CLINICAL MICROBIOLOGY

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

MARILDA C. VIDOTTO, RENATA K. T. KOBAYASHI and ANGELA M. G. DIAS*

Universidade Estadual de Londrina, Departamento de Microbiologia, Campus Universitário, Caixa Postal 6001, 86051-970, Londrina, PR and *Instituto Adolfo Lutz, São Paulo, SP, Brazil

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 entero-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 bacterium 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.

Received 25 June 1999; revised version accepted 26 Jan. 2000.

Corresponding author: Dr M. C. Vidotto (e-mail: macarlos@uel.br).

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 TTG CT-3') and EP2 (5'-GCC GCT TTA TCC AAC CTG GT-3') for the *bfp* probe [10], 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 [14], 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 *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'-GCG ACA AAT TAT ACC GTG CT-3') and LT2 (5'-CCG AAT TCT GTT ATA TAT GT-3'), STa1 (5'-CTG TAT TGT CTT TTT CAC CT-3') and STa2 (5'-GCA 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 pRP4 (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 untypable 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 *eaf*, *bfp* and *eaeA* probes, serotypes and adherence to HEp-2 cells of *E. coli* isolates from infants with acute diarrhoea

Isolate no.	Hybridisation with probes			Serotype	Adherence	<i>E. coli</i> classification
	<i>eaf</i>	<i>bfp</i>	<i>eaeA</i>			
29.1	+	+	+	O119: H6	LA	Typical EPEC
42.2	+	+	+	O119: H6	LA	Typical EPEC
43.1	+	+	+	O119: H6	LA	Typical EPEC
46.1	+	+	+	O119: H6	LA	Typical EPEC
48.2	+	+	+	O119: H6	LA	Typical EPEC
56.1	+	+	+	ONT: H7	LA	Typical EPEC
64.1	+	+	+	ONT: H7	LA	Typical EPEC
66.2	+	+	+	ONT: H7	LA	Typical EPEC
71.1	+	+	+	O111: H6	LA	Typical EPEC
92.1	+	+	+	ONT: H7	LA	Typical EPEC
95.1	+	+	+	O119: H6	LA	Typical EPEC

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

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).

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 *bfpA* gene, which encodes the pilin 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 serotyping 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 *bfpA* 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.

We thank Dr L. C. J. Gaziri for revising the manuscript, R. E. Linhares for cell culture and M. R. Perugini for help with strain isolation.

References

1. Gomes TAT, Rassi V, MacDonald KL *et al.* Enteropathogens associated with acute diarrheal disease in urban infants in São Paulo, Brazil. *J Infect Dis* 1991; **164**: 331–337.
2. Cravioto A, Molina J, Manjarrez A, Eslava C. Enteropatho-

- genic *Escherichia coli*: the Mexican experience. *Rev Microbiol* 1996; **27** Suppl 1: 21–24.
3. Levine MM, Prado V, Lior H, Lagos R. Epidemiologic studies of diarrhea associated with enteropathogenic *Escherichia coli* (EPEC) in infants and young children in Santiago, Chile. *Rev Microbiol* 1996; **27** Suppl 1: 40–44.
 4. Donnenberg MS, Kaper JB, Finlay BB. Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol* 1997; **5**: 109–114.
 5. Knutton S, Rosenshine I, Pallen MJ *et al.* A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* 1998; **17**: 2166–2176.
 6. Donnenberg MS, Girón JA, Nataro JP, Kaper JB. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. *Mol Microbiol* 1992; **6**: 3427–3437.
 7. Jerse AE, Kaper JB. The *eaeA* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. *Infect Immun* 1991; **59**: 4302–4309.
 8. Levine MM, Prado V, Robins-Browne R *et al.* Use of DNA probes and Hep-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. *J Infect Dis* 1988; **158**: 224–228.
 9. Nataro JP. Molecular diagnosis of EPEC. *Rev Microbiol* 1996; **27** Suppl 1: 54–57.
 10. Gunzburg ST, Tornieporth NG, Riley LW. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *J Clin Microbiol* 1995; **33**: 1375–1377.
 11. Tornieporth NG, John J, Salgado K *et al.* Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. *J Clin Microbiol* 1995; **33**: 1371–1374.
 12. Trabulsi LR, Campos LC, Whittam TS, Gomes TAT. Traditional and non-traditional enteropathogenic *Escherichia coli* serogroups. *Rev Microbiol* 1996; **27** Suppl 1: 1–6.
 13. Levine MM, Nataro JP, Karch H *et al.* The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. *J Infect Dis* 1985; **152**: 550–559.
 14. Franke J, Franke S, Schmidt H *et al.* Nucleotide sequence analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor probe and development of PCR for rapid detection of EPEC harboring virulence plasmids. *J Clin Microbiol* 1994; **32**: 2460–2463.
 15. Kobayashi RKT, Vidotto MC. Digoxigenin-labelled probe for the detection of enteropathogenic *Escherichia coli*. *J Microbiol Methods* 1998; **31**: 201–204.
 16. Scaletsky IC, Silva MLM, Toledo MRF *et al.* Correlation between adherence to HeLa cells and serogroups, serotypes, and bioserotypes of *Escherichia coli*. *Infect Immun* 1985; **49**: 528–532.
 17. Kado C, Liu ST. Rapid procedure for the detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365–1373.
 18. Nataro JP, Scaletsky ICA, Kaper JB, Levine MM, Trabulsi LR. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. *Infect Immun* 1985; **48**: 378–383.