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# Use of an Alkaline Phosphatase-Conjugated Oligonucleotide Probe for the Gene Encoding the Bundle-Forming Pilus of Enteropathogenic *Escherichia coli*

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**An alkaline phosphatase-conjugated 29-base oligonucleotide probe was developed to detect the gene encoding the bundle-forming pilus of enteropathogenic *Escherichia coli*. The sensitivity and specificity of the probe versus the results of localized adherence in the HEp-2 cell assay and fluorescent actin staining assay positivity were 95.7 and 100%, respectively.**

Enteropathogenic *Escherichia coli* (EPEC) is a significant cause of acute and persistent infant diarrhea, especially in developing countries (2, 4). It has been clear that EPEC can adhere to HEp-2 cells and HeLa cells in vitro in a manner described as localized adherence (LA) (7, 12). EPEC is capable of producing characteristic histopathogenic lesions, so-called attaching-and-effacing (AE) lesions, on infected cells both in vivo (13) and in vitro (10). By using electron microscope, Giron et al. (6) observed that EPEC expresses rope-like bundles of filaments, termed bundle-forming pili (BFP), that create a network of fibers that bind the individual organisms together. Expression of BFP is strongly associated with LA to HEp-2 cells and the presence of the EPEC adherence factor (EAF) plasmid (3, 6, 17). The structural gene of BFP, *bfpA*, has been cloned from the EAF plasmid and sequenced (3, 17). The *bfpA* gene was considered to be specific for EPEC, because the homologous sequences were found only in EPEC but not in other enteropathogens (5). The diagnosis of EPEC has been based on O:H serotyping. As understanding of the pathogenic mechanisms of EPEC has improved, new diagnostic methods based on genetic virulence determinants have been developed. Besides LA in the HEp-2 cell assay, Knutton et al. (10) developed a sensitive and specific fluorescent actin staining (FAS) test to identify the AE lesions produced by EPEC in cell culture. A radiolabeled 1-kbp DNA probe, the EAF probe, has been used extensively to diagnose EPEC in epidemiologic studies (4, 12, 14). Recently, radiolabeled 850- and 579-bp DNA probes were also developed to detect the *bfpA* gene (5, 17). Furthermore, a PCR method amplifying the *bfpA* gene sequences was used to identify EPEC which showed LA to HeLa cells (8). Here, we report on the development of a 29-base alkaline phosphatase (ALP)-conjugated oligonucleotide probe and the evaluation of its potential application in identifying EPEC.

We examined 162 *E. coli* organisms of classical EPEC serotypes, as defined in Table 2; 65 enterotoxigenic *E. coli* (ETEC) isolates identified with DNA probes for heat-labile enterotoxin (18) and heat-stable enterotoxins (15); 10 enterohemorrhagic *E. coli* (EHEC) isolates identified by DNA probes for Shiga-like toxins I and II, purchased from Toyobo Co., Tokyo, Japan; 58 *E. coli* isolates isolated from nondiarrheal stool specimens; 103 *Salmonella* spp. including *Salmonella typhi*, *Salmonella paratyphi* A and B, *Salmonella enteritidis*, and *Salmonella dublin*; 25 *Shigella* spp.; and 50 *Vibrio* spp., as listed in Table 1.

The bacterial strains were grown on Luria-Bertani (LB) agar (for *Vibrio* spp., LB agar was supplemented with 3% NaCl) for hybridization tests and in LB broth for the HEp-2 cell assay and the FAS tests.

A linker-armed oligonucleotide probe with ligands was synthesized by the phosphoramidite method with a DNA synthesizer (type 381A; Applied Biosystems Inc., Foster City, Calif.) (9) and was purified on a mono-Q column (Pharmacia, Uppsala, Sweden). The selected nucleotide base sequence for the probe is from positions 260 to 287 of the published *bfpA* gene sequence (3). The sequence is 5′-AGACCATTATAGXGGA TTGGACTCAAC-3′. X denotes the deoxyuridine where a side arm is linked. Purified oligonucleotide was linked by using the homobifunctional reagent disuccinimidyl substrate and was covalently cross-linked with ALP as described previously (9). Conjugates were then dissolved in conjugate buffer (30 mM Tris-HCl, 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.005% $NaN_3$ [pH 7.6]) and kept at 4°C in the dark until they were used. The procedure used for hybridization with the probe was essentially similar to the method described previously (20). A single colony grown on LB agar was transferred to a GeneScreen Plus hybridization membrane (Biotechnology System, Boston, Mass.). The membrane was treated with 0.5 N NaOH–1% sodium *N*-lauroylsarcosine for 10 min at 50°C to lyse the bacterial cells and to denature the DNA and was then neutralized with 1 M Tris-HCl (pH 7.4) for 10 min. The cell debris were gently removed in 5× SSC (0.75 M NaCl plus 0.075 M sodium citrate)–1% sodium dodecyl sulfate (SDS). The denatured DNA was hybridized with 50 ng of the ALP conjugate in 1 ml of hybridization buffer (5× SSC, 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, 1% SDS) for 15 min at 50°C. The membrane was washed in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) containing 1% SDS for 10 min at 50°C with gentle shaking. The washed membrane was immersed in 10 ml of substrate buffer (0.2 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, and 0.1 mM $ZnCl_2$ [pH 8.5]) containing 44 μl of nitroblue tetrazolium (75 mg/ml in 70% *N*,*N*-dimethylform-

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TABLE 1. Detection of the *bfpA* gene among *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp.

| Species | No. of strains tested | No. of strains hybridizing with probe |
|---|---|---|
| EPEC | 162 | 45 |
| ETEC | 65 | 0 |
| EHEC (O157:H7) | 10 | 0 |
| *E. coli* from nondiarrheal stools | 58 | 0 |
| *Salmonella* spp. | 103 | 0 |
| *Shigella* spp. | 25 | 0 |
| *Vibrio* spp. | 50 | 0 |

amide) and 33 μl of 5-bromo-4-chloro-3-indolylphosphate, *p*-toluidine salt (50 mg/ml in *N*,*N*-dimethylformamide), in a plastic bag, and the mixture was incubated at 37°C for 1 h. The result of color development (dark blue) was determined with the naked eye. Three hours was required for the completion of all the procedures.

Adherence to HEp-2 cells was examined by the method of Cravioto et al. (1). The FAS test was performed as described by Knutton et al. (11). Hybridization with the 1-kbp *Bam*HI-*Sal*I EAF probe (14) labeled with digoxigenin-dUTP was performed to detect the EAF plasmid by the Genius system (Boehringer Mannheim GmbH, Mannheim, Germany).

Examination by transmission electron microscopy was done after the bacterial cells were cultured by the method described previously (6).

In order to evaluate the specificity of the *bfpA* probe, other *E. coli* strains and different enteropathogens as well as *E. coli* strains belonging to classical EPEC serotypes were examined with the *bfpA* probe. As a result, the *bfpA* probe hybridized only to EPEC, not to any other *E. coli* or other enteropathogens examined (Table 1). Among 162 *E. coli* isolates of classical EPEC serotypes examined, the prevalence of the *bfpA* gene was 27.8% (45 isolates). The frequency of *bfpA* varied widely among EPEC serogroups and serotypes (Table 2). The *bfpA*-positive rate (43.2%) among strains of the class I serogroups (O55, O111, O119, O126, O127, O128, and O142) was much higher than that (9.5%) among strains of the class II serogroups (O18, O26, O44, O86, O114, O125, and O158) ($P < 0.01$; chi-square test) as described by Nataro et al. (14). We determined whether the *bfpA* probe assay correlates with the HEp-2 cell assay and FAS test in identifying EPEC. In the HEp-2 cell assay, 47 strains of EPEC showed LA. Among the 47 strains, 38 strains showed large microcolonies of adherent bacteria on HEp-2 cells, with >50% cells having microcolonies and good LA, as described by Knutton et al. (11). The rest of the strains showed smaller microcolonies than the ones shown by the strains with good LA, with 20 to 40% of the cells having microcolonies and moderate LA. None of the 47 LA-positive strains were classified as having poor LA, as described by Knutton et al. (11). The HEp-2 cell assay and FAS test showed 100% agreement (Table 3). Of the 47 LA- and FAS test-positive strains, 2 strains showing moderate LA did not hybridize to the *bfpA* probe. However, the EAF probe hybridized to all the 47 LA- and FAS test-positive strains. By transmission electron microscopy, the pilus structure morphologically consistent with BFP, as described by Giron et al. (6), was observed on both the 45 *bfpA* probe assay-, LA-, and FAS test-positive strains and the 2 *bfpA* probe-negative but LA- and FAS test-positive strains (data not shown). These results suggest that failure of the *bfpA* probe to hybridize to the two strains may be due to nucleotide sequence divergence in the region of the *bfpA* probe. The serotypes of these two strains were O55:NM and O126:H27, respectively. On the basis of the data obtained in the present study, we demonstrated that the sensitivity and specificity of the *bfpA* probe versus LA in the HEp-2 cell assay and FAS test positivity were 95.7 and 100%, respectively. Sohel et al. (17) used cloned 579-bp DNA probes to detect the *bfpA* gene and attempted to find a *bfpA* homolog in *Salmonella* strains. This probe hybridized to some *Salmonella* strains showing an adherence pattern resembling LA under low- and moderate-stringency conditions but not under high-stringency conditions. In the present study, none of 103 *Salmonella* strains including *S. typhi*, *S. paratyphi* A and B, *S. enteritidis*, and *S. dublin* hybridized with the *bfpA* probe. Recently, PCR-based detection of the *bfpA* gene was reported (8), the sequences of the *bfpA* probe used in the present study were within the amplicon (326 bp) targeted in the PCR. The *bfpA* probe might be useful for confirming the results of PCR for the *bfpA* gene.

TABLE 2. Frequency of the *bfpA* gene among EPEC strains

| Serotypes | No. of strains examined | No. (%) of strains positive for *bfpA* probe |
|---|---|---|
| O18ac:NM | 6 | 1 (16.7) |
| O26:NM | 13 | 2 (15.4) |
| O26:H11 | 9 | 0 |
| O44:NM | 10 | 0 |
| O44:H18 | 8 | 0 |
| O55:NM | 9 | 4 (44.4) |
| O55:H6 | 2 | 0 |
| O55:H7 | 7 | 3 (42.9) |
| O86:H34 | 7 | 3 (42.9) |
| O111:NM | 6 | 4 (66.7) |
| O86:H2 | 5 | 3 (60.0) |
| O86:H12 | 3 | 1 (33.3) |
| O114:NM | 6 | 0 |
| O114:H2 | 3 | 1 (33.3) |
| O119:NM | 4 | 1 (33.3) |
| O119:H6 | 9 | 8 (88.9) |
| O125ac:NM | 5 | 0 |
| O125ac:H21 | 4 | 0 |
| O126:NM | 8 | 3 (37.5) |
| O126:H21 | 7 | 1 (14.3) |
| O127:NM | 5 | 0 |
| O127:H6 | 3 | 2 (66.7) |
| O128ab:NM | 5 | 1 (20.0) |
| O128ab:H2 | 8 | 3 (37.5) |
| O128ab:H7 | 3 | 0 |
| O142:H6 | 4 | 4 (100) |
| O158:H23 | 3 | 0 |
| Total | 162 | 45 (27.8) |

Use of the *bfpA* probe could provide a rapid (3 h for completion) and specific method for the detection of EPEC for epidemiologic studies and would be useful in settings in which radioisotopes are unavailable or their use is impractical.

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TABLE 3. Comparison of *bfpA* probe with HEp-2 cell assay and FAS test in identifying EPEC

| Hybridization with *bfpA* probe | FAS test result | LA in HEp-2 cell assay | No. of strains |
|---|---|---|---|
| + | + | + | 45 |
| − | + | + | 2 |
| − | − | − | 115 |

## REFERENCES


1. **Cravioto, A., R. J. Gross, S. M. Scotoland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3:**95–99.
2. **Cravioto, A., A. Tello, A. Navarro, J. Ruiz, H. Villafan, F. Uribe, and C. Eslava.** 1991. Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. Lancet **337:**262–264.
3. **Donnenberg, M. S., J. A. Giron, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. **6:**3427–3437.
4. **Escheverria, P., F. Orskov, I. Orskov, S. Knutton, F. Scenttz, J. E. Brown, and U. Lexomboon.** 1991. Attaching and effacing enteropathogenic *Escherichia coli* as a cause of infantile diarrhea in Bangkok. J. Infect. Dis. **164:** 550–554.
5. **Giron, J. A., M. S. Donnenberg, W. C. Martin, K. G. Jarvis, and J. P. Kaper.** 1993. Distribution of the bundle-forming pilus structural gene (*bfpA*) among enteropathogenic *Escherichia coli*. J. Infect. Dis. **168:**1037–1041.
6. **Giron, J. A., A. S. Y. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
7. **Gomes, T. A. T., M. A. M. Vieira, I. K. Wachamuth, P. A. Blake, and L. R. Trabulsi.** 1989. Serotype specific prevalence of *Escherichia coli* strains with EPEC adherence factor gene in infants with and without diarrhea in Sao Paulo, Brazil. J. Infect. Dis. **160:**131–135.
8. **Gunzburg, S. T., N. G. Tornieporth, and L. W. Riley.** 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. J. Clin. Microbiol. **33:**1375–1377.
9. **Jablonski, E., E. W. Moomaw, R. T. Tullis, and J. L. Ruth.** 1986. Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. Nucleic Acids. Res. **14:**6115–6128.
10. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. Mcneish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cell: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
11. **Knutton, S., A. D. Philips, H. R. Amith, R. J. Gross, R. Show, P. Watson, and E. Price.** 1991. Screening of enteropathogenic *Escherichia coli* in infants with diarrhea by the fluorescent-actin staining test. Infect. Immun. **59:**365–371.
12. **Levine, M. M., V. Prado, R. Robins-Browne, H. Lior, J. B. Kaper, S. L. Moseley, K. Gicquelais, J. P. Nataro, P. Vial, and B. Tall.** 1987. Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. J. Infect. Dis. **158:**224–228.
13. **Moon, H. W., S. C. Whipp, A. Argenzio, M. M. Levine, and R. A. Giianella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41:**1340–1351.
14. **Nataro, P. N., M. M. Baldini, J. P. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152:**560–565.
15. **Nishibuchi, M., M. Arita, T. Honda, and T. Miwatani.** 1988. Evaluation of a nonisotopically labeled oligonucleotide probe to detect the heat-stable enterotoxins gene of *Escherichia coli*. J. Clin. Microbiol. **26:**784–786.
16. **Robins-Browne, R. M.** 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev. Infect. Dis. **9:**28–53.
17. **Sohel, I., J. L. Puente, W. J. Murray, J. Vupio-Varkila, and G. K. Schoolnik.** 1993. Cloning and characterization of the bundle-forming pilin gene of enteropathogenic *Escherichia coli* and its distribution in Salmonella serotypes. Mol. Microbiol. **7:**563–575.
18. **Tamatsukuri, S., K. Yamamoto, S. Shibata, F. Leano, T. Honda, and T. Miwatani.** 1991. Detection of a heat-labile enterotoxin gene in enterotoxigenic *Escherichia coli* by densitometric evaluation using highly specific enzyme-linked oligonucleotide probes. Eur. J. Clin. Microbiol. Infect. Dis. **10:** 1048–1055.
19. **Yamamoto, T., M. Kaneko, S. Changchawalti, O. Serichantalergs, S. Ijuin, and P. Echeverria.** 1994. Actin accumulation associated with clustered and localized adherence in *Escherichia coli* isolated from patients with diarrhea. Infect. Immun. **62:**2917–2929.
20. **Yoh, M., K. Miyagi, Y. Matsumoto, K. Hayashi, Y. Takarada, Y. Yamamoto, and T. Honda.** 1993. Development of an enzyme-labeled oligonucleotide probe for cholera toxin gene. J. Clin. Microbiol. **31:**1312–1324.