Prevalence of Colonization Factor Antigens (CFAs) and Adherence to HeLa Cells in Enterotoxigenic Escherichia coli Isolated from Feces of Children in São Paulo

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Abstract: Fifty-eight enterotoxigenic Escherichia coli (ETEC) strains, isolated from children with and without diarrhea in São Paulo, were examined for the presence of colonization factor antigens (CFAs) and their ability to adhere to HeLa cells. Antisera to CFA/I, the coli surface (CS) antigens CS1CS3, CS2CS3, and CS2 of CFA/II, CFA/III, and CS5CS6 and CS6 of CFA/IV were used. CFAs were identified in 43% of the ETEC strains: 40% of the strains with CFAs harbored CFA/I, 24% carried CFA/II (CS1CS3), 24% carried CFA/IV (CS4), and 12% carried CFA/IV (CS5CS6). CFAs occurred mainly among ETEC strains producing only heat-stable (ST-I) enterotoxin and in strains also producing heat-labile toxin (LT-I). No ETEC strains tested expressed CFA/III. A marked change in serotypes of ST-I-producing strains was found in São Paulo between 1979 and 1990. Adherence to HeLa cells was detected in 14% of the ETEC strains. All of them had a diffuse adherence pattern and produced only ST-I, and 88% carried CS6 antigen.

Key words: Escherichia coli, Colonization factor antigens, Adherence, Diarrhea

Enterotoxigenic Escherichia coli (ETEC) is an important cause of childhood diarrhea, especially in developing countries (22, 30, 31). The virulence determinants are heat-labile (LT) and/or heat-stable (ST) enterotoxins and colonization factor antigens (CFAs).

Distinct types of CFAs have been described in human ETEC strains. CFA/I, CFA/II, and CFA/IV have been well characterized (8, 10, 37), whereas others are considered putative colonization factors (PCFs) (17, 18, 24, 26, 36, 40).

Most CFAs are fimbrial proteins and consist of a single antigen (CFA/I), or a complex of different antigens (CFA/II and CFA/IV), named E. coli surface (CS) antigens. CFA/II consists of the CS1, CS2, and CS3 subcomponents (4, 32), and CFA/IV consists of the CS4, CS5, and CS6 subcomponents (39).

Most CFAs can agglutinate different species of erythrocytes in the presence of D-mannose (19), but some, such as CFA/III, CS6, PCF020 and PCF0159, are non-hemagglutinins and can be identified only by immunologic assays (18, 36, 39, 40).

Several studies have demonstrated a close association between the presence of a specific CFA, certain serotypes, and enterotoxigenic phenotypes of ETEC strains (4, 11, 22, 30). However, some ETEC serotypes have no reported CFA. Some reports have also shown ETEC adherence to

Abbreviations: CFA agar, Casamino acids-yeast extract agar; CFAs, colonization factor antigens; CS, coli surface antigen; ETEC, enterotoxigenic Escherichia coli; IgG, immunoglobulin G; LT, heat-labile enterotoxin; MRHA, mannose-resistant hemagglutination; MSHA, mannose-sensitive hemagglutination; PBS, phosphate buffered saline; PCFs, putative colonization factors; ST, heat-stable enterotoxin; TSB, tryptic soy broth.
different cultured cells (5, 29).
Previous studies of ETEC strains isolated in Brazil have only examined strains for CFA/I and CFA/II (15, 21, 30). Thus, the occurrence and the frequency of other CFAs among human ETEC strains in this country is unknown. The aim of the present study was to search for CFA/I, CFA/II (CS1CS3, CS2CS3), CFA/III, and CFA/IV (CS5CS6, CS6) in ETEC strains recently isolated from children with and without diarrhea in São Paulo city. The ability of these ETEC strains to adhere to HeLa cells and the characterization of their serotypes were also determined.

Materials and Methods

Bacterial strains and culture conditions. A total of 163 E. coli colonies characterized as enterotoxigenic by colony hybridization assays with LT-I and ST-I specific probes (28) were studied. They were isolated from 38 children in São Paulo seen as outpatients with acute diarrhea and from 16 with no gastrointestinal disorder. The study was part of a joint project on the etiology of diarrhea in children between 1 and 5 years old, conducted between 1989 and 1990, with the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, U.S.A. (Gomes, T.A.T. et al, manuscript in preparation). E. coli colonies isolated from most children had the same toxigenic phenotype and serotype and were homogeneous in other tests, and thus were considered as one ETEC strain. Therefore, the 163 E. coli colonies actually comprised 58 ETEC strains isolated from 54 children.

The production of LT-I and ST-I enterotoxins was confirmed by GM1-enzyme-linked immunosorbent assay (33) and the infant mouse test (6), respectively. The ETEC strains to be tested for CFAs were inoculated in Tryptic soy broth (TSB, DIFCO, Detroit, Mich., U.S.A.) and statically grown for 5 hr. Cultures were then streaked on Casamino acids-yeast extract agar (CFA agar) (10) with or without bile salts (24) and were incubated at 37°C overnight.

Reference strains for CFA/I (TR50/3); CFA/II CS1CS3 (PB176), CS2CS3 (E4833), CS2 (58R957); CFA/III (31-10); CFA/IV CS5CS6 (E17018A) and CS6 (E11881C) were included in all assays.

CFAs antisera. Absorbed rabbit antisera to CFA/I and CFA/II (CS1CS3) were previously prepared in São Paulo (30). Absorbed antisera to CFA/II (CS2CS3); CFA/II (CS2), CFA/III, CFA/IV (CS5CS6) and CFA/IV (CS6) were produced by immunizing rabbits with strains E4833, 31-10, E17018A and E11881C, respectively, and by absorbing several times with the corresponding CS-deficient mutants (38). The specificity of polyclonal rabbit antisera against CS1CS3, CS2CS3, CS2 and CS5CS6 was confirmed by agglutination tests using homologous and heterologous CFA reference strains. Immunodiffusion and dot blot assays were performed to confirm the specificity of CS6 and CFA/III antisera, respectively.

Hemagglutination pattern. Hemagglutination tests were performed as previously described (9) using a 5% suspension of washed human type A, bovine, and guinea pig erythrocytes in phosphate-buffered saline (PBS) 0.01 M, pH 7.2, with or without 1% D-mannose.

Agglutination with CFA-specific antisera. ETEC strains with a mannose-resistant hemagglutination (MRHA) pattern with human or human and bovine erythrocytes were tested with CFA/I and CFA/IV (CS5CS6) antisera, while strains with MRHA only with bovine erythrocytes were tested with CFA/II antisera. Slide agglutination tests were performed by mixing equal volumes of bacterial suspension in PBS (~10⁶ bacteria per ml) and CFA antiserum. CFA/II (CS2) and CFA/IV (CS5CS6) antisera were tested undiluted, while dilutions of 1:10 and 1:20 were used for CFA/I and CFA/II (CS1CS3) antisera, respectively.

Immunodiffusion tests. ETEC strains showing no hemagglutination activity were tested against CFA/IV (CS6) antiserum in an immunodiffusion assay. Heated extracts were prepared by suspending bacterial growth from CFA-bile salts agar plates (CFA-BS) (150 mm) in 1.5 ml of PBS. Bacterial suspensions were heated at 60°C for 30 min and centrifuged in a microcentrifuge at 8,000×g for 10 min. Supernatants were stored at −20°C and tested by the Ouchterlony immunodiffusion technique (4, 25).

Dot blot test. The dot blot assay was performed as described by Tacket et al (36) for detection of CFA/III as well as for confirming the immunodiffusion results with CS6 antisera. Briefly, heated extracts prepared in CFA-BS agar, as described for the immunodiffusion tests, were dotted on nitrocellulose filters and incubated at 37°C overnight. Reference strains for CFA/I (TR50/3); CFA/II CS1CS3 (PB176), CS2CS3 (E4833), CS2 (58R957); CFA/III (31-10); CFA/IV CS5CS6 (E17018A) and CS6 (E11881C) were included in all assays.

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peroxidase (Sigma, Inc. Coop., St. Louis, Mo., U.S. A.) for 2 hr at room temperature. Filters were again thoroughly washed and developed with hydrogen peroxidase substrate 3'3-diaminobenzedintetrahydrochloride.

**Adherence assay.** Adherence tests were performed with HeLa cells, as previously described (3).

**Serotyping.** The ETEC O and H antigens were determined by standard procedures (7) using antisera 01 to 0170 and H1 to H51 (CDC, Atlanta, Ga., U.S.A.).

**Statistical analyses.** P values were calculated using Fisher's exact test, 2-tailed.

**Results**

**Hemagglutination Pattern**

Twenty-five (43%) of the 58 ETEC strains did not agglutinate any of the erythrocytes tested, 21 (36%) displayed MRHA, and 12 (21%) displayed mannosensitive hemagglutination (MSHA) (Table 1). Most MRHA strains produced ST-I (52%) or LT-I/ST-I (33%), while most non-hemagglutinating strains (68%) and 50% of MSHA strains produced only LT-I.

Among the strains with MRHA, the toxin production pattern was related to the type of erythrocytes agglutinated. Ten (91%) of the 11 ST-I-producing strains with MRHA agglutinated both human and bovine erythrocytes, whereas only 2 (29%) of the 7 LT-I/ST-I-producing strains with MRHA had this agglutination pattern (P = 0.01).

**CFA Identification**

CFAs were identified in 25 (43%) of the 58 ETEC strains studied: 6 (24%) of 25 non-hemagglutinating strains, 19 (90%) of 21 with MRHA, and none of the 12 with MSHA. The highest CFA frequency was observed among ST-I-producing strains (75%), followed by LT-I/ST-I producers (58%), and LT-I producers (11%) (Table 2). CFA/I was found in 10 (40%) of 25 CFA-producing ETEC strains, and was the predominate CFA type among ST-I-producing strains. CFA/II (CS1CS3) was detected in 6 (24%) CFA-positive strains, mostly LT-I/ST-I producers. The CS6 antigen of CFA/IV was identified in 6 (24%) CFA producing strains, all of which produced only ST-I. The antigenic complex CS5CS6 of CFA/IV was detected in only 3 (12%) of 25 CFA strains (Table 2). None of the strains harbored CFA/III.

ETEC strains presenting CFAs occurred in 21 (55%) of 38 children with diarrhea, but in only 4 (25%) of 16 children with no gastrointestinal disorder (P = 0.07) (Table 3). A higher proportion of patients than controls had each CFA type.

**CFA Serotypes**

The relationships among CFA types, enterotoxin phenotypes, and serotypes are shown in Table 4. Five of 6 CFA/II-producing strains belonged to a single serotype (O6:H16) and they all produced LT-I/ST-I toxins. Three of 6 strains producing only CS6 belonged to serogroup O27, and were all ST-I producers.

**Adherence to HeLa Cells**

Only 8 ETEC strains adhered to HeLa cells. They all had a diffuse adherence pattern, produced only

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**Table 1. Hemagglutination patterns of E. coli strains of different enterotoxigenic phenotypes**

<table>
<thead>
<tr>
<th>Enterotoxin phenotype</th>
<th>No. of E. coli strains with hemagglutination pattern</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. H.</td>
<td>MRHA</td>
</tr>
<tr>
<td>LT-I</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>ST-I</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>LT-I/ST-I</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>21</td>
</tr>
</tbody>
</table>

* N. H., no hemagglutination; MRHA, mannose-resistant hemagglutination; MSHA, mannose-sensitive hemagglutination.

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**Table 2. Enterotoxin phenotype of E. coli strains of different CFA types**

<table>
<thead>
<tr>
<th>Type of CFA</th>
<th>No. of strains (n = 58)</th>
<th>No. of CFA strains producing LT-I (n = 26)</th>
<th>ST-I (n = 20)</th>
<th>LT-I/ST-I (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>0</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>II (CS1CS3)</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IV (CS6)</td>
<td>6*</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>IV (CS5CS6)</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Any CFA</td>
<td>25</td>
<td>3</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>No CFA</td>
<td>33</td>
<td>23</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* Non-hemagglutinating strains.

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**Table 3. Presence of CFAs in children with diarrhea and controls, São Paulo, Brazil**

<table>
<thead>
<tr>
<th>CFA type</th>
<th>Diarrhea (n = 38)</th>
<th>Control (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>II (CS1CS3)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>IV (CS6)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>IV (CS5CS6)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>4</td>
</tr>
</tbody>
</table>
Discussion

Wide variation in the number of ETEC strains harboring CFAs have been described in different parts of the world. In a recent study conducted in Burma, Central Africa, and Peru, ETEC strains were screened for the presence of CFA/I, CFA/II, CFA/III, CFA/IV and several PCFs (27). The highest frequency of ETEC strains with identifiable CFAs (71%) was found in Burma, followed by Central Africa (69%) and Peru (52%). However, the frequency with which ETEC in these areas displayed CFA/I, CFA/II, or CFA/IV were 53%, 45% and 23%, respectively. CFA/I, CFA/II, or CFA/IV was produced by 52% of ETEC isolates in Argentina (2), whereas in Chile (1) 75% of ETEC strains expressed CFA/I or CFA/II. In the present study, 43% of ETEC strains presented CFA/I, CFA/II, or CFA/IV. This frequency was similar to that reported by others and was higher than the 23% found in a previous study in São Paulo (30). CFA/I was also the most frequently identified type in Chile, Argentina, and Burma (1, 2, 27). However, in Central Africa and Peru CFA/IV was the most common type identified, and some PCFs also occurred in elevated frequencies (27).

Different CS combinations can be detected among CFA/II-producing strains. In previous studies, the antigens CS1 and CS2 were always produced together with CS3 and were associated only with serotypes O6:H16 or O6:H-; the presence of CS3 alone was never associated with these serotypes but occurred in others (4, 32). In this study, all 6 CFA/II strains expressed CS1CS3, and five belonged to serotype O6:H16. In Argentina, the pattern CS1CS3 was more common than any other CFA/II pattern, also occurring mostly in O6:H16 strains, but in Burma and Peru the CS1CS3 pattern did not occur, and all O6:H16 strains presented CS2CS3 (2, 27).

CFA/IV was expressed by 36% of ETEC strains showing any CFA type, and strains expressing CS6 only were more common than those expressing the CS5CS6 combination. CS6 predominance was also observed in ETEC strains isolated in Peru and Central Africa (27), while in Argentina most CFA/IV-producing strains showed CS5CS6 antigens (2). Although a variety of ETEC serotypes can express CS6, serogroup O27 was identified in most studies and in the present study it represented 3 of the 6 CS6-only producers.

Particular combinations of CFAs and enterotoxin types were associated with different serotypes, similar to the findings described by previous studies (2, 4, 13, 24, 27, 30). Most serotypes detected in this study have been isolated in other areas (2, 4, 24, 27), although several were not isolated before in São Paulo, including O114:H-, O153:H45, O154:H-, O163:H33, and O169:H-. None of the strains in this study presented CFA/III. This antigen is infrequently found among ETEC strains and has been found only in serotypes O25:H16 and O25:H- (23), which were not isolated in this study. Regarding the ETEC serotypes recently associated with different putative CFAs (17, 18, 24, 26, 36, 40), only serotypes O114:H- and O159:H4 (one strain each) occurred among the 33 ETEC strains in which no CFAs were identified (data not shown). They have been associated in the literature with CS7 and PCF O159:H4, respectively.

The result observed in this study showing that ETEC strains isolated from diarrheal stools were more likely than those from controls to display CFAs was not unexpected. However, in most other

<table>
<thead>
<tr>
<th>CFA type</th>
<th>Enterotoxin type</th>
<th>Total no. of strains</th>
<th>Serotype no. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ST-I</td>
<td>8</td>
<td>O153: H45 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O12: NT (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O32: H45 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O78: H12 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O163: H33 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O165: H9 (1)</td>
</tr>
<tr>
<td></td>
<td>LT-I/ST-I</td>
<td>2</td>
<td>O6: H16 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O78: H12 (1)</td>
</tr>
<tr>
<td>II (CS1CS3)</td>
<td>LT-I</td>
<td>1</td>
<td>O154: H-</td>
</tr>
<tr>
<td></td>
<td>LT-I/ST-I</td>
<td>5</td>
<td>O6: H16 (5)</td>
</tr>
<tr>
<td>IV (CS6)</td>
<td>ST-I</td>
<td>6</td>
<td>O27: H7 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O27: H-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O11: H4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O77: H7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O169: H-</td>
</tr>
<tr>
<td>IV (CS5CS6)</td>
<td>LT-I</td>
<td>2</td>
<td>NT: H4/H7</td>
</tr>
<tr>
<td></td>
<td>ST-I</td>
<td>1</td>
<td>O29: H4</td>
</tr>
</tbody>
</table>

ST-I, and 7 (88%) presented the CS6 complex of CFA/IV (6 strains had CS6 only, and one had CS5CS6). Seven (88%) strains were isolated from children with diarrhea.
reports on the prevalence of CFA, all ETEC strains studied were isolated from patients with diarrhea (1, 2, 4, 13, 27).

A wider range of ETEC serotypes was observed among ST-1 strains than in previous studies in São Paulo (14, 30). In 1979 (30), almost 70% of ST-1 producers belonged to serogroup O128ac; in 1982 (14) this serogroup accounted for 42% of ST-I-producing strains, while other serotypes, including O27 : H7 or H-1, and O78 : H12 were also isolated. In this study, none of the ST-1 producers belonged to serogroup O128ac, and except for O27 : H7 or H-, and O78 : H12, most of the ST-1 serotypes were not isolated before.

CFAs were identified in 90% of the ETEC strains showing an MRHA pattern. The two strains in which CFAs were not detected produced ST-I toxin; one of them (serotype O28ab : H8) hemagglutinated human and bovine erythrocytes, while the other (serotype O153 : H45) agglutinated only human erythrocytes. The three other ST-I-producing strains belonging to the O153 : H45 serotype isolated in this study presented CFA/I, and thus agglutinated both human and bovine erythrocytes. Probably CFAs different from the ones searched for may be present on these 2 ETEC strains or CFA/I was lost from that O153 : H45 strain.

The ability of ETEC strains possessing CFAs to adhere to different cell lines has been described by Darfeuille-Michaud et al (5). Studying ETEC strains presenting CFA/I, CFA/II, CFA/III and antigen 2230, they verified that among several cell lines tested, adherence was observed only in Caco-2 cells, irrespective of the adhesive factor. Adhesion to human intestinal enterocytes and to cultured human intestinal mucosa was described by Knutton et al (20) in ETEC strains producing CFA/III and CFA/IV, but the role of CS6 antigen in adhesion could not be confirmed. However, studies with an animal model have demonstrated the importance of CS6 as a colonization factor (35). The presence of a diffuse adherence pattern in ETEC strains producing ST-I toxin and belonging to serotype O29 : H21 has been previously described in Brazil (16). Recently, we found that all these strains presented CFA/IV composed of CS5 and CS6 (12) and that a CS5 deficient strain showed more pronounced adherence (Giraldi, R. et al, manuscript in preparation).

In the present study, adherence to HeLa cells was detected in 14% of the ETEC strains studied, and all of them presented a diffuse adherence pattern. Moreover, this characteristic was observed only in ST-I strains belonging to a few serotypes, and most of them (75%) presented only CS6 antigen. Taken together, these results and the ones obtained with O29 : H21 strains suggest a relationship between diffuse adherence and the presence of CS6 in ETEC strains producing ST-I toxin. Studies are in progress to test this hypothesis.

The knowledge of CFA distribution in different areas of the world is important since CFA-carrying ETEC are capable of inducing protective immunity and are candidates for inclusion in a multivalent vaccine (34). To our knowledge the present report is the most complete study of the prevalence of CFAs in ETEC strains isolated in Brazil.

We thank Dr. M.M. McConnel for providing the CFA reference strains E4833, 58R957, E17018A, E17018B, E11881C, E11881D, Dr. T. Honda for 31-10, and Dr. D.G. Evans for PB176. We thank Dr. A.M. Svennerholm for kindly providing the LT monoclonal antibody. We also thank Magda de Jesus Nisti and Gloria M.A. Thompson for secretarial assistance.

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Minneapolis.


