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Detection of Shiga Toxin-Producing *Shigella dysenteriae* Type 1 and *Escherichia coli* by Using Polymerase Chain Reaction with Incorporation of Digoxigenin-11-dUTP

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A technique has been developed for the detection of Shiga toxin- and Shiga-like toxin type I (ShT/SLT-I)-producing *Shigella dysenteriae* type 1 and *Escherichia coli* by using the polymerase chain reaction with the incorporation of digoxigenin-11-dUTP. Target DNA liberated from whole cells was amplified, using primer pairs homologous to the A-subunit genes of ShT/SLT-I. The TTP analog digoxigenin-11-dUTP was incorporated into the reaction mixture, permitting nonradioactive labeling of the amplified DNA. The labeled polymerase chain reaction products were hybridized to specific gene sequences immobilized on a nitrocellulose membrane and detected by using an alkaline phosphatase-conjugated antibody to digoxigenin and the enzyme substrates. Toxin-producing strains of *E. coli* and *S. dysenteriae* type 1 were identified as colored spots on the membrane. Because this technique does not require DNA purification, gel electrophoresis, or radioactive DNA probes, it is suitable for the clinical detection of ShT/SLT-I-producing strains of *S. dysenteriae* type 1 and *E. coli*.

Diarrheagenic *Escherichia coli* strains have been classified into five groups on the basis of the mechanism of pathogenesis (18). One of these groups, enterohemorrhagic *E. coli* (EHEC), causes hemorrhagic colitis, thrombotic thrombocytopenic purpura, and the potentially fatal hemolytic uremic syndrome, which is particularly severe in children and the elderly (14). EHEC strains produce one or more types of a cytotoxin which was originally characterized as a Vero cell toxin by Konowalchuk and coworkers (16). The designation Shiga-like toxin (SLT) was adopted for the cytotoxins produced by EHEC following the demonstration that they are closely related to Shiga toxin (ShT), which is produced by *Shigella dysenteriae* type 1 (21, 25). Members of the SLT family include SLT type I (SLT-I), the antigenically distinct SLT type II (SLT-II), and SLT type II variant (SLT-IIv) (22, 36). Sequence analysis revealed that the ShT (*stx*) and *slt*-I genes are essentially identical (34), while the *slt*-II (10) and *slt*-IIv (37) genes are approximately 55% related to *stx* and *slt*-I (hereafter referred to as *stx*/*slt*-I). Hemolytic uremic syndrome, which frequently follows a self-limiting diarrheal disease, may be due to SLT-induced damage to the vascular endothelium (14).

Outbreaks of EHEC-associated diarrhea in the United States, Canada, and developing nations are usually linked to the consumption of contaminated beef or milk, implicating cattle as a reservoir for these pathogens. Because the serious complications of EHEC infections might be prevented by early identification of the pathogen in contaminated food samples and fecal specimens, there is a need for a detection method suitable for the clinical laboratory. Unfortunately, the methods currently available for the detection of SLT-producing EHEC suffer from limited applicability or a requirement for radioactive DNA probes. Biochemical assays based on sorbitol fermentation are capable of detecting EHEC of only a single serotype (5, 9). The cytotoxicity assay (8, 15) requires tissue culture facilities and several days for accurate interpretation of the results, making it too time-consuming and expensive for screening large numbers of samples. Enzyme-linked immunosorbent assays for the detection of EHEC (1, 3, 35) necessitate isolation of monoclonal antibodies to each member of the SLT family. Southern blots or colony hybridizations using DNA probes derived from gene fragments (19, 24, 31, 32, 39) or synthetic oligonucleotides (2, 12) are very sensitive and specific but are less effective when a nonradioactive label is used. Recently, there have been reports that EHEC strains (13, 27) and invasive *Shigella* species (6, 17) can be detected by using the polymerase chain reaction (PCR), a technique which is relatively rapid, highly specific, and very sensitive (28). However, because these PCR methods may require purified DNA, visualization of the amplified product by agarose gel electrophoresis, or Southern blot hybridization with a radioactive DNA probe, they are not entirely appropriate for screening large numbers of samples in diagnostic laboratories. Olive (26) adapted the PCR technique for the identification of enterotoxigenic *E. coli* strains and used an alkaline phosphatase (AP)-labeled DNA probe in a spot blot assay to detect amplified products, making it better suited to clinical laboratories.

The purpose of this study was to develop a PCR technique which can be used for the identification of *S. dysenteriae* type 1 and EHEC strains which produce ShT and SLT-I (hereafter referred to as ShT/SLT-I). PCR products amplified with *stx*A and *slt*-IA (*stxA*/*slt*-IA) gene primers were labeled by direct incorporation of the TTP analog digoxigenin-11-dUTP (DIG) into the reaction mixture, hybridized with homologous DNA immobilized on a nitrocellulose membrane, and detected immunologically by using an AP-conjugated antibody to digoxigenin.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacterial strains used in this study. These include clinical isolates of EHEC and *S. dysenteriae* type 1, laboratory strains of *E. coli* which carry a recombinant plasmid with the *stx* operon, and laboratory strains lysogenized with SLT-I- and SLT-II-converting bacteriophage.

TABLE 1. Bacterial strains used in this study

Strain	EHEC serotype	Toxin type	Reference
E. coli			
H19	O26:H11	SLT-I	33
933	O157:H7	SLT-I + SLT-II	25
CL-40	O157:H7	SLT-I + SLT-II	30
E32511	O157:NM[a]	SLT-II	38
86-24	O157:H7	SLT-II	30
S1191	O139:ND[a]	SLT-IIv	22
C600			20
C600/H19A lysogen		SLT-I	36
C600/933W lysogen		SLT-II	36
HB101(pBR329)			20
HB101(pEW3)		ShT	11
S. dysenteriae			
60R		ShT	4
3818T		ShT	7
725-78[b]			7

[a] Abbreviations: NM, nonmotile; ND, not determined.
[b] *S. dysenteriae* 725-78 is a derivative of strain 3818T which does not produce ShT as a result of a deletion of the *stx* operon (7, 23).

Oligonucleotide primers. Oligonucleotides were synthesized by using an ABI model 380A DNA synthesizer with phosphoramidite chemistry. Two primers homologous to the A-subunit genes of *stx/slt*-I (34) were used to amplify a 680-bp fragment. The sequence of the 5′ primer is GACAG GATTTGTTAACAGG, and the 3′ primer is TTCCAGTTAC ACAATCAGGC. The 5′ and 3′ primers are 68 and 60% homologous, respectively, to the corresponding sequence of the *slt*-IIA gene (10).

Whole-cell DNA purification. Cells from a 30-ml culture grown overnight were collected by centrifugation and resuspended in 5 ml of a solution consisting of 25 mM Tris (pH 8.0), 10 mM EDTA, and 50 mM glucose and treated with 5 mg of lysozyme and 1% sodium dodecyl sulfate for 30 min at 4°C. Whole-cell DNA was purified from the bacterial cell lysate by extraction with equal volumes of 1 M Tris (pH 8.0)-saturated phenol, phenol-chloroform (50% [vol/vol]), and chloroform. Two volumes of 95% ethanol were added to the aqueous phase of the final extraction, and the DNA was collected by spooling on a glass rod.

PCR. One microgram of purified whole-cell DNA or a small portion of a bacterial colony (approximately 1 mm in diameter) was transferred from LB agar (20) to a 20-μl PCR mixture composed of 50 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 20 μM (each) of the 5′ and 3′ primers, and 125 μM (each) dATP, dGTP, dCTP, and TTP. The reaction mixture was covered with mineral oil and heated for 10 min at 95°C to lyse the bacterial cells and destroy proteases which may degrade the polymerase. One unit of thermostable DNA polymerase (Replinase; Dupont, NEN Research Products, Boston, Mass.) was added, and the samples were incubated for 30 cycles in a Perkin-Elmer-Cetus (Norwalk, Conn.) thermal cycler as follows: 94°C for 1 min, 55°C for 2 min, 72°C for 2 min with a 1-s extension at each cycle, and a final 10-min extension at 72°C.

The amplified DNA products were either labeled isotopically by including [α-^{32}P]dCTP (2 μCi; 3,000 Ci/mmol) (Dupont NEN) in a 20-μl PCR mixture which contained 125 μM dATP, 125 μM dGTP, 125 μM TTP, and 62.5 μM dCTP or labeled chemically by including 17.5 μM DIG (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in a 20-μl PCR mixture which contained 50 μM dATP, 50 μM dGTP, 50 μM dCTP, and 32.5 μM TTP.

Agarose gel electrophoresis and Southern blot analysis. [α-^{32}P]dCTP-labeled PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The agarose gel containing the labeled DNA was dried, covered with plastic wrap, and exposed to X-ray film for 3 min.

DIG-labeled PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Because immunologic detection of the DIG-labeled PCR products in a dried agarose gel was unsatisfactory, Southern blot analysis was used. The DIG-labeled DNA was alkali denatured and passively transferred from the agarose gel to a nitrocellulose membrane (20) which was processed for immunologic detection.

Selection of DIG-labeled PCR products by hybridization. A spot blot assay was developed for selection of the DIG-labeled PCR products. Plasmid pEW3 which carries the *stx/slt*-I operon (11) was purified by the alkaline sodium dodecyl sulfate minilysate procedure (20). Samples (5 μl each) of a solution containing pEW3 (5 mg/ml) in 30 mM Tris (pH 7.8) and 5 mM EDTA were denatured by boiling for 10 min and then were spotted onto a nitrocellulose membrane, which was baked for 2 h at 80°C in vacuo to immobilize the plasmid DNA. Unreacted sites on the membrane were blocked with 200 μg of denatured herring sperm DNA per ml as previously described for Southern blot analysis (20). The nitrocellulose membrane was air dried and 0.5-μl portions of the DIG-labeled PCR products were spotted onto immobilized plasmids to permit hybridization between the amplified DNA and the *stx/slt*-I gene sequences. The membrane was air dried briefly and washed for 1 h at 68°C in 0.1× SSC (0.1× SSC is 15 mM NaCl plus 1.5 mM sodium citrate) to remove the unincorporated DIG and nonspecifically labeled DNA fragments. DIG-labeled PCR products amplified by the *stxA/slt*-IA gene primers were identified as colored spots following immunologic development of the membrane.

Immunologic detection of DIG-labeled PCR products. DIG-labeled DNA transferred to a nitrocellulose membrane by Southern blotting or hybridized to membrane-bound pEW3 was identified by using a nucleic acid detection kit as described by the manufacturer (Boehringer Mannheim Biochemicals). The membranes were blocked for 30 min using 1% (wt/vol) nonfat dry milk in buffer 1 (100 mM Tris [pH 7.5], 150 mM NaCl). Following a brief wash in buffer 1, the membranes were incubated for 30 min with a 1:5,000 dilution of AP-conjugated anti-digoxigenin Fab fragments in buffer 1. The membranes were washed for 30 min in buffer 1, equilibrated for 2 min in buffer 2 (100 mM Tris [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$), and developed using 40 μl of the AP substrates nitroblue tetrazolium (75 mg/ml in dimethyl formamide) and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethyl formamide) per 10 ml of buffer 2. Color development was terminated in 2 to 3 min by briefly washing the membranes in 10 mM Tris (pH 8.0)–1 mM EDTA and air drying.

RESULTS

PCR with [α-^{32}P]dCTP incorporation. Isolated colonies of EHEC strains which produce SLT-I, SLT-II, or SLT-IIv, as well as ShT-producing and nontoxigenic strains of *S. dysenteriae* type 1, were transferred directly from solid medium to the PCR mixture to assess the feasibility of a rapid lysis step prior to amplification. The bacterial cells were lysed, and

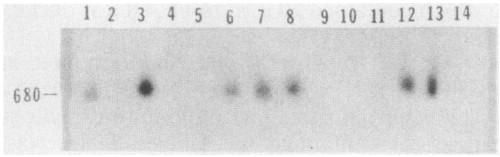

FIG. 1. Autoradiograph of dried agarose gel showing the 680-bp DNA fragment amplified by *stxA/slt*-IA primers with the incorporation of [α-^{32}P]dCTP. Lanes: 1, *E. coli* HB101(pEW3); 2, HB101(pBR329); 3, *E. coli* C600 lysogenized with SLT-I-converting phage H19A; 4, C600 lysogenized with SLT-II-converting phage 933W; 5, C600; 6, *E. coli* 933; 7, *E. coli* CL-40; 8, *E. coli* H19; 9, *E. coli* E32511; 10, *E. coli* 86-24; 11, *E. coli* S1191; 12, *S. dysenteriae* 60R; 13, *S. dysenteriae* 3818T; 14, *S. dysenteriae* 725-78.

whole-cell DNA was released by exposing the PCR mixture to 95°C for 10 min. Thirty PCR cycles were used to amplify a 680-bp fragment of DNA specified by two synthetic oligonucleotide primers homologous to the *stxA/slt*-IA gene sequence. Incorporation of [α-^{32}P]dCTP into the PCR mixture increased the sensitivity of the assay and permitted visualization of the amplified DNA fragment by autoradiography. As shown in Fig. 1, the predicted 680-bp band was detected in the whole-cell DNA of *E. coli* and *S. dysenteriae* strains which produce ShT/SLT-I but not from *E. coli* strains which produce SLT-II or SLT-IIv or do not produce toxin. This indicated that the incorporation of a labeled deoxynucleoside triphosphate into the PCR mixture had no effect on the amplification reaction and permitted the sensitive and rapid identification of toxigenic strains. However, this technique was of limited use because of its requirement for agarose gel electrophoresis and radioactive labeling.

PCR with DIG incorporation. A nonradioactive detection method was developed by incorporating the chemically labeled TTP analog DIG, rather than [α-^{32}P]dCTP, into the PCR mixture. The DIG-labeled PCR products were separated from the unincorporated label and nonspecifically amplified DNA fragments by agarose gel electrophoresis, transferred by Southern blotting to a nitrocellulose membrane, and developed by using an immunologic reagent. As shown in Fig. 2, exposure of the membrane to an AP-conjugated anti-digoxigenin antibody followed by development with the enzyme substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium revealed the predicted 680-bp band corresponding only to those strains which produce ShT/SLT-I and not the *E. coli* strains which produce SLT-II or the nontoxigenic controls. This indicated that the incorporation of DIG had no effect on the amplification reaction and that the specificity of this nonradioactive detection method was comparable with that of the method

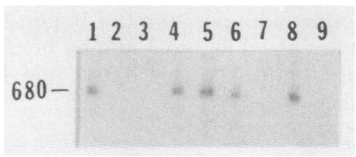

FIG. 2. Southern blot analysis showing 680-bp PCR product amplified by *stxA/slt*-IA primers with the incorporation of DIG. The Southern blot was developed with an AP-conjugated antibody to digoxigenin and the enzyme substrates. Lanes: 1, *S. dysenteriae* 60R; 2, *E. coli* E32511; 3, *E. coli* 86-24; 4, *E. coli* H19; 5, *E. coli* CL-40; 6, *E. coli* 933; 7, *E. coli* C600 lysogenized with SLT-II-converting phage 933W; 8, C600 lysogenized with SLT-I-converting phage H19A; 9, C600.

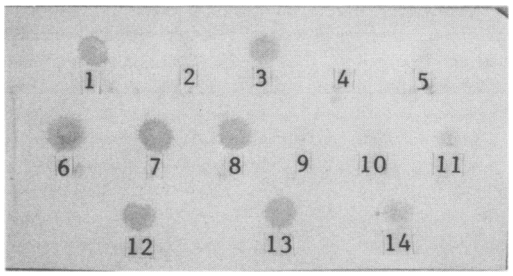

FIG. 3. Spot blot analysis of DIG-labeled PCR products amplified from whole-cell lysates by using *stxA/slt*-IA primers. Samples: 1, *E. coli* HB101(pEW3); 2, HB101(pBR329); 3, *E. coli* C600 lysogenized with SLT-I-converting phage H19A; 4, C600 lysogenized with SLT-II-converting phage 933W; 5, C600; 6, *E. coli* 933; 7, *E. coli* CL-40; 8, *E. coli* H19; 9, *E. coli* E32511; 10, *E. coli* 86-24; 11, *E. coli* S1191; 12, *S. dysenteriae* 60R; 13, *S. dysenteriae* 3818T; 14, *S. dysenteriae* 725-78.

using [α-^{32}P]dCTP. However, this technique was not entirely suitable for use in a clinical laboratory because of its requirement for agarose gel electrophoresis and Southern blot analysis.

Spot blot detection of DIG-labeled PCR products. Repeated ethanol precipitations or column chromatography failed to adequately separate the *stxA/slt*-IA primer-specified PCR products from the unincorporated DIG and nonspecifically amplified DNA sequences for a spot blot assay. Therefore, the DIG-labeled PCR products were selected by hybridization with homologous *stx/slt*-I gene sequences immobilized on a nitrocellulose membrane. Plasmid pEW3, a recombinant which carries the *stx/slt*-I operon (11), was heat denatured, spotted onto a nitrocellulose membrane, and immobilized by baking. The DIG-labeled PCR products of ShT/SLT-I-producing strains hybridized with the immobilized plasmid DNA, while the nonspecifically amplified DNA and unincorporated DIG were removed by a single high-stringency wash. As shown in Fig. 3, immunologic detection using an AP-conjugated antibody to digoxigenin and the AP substrates permitted the identification of ShT/SLT-I-producing strains of *S. dysenteriae* and *E. coli* as colored spots while nontoxigenic strains and those strains which produced SLT-II or SLT-IIV were faintly colored or colorless.

The PCR products of two strains, *S. dysenteriae* 725-78 (which is a nontoxigenic derivative of strain 3818T [23]) and *E. coli* S1191 (which produces SLT-IIv [22]), were detected as faintly colored spots on the membrane (Fig. 3). The intensity of the color reaction for strains 725-78 and S1191 was not reduced when the assay conditions were modified as follows: (i) increasing the PCR annealing temperature to 60°C (ii) altering the concentration of denatured pEW3 plasmid DNA immobilized on the nitrocellulose membrane (iii) altering the concentration of heterologous DNA and increasing the incubation time used to block unreacted sites on the membrane, (iv) pretreatment of the PCR products with proteinase K to eliminate AP in the bacterial lysates, or (v) increasing the stringency of the wash which followed hybridization of the PCR products to the immobilized plasmid. Therefore, whole-cell DNA purified by repeated phenol extractions from strains of *S. dysenteriae* and *E. coli* which produce ShT/SLT-I, SLT-II, and SLT-IIv was used in the amplification reaction. As shown in Fig. 4, substitution of the cell lysates with purified DNA significantly increased the contrast of staining intensity between the samples from the

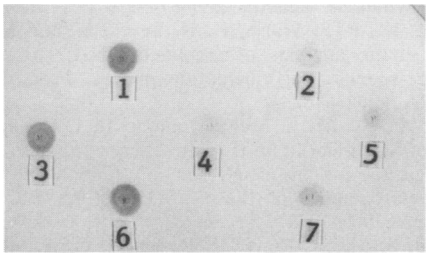

FIG. 4. Spot blot analysis of DIG-labeled PCR products amplified from purified DNA by using *stxA/slt*-IA primers. Samples: 1, *E. coli* HB101(pEW3); 2, HB101(pBR329); 3, *E. coli* CL-40; 4, *E. coli* E32511; 5, *E. coli* S1191; 6, *S. dysenteriae* 3818T; 7, *S. dysenteriae* 725-78.

ShT/SLT-I-producing strains and the nontoxigenic strain 725-78 and SLT-IIv-producing strain S1191.

DISCUSSION

A PCR technique has been developed for the identification of ShT/SLT-I-producing strains of *S. dysenteriae* type 1 and *E. coli*. Individual colonies were inoculated from solid medium directly into the PCR mixture, and the sensitivity of the assay was increased by incorporating [α-^{32}P]dCTP or DIG into the PCR mixture. The labeled DNA was detected by agarose gel electrophoresis and autoradiography or Southern blot analysis and immunologic development. The need for electrophoresis and Southern blotting was eliminated by hybridizing the DIG-labeled PCR products to homologous plasmid DNA sequences immobilized on a nitrocellulose membrane followed by development with an AP-conjugated antibody to digoxigenin and the AP substrates. This technique may be improved in future studies by the substitution of membrane-bound oligonucleotides for purified plasmid DNA in the hybridization step (29). As shown in Fig. 3, the PCR products of ShT/SLT-I-producing strains of *S. dysenteriae* type 1 and *E. coli* were identified as colored spots on the membrane while the products of SLT-II producers and nontoxigenic strains were differentiated as faintly colored or colorless spots. A significant contrast in staining intensity between the ShT/SLT-I-producing strains and the strains which produce SLT-II or SLT-IIv or do not produce toxin was observed when the whole-cell lysates were replaced with purified DNA (Fig. 4). This suggests that the cell lysates may have caused background staining due to nonspecific binding of the anti-DIG antibody or endogenous AP activity. Therefore, whole-cell lysates may be used in this assay to rapidly screen large numbers of clinical isolates, while purified DNA would be used for the analysis of strains which give equivocal results.

The oligonucleotide primers used in this investigation are approximately 65% homologous to the nucleotide sequences of the *slt*-II and *slt*-IIv genes (10, 34, 37). Therefore, only ShT/SLT-I-producing strains were detected with this primer pair and the appropriate annealing conditions. In future studies, the feasibility of this nonradioactive PCR detection method may be assessed using additional primer pairs capable of detecting strains which produce other SLT types. Nucleotide sequence analysis revealed limited regions of low (30%) and high (90%) homology among the *stx*, *slt*-I, *slt*-II, and *slt*-IIv operons (10, 34, 37). Oligonucleotide primers to the regions of high sequence homology among the *stx* and *slt* genes could be universally applied to screening fecal and contaminated food samples for the presence of any ShT or SLT producer. Conversely, primer pairs specific for a single *slt* gene, such as the primers used in this investigation, could be used to determine which SLT type is most commonly associated with outbreaks among a specific population or in a particular geographic area. Finally, although this method was developed for the detection of *S. dysenteriae* type 1 and EHEC, it can be adapted to other bacterial pathogens which are responsible for severe enteric diseases. For example, a primer pair homologous to the nucleotide sequences of the cholera toxin gene family could be used for the detection of *Vibrio cholerae* and enterotoxigenic *E. coli* by using the appropriate oligonucleotide primers.

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