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Unidentified serogroups of enteropathogenic Escherichia coli (EPEC) associated with diarrhoea in infants in Londrina, Parana, Brazil

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Digoxigenin-labelled DNA probes were used to characterise enteropathogenic Escherichia coli (EPEC) isolated in Londrina (Brazil) from faeces samples of 102 children with diarrhoea, and the results were compared with those obtained by serogrouping and adherence to HEp-2 cells. The probes employed detect the gene coding EPEC adherence factor (EAF) and the virulence genes for bundle-forming pilus (bfp) and enterobacterial-attaching-effacing (eae) factor. Twenty-one isolates hybridised with at least one probe, and 11 of them were classified as typical EPEC because they hybridised with all three probes, showed a pattern of localised adherence (LA) and carried no genes for enterotoxins (ST and LT) or invasion as detected by PCR. Six of the typical EPEC strains belonged to the classical serotype O119:H6 and one to O111:H6; O antigens could not be determined in four strains with antisera against O1–O173. All typical EPEC strains carried a 70-MDa plasmid plus two other large plasmids. These data showed that typical EPEC virulence traits may be found in strains not belonging to classical serogroups/serotypes and that molecular identification is required for studying the epidemiology of diarrhoea in children.

Introduction

The term enteropathogenic Escherichia coli (EPEC) refers to E. coli strains of specific serotypes, epidemiologically incriminated as pathogens in outbreaks of diarrhoea. They are one of the main causes of severe infantile diarrhoea in many developing countries [1–3]. EPEC strains can colonise the intestine and cause attaching and effacing (A/E) lesions, characterised by localised destruction (effacement) of brush border microvilli, intimate attachment of the bacteria to the cell membrane and formation of an underlying pedestal-like structure of polymerised actin in the host cell [4]. According to Knutton et al. [5], the A/E lesion begins with the presence of two adhesins, bundle-forming pilus (bfp) and intimin, and production of EspA filaments. Bfp, a type IV fimbrial protein, is responsible for binding of EPEC to epithelial cells and formation of microcolonies, a process termed localised adherence (LA) [6]. Intimin is a surface protein (94 kDa) encoded by the eae gene and regulated by genes located on plasmid EAF (EPEC adherence factor) [7]. The EspA filament is a novel EspA-associated surface organelle of EPEC involved in protein translocation into epithelial cells, which forms a physical bridge between the bacterium and the infected eukaryotic cell surface [5].

Characterisation of EPEC infections is routinely based on serological tests, but it can also be conducted easily by means of molecular diagnostic techniques, such as DNA probing and PCR. DNA probes have enabled the analysis of large numbers of specimens [8] and improved the understanding of their epidemiology [9]. PCR for EPEC is a rapid assay and gives high sensitivity and specificity [10,11]. Recently, several studies have demonstrated that EPEC serogroups are a heterogeneous group with regard to their virulence properties [12].

This study employed specific digoxigenin-labelled DNA probes to re-evaluate the correlation between serotyping and virulence-associated properties of EPEC.

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Materials and methods

Bacterial strains

Stool specimens from 102 infants, under 2 years of age, presenting with acute diarrhoea and from 46 healthy children of about the same age were inoculated on to MacConkey agar and incubated in air at 37°C for 24 h. Three-to-five colonies from each sample, identified by biochemical assays as *E. coli*, were selected for subculture and hybridisation with BFP, *eaeA* and EAF probes. Only *E. coli* strains that hybridised with at least one probe were further identified by serotyping and studied for virulence properties. EPEC serogrouping and flagellar antigen determination were performed at the Instituto Adolfo Lutz, São Paulo, Brazil, by agglutination tests with polyvalent and monovalent sera against O antigens (O1–O173) and flagellar H antigens.

*E. coli* 2348/69 O127:H6 strain (originally isolated from a nursery outbreak of gastro-enteritis in Taunton, UK) and expressing BFP, EAF and intimin as a prototype of EPEC [13] was used as a positive control in HEp-2 cell adherence assays and in colony hybridisation tests. The recombinant *E. coli* K12:HB101 was used as a negative control.

PCR and labelling of DNA probes with digoxigenin

PCR was performed with primers EP1 (5’-CAA TGG TGC TTG CGC TTA TCC AAC CTG GT-3’) and EP2 (5’-GCT GCT TTA TTC ACC CAT GTA TTA TCA-3’) for the *bfp* probe [10], EAF1 (5’-CAG GGT AAA AGA AAG ATG ATA A-3’) and EAF2 (5’-TAT GGT GCG CAT GTA TTA TCA-3’) for the *eae* probe [14], EPEC1 (5’-TGG TCA CAG TGG CAG TTC GGG T-3’) and EPEC2 (5’-CCG AAA GGT TCA TAT CCG TAA AGT-3’) for the *eaeA* probe [15]. *E. coli* E2348/69 was grown in Luria broth (LB) at 37°C overnight, suspended in 200 μl of sterile distilled water, boiled for 10 min and centrifuged at 12,000 rpm for 2 min. Supernate (5 μl) was added to PCR mixture (45 μl). Samples were incubated at 94°C for 1 min to denature the DNA, at 56°C for 2 min to anneal the primers and 72°C for 1 min to extend the annealed primers, for 30 cycles. Labelling of the PCR-generated fragment was performed with a digoxigenin labelling kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer’s instructions. The probes were estimated by detection of labelling efficiency in a direct assay as described by Boehringer and used at a concentration of 50 ng/ml.

Colony hybridisation assays

PCR-generated probes labelled with digoxigenin were used for colony hybridisation. Filters were prepared and hybridised as described previously [15]. Hybridisation was visualised by chemiluminescent detection CSPD (Boehringer Mannheim GmbH).

Adherence assays

Adherence to HEp-2 cells was assessed as described by Scaletsky et al. [16]. *E. coli* strains showing no adherence after a period of 30 min plus incubation for 3 h were submitted to a 6-h adherence test. D-Mannose (1% w/v) was added to the medium to inhibit adherence through mannose-sensitive pili [16].

PCR detection of the presence of DNA responsible for enterotoxins and invasion

EPEC strains were grown in LB broth and DNA was extracted by boiling. DNA sequences for LT and ST production and invasion were assayed by PCR with the specific primers: LT1 (5’-GCC ACA AAT TAT ACC GTG CT-3’) and LT2 (5’-CGG AAT TCT GTT ATA TAT GT-3’), Stal (5’-CGT TAT TGT CCT TTT CAC CT-3’) and Sta2 (5’-GCC CCC GGT ACA AGC AGG AT-3’), INV1 (5’-GCT GGA AAA ACT CAG TGC CT-3’) and INV2 (5’-CCA GTC CGT AAA TTC ATT CT-3’) [11]. *E. coli* HB101(K12) was used as a negative control.

Plasmid DNA profile

Plasmids were extracted by an alkaline lysis method [17], electrophoresed on vertical agarose 0.7% gels in TBE running buffer (89 mM Trizma base, 89 mM boric acid, 2 mM EDTA, pH 8.3) and visualised by ethidium bromide staining (0.5 μg/ml, 15 min). The following standard markers were used to estimate plasmid molecular mass: pR27 (110 MDa), pJPN11 (66 MDa) and pRM4 (34 MDa).

Results

A total of 442 *E. coli* colonies isolated from 102 faecal samples from children with diarrhoea and 177 *E. coli* colonies isolated from 46 controls was examined by means of probes for the presence of EPEC virulence characteristics. Twenty-one *E. coli* isolates (22%) hybridised with one or more of the probes for EAF, *eaeA* and BFP. Of these, 11 (52%) (i.e., 2.5% of the total isolates), hybridised with all probes (Table 1). Of the controls, seven strains (4%) hybridised with one or two probes but none hybridised with all.

The 11 *E. coli* strains that hybridised with EAF, *eaeA* and BFP probes also adhered to HEp-2 cells, showing a localised adherence patterns and were classified as typical EPEC. These EPEC belonged to serotypes O119:H6 (six strains), O111:H6 (one strain) and ONT:H7 (four strains untypeable for O1–O173) (Table 1).

The plasmid profiles of all typical EPEC strains were similar, presenting three plasmids of high molecular mass, whose size varied from 19 to 70 MDa (Fig. 1).
**Table 1.** Relationship of the results of colony hybridisation with *eae*, *bfp* and *eaeA* probes, serotypes and adherence to HEP-2 cells of *E. coli* isolates from infants with acute diarrhoea

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th><em>eae</em></th>
<th><em>bfp</em></th>
<th><em>eaeA</em></th>
<th>Serotype</th>
<th>Adherence</th>
<th><em>E. coli</em> classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>O119: H6</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>42.2</td>
<td>+</td>
<td>+</td>
<td></td>
<td>O119: H6</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>43.1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>O119: H6</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>46.1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>O119: H6</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>48.2</td>
<td>+</td>
<td>+</td>
<td></td>
<td>ONT: H7</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>56.1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>ONT: H7</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
<tr>
<td>64.1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>ONT: H7</td>
<td>LA</td>
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</tr>
<tr>
<td>71.1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>O111: H6</td>
<td>LA</td>
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<td>+</td>
<td>+</td>
<td></td>
<td>O119: H6</td>
<td>LA</td>
<td>Typical EPEC</td>
</tr>
</tbody>
</table>

+, hybridisation with probes, NT, non-typable with antisera O1–O173; LA, localised adherence.

**Discussion**

The main virulence characteristics of EPEC strains have been detected by molecular diagnostic techniques, including detection of the chromosomal locus, *eaeA* gene, and two plasmid-borne loci, the *bfp*A gene, which encodes the pilus subunit of the bundle-forming pilus, and the EAF. These target genes can be detected by DNA probes, which are more sensitive and specific, and less time-consuming than serogrouping and tissue culture assays [9].

In the present study, the three probes detected EPEC strains, that were untypable for O1–O173 and showed localised adherence to HEP-2 cells, suggesting the existence of new serogroups of typical EPEC. The high frequency (29%) of typical EPEC ONT:H7 strains corroborates the conclusion that the identification of EPEC strains must not be based only on serotyping, but that it is essential to use DNA probes detecting *bfp*A and *eaeA* genes, which are directly related to localised adherence to HEP-2 cells. The serotype O111:H6 has not been mentioned as a typical EPEC in previous studies. Plasmids of 70 MDA observed in most of the typical EPEC strains probably correspond to the EAF plasmid [18], which is related to adhesion to HEP-2 cells.

The results of the present study demonstrate that typical EPEC virulence traits may be found in strains not belonging to classical serogroups/serotypes, showing the need for molecular identification in studying the epidemiology of diarrhoea in children in different regions.

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**References**

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**Fig. 1.** Agarose gel electrophoresis of plasmid DNA of 11 EPEC isolates from children with diarrhoea. Lanes 1–11, isolates 29.1, 42.2, 43.1, 46.1, 48.2, 95.1, 56.1, 64.1, 66.2, 92.1, 71.1. A, B, C, reference plasmids: R27 (110 MDA), JPN (66 MDA), RP4 (34 MDA).