

Detection and Toxin Typing of *Clostridium perfringens* in Formalin-Fixed, Paraffin-Embedded Tissue Samples by PCR[∇]

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Since current microbiology methods are not suitable to detect *Clostridium perfringens* in formalin-fixed, paraffin-embedded tissue samples, we developed a PCR assay to detect toxin-encoding genes and the 16S rRNA 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 (15, 17). Based on the presence of four major lethal toxins (alpha-, beta-, epsilon-, and iota-toxins), *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 (4). 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 (16). The 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 (12). The pathogenicity of type E (alpha- and iota-toxins) strains is unclear and has rarely been isolated in humans (16). Similar toxins can be found in other *Clostridium* species, including *C. novyi*, *C. septicum*, and *C. histolyticum* (4).

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 (6, 8). 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 nonviable bacteria, such as those found in formalin-fixed tissue samples. 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 (17). PCR assays have been used to identify *C. perfringens* in animals (3, 6, 9, 13, 17). However, no studies describe methods to detect and differentiate the various toxigenic types in formalin-fixed, paraffin-embedded 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 tissue samples in situations when diagnosis of the infection by culture is unavailable.

In order to confirm the sensitivity and specificity of the assay, four 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 enterica* serovar 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 and focal necrosis of lungs were found. In case two, grossly visible air spaces (“Swiss cheese” appearance) (Fig. 1A) were found in the 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* (Fig. 1A 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 thioglycolate broth at 37°C for 48 to 72 h in an anaerobic or aerobic atmosphere. A 200- μ l 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 a thickness of 10 μ m, and five sections were placed in a 1.5-ml microcentrifuge tube. Precautions were taken to avoid cross-contamination. The blades were changed, and the holder was cleaned with a 10% sodium hypochlorite solution between each tissue block (7, 10). 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 the

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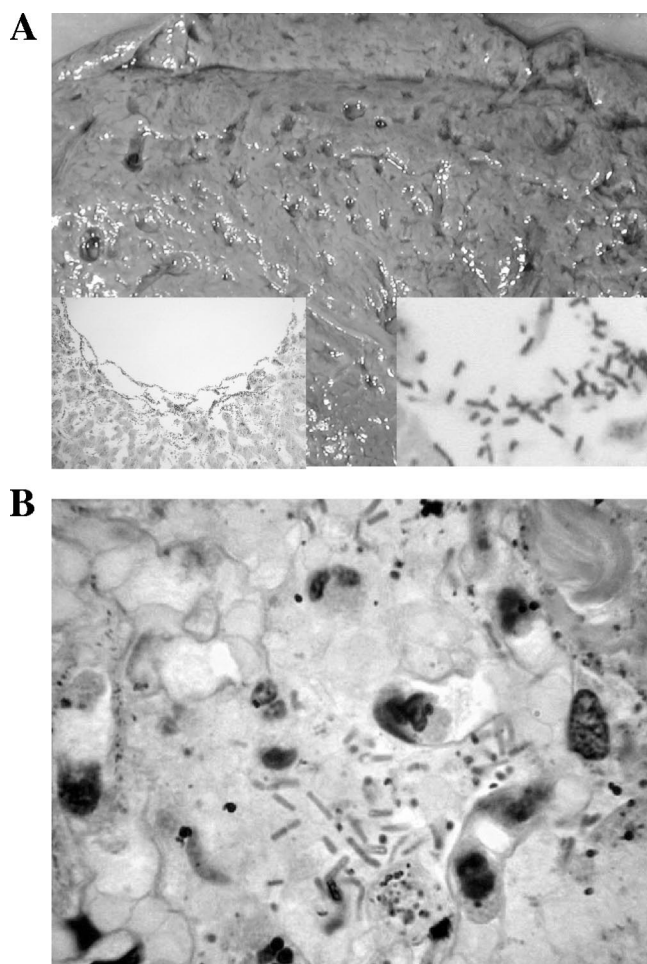


FIG. 1. Identification of *C. perfringens* by macroscopic and microscopic examination. (A) Cut surface of liver (case 2) exhibiting multiple cystic air spaces ("Swiss cheese" appearance) lined by bacteria (left and right insets, magnifications of $\times 10$ and $\times 40$, respectively; both insets stained with hematoxylin and eosin). (B) Section of stomach (case 1) demonstrating colonization of gastric epithelium by numerous boxcar-shaped, gram-positive organisms.

manufacturer's protocols. Briefly, 340 μl of buffer ATL and 60 μl of proteinase K (20 mg/ml) were added to the each tube, and the tubes were incubated at 56°C overnight. The supernatants were removed after a brief centrifuge, 400 μl buffer AL

was added to each tube, and the tubes were incubated at 70°C for an additional 20 min. At the end of incubation, 400 μl of ethanol was added to the reaction mixture, and the mixture was passed through a QIAamp spin column. DNA was eluted from the column with 100 μl of buffer AE and stored at -20°C for later use.

Specific primers corresponding to the 16S rRNA gene (GenBank accession no. Y12669) (15) and to the alpha-toxin gene or *cpa* (GenBank accession no. X13608) (8), beta-toxin gene or *cpb* (GenBank accession no. X83275) (8), and epsilon-toxin gene or *etx* (GenBank accession no. X60694) (8) 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 (12, 16). Furthermore, detection of these genes allows for the molecular genotyping of *C. perfringens* (13). Primer sequences, locations of the primers, and sizes of the products are shown in Table 1.

PCR was carried out in a 50- μl reaction mixture composed of 10 mM Tris-HCl (pH 8.3), 2 mM MgCl_2 , 50 mM KCl, 0.2 mM deoxynucleoside triphosphate, 0.5 μM 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 (1 cycle consists of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C) for the 16S rRNA and *cpb* genes and 35 cycles (1 cycle consists of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C) for the *cpa* and *etx* genes. Fifteen microliters of PCR products was examined by gel electrophoresis through a 2.0% agarose gel and visualized after staining with 0.5 $\mu\text{g/ml}$ of ethidium bromide.

PCR amplification yielded the expected products for the 16S rRNA (279-bp), *cpa* (324-bp), *cpb* (196-bp), and *etx* (665-bp) genes (Fig. 2A). The assay sensitivity was determined using a serial 10-fold dilution of genomic DNA isolated from cultured *C. perfringens*. Since the *cpb* and *etx* genes are on plasmids, the exact copy numbers of these plasmids in these strains are unknown. Therefore, the copy number of genomic DNA was used to determine analytical sensitivity. The analytical sensitivity of the PCR assay for the 16S rRNA gene was 50 copies of genomic DNA and ranged from 50 (*cpb* and *etx*) to 500 (*cpa*) copies for the other genes (Fig. 2A). The lower sensitivity for

TABLE 1. PCR primers used in this study

Gene ^a	Primer sequence (5' to 3')	Primer positions	Product size (bp)	Reference
16S rRNA gene	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	184–205 441–462	279	17
<i>cpa</i>	GCTAATGTTACTGCCGTTGACC TCTGATACATCGTGTAAG	1280–1300 1585–1602	324	9
<i>cpb</i>	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	737–757 911–932	196	9
<i>etx</i>	GCGGTGATATCCATCTATTC CCACTTACTTGTCTACTAAC	258–277 893–913	665	9

^a *cpa*, *cpb*, and *etx* are the alpha-, beta-, and epsilon-toxin genes of *C. perfringens*, respectively.

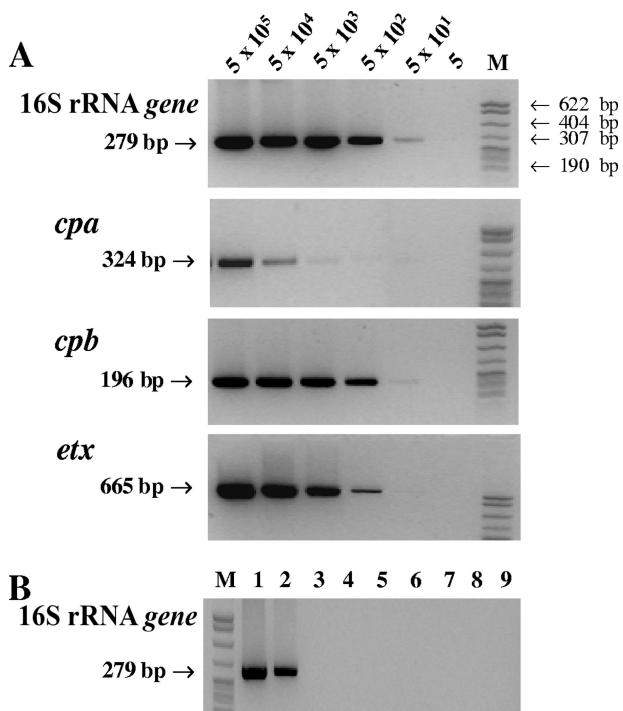


FIG. 2. Detection of 16S rRNA and toxin genes of *C. perfringens* by PCR. (A) Sensitivity of the PCR assay for the detection of 16S rRNA and toxin genes (*cpa*, *cpb*, and *etx*) of *C. perfringens*. Genomic DNA was serially diluted 10-fold from 5×10^5 to 5 copies. The results showed that the sensitivity of detecting each gene was between 50 and 500 copies. (B) Specific amplification of the 16S rRNA gene of *C. perfringens* (279 bp) by PCR. Lane 1, *C. perfringens* type B (5×10^5 copies); lane 2, *C. perfringens* type B (5×10^2 copies); lane 3, blank (no target); lane 4, *C. difficile*; lane 5, *C. sordelli*; lane 6, *P. aeruginosa*; lane 7, *E. coli*; lane 8, *S. enterica* serovar Typhimurium; lane 9, *P. vulgaris*. Lanes M in panels A and B contain DNA size markers (pBR322 DNA MspI digest). Genomic DNA (5×10^5 copies) was tested for bacteria other than *C. perfringens*.

cpa (500 copies) may be due to sequence variation of this gene in different strains or insufficient sensitivity of the primer (14).

The 16S rRNA gene primer set was specific for *C. perfringens*, and no cross-reactivity with other bacterial species was observed (Fig. 2B). This is consistent with a previous report that tested 11 other *Clostridium* 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* spp. (15). Therefore, this primer set can be used for primary screening of clostridial microorganisms.

As expected, the 16S rRNA gene (279 bp) and alpha-toxin gene (324 bp) were present in all types of *C. perfringens*, the beta-toxin gene (196 bp) was present in types B and C, and the epsilon-toxin gene (665 bp) was present in types B and D (Fig. 3A). These results indicate that selected toxin gene primers can reliably categorize *C. perfringens* into specific subtypes (8, 13).

The PCR using 16S rRNA gene 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 the 16S rRNA gene confirmed adequate DNA for PCR analysis and confirmed the presence of *C. perfringens* in

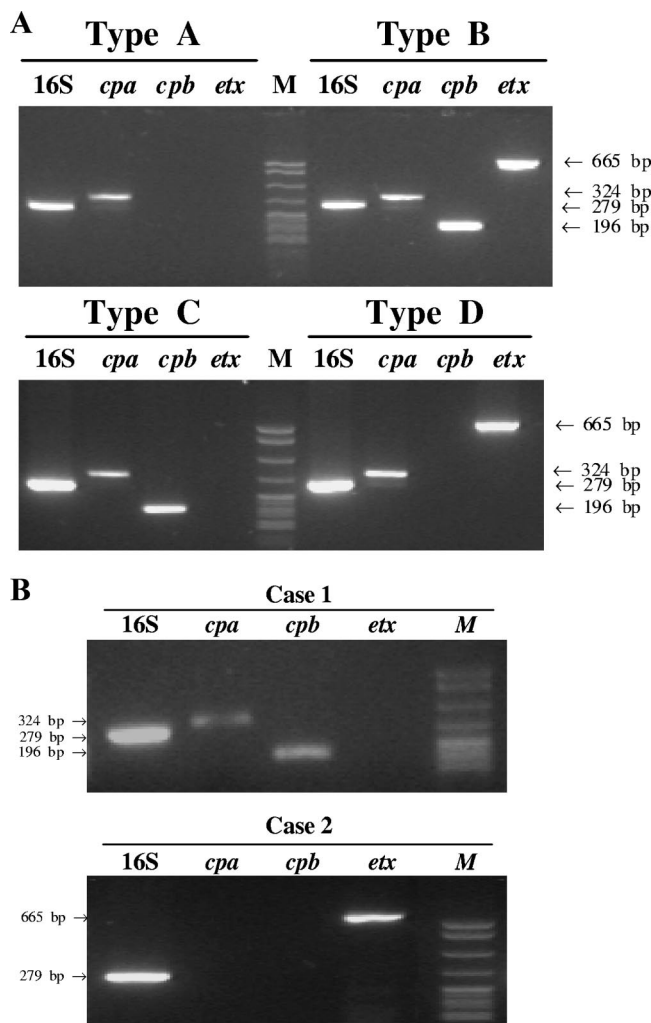


FIG. 3. Genotyping of *C. perfringens* by PCR. The DNAs extracted from *C. perfringens* types A to D (ATCC strains) (A) or from tissue sections of case 1 (stomach) and case 2 (liver) (B) were amplified using primers specific for the 16S rRNA gene and each toxin gene (*cpa*, *cpb*, and *etx*) by PCR. Lanes M contain DNA size markers (pBR322 DNA MspI digest).

these tissues, negating the need for the use of a housekeeping gene to assess inhibition due to formalin cross-link formation (Fig. 3B). Furthermore, the PCR products of the 16S rRNA genes from both cases were sequenced, which matched with 100% concordance the *C. perfringens* sequence (GenBank accession no. Y12669) obtained from GenBank. PCR analysis with primers specific to *cpa*, *cpb*, and *etx* toxin genes detected a 324-bp (alpha-toxin) band and a 196-bp (beta-toxin) band 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 (2, 5, 11) (Fig. 3A and B). The inability to detect the *cpa* and/or *cpb* genes in this bacterial species may be the result of high genetic variability in different strains (14) 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 *C. perfringens* (1), this assay can be used to confirm the diagnosis of *C. 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 with the described PCR assay does not rule out the involvement of other, non-*C. perfringens*, gas gangrene-causing *Clostridium* species, such as *C. welchii*, *C. septicum*, *C. novyi*, and *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 samples. These primer sets hold promise as a means of detecting *C. perfringens* and the genes that encode its principal toxins, but further optimization and confirmatory studies are needed. More importantly, to improve the clinical value of the assay, a similar PCR assay that includes other gas gangrene-causing *Clostridium* species (e.g., *C. welchii*, *C. septicum*, *C. novyi*, *C. histolyticum*) as well can now be developed.

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