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# Evaluation of Multiplex PCRs for Diagnosis of Infection with Diarrheagenic *Escherichia coli* and *Shigella* spp.

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**We have developed two multiplex PCR assays that detect typical and atypical enteropathogenic *Escherichia coli* (EPEC) isolates, enteroaggregative *E. coli* (EAEC) isolates, enterotoxigenic *E. coli* (ETEC) isolates, enteroinvasive *E. coli* (EIEC) isolates, Shiga toxin-producing *E. coli* (STEC) isolates, and *Shigella* spp. The targets selected for each group were *eae* and *bfpA* for EPEC isolates, the target of probe CVD432 for EAEC isolates, the genes encoding heat-labile and heat-stable toxins for ETEC isolates, $stx_1$ and $stx_2$ for STEC isolates, and *ipaH* for EIEC isolates and *Shigella* spp. These PCRs were specific and sensitive for rapid detection of target isolates in stools. Among 150 stool specimens from the acute diarrhea tested, 9 samples (6%) had atypical EPEC, 9 (6%) had typical EPEC, 7 (4.7%) had EAEC, 3 (2%) had EIEC, 3 (2%) had *Shigella* spp., and 1 (0.7%) had an O26 STEC strain; we also detected mixed infections, 2 (1.3%) with EAEC and *Shigella* spp., 1 (0.7%) with atypical and typical EPEC strains, and another with atypical EPEC and EAEC strains. One of the multiplex PCRs directly applied to 36 stool specimens correctly identified 100% of EPEC and EAEC isolates.**

Five categories of *Escherichia coli* have been well associated with diarrhea in several epidemiological studies (9): enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC). The virulence mechanisms that characterize these categories of *E. coli* are genetically encoded by chromosomal, plasmid, and bacteriophage DNAs and are represented by the following genes: *eae* (attaching and effacing lesions), *bfpA* (localized adherence), the gene encoding enteroaggregative adherence, *ipaH* (enteroinvasive mechanism), the genes encoding heat-labile toxin (LT) and heat-stable toxin (ST), and $stx_1$ and $stx_2$ (Shiga toxins). To correctly identify diarrheagenic *E. coli* strains, these organisms must 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 and of themselves to reliably identify a strain as diarrheagenic. Thus, the detection of diarrheagenic *E. coli* has focused increasingly on the identification of certain characteristics which 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 specific virulence traits or the genes encoding these traits. The first two types of assays require special expertise, employ cell culture and radioactive material, and are time-consuming.

We developed two multiplex PCR assays to detect the five categories of diarrheagenic *E. coli* organisms and *Shigella* spp. and assessed the direct application of those assays to human diarrheal stool samples.

The targets selected for each category were *eae* and *bfpA* for EPEC isolates, the target of probe CVD432 for EAEC isolates, the LT and ST genes for ETEC isolates, $stx_1$ and $stx_2$ for STEC isolates, and *ipaH* for EIEC isolates and *Shigella* spp. For each target gene, a different pair of primers was selected from the literature (Table 1). Multiplex PCR assay 1 utilizes three primer pairs and detects the presence of *eae*, *bfpA*, and the target of CVD432, generating amplification products of 917, 326, and 630 bp, respectively. Detection of *eae* confirms the presence of typical and/or atypical EPEC strains, while testing for *bfpA* confirms the presence of the bundle-forming pilus major subunit that is found only in typical EPEC strains (4, 5, 12). To include the identification of EAEC strains in the multiplex PCR, we selected a primer pair complementary to the EAEC probe sequence that detects 90% of EAEC strains (15). PCR assay 2 uses five primer pairs and detects the presence of the LT and ST genes, $stx_1$, $stx_2$, and *ipaH*, generating PCR products of distinct sizes which are easily distinguished after agarose gel electrophoresis. The primers detect the genes encoding LT and porcine and human ST in order to detect all types of ETEC in a single multiplex reaction (18). The $stx_1$ and $stx_2$ primers were designed to amplify $Stx_1$ and all $Stx_2$ variants (11). The *ipaH* sequences are present at multiple sites on both the large invasive plasmid and the chromosomes in *Shigella* spp. and EIEC strains (16).

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 deionized 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. Having confirmed the specificity of each primer set by monoplex PCR, we combined the primer sets in different ratios and tested the reference strains with several

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TABLE 1. PCR primers used in this study

| Primer designation | Primers (5′ to 3′) (reference)[a] | Target gene or probe | Amplicon size (bp) | Primer concn. (pmol) |
|---|---|---|---|---|
| *eae1*<br>*eae2* | CTGAACGGCGATTACGCGAA<br>CCAGACGATACGATCCAG (12) | *eae* | 917 | 5 |
| BFP1<br>BFP2 | AATGGTGCTTGCGCTTGCTGC<br>GCCGCTTTATCCAACCTGGTA (5) | *bfpA* | 326 | 5 |
| EAEC1<br>EAEC2 | CTGGCGAAAGACTGTATCAT<br>CAATGTATAGAAATCCGCTGTT (15) | CVD432 | 630 | 5 |
| LTf<br>LTr | GGCGACAGATTATACCGTGC<br>CGGTCTCTATATTCCCTGTT (18) | LT gene | 450 | 5 |
| STf<br>STr | ATTTTTMTTTCTGTATTRTCTT<br>CACCCGGTACARGCAGGATT (18) | ST gene | 190 | 6.47 |
| IpaH1<br>IpaH2 | GTTCCTTGACCGCCTTTCCGATACCGTC<br>GCCGGTCAGCCACCCTCTGAGAGTAC (16) | *ipaH* | 600 | 10 |
| Stx1f<br>Stx1r | ATAAATCGCCATTCGTTGACTAC<br>AGAACGCCCACTGAGATCATC (11) | $stx_1$ | 180 | 3.88 |
| Stx2f<br>Stx2r | GGCACTGTCTGAAACTGCTCC<br>TCGCCAGTTATCTGACATTCTG (11) | $stx_2$ | 255 | 2.5 |

[a] M, A/C; R, A/G.

PCR cycling protocols. The optimized protocol was carried out with a 50-μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 2 mM concentration 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 the five prototype *E. coli* strains was used as the PCR template. The concentration for each primer pair used in the final reactions is given in Table 1. The PCR mixtures were then subjected to the following cycling conditions: for assay 1, 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 40 cycles of 95°C (40 s), 58°C (1 min), and 72°C (2 min); and a final extension step at 72°C (7 min, 1 cycle); and for assay 2, 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 40 cycles of 95°C (45 s), 50°C (1 min), and 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. In all experiments, the DNA mixture from the prototype EPEC E2348/69, EAEC O42, ETEC H10407, EIEC EDL1284, and STEC EDL931 strains served as the positive control (9), while *E. coli* K-12 DH5α was the negative control.

The two multiplex PCRs were further validated with 270 additional reference strains. One hundred EPEC and 50 EAEC reference strains were identified in a previous case-control study by their reactivity with the *eae*, EPEC adherence factor, and EAEC probes (4, 14). Fifty ETEC, 20 EIEC, and 50 *Shigella* species reference strains were also identified by DNA hybridization by other laboratories. The strains were subjected to both multiplex PCRs, and the results were compared with those obtained by monoplex PCR. Both multiplex PCR assays showed 100% specificity in identifying the reference strains; most importantly, nonspecific bands were not visualized. The same results were seen when DNA from the reference *E. coli* strains was mixed and used in the multiplex PCR assays (Fig. 1 and 2).

To demonstrate the diagnostic usefulness of both multiplex PCR assays, we examined bacterial colonies isolated from stool specimens obtained from 150 children less than 5 years old who had been assisted in the emergency room of Hospital São Paulo, which provides public medical assistance to children of

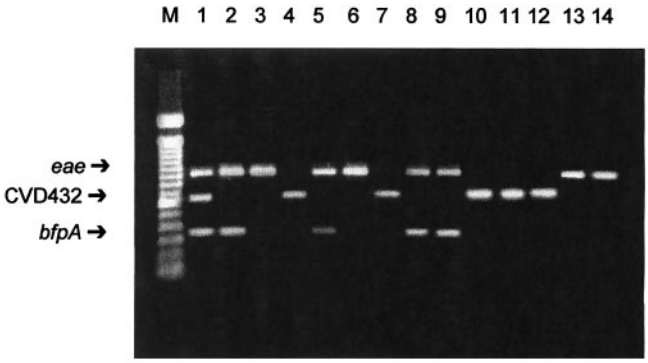


FIG. 1. Multiplex PCR assay 1 of reference strains and clinical and stool samples. Lane 1, EPEC E2348/69 (*eae* and *bfpA*) and EAEC 042 (CVD432); lane 2, EPEC HSP43-1 (*eae* and *bfpA*); lane 3, atypical EPEC MA343-4 (*eae*); lane 4, EAEC MA233-1 (CVD432); lane 5, patient sample 5 (*eae* and *bfpA*); lane 6, patient sample 6 (*eae*); lane 7, patient sample 29 (CVD432); lane 8, stool sample 2 (*eae* and *bfpA*); lane 9, stool sample 8 (*eae* and *bfpA*); lane 10, stool sample 3 (CVD432); lane 11, stool sample 5 (CVD432); lane 12, stool sample 12 (CVD432); lane 13, stool sample 33 (*eae*); lane 14, stool sample 34 (*eae*); lane M, DNA molecular size markers (100-bp ladder).

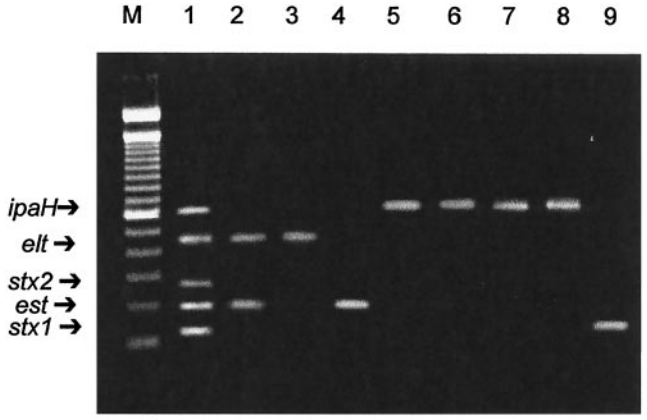


FIG. 2. Multiplex PCR assay 2 of reference strains and clinical samples. Lane 1, ETEC H10407 (LT gene and ST gene), EIEC EDL1285 (*ipaH*), and STEC EDL931 ($stx_1$ and $stx_2$); lane 2, ETEC 1111-1 (LT gene and ST gene); lane 3, ETEC 2811-1 (LT gene); lane 4, ETEC 2021-1 (ST gene); lane 5, EIEC 733/6 (*ipaH*); lane 6, *S. flexneri* 245-5 (*ipaH*); lane 7, patient sample 48 (*ipaH*); lane 8, patient sample 75 (*ipaH*); lane 9, patient sample 127 ($stx_1$); lane M, DNA molecular size markers (100-bp ladder).

low socioeconomic status in the city of São Paulo, Brazil. Every fecal specimen was examined by standard methods for the presence of *Shigella* spp., *Salmonella* spp., *Giardia lamblia*, *Yersinia enterocolitica*, *Campylobacter* spp., *Cryptosporidium* spp., and rotavirus. Four separate lactose-fermenting colonies and two non-lactose-fermenting colonies from each patient were cultivated in commercial test systems (PROBAC do Brasil, São Paulo) for biochemical confirmation of species or genus. All isolates of *E. coli* and *Shigella* spp. were screened by colony hybridization with EPEC adherence factor (8), the *E. coli* attaching and effacing gene encoding intimin (*eae*) (6), diffuse adherence factors (*daaC* and AIDA-I) (2, 3), EAEC adherence factor (1), enterotoxins LT and ST (7), the enteroinvasiveness factor (Inv) (17), and Shiga toxin probes (10). These probes were labeled with [$\alpha$-$^{32}$P]dCTP with a random primer extension kit (Rediprime DNA labeling system; Amersham). Colony hybridization assays were performed as previously described elsewhere (13). The identified strains of *E. coli* and *Shigella* spp. were subjected to the multiplex PCR assays, and the results were compared with those obtained by DNA probe hybridization.

A total of 267 *E. coli* and 17 *Shigella* species strains isolated from 150 patients were subjected to both multiplex PCRs and DNA hybridization assays (Table 2). Thirty-six (24%) of the 150 patients had a potentially diarrheagenic strain of *E. coli* or *Shigella* spp. in their stool samples. Atypical and typical EPEC strains were isolated from nine children each. Seven children were infected with an EAEC strain, three children were infected with an EIEC strain, and one child was infected with an O26 STEC strain. *Shigella* spp. were isolated from three children. One child (patient 66) was infected with atypical and typical EPEC strains, one child (patient 118) was infected with atypical EPEC and EAEC strains, and two children were infected with EAEC and *Shigella* species (patients 55 and 131) strains. For patient 127, an STEC strain of serogroup O26 was detected, which was positive for the *eae* and $stx_1$ genes. None of the other 114 patients with diarrhea had *E. coli* strains containing the target genes in their stools. There was agreement between results of the PCR multiplex and DNA hybridization assays for almost all strains. For patients 4 and 99, one more *E. coli* strain positive for the *eae* gene was detected by PCR assays, and, for patient 150, two more *E. coli* strains positive for the *eae* gene were detected by PCR assays. For patients 103 and 117, one more gene-positive strain was detected with the DNA probe.

To assess the sensitivity of the multiplex PCR assays, overnight cultures of prototype strains were suspended in phosphate-buffered saline at a MacFarland standard of 1, which is equivalent to $3 \times 10^8$ CFU of *E. coli*, and were serially diluted 10-fold. DNA was extracted from these samples and subjected to both multiplex PCRs. The sensitivity of detection was $10^3$ CFU per assay for all target genes. The PCR assays were then used to detect diarrheagenic *E. coli* strains directly from fecal samples spiked with different concentrations of prototype strains. The prototype strains, suspended in phosphate-buffered saline at different concentrations, were used to spike 180 mg of stool specimen. The DNA was isolated from the spiked stool specimens by use of the QIAamp stool mini kit (QIAGEN) and subjected to both multiplex PCRs. The method accurately detected the presence of all gene products in 180 mg of feces seeded with $3 \times 10^8$ CFU of a single strain and was unable to detect prototype strains at concentrations less than $10^8$ CFU. Also, the presence of several *E. coli* strains together (each of them at $3 \times 10^8$ CFU) in a fecal sample allowed visualization of bands specific for each strain (data not shown). The detection limit was found to be approximately $1.7 \times 10^6$ CFU per g of feces.

This method was then directly applied to 36 stool specimens to detect diarrheagenic *E. coli* and *Shigella* spp. These specimens were obtained from infants hospitalized in Hospital São Paulo with diarrhea or other gastrointestinal alterations. The stools were watery, and no mucus or blood was present. Most of the children were dehydrated and needed treatment with fluids and electrolytes. None of the 36 infants received antibiotics, and the duration of diarrhea was less than 1 week (median, 5 days), starting before hospitalization. Each fecal sample, obtained upon admission, was directly subjected to both multiplex PCRs and was analyzed by conventional assays, such as biochemical identification, serotyping, and DNA hybridization with specific DNA probes (Table 3). Seven (19.4%) of the 36 fecal specimens were multiplex PCR positive: 2 specimens were positive for typical EPEC isolates (O111 and O142 serogroups), 3 were positive for EAEC isolates, and 2 were positive for atypical EPEC isolates in assay 1 (Fig. 1). The same results were obtained by colony DNA hybridization with specific DNA probes. All fecal samples tested were negative by PCR assay 2.

Both multiple PCR assays showed very high specificity when compared to conventional methods for detecting the virulence genes. This high specificity was demonstrated using several reference strains as well as clinical isolates. There was complete agreement between the results of single and multiplex PCRs for all reference strains tested. In an epidemiological study, we compared standard methods, including colony blot hybridization, with multiplex PCR assays for the identification of diarrheagenic *E. coli* and *Shigella* spp. in the diarrhea of 150

TABLE 2. Diarrheagenic isolates of *E. coli* and *Shigella* spp. from patients

| Patient | Age | *E. coli* group and/or *Shigella* sp. | Gene(s) and/or probe | No. of tested strains/no. of positive strains | |
|---|---|---|---|---|---|
| | | | | PCR | DNA probe |
| 4 | 9 mo | Atypical EPEC | *eae* | 3/3 | 3/2 |
| 5 | 1 yr 4 mo | Typical EPEC | *eae, bfpA* | 5/2 | 5/2 |
| 6 | 1 yr 8 mo | Atypical EPEC | *eae* | 3/2 | 3/2 |
| 7 | 2 mo | Typical EPEC | *eae, bfpA* | 2/2 | 2/2 |
| 8 | 3 mo | Typical EPEC | *eae, bfpA* | 3/2 | 3/2 |
| 10 | 1 yr | Typical EPEC | *eae, bfpA* | 1/1 | 1/1 |
| 14 | 6 mo | Atypical EPEC | *eae* | 3/2 | 3/2 |
| 17 | 7 mo | Typical EPEC | *eae, bfpA* | 3/3 | 3/3 |
| 25 | 2 yr | EIEC | *ipaH* | 2/1 | 2/1 |
| 27 | 2 yr | EIEC | *ipaH* | 3/1 | 3/1 |
| 29 | 2 yr | EAEC | CVD432 | 4/1 | 4/1 |
| 30 | 2 yr | EAEC | CVD432 | 3/2 | 3/2 |
| 35 | 5 mo 11 days | Atypical EPEC | *eae* | 2/1 | 3/1 |
| 39 | 1 yr 6 mo | EAEC | CVD432 | 3/3 | 3/3 |
| 42 | 3 mo | Typical EPEC | *eae, bfpA* | 5/2 | 5/2 |
| 44 | 1 yr 8 mo | Atypical EPEC | *eae* | 5/1 | 5/1 |
| 48 | 10 mo | *Shigella flexneri* | *ipaH* | 5/1 | 5/1 |
| 55 | 8 mo | EAEC | CVD432 | 3/1 | 3/1 |
| | | *Shigella flexneri* | *ipaH* | 4/4 | 4/4 |
| 66 | 12 mo | Typical and atypical EPEC | *eae, bfpA* | 5/4 | 5/4 |
| 75 | 1 yr 6 mo | EIEC | *ipaH* | 5/2 | 5/2 |
| 77 | 5 mo 21 days | Atypical EPEC | *eae* | 3/2 | 3/2 |
| 78 | 46 days | EAEC | CVD432 | 4/2 | 4/2 |
| 80 | 2 yr | *Shigella sonnei* | *ipaH* | 3/1 | 3/1 |
| 82 | 3 mo | EAEC | CVD432 | 4/3 | 4/3 |
| 85 | 9 mo | Typical EPEC | *eae, bfpA* | 4/3 | 4/3 |
| 96 | 1 yr 2 mo | EAEC | CVD432 | 5/1 | 5/1 |
| 99 | 1 yr 10 mo | Atypical EPEC | *eae* | 3/2 | 3/1 |
| 103 | 2 yr 6 mo | Atypical EPEC | *eae* | 3/2 | 3/3 |
| 114 | 1 yr 8 mo | *Shigella sonnei* | *ipaH* | 2/1 | 2/1 |
| 117 | 5 mo | Typical EPEC | *eae, bfpA* | 3/2 | 3/3 |
| 118 | 2 mo | Atypical EPEC, EAEC | *eae*, CVD432 | 4/2 | 4/2 |
| 127 | 3 yr | STEC | *eae*, $stx_1$ | 3/3 | 3/3 |
| 131 | 1 yr 2 mo | EAEC | CVD432 | 3/2 | 3/2 |
| | | *Shigella flexneri* | *ipaH* | 3/1 | 3/1 |
| 140 | 10 mo | Typical EPEC | *eae, bfpA* | 4/1 | 4/1 |
| 144 | 2 mo | EAEC | CVD432 | 6/1 | 6/1 |
| 150 | 2 mo | Atypical EPEC | *eae* | 3/3 | 3/1 |

children. There was total agreement between the results of multiplex PCRs and DNA hybridization for all tested isolates.

The PCR multiplex assays were also sensitive for the detection of diarrheagenic *E. coli*. Stool specimens spiked with $10^8$ CFU from cultures of prototype *E. coli* strains generated a specific PCR product which was visible on an ethidium bromide-stained agarose gel. This corresponds to $10^6$ bacteria per g of feces. Of course, many more strains should be tested before concluding that the PCR assays have 100% sensitivity.

Most importantly, the two multiplex PCR assays were also found to be effective for direct detection of EPEC, EAEC, ETEC, STEC, EIEC, and *Shigella* spp. in stool specimens from 36 patients with diarrhea. The specificities of both multiplex PCRs were evidenced by the absence of nonspecific PCR products in feces from children without any diarrheagenic *E. coli* or *Shigella* spp.

In conclusion, the two multiplex PCR assays presented in this paper correctly determined the presence of corresponding diarrheagenic *E. coli* and *Shigella* species virulence genes in all strains tested. Multiplex PCR assay 1 correctly identified 100% of EPEC and EAEC isolates directly in stool specimens. Although assay 1 cannot detect all EAEC isolates and assay 2 cannot distinguish EIEC from *Shigella* species or one *Shigella* species from another, these multiplex PCR assays offer a practical possibility for rapid identification of diarrheagenic *E. coli* and *Shigella* spp. and could be used in the routine diagnostic laboratory.

TABLE 3. Comparison of results with multiplex PCR, DNA probe hybridization, and conventional assays to detect diarrheagenic *E. coli* strains

| Stool sample | Diarrheagenic *E. coli* type | Target(s) detected[a]: | | Serotyping result[b] |
|---|---|---|---|---|
| | | By multiplex PCR assay 1 | With DNA probe | |
| 2 | Typical EPEC | *eae*, *bfpA* | *eae*, EAF | *E. coli* O111 |
| 8 | Typical EPEC | *eae*, *bfpA* | *eae*, EAF | *E. coli* O142 |
| 3 | EAEC | CVD432 | EAEC target | *E. coli* OND |
| 5 | EAEC | CVD432 | EAEC target | *E. coli* OND |
| 12 | EAEC | CVD432 | EAEC target | *E. coli* OND |
| 33 | Atypical EPEC | *eae* | *eae* | *E. coli* OND |
| 34 | Atypical EPEC | *eae* | *eae* | *E. coli* OND |

[a] EAF, EPEC adherence factor.
[b] OND, serogroup not determined.

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