BACTERIAL PATHOGENICITY AND CHARACTERISATION

Enteropathogenicity markers in *Escherichia coli* isolated from infants with acute diarrhoea and healthy controls in Rio de Janeiro, Brazil

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Faeces from urban children <2 years old with acute diarrhoeal illness and from non-diarrhoeal infants (controls) were examined for *Escherichia coli* and other enteropathogens. A total of 990 *E. coli* isolates from 100 patients and 50 controls was tested for enteropathogenic *E. coli* (EPEC) serotype (O:H), adherence to HEp-2 cells after incubation for 3 and 6 h, fluorescent actin staining (FAS), DNA hybridisation with EAF, eaeA, StH, StP and EAggEC probes and production of heat-labile enterotoxin (LT) and verocytotoxin (VT) with Y1 and Vero cells. EPEC were the most prevalent enteropathogens in patients (32.7%; and 14% in controls). Enteroinvasive *E. coli* (EIEC) and Vero cytotoxin-producing *E. coli* (VTEC) were not detected. The rate of isolation of enterotoxigenic *E. coli* (ETEC) was identical in both groups. Among the EPEC isolates the prevalent serotypes were O111:H2, O55:NM and O119:H6. Localised adherence (LA) was found significantly more frequently in isolates from patients (19.6%; and 14% in controls). Enteroinvasive *E. coli* (EIEC) and Vero cytotoxin-producing *E. coli* (VTEC) were not detected. The rate of isolation of enterotoxigenic *E. coli* (ETEC) was identical in both groups. Among the EPEC isolates the prevalent serotypes were O111:H2, O55:NM and O119:H6. Localised adherence (LA) was found significantly more frequently in isolates from patients (19.6%) than controls (2.1%). All LA-positive EPEC isolates were FAS+ and eaeA+, but only 75.2% of them hybridised with the EAF probe. Diffusely adhering *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC) were found with equal frequency in patients and controls. Twenty-seven *E. coli* isolates were negative for EAF but positive for eaeA and FAS and produced LA in 6-h adherence tests. These EAF−/eaeA+ strains were the only putative enteropathogen identified in seven patients and were not found in controls. The ability of these strains to elicit ultrastructural cell alterations and cell-signalling events was evaluated in Caco-2 cells (human colon carcinoma cell line) by the gentamicin invasion assay and by transmission electron microscopy. The numbers of intracellular bacteria in cell invasion tests varied from 0.4% to 1.6% of the cell-associated bacteria after a 6-h incubation period. Tyrosine phosphorylation of host cell proteins was assessed in HEp-2 cells by immunofluorescence microscopy and all strains gave positive results. EAF−/eaeA+ *E. coli* strains express most of the virulence properties found among true EPEC strains and can be a relevant cause of infant diarrhoea in developing countries.

Introduction

Acute diarrhoea is a leading cause of mortality in developing countries. Among the diarrhoea-producing *Escherichia coli*, enteropathogenic *E. coli* (EPEC) are considered the main cause of epidemic outbreaks and endemic cases of infantile diarrhoea [1–3]. Illness caused by EPEC is often a clinically acute severe diarrhoea. On some occasions, persistent diarrhoea may develop [4]. Mannose-resistant adhesiveness to epithelial cells is considered to be a strong indication of enteropathogenicity for *E. coli*. Three patterns of adherence are recognised: localised, diffuse and aggregative [5, 6]. The localised adherence (LA) pattern is often exhibited by strains belonging to certain serotypes of EPEC frequently associated with acute diarrhoea in infants [3, 7–9]. Diffusely adhering *E. coli*
(DAEC) and enterohaemorrhagic E. coli (EAggEC) strains are usually not related to EPEC serotypes and their role in acute diarrhoeal disease is controversial [6,8–15]. In most EPEC strains, LA correlates with the presence of an EPEC adherence factor (EAF) plasmid [16]. A specific DNA probe has been used for the identification of localised adhering E. coli strains [17,18]. Associated with the LA phenotype is the induction of the attaching and effacing (A/E) lesions characterised by intimate attachment of bacteria to the apical enterocyte membrane and localised destruction of brush border microvilli. Accumulation of polymerised actin beneath bacteria results in the formation of cup-like pedestal structures [19]. The fluorescent actin staining (FAS) test is a diagnostic assay that detects polymerised actin associated with the A/E lesions [20]. Actin re-arrangement is also involved in the internalisation of a subpopulation of EPEC by epithelial cells [21–24]. Signal transduction, including induction of tyrosine phosphorylation of a 90-kDa host cell protein (Hp 90) by the attached EPEC, is the initial event that leads to formation of A/E lesions [25]. The genes implicated in the formation of A/E lesions have been localised in a large (35-kb) region of the EPEC chromosome termed the locus of enterocyte effacement (LEE) [26]. Several loci within the LEE, including eaeA, espA, espB (formerly eaeB) and sep, have been characterised. A DNA probe derived from the chromosomal gene eaeA encoding intimin can also be used to detect EPEC strains [27]. In the present study, virulence markers such as EPEC serotyping (O:H), adherence to HEp-2 cells, FAS test, DNA hybridisation with EAF, eaeA, Sth, Stp and EAggEC probes and production of heat-labile enterotoxin (LT) and verocytotoxin (VT) were analysed in E. coli isolates from children <2 years old with acute diarrhoea and healthy controls in Rio de Janeiro, Brazil.

Materials and methods

Bacterial strains

E. coli isolates from diarrhoeal stool specimens from 150 urban infants (<2 years old) attending the outpatient unit or admitted to the paediatric wards of Hospital Universitario Pedro Ernesto in Rio de Janeiro from July 1990 to April 1993 were examined. Diarrhoea was defined as the occurrence of one or more liquid or watery stools in a 24-h period. E. coli isolates from 50 non-hospitalised healthy infants belonging to the same age group and urban areas as the patients were also examined. Patients and controls in whom E. coli was not detected in the stools were excluded from the study. E. coli strains were isolated on Eosin Methylene-blue Agar (Difco Laboratories). Five randomly selected lactose-fermenting colonies were examined by biochemical tests [28] and E. coli isolates were stored on nutrient agar slants at 15°C until further testing. Shigella spp., Salmonella spp., Aeromonas spp., Yersinia enterocolitica, Campylobacter spp. and Vibrio cholerae were isolated and identified according to Lennette et al. [29]. Rotavirus was identified by a monoclonal latex agglutination test (SlideX Rota-Kit 2, bioMérieux, Lyon, France). E. coli strains E2348/69 (O127:H6; EAF+, eaeA+), E40705 (O157:H7;VT1+), H1/1 (O2:H?; DAEC), 239 (O111:H2; EAggEC), 40 T (O2:H7; LT+ – obtained from Dr A. F. P. Castro, Universidade Estadual de Campinas, UNICAMP, Brazil), 4871-1 (O78:H12, STH+) and 4511-2 (O27:H+, STp+) were used as positive control strains in cell adhesion and invasion tests, cytototoxicity assays and colony hybridisation assays. E. coli DH5α (K12) was used as the negative control in all tests.

E. coli serotyping

For the detection of EPEC serogroups, all E. coli colonies were tested for slide and tube agglutination with polyvalent and monovalent antisera (Difco; Probac, São Paulo, Brazil). The serogroups investigated were as follows: O18, O20, O26, O28, O44, O55, O86, O111, O112, O114, O119, O125, O126, O127, O129, O142 and O158. The actively motile cultures were tested by tube agglutination to determine H antigen with H1, H2, H4–H9, H11, H12, H15, H17, H18, H20, H21, H23, H25, H27, H29, H31–H35, H37, H40 and H41 antisera from the Centers for Disease Control, Atlanta, GA, USA. O:H serotyping of a few selected strains was done at the Laboratory of Enteric Pathogens, Central Public Health Laboratory, London. Sorbitol-negative E. coli isolates were screened with O157 and H7 antisera, raised in rabbits in our laboratory. Non-motile, lysine-negative E. coli isolates were tested with EIEC polyvalent antisera (Probac). The EIEC serogroups sought were as follows: O28ac, O29, O112ac, O124, O136, O143, O144, O152, O164 and O167. Agglutinating isolates were subjected to the Serény test [30].

Cytotoxicity assays

Cytotoxicity assays were done essentially as described by Smith and Scotland [31]. Vero and Y-1 adrenal cells were grown in 96-well tissue culture plates (Costar, Cambridge, MA, USA) respectively in Eagle’s Minimum Essential Medium (MEM; Sigma) supplemented with fetal calf serum (FCS) 5% v/v and Ham F-10 medium (Sigma) supplemented with L-glutamine 0.5% w/v and FCS 10% v/v. E. coli isolates were inoculated into 10 ml of Tryptic Soy Broth (TSB; Difco) in a 250-ml Erlenmeyer flask and incubated with shaking at 200 rpm for 18 h. The cultures were centrifuged at 9000 g for 10 min. The supernates were filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, USA) and stored at 4°C. Sterile culture supernates were then tested in the Y-1 cell assay for LT [32] and in the Vero cell assay for VT [31].
HEp-2 adherence assay

All E. coli isolates from patients and controls were tested individually for adherence to HEp-2 cells by the method of Cravioto et al. [33]. The HEp-2 human epithelial cell line CCL 23 (American Type Culture Collection, MD, USA) was cultivated in MEM supplemented with FCS 5% v/v, gentamicin 50 μg/ml and amphotericin B 2.5 μg/ml. Subconfluent cell monolayers were obtained on 13-mm diameter glass coverslips placed in 24-well tissue culture plates (Costar), washed twice with Dulbecco’s phosphate-buffered saline (PBS-D) pH 7.2, and covered with 1 ml of fresh MEM without antibiotics and containing D-mannose 1%. Samples (35 μl) of each bacterial culture monolayers were obtained on 13-mm diameter glass coverslips mounted on glass slides and examined by oil immersion microscopy by two independent examiners.

FAS test

All E. coli isolates were examined by the fluorescent actin staining (FAS) test by the method described by Knutton et al. [20]. After 3-h and 6-h HEp-2 adherence assays the cells were washed three times with PBS-D and fixed for 15 min in formalin 3% v/v. Fixed and washed cells were permeabilised by treating coverslips with Triton X-100 0.1% in PBS for 4 min. After washing three times in PBS-D the cells were treated with a solution of fluorescein isothiocyanate-phalloidin (Sigma) 5 μg/ml in PBS-D for 30 min to specifically stain filamentous actin. Coverslips were washed three times and mounted in glycerol-PBS-D. Specimens were examined by alternating fluorescence and phase-contrast microscopy with a Labophot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence. The FAS test was positive when the foci of intense fluorescence corresponded to areas of bacterial adhesion observed under phase-contrast.

DNA probes and hybridisation analyses

All strains were spotted on to MacConkey Agar (Difco) and incubated at 37°C overnight. Colonies were then transferred to Whatman 541 paper (Whatman, Clifton, NJ, USA) and the filters were processed and hybridised as described by Maas [34]. The probe fragments were radio-labelled with [32P]dATP by the random primer method. Results were revealed by autoradiography. The filters were hybridised with radio-labelled probes that detect the EAF (1-kb BamH1-SalI fragment of pJPN 16) [18], eaeA (1-kb SalI-KpnI fragment of pCVD 434) [27], human (216-bp EcoRl fragment of pCVD 427) and porcine (157-bp PstI fragment of pCVD 426) heat-stable enterotoxin (ST) of E. coli [35], and EAgoEC (1-kb EcoRl-PstI fragment of pCVD 432) [36].

Bacterial invasion

Caco-2 cells from a human colon carcinoma [37] were kindly provided by A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Unité 178, Villejuif, France). The cells were grown to post-confluence in 24-well plates containing Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma) supplemented with antibiotics, FCS 10% v/v, essential amino acids mixture (Sigma) 1% and L-glutamine 0.5% at 37°C in CO2 5%. The cell monolayers were washed twice in PBS-D and covered with 1 ml of fresh medium containing D-mannose 1%. The cells were incubated at 37°C for 6 h with medium change at 3 h to minimise extracellular bacterial growth. For each bacterial isolate, half of the Caco-2 monolayers were washed twice with PBS-D and incubated for 1 h in the same medium containing gentamicin 250 μg/ml. Control wells were incubated with medium without antibiotics. After removal of the antibiotic-supplemented medium the cells were washed twice and lysed with trypsin (Trypsin 1:250, Difco) 0.1% and Tween 20 (Sigma) 1% for 30 min at 37°C. Samples of the cell lysates were diluted and plated on Tryptic Soy Agar (TSA; Difco) to quantify viable bacteria. The percentage invasion was calculated by dividing the number of surviving (intracellular) bacteria by the number of bacteria in the controls (cell-associated bacteria) [38]. Experiments were run in duplicate and repeated independently at least three times with positive (EPEC E2348/69) and negative (DH5α K12) controls. All the strains were susceptible to killing by gentamicin as previously established by broth dilution techniques.

Detection of tyrosine phosphorylated proteins

EAF-/eaeA+ E. coli isolates were tested for tyrosine phosphorylation of eukaryotic proteins by immunofluorescent microscopy [39]. After 6-h HEp-2 adherence assays the coverslips were washed three times with PBS-D to remove non-adherent bacteria. The cells were then fixed in paraformaldehyde 2% for 15 min and washed. The cells were permeabilised by treatment with Triton X-100 0.1% in PBS-D for 4 min and then washed. Accumulation of tyrosine-phosphorylated proteins was detected by incubation of cells with a 1 in 2000 dilution of murine monoclonal antiphosphotyrosine IgG (Sigma) as the primary antibody for 60 min. Following three washes in PBS-D the cells were incubated with a 1 in 100 dilution of fluorescein isothiocyanate-conjugated anti-mouse rabbit IgG (Sigma) for 60 min. Following further washes and mounting in glycerol-PBS-D the coverslips were examined by alternating phase-contrast and fluorescence microscopy in a Labophot microscope equipped with epifluorescence.
Transmission electron microscopy

Caco-2 cells were grown to post-confluence on the surface of a thin layer of polymerised type I collagen spread on coverslips placed in 24-well tissue culture plates. After incubation for 6 h, infected Caco-2 cells were washed with PBS-D and fixed in glutaraldehyde 2.5% in cacodylate-sucrose buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M MgCl₂, 0.01 M CaCl₂, pH 7.2) for 2 h at 4°C. Cells were post-fixed in OsO₄ 1%, dehydrated through a series of graded ethanol solutions and embedded in Epon. Small blocks were cut and oriented before resin polymerisation at 60°C for 72 h. Ultra-thin sections were cut with a Reichert Ultracut S microtome, stained in aqueous solutions of uranyl acetate and lead citrate 2.5% and examined in an EM 906 Zeiss transmission electron microscope at 80 Kv.

Statistical analysis

The χ² test was used to determine the statistical significance of the data. A p value of <0.05 was considered significant.

Results

A total of 990 E. coli isolates from stool cultures, comprising 740 from 150 patients and 250 from 50 controls, was assayed for adherence to HEp-2 cells, FAS test, DNA hybridisation with EAF, eaeA, STh, STp and EAggEC probes and production of verocytotoxin (VT) and heat-labile enterotoxin (LT). The occurrence of enteropathogens in 150 infants with diarrhoea and 50 controls is shown in Table 1. EPEC were far more common in patients than controls (32.7% versus 14.0%; p < 0.025). There were no significant differences in the rates of isolation of EAggEC, DAEC and ETEC between patients and controls. EIEC, VTEC and enterohaemorrhagic E. coli (EHEC) strains of the serotype O157:H7 were not found in either group.

Table 1. Isolation of enteropathogens from the stools of 150 children <2 years old with acute diarrhoea and 50 healthy controls

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number (% of isolates for</th>
<th>Patients</th>
<th>Controls</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPEC</td>
<td>49 (32.7)</td>
<td>7 (14.0)</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td>EAggEC</td>
<td>18 (12.0)</td>
<td>12 (24.0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>DAEC</td>
<td>15 (10.0)</td>
<td>2 (4.0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td>14 (9.3)</td>
<td>ND</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>5 (3.3)</td>
<td>2 (4.0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>2 (1.3)</td>
<td>ND</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>1 (0.7)</td>
<td>ND</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>1 (0.7)</td>
<td>ND</td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

EPEC, enteropathogenic E. coli; EAggEC, enterogastric E. coli; DAEC, diffusely adhering E. coli; ETEC, enterotoxigenic E. coli; ND, not determined; NS, not significant.

* Determined by χ² test; p < 0.05 was considered significant.

Patients

Among the 740 E. coli isolates from diarrhoeal specimens, 158 gave positive agglutination with EPEC antisera. Table 2 presents the serotypes and the virulence markers of these isolates. Among the EPEC serotypes found, the most common serotypes were O111:H2 (five children), O55:NM (four) and O119:H6 (four). Of the EPEC isolates, 80 (50.6%) showed localised adherence. All isolates with LA gave a positive FAS assay and hybridised with the eaeA probe. Of these 80 LA-positive EPEC strains, 60 (75%) hybridised with the EAF probe. In this study, the serotypes that produced LA were O55:NM, O55:H40, O55:H4, O86:H34, O111:H2, O111:H1, O119:NM, O119:H2, O119:H6, O119:H1 and O142:H6. Twenty EAF−/eaeA+ E. coli isolates that belonged to serotypes O55:NM (three), O119:H2 (nine), O119:H1 (two), O119:H6 (one) and O142:H6 (five) were isolated from six patients (Table 2). Diffuse adherence was displayed by eight EPEC (5.0%) isolates. Thirty-one EAggEC isolates were from 18 (12.0%) patients and most of them belonged to EPEC serotypes (O26:H27, O86:NM, O86:H7, O86:H11, O86:H18, O111:H1, O119:H17, O125:H11, O125:H31, O127:H7 and O128:H7) lacking the EPEC virulence factors. All EAggEC probe-positive isolates that belonged to EPEC serotypes showed the aggregative pattern of adherence in HEp-2 cells. However, EAggEC isolates of serotype O125:H11 produced a typical aggregative phenotype but were negative with the EAggEC probe. On three occasions, EAggEC isolates were associated with rotavirus (one), EPEC (one) or Shigella sonnei (one). Among the 582 E. coli isolates that did not agglutinate with EPEC antisera, 23 (3.9%) showed LA, gave a positive FAS test and hybridised with the eaeA probe (Table 3). Of these, only 16 (69.5%) isolates hybridised with the EAF probe. All EAF−/eaeA+ EPEC and non-EPEC isolates were LA+ only after 6 h, although a positive FAS test had already been observed after incubation for 3 h. EAF−/eaeA+ isolates were the only putative enteropathogens identified in seven patients (Tables 2 and 3). In one patient, EAF− and EAF+ isolates of the same EPEC serotype were co-isolated. Diffuse adherence was observed in 48 (8.2%) non-EPEC isolates. Among the eight (1.3%) EAggEC probe-positive isolates, two showed the aggregative pattern of adherence in HEp-2 cells. However, four probe-positive strains attached to cells without any recognisable pattern of adherence and two strains caused complete detachment of cell monolayer even during 3-h adherence tests.

Controls

Of the 250 E. coli isolates from controls, 29 (11.6%) belonged to EPEC serotypes (Table 4). Five EPEC isolates (O55:NM) showed LA and hybridised with the EAF and eaeA probes. EAF−/eaeA+ E. coli isolates were not found in the control group. Diffuse adherence
Table 2. Virulence markers in isolates of EPEC serogroups from 150 patients < 2 years old in Rio de Janeiro, Brazil

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Isolates (patients)</th>
<th>HEp-2 adherence</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LA</td>
<td>DA</td>
<td>AA</td>
</tr>
<tr>
<td>O26:H27</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O55:NM</td>
<td>15 (4)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>O55:H21</td>
<td>4 (1)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>O55:H40</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O55:H7</td>
<td>2 (2)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O86:NM</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O86:H11</td>
<td>5 (1)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>O86:H18</td>
<td>5 (2)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>O86:H34</td>
<td>3 (1)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>O86:H7</td>
<td>7 (3)</td>
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<td>O111:H2</td>
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<td>O111:H12</td>
<td>3 (1)</td>
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<td>3</td>
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<tr>
<td>O111:H21</td>
<td>2 (1)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O111:H7</td>
<td>10 (4)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>O119:NM</td>
<td>1 (1)</td>
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<td>1</td>
</tr>
<tr>
<td>O119:H2</td>
<td>9 (2)</td>
<td>9</td>
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<tr>
<td>O119:H6</td>
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</tr>
<tr>
<td>O119:H17</td>
<td>1 (1)</td>
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<tr>
<td>O119:H?</td>
<td>19 (5)</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>O124:H31</td>
<td>5 (1)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>O125:NM</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
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<tr>
<td>O125:H4</td>
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<tr>
<td>O125:H11</td>
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<td>O128:H40</td>
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<td>O132:H4</td>
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<tr>
<td>O142:H6</td>
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<td>5</td>
</tr>
<tr>
<td>O142:H11</td>
<td>5 (1)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>O142:H7</td>
<td>2 (1)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>158 (60)*</td>
<td>80 (50.6)</td>
<td>8 (5.0)</td>
</tr>
</tbody>
</table>

LA, localised adherence; DA, diffuse adherence; AA, aggregative adherence; A+, adherence without a recognisable pattern; NA, non-adherent.


Table 3. Virulence markers in non-EPEC isolates from 150 patients < 2 years old in Rio de Janeiro, Brazil

<table>
<thead>
<tr>
<th>Non-EPEC isolates</th>
<th>HEp-2 adherence</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>LA</td>
<td>DA</td>
</tr>
<tr>
<td>4</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>85</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>110</td>
</tr>
</tbody>
</table>

LA, localised adherence; DA, diffuse adherence; AA, aggregative adherence; A+, adherence without a recognisable pattern; NA, non-adherent.

*Four EAggEC probe-positive isolates from three patients exhibited the A+ adherence phenotype and two isolates from two patients caused detachment of cell monolayer.

was detected in three (1.2%) EPEC isolates and one (0.4%) non-EPEC isolate. Twenty EAggEC probe-positive isolates were detected in nine (18%) control subjects. All of them produced the aggregative phenotype in HEp-2 cells, but none reacted with the EPEC antisera. On the other hand, five EAggEC probe-negative isolates that produced a typical aggregative phenotype were isolated from three controls.
Comparison of patients and controls

Of 150 infants with diarrhoea, 138 were selected from whom no recognised enteropathogens, except classic enteropathogenic E. coli, were identified. For comparison, 48 controls were also examined (Table 5). E. coli isolates that agglutinated with EPEC antisera were far more common in patients than controls (31.9% versus 14.6%; p < 0.025) and isolates exhibiting LA were more often isolated from patients than controls (19.6% versus 2.1%; p < 0.005). Diffusely adhering and EAggEC isolates were found with equal frequency in patients and controls, thereby showing no association with diarrhoea. E. coli isolates exhibiting adherence without a recognisable pattern (A+ strains) were slightly more frequent in patients than controls (10.8% versus 4.2%; not significant). On the other hand, non-adherent E. coli isolates were significantly associated with children without diarrhoea (Table 5). LA was strongly correlated with a positive FAS assay and eaeA probe, whereas hybridisation with the EAF probe showed partial correlation with the same virulence markers. In seven (5.1%) children with diarrhoea, E. coli isolates positive for LA, FAS and eaeA but negative for EAF were detected. E. coli exhibiting this combination of markers were not detected among controls.

Toxin production

LT+ ETEC were isolated from five (3.6%) diarrhoea cases and one (2.1%) control, whereas LT+/STh+ strains were isolated from one (2.1%) control. STp+ ETEC and VTEC were not found in either group. However, in 10 (6.7%) patients, 13 EPEC and nine non-EPEC isolates were found whose culture filtrates, even after heating (100°C, 30 min) produced cytotoxic effects in Vero cells characterised by complete detachment of the cell monolayer even during 3-h adherence tests. Among these isolates, 10 EPEC and one non-EPEC showed all the EPEC virulence markers (LA+, FAS+, EAF+ and eaeA+).

Pathogenicity of EAF−/eaeA+ isolates

To evaluate the pathogenic potential of the EAF−/eaeA+E. coli isolates, the invasive ability of these isolates for Caco-2 cells was investigated by the gentamicin invasion assay and by transmission electron microscopy. After incubation for 6 h, all EAF−/eaeA+ isolates were found in the intracellular compartment. The percentages of intracellular bacteria varied from 0.4% to 1.6% of the cell-associated bacteria (Table 6). Transmission electron microscopy was performed on Caco-2 monolayers after a 6-h invasion assay. As shown in Fig. 1, an intimate adherence was demonstrated by the close association of the bacteria to the cell membrane. Intracellular EAF−/eaeA+E. coli were seen in membrane-bound vesicles. Marked cytoskeletal changes were seen directly beneath the adherent bacterium, including the accumulation of polymerised actin, and the bacteria sometimes sat upon a pedestal-like structure (Fig. 2). Accumulation of tyrosine-phosphorylated proteins was assayed by im-
Table 6. Invasion of Caco-2 cells by EAF−/eaeA+ E. coli isolates from children with diarrhoea in Rio de Janeiro, Brazil

<table>
<thead>
<tr>
<th>E. coli isolate</th>
<th>Serotype (SD)*</th>
<th>Percent invasion (SD)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>I31/5</td>
<td>O119:H2?</td>
<td>1.0 (0.2)+</td>
</tr>
<tr>
<td>H32/5</td>
<td>O119:H2?</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>H98/3</td>
<td>O119:H2</td>
<td>1.6 (0.1)</td>
</tr>
<tr>
<td>H100/1</td>
<td>O119:H2</td>
<td>1.4 (0.2)</td>
</tr>
<tr>
<td>H104/2</td>
<td>O45:NM</td>
<td>0.6 (0.2)</td>
</tr>
<tr>
<td>H111/2</td>
<td>O55:NM</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>H112/5</td>
<td>O7:H33†</td>
<td>0.5 (0.1)</td>
</tr>
<tr>
<td>H152/2</td>
<td>O142:H6</td>
<td>0.6 (0.1)</td>
</tr>
<tr>
<td>E2348/69</td>
<td>O127:H6</td>
<td>1.5 (0.2)</td>
</tr>
<tr>
<td>DH50(912)</td>
<td></td>
<td>0.04 (0.03)</td>
</tr>
</tbody>
</table>

*Percentage of intracellular bacteria relative to the cell-associated (intracellular plus extracellular) bacteria after 6-h infection.
†Results are the mean (SD) of at least three independent tests, performed in duplicate.
‡Untypable with O1–O173 antisera.

munofluorescence in HEp-2 cells infected with EAF−/eaeA+ E. coli isolates. Results from microscopy showed foci of increased fluorescence in HEp-2 cells after infection for 6 h. These foci of fluorescence corresponded with areas of bacterial adhesion observed under phase-contrast microscopy.

Discussion

EPEC were isolated from 49 (32.7%) patients compared with seven (14.0%) controls, a statistically significant difference (p < 0.025). No pathogen other than EPEC was isolated from 44 of these 49 cases. These findings as well as others obtained in Brazil [3, 40, 42] implicate EPEC as the main cause of infantile diarrhoea in the large Brazilian urban centres and emphasise the role of this micro-organism as an important cause of acute diarrhoea in developing countries [2, 13, 43]. The incidence of ETEC was very low. The results of the present study differ from those in many developing countries, where ETEC are often the most frequent pathogen found [43–45]. VTEC and O157:H7 (EHEC) strains were not detected. In São Paulo, Brazil, Giraldi et al. [46] found only one (0.2%) non-O157 VTEC in 466 isolates from 95 children < 1 year of age with acute diarrhoea. This result suggests that VT production is an uncommon feature among E. coli isolates from endemic acute infantile diarrhoea in Brazil. In fact, both VTEC and EHEC are usually associated with food-borne outbreaks involving patients of different age groups. EHEC strains probably do not have a universal distribution and are presently restricted to some geographical areas. EIEC was not found. In Brazil EIEC has been isolated most frequently in children >2 years of age [47].

Among the EPEC serotypes, the most commonly isolated were O111:H2, O55:NM and O119:H6. These O:H types were also prevalent in several studies conducted in Brazil and other developing nations [3, 40, 41, 48].

![Fig. 1. Electron micrograph of a confluent monolayer of Caco-2 cells 6 h after infection with E. coli EAF−/eaeA+ H104/2 (O45:NM). Bacterial microcolonies are transversely sectioned. Membrane folding and production of digitiform projections suggests intense mobilisation of cell membrane at the sites of bacterial colonisation. Bacteria closely associated with cell membrane and bacteria undergoing internalisation can be seen (×9150).](image-url)
Fig. 2. Electron micrograph of Caco-2 cell 6 h after infection with *E. coli* EAF-/eaeA+ H152/2 (O142: H6). Intimate adherence and bacterial internalisation can be seen. Membrane cupping and formation of discrete pedestals can be seen at the sites of bacterial attachment (×25 200).

With regard to the adhesive properties of isolates, LA was found both in EPEC and non-EPEC isolates from patients. Among 103 LA+ isolates, 77% belong to a restricted number of EPEC O:H serotypes. A significant association between some EPEC serotypes and LA has been shown by several authors [7, 8, 41, 49, 50]. Serotypes O55:NM, O86:H34, O111:H2, O119:H6 and O142:H6 were strongly associated with LA in Brazil [7, 40–42] and other countries [51, 52]. In the control group only one child (2.0%) harboured EPEC LA+ isolates belonging to the O55:NM serotype. Non-EPEC isolates producing LA were detected in patients only at low frequency (3.9%). All these isolates were FAS+ and hybridised with *eaeA* probe, but 30% of them were negative for the EAF sequence. Other authors have also reported the isolation of these non-EPEC strains in association with diarrhoea [12, 13].

In the present work, complete correlation was found between LA, FAS test and hybridisation with the *eaeA* probe, as also verified by several authors in many parts of the world [27, 48, 52]. However, a weaker correlation (89%) between EAF and the other virulence markers was found, especially for EPEC strains of serotypes O55:NM, O119:H2, O119:H6, O119:H7, O142:H6 and for non-EPEC strains. EPEC strains cured of the EAF plasmid retain the ability to form A/E lesions in tissue culture and cause actin aggregation, although much less efficiently than the EAF- expressing parent [20, 52]. EPEC serotypes that do not possess the EAF plasmid have also been incriminated by epidemiological studies and studies in volunteers as causes of diarrhoea, even though their pathogenesis at the molecular level is not as precisely known as that of the EAF-positive EPEC [1]. In the UK, Scotland et al. [12] proposed that EAF probe-negative EPEC may be of greater importance than EAF probe-positive strains.

DAEC isolates showed no association with diarrhoea. All isolates were negative in the FAS test and did not hybridise with the *eaeA*, EAF and EAggEC probes. Several authors did not find any association of DAEC with acute infantile diarrhoea [9, 13, 42, 49]. However, Girón et al. [14] and Jallat et al. [53] reported its isolation with significant frequency from cases of acute diarrhoea.

The small number of epidemiological studies performed to date give conflicting results with respect to differences in the rate of isolation of EAggEC from patients versus controls and their association with acute and persistent diarrhoea. In this study and in previous work performed in Brazil [9], EAggEC were isolated with similar frequency from cases of acute diarrhoea and controls. Children with persistent diarrhoea were not included in these studies. Among the strains that produced the aggregative phenotype, the pattern was identified only in 6-h adhesion tests. In 3-h tests, bacteria could occasionally be seen scattered on the periphery of HEp-2 cells, but without the
brane alterations, with the formation of discrete cup-of-A/E lesions, such as intimate adherence of
produced a positive FAS test in 3-h tests. The
showed a significant invasive capability. However, the
control studies in Chile [8], Brazil [3,41], Thailand
interaction of these strains with Caco-2 cells was
studied further by TEM and by the gentamicin
fall into well-recognised O:H types, carry EAF,
EPEC recently held in Brazil, most true EPEC strains
different adherence phenotypes and were uniformly
negative for the virulence markers investigated.
Despite their Hep-2 adherence ability, their isolation
rates were similar in patients and controls, indicating
that this adhesion is not a characteristic of pathogenic
E. coli.

As defined in the 2nd International Symposium on
EPEC recently held in Brazil, most true EPEC strains
fall into well-recognised O:H types, carry EAF, bfpA
and eaeA DNA sequences and do not produce VT [55]. In this study, EPEC and non-EPEC isolates from
eight patients were EAF−/eaeA+. These isolates were
the sole pathogen isolated from seven patients and
were not found in the control group. Previous studies have shown E. coli EAF−/eaeA+ strains significantly
associated with diarrhoea [12,56]. However, case-
control studies in Chile [8], Brazil [3,41], Thailand
[48] and Bangladesh [43] found that only EAF
positive strains were associated with diarrhoea. At the above-mentioned International Symposium, these
EAF−/eaeA+ strains were referred to provisionally as
‘atypical EPEC’. The EAF−/eaeA+ isolates in the
present study showed LA in 6-h adhesion tests, but
produced a positive FAS test in 3-h tests. The
interaction of these strains with Caco-2 cells was
studied further by TEM and by the gentamicin
invasion assay – widely used to assess bacterial
internalisation by cultured epithelial cells. All isolates
showed a significant invasive capability. However, the
uptake of most EAF−/eaeA+ E. coli strains is far
more discrete than with true EPEC strains. TEM
observation of infected Caco-2 cells showed evidence
of A/E lesions, such as intimate adherence of
bacteria, cytoskeletal re-arrangement and cell mem-
brane alterations, with the formation of discrete cup-
like pedestals. Internalised bacteria were seen inside
membrane-bound cell vacuoles. In EPEC, the A/E
lesion results from the co-ordinated expression of
genes encoding a type III protein secretion system and
esp genes involved in signal transduction events, such
as inositol phosphate release and induction of tyrosine
phosphorylation of host cell proteins. The atypical
EPEC isolates studied showed ability to induce A/E
lesions and to trigger cell signalling events character-
istic of fully virulent EPEC. The results of this study
confirm that EPEC remain the leading pathogen
associated with acute diarrhoea in Brazilian children,
and also raise questions as to the role of EAF−/eaeA+ E. coli strains as an important agent
of infantile diarrhoea.

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