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Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children

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Abstract

A multiplex PCR to differentiate typical and atypical enteropathogenic *Escherichia coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic (ETEC), enteroinvasive *E. coli* (EIEC) and Shiga toxin-producing *E. coli* (STEC) strains was developed and evaluated. The targets selected for each group were *eae* and *bfpA* for EPEC, *aggR* for EAEC, *elt* and *est* for ETEC, *ipaH* for EIEC and *stx* for STEC isolates. This PCR was specific and sensitive for rapid detection of target isolates in stools. Among 79 children with acute diarrhea, this technique identified 13 (16.4%) with atypical EPEC, four (5%) with EAEC, three (3.8%) with typical EPEC, one (1.3%) with ETEC and one (1.3%) with EIEC.

Introduction

Diarrhea remains an important public health problem for children of low socio-economic status in Brazil (Trabulsi *et al.*, 1985). In an etiologic study of acute diarrhea in children less than 2 years of age from different regions of Brazil, Scaletsky *et al.* (2002a, b) showed that diarrheagenic *Escherichia coli* organisms have an important role as a cause of enteric diseases. However, these pathogens are probably underestimated due to inappropriate diagnostic methods in clinical practice.

Diarrheagenic *E. coli* can be divided into five main categories on the basis of distinct epidemiological and clinical features, specific virulence determinants and an association with certain serotypes (Nataro & Kaper, 1998). The most commonly reported diarrheagenic *E. coli* strains in Brazilian children are enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC). EPEC harbor the 'locus of enterocyte effacement' (LEE) pathogenicity island, which encodes factors responsible for the attaching and effacing (A/E) phenotype on host enterocytes (Jerse *et al.*, 1990). These EPEC strains can also harbor the EPEC adherence plasmid (EAF) comprising the cluster of genes encoding the bundle-forming pilus (BFP) (Girón *et al.*, 1993). EPEC strains with the EAF plasmid are classified as 'typical EPEC', whereas EPEC strains that do not possess the EAF plasmid are classified as 'atypical EPEC' (Kaper, 1996). EAEC are characterized by an aggregative adherence (AA) pattern on cultured epithelial cells, and produce fimbrial colonization factors called aggregative adherence factors (AAFs). The three other categories seem to be less prevalent: enterotoxigenic *E. coli* (ETEC), which produces the heat labile (LT) and/or heat-stable (ST) enterotoxins; enteroinvasive *E. coli* (EIEC) invades the colonic epithelium; and Shiga toxin-producing *E. coli* (STEC), which produces Shiga toxins 1 and 2 (Stx 1 and Stx 2) and in some strains the presence of the LEE region (Nataro & Kaper, 1998).

Identification of diarrheagenic *E. coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. Serotypic markers correlate, sometimes very closely, with specific categories of diarrheagenic *E. coli*; however, these markers are rarely sufficient in themselves to identify strains reliably as diarrheagenic. Thus, the detection of diarrheagenic *E. coli* has focused increasingly on the identification of certain characteristics that themselves determine the virulence of these organisms. This identification process may include HEp-2 cell adherence, DNA hybridization and PCR assays to detect the presence of

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specific virulence traits or the genes encoding these traits. The first two types of assays require special expertise, use cell culture and radioactive material and are time-consuming.

Considering the importance of diarrheagenic *E. coli* as a cause of childhood diarrhea in Brazil, we previously designed two multiplex PCRs to detect EPEC, ETEC, EIEC, STEC and EAEC, in stool samples (Aranda *et al.*, 2004). In order to simplify diagnosis, we set up a single multiplex PCR assay by combining seven primer pairs to detect these types of *E. coli* strains simultaneously in a single reaction. The present study was undertaken to evaluate the application of this new assay to categorized pathogenic *E. coli* isolates and determine their distribution among children with and without diarrhea.

Materials and methods

Clinical specimens

From 1 January to 31 March 2005, all children under 5 years of age with acute diarrhea who were brought to the emergency room of Hospital São Paulo, Monday through Friday, were enrolled in the study. Every fecal specimen was examined by standard methods for the presence of *Shigella* spp. *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter* spp. and rotavirus. Four separate lactose-fermenting colonies and two nonlactose-fermenting colonies from each child were selected from MacConkey agar plates for testing. A total of 277 *E. coli* isolates were obtained from the 79 children. In addition, 210 *E. coli* isolates from 60 children without diarrhea were tested as controls. In total, 487 *E. coli* isolates were individually tested by the multiplex PCR assay and also screened by colony hybridization with specific DNA probes designed to detect EPEC (*eae* and *bfpA* probes), EAEC (CVD432 probe), ETEC (LT and ST probes), EIEC (Inv probe) and STEC (*stx1* and *stx2* probes) (Nataro & Kaper, 1998). These probes were labeled with [α-^{32}P]dCTP, and colony hybridization assays were performed as described previously (Scaletsky *et al.*, 2002a).

Bacterial strains

Diarrheagenic *E. coli* reference strains used as positive controls in the PCR assays included the EPEC E2348/69 (*eae*, *bfpA*), EAEC O42 (*aggR*), ETEC H10407 (*elt*, *est*), EIEC EDL1284 (*ipaH*) and EHEC EDL931 (*eae, stx1*, *stx2*) strains. The nonpathogenic *E. coli* K12 DH5αstrain was used as a negative control. We used 330 additional reference strains obtained from our laboratory collection to evaluate the multiplex PCR assay. These included 110 nondiarrheagenic *E. coli* isolates and 220 diarrheagenic *E. coli* isolates, divided into 50 EAEC, 50 atypical EPEC, 50 typical EPEC, 50 ETEC and 20 EIEC. We also included 115 *E. coli* and 17 *Shigella* species strains isolated from 150 diarrheic patients in a previous study (Aranda *et al.*, 2004).

Preparation of DNA templates for PCR

All strains examined by PCR were grown on MacConkey agar plates at 37 °C. DNA was extracted from bacteria by resuspending one bacterial colony in 50 μL of sterile water, boiling the suspension for 5 min and centrifuging it at 10 000 ***g*** for 1 min. The supernatant was then used as the DNA template for PCR.

Development of multiplex PCR assay

The DNA templates were subjected to multiplex PCR with specific primers for the detection of the following virulence markers: *eae* (structural gene for intimin of EPEC and EHEC), *bfpA* (structural gene for the BFP of typical EPEC), *aggR* (transcriptional activator for the AAFs of EAEC), *elt* and *est* (enterotoxins of ETEC), *ipaH* (invasion plasmid antigen H found in EIEC and *Shigella*), and *stx* (Shiga toxins 1, 2 and variants). Primers for *stx* and *aggR* were previously described by Toma *et al.* (2003), and those for *eae*, *bfpA*, *elt*, *est* and *ipaH* were described by Aranda *et al.* (2004). DNA sequences and sizes of PCR products are shown in Table 1.

The multiplex PCR assay was carried out with a 50-μL of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM of each deoxynucleoside triphosphate, 1.5 U of AccuPrime *Taq* DNA polymerase (Invitrogen), 2 μL of the DNA template and the PCR primers. The optimal concentration of each primer pair in the reaction mixture was determined empirically. Each primer pair concentration was varied independently until the PCR products exhibited equal intensities on 2% agarose gels when a DNA mixture of five prototype *E. coli* strains was used as the PCR template (Fig. 1). The concentration for each primer pair in the final reaction is given in Table 1. The PCR mixtures were then subjected to the following cycling conditions: 50 °C (2 min, 1 cycle), 95 °C (5 min, 1 cycle), 40 cycles of 95 °C (1 min), 50 °C (1 min), 72 °C (1 min) and 72 °C (7 min, 1 cycle) in a thermal cycler (model system 2400; Perkin-Elmer Corporation, Norwalk, Conn.). PCR products (10 μL) were visualized after electrophoresis in 2% agarose gels in Tris-borate-EDTA buffer and ethidium bromide staining, and the amplicons were identified based only on the size of the amplified product. In all further experiments, the DNA mixture from the five diarrheagenic reference strains from pure cultures served as the positive control, while *E. coli* K12 DH5α was the negative control.

In order to determine the limit detection of the multiplex PCR, stool samples negative for diarrheagenic *E. coli* were spiked with a phosphate-buffered saline (PBS) suspension of

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Table 1. PCR primers used in this study

Primer designation	Primers (5′–3′)	Target gene	Amplicon size (pb)	Primer conc. (pmol)
eae1	CTGAACGGCGATTACGCGAA	*eae*	917	10
eae2	CGAGACGATACGATCCAG			
BFP1	AATGGTGCTTGCGCTTGCTGC	*bfpA*	326	1.25
BFP2	GCCGCTTTATCCAACCTGGTA			
aggRks1	GTATACACAAAAGAAGGAAGC	*aggR*	254	2.5
aggRksa2	ACAGAATCGTCAGCATCAGC			
LTf	GGCGACAGATTATACCGTGC	*elt*	450	0.25
LTr	CGGTCTCTATATTCCCTGTT			
STf	ATTTTTMTTTCTGTATTRTCTT	*est*	190	6.47
STr	CACCCGGTACARGCAGGATT			
IpaH1	GTTCCTTGACCGCCTTTCCGATACCGTC	*ipaH*	600	1
IpaH2	GCCGGTCAGCCACCCTCTGAGAGTAC			
VTcom-u	GAGCGAAATAATTTATATGTG	*stx1/stx2*	518	6
VTcom-d	TGATGATGGCAATTCAGTAT			

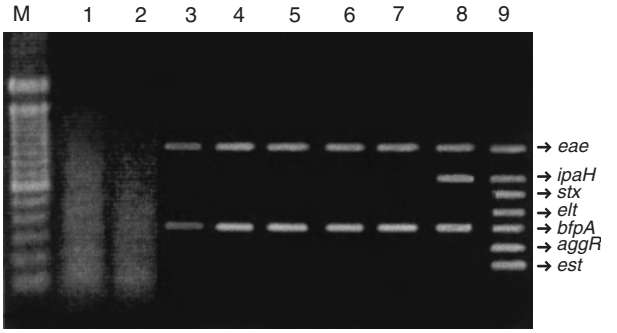

Fig. 1. Sensitivity of multiplex PCR of EPEC E2348/69 (*eae*, *bfpA*) from a spiked stool sample. Lane M, DNA molecular size markers (100-bp ladder); lane 1, nonspiked stool sample; lanes 2–7, dilutions from 10^2 to 10^7 CFU mL^{-1}; lane 8, EPEC E2348/69 (*eae*, *bfpA)* and EIEC EDL1284; lane 9, positive control (DNA mix from the five prototype *Escherichia coli:* EPEC E2348/69 (*eae*, *bfpA*), EAEC O42 (*aggR*), ETEC H10407 (*elt*, *est*), EIEC EDL1284 (*ipaH*) and EHEC EDL931 (*stx1*, *stx2*, *eae*).

reference diarrheagenic *E. coli* strains in serial 10-fold dilutions to give 10^2–10^8 CFU mL^{-1}. Each serial dilution of the spiked stool sample was spread on MacConkey agar plates at 37 °C and colonies were subjected to multiplex PCR. The sensitivity of the assay was defined as the lowest concentration of diarrheagenic *E. coli* that yielded positive results for each dilution. There may be a possibility that two diarrheagenic *E. coli* strains are in the same sample, we therefore mixed each pair of two reference strains and carried out the multiplex PCR to detect these *E. coli* strains.

Results

Sensitivity of multiplex PCR

The sensitivity of the diagnostic multiplex PCR assay was determined from the number of diarrheagenic *E. coli* cells (in CFU per milliliter) spiked into each milliliter of stool sample that could be detected by this method. Repeat experiments confirmed the limit of detection of diarrheagenic *E. coli* was *c.* 10^3 CFU mL^{-1} of stool suspension. Figure 1 shows the limit of detection of typical EPEC E2348/69 (*eae*, *bfpA*) from a spiked stool sample (the results of other diarrheagenic *E. coli* strains are not shown), and that the multiplex PCR could detect two diarrheagenic *E. coli* (typical EPEC and EIEC) strains in a spiked stool sample.

Table 2. Results of multiplex PCR with 330 verified *Escherichia coli* strains

E. coli strain and gene	No. of strains	Number of positives by multiplex PCR
Diarrheagenic *E. coli* (*n* = 220)		
EPEC		
eae	50	50
eae+*bfp*	50	50
EAEC	50	50
ETEC		
elt	13	13
est	21	21
elt+*est*	16	16
EIEC	20	20
Nondiarrheagenic *E. coli*	110	0

Specificity of multiplex PCR

The specificity of multiplex PCR was tested with 330 additional reference strains. The strains included 220 diarrheagenic *E. coli* isolates and 110 nondiarrheagenic *E. coli* isolates. The multiplex PCR showed positive results for the diarrheagenic *E. coli* strains and negative results for the nondiarrheagenic *E. coli* strains (Table 2).

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Table 3. Results of monoplex and multiplex PCR assays with *Escherichia coli* and *Shigella* strains obtained from clinical isolates

Strain	Number of strains	Gene(s)	Number of positive strains by PCR	
			multiplex	monoplex
Typical EPEC	20	*eae*, *bfpA*	20	20
Atypical EPEC	21	*eae*	21	21
EAEC	17	*aggR*	17	17
EIEC	4	*ipaH*	4	4
STEC	3	*eae*, *stx1*	3	3
Shigella flexneri	6	*ipaH*	6	6
Shigella sonnei	2	*ipaH*	2	2
E. coli	50		0	0
Shigella spp	9		0	0

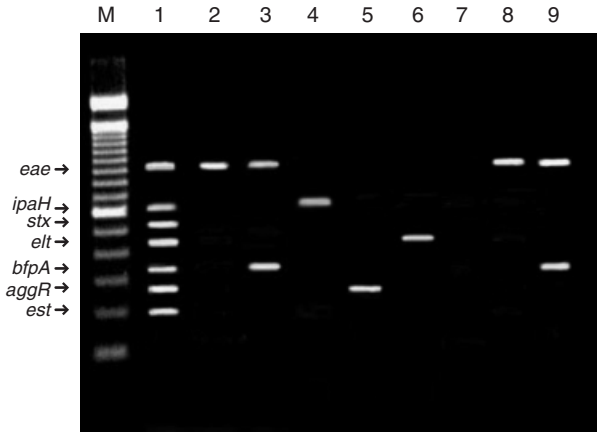

Fig. 2. Multiplex PCR of clinical *Escherichia coli* isolates and stool samples. Lane M, DNA molecular size markers (100-bp ladder); lane 1, positive control (DNA mix from the five prototype *E. coli*): lane 2, EPEC HSP 7-1 (*eae);* lane 3, EPEC HSP 43-1 (*eae* and *bfpA*); lane 4, EIEC MA 245/5 (*ipaH*); lane 5, EAEC MA233-1 (*aggR*); lane 6, *Shigella flexneri* (*ipaH*); lane 7, negative control (*E. coli* DH5α); lanes 8 and 9, atypical and typical EPEC isolated from children with diarrhea.

Validation of the multiplex PCR with clinical isolates

To demonstrate the utility of the multiplex PCR assay, 115 *E. coli* and 17 *Shigella* strains isolated from diarrheic patients were subjected to the multiplex PCR, and the results were compared with those obtained by monoplex PCRs (Table 3; Fig. 2). The comparison of the analysis of 65 pathogenic *E. coli* comprising typical and atypical EPEC, EAEC, EIEC and STEC by multiplex PCR and monoplex PCRs targeting *eae*, *bfpA*, *aggR*, *ipaH* and *stx* as single genes demonstrated that both assays yielded the same virulence genes. Moreover, eight of 17 *Shigella* strains carrying the *ipaH* gene were also detected by the multiplex PCR. The 50 nonpathogenic *E. coli* strains and the nine *Shigella ipaH*-negative tested negative in the multiplex PCR.

Validation of multiplex PCR with stool samples from children with and without diarrhea

A total of 24 diarrheagenic *E. coli* strains were isolated from 79 stool samples from children with diarrhea and from 60 samples from children without diarrhea. The prevalence of diarrheagenic *E. coli* in both groups was significantly different ($P < 0.003$). The PCR assays detected 13 (16.4%) atypical EPEC isolates (*eae* PCR positive), three (3.8%) typical EPEC isolates (*eae* and *bfpA* PCR positive), four (5.1%) EAEC isolates (*aggR* PCR positive) and one (1.3%) EIEC isolate (*ipaH* PCR positive) and one (1.3%) ETEC isolate (*elt* PCR positive) from the group with diarrhea. The prevalences of atypical EPEC and EAEC were similar (1.7%) in the healthy group. No STEC strains were isolated from any of the children examined. There was agreement between results of the PCR multiplex and DNA hybridization assays for all strains (Table 4). EPEC and EAEC strains detected by multiplex PCR were serotyped by an agglutination test using a commercial antiserum (PROBAC do Brasil, São Paulo). The typical EPEC strains detected belonged to serogroups O111, O127 and O142. One atypical EPEC belonged to serogroup O26 and one EAEC was of serogroup O126; both strains were isolated from children with diarrhea.

No *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter* spp. or rotavirus were isolated from the diarrhea or the control group.

Discussion

Numerous multiplex PCRs methods have been developed for the identification of *E. coli* pathotypes (Pass *et al.*, 2000; Toma *et al.*, 2003; Kimata *et al.*, 2005; Müller *et al.*, 2006). However, most of the assays harbor limitations in terms of the number of targeted genes, specificity, the resolution of amplified fragments in agarose electrophoresis, nonspecific amplification and the inability to differentiate all categories of diarrheagenic *E. coli* strains. Recently, Vu Nguyen *et al.* (2005) reported a multiplex PCR assay to detect eight genes for identification of EPEC, EAEC, ETEC, EIEC and STEC. However, it appears that a differentiation between typical and atypical EPEC and ETEC-LT and EIEC might not be straightforward. As a result of the similarity of the sizes of the DNA fragments amplified, from *eae* and *bfpA* (376 and 367 bp, respectively) and from *elt* and *ial* (322 and 320 bp, respectively), it has been necessary to perform PCRs with specific primers after the multiplex PCR in order to verify the result.

Therefore, in the present study, a novel multiplex PCR assay has been developed that allows the simultaneous detection of virulence genes from typical and atypical EPEC (*eae* and *bfpA*), ETEC (*elt* and *est*), EIEC (*ipaH*), EHEC (*stx1*, *stx2* and variants), and EAEC (*aggR*) in a single reaction. In order to identify these virulence genes, we must

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Table 4. Diarrheagenic isolates of *Escherichia coli* from children with diarrhea or without diarrhea

					Number of tested strains/number of positive strains		
Child	Age (years)	Diarrhea	*E. coli* group	Gene(s) and/or probe	PCR	DNA probe	Serogroup*
4	4	Yes	Typical EPEC	*eae, bfpA*	2/2	2/2	O111
6	2	Yes	Atypical EPEC	*eae*	3/3	3/3	ND
8	1	Yes	Atypical EPEC	*eae*	3/3	3/3	ND
14	2	Yes	Typical EPEC	*eae*, *bfpA*	3/3	3/3	O127
17	2	Yes	EAEC	*aggR*, pCVD432	3/2	3/2	ND
20	3	Yes	EAEC	*aggR*, pCVD432	3/2	3/2	ND
22	3	Yes	Atypical EPEC	*eae*	4/3	4/3	ND
25	2	Yes	Atypical EPEC	*eae*	2/1	2/1	ND
32	2	Yes	Atypical EPEC	*eae*	2/2	2/2	ND
33	4	Yes	EAEC	*aggR*, pCVD432	3/3	3/3	O126
34	2	No	EAEC	*aggR*, pCVD432	4/4	4/4	ND
39	4	Yes	Atypical EPEC	*eae*	3/2	3/2	O26
41	5	Yes	ETEC	*elt*	4/4	4/4	OND
42	1	Yes	Atypical EPEC	*eae*	4/3	4/3	ND
45	3	No	Atypical EPEC	*eae*	3/2	3/2	ND
49	2	Yes	Atypical EPEC	*eae*	3/2	3/2	ND
57	1	Yes	EAEC	*aggR*, pCVD432	3/2	3/2	ND
58	1	Yes	Atypical EPEC	*eae*	2/2	2/2	ND
59	5	Yes	EIEC	*ipaH, pInv*	2/2	2/2	ND
62	3	Yes	Typical EPEC	*eae, bfpA*	3/3	3/3	O142
65	1	Yes	Atypical EPEC	*eae*	3/2	3/2	ND
69	1	Yes	Atypical EPEC	*eae*	3/3	3/3	ND
70	2	Yes	Atypical EPEC	*eae*	3/3	3/3	ND
72	1	Yes	Atypical EPEC	*eae*	4/2	4/2	ND

*ND, serogroup not determined.

compare the sizes of the DNA fragments amplified with those of the positive controls or DNA molecular markers.

It was estimated that the limit of detection of diarrheagenic *E. coli* by the multiplex PCR was *c.* 10^3 CFU mL^{-1} in a stool sample. It was also shown that the presence of two types of diarrheagenic *E. coli* in a stool sample could be detected by multiplex PCR.

The specificity of the multiplex PCR assay was demonstrated using several reference strains, as well as with well-characterized clinical isolates. It showed positive results for the diarrheagenic *E. coli* strains tested and negative results for all nondiarrheagenic *E. coli* strains. There was complete agreement between the results of single and multiplex PCRs for all clinical isolates tested, indicating the high degree of specificity of the assay.

Using the multiplex PCR assay, we detected pathogenic *E. coli* (typical and atypical EPEC, EAEC, ETEC, and EIEC) in the fecal samples of 22 (27.8%) children with acute diarrhea, whereas only two (0.3%) asymptomatic children were found to harbor atypical EPEC and EAEC strains. The same results were obtained by colony DNA hybridization with specific DNA probes.

In the field study, atypical EPEC was the most commonly isolated category of diarrheagenic *E. coli* and significantly associated with diarrhea ($P < 0.005$). Furthermore, our findings demonstrate the previously recognized importance of atypical EPEC as a cause of childhood diarrhea in the field study and in other cities (Scaletsky *et al.*, 1999; Gomes *et al.*, 2004; Rodrigues *et al.*, 2004). Moreover, our results also support the evidence from recent prospective case–control studies showing a reduced etiological role for typical EPEC in cases of diarrhea in children (Gomes *et al.*, 2004; Rodrigues *et al.*, 2004). Indeed, this pathogenic category was detected only in three children.

We diagnosed only four EAEC infections by PCR using *aggR*-derived primers. This primer set had previously been found to be very sensitive (Toma *et al.*, 2003). One ETEC-LT and one EIEC strain were detected, but they were less common categories of diarrheagenic *E. coli* found in Brazil. We did not diagnose any STEC infection by the multiplex PCR and believe that STEC strains are relatively rare in Brazil (Vaz *et al.*, 2004).

In conclusion, the multiplex PCR presented in this paper offers a practical possibility for rapid identification of diarrheagenic *E. coli* in a single reaction tube and could be used in a routine diagnostic laboratory.

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