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MOLECULAR IDENTIFICATION OF ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) ASSOCIATED WITH INFANT DIARRHEA IN LONDRINA, PARANA, BRAZIL

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ABSTRACT

In this work, the prevalence of enteropathogenic *Escherichia coli* (EPEC) in children in Londrina-PR, Brazil, was evaluated by means of digoxigenin-labelled DNA probes which identify the plasmid responsible for EPEC adherence factor (EAF), and virulence genes for EPEC as bundle-forming pilus (*bfp*) and *E. coli* attaching-effacing factor (*eae*). In addition, the isolated strains were serotyped and tested for adherence to HEp-2 cells. From 102 children with diarrhoea, 19 strains hybridized with at least one probe, and eleven of them were identified as typical EPEC because they hybridized with the three probes used, showed a localized adherence (LA) pattern, and presented 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 (43%); in four strains O antigens could not be determined using antisera against O1 to O173, they were all ONT:H7 (29%); one strain belonged to O111:H6. Three strains were classified as atypical EPEC: O26H$^-$, O111:H9 and O119:HNT. Strains O26H$^-$ and O111:H9 hybridized with the *eae* probe only and showed localized adherence like (LAL) pattern; strain O119:HNT hybridized with the *bfp* and *eae* probes, and showed a localized adherence/diffuse adherence (LA/DA) pattern after 6 h. A DA pattern was observed in two strains isolated from children with diarrhoea (ONT:H11 and O142:H34), which hybridized with the *eae* probe. From 46 controls, five strains hybridized with one or two probes, but none hybridized with all probes or presented the LA pattern. Three strains with the DA pattern hybridized with the *eae* probe. No EPEC strain belonging to classical EPEC serotypes was isolated from faeces of control children.

Kew words: Enteropathogenic *Escherichia coli*, EPEC, digoxigenin-labelled DNA probes, virulence factors, serotypes, diarrhoea

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) strains are diarrheagenic *E. coli* belonging to specific serotypes, historically associated with outbreaks of infantile diarrhea, which are one of the main causes of severe diarrhea in many developing countries (4, 14, 26). EPEC produce diarrhea by an attaching and effacing (A/E) type of lesion (31), characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacteria to the enterocyte membrane, and formation of an underlying pedestal-like structure of polymerized actin in the host cell (7).

Chromosomal and plasmid-encoded genes are necessary for A/E lesions formation. The genes *eae*, that encodes the surface protein intimin (22), and *esp*, responsible for secreted proteins involved in cell signalling, belong to a chromosomal pathogenicity island, called the LEE region (for locus of enterocyte effacement) (30). A large plasmid, referred to as EPEC adherence factor (EAF) plasmid, harbours the bundle-forming pili gene (*bfp*) cluster (12), and regulates expression of *eae* (18). The A/E lesion begins with the presence of the two adhesins, BFP and intimin, and production of EspA filaments (24). BFP is a type IV fimbria, responsible for binding of EPEC to epithelial cells and formation of microcolonies, a process termed

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localized adherence (LA) (8). EspA filaments are a novel EspA-associated surface organelle of EPEC, involved in protein translocation into epitelial cells, which forms a physical bridge between the bacterium and the infected eukaryotic cell surface (24).

The adhesive properties of EPEC have been examined *in vitro* with cell cultures, and in HEp-2 cells the LA pattern was clearly differentiated from two other patterns, diffuse adherence (DA) and aggregative adherence (AA) (32).

The diagnosis of EPEC infections is usually performed by conventional methods such as serological tests, which suffer from a somewhat restricted specificity and sensitivity (45). Therefore, the use of DNA probes (13, 16, 33) and PCR (10, 21, 42), which are methods with high specificity and sensitivity, is required for an adequate characterization of the EPEC strains associated with cases of diarrhea in particular regions.

In Brazil, diarrhea is still a major health problem and EPEC strains have been recovered from cases of severe diarrhea in infants of low socioeconomic level, in some parts of the country. The purpose of this work was to study the prevalence of EPEC in children in Londrina-PR, Brazil, by means of DNA probes.

MATERIALS AND METHODS

Patients. From February 1995 to September 1996, 102 infants under 2 years of age presenting acute diarrhea and 46 controls were studied. This study was conducted in Londrina, an urban area located in southern Brazil, at the University Hospital. Acute diarrhea was defined as bowel movement of three or more liquid or semiliquid stools per day. The controls were healthy children under 2 years of age, with no symptoms of gastrointestinal disorders. Stool specimes were collected on swabs, which were then transported to the laboratory in Cary Blair medium at 22°C and processed within 24 h.

Bacterial strains. The stool specimens were inoculated onto MacConkey agar plates and incubated at 37°C for 24 h. Three to five colonies of each sample, identified by biochemical assays (40, 41) as *E. coli*, were selected for subculture and hybridization with BFP, *eae* and EAF probes. Only *E. coli* strains that hybridized with at least one probe were further identified by serotyping and studied for virulence properties. Determination of the EPEC serogroups and the flagellar antigens was performed at the Instituto Adolfo Lutz, São Paulo, by agglutination tests using polyvalent and monovalent sera against O antigens O1 to O173 and flagelar H antigens (H1 to H56).

Prototype EPEC strain E2348/69 (serotype O127: H6), originally isolated from a nursery outbreak of gastroenteritis in Taunton, England, and which expressed BFP, EAF and intimin (28) was used as positive control in adherence to HEp-2 cells assays and in colony hybridization tests, and *E. coli* K12 HB101 as a negative control.

Colony hybridization assays. The BFP, *eae* and EAF probes were prepared by PCR amplification of the corresponding genes present in strain E2348/69. The following specific primers were used: EP1 (5'-CAA TGG TGC TTG CGC TTG CT-3') and EP2 (5'- GCC GCT TTA TCC AAC CTG GT-3') for the BFP probe (21), EPEC1 (5'- TCG TCA CAG TTG CAG GCC TGG T-3') and EPEC2 (5'-CCG AAG TCT TAT CAG CCG TAA AGT-3') for the *eae* probe (25), and EAF1 (5'- CAG GGT AAA AGA AAG ATG ATA A-3') and EAF2 (5'-TAT GGG GAC CAT GTA TTA TCA-3') for the EAF probe (10) as previously described (25). Labelling of the PCR-generated fragments was performed with a digoxigenin labelling kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions.

PCR-generated probes labelled with digoxigenin were used for colony hibridization. Filters were prepared and hybridized as previously described (25). Hybridization was visualized by chemioluminescent detection CSPD (Boehringer Mannheim GmbH, Germany)

Adherence assays. The test to detect adherence to HEp-2 cells was performed as described by Cravioto *et al.* (1979) (5). *E. coli* strains showing no adherence after a period of 3 h of incubation were submited to a 6 h adherence test (5). D-mannose (1% wt/vol) was present throughout the tests.

Detection of DNA sequences associated with enterotoxins and invasion by PCR. EPEC strains were grown in Luria Broth and DNA extracted by boiling. DNA sequences for LT and ST production and invasion were assayed by PCR using the specific primers: LT1 (5´- GCG ACA AAT TAT ACC GTG CT - 3´) and LT2 (5´- CCG AAT TCT GTT ATA TAT GT–3´) (42), STa1 (5´- CTG TAT TGT CTT TTT CAC CT –3´) and STa2 (5´- GCA CCC GGT ACA AGC AGG AT –3´) (42), INV1 (5´- GCT GGA AAA ACT CAG TGC CT–3´) and INV2 (5´- CCA GTC CGT AAA TTC ATT CT – 3´) (42). *E. coli* HB101(K12) was used as a negative control.

Immunoblotting. EPEC strains grown overnight in Dulbecco's modified Eagle's medium containing 2% bovine fetal serum, at 37°C, were centrifuged (10,000 x g, 5 min), and the cells were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (0.06M Tris-HCl, pH6.8; 2% SDS and 5% 2-mercaptoethanol). Samples were denatured by boiling for 5 min and the proteins were separated by 15% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes as described previously (43) and the membranes were blocked with PBS containing 0.5% (vol/vol) Tween 20 and 1% bovine serum albumin. The membranes were incubated with a BFP-specific rabbit antiserum (kindly provided by Jorge A. Girón) at a 1: 1,000 dilution, and bound antibody was detected with antiserum conjugated with peroxidase and enhanced chemioluminescence (Amersham International, Amersham, United Kingdom).

RESULTS

A total of 442 *E. coli* colonies isolated from 102 faecal samples from children with diarrhea and 177 *E. coli* colonies isolated from 46 controls were examined for the presence of EPEC virulence characteristics. Nineteen (18.6%) *E. coli* strains isolated from different faecal specimens from children with diarrhea hybridized with one or more of the EPEC probes tested. Of these, 11 (58%) strains, i.e. 2.5% of the total isolates, hybridized with all three probes, and eight strains hybridized with one or two probes (Table 1). From the controls, five (10.8%) strains isolated from five control cases hybridized with one or two probes, but none with all probes (Table 1).

The 11 *E. coli* strains that hybridized with the BFP, *eae* and EAF probes also adhered to HEp-2 cells, showing the LA pattern, and were classified as typical EPEC. Out of eight strains that hybridized with one or two probes, seven showed adherence in different patterns: the two strains presenting LAL (localized adherence like) and one strain presenting LA/DA (Fig.1) were classified as atypical EPEC, whereas the two strains showing DA and two strains showing AA were classified as diffusely adhering *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC), respectively (Table 1). Of the five *E. coli* isolated from controls, that hybridized with one or two probes, three strains adhered to HEp-2 cells showing DA and were classified as DAEC (Table 1). No strains showed DNA sequences associated with enterotoxins and invasion by PCR.

The typical EPEC strains belonged to serotypes O119: H6 (six strains), O111: H6 (one strain), and ONT: H7 (four strains; they were untypable for O1 to O173). The three atypical EPEC belonged to serotypes O26: H^-, O111: H9, and O119: HNT. The DAEC strains isolated from diarrheal cases belonged to serotypes O142: H34 and ONT: H11. The DAEC strains from controls belonged to serotypes O142: H34 (three strains) (Table 1).

Table 1. Relationship among the results of colony hybridization with 3 EPEC probes (*bfp*, *eae* and EAF) serotypes and adherence to HEp-2 cells of *E. coli* strains isolated from children with acute diarrhea and controls.

strains	Probes EAF	Probes BFP	Probes *eae*	Serotypes	Adherence 30 min + 3 h	Adherence 3 h + 3 h	*E. coli* classification
13.4	-	-	+	ONT: H11	DA	ND	DAEC
18.4	-	-	+	ONT: H33	NA	NA	-
29.1	+	+	+	O119: H6	LA	ND	typical EPEC
36.7	-	-	+	O26: H^-	NA	LAL	atypical EPEC
42.2	+	+	+	O119: H6	LA	ND	typical EPEC
43.1	+	+	+	O119: H6	LA	ND	typical EPEC
46.1	+	+	+	O119: H6	LA	ND	typical EPEC
48.2	+	+	+	O119: H6	LA	ND	typical EPEC
56.1	+	+	+	ONT: H7	LA	ND	typical EPEC
57.2	-	-	+	O111: H9	NA	LAL	atypical EPEC
64.1	+	+	+	ONT: H7	LA	ND	typical EPEC
66.2	+	+	+	ONT: H7	LA	ND	typical EPEC
71.1	+	+	+	O111: H6	LA	ND	typical EPEC
86.5	-	+*	+	ONT: HNT	AA	ND	EAEC
88.3	-	+*	+	O111: H6	AA	ND	EAEC
92.1	+	+	+	ONT: H7	LA	ND	typical EPEC
94.1	-	-	+	O142:H34	DA	ND	DAEC
95.1	+	+	+	O119:H6	LA	ND	typical EPEC
103.2	-	+*	+	O119:HNT	NA	LA/DA	atypical EPEC
C4.4	-	-	+	O142:H34	DA	ND	DAEC
C6.1	-	+*	-	ONT:H18	NA	NA	-
C7.1	-	+*	-	O157:H-	NA	NA	-
C21.4	+	-	+	O142:H34	DA	ND	DAEC
C22.3	-	-	+	O142:H34	DA	ND	DAEC

+: Hybridization with probes, -: no hybridization and no classical EPEC serogroups, H^-: nonmotile, NT: non-typable with antisera O1a O173, HNT: non-typable with antisera H1 to H56, ND: not determined, DA: diffuse adherence, LA: localized adherence, LAL: localized adherence like, LA/DA: localized/diffuse adherence, NA: non adherent, *: did not present the expression of fimbria BFP.

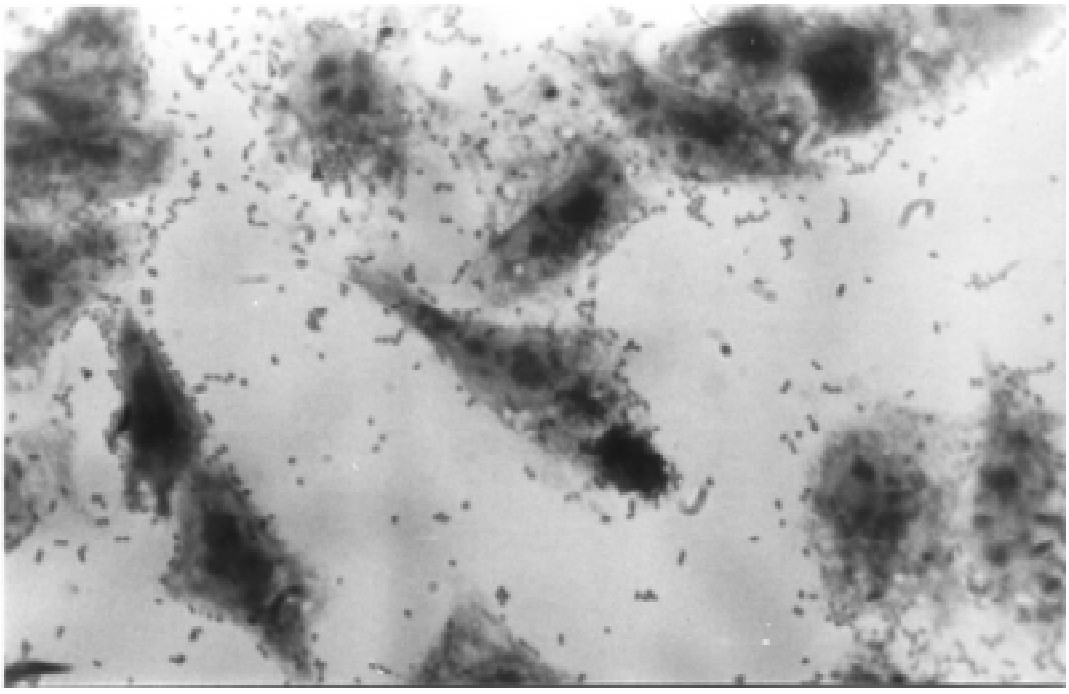

Figure 1. *E. coli* showing a LA/DA pattern of attachment to HEp-2 cells after incubation for 6 h.

Different virulence markers were found in the *E. coli* O111: H6, one EPEC strain with the LA pattern and hybridization with all probes, and one EAEC strain, that hybridized with *bfp* and *eae*, but did not hybridize with the EAF probe (Table 1).

Expression of *bfp* was tested by immunoblotting with antiserum against purified BFP, and all typical EPEC strains presented a protein band with an apparent molecular mass of 19.5 kDa corresponding to BFP. The EAEC strains (O111: H6 and ONT: HNT), the atypical EPEC strain (O119:HNT) and the control strains that hybridized with the *bfp* probe did not express BFP.

The most prevalent serotype, O119:H6, represented 43% of all EPEC (typical and atypical) and the four strains of typical EPEC untypable for O1 to O173 represented 29%, not belonging to classical EPEC serogroup.

The incidence of typical EPEC strains in the specimens used in this study was 11%, and considering all EPEC (typical and atypical) the incidence was 14%. The isolation of EPEC was found only in samples from cases of acute diarrhea, and no EPEC strain was isolated from controls. All typical EPEC strains were isolated from infants aged 0 to 9 months, and the atypical EPEC, DAEC and EAEC strains were isolated from children aged 9 months to 2 years.

DISCUSSION

In this work, strains classified as typical EPEC, which hybridized with the three probes, EAF, *bfp* and *eae*, and showed the LA pattern to HEp-2 cells, belonged to two classical serotypes, O119:H6 (six strains) and O111:H6 (one strain), and to unidentified serotype ONT: H7 (four strains). The identification of EPEC strains must not be based only on serotyping, but it is essential to use DNA probes to detect the *bfp* and *eae* genes (45).

O119:H6 has also been a prevalent EPEC serotype in São Paulo, Brazil, accounting for 6.2% of cases of diarrhea, and those bacteria were shown to carry EAF, *eae* and *bfp* DNA sequences (15, 44). This EPEC serotype was found in sporadic cases of infection in the UK, where it reacted with the same probes used in this study (39). The serotype O111:H6 has not been mentioned as a typical EPEC on previous works.

Atypical EPEC strains, recently described, hybridize with the *eae* probe but not with the EAF probe (23); they are found in several EPEC serotypes, but belong to different electrophoretic types (ETs) from those of typical EPEC (2, 19, 34, 36, 37).

In this work, three strains classified as atypical EPEC belonged to different serotypes: O26:H^-, O111:H9 and O119:HNT. Strains O26: H^- and O111: H9 hybridized only with the *eae* probe and showed LAL pattern in HEp-2 cells. These serotypes were also isolated in São Paulo-Brasil, USA and Switzerland as mentioned by Scaletsky *et al.* (38), who classified them as atypical EPEC strains, because the strains hybridized with the *eae* but not with the EAF and *bfp* probes, and showed the LAL pattern (15, 34). LAL pattern, characterized by delayed appearance and weaker density of the LA pattern, is probably

due to the lack of BFP production and can be mediated by intimin (34).

We found an atypical EPEC strain of serotype O119:HNT, that hybridized with the *bfp* and *eae* probes, but BFP was not expressed as verified by Western blott with anti-BFP polyclonal serum. This strain showed a LA/DA pattern in the 6 h assay (Fig 1).

Other categories of *E. coli* were found in strains isolated from children with diarrhoea. Two of them (ONT:HNT and O111:H6) were classified as EAEC due to the typical pattern observed in the adherence assay; they hybridized with the *eae* and *bfp* probes, but did not express BFP. Two strains (ONT:H11 and O142:H34) were classified as DAEC and hybridized with the *eae* probe. Three strains classified as DAEC (O142:H34) were also found in controls; these strains hybridized with *eae* probe and one of them also hybridized with the EAF probe. These results suggest that DAEC strains harbouring *eae* genes were not associated with diarrhea. Other categories of *E. coli*, such as EAEC, enterohaemorrhagic *E. coli* (EHEC) and DAEC, showing various combinations of virulence genes and belonging to different classical EPEC serogroups (11, 17, 36, 39, 44) were also observed.

Epidemiological data of EPEC diarrhea indicates that case/control studies generally show a significant difference in isolation rates of EPEC when infants less than 12 months of age are examined (27). Also, EPEC are more frequent in newborns with diarrhea mainly in those younger than 6 months (6, 8, 14). In this work, EPEC strains were isolated more often from patients (14%) than from controls (0%), and the frequency of isolation of EPEC decreased as group age increased. The typical EPEC strains were isolated from infants aged 0 to 9 months, in contrast to the atypical EPEC, DAEC and EAEC strains that were isolated from infants 9-months to 2-years-old.

EPEC is the most important cause of acute childhood diarrhea in several large urban centers in Brazil, with detection rates of 26% in São Paulo (14), 17.1% in Rio de Janeiro (35), and 37% in Rio Grande do Sul (3). In this study, EPEC (typical and atypical) was isolated in 14% of children with diarrhea in Londrina. Similar results were found in other developing countries like Bangladesh - 15.5% (1), Chile - 19% (26), and Mexico- 19% (4).

The most prevalent serogroup in Londrina (O119) has also been isolated in other parts of Brazil (9, 14), and elsewhere in the world (4, 26, 39). However, serogroup O55 commonly isolated in São Paulo, Brazil (14), Mexico (4), Chile (29) and Venezuela (20), was not detected in Londrina.

In this study, the prevalence of EPEC associated with infant diarrhea was assessed by means of molecular probes of putative virulence traits of EPEC, and typical EPEC strains, not belonging to classical serogroups/or serotypes, were found in children in Paraná-Brazil.

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RESUMO

Identificação molecular de *Escherichia coli* enteropatogênica (EPEC), associada com diarréia infantil, em Londrina, Paraná-Brazil

A prevalência de *Escherichia coli* enteropatogênica (EPEC) em crianças em Londrina-PR, Brazil, foi avaliada através de sondas de DNA marcadas com digoxigenina, as quais identificam o plasmidio EAF (EPEC adherence factor) e os genes de virulência para EPEC, *bfp* (bundle-forming pilus) e *eae* (E. coli attaching and effacing). As amostras de *E. coli* foram também sorotipadas e testadas para a aderência às células HEp-2. Das 102 crianças com diarréia, 19 colonias de *E. coli* hibridizaram com pelo menos uma sonda, e destas, 11 foram identificadas como EPEC típica pois hibridizaram com as 3 sondas testadas. Todas as EPEC típicas apresentaram o padrão de aderência localizada e não apresentaram os genes para enterotoxinas (ST e LT) e invasão por PCR. Seis EPEC típica (43%) pertenceram ao sorotipo clássico O119:H6, uma ao sorotipo O111:H6 e em quatro amostras (29%) o antígeno O não foi determinado com os soros contra O1-O173 (ONT:H7). Três amostras foram classificadas como EPEC atípicas: O26H$^-$, O111:H9 e O119:HNT. *E. coli* O26H$^-$ e O111:H9 hibridizaram somente com *eae* e mostraram padrão de aderência localizada "like" (LAL); O119:HNT hibridizou com as sondas *bfp* e *eae*, e mostrou padrão de aderência localizada/difusa (LA/DA) após 6 h. O padrão DA foi observado em duas amostras (ONT:H11 e O142:H34) isoladas de crianças com diarréia, as quais hibridizaram com *eae*. Dos 46 controles, cinco colônias hibridizaram com pelo menos uma sonda, mas nenhuma hibridizou com as 3 sondas testadas ou apresentou LA. Três amostras com padrão DA hibridizaram com sonda *eae*. Nenhuma EPEC com sorotipo clássico foi encontrada nas fezes de crianças controle.

Palavras-chave: *Escherichia coli* enteropatogênica, EPEC, sondas de DNA, fatores de virulência, sorotipos, diarréia.

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