clarified. In this paper, 112 diffusely adherent *E. coli* strains isolated in a case control study (61 from patients and 51 from healthy control subjects) (25) were tested for a number of phenotypic and genotypic characteristics associated with potential *E. coli* virulence factors. We further characterized these strains by

determining their serotypes, antibiotic resistance patterns, and the role of plasmids in mediating the diffuse adherence.

Strains were screened for hemolytic activity on blood agar base plates containing 5% sheep erythrocytes washed with phosphate-buffered saline. The plates were observed for lysis of erythrocytes after 24 h of growth at 37°C. Strains were tested for enterotoxin production by the Y1 adrenal cell culture method of Donta et al. (10) and by the infant mouse assay of Dean et al. (9). Cytotoxin production was detected by the Vero cell culture assay (15). Colicin production was detected by using the agar overlay method of Azevedo and Costa (1).

DNA probes for virulence factors associated with other pathogenic *E. coli* strains were employed in hybridization experiments as described previously (25) (Table 1).

Serotyping was performed by the method of Ørskov and Ørskov (21) with antisera against *E. coli* O antigens 1 to 181 and H antigens 1 to 53.

Antibiotic susceptibility testing was performed by the standard disk diffusion method of Bauer and Kirby (3). The antibiotic disks used were ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), cotrimoxazole (10 µg), lomefloxacin (10 µg), ceftazidime (30 µg), ofloxacin (5 µg), streptomycin (10 µg), gentamicin (10 µg), nalidixic acid (30 µg), sulfonamide (300 µg), and tetracycline (30 µg).

To test for the presence of DA conjugative plasmids, diffusely adherent *E. coli* isolates that were resistant to ampicillin, streptomycin, and tetracycline were mated with the *E. coli* K-12 C600 strain (*supE44 hsdR thr-1 leuB6 lacY1 tonA21 Na^r Fu^r*), as described elsewhere (23). The donor and recipient strains were grown in Luria broth for 18 h, mixed on the surface of Mueller-Hinton agar plates, and incubated for 24 h at 37°C. Transconjugants were selected on plates containing nalidixic acid (100 µg/ml) with ampicillin (100 µg/ml), tetracycline (30 µg/ml), and strepto-

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TABLE 1. DNA probes hybridizing to colony blots of diffusely adherent *E. coli* isolates

Probe	Description of probe	Properties of probe (reference)	No. (%) of DAEC strains isolated from:		
			Cases (n = 61)	Controls (n = 51)	Cases and controls (total) (n = 112)
<i>daaE</i>	419-bp amplified fragment	F1845 fimbrial subunit (7)	6 (9.8)	9 (17.6)	15 (13.4)
<i>afa</i>	750-bp amplified fragment	Afimbrial adhesin (16)	9 (14.7)	8 (15.7)	17 (15.2)
<i>aggA</i>	450-bp amplified fragment	AAF/I fimbrial subunit (8)	1 (1.6)	0	1 (0.9)
<i>aafA</i>	550-bp amplified fragment	AAF/II fimbrial subunit (8)	0	0	0
<i>agg-3A</i>	485-bp amplified fragment	AAF/III fimbrial subunit (5)	10 (16.4)	10 (19.6)	20 (17.8)
<i>fimH</i>	527-bp amplified fragment	Type 1 fimbriae (14)	26 (42.6)	28 (54.9)	54 (48.2)
<i>sfa</i>	410-bp amplified fragment	S fimbriae (16)	1 (1.6)	2 (3.9)	3 (2.7)
<i>pap</i>	328-bp amplified fragment	P fimbriae (16)	3 (4.9)	3 (5.9)	6 (5.3)
<i>iucA</i>	1,100-bp amplified fragment	Aerobactin (20)	31 (50.8)	25 (49)	56 (50)
<i>hly</i>	7,000-bp fragment of pSF4000	Alpha-hemolysin (29)	4 (6.5)	2 (3.9)	6 (5.4)
<i>pet</i>	1,037-bp amplified fragment	104-kDa cytotoxin (28)	0	0	0
<i>astA</i>	111-bp amplified fragment	EAST1 toxin (30)	11 (18)	6 (11.8)	17 (15.2)
<i>shET1</i>	309-bp amplified fragment	<i>Shigella</i> enterotoxin 1 (28)	7 (11.5)	1 (1.9)	8 (7.1)
<i>shET2</i>	799-bp amplified fragment	<i>Shigella</i> enterotoxin 2 (28)	0	0	0
<i>pic</i>	5,800-bp amplified fragment	Mucinase (28)	0	0	0
<i>cnf</i>	498-bp amplified fragment	Cytotoxic necrotizing factor (22)	1 (1.6)	1 (1.9)	2 (1.8)
<i>cdt</i>	175-bp fragment of pCVD448	Cytolethal distending toxin (26)	0	0	0
<i>espC</i>	2,000-bp fragment of pMS1	Enterotoxin (27)	0	0	0

mycin (100 µg/ml) and were tested for HEp-2 cell adherence using the assay of Scaletsky et al. (24).

Colicin production was found in eight strains isolated from six patients and two healthy control subjects. Six strains isolated from four patients and two controls were hemolysin producers and hybridized with the *hly* probe. No strains produced enterotoxins as tested by the Y1 adrenal cell culture method and by the infant mouse assay. Eight strains isolated from six patients and two controls showed a cytotoxic effect on Vero cells after 3-h incubation (data not shown). A similar cytotoxic effect on HEp-2 cells was observed by Jallat et al. (13) in 16 diffusely adherent *E. coli* strains, 14 from patients and 2 from controls, in France.

According to the hybridization studies, 48% of strains harbored the *iucA* gene that is involved in the synthesis of the aerobactin siderophore (Table 1). In a recent study, it has been reported that 50% of the diffusely adherent *E. coli* strains hybridize with the *irp2* probe, which is part of the yersiniabactin operon, encoding a siderophore-dependent iron transport system (8). Therefore, it appears that the genes for aerobactin and yersiniabactin siderophore production are widely distributed among diffusely adherent *E. coli* strains, as they are for enteroaggregative *E. coli* (20).

In addition, some strains possessed genes encoding the EAST1 toxin (*astA*) and eight strains had the *shET1* sequence, virulence factors found frequently in enteroaggregative *E. coli* strains (28). The *astA* gene was detected more frequently in strains from patients (18%) than in strains from control subjects (12%). Of the eight strains with the *shET1* profile, seven (87.5%) were isolated from children with diarrhea who carried no other well-established enteropathogen. To our knowledge, this is the first report of the prevalence of *shET1* in diffusely adherent *E. coli* strains. Further studies are necessary to determine whether other virulent factors play a role in the pathogenicity of the diffusely adherent *E. coli* strains.

The *cnf1* gene encoding cytotoxic necrotizing factor 1 was found in two strains from both patients and healthy controls.

This virulence factor has been found in cell-detaching *E. coli* strains from children with and without diarrhea (19). None of the 112 strains hybridized with the *cdt*, *pet*, *pic*, *shET2*, and *espC* probes.

Besides the F1845 fimbriae and AIDA-I, little is known about possible adhesins expressed by diffusely adherent *E. coli* strains. We investigated the presence of putative adhesins in an attempt to identify the factor responsible for the DA phenotype. As shown in Table 1, 15 (13%) strains carried the *daaE* (F1845 major fimbrial subunit), and 17 (15%) other strains carried the *afa* (afimbrial adhesin) sequence, an afimbrial adhesin of the Dr family. The *fimH* gene, which encodes adhesin subunit type 1 fimbriae, was the most frequently found (54 strains [48%]). This adhesin is present in nearly all *E. coli* strains, including commensal strains (14). Our results for *afa*, *agg-3A*, *sfa*, and *pap* extended the observations of previous reports that investigated the distribution of some of these adhesins in a limited number of strains (16). Indeed, 20 strains carried the *agg-3A* sequence that encodes the aggregative adhesion fimbria type III (AAF/III) fimbrial subunit, 6 strains hybridized with *pap* (P fimbria), 3 strains hybridized with *sfa* (S fimbria), and one strain hybridized with *aggA* (AAF/I fimbria) probes. Thus, at least half of the strains must have adhered by means of an adhesin different from those described so far. Further studies are necessary to characterize the adhesin(s) of diffusely adherent *E. coli* strains.

The serotypes of the 112 diffusely adherent *E. coli* strains are indicated in Table 2. In total, 45 different serotypes were found. Thirty-five strains were O nontypeable, and three were rough.

A variety of different combinations of the specific virulence DNA sequences and serotypes was observed in diffusely adherent *E. coli* isolates from both patients with diarrhea and healthy controls (Table 2). However, an interesting combination of virulence markers was found in some of the strains studied: three strains isolated from patients of serotypes O89:HNT, O99:H33, and O126:H16, for example, carried *astA*

TABLE 2. Characteristics of 112 diffusely adherent *E. coli* strains carrying different putative virulence DNA sequences

Genetic profile	No. (%) of strains from:		Serotype(s) ^a
	Patients (n = 61)	Controls (n = 51)	
<i>hly shET1 cnf sfa fimH</i>	1 (1.6)	0	O2:H6
<i>hly cnf sfa pap fimH</i>	0	1 (2)	O6:H27
<i>iucA daaE agg-3A fimH</i>	0	2 (3.9)	O21:HNT, ONT:H18
<i>iucA hly afa pap</i>	2 (3.3)	0	O21:HNT
<i>iucA astA shET1 agg-3A</i>	1 (1.6)	0	O89:HNT
<i>iucA astA shET1 fimH</i>	2 (3.3)	0	O99:H33, ONT:H18
<i>iucA agg-3A fimH</i>	1 (1.6)	3 (5.9)	O15:H16, O21:HNT, O158:H4, ONT:NM
<i>daaE afa fimH</i>	0	1 (2)	O1:H1
<i>iucA afa fimH</i>	2 (3.3)	0	O15:NM, O99:NM
<i>agg-3A pap fimH</i>	1 (1.6)	0	O21:HNT
<i>iucA pap fimH</i>	0	1 (2)	O21:H18
<i>iucA sfa fimH</i>	0	1 (2)	O23:HNT
<i>astA daaE fimH</i>	1 (1.6)	0	O86:H18
<i>astA shET1 fimH</i>	1 (1.6)	0	O99:H33
<i>iucA astA shET1</i>	1 (1.6)	0	O126:H26
<i>hly astA fimH</i>	0	1 (2)	O141:H19
<i>iucA daaE agg-3A</i>	0	2 (3.9)	O153:H2, ONT:H17
<i>iucA daaE afa</i>	0	1 (2)	ONT:NM
<i>iucA daaE fimH</i>	1 (1.6)	0	ONT:NM
<i>aggA agg-3A fimH</i>	1 (1.6)	0	ONT:HNT
<i>iucA afa agg-3A</i>	1 (1.6)	0	R
<i>astA afa fimH</i>	0	1 (2)	R
<i>iucA astA</i>	2 (3.3)	0	O5:H2, O86:H11
<i>iucA afa</i>	2 (3.3)	1 (2)	O11:NM, O86:H18, O101:H9
<i>iucA shET1</i>	1 (1.6)	0	O15:HNT
<i>iucA fimH</i>	6 (9.8)	5 (9.8)	O2:H4, O21:NM, O93:HNT, O153:H18, O158:NM, O164:NM, ONT:NM, ONT:H18, ONT:HNT, R:H45
<i>shET1 fimH</i>	0	1 (2)	O33:H32
<i>iucA daaE</i>	1 (1.6)	1 (2)	O86:H18, O117:H4
<i>daaE fimH</i>	0	1 (2)	O86:H18
<i>astA agg-3A</i>	1 (1.6)	0	O89:H33
<i>afa fimH</i>	1 (1.6)	1 (2)	O86:H18, O117:NM
<i>iucA agg-3A</i>	1 (1.6)	2 (3.9)	O128:NM, O153:H2
<i>agg-3A fimH</i>	0	1 (2)	ONT:NM
<i>afa pap</i>	0	1 (2)	ONT:H7
<i>astA daaE</i>	0	1 (2)	ONT:H18
<i>astA fimH</i>	1 (1.6)	1 (2)	O99:H33
<i>hly afa</i>	1 (1.6)	0	O102:HNT
<i>daaE agg-3A</i>	2 (3.3)	0	ONT:HNT
<i>astA</i>	1 (1.6)	2 (3.9)	O5:H10, O86:11, ONT:H34
<i>agg-3A</i>	1 (1.6)	0	O15:H18
<i>daaE</i>	1 (1.6)	0	ONT:HNT
<i>fimH</i>	7 (11.5)	7 (11.8)	O1:H45, O2:H17, O75:HNT, O78:HNT, O86:H18, O99:H7, O117:H7, O164:H4, ONT:H18, ONT:H23, ONT:H27, ONT:HNT
<i>iucA</i>	7 (11.5)	6 (11.8)	O5:H2, O5:H10, O11:H16, O83:HNT, O153:H2, O153:H18, ONT:H17, ONT:NM
<i>afa</i>	0	2 (3.9)	O86:H18
None	9 (13.1)	4 (9.8)	O11:H16, O86:H18, O107:NM, O153:HNT, ONT:NM, ONT:H2, ONT:H9, ONT:H10, ONT:H16, ONT:H18, ONT:HNT

^a HNT, serotype H nontypeable; ONT, serotype O nontypeable; NM, nonmotile.

shET1 sequences, which suggests that these strains are potentially pathogenic *E. coli* strains.

Frequent antibiotic use could predispose or select for infection with antibiotic-resistant diffusely adherent *E. coli* strains. In addition, antibiotic resistance patterns could serve as markers for virulent strains whose virulence factors have not been identified. For these reasons, we sought to characterize antibiotic resistance rates in our diffusely adherent *E. coli* isolates. The frequencies of resistance to ampicillin, cephalothin, cotrimoxazole, streptomycin, sulfonamide, and tetracycline were

each >50%. Resistance to chloramphenicol (20%) was less frequent. All the isolates were susceptible to ceftazidime, gentamicin, lomefloxacin, ofloxacin, and nalidixic acid, and two strains were susceptible to all antibiotics tested.

Since it is well-known that plasmids are responsible for the horizontal spread of antibiotic resistance between microorganisms, we wanted to know whether the DA phenotype could be cotransferred by conjugation into an *E. coli* K-12 recipient strain. It was not possible to examine all isolates for plasmids, but in conjugation experiments, the transfer of multiple drug

TABLE 3. Results of conjugation between 10 diffusely adherent *E. coli* strains and *E. coli* K-12 C600

Diffusely adherent <i>E. coli</i> strain	Source	Resistance profile ^a	Size (MDa) of plasmid(s)	Resistance profile of the transconjugant	DA phenotype	Size (MDa) of transconjugant plasmid(s)
10	Control	Ap ^r Cf ^r Co ^r Sm ^r Su ^r Tc ^r	44, 80, 91	Ap ^r Nal ^r	—	91
53	Diarrhea	Ap ^r Ci ^r Co ^r Sm ^r Su ^r Tc ^r	38, 75, >98	Ap ^r Tc ^r Nal ^r	—	75
64	Control	Ap ^r Cf ^r Co ^r Sm ^r Su ^r Tc ^r	78, 98	Ap ^r Sm ^r Nal ^r	—	78
66	Diarrhea	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	73, >98	Ap ^r Sm ^r Nal ^r	—	73
67	Control	Ap ^r Cf ^r Cc ^r Sm ^r Su ^r Tc ^r	75, >98	Ap ^r Sm ^r Tc ^r Nal ^r	+	75, >98
69	Diarrhea	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	75, >98	Ap ^r Sm ^r Tc ^r Nal ^r	+	75, >98
73	Control	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	31, 73	Ap ^r Sm ^r Nal ^r	—	73
79	Diarrhea	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	68, >98	Ap ^r Sm ^r Tc ^r Nal ^r	+	68, >98
96	Diarrhea	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	75, >98	Ap ^r Sm ^r Tc ^r Nal ^r	+	75
97	Diarrhea	Ap ^r Co ^r Sm ^r Su ^r Tc ^r	66, 95, >98	Ap ^r Sm ^r Nal ^r	—	66

^a Ap^r, ampicillin resistant; Cf^r, cephalothin resistant; Ci^r, ciprofloxacin resistant; Co^r, cotrimoxazole; Sm^r, streptomycin resistant; Su^r, sulfonamide resistant; Tc^r, tetracycline resistant; Nal^r, nalixidic acid resistant.

resistance was observed in 10 isolates from 6 patients and 4 controls (Table 3). In these in vitro conjugation experiments, 6 of the 10 isolates transferred only drug resistance to the recipient strain. Four isolates, however, transferred drug resistance determinants and DA phenotype to *E. coli* recipient C600. By transforming *E. coli* K-12 DH5 α (*supE44* Δ *lacU169* *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1* Nal^r) (23) with plasmid DNA from two isolates (strains 67 and 79), we were able to demonstrate that genes encoding ampicillin, streptomycin, and tetracycline resistance and diffuse adherence were encoded on a 67- to 75-MDa conjugative plasmid. Curing an Ap^r Sm^r Tc^r DA transconjugant with acridine orange showed that both resistance and adherence traits were lost simultaneously.

In conclusion, our results show that diffusely adherent *E. coli* strains isolated from patients and control subjects present very different profiles when virulence markers and serotypes are considered. Among the virulence factors, *Shigella* enterotoxin 1 was noted most frequently in strains isolated from patients. The present study also demonstrated the high rate of resistance to certain antimicrobial agents in diffusely adherent *E. coli* strains in which resistance is apparently associated with conjugative plasmids. We also found plasmids encoding multiple drug resistance along with adhesion genes. The transfer of these potential virulence markers could be important in the epidemiology of diffusely adherent *E. coli* strains.

We thank Rosa M. Silva for critically reading the manuscript and helpful comments.

This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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