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Colorimetric Detection of Heat-Labile Toxin-Encoding Gene of Enterotoxigenic *Escherichia coli* by PCR

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In the developing world, enterotoxigenic *Escherichia coli* (ETEC) strains which produce enterotoxins are a significant cause of morbidity and mortality. Heat-labile (LT) toxin PCR detection methods have been described, but they have limited applications in a routine laboratory setting. A colorimetric DNA method for the rapid amplification and detection of the LT toxin gene in ETEC strains is described. Target amplification together with colorimetric detection would overcome many of the limitations of conventional PCR. This paper describes a colorimetric PCR detection method specific for LT-gene-encoding ETEC strains. DNA was extracted from two representative colonies from each bacterial isolate and amplified by PCR. Digoxigenin was incorporated into the amplification product, permitting a one-step direct detection using anti-digoxigenin alkaline phosphatase-conjugated antibody. This technique was applied to the investigation of 70 *E. coli* isolates derived from clinical fecal samples obtained from an Irish population. Eleven percent of the samples were LT positive, confirming the applicability of this method. All LT-positive ETEC strains (controls and clinical isolates) were detected, and no false-positive results occurred.

The importance of enterotoxigenic *Escherichia coli* (ETEC) as a causative agent in childhood diarrhea is well recognized (18). Two groups of plasmid-encoded enterotoxins have been described: a heat-labile (LT) toxin (3) and a heat-stable toxin (10, 11, 16). To reduce the impact of ETEC strains, their epidemiology must be fully established. Epidemiological studies would be greatly facilitated by the availability of a technique which reliably detects low numbers of ETEC pathogens in food, water, and environmental materials.

The development of PCR permits direct detection of a nucleic acid target (12), and numerous reports describe its application in clinical diagnostics (9). PCR can be used to screen for numerous pathogens simultaneously by the multiplex approach (4, 5). A great need exists for a highly specific, rapid and nonisotopic PCR assay to overcome the practical difficulties associated with current detection formats. This paper describes a simple one-step amplification-colorimetric assay capable of identifying LT-gene-encoding ETEC strains with a detection range of between 10 and 100 CFU. Seventy *E. coli* isolates from clinical fecal samples from an Irish population were analyzed by this approach, and these results are discussed.

ETEC control strains used in this study were obtained from the Central Public Health Group, Collindale (United Kingdom), and are listed in Table 1. Clinical isolates of ETEC 73, *Vibrio cholerae*, *Salmonella enteritidis*, *Shigella sonnei*, and *Campylobacter jejuni* were also studied (Table 1) in order to confirm oligonucleotide primer specificity. All organisms were examined for LT toxin production by the Bicken agar diffusion method (7a) (Sigma) and the Y1 mouse adrenal cell assay (14). To amplify the LT gene target, the following oligonucleotide primers were used (6): LT-1 (forward direction), 5′-TTACGG CGTTACTATCCTCTCTA-3′ (23-mer); and LT-2 (reverse direction), 5′-GGTCTCGGTCAGATATGTGATTC-3′ (23-mer). LT-1 and LT-2 anneal to the B subunit of the LT gene at nucleotide positions 44 to 66 and 298 to 318 respectively. (Primers were purchased from Research and Development Systems, Abingdon, United Kingdom, and purified by polyacrylamide gel electrophoresis before use.) Both primers have a calculated G+C content of 46%. Each amplification was carried out in a total reaction volume of 50 μl containing 25 pmol each of primers LT-1 and LT-2, 5 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9], 1% Triton X-100), 3 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 8 μl of deoxyribonucleotide mixture containing 1.25 mM dATP, dCTP, or dGTP or 1.21 mM dTTP, and 0.04 mM digoxigenin (DIG)-dUTP (Boehringer, Mannheim, Germany). A crude sample of target DNA was prepared by resuspending two colonies of each test organism, from an overnight tryptic soy agar plate in 10 μl of lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.1% Tween 20). After the sample was heated to 99°C for 5 min, cell debris was removed by centrifugation (20,000 × *g*) in a microcentrifuge for 2 min. Two microliters of this supernatant was used for each amplification reaction. The final reaction mixture was overlaid with 50 μl of sterile mineral oil. All samples were amplified in a Techne Programmable Dry Block Thermocycler (Techne PHC-2/UK), with the following cycling parameters: 4 min at 92°C (1 cycle); 1 min at 92°C, 30 s at 60°C, and 1 min at 72°C (25 cycles); and 4 min at 72°C (1 cycle) to complete the extension. In order to minimize the risk of contamination by the previously amplified products, reagents were divided into aliquots and stored at −20°C and the DNA preparation-electrophoresis process was physically separated from the PCR reagent mixing area. These measures together with inclusion of appropriate controls for each assay ensured the authenticity of data obtained.

The DIG-labelled PCR product was recovered by precipitation in 0.14 volumes of 4 M LiCl and 4 volumes of cold ethanol at −20°C, as recommended by the manufacturer. The labelled DNA pellet was then resuspended in a final volume of 400 μl of sterile dH_2O. For solid-phase detection, 100 μl of DIG-

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TABLE 1. Bioassay and PCR amplification results of strains in this study

Bacterium and strain	Bioassay result	PCR result	Reference or source
E. coli			
PHLS 8068	LT^+	LT^+	6
PHLS 5541	LT^+	LT^+	6
73	LT^+	LT^+	Clinical isolate
PHLS 7539	LT^-	LT^-	6
PHLS 9060	LT^-	LT^-	6
S. enteritidis	LT^-	LT^-	Clinical isolate
S. sonnei	LT^-	LT^-	Clinical isolate
C. jejuni	LT^-	LT^-	Clinical isolate
V. cholerae	LT^+	LT^-	Clinical isolate

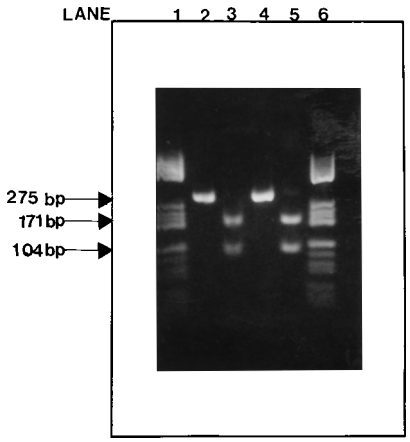

FIG. 1. Agarose gel electrophoresis of amplification products. Lane 1, molecular weight markers; lane 2, ETEC PHLS 5541; lane 3, ETEC PHLS 5541 digested with *Cfo*I; lane 4, ETEC 73; lane 5, ETEC 73 digested with *Cfo*I; lane 6, molecular weight markers. The lengths of the amplification products (arrows) are indicated on the left.

labelled DNA sample was applied to a nylon membrane (Millipore) with a dot blot apparatus (Millipore Milliblot D System). DIG-labelled DNA was directly detected with a polyclonal anti-DIG antibody conjugated to alkaline phosphatase as described by the manufacturer (DNA labelling and detection kit; Boehringer). Alkaline phosphatase (Boehringer) catalyzed the oxidation and reduction of 5-bromo-4-chloro-3-indolylphosphate and 4-nitroblue tetrazolium chloride, respectively, resulting in an enzyme-linked color reaction. This detection phase can also be adapted to a microtiter plate format with spectrophotometrically determined endpoints. For this process, DIG-labelled amplicons are captured directly on a microtiter plate previously coated with polyclonal anti-DIG antibody, thereby facilitating detection of the immobilized amplicons via an anti-DIG–alkaline phosphatase-conjugated antibody. The color produced may subsequently be further amplified by using the NADPH-diaphorase system (4a).

A 275-bp internal DNA fragment from the LT gene delineated by primers LT-1 and LT-2 was amplified by PCR from all LT-toxin-containing control organisms, as demonstrated by agarose gel electrophoresis (Fig. 1). This 275-bp band was also detected in the ETEC 73 clinical isolate (Table 1). ETEC PHLS 7539 which does not contain the LT-toxin-encoding gene (Table 1) together with the four enteric pathogens *S. enteritidis*, *S. sonnei*, *C. jejuni*, and *V. cholerae* did not produce this 275-bp band. It is interesting that at the DNA level, no cross amplification between the ETEC LT gene and *V. cholerae* occurred, despite the fact that the toxins are immunologically similar (3). As previously reported (6), the amplified fragment had a *Cfo*I restriction site (5′-GCGC-3′) and in order to authenticate the amplification product, the 275-bp DNA fragment was tested for the presence of this restriction site. Digestion with *Cfo*I generates two DNA bands of 104 and 171 bp. All LT-gene-encoding ETEC strains produced the expected digest products (Fig. 1, lanes 2 and 4).

Incorporation of DIG-dUTP into the PCR product allowed direct colorimetric detection. ETEC strains PHLS 8068, PHLS 5541, and 73 yielded a colored precipitate, indicating a positive result (Fig. 2, dots 1 to 3). The four enteric pathogens along with ETEC PHLS 7539 were all LT gene negative when tested (Fig. 2, dots 4 to 8). These results clearly demonstrate the ability of the assay to discriminate between LT-gene-positive and -negative enteric pathogens. In particular, unlike the multiplex PCR reported by Abe et al. (1), no cross-reaction with the *V. cholerae* toxin gene was detected, which is obviously important in epidemiological studies in areas where cholera is endemic. To determine the sensitivity of the assay, an LT-gene-positive ETEC strain was cultured in nutrient broth for 12 h. The culture was serially diluted, and the cell numbers were enumerated. Two-microliter aliquots were removed from each sample and lysed in 10 μl of lysis buffer (as described above) prior to PCR and dot blot detection. The results obtained demonstrate that as few as 14 bacterial CFU are reliably detected.

To demonstrate the usefulness of this approach, 70 *E. coli* fecal isolates (identified by API-20 E, bioMerieux, Marcy l'Etoile, France) were tested for the presence of the LT-toxin-encoding gene. All isolates were cultured individually, and DNA was extracted as described above. At this point and for economic reasons, the collection was divided into seven groups, each containing pooled DNA extracts from 10 individual isolates. Supernatant (2 μl) containing DNA was subjected to PCR, following which the DIG-labelled product was detected directly. Results showed (Fig. 3A) that six of the seven groups gave a positive color signal. The isolates in these groups were then individually tested for the LT gene. Positive LT-gene-containing organisms were identified (Fig. 3B), and they accounted for 11% of the collection. All of the 70 ETEC isolates were assayed by Bicken and Y1 adrenal cell methods (14). The PCR and bioassay results concurred. A similar ob-

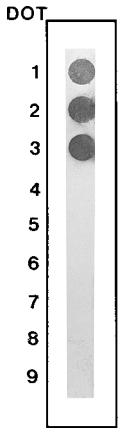

FIG. 2. Dot blot hybridization. Dot 1, ETEC PHLS 8068; dot 2, ETEC PHLS 5541; dot 3, ETEC 73; dot 4, ETEC PHLS 7539; dot 5, *S. enteritidis*; dot 6, *S. sonnei*; dot 7, *C. jejuni*; dot 8, *V. cholerae*; dot 9, reaction blank.

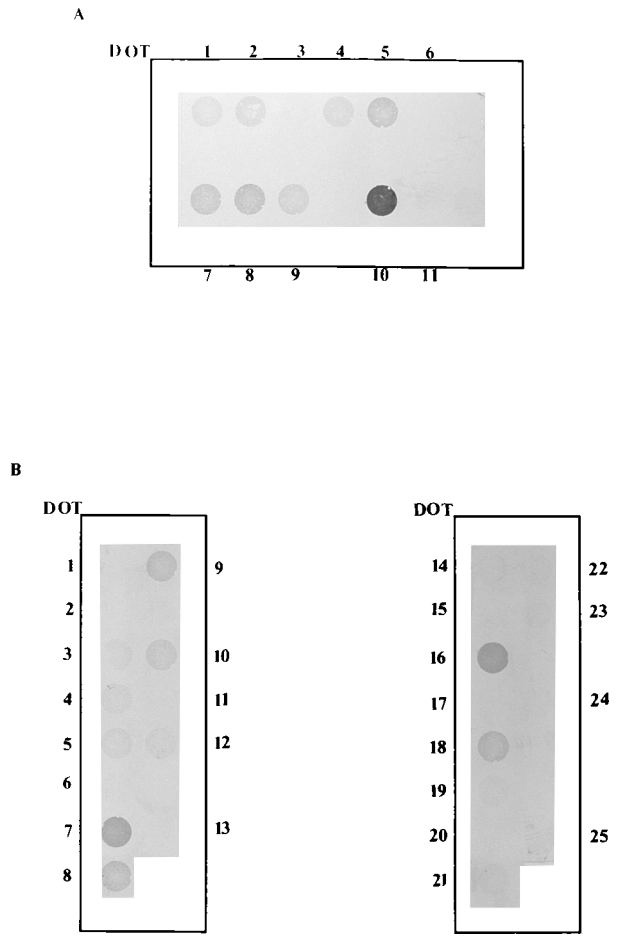

FIG. 3. (A) Pooled PCR. Dot 1, *E. coli* 1-10; dot 2, *E. coli* 11-20; dot 3, *E. coli* 21-30; dot 4, *E. coli* 31-40; dot 5, positive control (ETEC PHLS 5541); dot 6, reaction blank; dot 7, *E. coli* 41-50; dot 8, *E. coli* 51-60; dot 9, *E. coli* 61-70; dot 10, positive control (ETEC PHLS 5541); dot 11, reaction blank. (B) Individual PCR of *E. coli* isolates. Dots 1 to 10, *E. coli* 1-10; dot 11, reaction blank; dot 12, positive control (ETEC PHLS 5541); dot 13, negative control (ETEC PHLS 9060); dots 14 and 15, *E. coli* 11-12; dot 16, positive control (ETEC PHLS 5541); dots 17 to 24, *E. coli* 13-20; dot 25, negative control (ETEC PHLS 9060).

servation was previously noted by other investigators (17). In this assay, variations in the intensity of the positive controls due to the variability encountered during the extraction of template DNA were noted. Since crude DNA preparations were analyzed in all cases, this observation can be expected (Fig. 3A and B). This method was applied to seeded chlorinated and nonchlorinated water samples, and the DNA target was recovered in each instance (data not shown). One-liter volumes of water were filtered, and the filters were placed on nutrient media and incubated overnight at 37°C. DNA was extracted from six *E. coli* colonies from each filter (as described above), and a pooled PCR was performed, resulting in a detection limit of between 10 and 30 CFU/100 ml for both chlorinated and nonchlorinated water. However, a drawback of using PCR on environmental samples such as fecal and wastewater samples is the presence of inhibitors and contaminants that interfere with the PCR process. Methods of removing the majority of environmental contaminants have been reported, but as yet a simple and universal protocol for removing all possible inhibitors needs to be described (2). The inclusion of positive internal controls in such samples may allow the monitoring of inhibition, preventing false-negative results.

In conclusion, we describe a colorimetric PCR which is both specific (no reaction with other enteric pathogens or LT-gene-negative ETEC) and sensitive (detection limit of 10 to 100 CFU) for LT and can be applied to isolates from clinical material. The incorporation of a DIG-labelled nucleotide in the assay allowed detection of the amplified DNA target by a simple and direct colorimetric assay (8). Many diagnostic laboratories are familiar with the use of alkaline phosphatase conjugate-based detection formats. As an alternative label, DIG provides signal-to-noise ratios superior to those of biotin-labelled DNA (13). The use of DIG-labelled amplicons rather than DIG-labelled probes results in an assay with increased sensitivity because of the high yield of PCR product. This assay is also less time-consuming, in that solid-phase detection of labelled amplicons can be completed in less than 2 h, than probe hybridization, which involves many steps including an overnight incubation. Other solid-phase PCR formats (15) detect positive organisms by hybridization with labelled probes. Not only does our assay employ a simulated-enzyme-linked immunosorbent assay-based methodology common to many diagnostic disciplines, it also offers the methodology in a potentially automated format (7) which may be readily applied in a clinical laboratory setting. The sensitivity of this method could be particularly relevant in epidemiological investigations, wherein low organism numbers may be encountered in food, water, and human or animal carriers.

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