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Detection of Enterotoxigenic *Escherichia coli* after Polymerase Chain Reaction Amplification with a Thermostable DNA Polymerase

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The direct identification of enterotoxigenic *Escherichia coli* from clinical specimens was examined by using the polymerase chain reaction (PCR) for amplifying the heat-labile toxin (LT) gene. Two synthetic primers, each of which was 20 bases in length, were used with the thermostable DNA polymerase from *Thermus aquaticus* to amplify the LT gene. The amplified PCR products were detected by either gel electrophoresis or hybridization to a 24-base synthetic oligonucleotide probe conjugated to alkaline phosphatase. The PCR method detected LT-positive bacteria but did not react with *E. coli* producing the heat-stable toxin, enteroinvasive *E. coli*, *Salmonella typhi*, *Salmonella typhimurium*, or *Shigella dysenteriae*. By the PCR method, a single bacterium could be detected following 30 cycles of amplification. The *T. aquaticus* DNA polymerase was inhibited by more than 10^3 organisms in the amplification reaction mixture. A group of 40 clinical specimens consisting of 16 LT bioassay-positive and 24 LT bioassay-negative stool specimens were tested by PCR for the presence of toxigenic *E. coli*. The total DNA from 100 μl of stool specimen was extracted and partially purified with a commercially available ion-exchange column. All 16 of the bioassay-positive stool specimens were positive by PCR. In addition, one stool specimen which was bioassay negative for LT but positive for LT in a previous hybridization assay with a different LT probe was also positive by PCR. This may indicate that the LT gene is present but either is not expressed or is expressed below detectable levels. Amplification of specific DNA sequences by PCR provides a highly sensitive and specific tool for the detection of pathogenic microorganisms directly from clinical specimens without the need for prior isolation. This technique may find wide application in the detection of other organisms in addition to enterotoxigenic *E. coli*.

The purpose of the clinical microbiology laboratory is to rapidly and accurately provide the clinician with information concerning the presence or absence of a microbial agent that may be involved in an infectious disease process. Traditionally, this involves the isolation of a suspected pathogen from a clinical specimen followed by identification of the organism by serological or biochemical methods. Depending on the organism, the entire identification process can take anywhere from several days to weeks. Most of these tests are contingent on the isolation of a pure culture of the suspected bacterium.

The use of nucleic acid hybridization probes is becoming increasingly common as an alternative means for rapidly identifying infectious microorganisms (2, 5, 6, 11, 18). This method is particularly useful for screening large numbers of specimens. However, nucleic acid hybridization suffers from several disadvantages. Traditionally, nucleic acid hybridization has relied on radioactive labels for detection. Alternative, nonradioactive labels have been developed (7, 8) and have been used successfully (1, 12, 13, 17); however, the sensitivity of the assays requires large numbers of organisms for detection (12, 13, 17). Thus, the currently used hybridization assays are generally for culture confirmation rather than direct detection and identification.

In spite of this, the detection of organism-specific DNA sequences by nucleic acid hybridization offers the possibility of detecting pathogenic microorganisms without the tedious process of prior isolation of a pure culture. The development of a standardized, highly sensitive and specific, nonradioactive detection system in which organism-specific gene sequences are used would facilitate rapid diagnosis.

Saiki and co-workers (15, 16) have described a system for amplifying the concentration of specific nucleic acid sequences as much as 10^6-fold. I have previously used the polymerase chain reaction (PCR) described by Saiki et al. (15) to increase the sensitivity of a hybridization assay for enterotoxigenic *Escherichia coli* (12). In this report I describe significant improvements that I have made on the previous system in terms of increased sensitivity and simplicity. The PCR system can detect a single microorganism and can be used on mixed microbial specimens without the isolation of individual *E. coli*. Specimen processing consists of the purification of total nucleic acid with a simple ion-exchange chromatography system followed by PCR amplification and detection by agarose gel electrophoresis, nucleic acid hybridization, or both.

MATERIALS AND METHODS

Bacterial strains and clinical specimens. *E. coli* 222, 230, and 112 are clinical isolates which were determined to have a heat-labile enterotoxin (LT^+), a heat-stable enterotoxin (ST^+), and both enterotoxins, respectively, by bioassays and hybridization assays (3, 13, 14). The enteroinvasive *E. coli* was a gift from S. Formal. *Salmonella typhi*, *Salmonella typhimurium*, and *Shigella dysenteriae* were isolated from patients with gastroenteritis and were identified by standard biochemical procedures. Stool specimens were collected from adults who were hospitalized for gastroenteritis and were screened for the presence of LT^+ *E. coli* by a bioassay and a probe assay (13).

Synthetic oligonucleotide probes. All oligonucleotide probes were purchased from O.C.S. Laboratories. The sequences of the probes and the homologous sites for hybridization to the LT gene are shown in Fig. 1 (20). The LT detection (LTd) probe was end-labeled with alkaline phosphatase.

DNA extraction from stool specimens. Total DNA from stool specimens was extracted and partially purified by using

Extractor columns (Molecular Biosystems Inc.). The procedure and all buffers and solutions used were those supplied with the columns by the manufacturer. Briefly, stool specimens were combined with 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE buffer) to make a 10% suspension. From this, 100 μl was combined with 1 ml of specimen dilution buffer. The stool suspension was combined with 2 ml of lysing solution and 100 μl of a 2-mg/ml solution of proteinase K. The mixture was vortexed and incubated at 60°C for 30 min. The stool suspension was centrifuged at 750 × *g* for 5 min, and the liquid was applied to an Extractor column. The sample was allowed to enter the column by gravity flow, and the column was washed with 15 ml of wash reagent 1. This was followed by a wash with 5 ml of wash reagent 2. The nucleic acid was eluted with 2 ml of elution reagent, and 20 μl of the nucleic acid was used for amplification.

PCR. For amplification (16), either 20 μl of nucleic acid or 10 μl of a culture of bacteria in L broth was combined with 10 μl of 10× *Thermus aquaticus* salts (500 mM KCl, 100 mM Tris hydrochloride [pH 8.4], 25 mM $MgCl_2$, 0.1% gelatin), 10 μl of 8 mM deoxynucleoside triphosphate stock (2 mM each of dATP, dGTP, dTTP, and dCTP), 4 μl of each primer stock (10 μM each), and water to a final volume of 99 μl. The reaction mixtures were overlaid with mineral oil and heated to 95°C for 10 min to lyse the bacteria and denature the DNA. The primers were allowed to anneal at 55°C for 1.5 min, and then 2.5 U of *T. aquaticus* DNA polymerase (New England BioLabs, Inc.) was added. The reaction was incubated for 1.5 min at 93°C and shifted to 55°C for 1.5 min. The heating and cooling cycles were carried out by using two heat blocks (Labline, Inc.). The sample holes of each heat block were filled with water to ensure even heat transfer. The denaturation-renaturation-polymerization process was continued until 30 cycles were completed. After 12 cycles, an additional 2.5 U of *T. aquaticus* DNA polymerase was added. The amplified DNA was analyzed by electrophoresis of 50 μl of the reaction mixture on a composite agarose gel consisting of 3% Nuseive agarose and 1% HGT agarose (FMC Corp.). The remainder of the DNA was analyzed by hybridization to a 24-base alkaline phosphatase-conjugated synthetic probe.

DNA hybridization. For hybridization analysis, 50 μl of amplified DNA was combined with 50 μl of 0.6 M NaOH. After 15 min at room temperature, the pH was neutralized by the addition of 100 μl of 2 M ammonium acetate. The samples were applied to a nylon membrane (Transphor; Hoefer Scientific, Inc.) by using a filtration manifold, and the filters were dried for 60 min at 80°C. The filters were hybridized as described previously (13).

Detection of the hybridization reactions. The hybridization reactions with the alkaline phosphatase-conjugated probe were visualized by reaction with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as described previously (13).

RESULTS

Specificity of the PCR. To demonstrate the specificity of the PCR and the synthetic primers for LT^+ *E. coli*, cultures of common diarrhea-causing bacteria were grown for 6 h in L broth (10), and each culture was subjected to amplification. The cultures were amplified directly, without DNA extraction. A 10-μl portion containing approximately 500 bacteria from each culture was combined with the PCR mixture and amplified for 30 cycles. The synthetic primers

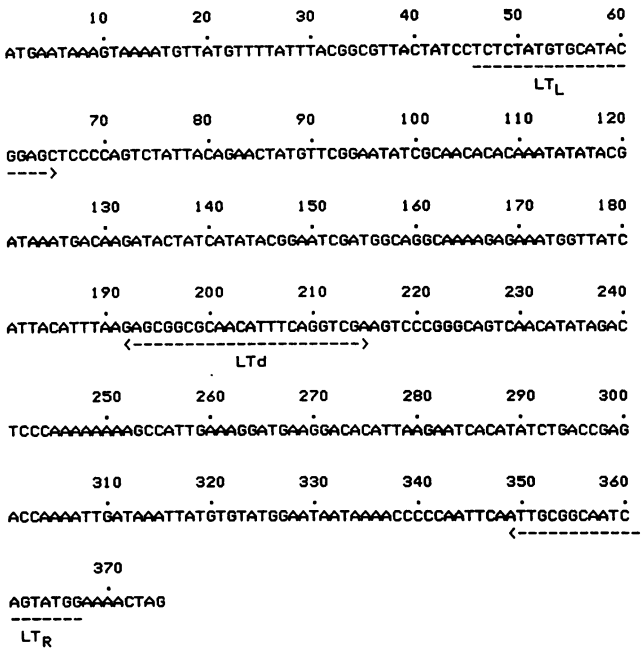

FIG. 1. DNA sequence of *E. coli* LT gene and sequences of the amplification and detection primers. The primers LT_R and LT_L were used to amplify the LT gene sequence from *E. coli*. The primers covered a 322-bp region of the LT gene. The PCR products were detected with the LTd probe, which was conjugated to alkaline phosphatase.

used for amplification were positioned over a DNA sequence covering 322 base pairs (bp) (Fig. 1). A 322-bp fragment resulted from the amplification of LT^+ *E. coli* 222 (Fig. 2, lane 2). The amplified DNA also hybridized with the LTd probe, confirming correct amplification. Similarly, LT^+ ST^+ *E. coli* 112 also gave rise to a 322-bp DNA fragment and a confirmatory positive hybridization reaction (Fig. 2, lane 4). However, *E. coli* 230 (ST^+), enteroinvasive *E. coli*, *Salmonella typhi*, *Salmonella typhimurium*, and *Shigella dysenteriae* all gave negative PCR results. Thus, the primers were specific for the LT gene.

Quantitation of the number of bacteria detected. *E. coli* 222 (LT^+) was grown for 6 h and serially diluted in L broth. Portions (100 μl) of each serial dilution were plated onto L-agar plates and grown overnight at 37°C to determine the number of bacteria in each dilution. Portions (10 μl) from the serially diluted cultures were simultaneously subjected to PCR analysis. A single LT^+ bacterium in 10 μl was detected (Fig. 3). However, samples containing more than 10^3 bacteria per 10 μl exhibited an inhibitory effect on the PCR.

Detection of LT^+ bacteria from stool specimens. Stool specimens obtained from patients with gastroenteritis were subjected to PCR analysis both directly and following partial purification of the DNA by chromatography. Stool specimens could not be consistently amplified directly (unpublished data). Some of the stool specimens exhibited an inhibitory effect on the PCR. Following chromatographic purification of the total nucleic acid, however, all stool specimens were successfully analyzed by PCR. Stool specimens that were positive for LT by the bioassay gave positive results by PCR following gel electrophoresis or the hybridization assay on the PCR products (Fig. 4, lanes 3 and 4). One specimen was negative by the bioassay yet was positive by PCR analysis (Fig. 4, lane 5). Previously, this

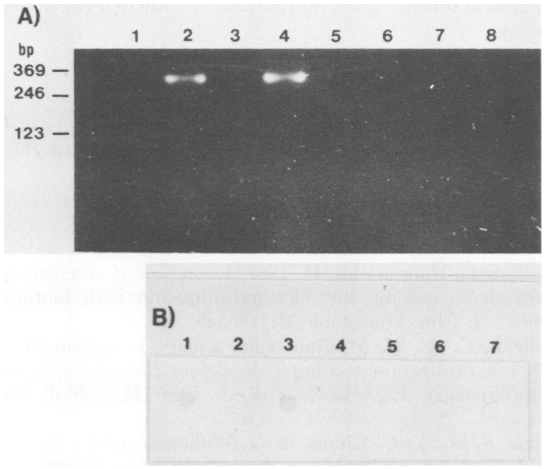

FIG. 2. Specificity of PCR amplification. Common diarrhea-causing bacteria were subjected to PCR amplification. (A) PCR products were detected by electrophoresis on an agarose gel. The samples were marker DNA (lane 1), *E. coli* 222 (LT^+) (lane 2), *E. coli* 230 (ST^+) (lane 3), *E. coli* 112 (LT^+ ST^+) (lane 4), enteroinvasive *E. coli* (lane 5), *Salmonella typhi* (lane 6), *Salmonella typhimurium* (lane 7), and *Shigella dysenteriae* (lane 8). (B) Hybridization of the PCR products to the LTd probe. The samples were *E. coli* 222 (LT^+) (lane 1), *E. coli* 230 (ST^+) (lane 2), *E. coli* 112 (LT^+ ST^+) (lane 3), enteroinvasive *E. coli* (lane 4), *Salmonella typhi* (lane 5), *Salmonella typhimurium* (lane 6), and *Shigella dysenteriae* (lane 7).

specimen was analyzed and found to hybridize with a commercially available DNA probe to LT (12). Based on a hybridization test with a probe targeted to a different region of the LT gene, this specimen was judged to be a true positive in which LT was either not expressed or expressed below detectable levels. The results of the PCR analysis of

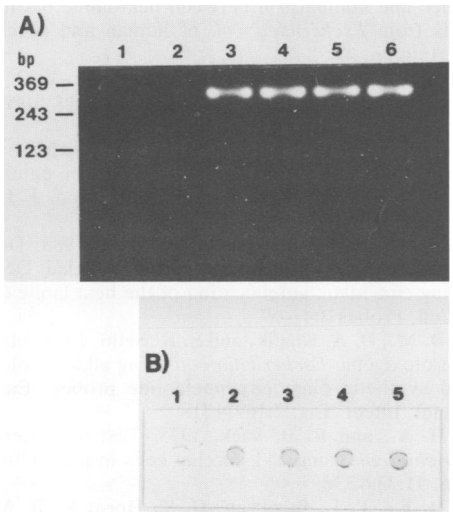

FIG. 3. Quantitation of the number of bacteria detected. *E. coli* 222 (LT^+) was grown and titrated, and serial dilutions were subjected to PCR amplification. (A) Analysis of the PCR products by gel electrophoresis. The numbers of bacteria amplified in each sample were 10^4 (lane 2), 10^3 (lane 3), 10^2 (lane 4), 10^1 (lane 5), and 1 (lane 6). Lane 1 contained DNA molecular weight markers. (B) Analysis of the PCR products by hybridization to the LTd probe. The numbers of bacteria amplified in each sample were 10^4 (lane 1), 10^3 (lane 2), 10^2 (lane 3), 10^1 (lane 4), and 1 (lane 5).

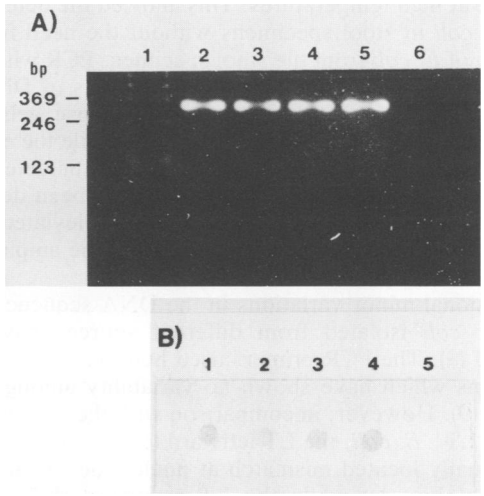

FIG. 4. Detection of LT^+ *E. coli* from stool specimens. (A) The size of the PCR products is shown in the gel. Lane 1, Molecular weight markers; lane 2, *E. coli* 222 (LT^+); lanes 3 and 4, LT bioassay-positive stool; lanes 5 and 6, LT bioassay-negative stool. (B) The filter shows the results of hybridization of the PCR products to the LTd probe. Lane 1, *E. coli* 222 (LT^+); lanes 2 and 3, LT bioassay-positive stool; lanes 4 and 5, LT bioassay-negative stool.

clinical stool specimens were as follows. All 16 of the LT bioassay-positive specimens were positive by PCR, and all 23 of the LT bioassay-negative specimens were negative by PCR (sensitivity, 100%; specificity, 100%). The positive and negative predictive values for PCR were both 100%.

DISCUSSION

Saiki et al. (15) originally described a method for using the Klenow fragment of DNA polymerase to amplify the concentration of a specific DNA sequence by using what is now referred to as PCR. I and others have described previously (12) the use of PCR for the detection of enterotoxigenic *E. coli* by using the Klenow fragment enzyme. Although the previous study (12) resulted in sensitive detection of LT^+ *E. coli*, the procedure was tedious because of the requirement for purified DNA. Recently, Saiki et al. (16) have described an improved procedure for amplifying DNA sequences by using a thermostable DNA polymerase isolated from *T. aquaticus*. Using this enzyme, Saiki and co-workers (16) were able to achieve a 10^6-fold amplification of specific DNA sequences. In this study, I used *T. aquaticus* DNA polymerase to amplify the LT gene from enterotoxigenic *E. coli*. Previously, using the Klenow polymerase, I and others (12) were able to detect 1,000 LT^+ bacteria by hybridization following amplification. By using *T. aquaticus* DNA polymerase, a single LT^+ bacterium was detected. In addition, bacterial cultures could be used directly without the need for DNA purification as long as the number of bacteria in the culture was below 10^3/10 μl. High levels of bacterial proteins may inhibit the *T. aquaticus* DNA polymerase, as they do the Klenow fragment polymerase (12, 15). Nucleic acids from stool specimens had to be partially purified prior to PCR analysis in order to achieve consistent results. The column purification step, however, was simple to perform and yielded pure DNA in 1 h.

PCR is highly specific, as it relies on the selective hybridization of three different oligonucleotides to regions of the

LT gene at high temperatures. This allowed the detection of LT^+ *E. coli* in stool specimens without the need for prior isolation of *E. coli* from the stool specimen. PCR with the *T. aquaticus* DNA polymerase yielded amounts of DNA that could be analyzed either directly on agarose gels or by hybridization with a gene-specific probe. While the enzyme-labeled oligonucleotide probe system used for detection is not as sensitive as other systems that have been described previously (12), the need for sensitivity is alleviated by the high yields of PCR product resulting from the amplification reaction.

Occasional minor variations in the DNA sequences from LT^+ *E. coli* isolated from different sources have been reported (9). The PCR primers used here were homologous to regions which have shown no variability among human isolates (9). However, in comparison with the sequence of a porcine LT^+ *E. coli*, the LT leftward (LT_L) primer contains an internally located mismatch at nucleotide 53 on the LT sequence (Fig. 1), while the LT rightward (LT_R) primer contains a mismatch at the 5′-terminal nucleotide (9). Kinetic studies with oligonucleotides have shown that a single internal base mismatch between an oligonucleotide and its complementary template can significantly lower the melting temperature of the duplex (19). While the PCR was successful for all human isolates reported here, it was unsuccessful for amplifying two porcine strains of LT^+ *E. coli* (unpublished data). The lack of amplification was presumably due to the destabilization of the duplexes between the LT_L primer and the target DNA. Because of the extreme specificity of the PCR reaction, this may be a potential disadvantage. This problem can be avoided, however, by choosing primers that are homologous to invariable regions of the DNA sequences.

Duggan et al. (4) have recently described a patient who was misdiagnosed as having Hodgkin's disease. The patient was seronegative for human T-cell lymphotropic virus type I antibodies by enzyme-linked immunosorbent assay, Western blot (immunoblot), and radioimmunoprecipitation. Tumor tissue DNA was negative for human T-cell lymphotropic virus type I by Southern blot analysis but was positive for distinct human T-cell lymphotropic virus type I sequences following PCR amplification. This demonstrates the extreme sensitivity of PCR for the detection of specific DNA sequences. Similarly, in the present study the presence of the LT gene was verified in a specimen which was previously suspected to express LT either not at all or below detectable levels (13).

By using the PCR technique, 16 of 16 LT bioassay-positive stool specimens were detected. If the single specimen which was LT negative by bioassay but LT positive by PCR was considered to be a true positive, then the positive and negative predictive values of the PCR were 100%. In all specimens that were positive for the LT gene, both a gel band and a positive hybridization reaction were observed. While either criterion may be sufficient alone to detect LT^+ *E. coli*, both criteria were used for confirmation.

The PCR can be used as a highly sensitive and specific test for the presence of pathogenic bacteria in clinical specimens. The PCR should be useful in clinical situations in which extreme sensitivity is required, such as for the analysis of food specimens. The ability to analyze specimens without the isolation of specific organisms should facilitate rapid diagnosis and treatment. In this report, the detection of enterotoxigenic *E. coli* was used as a model system. PCR can conceivably be adapted to any microbial system in which characteristic gene sequences are known for a specific pathogen.

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