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Identification of Enteropathogenic *Escherichia coli* by PCR-Based Detection of the Bundle-Forming Pilus Gene

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A rapid and simple method of detecting enteropathogenic *Escherichia coli* (EPEC) was developed. The procedure is based on amplifying by the PCR method a 326-bp region of the bundle-forming pilus gene of EPEC. The oligonucleotide DNA primers used in this procedure did not amplify DNA of any other bacterial enteropathogens tested. The procedure was 100% specific for EPEC strains that exhibit a characteristic pattern of attachment (localized adherence) to HeLa cells.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea among children in developing countries (3, 9). Techniques to differentiate EPEC strains from nonpathogenic strains or other strains of *E. coli* associated with diarrhea have been confined to research laboratories. In vitro, EPEC strains establish characteristic microcolonies on the surface of tissue culture cells (13). This pattern of adherence, termed localized adherence (LA), is significantly associated with diarrhea production, and a tissue culture assay to specifically detect EPEC was developed (6, 10, 13, 14). A 50- to 60-MDa plasmid called the enteroadherence factor (EAF) plasmid is necessary for most EPEC strains to exhibit LA on tissue culture cells (1). A probe to detect the EAF plasmid, called the EAF probe, was therefore developed to identify EPEC strains (11). Recently, the EAF plasmid was found to encode inducible fimbrial structures called bundle-forming pili (BFP) (5). BFP were suggested to mediate microcolony formation on HeLa cells in vitro (16). The BFP gene (*bfpA*) sequences from different EPEC strains show a high degree of homology and appear to be specific to EPEC (2, 15).

Recently, Giron and coworkers (4) used a probe derived from the structural gene of the BFP to determine the distribution of BFP among EPEC strains. The BFP gene probe proved to be more specific than the EAF gene probe in the identification of EPEC strains. No cross-reactivity with other enteric *E. coli* strains or other enteropathogens was noted with the BFP gene probe under high-stringency conditions. It was further suggested that the BFP gene probe may prove to be more useful than the EAF gene probe, as the *bfpA* gene has a defined role, in contrast to the as-yet-undefined role of the EAF probe sequence.

A PCR procedure has previously been applied to identify gene sequences from pathogenic enteric *E. coli* strains (7, 12, 17). This procedure relies on detecting DNA sequences of interest amplified by a set of synthetic oligonucleotide primers. The present study examined the use of specific PCR primers in the amplification of BFP gene sequences as a method of detecting EPEC organisms.

All bacterial strains examined by PCR were grown on tryptic soy agar overnight at 37°C. DNA was extracted from bacteria by resuspending the bacteria in a small volume (1 ml) of sterile deionized water and boiling the suspension for 10 min. Each set of PCR mixtures with the samples was accompanied by a sample of *E. coli* B171 (O111:NM) as a positive control and the EAF plasmidless derivative strain B171-4 as a negative control. The *E. coli* strains were kindly provided by Joy Wells at Centers for Disease Control and Prevention in Atlanta, Ga., and one strain of *E. coli* O55:H7 was provided by Luis R. Trabulsi at University of São Paulo, São Paulo, Brazil.

Primers capable of amplifying an internal 326-bp region of the gene sequence encoding the mature 19-kDa BFP protein were constructed. The primers used for PCR (EP1 [5′-AAT GGTGCTTGCGCTTGCTGC and EP2 [5′-GCCGCTTTA TCCAACCTGGTA]) were based on the published *bfpA* gene sequence (15). A 10-μl aliquot of the boiled bacterial suspension was mixed with 90 μl of a PCR mixture (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, and 0.5 U of *Taq* polymerase [Boehringer Mannheim, Indianapolis, Ind.]). DNA was amplified for 29 cycles (each cycle consisted of 30 s of denaturation at 94°C, 1 min of annealing at 56°C, and 2 min of primer extension at 72°C). The amplified DNA product was resolved by agarose gel electrophoresis and visualized under UV transillumination after the gel was stained with ethidium bromide.

The HeLa cell attachment assay was performed by the method described by Scaletsky et al. (13); a 3-h-incubation assay was used. The DNA hybridization using the EAF probe (kindly provided by Jim Kaper of University of Maryland) followed the method described by Nataro et al. (11).

Fifty-five *E. coli* and 51 non-*E. coli* bacterial strains were examined (Tables 1 and 2). Of the *E. coli* strains, only strains belonging to the classic EPEC serotypes produced an expected amplified DNA product of approximately 330 bp (Fig. 1). However, several strains belonging to nine different EPEC serotypes or serogroups failed to produce a PCR-amplified product (Table 2). None of these BFP-PCR-negative strains exhibited LA on HeLa cells. One strain of O55:H7, however, yielded a PCR-amplified product and was LA positive by the HeLa cell attachment assay.

The remaining strains, belonging to seven other EPEC serotypes, were BFP-PCR positive, hybridized with the EAF gene probe, and exhibited the typical LA to HeLa cells. None of the other non-EPEC groups of *E. coli* associated with diarrhea yielded a positive result by PCR. Strain B171-4 (which lacks the EAF plasmid and fails to show LA or hybridize with the EAF gene probe) was BFP-PCR negative.

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TABLE 1. PCR test results for bacteria other than *E. coli*

Species	No. of strains tested (% positive)
Streptococcus spp.	14 (0)
Pseudomonas spp.	9 (0)
Staphylococcus spp.	7 (0)
Salmonella spp.	6 (0)
Yersinia enterocolitica	6 (0)
Shigella spp.	5 (0)
Klebsiella spp.	2 (0)
Aeromonas hydrophila	1 (0)
Proteus mirabilis	1 (0)

A previous study reported hybridization of diffuse adherent *E. coli* DNA with a BFP gene probe under moderate-stringency conditions (15). Furthermore, DNA from *Salmonella* strains showed a moderate level of hybridization with a BFP gene probe (15). Several of these strains attached to HeLa cells in a pattern resembling LA. However, in this study, two of the *Salmonella* strains that produced microcolonies on HeLa cells (results not shown) failed to produce an amplified DNA product by PCR (Table 1).

The BFP primers used in this study proved to be specific (100%) for the identification of EPEC strains that exhibited the LA phenotype. Giron et al., in validating the use of *bfpA* as a probe to detect EPEC strains, found that among 103 strains that were LA positive, the *bfpA* probe hybridized to 102 (99%), while the EAF probe hybridized to 99 strains (96%) (4). For strains belonging to EPEC serotypes that we found were usually LA negative, Giron's group obtained similar results: among five strains of O55:H7, one was LA and *bfpA* positive, one was LA negative and *bfpA* positive, and the others were negative by both tests; among six O128:H2 strains, the only two that were LA positive were those that were *bfpA* positive; and three O26:H11 strains were all LA and *bfpA* negative (4).

The BFP of EPEC strains have been proposed to belong to the type IV fimbrial group. A number of different organisms express type IV pili, including *Vibrio cholerae*, *Neisseria* spp., and *Pseudomonas aeruginosa*. Although strains belonging to the former two species were not examined in this study, no amplification product was detected among any of the nine *P. aeruginosa* strains examined.

Interestingly, the BFP-PCR method failed to detect most strains belonging to nine distinct EPEC serotypes or serogroups: O26:NM, O44:H18, O55:H7, O111ab:H21, O111ab:H25, O114:NM, O119:NM, O126:H27, and O128. Except for one strain, none of these attached to HeLa cells in an LA pattern. Scotland et al. and Knutton et al. (8, 14) reported EAF probe-negative strains that belonged to these same groups. They reported that some of the EAF probe-negative strains showed LA or poor LA to HEp-2 cells while others showed an enteroaggregative pattern of attachment (8, 14). In our study, a 3-h-incubation attachment assay was used and no distinct patterns of attachment could be discerned for most of the strains tested, while Scotland et al. used a 6-h-incubation assay; this difference may account for the different patterns of attachment observed in the respective studies. One strain of O55:H7 that was PCR positive was also LA positive in our study. Hence, the BFP-PCR test appears to be specific for EPEC strains that exhibit LA on HeLa cells. It is possible that the failure to detect some of these EPEC strains with the EAF probe, the BFP-PCR test, or the tissue culture assay may be due to loss of the EAF plasmid or due to a deletion occurring within the plasmid during storage, passage, or natural infection.

TABLE 2. PCR and HeLa cell attachment assay results for *E. coli* strains

E. coli type and/or strain (*n*)	Serotype	PCR result	LA to HeLa cells
Enteropathogenic (32)			
1914-55	O26:NM	−	−
1176-83	O26:NM	−	−
1178-83	O26:NM	−	−
9082-83	O44:H18	−	−
2856-68	O44:H18	−	−
3138-87	O44:H18	−	−
5513-56	O55:NM	+	+
660-79	O55:H7	−	−
5644-62	O55:H7	−	−
9095-83	O55:H7	+	+
340.2	O55:H7	−	−
2861-59	O86:H34	+	+
B171	O111:NM	+	+
2966-56	O111:H2	+	+
6170-50	O111:H2	+	+
4012-76	O111ab:H21	−	−
3751-76	O111ab:H21	−	−
4016-76	O111ab:H21	−	−
4013-76	O111ab:H25	−	−
91-77	O111ab:H25	−	−
157-77	O111ab:H25	−	−
2858-77	O111ab:H25	−	−
4393-57	O114:NM	−	−
1174-83	O119:H6	+	+
1175-83	O119:H6	+	+
9108-83	O119:NM	−	−
362-72	O119:NM	−	−
270-72	O119:NM	−	−
1929-50	O126:NM	+	+
5194-55	O126:H27	−	−
764-55	O128ab:H12	−	−
1179-83	O142:NM	+	+
B171-4[c]		−	−
Enterotoxigenic (6)			
EDL1493	O6:H16	−	ND[a]
E2539-C1	O25:NM	−	ND
EDL1257	O27:H20	−	ND
TX-1	O78:H12	−	ND
EDL899	O148:H28	−	ND
423-70	O148:H28	−	ND
Enteroaggregative (5)[b]		−	−
Enteroinvasive (5)			
3187-87	O28ac:NM	−	−
978-77	O29:NM	−	−
3173-88	O121:NM	−	−
E11	O124:NM	−	−
3194-87	O144:H25	−	−
Enterohemorrhagic (3)[b]		−	−
Diffuse adherent (3)[b]		−	−

[a] ND, not done.
[b] All strains tested were negative in both assays.
[c] Derivative of *E. coli* B171 (O111:NM) lacking the 60-MDa EAF plasmid.

Since the LA phenotype of EPEC strains is strongly associated with the diarrheagenic potential of the strains (6, 10, 14), the primers used in this study offer a new advantage of more specifically detecting strains of EPEC associated with diarrhea. In areas of Brazil where EPEC strains are endemic, the EAF-negative classic EPEC serotypes account for <10% of the EPEC-associated diarrhea cases (6). In the study of Scotland et al., they constituted 20% of EPEC strains examined (14).

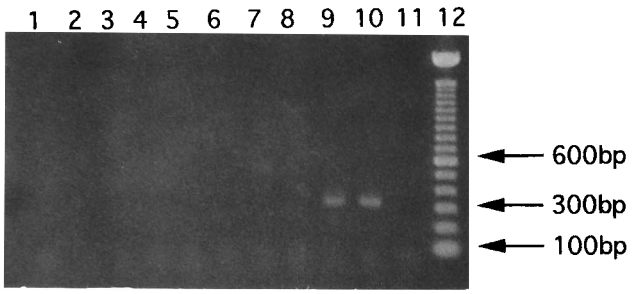

FIG. 1. Ethidium bromide-stained agarose gel of PCR-amplified products from extracted *E. coli* DNA amplified with primers EP1 and EP2. Lanes 1 and 3, diffuse adherent *E. coli*; lane 2, enteroaggregative *E. coli*; lanes 4 and 5, enterotoxigenic *E. coli* strains TX-1 and EDL899, respectively; lane 6, enterohemorrhagic *E. coli*; lane 7, enteroinvasive *E. coli* strain E11; lanes 8 to 11, enteropathogenic *E. coli* strains 4393-57, 1929-50, B171, and B171-4, respectively; lane 12, DNA molecular size marker (100-bp ladder).

Hence, this PCR test should be quite sensitive in detecting epidemiologically important EPEC strains in most settings.

In addition, by the PCR procedure, it took <5 h to process more than a dozen bacterial strains. The standard hybridization procedure with the EAF or *bfpA* probe may take 2 to 3 days to complete, depending on the availability of the plasmid-derived probe. The sample DNA extraction procedure has been reduced to simply boiling in water for 10 min in this PCR procedure, whereas the preparation of sample DNA for the standard hybridization procedure may take several hours. Finally, the PCR procedure required no radioisotopes or nonradioactive reporter constructs to label a hybridization probe. The technical advantages of the PCR-based detection of EPEC strains offer a renewed opportunity to study the epidemiology of this organism in developing countries where EPEC is a major cause of infantile diarrhea.

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