Evaluation of a Technique for Identification of Shiga-Like Toxin-Producing *Escherichia coli* by Using Polymerase Chain Reaction and Digoxigenin-Labeled Probes

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A polymerase chain reaction (PCR) technique for the identification of Shiga-like toxin (SLT)-producing *Escherichia coli* was assessed by using 95 strains of SLT-producing *E. coli* and 5 *Shigella dysenteriae* type 1 strains. PCR was used for the amplification of *slt* gene sequences from whole bacterial colonies. A digoxigenin-labeled DNA probe was used for identification of the PCR products in a spot blot hybridization assay. Modifications were made to adapt this technique for the proper identification of 10 SLT-producing isolates which were refractory to the heat lysis step that was used to liberate whole-cell DNA for PCR and 6 isolates which gave nonspecific amplification products. The sensitivity and specificity of this assay were each 99% when compared with toxin neutralization results by using SLT-specific monoclonal antibodies. These values indicate that this detection technique could be suitable for use in a clinical laboratory.

In January 1993 a serious outbreak of diarrheal disease associated with the consumption of beef in Washington State was shown to be caused by enterohemorrhagic *Escherichia coli* (EHEC) (2). EHEC is a class of diarrheagenic *E. coli* which carries the genetic determinants for attaching-effacing lesions and Shiga-like toxin (SLT) production (12, 13). Although the pathogenesis of EHEC has not been definitively established, the existing hypothesis is that the attaching-effacing lesion may cause hemorrhagic colitis, while SLT has been implicated in the hemolytic uremic syndrome (HUS). Shiga toxin (STX), which is produced by *Shigella dysenteriae* type 1 (15), is the prototype for a family of SLTs which have been implicated as the causative agents of the HUS which follows an EHEC infection (11). Nucleotide sequence analysis has shown that SLT type I (SLT-I) is essentially identical to STX (21), while SLT-II is approximately 55% homologous to STX (SLT-I) (8). Although the frequency of EHEC infection in the United States is unknown, some areas such as Washington State have a relatively high incidence, as demonstrated recently when over 300 individuals with bloody diarrhea as well as some cases of HUS were reported (2). Therefore, a significant need exists for a technique which could be used to detect EHEC strains in clinical samples. In addition, because EHEC strains that produce potentially fatal complications in children and elderly people is a consequence of toxemia, the detection method should be based on the identification of SLT-producing strains.

A detection technique for the identification of bacteria carrying the *slt* gene sequences using the polymerase chain reaction (PCR) with incorporation of the TTP analog digoxigenin-dUTP (DIG) has been developed (6). In this procedure, DIG was included in the PCR mixture of all samples, and the labeled amplification products were hybridized to homologous *slt* gene sequences immobilized on a nitrocellu-

lose membrane. This technique was subsequently modified by immobilizing unlabeled amplification products to a nitrocellulose membrane and using a DIG-labeled gene probe in the hybridization step (7). This modification made the technique better suited for the detection of SLT producers in a clinical setting by increasing the specificity and reducing the cost of the assay by decreasing the amount of DIG reagent required.

Because this PCR detection technique was initially developed with a limited number of *E. coli* strains with well-defined SLT phenotypes, the purpose of the present study was to assess the feasibility of the method with a collection of clinical isolates. The PCR products of 95 strains of SLT-producing *E. coli* (SLTEC) from 15 different serotypes and 5 STX-producing strains of *S. dysenteriae* type 1 were amplified by using *slt-I* and *slt-II* gene-specific primers that were immobilized on nitrocellulose membranes and detected by hybridization with DIG-labeled probes. The technique was adapted to the identification of toxigenic clinical strains by modifying the cell lysis step to optimize sensitivity and the stringency of the PCR annealing step and hybridization with the DIG-labeled probe to optimize specificity.

MATERIALS AND METHODS

Bacterial strains. A total of 95 SLTEC and 5 *S. dysenteriae* type 1 clinical isolates collected at the Centers for Disease Control and Prevention (Atlanta, Ga.) were used in the study. Ninety-one of the SLTEC strains comprised 15 different serotypes (Table 1) from human cases of enteritis, gastroenteritis, diarrhea, bloody diarrhea, hemorrhagic colitis, and HUS. Four of the SLTEC strains were bovine isolates. All of the SLTEC strains were collected in the United States, and the five *S. dysenteriae* type 1 strains were from India, Bangladesh, Thailand, Guatemala, and Mexico, respectively.

Oligonucleotide primers. The sequences of the forward and reverse oligonucleotide primers which were used for the

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TABLE 1. SLTEC serotypes and toxin phenotypes

SLTEC serotype	No. of isolates	Neutralization (PCR) result ^a		
		SLT-I	SLT-II	SLT-I and SLT-II
O157:H7	63	15	13	35
O157:NM	15	5	2 (3)	8 (7)
O171:H2	1		1	
O171:NM	1		1	
O22:H40	1		1	
O22:H8	1		1	
O145:NM ^b	1		1	
O145:NM	1			1
O111:NM	4			4
O50:H7	1		1	
OX3:H21	1		1	
O172:NM	1		1	
O165:H25	1		1	
O4:NM	1		1	
O83:H1 ^b	1			1
O37:H41	1		1	
<i>S. dysenteriae</i> type 1	5	5 (4)		
Total	100	25 (24)	26 (27)	49 (48)

^a PCR results are shown in parentheses only when they differed from the toxin phenotype characterized by neutralization.

^b The presence of *slt-II* sequences in these strains was established by using a gene probe isolated from the *slt-II* gene sequence in pMJ100.

amplification of the *slt-I* and *slt-II* genes in the clinical isolates and for synthesis of the DIG-labeled hybridization probes are given in Table 2. The reverse oligonucleotide primers, designated SLT-IR and SLT-IIR in Table 2, are listed in the 5' to 3' orientation with respect to the noncoding strand. Therefore, these primers appear as the reverse complement of the sequences given at positions 1091 to 1108 in the *slt-I* gene (21) and positions 1427 to 1445 in the *slt-II* gene (8).

In some experiments, the SLT-I and SLT-II primers were mixed together for coamplification of the *slt-I* and *slt-II* genes.

Cytotoxicity assays and toxin neutralization. A cytotoxicity assay (5) with neutralizing monoclonal antibodies to either the B subunit of SLT-I (22) or the B subunit of SLT-II

(3) was used to evaluate toxin production by the clinical isolates.

PCR. The PCR technique (17) was performed as described previously (6, 7). A 20- μ l PCR mixture was inoculated with a small bacterial colony and was incubated for 10 min at 95°C to cause cell lysis. Either SLT-I, SLT-II, or a mixture of the SLT-I and SLT-II primer pairs was used for each amplification reaction. Those clinical isolates which were refractory to lysis under these conditions were incubated at 100°C for 20 min. One unit of thermostable DNA polymerase was added, and amplification was conducted for 30 cycles. It was necessary to increase the annealing temperature from 52 to 60°C for those isolates which exhibited false hybridization reactions.

Preparation of DIG-labeled probe. A DNA probe for detection of the amplified SLT-I sequences was prepared by PCR by using the SLT-IF and SLT-IR primer pair and purified pNAS10 DNA, which contains the *stx* operon (21). A probe for detection of the amplified SLT-II sequences was prepared by using the SLT-IIF and SLT-IIR primer pair and purified pMJ100 DNA, which contains the *slt-II* operon (24). The SLT-I and SLT-II probes were labeled with DIG by incorporating the nucleotide analog in a TTP-deficient PCR as described previously (6, 7). The 821-nucleotide SLT-I probe was 59% homologous to the *slt-II* operon, while the 620-nucleotide SLT-II probe was 60% homologous to the *slt-I* operon.

Hybridization and immunologic detection. One-half of a microliter of the amplification products was transferred from the reaction tube on a dry nitrocellulose membrane for the spot blot hybridization. The samples were immobilized by baking and were prehybridized as described previously (7). Five microliters of the DIG-labeled DNA probe was denatured by boiling for 10 min and was added to the prehybridization mixture, which was incubated overnight. Either SLT-I, SLT-II, or a mixture of the SLT-I and SLT-II probes was used for the hybridizations. The blots were washed in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min to remove nonspecifically bound DIG-labeled probe from the membranes. The hybridization and wash temperatures were varied at 68, 72, 76, and 80°C to optimize specificity. PCR products which hybridized with the DIG-labeled probes were identified by using the Fab fragment of an alkaline phosphatase (AP)-conjugated anti-DIG monoclonal antibody and the AP color development reagent (Boehringer Mannheim Biochemicals) (7).

RESULTS

Strain collection evaluation. A total of 95 SLTEC strains from 12 different serogroups and 5 strains of *S. dysenteriae* type 1 were chosen to evaluate the PCR detection technique. Because the O157 serogroup is most frequently isolated from EHEC-associated outbreaks (11), 82% of the *E. coli* strains used in the study were either O157:H7 or O157:NM (Table 1). The majority of the isolates were from human patients with diarrheal disease and HUS, although four bovine isolates were also included for analysis. SLTEC strains which produced only SLT-I (25%) or SLT-II (26%) were used for analysis, while 49% produced a combination of SLT-I and SLT-II (Table 1). The five *S. dysenteriae* type 1 strains were included for analysis to assess the flexibility of this technique for the detection of SLT producers other than *E. coli*.

Optimization of specificity. While a majority of the SLTEC isolates used in the study produced both SLT-I and SLT-II, 25% produced only SLT-I and 26% produced only SLT-II

TABLE 2. PCR primers

Designation	Sequence ^a	Position ^b	% Homology to:		<i>T_m</i> ^c
			<i>slt-I</i>	<i>slt-II</i>	
SLT-IF	TGCAAAGACGTATGTAGA	253–270	100	33	48
SLT-IR ^d	TCAACTGCTAATAGTTCT	1091–1108	100	11	48
SLT-IIF	CAGAAGCCTTACGCTT	798–813	81	100	48
SLT-IIR ^d	CCGGAGCCTGATTCACAGG	1427–1445	53	100	61

^a The sequences for all oligonucleotides are given in the 5' to 3' orientation.

^b The sequences and positions of the SLT-I primers were taken from Strockbine et al. (21). The sequences and positions of the SLT-II primers were taken from Jackson et al. (8).

^c Thermal denaturation (*T_m*) values were calculated from the nucleotide sequences (18).

^d Reverse primers designated SLT-IR and SLT-IIR are given in the 5' to 3' orientation of the noncoding strand of the *slt-IA* and *slt-IIB* gene sequences, respectively.

(Table 1). Therefore, it was possible to calculate the specificity of this assay by using the SLT-I PCR primers and probe to amplify the DNAs of strains which produced SLT-II only and the SLT-II PCR primers and probe to amplify the DNAs of strains which produced SLT-I only. Previously, the annealing temperature which was used for PCR was 52°C and the blots were hybridized and washed at 68°C (7). While these amplification and hybridization conditions were appropriate for most of the isolates, it was necessary to increase the stringencies for two SLTEC strains which produced only SLT-II in the neutralization assay but which reacted with the SLT-I probe and four SLTEC strains which were completely neutralized with antiserum to SLT-I but which reacted with the SLT-II probe. The PCR values given in Table 1 reflect the optimized conditions which were used to increase the specificity of this detection technique. Because the SLT-I primers were only 11 and 33% homologous to *slt-II*, raising the PCR annealing temperature to 55°C and the hybridization temperature to 72°C was sufficient to eliminate reaction of the SLT-II-producing strains with the SLT-I probe. In contrast, the SLT-II primers were 53 and 81% homologous to the *slt-I* gene sequence, making it necessary to increase the PCR annealing temperature to 60°C and the temperature of hybridization to 80°C to eliminate reaction of the four SLT-I-producing strains with the SLT-II probe. Although these modifications were sufficient to eliminate the false-positive reactions for three of these four strains, one O157:NM strain which was shown by neutralization to produce only SLT-I reacted with the SLT-II probe (Table 1). Therefore, the specificity for this detection technique was 99%.

Optimization of sensitivity. The PCR detection technique with a DIG-labeled hybridization probe was initially established by using a 95°C, 10-min incubation step which caused bacterial lysis and the liberation of whole-cell DNA for amplification (7). In the current study, several clinical isolates were not lysed under these conditions, yielding unacceptably low sensitivity levels. Therefore, it was necessary to modify the lysis conditions by increasing the preincubation step to 100°C for 20 min to permit proper identification of those strains which were not reactive with the DIG-labeled probe but which were toxinogenic in the cytotoxicity assay. Although this step did not substantially increase the time or complexity of the technique, it significantly enhanced its capacity to detect toxinogenic strains.

A total of 10 SLTEC isolates and 1 strain of *S. dysenteriae* type 1 which were either partially reactive or unreactive by the PCR detection technique were subjected to lysis by boiling. The majority of the strains which were not efficiently lysed by the 95°C, 10-min incubation produced both SLT-I and SLT-II. Therefore, these strains were detected by using at least one of the probes or a combination of SLT-I and SLT-II primers and probes (Table 3). Although the PCR results corresponded to the toxin phenotypes previously established by neutralization, there remained three strains which were not fully characterized by the PCR detection technique (Table 3): (i) one O157:NM SLTEC which was shown to produce both SLT-I and SLT-II by neutralization but which failed to react with the SLT-II probe and the mixed probe, (ii) the single O83:H1 strain whose SLT-I and SLT-II phenotype was established previously but which failed to react with the SLT-I probe, and (iii) one *S. dysenteriae* type 1 strain which was not identified as toxinogenic by using the SLT-I probe. Although the toxin genotypes of the two SLTEC strains were not fully characterized by the PCR detection technique, they were identified as

TABLE 3. Isolates which required lysis by boiling

Serotype	Neutralization	95°C ^a	100°C ^a
O157:H7	I	Tox ⁻	I
O157:H7	I + II	I	I, II
O157:H7 ^b	I + II	II	I, II
O157:NM	I + II	Tox ⁻	I
O111:NM	I + II	I	I, II
O111:NM	I + II	II	I, II
O145:NM	I + II	Tox ⁻	I, II
O50:H7	II	Tox ⁻	II
O83:H1	I + II	II	II
<i>S. dysenteriae</i> type 1	STX	Tox ⁻	Tox ⁻

^a Cell lysis at 95°C for 10 min or 100°C for 20 min prior to PCR. Tox⁻, unreactive with any toxin probe.

^b Two O157:H7 strains were in this category.

toxinogenic strains by using at least one of the probes (Table 3). In addition, the O83:H1 strain was also reactive when mixed primers and probes were used. Therefore, if these three strains are not considered to be fully reactive with the appropriate probes, the overall sensitivity of the assay was 97%. However, if the identification of a toxin producer regardless of the SLT type is considered as the only criterion for this assay, then the sensitivity for the detection of SLTEC by this PCR technique would be raised to 100% (Table 3). Therefore, only the single *S. dysenteriae* type 1 strain would have totally escaped detection (Table 1).

DISCUSSION

Despite the potentially severe toxemia which may follow an EHEC infection, many of the sporadic cases remain undiagnosed. Therefore, a convenient detection method which has the capacity to identify the presence of an SLT-producing clinical isolate would be a beneficial diagnostic tool. There have been reports of SLT detection methods which use synthetic oligonucleotides or gene fragments as specific hybridization probes (1, 9, 13, 14, 19, 20). With the advent of PCR came the development of detection methods based on the amplification of the *slt* genes (4, 10, 16, 23). Unfortunately, these techniques are not well-suited to screening large numbers of bacteria in a clinical laboratory because they require DNA purification, identification of the amplification product by agarose gel electrophoresis, or radiolabeled DNA probes. Therefore, the purpose of the present study was to evaluate a technique which is better suited to practical application because it has none of these requirements.

Initially, this technique was developed with the incorporation of DIG into each amplification reaction (6). The DIG-labeled amplification products were hybridized to specific sequences immobilized on a nitrocellulose membrane and detected by using an anti-DIG monoclonal antibody conjugated to AP. Subsequently, that technique was modified to increase specificity and decrease cost by omitting DIG from the individual amplification reactions, immobilizing the unlabeled PCR products directly on a nitrocellulose membrane, and using a DIG-labeled probe for hybridization and immunologic detection (7). Separate *slt-I* gene and *slt-II* gene PCR primers were used in these previous two studies (6, 7). These earlier adaptations were incorporated into the current study to assess the practicality of this nonradioactive PCR detection method by using a collection of 95 SLTEC

and 5 *S. dysenteriae* type 1 isolates which produced STX, SLT-I, and SLT-II.

Some strains were refractory to the heat lysis step which was used to liberate whole-cell DNA for amplification, perhaps because the various SLTEC and *S. dysenteriae* type 1 isolates used in the present study differed with respect to the lipopolysaccharide content of the outer membrane. Modification of the reaction conditions to increase the time and temperature used to induce cell lysis permitted detection of these strains with at least one of the *slt* gene probes. Although the SLT genotypes for most isolates were properly characterized by this treatment, the PCR products of one O157:NM strain and one O83:H1 strain which both produced SLT-I and SLT-II in neutralization assays were detected with only one SLT probe (Table 3). In addition, a single *S. dysenteriae* type 1 isolate was not detected by the PCR assay with this modification, although it did produce STX in the microcytotoxicity assay. These findings indicate that PCR with mixed SLT primers and DIG-labeled probes would be most suitable for the identification of SLTEC.

The modifications incorporated into the PCR assay described here resulted in a sensitivity and a specificity of 99% each for the detection of all toxinogenic strains by using mixed primers and probes. If only the SLTEC were assessed in this analysis, then the sensitivity would have been raised to 100%. Therefore, the present study extended the previous reports (6, 7) by establishing the optimum conditions for the PCR detection technique as a 100°C, 20-min incubation step to promote cell lysis prior to amplification and the use of mixed *slt-I* and *slt-II* gene primers and probes to ensure the detection of all toxinogenic strains without SLT type-specific differentiation.

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