Detection of Enterotoxigenic Clostridium perfringens in Food and Fecal Samples with a Duplex PCR and the Slide Latex Agglutination Test

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A duplex PCR procedure was evaluated for the detection of Clostridium perfringens in food and biological samples and for the identification of enterotoxigenic strains. This method uses two sets of primers which amplify in the same reaction two different DNA fragments simultaneously: the 283-bp C. perfringens phospholipase C gene fragment and the 426-bp enterotoxin gene fragment. Internal primers within the two primer sets confirmed the specificity of the method by DNA-DNA hybridization with the PCR products. No cross-reaction was observed with other Clostridium species or with other bacteria routinely found in food. The detection level was approximately 10³ C. perfringens cells per g of stool or food sample. When overnight enrichment culture was used, 10 C. perfringens cells per g was detected in 57 artificially contaminated food samples. The duplex PCR is a rapid, sensitive, and reliable method for the detection and identification of enterotoxigenic C. perfringens strains in food samples. A slide latex agglutination test was also evaluated as a rapid, simple technique for the detection of C. perfringens enterotoxin in stool samples.

Clostridium perfringens type A is widely distributed in soil, sewage, and intestinal tracts of humans and animals. The enterotoxigenic strains are a common cause of food poisoning outbreaks worldwide (10, 14). The symptoms, predominantly diarrhea and abdominal pain, appear 6 to 24 h after ingestion of contaminated food. Vomiting and fever are unusual. Death occurs occasionally among debilitated patients, particularly the elderly (13). Enterotoxigenic C. perfringens has also been associated with sporadic cases of diarrhea and with some cases of sudden infant death syndrome (3, 15, 19, 20).

C. perfringens enterotoxin (CPE), which is produced during the sporulation phase, causes the symptoms of C. perfringens food poisoning (13, 17). The illness is caused by ingestion of food containing a large number (>10⁵ bacteria per g) of vegetative enterotoxigenic C. perfringens cells (24). The ingested bacteria multiply and sporulate, releasing CPE into the intestine.

A C. perfringens count of >10⁶ cells/g in fecal samples of patients is indicative of C. perfringens food poisoning (24). Direct detection of CPE in fecal samples is also a valuable diagnostic technique (1, 2, 9).

Epidemiological investigations involve enumerating C. perfringens in suspected food. Characterization of enterotoxigenic C. perfringens strains is not performed routinely, since C. perfringens sporulation, which is a prerequisite for CPE production, is limited in the usual culture media (17).

CPE and phospholipase C gene sequences have been determined (26, 28, 29). The phospholipase C gene is located on the chromosomal DNA in all C. perfringens toxin types (4), whereas distribution of the CPE gene is restricted. DNA-DNA hybridization experiments showed that only 6% of C. perfringens isolates from various origins carried the CPE gene (28). This percentage is higher (59%) among C. perfringens strains isolated from confirmed outbreaks of food poisoning (30).

We describe here a duplex PCR for the rapid detection and identification of enterotoxigenic C. perfringens strains in food and fecal samples. A slide agglutination test for the detection of CPE in stool samples was also evaluated.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used in this study are listed in Table 1. The Clostridium strains were grown in TYG medium under anaerobic conditions by using anaerobic jars containing 95% H₂ and 5% CO₂; this medium contained Trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine HCl (0.5 g/liter), and its pH was 7.2.

Standard bacteriological methods. For bacteriological analysis of food and fecal samples, 10-g samples were weighed aseptically, placed into sterile stomacher bags, and homogenized for 2 min in 90 ml of peptone water. One-milliliter aliquots of 1:10 dilutions of the suspensions were mixed with 9 ml of SPS agar, which contained tryptone (15 g/liter), yeast extract (10 g/liter), ferric citrate (0.5 g/liter), sodium sulfate (0.5 g/liter), sodium thiglycollate (0.1 g/liter), Tween 80 (0.005 g/liter), polymyxin B sulfate (0.001 g/liter), disodium sulfadiazine (0.12 g/liter), and agar (14 g/liter) and had a pH of 7. After a 18 h of anaerobic incubation at 40°C, the sulfate-reducing Clostridium cells were counted. Colonies surrounded by the characteristic black precipitate were identified biochemically (by using lactose fermentation, nitrate reduction, gelatinase production, and motility tests).

Anti-CPE immunoglobulins. CPE was purified from C. perfringens 8-6, and rabbit anti-CPE antibodies were prepared as described previously (21). Anti-CPE immunoglobulins were purified by an immunoadfinity procedure. CPE (3 to 5 mg) was coupled with 1 g of cyanogen bromide-activated Sepharose 4B (Pharmacia, Paris, France) according to the instructions of the manufacturer. Rabbit anti-CPE serum (5 ml) was passed over the immunoadfinity column. The column was washed with phosphate-buffered saline (PBS) until no further protein was detected in the eluate. The anti-CPE antibodies were then eluted with 1 M acetic acid. Fractions (200 μl) were collected in tubes containing 100 μl of 3 M Tris-HCl (pH 8). The fractions containing purified anti-CPE immunoglobulins were dialyzed against PBS.

Preparation of latex beads. Coating of latex beads (Bacto Latex 0.81; Difco, Detroit, Mich.) was performed as described previously (24). The latex suspension (2 to 5 ml) was diluted in 15 ml of glycine buffer (0.1 M glycine, 0.15 M NaCl, pH 8.2) containing 200 μg of purified anti-CPE immunoglobulin and homogenized for 1 min at room temperature. An equal volume of PBS-0.1% bovine serum albumin (BSA) was then added, and the mixture was vortexed and stored at 4°C. A negative latex control was prepared in the same way by using nonimmune immunoglobulin G (Sigma, Paris, France).
TABLE 1. Specificity of the duplex PCR: use of the duplex PCR to distinguish between enterotoxigenic C. perfringens strains and other Clostridium and enterobacterial strains

<table>
<thead>
<tr>
<th>Strains yielding positive results with the PCR</th>
<th>Strains yielding negative results with the PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. perfringens type A strains ATCC 13124, 6-3, 1088.0, 4012, 4086, 4009, 4010, 4011, 1089.1, 1089.2, 1089.3, 1089.4, 1122, and 1513</td>
<td>C. perfringens type B strain CN39.22</td>
</tr>
<tr>
<td>C. perfringens type C strain CWC236</td>
<td>C. perfringens type D strains 2534, 250, A0, 48, 76, and 64/1</td>
</tr>
<tr>
<td>C. perfringens type E strain NCIB 10748</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Sequences of the primers and probes used in the PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL3</td>
<td>AAG TTA CCT TTG CTG CAT AAT CCC</td>
<td>1676-1699</td>
</tr>
<tr>
<td>PL7</td>
<td>ATG GAT ACT CCA TAT CAT CCT GCT</td>
<td>1418-1440</td>
</tr>
<tr>
<td>PLC</td>
<td>TCA AAA GAA TAT GCA AGA GGT</td>
<td>1591-1611</td>
</tr>
<tr>
<td>P145</td>
<td>GAA AGA TCT GTA TCA TCT AGC GCT GG C</td>
<td>472-500</td>
</tr>
<tr>
<td>P146</td>
<td>GCT GGC TAA GAT TCT ATA TTT TTC TGC ATG</td>
<td>868-897</td>
</tr>
<tr>
<td>EntA</td>
<td>GAA CGC CAA TCA TAT AAA TTT CCA GCT GGG</td>
<td>783-812</td>
</tr>
</tbody>
</table>

*Complementary DNA strand.*

**RESULTS**

**C. perfringens duplex PCR specificity and characterization of enterotoxigenic strains.** The specificity of the duplex PCR with the two sets of primers derived from the phospholipase C and CPE genes was assessed with different Clostridium species and enterobacterial strains.
other bacterial strains frequently associated with food (Table 1).

The 283-bp amplified fragment from the 24 C. perfringens strains tested was observed by agarose gel electrophoresis (Table 1). This PCR product had the expected size of the phospholipase C gene DNA fragment amplified with PL3 and PL7, and it hybridized with internal primer PIC. The other bacterial strains tested, including the Clostridium biformentans strain, which produces a phospholipase related to that of C. perfringens (27) (Table 1), did not yield amplified fragments with PL3 and PL7 or with P145 and P146.

Seven of the 24 C. perfringens strains tested yielded an additional 426-bp PCR product, a product having the predicted size for the CPE gene DNA fragment amplified with P145 and P146. This 426-bp PCR product hybridized with the EntA primer located on the CPE DNA sequence between P145 and P146 (29), but not with PIC.

The specificity of the duplex PCR was confirmed by studying C. perfringens 8-6 broth culture to determine the sensitivity of the technique. Bacteria in the culture sample were counted microscopically in a Petrov chamber. As few as 50 bacteria in the reaction mixture yielded a positive result as determined by agarose gel electrophoresis. This sensitivity was increased 10-fold by Southern blotting with digoxigenin-labeled internal probes EntA and Plc.

Application of the duplex PCR to naturally and artificially contaminated food samples. The food in the school restaurant responsible for the outbreak of food poisoning contained enterotoxigenic C. perfringens as determined by the duplex PCR technique (Table 3) and yielded two amplified fragments of the sizes predicted for phospholipase C and CPE gene DNA fragments amplified with PL3-PL7 and P145-P146 and hybridized with internal primers PIC and EntA, respectively. The number of sulfite-reducing bacteria as determined by the standard method ranged from <10^4 to 10^7 bacteria per g. Five feces samples did not contain enterotoxigenic C. perfringens as determined by the duplex PCR technique and Southern blotting. The corresponding sulfite-reducing bacterial counts were less than 10^4 bacteria per g for the two other samples (Table 3).

CPE SLAT with stool samples. CPE was detected in 18 of 23 stool samples with the SLAT (Table 3). Enterotoxigenic C. perfringens was identified in 17 of these 18 SLAT-positive stool samples by the duplex PCR. One stool sample (Table 3, sample 4) contained enterotoxigenic C. perfringens as determined by the duplex PCR but not by the SLAT. The low number of sulfite-reducing bacteria in this sample (<10^4 bacteria per g) suggested that CPE was absent. The other SLAT-positive samples contained at least 10^4 sulfite-reducing bacteria per g (Table 3).

CPE was detected in one stool sample by the SLAT (Table 3, sample 11), but this sample was negative as determined by the duplex PCR. There were 10^4 sulfite-reducing bacteria per g in this sample. Further analysis of 20 C. perfringens clones from this sample grown on sheep blood agar identified them as enterotoxigenic C. perfringens as determined by the PCR (data not shown).

The specificity of the duplex PCR was confirmed by studying

**TABLE 3. Stool sample analysis performed with the PCR and SLAT**

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of sulfite-reducing bacteria per ml</th>
<th>Results with the following detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duplex PCR</td>
</tr>
<tr>
<td>1</td>
<td>&lt;10^4</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>&lt;10^4</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>&lt;10^4</td>
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</tr>
<tr>
<td>4</td>
<td>&lt;10^4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>6</td>
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<tr>
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</tr>
<tr>
<td>8</td>
<td>2 × 10^4</td>
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<tr>
<td>9</td>
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<tr>
<td>10</td>
<td>5 × 10^4</td>
<td>+</td>
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<tr>
<td>11</td>
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<td>+</td>
</tr>
<tr>
<td>23</td>
<td>10^7</td>
<td>+</td>
</tr>
</tbody>
</table>

* – C. perfringens not present in sample; +, C. perfringens present in sample.

**DISCUSSION**

We developed a duplex PCR for detecting C. perfringens and identifying which strains are enterotoxigenic. One pair of primers (PL3 and PL7) was derived from the phospholipase C gene present in all strains of C. perfringens (7). The other pair (P145 and P146) was derived from the enterotoxin gene found predominantly in C. perfringens strains associated with outbreaks of food poisoning (29, 30). The PCR products were analyzed by DNA-DNA hybridization by using specific probes for the phospholipase C gene (PLC) and CPE (EntA).

The specificity of the duplex PCR was confirmed by studying

**Figures and Tables**

- Table 3: Stool sample analysis performed with the PCR and SLAT
- Figures: Sensitivity of the duplex PCR with a broth culture, Application of the duplex PCR to naturally and artificially contaminated food samples, Application of the duplex PCR to stool samples.

**Acknowledgments**

We would like to thank Dr. John F. Poopoff for his helpful comments and suggestions.

**References**

1. Application of the duplex PCR to stool samples.

2. Sensitivity of the duplex PCR with a broth culture.

3. Application of the duplex PCR to naturally and artificially contaminated food samples.

4. The sensitivity of the duplex PCR method was investigated by using naturally and artificially contaminated food samples.

5. Discussion.

6. We developed a duplex PCR for detecting C. perfringens and identifying which strains are enterotoxigenic.

7. One pair of primers (PL3 and PL7) was derived from the phospholipase C gene present in all strains of C. perfringens.

8. The other pair (P145 and P146) was derived from the enterotoxin gene found predominantly in C. perfringens strains associated with outbreaks of food poisoning.

9. The PCR products were analyzed by DNA-DNA hybridization by using specific probes for the phospholipase C gene (PLC) and CPE (EntA).

10. The specificity of the duplex PCR was confirmed by studying

**Appendix**

- Additional methods and materials.

**Supplementary Information**

- Additional data and figures.

**Supplementary Tables**

- Table 4: Additional data and figures.
24 *C. perfringens* strains, 27 different *Clostridium* species, and 20 bacterial strains commonly found in food samples. The identification of enterotoxigenic *C. perfringens* isolates by immunological methods requires in vitro sporation to obtain detectable levels of CPE, and since *C. perfringens* sporulates poorly in culture medium, this technique is unsatisfactory (6, 21). The duplex PCR and DNA-DNA hybridization methods are rapid, simple techniques for the identification of enterotoxigenic *C. perfringens* isolates (12, 23, 27, 30). The PCR and hybridization analysis have also been reported to be useful methods for *C. perfringens* typing (5, 18).

Our results show that the PCR is a useful technique for the analysis of biological and food samples. Enterotoxigenic *C. perfringens* was detected directly in 18 of 23 stool samples from patients suffering from food poisoning. These results were consistent with results obtained by using the standard method of counting sulfite-reducing bacteria with detection of CPE by the SLAT, except in two cases. One stool sample that contained $10^5$ sulfite-reducing bacteria per g and was CPE positive as determined by the SLAT gave a negative result with the duplex PCR. However, *C. perfringens* clones isolated from this stool sample were subsequently identified as enterotoxigenic *C. perfringens* by using the duplex PCR. The negative result may have been due to PCR inhibitors in the sample, and a more appropriate method of DNA extraction could have been used. The other stool sample contained $10^4$ sulfite-reducing bacteria per g and no detectable CPE as determined by the SLAT but was positive as determined by the duplex PCR technique.

The duplex PCR is a sensitive diagnostic technique; enterotoxigenic *C. perfringens* is detectable in stool samples at a concentration of $10^3$ to $10^5$ bacteria per g. Since stools of patients suffering from *C. perfringens* food poisoning harbor $10^6$ or more bacteria per g (2, 24), the duplex PCR is an appropriate diagnostic tool. The SLAT is also a sensitive, reliable, and rapid diagnostic technique for CPE detection (detection limit, 0.1 ng of CPE per ml [data not shown]). The use of the SLAT and latex agglutination on microplates for specific CPE detection has been described previously (1, 16).

Monitoring levels of *C. perfringens* food contamination is important in the food industry for preventing food poisoning. The standard method used routinely detects sulfite-reducing bacteria, including *C. perfringens* strains and other *Clostridium* strains. The duplex PCR method reported here is specific to *C. perfringens* and discriminates the enterotoxigenic strains of this species. The sensitivity of this method (10 *C. perfringens* cells per g) with culture enrichment of food samples is comparable with the detection levels required for food testing. Without culture enrichment, $10^3$ *C. perfringens* cells per g is detectable by the duplex PCR. Since food samples responsible for *C. perfringens* intoxication usually contain more than $10^5$ bacteria per g (24), the duplex PCR may be used directly in the first instance when *C. perfringens* food poisoning is suspected. The PCR method as described here can be used for rapid screening for *C. perfringens* in routine testing of food but does not provide quantitative results. A quantitative detection technique based on the most-probable-number method, consisting of inoculating serial dilutions of food samples into enrichment medium and performing PCR with each dilution, has been proposed for enumeration of *Clostridium botulinum* (11) and could be used for *C. perfringens* enumeration.

In conclusion, the duplex PCR method is a rapid, sensitive detection method for enterotoxigenic *C. perfringens* present in stool samples and in contaminated food. For routine food tests, culture enrichment is necessary to obtain a detection level of $10^3$ *C. perfringens* cells per g. Detection of CPE in stools by the SLAT allows early diagnosis of *C. perfringens* intoxication and confirms the production of toxin by enterotoxigenic *C. perfringens* as detected by the duplex PCR.

REFERENCES

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