Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from children with and without diarrhoea in Londrina, Brazil

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Attaching and effacing *Escherichia coli* (AEEC) have been implicated in diarrhoea in humans in several countries. A total of 919 *E. coli* strains, isolated from 125 children with diarrhoea and 98 without diarrhoea, was investigated by PCR for the presence of the EAF, *bfp*, *eae* and *stx* genes. Thirty-four of these isolates were found to carry the *eae* gene; they were isolated from 27 (79.4 %) children with diarrhoea and seven (20.6 %) controls, in the city of Londrina, Brazil. These strains were investigated for their genotypic and phenotypic characteristics. Different genetic profiles were observed; strains containing the *eae* gene alone were most common (47.1 %). The characteristic genetic profile of typical enteropathogenic *E. coli* (EPEC), *eae*, *bfp* and EAF, was only found in isolates from children with diarrhoea. The *stx* gene was not detected in any of the 34 strains studied. Ten (29.4 %) strains were negative in the fluorescent actin-staining test. Localized adhesion (LA) was the most common pattern of adhesion (44.1 %), followed by the aggregative adhesion (AA) (23.5 %) and localized adhesion-like (LAL) (14.7 %) patterns. The results showed a strong association between strains presenting the LA pattern and diarrhoea. Forty-seven per cent of the strains studied belonged to classical O-serogroups of EPEC. The most common serotype found was O119:H6; these isolates all showed the LA pattern, were positive for fluorescent actin-staining and were associated with diarrhoea. Intimin β was detected in seven strains, four of which belonged to serotype O119:H6 and three to serotype O119:H7; all were associated with diarrhoea. On the other hand, intimin ϵ was detected in two strains of serotype O111:H38 and one of serotype O119:H7, isolated from children without diarrhoea. To our knowledge, this is the first report of the occurrence of intimin ϵ in strains of *E. coli* isolated from humans in Brazil.

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INTRODUCTION

Attaching and effacing *Escherichia coli* (AEEC) are characterized by their ability to cause intestinal lesions, known as attaching and effacing (AE) lesions. These are characterized by intimate attachment of the bacterium to the host cell, the effacement of enterocyte microvilli and formation of pedestal-like structures (Knutton *et al.*, 1989).

The genes necessary for AE lesion formation are located on

Abbreviations: AA, aggregative adhesion; AEEC, attaching and effacing *E. coli*; DA, diffuse adhesion; EAF, EPEC adherence factor; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; FAS, fluorescent actin-staining; IS, isolated bacteria; LA, localized adhesion; LAL, localized adhesion-like; LEE, locus of enterocyte effacement; NA, non-adherent.

the bacterial chromosome, forming a pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). The LEE contains genes encoding a type-III secretion system (*sep* and *esc*), a number of secreted proteins [EspA, EspD, EspB and Tir (translocated intimin receptor)] and intimin (*eae*), a 94 kDa outer-membrane protein that mediates the intimate adhesion between the bacterium and the epithelial cell membrane (Kenny *et al.*, 1997; Nataro & Kaper, 1998). The AE lesion can be observed by the fluorescent actin-staining (FAS) test; it gives rise to intense fluorescence of filamentous actin, which can be observed in the host cell beneath the site of bacterial attachment (Knutton *et al.*, 1989).

Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* are often grouped together as AEEC. In addition to

their ability to induce AE lesions, EPEC possess the large EPEC adherence factor (EAF) plasmid and the cluster of genes that encodes bundle-forming pili (BFP) (Baldini *et al.*, 1983; Girón *et al.*, 1991). EPEC are a common cause of severe infantile diarrhoea, particularly in developing countries (Nataro & Kaper, 1998). These strains are characterized by a pattern of adhesion to epithelial cells called localized adhesion (LA), and are found among 12 different O-serogroups (O26, O55, O86, O111, O114, O119, O125–O128, O142 and O158) (Scaletsky *et al.*, 1984).

EPEC strains carrying the *eae* gene, but lacking the EAF plasmid and *stx* (Shiga toxin) genes, were described as atypical EPEC (Kaper, 1996) at the Second International Symposium on EPEC (São Paulo, Brazil, 1995). Atypical EPEC strains were found in several EPEC O-serogroups, but belonged to serotypes different from those of typical EPEC (Pelayo *et al.*, 1999). These strains present an adhesion pattern called localized adhesion-like (LAL), characterized by the presence of less compact microcolonies or clusters of bacteria in a few cells (Scaletsky *et al.*, 1996, 1999).

EHEC are typically characterized by the ability to cause the AE lesion, possession of a 60 MDa plasmid encoding enterohaemolysin and production of the Shiga toxins (Stx1 and/or Stx2), which are responsible for the major symptoms of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Nataro & Kaper, 1998). Serotype O157:H7 is more frequently associated with disease in humans and animals, but other serotypes, such as O26:H11, O111:H[−], O111:H8, O103:H2, O118:H[−] and O118:H16, have also been implicated (Nataro & Kaper, 1998). In a recent study performed in São Paulo, Brazil, Vieira *et al.* (2001) identified *E. coli* associated with infantile diarrhoea that belonged to non-EPEC serogroups, carried the *eae* gene and lacked the EAF and *stx* probe sequences.

The *eae* gene has been cloned and sequenced from several EPEC and EHEC strains isolated from humans and animals, RDEC (a rabbit EPEC-like *E. coli* strain), *Citrobacter freundii* and *Hafnia alvei* (Jerse *et al.*, 1990; Beebakhee *et al.*, 1992; Frankel *et al.*, 1994; Agin & Wolf, 1997). These genes possess a highly conserved N-terminal region, whereas the C-terminal region shows much less similarity (Frankel *et al.*, 1994). Frankel *et al.* (1995) have demonstrated that the binding activity of intimin is located in the C-terminal region.

Adu-Bobie *et al.* (1998) used PCR and antiserum against the C-terminal region of intimin to investigate the antigenic variation in this region, and identified four distinct intimin

types: intimins α , β , γ and δ . More recently, Oswald *et al.* (2000) characterized a new intimin, referred to as intimin ϵ , which was present in human and bovine EHEC strains.

The aim of this study was to determine the genotypic and phenotypic characteristics of AEPEC isolated from children with and without diarrhoea in Londrina, Brazil. The investigation has been prompted by the lack of data concerning diarrhoea caused by these micro-organisms in children from this region, and by the need for better understanding of their enteropathogenic potential.

METHODS

Bacterial strains. A total of 919 *E. coli* isolates was examined in this study; 524 of these were selected from 125 children with symptoms of gastrointestinal disorders (patients) and 395 from 98 children without diarrhoea (controls). The population for analysis was selected from children attending the emergency room of public hospitals (patients), day-care centres and a clinical laboratory (controls) in Londrina, in the north of Paraná state, Brazil. Strains were isolated between February 1995 and May 1996, and between July 1998 and January 1999, on MacConkey agar plates (Biobrás, Brazil). From each faecal sample, five lactose-fermenting colonies and one non-lactose-fermenting colony were identified by biochemical tests (Edwards & Ewing, 1972). *E. coli* strains were stored at −70 °C in tryptic soy broth (Biobrás) with 15 % (v/v) glycerol. Bacterial strains used as positive controls in this study are listed in Table 1. Strain HB101 (*E. coli* K-12) was used as the negative control in all tests.

PCR. All 919 *E. coli* isolates were screened for the presence of the genetic sequences of EAF, *bfp*, *eae* and *stx*. Strains positive for *eae* by PCR were analysed further to identify the intimin type present. Table 2 shows the primer sequences, sizes of amplified DNA fragments and amplification conditions for all sequences studied. Amplification reactions were performed as follows: cultures on tryptic soy agar (Difco) were suspended in 300 µl Milli-Q water (Millipore) and boiled for 10 min to release and denature the bacterial DNA. Amplification of bacterial DNA was carried out in volumes of 25 µl, containing 10 µl bacterial lysate, 200 µM dNTPs, 1.5 mM MgCl₂, 20 pmol each primer and 1.5 U *Taq* DNA polymerase (all Gibco-BRL). After amplification, products were electrophoresed in 1 % agarose gel (Difco), stained with ethidium bromide (Gibco-BRL) and visualized using UV light.

HEp-2 cell adherence assay. The *eae*-positive strains were tested for adhesion to HEp-2 cells, as described by Cravioto *et al.* (1979). When the adhesion pattern was not defined after 3 h incubation, the test was repeated with a 6 h incubation period.

DNA hybridization. *E. coli* strains carrying *eae* and displaying an aggregative adhesion (AA) pattern were tested for hybridization with the specific DNA probe EAEC (enteroaggregative *E. coli* adherence

Table 1. Bacterial strains used as positive controls in this study

Strain	Description	Reference
E2348/69	Human EPEC O127:H6	Jerse <i>et al.</i> (1990)
EDL933	Human EHEC O157:H7	Yu & Kaper (1992)
H19	Human EHEC O26:H11	Agin & Wolf (1997)
PMK5	Human EHEC O103:H2	Oswald <i>et al.</i> (2000)

Table 2. Primer sequences, sizes of amplified DNA fragments and amplification conditions used in this study

Target	Primer sequence (5'→3')		Amplified fragment size (bp)	Amplification conditions	Cycles (n)	Control <i>E. coli</i> strains positive	Reference
	Forward	Reverse					
<i>eae</i>	TCGTCACAGTTGCAGGCCTGGT	CCGAAGTCTTATCAGCCGTAAAGT	1100	94 °C (1 min), 60 °C (1 min), 72 °C (1 min)	30	E2348/69	Louie <i>et al.</i> (1993)
EAF	CAGGGTAAAAGAAAGATGATAA	TATGGGGACCATGTATTATCA	397	94 °C (1 min), 56 °C (2 min), 72 °C (1 min)	30	E2348/69	Franke <i>et al.</i> (1994)
<i>bfp</i>	CAATGGTGTCTTGCCTTGCT	GCCGCTTTATCCAACCTGGT	326	94 °C (1 min), 60 °C (1 min), 72 °C (1 min)	30	E2348/69	Gunzberg <i>et al.</i> (1995)
<i>stx</i>	CAACGAAATAATTTATATGT	TTTGATTGTTACAGTCAT	887	94 °C (1 min), 45 °C (90 s), 72 °C (90 s)	39	EDL933	Lin <i>et al.</i> (1993)
Intimin gene typing:							
α	CCCGAATTCGGCACAAGCATAAGC	CCCGAATTCTTATTTACACAAGTGGC	2807	94 °C (45 s), 48 °C (1 min), 72 °C (150 s)	30	E2348/69	Oswald <i>et al.</i> (2000)
β	CCCGAATTCGGCACAAGCATAAGC	CCCGTGATACCAGTACCAATTACGGTC	2287	94 °C (45 s), 59 °C (1 min), 72 °C (150 s)	30	H19	Oswald <i>et al.</i> (2000)
γ	CCCGAATTCGGCACAAGCATAAGC	CCCGAATTCTTATTCTACACAAACCGC	2792	94 °C (45 s), 59 °C (1 min), 72 °C (150 s)	30	EDL933	Oswald <i>et al.</i> (2000)
ϵ	CCCGAATTCGGCACAAGCATAAGC	AGCTCACTCGTAGATGACGGCAAGCG	2608	94 °C (45 s), 65 °C (1 min), 72 °C (150 s)	30	PMK5	Oswald <i>et al.</i> (2000)

plasmid), a 1 kb *EcoRI*–*PstI* fragment of pCVD432 (Baudry *et al.*, 1990) labelled with [α - 32 P]dATP, in a colony hybridization assay.

FAS test. The FAS test, which demonstrates the presence of filamentous actin beneath attached bacteria, was performed as described by Knutton *et al.* (1989). The strains were tested after 3 and 6 h incubation.

Haemolysin assay. All *eae*-positive strains were tested for production of haemolysin as described by Beutin (1991). The strains were cultivated on blood agar base (Difco), supplemented with 10 mM CaCl₂ and 5 % defibrinated sheep erythrocytes washed three times in PBS. Plates were examined for zones of haemolysis around bacterial growth after 3 and 24 h incubation at 37 °C.

Serotyping. Identification of somatic (O) and flagellar (H) antigens was performed in all *eae*-positive strains in the Enteric section of Instituto Adolfo Lutz, São Paulo, with specific antisera O1–O173 and H1–H52.

RESULTS AND DISCUSSION

AEEC are characterized by their ability to induce the AE lesion in the intestine; this process is mediated by an outer-membrane protein encoded by the *eae* gene. This category of *E. coli* has been implicated in diarrhoea in humans and animals in several countries (Pelayo *et al.*, 1999; Aidar *et al.*, 2000; Vieira *et al.*, 2001).

In this study, of 919 *E. coli* strains isolated from 125 children with diarrhoea and 98 without diarrhoea, 34 strains possessed the *eae* gene, either alone or associated with the other genes investigated. Twenty-seven of these strains (79.4 %) were isolated from cases of diarrhoea and seven (20.6 %) from controls. A summary of the genotypic and phenotypic characteristics of these strains is shown in Table 3.

Four different genetic profiles were found (Table 3). Strains

Table 3. Genotypic and phenotypic characteristics of *E. coli* strains possessing the *eae* gene

Genetic profile	Type of adhesion	FAS reactivity	Intimin type	Serotype	Origin
<i>eae</i>	LAL	+	NT	ONT:H11	P
<i>eae</i>	LAL	+	NT	ONT:H19	P
<i>eae</i>	LAL	+	NT	O86:H8	P
<i>eae</i>	LAL	—	NT	O78:H49	P
<i>eae</i>	AA	+	NT	ONT:H19	P
<i>eae</i>	AA	—	NT	O24:H40	P
<i>eae</i>	AA	+	NT	O26:H [—]	P
<i>eae</i>	AA	—	NT	O111:H9	P
<i>eae</i>	AA	—	NT	O114:HNT	P
<i>eae</i>	DA	—	NT	O12:H [—]	P
<i>eae</i>	NA	—	NT	O1:H11	P
<i>eae</i>	AA	+	ε	O111:H38	C
<i>eae</i>	LAL	+	ε	ONT:H19	C
<i>eae</i>	IS	+	ε	O111:H38	C
<i>eae</i>	IS	—	NT	O127:H40	C
<i>eae</i>	NA	—	NT	ONT:H7	C
<i>eae</i> , EAF	AA	—	NT	ONT:H [—]	P
<i>eae</i> , EAF	LA	+	NT	O142:H34	C
<i>eae</i> , <i>bfp</i>	LA	+	NT	O107:H [—]	P
<i>eae</i> , <i>bfp</i>	LA	+	NT	O107:H [—]	P
<i>eae</i> , <i>bfp</i>	LA	+	NT	O111:H6	P
<i>eae</i> , <i>bfp</i>	LA	+	NT	O119:H2	P
<i>eae</i> , <i>bfp</i>	LA	+	β	O119:H6	P
<i>eae</i> , <i>bfp</i>	IS	—	NT	ONT:HNT	P
<i>eae</i> , <i>bfp</i>	AA	+	NT	O148:H28	C
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	ONT:H7	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	ONT:H7	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	ONT:H7	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	NT	ONT:H7	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	NT	O111:H6	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	O119:H6	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	O119:H6	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	NT	O119:H6	P
<i>eae</i> , <i>bfp</i> , EAF	LA	+	β	O119:H6	P

Abbreviations: NT, not typable; P, patient; C, control.

possessing the *eae* gene alone (47.1 %) were most frequent, and were found in children both with and without gastro-intestinal disease. In contrast, the characteristic genetic profile of typical EPEC (*eae*, *bfp* and EAF) was found only in children with diarrhoea.

The *stx* gene was not detected in any of the 34 strains identified as *eae*-positive. In Brazil, the incidence of EHEC in humans is very low in contrast with that of EPEC (Giraldi *et al.*, 1990; Gomes *et al.*, 1991; Saridakis, 1994). In fact, it is possible that EPEC infections in early childhood confer cross-reactive protective immunity against EHEC types that share common antigens (such as LPS and intimin) with classical EPEC strains (Beutin *et al.*, 1998). This could explain why human infections with *eae*-positive EHEC occur less frequently in developing countries where infections of children by EPEC are common.

Five distinct adhesion patterns were observed (Table 3): LA, AA, LAL, DA (diffuse adhesion) and IS (isolated bacteria). Two strains were non-adherent (NA). LA was the most frequent pattern of adhesion (44.1 %), followed by AA (23.5 %), LAL (14.7 %), IS (8.8 %) and DA (2.9 %). Strains with AA, LAL and IS were found in children both with and without diarrhoea. The only strain that presented the DA pattern was isolated from a child without diarrhoea. All strains that presented the genetic profile of typical EPEC showed the LA pattern. Our results show a strong association between strains that presented the LA pattern and diarrhoea (one from the control group and 14 from patients). The strains that displayed the AA pattern did not hybridize with the EAEC probe.

Of all the *eae*-positive strains, 10 (29.4 %) were FAS-negative after incubation for 3 or 6 h. The majority of the *eae*-positive, FAS-negative strains possessed the *eae* gene alone (80 %). Vieira *et al.* (2001) studied 59 *E. coli* strains that carried *eae* but lacked the EAF plasmid probe sequence and the *stx* genes, and found that 25.9 % of the strains were FAS-negative. It is possible that *eae*-positive, FAS-negative strains possess some deficiency in expression of the genes encoding the type-III secretion system proteins and/or the secreted LEE proteins. Two *eae*-positive, FAS-negative strains were unable to attach to HEp-2 cells. According to Cantey & Moseley (1991), non-adherent, *eae*-positive strains can possess adhesins capable of recognizing the intestinal mucosal surface, but not the cultured cells used in laboratory assays, for adhesion.

Fourteen different serogroups were detected. Forty-seven per cent of the strains belonged to classical O-serogroups of EPEC (O26, O86, O111, O114, O119, O127 and O142). The most frequent serotype found in our study was O119:H6 (14.7 %). All strains of serotype O119:H6 presented the LA pattern, were FAS-positive and were isolated from children with diarrhoea. Eleven strains were non-O-typable, and two were non-H-typable.

Adu-Bobie *et al.* (1998) revealed the existence of four distinct intimin types: intimins α , β , γ and ϵ . Oswald *et al.* (2000) typed the intimin of the serotype O86:H34 EPEC strain

ICC95 as type β , although Adu-Bobie *et al.* (1998) had previously classified this intimin as type δ . Restriction analysis of the PCR product obtained from strain ICC95 with β -specific primers as described by Oswald *et al.* (2000) showed a digestion profile, termed β_2 , that differed from the β_1 pattern shared by all other intimin β -positive strains. According to Oswald *et al.* (2000), these data confirm that there are differences between the *eae* DNA sequences of serogroup O86 EPEC and the other β -type strains, and indicate that the β_2 subtype corresponds to the δ type of Adu-Bobie *et al.* (1998). Oswald *et al.* (2000) also revealed the existence of a new intimin type, referred to as intimin ϵ .

It is interesting to note that intimin β was detected in seven strains in this work, four of which belong to serotype O119:H6 (a classical EPEC serotype), and all of which were isolated from children with diarrhoea. According to the literature, intimin β is the most common subtype among human strains of *E. coli*, and it seems that it is also the most common in bovine *E. coli* (Aidar *et al.*, 2000; Vieira *et al.*, 2001). On the other hand, intimin ϵ was found in two strains of serotype O111:H38 and one of serotype ONT:H19, isolated from children without diarrhoea. Oswald *et al.* (2000) detected intimin ϵ in human and bovine EHEC strains of serogroups O8, O11, O45, O103, O121 and O165. To our knowledge, this is the first report of the occurrence of intimin ϵ in strains isolated from humans in Brazil and the first report of *eae*⁺, *stx*⁻ strains in the literature. The majority of strains studied (70.6 %) produced an intimin that could not be typed.

Haemolysin expression on blood agar was observed for only five strains (four after 24 h incubation and one after 3 h), all of which were isolated from cases of diarrhoea. The low frequency of haemolysin expression among the *eae*-positive strains did not support a major role in enteropathogenicity.

The results obtained in this study indicate that AECC are a heterogeneous group of organisms. However, typical EPEC continue to be important pathogens associated with diarrhoea in Brazilian children. EAF⁻, *bfp*⁻, *eae*⁺ *E. coli* strains have the potential, as a new enteropathogenic group, to become an important cause of diarrhoea in children.

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