Detection and toxin-typing of *Clostridium perfringens* in formalin-fixed paraffin embedded tissue by PCR

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Abstract

Since current microbiology method is not suitable to detect *Clostridium perfringens* in formalin-fixed paraffin-embedded-tissue samples, we developed a PCR assay to detect toxin-genes and the 16S ribosomal-DNA-gene of *C. perfringens*. We successfully detected and genotyped *C. perfringens* in tissue sections from two autopsy cases.
*Clostridium perfringens* causes several forms of enteric diseases, including food poisoning and fatal enterotoxemia (14;16). Based on the presence of four major lethal toxins (alpha, beta, epsilon and iota), *C. perfringens* is classified into five toxigenic types (A through E) and each type can cause different diseases. The most commonly encountered type A (alpha toxin) strain causes gas gangrene (myonecrosis), diarrhea and food borne illness in humans (3). Type B (alpha, beta, and epsilon toxins) and type D (alpha and epsilon toxins) strains are the causative agents of fatal enterotoxemia in domestic animals and occasionally humans (15). Type C (alpha and beta toxins) strain causes severe necrotic enteritis in humans and is called “Darmbrand” in Germany and “pig-bel” in New Guinea (11). The pathogenicity of type E (alpha and iota toxins) is unclear and has rarely been isolated in humans (15). Similar toxins can be found in other clostridium species including *C. novyi*, *C. septicum* and *C. histolyticum* (3).

While the presumptive diagnosis of *C. perfringens* can be achieved by clinical and pathological findings, confirmation is routinely performed by conventional microbiological isolation and characterization methods including bacterial culture, biochemical analysis, and enzyme linked immunosorbent assay (ELISA) (5;7). Conventional culture procedures are expensive and time consuming, and detect only live microorganisms. Therefore, current conventional detection techniques are not applicable for the detection of non-viable bacteria such as those found in formalin-fixed tissues. PCR is a well-accepted rapid and sensitive technique for the detection of microbial pathogens, particularly in situations in which low bacterial copy numbers are present (16). PCR assays have been reported for the identification of *C. perfringens* in animals (2;5;8;12;16). However, no studies describe methods to detect and differentiate the various toxigenic types in formalin-fixed paraffin embedded in human tissue samples. Therefore, we developed a sensitive PCR-based method for the detection of *C. perfringens* strains and their associated toxin genes in
formalin-fixed paraffin embedded tissues in situations when diagnosis of the infection by culture is unavailable.

In order to confirm the assay sensitivity and specificity, 4 types of *C. perfringens* reference strains were obtained from American Type Culture Collection (ATCC; Manassas, VA): type A (ATCC 13124), type B (ATCC 3626), type C (ATCC 3628), and type D (ATCC 3629). Other bacterial strains including *Clostridium difficile* (ATCC 9689), *Clostridium sordellii* (ATCC 9714), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 13315), and *Salmonella typhimurium* (ATCC 4924) were also obtained. We then studied two autopsy cases in which bacterial infection was clinically unsuspected as the cause of death. In case one, emphysematous gastritis involving the proximal half of the stomach and distal esophagus as well as focal necrosis of lungs was found. In case two, grossly visible air spaces (“Swiss cheese” appearance) (Figure 1A) were found in liver, brain, heart, kidneys, spleen, and intestine. Microscopic examination of the tissue sections from various organs of both cases revealed spore-forming boxcar-shaped gram-positive bacilli, morphologically consistent with *C. perfringens* (Figure 1 A and B). PCR was performed to confirm the diagnosis of *C. perfringens* and for further toxinogenic genotyping of the bacteria using formalin-fixed paraffin-embedded tissue blocks of the stomach (case 1) and liver (case 2) in which gram-positive bacilli were identified.

ATCC bacterial strains were cultivated in a thioglycollate broth at 37°C for 48-72 hours in an anaerobic or aerobic atmosphere. A 200 ul aliquot of each culture was centrifuged (14,000 rpm) and the resultant pellets were used to extract total DNA. Formalin-fixed, paraffin embedded tissue blocks were sectioned using a microtome with a disposable blade to 10 µm in thickness and 5 sections were placed into a 1.5-ml microcentrifuge
tube. Precautions were taken to avoid cross-contamination. The blades were changed and holder cleaned with a 10% sodium hypochlorite solution between each tissue block (6;9). The sections were deparaffinized twice in 1 ml xylene at 60°C for 30 min and washed in 1 ml of absolute ethanol once. Total DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA) according to manufacturer’s protocols. Briefly, 340 ul of Buffer ATL and 60 ul of proteinase K (20 mg/ml) were added to the each tube and incubated at 56°C overnight. The supernatants were removed after a brief centrifuge and 400 ul Buffer AL were added to each tube and incubated at 70°C for an additional 20 min. At the end of incubation, 400 ul of ethanol were added to the reaction and the mixture was passed through a QIAmp spin column. DNA was eluted from the column with 100 ul of Buffer AE and stored at -20°C for later use.

Specific primers corresponding to the 16S rDNA gene (Y12669) (14) and the alpha or cpa (X13608) (7), beta or cpb (X83275) (7), and epsilon or etx (X60694) (7) toxin genes of C. perfringens were designed by using the sequence data obtained from GenBank (National Institute of Health, Bethesda, MD) and synthesized by Gene Link (Hawthorne, NY). Primers were designed for alpha, beta, and epsilon toxin genes of C. perfringens as these toxins are the major cause of human diseases (11;15). Furthermore, detection of these genes allows for the molecular genotyping of C. perfringens (12). Primer sequences, location of the primers and size of the products are indicated in Table 1.

PCR was carried out in a 50-µl reaction composed of 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 50 mM KCl, 0.2 mM dNTP, 0.5 uM of each primer, 15 ng of DNA, and 4 units of AmpliTaq Gold DNA polymerase (Roche, Indianapolis, IN). The reaction was performed in a thermal cycler (GeneAmp 9600 PCR system, Perkin Elmer, Boston, MA) for 35 cycles of 94°C, 53°C and 72°C for 1 min each for the 16S rDNA and cpb genes and
94°C, 50°C and 72°C for 1 min each for the cpa and etx genes. Fifteen ul of PCR products were examined by gel electrophoresis through a 2.0% agarose gel and visualized after staining with 0.5 ug/ml of ethidium bromide.

PCR amplification yielded the expected products for the 16S rDNA gene (279 bp), cpa (324 bp), cpb (196 bp), and etx (665 bp) genes (Figure 2A). The assay sensitivity was determined using a serially 10-fold dilution of genomic DNA isolated from cultured C. perfringens. Since the genes for cpb and etx are on plasmids, the exact copy numbers of these plasmids in these strains are unknown. Therefore, the copy of genomic DNA was used to determine analytical sensitivity. The analytical sensitivity of the PCR assay for 16S rDNA was 50 copies of genomic DNA, and ranged from 50 (cpb and etx) to 500 (cpa) copies for the other genes (Figure 2A). The lower sensitivity for cpa (500 copies) may be due to sequence variation of this gene in different strains or insufficient sensitivity of the primer (13).

The 16S rDNA primer set was specific for C. perfringens and no cross-reactivity with other bacterial species was observed (Figure 2B). This is consistent with a previous report that tested eleven other clostridial isolates including C. butyricum, C. difficile, C. sporogenes, and C. novyi as well as 38 other clinically important species of common bacteria such as Listeria and Bacillus (14). Therefore, this primer set can be used for primary screening of clostridial microorganisms.

As expected, the 16S rDNA (279 bp) and alpha toxin (324 bp) genes were present in all types of C. perfringens, beta toxin (196 bp) gene was present in types B and C, and epsilon toxin (665 bp) gene was present in types B and D (Figure 3a). These results indicate that selected toxin gene primers can reliably categorize C. perfringens into specific subtypes (7;12).
The PCR using 16S rDNA and toxin gene primers reliably detected *C. perfringens* in formalin-fixed paraffin embedded tissue blocks from two patients (stomach from case 1 and liver from case 2). An initial PCR assay using primers specific for 16S rDNA confirmed adequate DNA for PCR analysis and confirmed the presence of *C. perfringens* in these tissues, negating the need for the use of a housekeeping gene to assess inhibition due to formalin crosslink formation (Figure 3b). Furthermore, the PCR products of the 16S rDNA from both cases were sequenced, which matched with 100% concordance to the *C. perfringens* sequence (Y12669) obtained from GenBank. PCR analysis with primers specific to *cpa*, *cpb*, and *etx* toxin genes detected a 324-bp (alpha toxin) and a 196-bp (beta toxin) bands in case one, consistent with *C. perfringens* type C. In case two, only a 665 bp (epsilon toxin) PCR product was detected, suggesting the presence of either type B or type D as only these types carry the *etx* gene (1;4;10) (Figure 3a, 3b).

Inability to detect the *cpa* and/or *cpb* genes in this bacteria may be the result of high genetic variability in different strains (13) in which only the *etx* gene is present. Alternatively, primer selection may lack sufficient sensitivity for the detection of the *cpa* and *cpb* genes.

As the majority of cases of gas gangrene are caused by *Cl. perfringens* (17) this assay can be used to confirm the diagnosis of *Cl. perfringens* gas gangrene, particularly in a situation where the only material available for microbiological testing is formalin fixed paraffin embedded tissue. However, it has to be acknowledged that a negative result in the described PCR assay does not rule out the involvement of other, non *C. perfringens*, gas gangrene causing clostridial species such as *C. welchii, C. septicum, C. novyi, C. histolyticum*. 
In conclusion, the described PCR assay could be used to detect *C. perfringens* and simultaneously detect the presence of its specific toxin genes in formalin-fixed, paraffin embedded tissue. These primer sets hold promise as a means of detecting *C. perfringens* and the genes that encode its principle toxins, but further optimization and confirmatory studies are needed. More importantly, to improve the clinical value a similar PCR assay can now be developed to include other gas gangrene causing Clostridium species (e.g. *C. welchii*, *C. septicum*, *C. novyi*, *C. histolyticum*) as well.
Table 1. Sequence of PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences 5' to 3'</th>
<th>Primer Position</th>
<th>Product Size</th>
<th>Reference</th>
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<tbody>
<tr>
<td>16S rDNA</td>
<td>AAAGATGGCATCATCATTCAAC TACCGTCAATTATCTTCCCCAAA</td>
<td>184 - 205</td>
<td>279 bp</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>TACCGTCAATTATCTTCCCCAAA</td>
<td>441- 462</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpa</td>
<td>GCTAATGTTACTGCGGTGGACC TCTGATACATCGTGTAAG</td>
<td>1280 - 1300</td>
<td>324 bp</td>
<td>8</td>
</tr>
<tr>
<td>cpe</td>
<td>GCAGAAACATTAGTATATCTTTC</td>
<td>737 - 757</td>
<td>196 bp</td>
<td>8</td>
</tr>
<tr>
<td>etx</td>
<td>GCGGTGATATCCATCTATTC</td>
<td>911 - 932</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCACCTCTGGTCCTACTAAC</td>
<td>258 - 277</td>
<td>665 bp</td>
<td>8</td>
</tr>
</tbody>
</table>

*cpa, cpe, and etx indicate the alpha, beta and epsilon toxin genes of C. perfringens, respectively.*
Figure 2

A

16S rDNA
279 bp →

cpa
324 bp →

cpb
196 bp →

etx
665 bp →

B

16S rDNA
279 bp →
Figure 3

A

<table>
<thead>
<tr>
<th>Type A</th>
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<tbody>
<tr>
<td>16S</td>
<td>cpa</td>
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![Image of gel electrophoresis bands for Type A and Type B]

Type C

<table>
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<th>Type D</th>
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![Image of gel electrophoresis bands for Type C and Type D]

B

**Case 1**

<table>
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<th>cpb</th>
<th>etx</th>
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<tbody>
<tr>
<td></td>
<td>324 bp</td>
<td>→</td>
<td>279 bp</td>
<td>→</td>
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</table>

![Image of gel electrophoresis bands for Case 1]

**Case 2**

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<th>cpb</th>
<th>etx</th>
<th>M</th>
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<tbody>
<tr>
<td>665 bp</td>
<td>→</td>
<td>279 bp</td>
<td>→</td>
<td></td>
</tr>
</tbody>
</table>

![Image of gel electrophoresis bands for Case 2]
Figure Legend:

**Figure 1:** Identification of *C. perfringens* by macroscopic and microscopic examination. (A) Cut surface of liver (case 2) exhibiting multiple cystic air spaces (Swiss cheese” appearance) lined by bacteria (*left and right inset, H & E stain, 10X and 40X magnification, respectively*) (B). Section of stomach (case 1) demonstrating colonization of gastric epithelium by numerous boxcar-shaped gram-positive organisms.
Figure 2: Detection of 16S rDNA and toxin genes of *C. perfringens* by PCR. (A) Sensitivity of the PCR assay for the detection of 16S rDNA and toxin genes (*cpa, cpb* and *etx*) of *C. perfringens*. Genomic DNA was diluted in 10 fold from $5 \times 10^5$ to 5 copies. The results showed that the sensitivity of detecting each gene was between 50 and 500 copies. (B) Specific amplification of the 16S rDNA gene of *C. perfringens* (279 bp) by PCR. Lane 1: *C. perfringens* Type B ($5 \times 10^5$ copies); lane 2: *C. perfringens* Type B ($5 \times 10^2$ copies); lane 3: blank (0 target); lane 4: *C. difficile*; lane 5: *C. sordelli*; lane 6: *P. aeruginosa*; lane 7: *E. coli*; lane 8: *S. typhimurium*; lane 9: *P. vulgaris*; M: DNA size marker (pBR322 DNA *Msp* I Digest). $5 \times 10^5$ copies of genomic DNA were tested for bacteria other than *C. perfringens*. 

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Figure 3: Genotyping of *C. perfringens* by PCR. The DNAs extracted from *C. perfringens* types A to D (ATCC strains) (A) or from tissue sections of case 1 (stomach) and case 2 (liver) (B) were amplified using primers specific for 16S rDNA gene and each toxin gene (*cpa, cpb* and *etx*) by PCR. M: DNA size marker (pBR322 DNA *Msp* I Digest).
References


