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# Comparison of Western Immunoblots and Gene Detection Assays for Identification of Potentially Enterotoxigenic Isolates of *Clostridium perfringens*

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***Clostridium perfringens* enterotoxin (CPE) is an important sporulation-associated virulence factor in several illnesses of humans and domestic animals, including *C. perfringens* type A food poisoning. Therefore, the ability to determine the enterotoxigenicity of food or fecal *C. perfringens* isolates with simple, rapid assays should be helpful for epidemiologic investigations. In this study, Western immunoblotting (to detect CPE production in vitro) was compared with PCR assays and digoxigenin-labeled probe assays (to detect all or part of the *cpe* gene) as a method for determining the enterotoxigenicity of *C. perfringens* isolates. The *cpe* detection assays yielded reliable results with DNA purified from vegetative *C. perfringens* cultures, while Western immunoblots required in vitro sporulation of *C. perfringens* isolates to detect CPE production. Several *cpe*-positive *C. perfringens* isolates from diarrheic animals did not sporulate in vitro under commonly used sporulation-inducing conditions and consequently tested CPE negative. This result indicates that *cpe* gene detection and serologic CPE assays do not necessarily yield similar conclusions about the enterotoxigenicity of a *C. perfringens* isolate. Until further studies resolve whether these *cpe*-positive isolates which do not sporulate in vitro can or cannot sporulate and produce CPE in vivo, it may be preferable to use *cpe* detection assays for evaluating *C. perfringens* isolate enterotoxigenicity and thereby avoid potential false-negative conclusions which may occur with serologic assays.**

Laboratory identification of *Clostridium perfringens* food poisoning outbreaks is complicated by the presence of *C. perfringens* as normal fecal flora and by the ubiquitous distribution of this bacterium in the environment (9). Consequently, merely demonstrating the presence of *C. perfringens* in food or feces is not sufficient to establish *C. perfringens* as the cause of a particular food poisoning outbreak. Instead, public health agencies have traditionally relied upon a more stringent set of bacteriologic criteria (9, 12) to establish the identity of *C. perfringens* food poisoning outbreaks. While these bacteriologic criteria can be useful in some situations, a series of recent studies (7, 22, 23) has shown that these bacteriologic criteria also have significant limitations.

Symptoms associated with *C. perfringens* food poisoning are caused by *C. perfringens* enterotoxin (CPE), a 35-kDa polypeptide which is expressed during sporulation (4). Recent studies (26) suggest that only 6% of global *C. perfringens* isolates carry the *cpe* gene, while the percentage of *cpe*-positive *C. perfringens* isolates in feces from individuals affected by *C. perfringens* type A food poisoning appears to be considerably higher (25). These relationships would suggest that determining the enterotoxigenicity of food or fecal *C. perfringens* isolates by either (i) serologic assay to demonstrate the isolate's ability to produce CPE in vitro or (ii) *cpe* gene probe assay to show the isolate carries the *cpe* gene may be helpful in epidemiologic investigations of food poisoning outbreaks. Several different serologic assays have proven useful in epidemiologic investigations of food poisoning outbreaks (3, 17, 25), but all of these existing assays (e.g., enzyme-linked immunosorbent assay [ELISA] or reverse passive latex agglutination) can yield false-positive results (2, 25). Theoretically, Western immunoblots should fill the need for a more specific CPE serologic assay for demonstrating isolate enterotoxigenicity since this assay can demonstrate the specific presence in *C. perfringens* lysates of a 35-kDa species which comigrates with purified CPE and specifically reacts with antibodies prepared against purified CPE. Consistent with this expectation, a CPE Western immunoblot assay was recently shown to distinguish a CPE-positive *C. perfringens* strain from a CPE-negative *C. perfringens* strain (4).

At least three *cpe* gene detection assays, including PCR (19), radioactive gene probe (25), and digoxigenin (dig)-labeled gene probe procedures (24), have now been reported for use in identifying *cpe*-positive *C. perfringens* isolates. Unlike CPE serologic assays, which require isolates to sporulate in vitro to obtain detectable levels of CPE expression, *cpe* gene assays should be applicable to properly prepared samples from vegetative *C. perfringens* cultures. This may represent a significant advantage for gene probe assays, since fresh *C. perfringens* isolates often sporulate poorly, or not at all, in laboratory media (25), raising the theoretical possibility that serologic assays could yield false-negative results if some of these purportedly nonsporulating isolates are *cpe*-positive isolates which sporulate in vivo but not in vitro.

An important step in evaluating the usefulness of any diagnostic assay is to confirm that assay's accuracy for a large collection of samples with known characteristics. Somewhat surprisingly, neither *cpe* gene detection assays nor CPE Western immunoblots have been evaluated for a large collection of well-characterized CPE-positive and -negative *C. perfringens*

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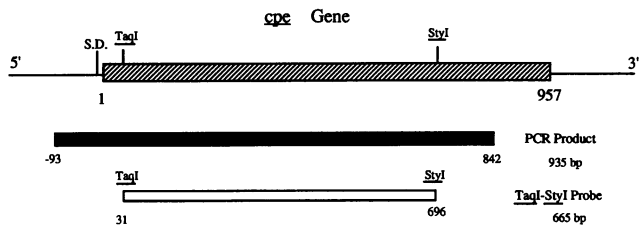


FIG. 1. Comparison of the PCR product and *Taq*I-*Sty*I probe with the *cpe* gene. The top drawing represents the ORF of the *cpe* gene, which is 957 nucleotides long, and the adjacent flanking regions (4). The sites of the single *Taq*I and *Sty*I sites within the *cpe* ORF are shown. S.D. represents the putative Shine-Dalgarno ribosome binding site for the *cpe* gene (4). The numbers shown for the PCR product and *Taq*I-*Sty*I probe reflect corresponding sequences of the *cpe* ORF.

reference strains. The current study reports a direct comparative analysis of these assays, using both well-characterized *C. perfringens* reference strains and fresh *C. perfringens* isolates.

## MATERIALS AND METHODS

***C. perfringens* reference strains.** Twenty *C. perfringens* strains previously characterized for CPE production by serologic or biologic activity assays were used in the initial portion of the study (Table 1). All of these reference strains were type A, except for CN-5383, which is a type C strain.

**Western immunoblot analysis of *C. perfringens* lysates.** A 0.1-ml aliquot of a cooked meat medium stock of each *C. perfringens* reference strain or fresh isolate was transferred to 6 ml of fluid thioglycollate (FTG) and then heat shocked for 20 min at 70°C. Each heat-shocked culture was incubated for 14 h at 37°C, and 0.4 ml of this starter culture was transferred to a second 6 ml of FTG before this culture was incubated for 9 h at 37°C. An aliquot (0.4 ml) of this FTG culture was then added to 20 ml of either Duncan-Strong (DS) sporulation medium (15) or raffinose-modified DS medium supplemented with 1 mM caffeine (RC; raffinose and caffeine are reported to enhance sporulation levels for some *C. perfringens* strains [10, 11]). RC cultures were incubated for 5 h at 43°C (6), while DS cultures were incubated for 8 h at 37°C (15). After the cultures were chilled to 4°C, sporulating or vegetative (FTG) cell lysates were prepared by sonicating 3-ml aliquots of DS, RC, or FTG culture until >95% of all cells were lysed (with lysis monitored by phase-contrast microscopy). Sonicated culture lysates were microcentrifuged to remove debris and unlysed cells. For some experiments, supernatants from sonicated sporulating cultures of CPE-negative strains or from sonicated FTG cultures of CPE-positive and CPE-negative reference strains were concentrated 100-fold by lyophilization prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Samples of *C. perfringens* lysates (3 μl of RC lysate or 20 μl of DS lysate) were subjected to SDS-PAGE and electroblotting, as described previously (4). The nitrocellulose membranes were incubated for 2 h at 24°C in BLOTTO (4) containing 0.2% purified immunoglobulin G prepared from rabbit polyclonal anti-CPE serum (13). After five washes in Tris-buffered saline (20 mM Tris buffer, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20), each nitrocellulose sheet was incubated for 1 h at 24°C in BLOTTO containing $^{125}$I-protein A (0.5 μCi/ml; specific activity, 4.5 μCi/mg; ICN). After a final five washes in Tris-buffered saline, each nitrocellulose membrane was then exposed on X-ray film overnight at −80°C.

**Isolation and purification of *C. perfringens* DNA.** In preliminary studies (data not shown), the boiling of cultures did not provide reliable lysis or *cpe* gene detection with vegetative cultures of *C. perfringens*. Therefore, the following combination of enzymatic and chemical treatments was used for lysis of *C. perfringens* cells. Each *C. perfringens* reference strain or fresh isolate was inoculated into 10 ml of brain heart infusion broth, which was then incubated anaerobically overnight at 37°C. This culture was centrifuged and washed in sterile double-distilled $H_2O$, and the cell pellet was resuspended in 1.5 ml of lysozyme solution (50 mM Tris-HCl–12.5 mM EDTA–500 mM sucrose, pH 8.0, containing lysozyme [20 mg/ml] and proteinase K [100/μg/ml]) and incubated for 2 to 4 h at 37°C. The solution was microcentrifuged, and pellets were incubated for 1 h at 37°C in 700 μl of 130 mM Tris-HCl–100 mM EDTA, pH 8.0, before the addition of 22.5 μl of 20% SDS and 4.5 μl of proteinase K solution (20 mg/ml).

The direct use of crude culture lysates for PCR or gene probe assays was investigated in preliminary experiments (data not shown), but some assay interference was noted; i.e., crude lysates from some CPE-positive strains sometimes tested *cpe* negative. Similar assay interference was noted (data not shown) when *cpe*-positive colonies were grown and lysed on membranes by a previously described procedure (25). Considering these unreliable preliminary results with crude lysates or colonies lysed on membranes, purified DNA was isolated to reduce the presence of substances which interfere with *cpe* gene detection assays. To obtain purified DNA, each tube of cell lysate (prepared as described above) received 150 μl of 5 M NaCl and 120 μl of 274 mM hexadecyltrimethylammonium bromide (Sigma) in 1 M NaCl and the mixtures were incubated for 10 min at 65°C. The cell lysate mixtures were then extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous layer was incubated with 3 μl of RNase (5 mg/ml) for 1 h at 24°C and then extracted with an equal volume of phenol-chloroform before extraction with an equal volume of chloroform-isoamyl alcohol. The DNA was precipitated by adding 0.6 volume of 100% isopropanol, dried under a vacuum, and dissolved in 100 μl of double-distilled $H_2O$. DNA concentrations were estimated from $A_{260}$ readings for each sample.

**PCR protocol for *cpe* detection.** The primer sequences used in our standard PCR protocol are 5′-TGTTAATACTTTAAG GATATGTATCC-3′ and 5′-TCCATCACCTAAGGACTG-3′. This primer pair was designed (see Fig. 1) to decrease the probability of identifying isolates carrying only portions of the *cpe* open reading frame (ORF) or carrying transcriptionally and/or translationally silent copies of the *cpe* gene by directing the amplification of a large 935-bp PCR product which includes sequences immediately upstream of *cpe* plus most of the *cpe* ORF. PCR amplification involved incubation of ~500 ng of template DNA, 0.4 μM (each) primer, 0.4 mM deoxynucleoside triphosphates, 2.0 mM $MgCl_2$ (determined as the optimal $MgCl_2$ concentration for amplification of the *cpe* gene; data not shown), and 1.5 U of *Taq* polymerase (Promega), in a total reaction volume of 50 μl, in a thermal cycler (Perkin-Elmer Cetus 480) for 34 cycles, each consisting of the following: 1.5 min at 94°C, 1 min at 50°C, and 1 min at 72°C. DNA from the *cpe*-positive *C. perfringens* strain NCTC 8239 was always included as a positive control in each set of PCRs. After PCR cycling, 20-μl aliquots of the reaction products were loaded onto a 1.5% agarose gel in the presence of ethidium bromide for electrophoresis. When present, the 935-bp PCR product was visualized with UV light.

To confirm their identity as *cpe* amplification products, the 935-bp PCR products from all *C. perfringens* reference strains

TABLE 1. Comparison of PCR and *Taq*I-*Sty*I gene probe results with Western immunoblot results for reference *C. perfringens* strains

| CPE classification and strain | Sporulation[a] | | Western blot[b] | | *Taq*I-*Sty*I[b] gene probe | PCR[b] |
|---|---|---|---|---|---|---|
| | DS | RC | DS | RC | | |
| CPE positive[c] | | | | | | |
| NCTC 8235 | sp− | Low | − | + | + | + |
| NCTC 8238 | Med | Med | + | + | + | + |
| NCTC 8239 | High | High | + | + | + | + |
| NCTC 8359 | Med | Low | + | + | + | + |
| NCTC 8798 | High | sp− | + | − | + | + |
| NCTC 8799 | sp− | Med | − | + | + | + |
| NCTC 10239 | Med | Med | + | + | + | + |
| C-1841 | Low | High | + | + | + | + |
| C-1849 | Low | High | + | + | + | + |
| C-1851 | Low | High | + | + | + | + |
| C-1869 | Low | High | + | + | + | + |
| C-1881 | Low | High | + | + | + | + |
| C-1887 | High | High | + | + | + | + |
| FD 1041 | High | High | + | + | + | + |
| CPE negative[c] | | | | | | |
| ATCC 3624 | Low | Med | − | − | − | − |
| CN-5383 | High | sp− | − | − | − | − |
| FD-1 | High | Low | − | − | − | − |
| F-42 | Med | Med | − | − | − | − |
| 215b | sp− | sp− | − | − | − | − |
| 8-1 | sp− | sp− | − | − | − | − |

[a] Degree of sporulation (as assessed by phase-contrast microscopy): sp−, no sporulation; low, <25% of cells in culture; med, 25 to 75% of cells in culture; high, >75% of cells in culture (average of four experiments for each strain).

[b] Results shown are based upon at least three independent determinations. Consistent results were obtained for all samples tested by each assay.

[c] Based upon previous classification (3, 5, 6, 13, 16, 21).

and eight randomly selected PCR-positive fresh isolates were analyzed by restriction endonuclease digestion. Each PCR product (8 μl) was digested overnight at 37°C with 1 μl of either *Pst*I, *Pvu*II, *Bgl*II, or *Kpn*I and 1 μl of the appropriate 10× buffer. Restriction enzyme-digested samples were loaded onto a 0.8% agarose gel and run at 80 V for 1 h. After ethidium bromide staining, bands were visualized by UV illumination.

**dig-labeled *cpe* gene probe protocol.** On the basis of the published *cpe* sequence (4), two unique restriction sites, one for *Taq*I and the second for *Sty*I, were predicted to lie within the *cpe* ORF (Fig. 1). Therefore, cesium-purified DNA from a recombinant pUC19 plasmid carrying the *cpe* gene (4) was *Xba*I digested to remove a clostridial DNA insert. This insert was gel purified and then double digested with *Taq*I-*Sty*I to generate a 665-bp DNA fragment containing internal sequence corresponding to 70% of the *cpe* ORF (Fig. 1). The *Taq*I-*Sty*I *cpe* fragment was gel purified, and its identity was confirmed (data not shown) by restriction enzyme digestion and Southern blotting with the recombinant pUC19 plasmid carrying the *cpe* gene (4). After ethanol precipitation, 100 ng of the *Taq*I-*Sty*I fragment was labeled by random priming with dig-dUTP, using the Genius system per the supplier's instructions; the dig-labeled *Taq*I-*Sty*I probe was stable for at least 6 months (data not shown).

Approximately 500 ng of purified DNA from each *C. perfringens* strain or isolate was spotted onto a Nytran-Plus nylon membrane (Schleicher and Schuell). This DNA was then denatured by NaOH treatment and fixed to the membrane by UV cross-linking. The DNA dot blots were prehybridized and then hybridized with the dig-labeled *cpe* probe, per the Genius system instructions. When present, the hybridized probe was detected with an anti-dig immunoglobulin G-alkaline phosphatase conjugate (Boehringer Mannheim) and Lumi-Phos 530 (Boehringer Mannheim) alkaline phosphatase substrate, per the Genius system instructions, prior to exposure on X-ray film for 10 to 15 min.

## RESULTS

**Evaluation of the ability of Western immunoblots to specifically identify enterotoxigenic *C. perfringens* reference strains.** While a recent study (4) suggests that Western immunoblots may be useful for distinguishing enterotoxigenic from nonenterotoxigenic *C. perfringens*, only a single CPE-positive strain and a single CPE-negative strain were examined in that study. Therefore, the usefulness of Western immunoblots for identifying enterotoxigenic *C. perfringens* was more thoroughly evaluated in the current study by using a large collection of *C. perfringens* reference strains which are well characterized for their enterotoxigenicities. As shown in Fig. 2A and B and Table 1, CPE Western immunoblots clearly and consistently distinguished between sporulating lysates from CPE-positive and -negative reference strains; i.e., sporulating lysates from all known CPE-positive strains contained a 35-kDa polypeptide which reacted with CPE antibodies. This immunoreactive species was absent from (i) sporulating lysates from all CPE-negative strains, even when 100-fold-concentrated sporulating lysates from these strains were assayed (data not shown), and (ii) 100-fold-concentrated vegetative (FTG) lysates from all reference strains (data not shown).

Additional studies were performed to further define the sensitivity and specificity of the CPE Western immunoblot procedure. Our standard CPE Western immunoblot assay is capable of detecting at least 10 ng of CPE (data not shown), as evaluated by adding known amounts of purified CPE to a CPE-negative culture lysate background; this sensitivity can be easily increased, if desired, by using longer exposure of X-ray films during autoradiography (data not shown). Two additional experiments further demonstrated the specificity of the CPE Western immunoblot suggested by the results shown in Fig. 2 and Table 1: (i) a double-blind evaluation of the CPE Western immunoblot in which lysate samples from 18 different *C. perfringens* reference strains (including lysates from 12 CPE-positive and 6 CPE-negative strains listed in Table 1) were correctly and reproducibly distinguished and (ii) tests of CPE Western immunoblot reactivity with lysates from other potential enteropathogens (including *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Clostridium difficile*, and *Bacillus cereus*), which showed that these lysates all tested consistently negative with the CPE Western immunoblot assay (data not shown).

**Evaluation of the ability of *cpe* gene detection assays to specifically identify enterotoxigenic *C. perfringens* reference strains.** Since dig-labeled *cpe* gene probe and *cpe* PCR assays have never been evaluated for a large collection of well-characterized *C. perfringens* reference strains, the same strains used to evaluate the Western immunoblot were also tested with these two *cpe* gene detection assays. With purified DNA from vegetative cultures of each reference strain, both our dig-labeled *cpe* gene probe assay and *cpe* PCR protocols yielded reproducible results for each strain (Table 1); positive versus negative results could be clearly distinguished for both of our *cpe* gene detection protocols (Fig. 3 and 4). The specificity of both *cpe* gene detection assays was further demonstrated by (i) double-blind experiments, in which DNAs from 18 different *C. perfringens* reference strains (including DNAs from 12 CPE-positive and 6 CPE-negative strains listed

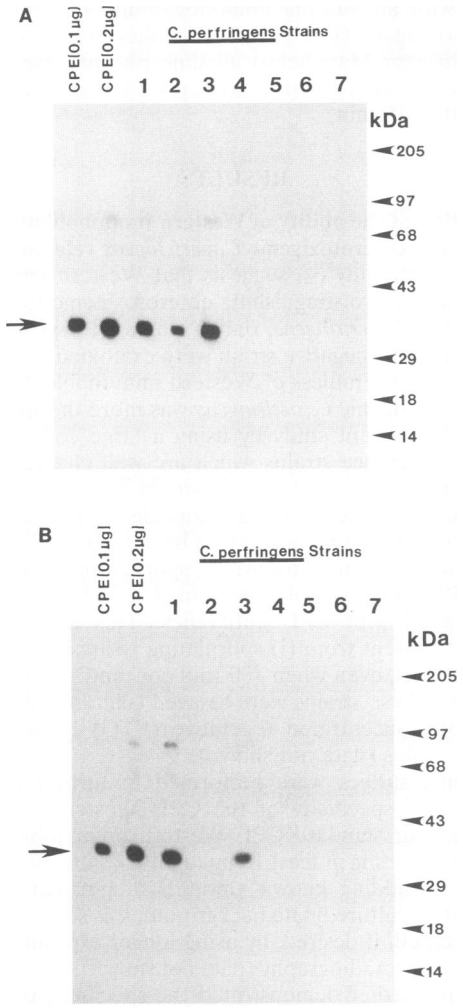


FIG. 2. Representative Western immunoblot results for CPE detection in cell lysates from *C. perfringens* reference strains. (A) Lysates from representative reference strains grown in DS sporulation medium, including NCTC 8239 (lane 1), NCTC 8798 (lane 2), NCTC 10239 (lane 3), ATCC 3624 (lane 4), FD-1 (lane 5), F-42 (lane 6), and 215b (lane 7). Reference strains corresponding to samples in lanes 1 through 3 have been previously classified as enterotoxin producing, while those of samples in lanes 4 through 7 have been classified as nonenterotoxigenic (see Table 1). For comparison, specified concentrations of purified native *C. perfringens* enterotoxin are shown in the leftmost two lanes (with the location of purified CPE on the Western immunoblots highlighted by the arrow on the left). The small amounts of high-$M_r$ immunoreactive material present in samples containing high concentrations of purified CPE, or in some CPE-positive lysates, result from CPE aggregation in SDS-PAGE (14). (B) Lysates from the same representative reference strains grown in RC sporulation medium. Samples are identical to those described for panel A. Identical results were obtained with three repetitions of both panel A and B Western immunoblots, each repetition using lysates from a freshly grown culture. No immunoreactivity was detected on immunoblots identical to those in panels A and B developed with normal rabbit immunoglobulin G (data not shown). The positions of molecular mass markers are indicated at the right of each blot.

in Table 1) were correctly and reproducibly distinguished by both *cpe* gene detection assays and (ii) testing of DNAs from other enteropathogens (including the same species used to evaluate the CPE Western immunoblot), which produced

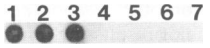


FIG. 3. Representative *Taq*I-*Sty*I gene probe results for *cpe* detection with DNA from *C. perfringens* reference strains. Samples contained 500 ng of DNA isolated from vegetative cultures of reference DNA strains. Dots: 1, NCTC 8239; 2, NCTC 8798; 3, NCTC 10239; 4, ATCC 3624; 5, FD-1; 6, F-42; 7, 215b. Reference strains used for samples 1 through 3 have been previously classified as enterotoxigenic, while those used in samples 4 through 7 have been classified as nonenterotoxigenic (see Table 1). Similar results were obtained for two repetitions with the samples shown in this figure. An identically clear distinction between positive and negative results was reproducibly noted for all results shown in Tables 1 and 3.

consistently negative results with both *cpe* assays. Finally, the PCR assay's specificity was also verified by restriction endonuclease digestions which authenticated the 935-bp PCR product observed for each PCR-positive reference strain as a *cpe* amplification product (data not shown). The absolute sensitivity limit of the dig-labeled *cpe* gene probe assay was determined to be 1 to 10 ng of purified DNA from a CPE-positive *C. perfringens* strain, while the *cpe* PCR was more sensitive; i.e., it reproducibly showed a visible 935-bp product with 50 to 500 pg of template DNA.

**Comparison of Western immunoblots and *cpe* gene detection assays using fresh *C. perfringens* isolates.** Since the results shown in Table 1 indicate that both the CPE Western immunoblot and *cpe* gene detection assays reliably identified the enterotoxigenic *C. perfringens* reference strains, the diagnostic abilities of these assays were then tested for a collection of fresh *C. perfringens* isolates from a variety of sources; only a low percentage of these isolates initially tested PCR positive for the *cpe* gene (Table 2). Restriction endonuclease digestions confirmed that the 935-bp PCR product amplified from the DNAs of eight randomly selected PCR-positive isolates represented an authentic *cpe* amplification product; i.e., predicted digestion patterns similar to those of the *cpe* gene of strain NCTC 8239 were observed with each isolate's PCR product (data not shown).

Twelve isolates testing PCR positive and twelve isolates

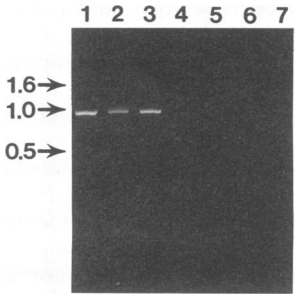


FIG. 4. Representative PCR results for *cpe* detection with DNA from *C. perfringens* reference strains. Samples contained DNA isolated from vegetative cultures of reference strains. Lanes: 1, NCTC 8239; 2, NCTC 8798; 3, NCTC 10239; 4, ATCC 3624; 5, FD-1; 6, F-42; 7, 215b. Reference strains used for samples in lanes 1 through 3 have been previously classified as enterotoxigenic, while those used for samples in lanes 4 through 7 have been classified as nonenterotoxigenic (see Table 1). Similar results were obtained when these samples were retested twice by PCR. An identically clear distinction between positive and negative results was reproducibly noted for all results shown in Tables 1 and 3. The positions of size markers (in kilobases) are indicated at the left of the gel.

TABLE 2. PCR testing of previously uncharacterized *C. perfringens* isolates to identify *cpe*-positive isolates[a]

| Isolate origin | No. of isolates | |
|---|---|---|
| | Tested | Positive[b] |
| Avian | 18 | 2 |
| Bovine | 96 | 0 |
| Canine | 125 | 7 |
| Caprine | 3 | 0 |
| Deer | 7 | 0 |
| Equine | 20 | 0 |
| Feline | 7 | 0 |
| Ferret | 2 | 0 |
| Goat | 1 | 0 |
| Horse | 1 | 0 |
| Human | 41 | 2 |
| Kangaroo | 1 | 0 |
| Lizard | 1 | 0 |
| Murine | 1 | 0 |
| Ovine | 22 | 0 |
| Porcine | 70 | 3 |
| Puma | 3 | 0 |
| Rabbit | 2 | 0 |
| Red panda | 1 | 0 |
| Seal | 3 | 0 |
| Silver leaf langur | 1 | 0 |
| Tapir | 1 | 0 |
| Woodchuck | 1 | 0 |
| Animal food | 4 | 0 |
| Unknown[c] | 22 | 2 |
| Total | 454 | 16 (3.5%) |

[a] No two isolates were obtained from the same individual animal or food sample.

[b] All samples initially testing PCR positive were retested at least twice, with similar results. Representative randomly selected DNA samples from 174 isolates initially testing PCR negative were rescreened by PCR and remained negative.

[c] Source not submitted with isolate.

TABLE 3. Comparison of PCR and *Taq*I-*Sty*I gene probe results with Western immunoblot results for previously uncharacterized *C. perfringens* isolates

| Strain | Sporulation[a] | | Western blot[b] | | *Taq*I-*Sty*I[b] gene probe | PCR[b] | Isolate origin |
|---|---|---|---|---|---|---|---|
| | DS | RC | DS | RC | | | |
| 5 | Low | Low | + | + | + | + | Human |
| 153 | High | Low | + | + | + | + | Porcine |
| 155 | Med | Med | + | + | + | + | Porcine |
| 222 | High | Med | + | + | + | + | Canine |
| 382 | Low | Med | + | + | + | + | Canine |
| 452 | Low | Low | + | + | + | + | Canine |
| 458 | Med | Low | + | + | + | + | Canine |
| CP-1 | sp− | sp− | − | − | + | + | Porcine |
| 157 | sp− | sp− | − | − | + | + | Canine |
| 455 | sp− | sp− | − | − | + | + | Canine |
| 456 | sp− | sp− | − | − | + | + | Canine |
| 457 | sp− | sp− | − | − | + | + | Canine |
| 75 | Med | Med | − | − | − | − | Avian |
| 406 | sp− | Low | − | − | − | − | Porcine |
| 424 | High | Low | − | − | − | − | Canine |
| 425 | Low | sp− | − | − | − | − | Bovine |
| 427 | High | Low | − | − | − | − | Bovine |
| 428 | Low | Med | − | − | − | − | Feline |
| 125 | sp− | sp− | − | − | − | − | Human |
| 126 | sp− | sp− | − | − | − | − | Human |
| 127 | sp− | sp− | − | − | − | − | Human |
| 150 | sp− | sp− | − | − | − | − | Avian |
| 414 | sp− | sp− | − | − | − | − | Ovine |
| 416 | sp− | sp− | − | − | − | − | Equine |

[a] Degree of culture sporulation (as assessed by phase-contrast microscopy): sp−, no sporulation; low, <25% of cells in culture; med, 25 to 75% of cells in culture; high, >75% of cells in culture (average of four experiments for each strain).

[b] Results shown are based upon at least three independent determinations. Consistent results were obtained for all samples tested by each assay.

testing PCR negative were then randomly selected for further evaluation by the dig-labeled *cpe* gene probe assay and CPE Western immunoblot assay to permit direct comparisons between all three enterotoxigenicity assays using the same fresh *C. perfringens* isolates. Similarly to results observed in Table 1 for the reference strains, there was complete agreement between the *cpe* PCR and dig-labeled *cpe* gene probe assays using fresh isolates (Table 3). Further, all *cpe*-positive fresh isolates which sporulated in vitro produced detectable amounts of CPE on Western immunoblots, while all *cpe*-negative fresh isolates tested CPE-negative by Western immunoblots (Table 3). However, several *cpe*-positive isolates did not sporulate in vitro and thus did not produce detectable levels of CPE on Western immunoblots (Table 3).

## DISCUSSION

This study evaluated the ability of CPE Western immunoblots, *cpe* PCR, and dig-labeled *cpe* gene probe assays to identify enterotoxigenic *C. perfringens* isolates. When performed as described, all three assays were 100% specific and 100% sensitive for distinguishing between enterotoxigenic and nonenterotoxigenic reference strains; i.e., the results from each assay were consistent with the known phenotypes of these strains. It might be noted that while the absolute sensitivity of our *cpe* PCR, i.e., the smallest amount of DNA which produces a visible PCR product, is somewhat low compared with the sensitivities of some other PCR assays (1, 20), our *cpe* PCR assay still provides reliable results since 1,000-fold more template DNA than is required for minimum sensitivity is included in the PCR. The reason for the relatively low sensitivity of our *cpe* PCR is not clear, but it does not appear to involve limitations imposed by the size of the target sequence, since similar low sensitivity was also observed with a different primer concentration which amplified a *cpe* PCR product of only 343 bp, rather than our standard 935-bp PCR product (data not shown).

When fresh *C. perfringens* isolates were grown to evaluate these three assays, 40% of the fresh *cpe*-positive *C. perfringens* isolates failed to sporulate under either of two growth conditions commonly used to induce in vitro sporulation of *C. perfringens*. This finding has obvious diagnostic significance, since these *cpe*-positive isolates which failed to sporulate in vitro also tested CPE negative on sporulation-dependent CPE Western immunoblots. Therefore, our results have now confirmed the theoretical possibility that CPE serologic assays and *cpe* gene detection assays using purified DNA from vegetative *C. perfringens* cultures may not necessarily produce similar conclusions about the enterotoxigenicity of *C. perfringens* isolates. Should any *cpe*-positive isolates which do not sporulate well in vitro be capable of sporulating and producing CPE in vivo, then *cpe* gene detection assays using DNA purified from vegetative cultures would hold a significant advantage over CPE serologic assays, which would be generating false-negative results with these isolates. Given the limited information available concerning the relationship between in vivo and in vitro sporulation for *cpe*-positive *C. perfringens*, animal challenge studies will be necessary to definitively evaluate whether

any *cpe*-positive *C. perfringens* isolates failing to sporulate in vitro actually sporulate and produce CPE in vivo. Finally, our discovery of significant numbers of *cpe*-positive isolates which do not sporulate in vitro also brings the use of sporulation-based lysis procedures for *cpe* gene assays (as used recently [19]) into question, since this lysis procedure may yield false-negative conclusions about *cpe*-positive isolates which do not sporulate in vitro.

While it remains to be conclusively determined whether any *cpe*-positive isolates which do not sporulate in vitro are capable of sporulating and producing CPE in vivo, there is some indirect evidence supporting this possibility. First, several of the nonsporulating *cpe*-positive isolates identified in our study came from the feces of diarrheic dogs (Table 3). CPE made in vivo by these isolates could be responsible for this diarrhea, since previous studies (8) have closely linked enterotoxigenic *C. perfringens* to canine diarrhea. Second, in our laboratory we have encountered several instances in which large numbers of *C. perfringens* were detected (in the apparent absence of other enteropathogens) in the feces of diarrheic humans. However, despite repeated attempts with the use of different sporulation media, *C. perfringens* isolates from some of these individuals failed to sporulate in vitro and, as would be expected, tested CPE negative by serologic assay. In retrospect, this pattern is fully consistent with the possibility that these nonsporulating isolates may be *cpe* positive and capable of in vivo sporulation and CPE production. Unfortunately, since these samples were studied prior to the recent development of *cpe* gene detection assays, it was not possible to determine whether any of these *C. perfringens* isolates were *cpe* positive. Nonetheless, these experiences suggest that it is now important to determine whether there are human diarrheic isolates of *C. perfringens* which are *cpe* positive but do not sporulate in vitro.

As a final comment on comparing *cpe* gene detection and CPE serologic detection assays, it is clear that there is also at least one potential disadvantage (irrespective of the outcome of animal challenge experiments) shared by all *cpe* detection assays compared with serologic assays. These gene detection assays could theoretically generate false-positive conclusions regarding the ability of a *cpe*-positive isolate to actually produce CPE if the isolate (which may even be able to sporulate) does not also produce regulatory factors required for CPE expression. While the current study does not offer any support for this possibility (i.e., no sporulation-positive, CPE-negative isolates carrying the *cpe* gene were identified), the limited sampling in our study does not completely eliminate this possibility. Therefore, it is important to appreciate that isolates identified as *cpe* positive by gene detection assays should be referred to only as potentially enterotoxigenic unless subsequent CPE serologic testing confirms their ability to actually produce CPE.

Pending results of proposed animal challenge studies, the best current approach for evaluating the potential enterotoxigenicity of *C. perfringens* isolates appears to involve the use of gene detection assays with purified DNA from vegetative *C. perfringens* cultures to detect any *cpe*-positive isolates in food or feces and thus avoid the possible false-negative results inherent to serologic assays. Those *C. perfringens* isolates testing *cpe* positive should then be evaluated by serologic assay to attempt to confirm their ability to actually produce CPE. Given the longer time and more complex procedure involved in Western immunoblotting, commercially available ELISAs or reverse passive latex agglutination CPE assays should be used for routine screenings of most sporulating isolates (while nonsporulating *cpe*-positive isolates will still be identified only as potentially enterotoxigenic). However, given their high specificity, Western immunoblots can be used for definitive confirmation of positive (particularly weak-positive) results obtained by other CPE serologic assays. Regardless of the type of serologic assay used, our results clearly demonstrate the desirability of using more than one type of sporulation medium for CPE serologic assays. Instructions included with commercially available CPE detection kits may be misleading and imply that a single sporulation medium will induce sporulation in all *C. perfringens* isolates.

Finally, while this report establishes the reliability and limitations of *cpe* gene detection assays and CPE Western immunoblots for identifying potentially enterotoxigenic *C. perfringens* isolates, it is already becoming apparent that merely demonstrating the presence of some enterotoxigenic or potentially enterotoxigenic isolates in food or feces will not be sufficient, by itself, to establish reliable laboratory identification of CPE-linked diseases, since CPE-positive *C. perfringens* isolates have apparently been identified in feces from some healthy individuals (18). This observation also implies that sensitive *cpe* or CPE detection assays should not be used with crude cultures obtained from feces, as recently suggested (19). Instead, the technology evaluated in this report should now be used to specifically establish the numbers of enterotoxigenic (or potentially enterotoxigenic) *C. perfringens* cells, rather than the total numbers of *C. perfringens* cells, associated with (i) normal food versus contaminated food and (ii) feces from healthy individuals versus feces from individuals with CPE-mediated disease. These threshold levels for enterotoxigenic *C. perfringens* in food or feces can then be used to generate epidemiologically meaningful criteria for laboratory identification of CPE-linked diseases.

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