Journal of Clinical Laboratory Analysis 27: 155–161 (2013)

Detection of Diarrheagenic *Escherichia coli* Using a Two-System Multiplex-PCR Protocol

Octaviana Baccin Fialho,[1] Emanuel Maltempi de Souza,[2] Cibelle de Borba Dallagassa,[1] Fábio de Oliveira Pedrosa,[2] Giseli Klassen,[3] Kinue Irino,[4] Katia Sabrina Paludo,[5] Flávia Emanoelli Araújo de Assis,[1] Monica Surek,[1] Sônia Maria de Souza Santos Farah,[6] and Cyntia Maria Telles Fadel-Picheth[1]*

[1]*Departamento de Patologia Médica, Universidade Federal do Paraná, Curitiba-PR, Brazil*
[2]*Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba-PR, Brazil*
[3]*Departamento de Patologia Básica, Universidade Federal do Paraná, Curitiba-PR, Brazil*
[4]*Instituto Adolfo Lutz, São Paulo-SP, Brazil*
[5]*Departamento de Biologia Estrutural Molecular e Genética, Universidade Estadual de Ponta Grossa, Ponta Grossa-PR, Brazil*
[6]*Laboratório Central do Estado, São José dos Pinhais-PR, Brazil*

Background: Diarrheagenic *Escherichia coli* (DEC) strains are important causes of diarrhea. However, they cannot be distinguished from *E. coli* of the intestinal microbiota by conventional microbiological tests. *Methods:* This work presents a two-system multiplex PCR for detection of DEC. Primers for 16S rRNA gene were added as internal amplification control to validate negative reactions. The multiplex-PCR system 1 contains primers for detection of Shiga toxin producing *E. coli* (STEC; stx1, stx2), enteropathogenic *E. coli* (EPEC; eae, bfpA), atypical enteropathogenic *E. coli* (aEPEc; eae), enteroinvasive *E. coli* (ETEC; lt, st), enteroinvasive *E. coli* (EIEC; ial), and the internal amplification control 16S rRNA. The system 2 contains primers for EIEC (ipaH), enteroaggregative *E. coli* (CVD432), diffusely adherent *E. coli* (daaE), and 16S rRNA. The protocol was tested with *E. coli* reference strains, and also with cultures of fecal specimens of people with diarrhea and healthy controls. *Results:* The protocol correctly identified the DEC reference strains. No DEC marker was amplified for negative controls; these results were validated by the amplification of a fragment of the 16S rRNA gene. The frequency of DEC was 7.6% for both patients and healthy controls; two *Shigella sonnei* strains were detected in the group with diarrhea. The identity of the amplicons was confirmed by DNA sequencing. *Conclusion:* The protocol is specific for DEC *Shigella* and is suitable for clinical laboratories. J. Clin. Lab. Anal. 27:155–161, 2013.

Key words: diarrhea; enteropathogens; molecular diagnosis; internal PCR control; molecular method

INTRODUCTION

Escherichia coli is the most abundant facultative anaerobe of the human intestinal microflora. However, several diarrheagenic pathotypes of *E. coli* are recognized: enteropathogenic *E. coli* (EPEC), atypical enteropathogenic *E. coli* (aEPEC), Shiga toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC; (1, 2). Recently, strains with mixed characteristics of STEC and EAEC have also been described and associated with severe disease (3). The diar-

Grant sponsor: Fundação Araucária and Brazilian Program of National Institutes of Science and Technology - INCT/Brazilian Research Council - CNPq/MCT.

*Correspondence to: Dr. Fadel-Picheth, Departamento de Patologia Médica, Universidade Federal do Paraná, Campus Jardim Botânico, Av. Prefeito Lothário Meissner, 632, Jardim Botânico-Curitiba-Paraná, CEP 80210-170, Brazil.
E-mail: fpicheth@ufpr.br

Received 20 October 2012; Accepted 4 January 2013
DOI 10.1002/jcla.21578
Published online in Wiley Online Library (wileyonlinelibrary.com).

rheagenic strains are distinguished by their specific virulence factors (1). EPEC contain the pathogenicity island LEE, associated with the attaching and effacing (A/E) lesions on intestinal cells, and the EAF virulence plasmid (EPEC adherence factor) that encodes the bundle forming pilus (BFP), associated with the localized adherence pattern of EPEC in HeLa and HEp-2 cells (1, 4). aEPEC contain the LEE island but lack the EAF plasmid (1, 2, 5). ETEC is distinguished by the production of the heat-labile enterotoxin (LT) and/or the heat-stable enterotoxins (ST; (4). In *Shigella*, which are now considered as forms of *E. coli* (6), and EIEC virulence is largely due to a 220 kb virulence plasmid that encodes a T3SS on the Mxi-Spa locus that is required for invasion, cell survival, and apoptosis of macrophages (7). STECs are characterized by the production of Shiga toxins Stx1 and/or Stx2, which have the ability to inhibit protein synthesis in eukaryotic cells, can be detected by the cytotoxic effect on Vero cells (1, 8, 9). Some STEC strains may also contain the LEE pathogenicity island (1, 9). EAECs are defined as *E. coli* that do not secrete LT or ST and adhere to HEp-2 cells in a pattern known as autoaggregative that is mediated by aggregative adherence fimbriae (AAFs), related to the Dr family of adhesins, encoded in virulence plasmids called pAA. EAEC adherence to intestinal mucosa is characterized as a biofilm composed of aggregates of bacteria in association with a thick mucus layer that may promote persistent infection (4, 7). Dispersin is a protein produced by EAEC that decreases bacterial autoaggregation, allowing its dispersion along the intestinal mucosa. Dispersin is secreted by an ABC protein transport system coded by a cluster of genes designated *aat-PABCD*, present in plasmid pAA. Transcription of the dispersin gene and the *aat* cluster is dependent on AggR, a regulator of virulence genes in EAEC (10). It was shown that the sequence of the probe CVD432, originally used to detect EAEC in hybridization assays, corresponds to a region in the pAA coding for the dispersin secretion apparatus (10, 11).

DAEC are characterized by the presence of a diffuse pattern of adherence to HEp-2 cells (4), which is mediated by fimbrial (F1845, Dr) and afimbrial (Afa) adhesins. Unlike other pathogenic *E. coli*, the pathogenesis of DAEC seems to be predominately mediated through Afa–Dr adhesin interactions with host cells (7).

The diagnosis of diarrheagenic *E. coli* (DEC) is hampered by the fact that they are indistinguishable from commensal strains based on biochemical tests and serotypic markers are rarely sufficient to reliably identify a strain as diarrhoeagenic. Identification of DEC requires the use of immunological assays, cell culture, or molecular techniques (4, 12, 13). We have developed a two-system multiplex assay containing primers targeting markers of each DEC category and an internal amplification control to validate the negative results. The assay was tested with *E. coli* reference strains, and also with cultures of fecal specimens of people with diarrhea and healthy controls.

MATERIALS AND METHODS

Bacterial Strains

Reference strains used as positive controls were IAL 307 (O124:K72, EIEC), IAL 2391 (EAEC), C1845 (DAEC), E2348/69 (EPEC), H10407 (ETEC), and EDL 933 (STEC). Strains *E. coli* ATCC 25922 and *E. coli* DH10B were used as negative controls. Clinical isolates of STEC, M03, and J307 (14), and of *Klebsiella pneumoniae*, *Shigella sonnei*, *Salmonella* ser. Thyphimurium, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Providencia rettgeri*, *Citrobacter freundii*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were also tested.

Feces Specimens

We analyzed the fecal samples of 250 sequential outpatients (children and adults) with diarrhea, which were submitted to feces culture in a clinical laboratory at Curitiba-PR, Brazil. The 250 controls (children and adults) were selected among healthy subjects, without diarrhea and disease complaints, submitted to routine checkup. Feces samples were collected in Cary Blair transport medium and maintained under refrigeration.

Preparation of DNA Samples

Fecal samples were inoculated on MacConkey agar (Oxoid; Basingstoke, UK) plates and, after incubation overnight at 36°C, a loopful of the confluent region of growth was resuspended in 500 μl of sterile water and used for DNA extraction by the boiling method. The extracts were centrifuged at 14,000 rpm for 2 min and 3 μl of the supernatants were used in each one of the multiplex-PCR systems. Screening for DEC was performed using a pool of colonies. For samples positive in the screening step, that is, presenting bands of DNA amplification according to Table 1, a second step was performed to identify the colonies containing the DEC markers. In this step up to 100 isolated colonies of each sample, including lactose positive and lactose negative, were selected, used for DNA extraction, and tested individually by single PCR for the specific DEC marker (Table 1), according to the result in the screening step. The colonies confirmed as DEC were then stored at −20°C for additional testing.

Multiplex PCR

Primers for DEC strains described in literature were analyzed and selected to compose the two PCR systems

TABLE 1. Primers Used for Detection of Diarrheagenic *E. coli* and Internal Amplification Control

DEC group	Primer (concentration μM)	Sequence (5′–3′)	Amplicon size (pb)	Reference
STEC	stx1	F-CTGGATTTAATGTCGCATAGTG	150	(15,16)
	(0.4)	R-AGAACGCCCACTGAGATCATC		
	stx2	F-GGCACTGTCTGAAACTGCTCC	255	(15)
	(0.48)	R-TCGCCAGTTATCTGACATTCTG		
EPEC	bfpA	F-AATGGTGCTTGCGCTTGCTGC	324	(17)
	(0.48)	R-GCCGCTTTATCCAACCTGGTA		
	eae	F-GACCCGGCACAAGCATAAGC	384	(15)
	(0.48)	R-CCACCTGCAGCAACAAGAGG		
ETEC	lt	F-GGCGACAGATTATACCGTGC	450	(16)
	(0.48)	R-CGGTCTCTATATTCCCTGTT		
	st	F-ATTTTTMTTTCTGTATTRTCTT	190	(17)
	(0.48)	R-CACCCGGTACARGCAGGATT		
EIEC	ial	F-GGTATGATGATGATGAGTCCA	650	(16)
	(0.6)	R-GGAGGCCAACAATTATTTCC		
	ipaH	F-GTTCCTTGACCGCCTTTCCGATACCGTC	600	(18)
	(0.48)	R-GCCGGTCAGCCACCCTCTGAGAGTAC		
EAEC	CVD432	F-CTGGCGAAAGACTGTATCAT	630	(18)
	(0.48)	R-CAATGTATAGAAATCCGCTGTT		
DAEC	daaE	F-GAACGTTGGTTAATGTGGGGTAA	542	(19)
	(0.48)	R-TATTCACCGGTCGGTTATCAGT		
Internal Control	16S (0.48)	F-CCAGCAGCCGCGGTAATACG R-ATCGGYTACCTTGTTACGACTTC	996	(20)

according to the ΔG value calculated by OligoAnalyzer (www.idtdna.com) to avoid heterodimer formation. The sequences of the primers selected are indicated in Table 1. PCR reactions were performed in a final volume of 25 μl, containing Taq DNA Polymerase buffer 1× (Invitrogen, Foster City, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U of Taq DNA polymerase platinum (Invitrogen), and 3 μL of template DNA prepared as above. The concentration of each pair of primers (Table 1) was adjusted empirically to obtain DNA bands of similar intensities with controls. The multiplex-PCR system 1 contained primers for the detection of STEC (*stx1*, *stx2*), EPEC (*eae*, *bfpA*), aEPEc (*eae*), ETEC (*lt*, *st*), EIEC (*ial*, which corresponds to the *spa 9* gene, a component of the Mxi-Spa secretion machinery), and the internal control 16S rRNA. The system 2 contained primers for EIEC (*ipaH*, invasion plasmid antigen), EAEC (CVD432, *aatA* gene), DAEC (*daaE*, gene required for expression of the F1845 fimbriae), and 16S rRNA. The cycling programs used were 1 cycle at 94°C (4 min), 35 cycles at 94°C (1 min), 55°C (1 min), 72°C (1 min), and a final cycle at 72°C (5 min) for multiplex-PCR system 1; for system 2, the conditions were the same except the annealing temperature that was 58°C (1 min). The reactions were performed in a Biocycler MJ96G thermocycler. Detection of PCR products was by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light. The sensitivity of the multiplex-PCR protocol was tested with serial dilutions of reference DEC strains in the range of 10^8–10 CFU/ml.

Biochemical Identification of the Strains

Strains harboring virulence markers of DEC were identified using the API-20E strips and APIWEB (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions.

DNA Sequencing

For each strain, amplified DNA samples containing DEC markers were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing kit (GE Healthcare, Piscataway, NJ) and the automatic DNA sequencer ABI Prism 377 (Applied Biosystems, Foster City, CA) to confirm the identity of the fragment. Sequences were analyzed using BioEdit Sequence Alignment Editor (21), BLASTn (22) and CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Serotyping

Escherichia coli strains were serotyped using O (O1–O181) and H (H1–H56) antisera prepared at the Adolfo Lutz Institute (São Paulo, Brazil).

Cell Cytotoxicity Assay

stx production was determined by the Vero cell cytotoxicity assay (23). Briefly, the strains were grown overnight in Penassay broth (Antibiotic Medium no. 3, Difco, Sparks,

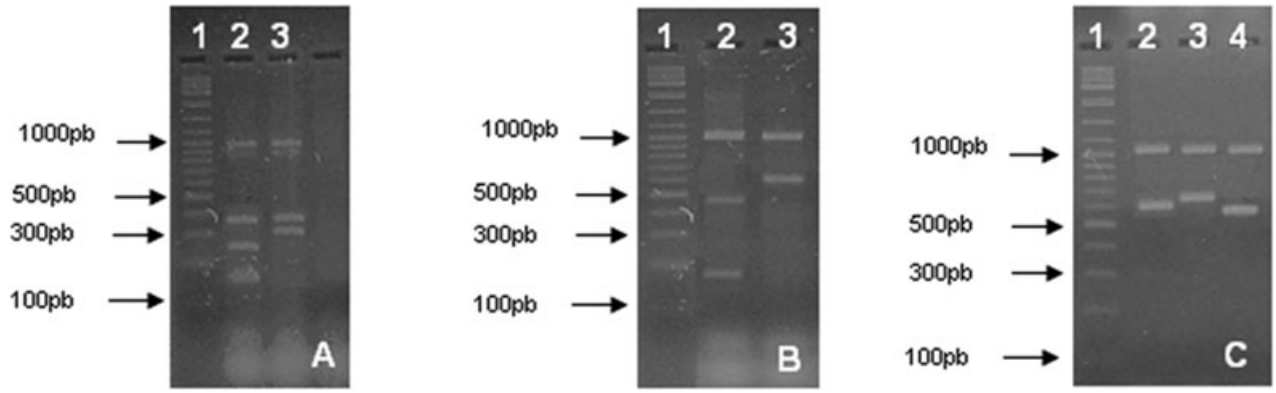

Fig. 1. Multiplex-PCR amplification pattern for DEC positive controls. (A) (1) 100 pb DNA ladder (Fermentas), (2) STEC (stx1 stx2 eae), (3) EPEC (eae, bfpA). (B) (1) 100 pb DNA ladder (Fermentas), (2) ETEC (lt, st), (3) EIEC (ial). (C) (1) 100 pb DNA ladder (Fermentas), (2) EIEC (ipaH), (3) EAEC (CVD432), (4) DAEC (daaE). A band of approximately 1,000 pb seen in all gels correspond to the internal amplification control *16S rRNA* gene. (A) and (B) correspond to the multiplex-PCR system 1 target genes, and (C) to system 2 target genes.

NV), and then centrifuged (14,000 rpm, 2 min). The resulting cell-free supernatants were filtered with a 0.22-μm membrane and stored at −20°C. Vero cells were seeded (1×10^3) into 96-well plates and maintained in RPMI medium (Gibco, Paisley, UK) containing 2 mM glutamine, 40 μg/ml of gentamicin, 2.5 μg/ml amphotericin B, 10 μg/ml of ciprofloxacin, and 10% fetal bovine serum. The cells were grown at 37°C in a 5% CO_2 atmosphere for 24 hr. Sterile supernatants (25 μl) were added to the wells and incubated under the same cell growth conditions described above for 72 hr. Cytotoxic effects such as cell disruption and detachment were observed under the microscope. STEC EDL 933 and *E. coli* DH10B strains were used as positive and negative control, respectively. This study was approved by the Ethical Committee of our institution.

RESULTS

Sensitivity and Specificity of the Method

The multiplex-PCR protocol was able to detect all the *E. coli* diarrheagenic pathotypes (Fig. 1). The detection limit was 10^4 CFU/ml for both PCR systems. STEC clinical control strains M03 and J307, and the *S. sonnei* strain were also correctly identified. As expected, the protocol did not distinguish between EIEC and *Shigella*, since both are positive for *ipaH* and *ial*. No amplification for DEC gene markers was seen for other clinical control strains tested, showing the specificity of the method. The negative results were validated by the amplification of the internal control.

Rate of Detection of DEC Among Fecal Specimens

Among diarrheal samples a total of 19 DEC (7.6%), belonging to aEPEC (ten strains), EAEC (six strains), STEC, DAEC, and EIEC (one strain each), and two *S. sonnei* strains (0.8%) were recovered. Nineteen DEC strains (7.6%) were also isolated from the control samples, which corresponded to aEPEC (seven strains), EAEC (eight strains), STEC (three strains), and DAEC (one strain). The same rate of isolation of DEC was seen in both groups. Figure 2 shows the DNA amplification pattern of some of these strains.

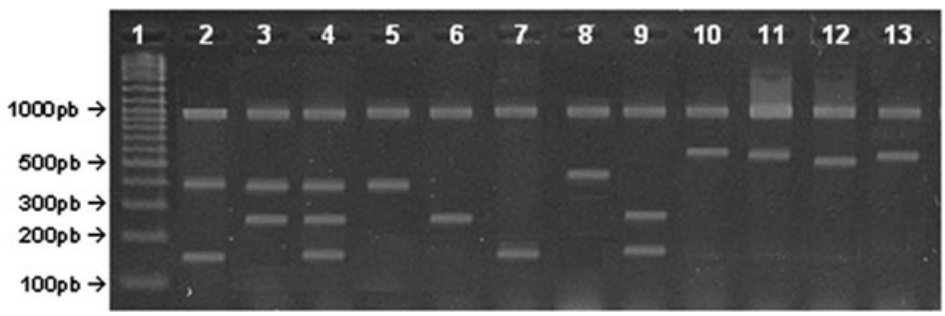

Fig. 2. Multiplex-PCR amplification pattern observed for some DEC strains detected. (1) 100 pb DNA ladder (Fermentas). (2–4), (6 and 7), and (9), respectively, STEC strains 107 CS, 44 CS, 62 CS, 150 CS, 149 D, and 10 D. (5) Strain 49 D (aEPEC). (8) Strain 116 D (ETEC). (10 and 11) Strain 29 D (EIEC, systems 1 and 2, respectively). (12) Strain 133 D (DAEC). (13) Strain 239 D (EAEC).

TABLE 2. Characteristics of Diarrheagenic *E. coli* Isolates

Strain[a]	DEC pathotype	Serotype
3 CS	eae/aEPEC	ONT:H-
18 CS	CVD432/EAEC	ONT:H2
21 CS, 56 CS, 66 CS	CVD432/EAEC	OR:H-
65 CS	eae/aEPEC	ONT:H8
77 CS, 133 D	daaE/DAEC	O25:H4
78 CS	eae/aEPEC	OR:H4
88 CS	stx2/STEC	ONT:H16
94 CS	eae/aEPEC	ONT:H6
107 CS	stx1, eae/STEC	O177:H-
128 CS	eae/aEPEC	O177:H11
132 CS	eae/aEPEC	O51:H40
150 CS	stx2/STEC	ONT:H9
173 CS, 141 D	CVD432/EAEC	O3:H2
179 CS, 245 D	CVD432/EAEC	ONT:H4
191 CS	CVD432, EAEC	OR:H2
225 CS	eae/aEPEC	ONT:H7
226 CS, 75 D	CVD432/EAEC	O25:H4
10 D	stx1, stx2/STEC	ONT:H16
28 D	eae/aEPEC	O157:H16
29 D	ipaH, ial/EIEC	O144:H-
49 D	eae/aEPEC	O177:H-
85 D	eae/aEPEC	O91:H14
86 D	eae/aEPEC	O145:H28
171 D	CVD432/EAEC	ONT:H25
172 D	eae/aEPEC	O25:H4
190 D	eae/aEPEC	O127:H40
200 D	eae/aEPEC	ONT:HNT
213 D	eae/aEPEC	O108:H21
216 D	eae/aEPEC	O137:H6
217 D	CVD432/EAEC	O3:H-
237 D	eae/aEPEC	OR:H40
239 D	CVD432/EAEC	ONT:H28

Note: CS indicates the isolates from healthy controls; D indicates isolates from person with diarrhea; aEPEC, atypical enteropathogenic *E. coli;* EAEC, enteroaggregative *E. coli*; DAEC, diffusely adherent *E. coli*; STEC, Shiga toxin producing *E. coli*; EIEC, enteroinvasive *E. coli* (EIEC).

[a]Two additional strains (25 D and 27 D) were isolated from people with diarrhea, both were positive for *ipaH* and *ial* but were identified as *Shigella sonnei* by biochemical tests.

Characterization of the DEC Strains

DNA sequences of the amplified fragments confirmed the presence of virulence genes in these bacteria. Sequences were submitted to GenBank under accession numbers for aEPEC, JQ638605–JQ638623; EAEC, JQ638624–JQ638637; EIEC, JQ638638 and JQ638641; and *Shigella*, JQ638639, JQ638640, JQ638642, and JQ638643. Sterile supernatants of all but one STEC strains (150 CS, where CS indicates the isolates from healthy controls) were cytotoxic to Vero cells (detachment/disruption). Virulence markers and serotype are indicated in Table 2.

DISCUSSION

DECs are important causes of diarrhea (24–30), however their detection depends on the use of methods able to distinguish them from the intestinal commensal *E. coli.* Several strategies have been described for detection and characterization of DEC based mainly on the detection of the virulence-associated characteristics (4, 13). There are several multiplex-PCR protocols described, some of them are able to detect most of DEC (16, 18, 25, 31–34) but do not include primers for identification of EAEC and/or DAEC. Other systems detect all DEC (19, 35) but do not include an internal PCR control.

This work describes a two-system multiplex PCR for detection of DEC, which includes an internal PCR control. Together the two PCR systems contain ten pairs of primers targeting markers of the seven DEC pathotypes. In both systems, the internal amplification control consists of a pair of primers for the *16S rRNA* gene (20), a nontarget DNA sequence present in the same sample tube, which is co-amplified simultaneously with the target sequence. The presence of primers for nontarget DNA sequences is essential to validate a negative result, which may be due to inhibition of the PCR by malfunction of the thermocycler, incorrect PCR mixture, poor DNA polymerase activity, or the presence of inhibitors in the sample. When a PCR containing an internal amplification control is used, the corresponding control band should always be produced, and its absence reveals failure in the PCR (36). Persson et al. (37) developed a multiplex PCR for DEC including the *16S rRNA* gene as an internal PCR control, however their protocol does not detect EAEC and DAEC, which is possible in the system presented in this study.

The multiplex-PCR system used in the present work correctly identified the DEC reference strains (Fig. 1), and reactions without DNA amplification for DEC markers were observed in the negative controls. The correct functioning of the PCR in these negative reactions was monitored by the amplification of a DNA band of 996 bp corresponding to a fragment of the bacterial *16S rRNA* gene (20). Besides, the multiplex-PCR protocol was used to test cultures of 250 feces specimens of people with diarrhea and 250 healthy controls. The frequency of DEC was 7.6% for both patient and healthy control groups (Table 2). Two strains of *S. sonnei* were also found in the group with diarrhea. DNA sequencing confirmed the identity of the amplicons indicating that the multiplex-PCR protocol is specific for DEC markers. The similar frequencies of DEC found between the two groups analyzed is in contrast with other studies that found higher DEC frequencies in patients with diarrhea (29, 38, 39). This is probably due to differences in the sample characteristics, since in those studies restrictive criteria, such as 3–4 emissions per day, blood presence in feces, among others, were used to

include a sample in the diarrheal group. The DEC frequency found in the diarrheal group (7.6%) was lower than those observed in some studies (24, 25, 29, 38, 39), which reported frequencies ranging from 16% to 33%. This may also be due to the criteria used to define diarrhea and to the sample composition since those studies included only children, the main age group affected by enteropathogens. The most frequent pathotypes recovered were aEPEC (4% and 2.8%, respectively, for diarrheal and nondiarrheal samples) and EAEC (2.4% and 3.2%, respectively, for diarrheal and nondiarrheal samples). These results are in agreement with others indicating that aEPEC and EAEC are the most prevalent pathotypes (25–27, 29, 39). Other pathotypes were found in low frequencies. STEC strains were found in 0.4% and 1.2% of diarrheal and nondiarrheal samples, respectively. Interestingly, STEC strain 150 CS that contained a *stx2* type gene did not show cytotoxic effects in the Vero cells assay suggesting that it does not express stx2. This was also observed in other studies (40–43), some of which detected the presence of the insertion sequence IS1203 interrupting the coding region of the *stx2* genes resulting in the inactivation of the genes and absence of cytotoxic effects (40, 41, 43). DAEC frequency was 0.4% in both groups and EIEC was found only in the diarrheal samples with a frequency of 0.4% (Table 2). ETEC and typical EPEC were not found. The absence of typical EPEC is in agreement with other studies undertaken in Brazil, which found low frequencies or absence of this DEC pathotype (24, 26). Most of the DEC isolates belonged to distinct serogroups (Table 2). Interestingly, among the 19 strains isolated from the control group 13 were ONT (nontypeable) or OR (rough), while among those recovered from diarrheal group, 13 had their somatic antigens identified (Table 2) and only six were ONT (nontypeable) or OR (rough). The most common serotype identified was O25:H4 that was found in five strains isolated from control and diarrheal groups, which belonged to DAEC, EAEC, and aEPEC categories (Table 2).

CONCLUSION

In summary, the protocol described here allowed the detection of DEC pathotypes and the use of an internal amplification control allows validation of negative reactions. The use of multiplex-PCR systems allows the detection of several targets simultaneously and is an alternative for DEC screening and identification in microbiology laboratories, saving time, cost, and effort to diagnose these bacteria.

ACKNOWLEDGMENTS

We wish to thank Dr Isabel Scaletsky and Dr Katia Aranda who kindly send us EPEC, ETEC, and DAEC reference strains, and Dr M.G. Yates for reading the manuscript and for suggestions. We thank CAPES/REUNI for scholarships.

REFERENCES

1. Kaper JB, Nataro JP, Mobley HLT. Pathogenic *Escherichia coli*. Nature Rev Microbiol 2004;2:123–140.
2. Schmidt MA. LEEways: Tales of EPEC, ATEC and EHEC. Cell Microbiol 2010;12:1544–1552.
3. Bielaszewska M, Mellmann A, Zhang W, et al. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: A microbiological study. Lancet Infect Dis 2011;11:671–676.
4. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998;11:142–201.
5. Trabulsi LR, Keller R, Gomes TAT. Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis 2002;8:508–513.
6. Pupo GM, Lan R, Reeves PR. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. PNAS 2000;97:10567–10572.
7. Croxen MA, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. Nature Rev Microbiol 2010;8:26–38.
8. Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. Infect Immun 1977;18:775–779.
9. Guth BEC, Prado V, Rivas M. Shiga toxin-producing *Escherichia coli*. In: AG Torres, editor. Pathogenic *Escherichia coli* in Latin America. Bentham Science Publishers Ltd., Chicago, IL, 2010. p 65–83.
10. Nishi J, Sheikh J, Mizuguchi K, et al. The export of coat protein from Enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system. J Biol Chem 2003;278:45680–45689.
11. Navarro-Garcia F, Elias WP, Flores J, Okhuysen PC. Enteroaggregative *Escherichia coli*. In: AG Torres, editor. Pathogenic *Escherichia coli* in Latin America. Bentham Science Publishers Ltd., Chicago, IL, 2010. p 48–64.
12. Yang JR, Wu FT, Tsai JL, et al. Comparison between O serotyping method and multiplex real-time PCR to identify diarrheagenic *Escherichia coli* in Taiwan. J Clin Micro 2007;45:3620–3625.
13. Piazza RMF, Abe CM, Horton DSPQ, Miliwebsky E, Chinen I, Vaz TMI, Irino K. Detection and subtyping methods of diarrheagenic *Escherichia coli* strains. In: AG Torres, editor. Pathogenic *Escherichia coli* in Latin America. Bentham Science Publishers Ltd., Chicago, IL, 2010. p 95–115.
14. De Toni F, Souza EM, Pedrosa FO, et al. A prospective study on Shiga toxin-producing *Escherichia coli* in children with diarrhoea in Paraná State, Brazil. Lett Appl Microbiol 2009;48:645–647.
15. Paton AW, Paton JC. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx*1, *stx*2, *eaeA*, Enterohemorrhagic *E. coli hlyA*, *rfb*O111, and *rfb*O157. J Clin Microbiol 1998;36:598–602.
16. López-Saucedo C, Cerna JF, Villegas-Sepulveda N, et al. Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg Infect Dis 2003;9:27–131.
17. Stacy-Phipps S, Mecca JJ, Weiss JB. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxaigenic *Escherichia coli* DNA during course of infection. J Clin Microbiol 1995;33:1054–1059.
18. Aranda KRS, Fagundes-Neto U, Scaletsky ICA. Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella* spp. J Clin Microbiol 2004;42:5849–5853.

19. Vidal M, Kruger E, Durán C, et al. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. J Clin Microbiol 2005;43:5362–5365.
20. Lu JJ, Perng CL, Lee SY, Wan CC. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J Clin Microbiol 2000;38:2076–2080.
21. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–98.
22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–410.
23. Gentry MK, Dalrymple JM. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J Clin Microbiol 1980;12:361–366.
24. Franzolin MR, Alves RCB, Keller R, et al. Prevalence of diarrheagenic *Escherichia coli* in children with diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 2005;100:359–363.
25. Aranda KRS, Fabbricotti SH, Fagundes-Neto U, Scaletsky ICA. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. FEMS Microbiol Lett 2007;267:145–150.
26. Araujo JM, Tabarelli GF, Aranda KRS, et al. Typical enteroaggregative and atypical enteropathogenic types of *Escherichia coli* are the most prevalent diarrhea-associated pathotypes among Brazilian children. J Clin Microbiol 2007;45:3396–3399.
27. Spano LC, Sadovsky ADI, Segui PN, et al. Age-specific prevalence of diffusely adherent *Escherichia coli* in Brazilian children with acute diarrhoea. J Med Microbiol 2008;57:359–363.
28. Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, et al. Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhea. J Clin Microbiol 2009;47:93–98.
29. Moreno ACR, Fernandes Filho A, Gomes TAT, et al. Etiology of childhood diarrhea in the northeast of Brazil: Significant emergent diarrheal pathogens. Diagn Microbiol Infect Dis 2010;66:50–57.
30. Liebchen A, Benz I, Mellmann A, et al. Characterization of *Escherichia coli* strains isolated from patients with diarrhea in São Paulo, Brazil: Identification of intermediate virulence factor profiles by multiplex PCR. J Clin Microbiol 2011;49:2274–2278.
31. Toma C, Lu Y, Higa N, et al. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J Clin Microbiol 2003;41:2669–2671.
32. Müller D, Greune L, Heusipp G, et al. Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. Appl Environ Microbiol 2007;73:3380–3390.
33. Fujioka M, Kasai K, Miura T, Sato T, Otomo Y. Rapid diagnostic method for the detection of diarrheagenic *Escherichia coli* by multiplex PCR. Jpn J Infect Dis 2009;62:476–480.
34. Antikainen J, Tarkka E, Haukka K, Siitonen A, Vaara M, Kirveskari J. New 16-plex PCR method for rapid detection of diarrheagenic *Escherichia coli* directly from stool samples. Eur J Clin Microbiol Infect Dis 2009;28:899–908.
35. Gómez-Duarte OG, Bai J, Newell E. Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn Microbiol Infect Dis 2009;63:1–9.
36. Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 2004;42:1863–1868.
37. Persson S, Olsen KEP, Scheutz F, Krogfelt KA, Gerner-Smidt P. 2007. A method for fast and simple detection of major diarrhoeagenic *Escherichia coli* in the routine diagnostic laboratory. Clin Microbiol Infect 13:516–524.
38. Orlandi PP, Magalhães GF, Matos NB, et al. Etiology of diarrheal infections in children of Porto Velho (Rondonia, Western Amazon region, Brazil). Braz J Med Biol Res 2006;39:507–517.
39. Bueris V, Sircili MP, Taddei CR, et al. Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 2007;102:839–844.
40. Kusumoto M, Nishiya Y, Kawamura Y, Shinagawa K. Identification of an insertion sequence, IS1203 variant, in a Shiga toxin 2 gene of *Escherichia coli* 0157:H7. J Biosci Bioeng 1999;87:93–96.
41. Jinneman KC, Weagant SD, Johnson JM, et al. A large insertion in the Shiga-like toxin 2 gene (*stx2*) of an *Escherichia coli* O157:H7 clinical isolate. Int J Food Microbiol 2000;57:115–124.
42. Rodolpho D, Marin JM. Isolation of Shiga toxigenic *Escherichia coli* from butcheries in Taquaritinga city, State of São Paulo, Brazil. Braz J Microbiol 2007;38:599–602.
43. Ding H, Huang L, Mao X, Zou Q. Characterization of *stx2* and its variants in *Escherichia coli* O157:H7 isolated from patients and animals. Afr J Biotechnol 2011;10:2991–2998.