Development of a Duplex PCR Genotyping Assay for Distinguishing *Clostridium perfringens* Type A Isolates Carrying Chromosomal Enterotoxin (*cpe*) Genes from Those Carrying Plasmid-Borne Enterotoxin (*cpe*) Genes

Qiyi Wen,[1,2] Kazuaki Miyamoto,[1,3] and Bruce A. McClane[1]*

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261[1]; Department of Veterinary Medicine, University of Yangzhou School of Veterinary Medicine, Yangzhou, Jiangsu 225009, China[2]; and Department of Microbiology, Wakayama Medical College, Wakayama, Japan 641-0021[3]

About 5% of *Clostridium perfringens* type A isolates carry the *cpe* gene encoding the *C. perfringens* enterotoxin. Those *cpe*-positive type A isolates are important causes of food-poisoning and non-food-borne cases of diarrheas in humans, as well as certain veterinary cases of diarrhea. Previous studies have determined that the enterotoxigenic type A isolates causing both non-food-borne human gastrointestinal disease and veterinary disease carry their *cpe* genes on plasmids, while the type A isolates causing human food poisoning carry a chromosomal *cpe* gene. The present study reports on the successful development of a duplex PCR assay that can rapidly genotype enterotoxigenic type A isolates (i.e., determine whether those *cpe*-positive isolates carry a chromosomal or a plasmid-borne *cpe* gene). The availability of this rapid *cpe* genotyping assay capable of handling large numbers of samples provides a powerful new investigative tool for diagnostic, epidemiologic, and basic research purposes.

Isolates of the gram-positive spore former *Clostridium perfringens* are classified into five types (types A to E), depending upon their ability to express alpha, beta, epsilon, and iota toxins (16). Type A isolates, which predominate in the global *C. perfringens* population (11, 13), rank as important histotoxic and enteric pathogens of humans and domestic animals. Approximately 5% of all type A isolates produce (11, 13) another biomedically important toxin, named *C. perfringens* enterotoxin (CPE). Those CPE-positive type A isolates are traditionally recognized as the cause of *C. perfringens* type A food poisoning, the third most commonly reported food-borne illness in the United States (15). More recent studies (1–6, 12, 18) have also identified CPE-positive type A strains as an underappreciated cause of up to 5 to 20% of all cases of non-food-borne human gastrointestinal (GI) disease. CPE-associated non-food-borne GI disease cases are strongly associated with antibiotic use and involve enteric disease symptoms more severe and persistent than those typically seen with *C. perfringens* type A food poisoning (1–6, 12, 18). Instead of food-borne transmission, CPE-associated non-food-borne GI diseases appear to spread person to person or via ingestion of environmental contaminants (1–6, 12, 14, 18). Finally, CPE-associated type A isolates are also responsible for certain veterinary cases of diarrhea (14, 19).

CPE-positive *C. perfringens* type A isolates have been shown to carry their enterotoxin gene (*cpe*) on either the chromosome or a plasmid (8, 9, 20). The *cpe* gene is present on the chromosomes of most (or all) type A food-poisoning isolates (8, 20) but resides on large, single- or low-copy-number plasmids in most (or all) type A non-food-borne human GI disease or veterinary isolates (8, 9, 20). The recent discovery of these specific *cpe* genotype-disease associations has spurred considerable clinical and epidemiologic interest in the genotyping of disease-associated food or fecal *cpe*-positive *C. perfringens* type A isolates to determine whether they carry a chromosomal or a plasmid-borne *cpe* gene.

To date, *cpe* genotyping assays are limited to *cpe* Southern blot analyses of *C. perfringens* DNA subjected to pulsed-field gel electrophoresis (PFGE) or restriction fragment length polymorphism (RFLP) analysis (8, 9, 20). Those Southern blot analysis-based *cpe* genotyping procedures are slow and technically challenging, require expensive equipment and considerable technical expertise, and can handle only limited numbers of samples.

To address the need for an improved *cpe* genotyping assay, the present study reports on the development of a new PCR-based assay that can rapidly and simply determine whether a *cpe*-positive type A isolate carries a chromosomal or a plasmid-borne *cpe* gene.

MATERIALS AND METHODS

***C. perfringens* strains.** This study analyzed 84 *cpe*-positive *C. perfringens* type A isolates that had previously been genotyped for determination of the location of their *cpe* genes; i.e., their *cpe* genes had been localized to the chromosome or a plasmid by *cpe* Southern blot analysis-based RFLP or PFGE genotyping approaches (8, 17, 20). Those isolates tested included 57 isolates carrying plasmid-borne *cpe* genes from the following sources: 7 isolates from human antibiotic-associated diarrhea cases in the United Kingdom in the 1980s, 10 isolates from human sporadic diarrhea cases in the United Kingdom in the 1990s, 29 isolates from human antibiotic-associated diarrhea cases occurring in several different

* Corresponding author. Mailing address: E1240 BST, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. Phone: (412) 648-9022. Fax: (412) 624-1401. E-mail: bamcc@pitt.edu.
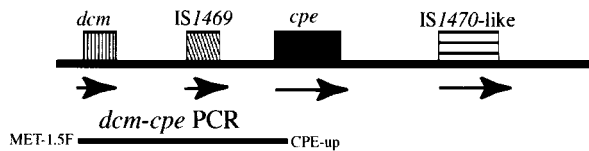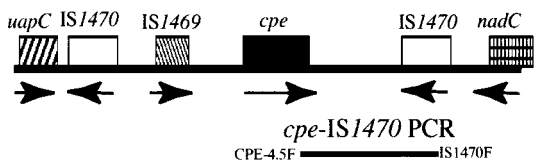
(A) PCR detection of the plasmid *cpe* locus in type A isolates

(B) PCR detection of the chromosomal *cpe* locus in type A isolates

FIG. 1. Duplex PCR strategy for amplifying products from the chromosomal *cpe* locus and the plasmid-borne *cpe* locus of *C. perfringens* type A isolates. (A) Organization of the plasmid *cpe* locus of type A isolates (17); the bar underlying the open reading frames depicts the *dcm-cpe* PCR product specifically amplified from the plasmid *cpe* locus by use of the MET-1.5F and CPE-up primer pair. (B) Organization of the chromosomal *cpe* locus of type A isolates (17); the bar underlying the open reading frames depicts the *cpe*-IS*1470* PCR product specifically amplified from the chromosomal *cpe* locus by use of the CPE-4.5F and IS1470F primer pair. Arrows indicate the orientations of the open reading frames.

hospitals in the United States or Canada in the 1990s, 7 isolates from canine diarrhea cases in the United States in the 1990s, 1 isolate from a porcine enteritis case in the United Kingdom in the 1990s, 1 isolate from the peritoneal fluid of a canine in the 1990s, 1 fecal isolate from a nonsymptomatic dog in the United States in the 1990s, and 1 fecal isolate from a nonsymptomatic human in Japan in 2000.

Also examined were 27 type A isolates with chromosomal *cpe* obtained from the following sources: 8 isolates from human food-poisoning cases in the United Kingdom in the 1950s, 2 isolates from human food-poisoning outbreaks in the United States in the 1960s, 8 isolates from two different human food-poisoning outbreaks in the United States in the 1980s, 6 isolates from two different food-poisoning outbreaks in the United States in the 1990s, and 1 isolate from a porcine enteritis case in the United Kingdom in the 1990s.

As negative controls, this study also used ATCC 3624 and ATCC 297442, two *C. perfringens* type A isolates previously shown to be *cpe* negative (10, 17).

DNA isolation. Starter vegetative cultures (10 ml) of each *C. perfringens* type A isolate were prepared by overnight growth at 37°C in fluid thioglycolate (FTG) broth (Difco). A 0.2-ml aliquot of each FTG broth culture was then inoculated into 10 ml of TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine [10]), which was then incubated at 37°C overnight. Genomic DNA was extracted from each overnight TGY culture, as described previously (10), and then quantified by measuring the UV absorption at 280 and 260 nm with a Bio-Rad Smartspec spectrophotometer. Aliquots of each extracted DNA preparation were then diluted to 10 ng/μl.

Single *dcm-cpe* PCR for detection of plasmid-borne *cpe* gene. On the basis of recent results of studies describing the organization of the *cpe* locus in 13 type A isolates carrying a plasmid-borne *cpe* gene (17), the following primer pair was designed to amplify by PCR an ~3.3-kb product from the apparently conserved DNA region (Fig. 1) between the upstream *dcm* sequences and the plasmid-borne *cpe* gene in type A isolates: 5′-CTCAGAGTTAGGGAGCTAGCCCAAC CC-3′ (primer MET-1.5F) and 5′-CCTAATATCCAACCATCTCC-3′ (primer CPE-up). The two primers were added (final concentration, 1 μM each) to a 50-μl PCR mixture containing 10 ng of purified DNA extracted from each *C. perfringens* type A isolate, 0.2 μM deoxynucleotide triphosphates (Promega), 5 μl of 10× Advantage 2 PCR buffer (Clontech), and 1 μl of Advantage 2 polymerase mix (Clontech). Those PCR mixtures were placed in a thermal cycler (Techne) and subjected to the following amplification conditions: 1st cycle, 94°C for 5 min; 2nd to 34th cycles, 94°C for 30 s, 63°C for 60 s, and 68°C for 180 s; and 35th cycle, 94°C for 90 s, 60°C for 90 s, and 68°C for 7 min. Aliquots (20 μl) of each PCR

sample were then electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Single *cpe*-IS*1470* PCR for detection of chromosomal *cpe* gene. On the basis of recent results of studies describing the organization of the *cpe* locus in five type A food-poisoning isolates carrying a chromosomal *cpe* gene (7, 17), the following primer pairing was designed to PCR amplify an ~2.1-kb product from the apparently conserved DNA region (see Fig. 1) between the chromosomal *cpe* gene and downstream IS*1470* sequences in type A isolates: 5′-CAGTCCTTAGGTGATG GA-3′ (primer CPE-4.5F) and 5′-AACTAAATAGGCCTATAAATACC-3′ (primer IS*1470*F). The two primers were added (final concentration, 1 μM each) to a 50-μl PCR mixture containing the same concentrations of template and reagents described above for the *dcm-cpe* PCR. Those reaction mixtures were then subjected to similar PCR amplification conditions as described above for the *dcm-cpe* PCR. The resultant PCR products were visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

Duplex PCR assay for genotyping *cpe*-positive *C. perfringens* type A isolates. The *dcm-cpe* and *cpe*-IS*1470* primer pairs (described above) were combined to create a duplex PCR genotyping assay capable of distinguishing between type A isolates carrying a chromosomal and a plasmid-borne *cpe* gene. The optimal final concentrations of the primers for this duplex PCR assay were determined by checkerboard titration (data not shown) to be 0.6 μM each for MET-1.5F and CPE-up and 0.8 μM each for CPE-4.5F and IS1470F. The duplex PCR was performed in a total volume of 50 μl under the same amplification conditions and with the same concentrations of purified DNA template and reagents described above for the two single PCRs for the amplification of sequences from either the type A chromosomal *cpe* locus or the type A plasmid-borne *cpe* locus. The PCR amplification conditions were also the same as those used for the single *dcm-cpe* and *cpe*-IS*1470* PCRs. Duplex PCR products were detected by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

Duplex PCR *cpe* genotyping assay with lysates from *C. perfringens* colonies. To test whether the duplex PCR *cpe* genotyping assay can use crude *C. perfringens* colony lysates as a template DNA source, a 0.1-ml aliquot of a cooked meat medium stock culture was transferred to 10 ml of FTG broth, which was then heat shocked at 72°C for 20 min before being incubated overnight at 37°C. A 0.2-ml aliquot of that FTG broth starter culture was inoculated into a fresh tube of FTG broth, which was incubated overnight at 37°C. A loopful of that overnight culture was then streaked onto a brain heart infusion (BHI) agar plate, which was then incubated for ~18 h at 37°C in an anaerobic jar (BBL). About four to five colonies were taken from each BHI agar plate and suspended in 200 μl of sterile phosphate-buffered saline (PBS). Those suspended cells were microcentrifuged at 14,000 × *g* for 5 min, and the resultant pellet was washed twice with PBS prior to resuspension in 200 μl of PCR-grade H$_2$O (Sigma). The microcentrifuge tube containing the resuspended cells was sealed with Parafilm and then microwaved (700 W) for a total of 20 min (administered as four 5-min heating treatments with 1-min cooling intervals) to induce bacterial lysis. The resultant lysate was cleared by microcentrifugation at 14,000 × *g* for 5 min, and 10 μl of that supernatant was used as template DNA for the duplex *cpe* genotyping PCR assay, as described above.

RESULTS

Development of a single *dcm-cpe* PCR assay for specific identification of *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene. A recent study comparing the organization of the plasmid-borne and the chromosomal *cpe* loci in *C. perfringens* type A isolates (17) had determined that *dcm* sequences are present ~3 kb upstream of the *cpe* gene in all 13 examined type A isolates carrying a plasmid-borne *cpe* gene (Fig. 1). However, *dcm* sequences were not detected in any of the five examined type A isolates carrying a chromosomal *cpe* gene (Fig. 1). Those previous observations suggested that primers specific for *dcm* and *cpe* sequences might be useful for the development of a PCR that could amplify a product (of ~3.3 kb) from type A isolates carrying a plasmid-borne *cpe* gene but not from type A isolates carrying a chromosomal *cpe* gene.

This hypothesis was tested by using purified DNA extracted from one *cpe*-negative isolate and the same 18 *cpe*-positive type
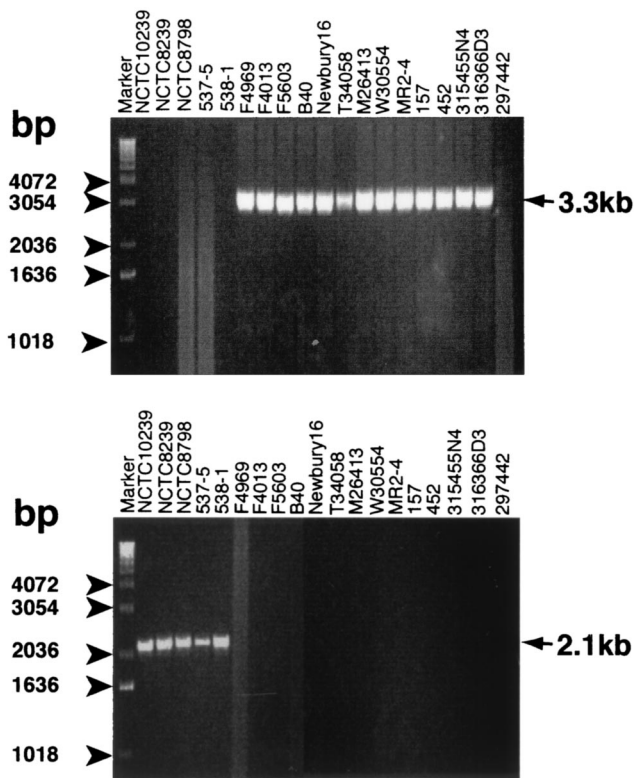
FIG. 2. Development of single PCR for identifying the plasmid-borne *cpe* locus or the chromosomal *cpe* locus of *C. perfringens* type A isolates. (A) Representative results obtained by the single *dcm-cpe* PCR (the expected product of 3.3 kb is indicated) with primers MET-1.5F and CPE-up. (B) Representative results obtained by the single *cpe*-IS*1470* PCR (the expected product of 2.1 kb is indicated) with primers CPE-4.5F and IS1470F. The numbers on the left of each gel indicate the migration of molecular size markers.

A isolates examined in the previous study. As indicated by the representative results shown in Fig. 2A, the expected ~3.3-kb PCR product was amplified by using purified DNA extracted from all 13 type A isolates whose plasmid-borne *cpe* loci had previously been investigated (17). However, no PCR product was amplified by using purified DNA extracted from any of the five previously examined type A isolates carrying a chromosomal *cpe* gene or from ATCC 297442, a type A isolate previously shown to be *cpe* negative (17).

Development of a single *cpe*-IS*1470* PCR assay for specific identification of *C. perfringens* type A isolates carrying a chromosomal *cpe* gene. The recent study comparing the organization of the plasmid-borne and chromosomal *cpe* loci in *C. perfringens* type A isolates (17) also determined that IS*1470* sequences are present ~2 kb downstream of the *cpe* gene in the five type A isolates carrying a chromosomal *cpe* gene examined previously. However, similarly oriented IS*1470* sequences were not detected in the plasmid-borne *cpe* locus of the 13 type A isolates examined (Fig. 1). Those observations suggested that primers specific for IS*1470* and *cpe* sequences might be useful for the development of a PCR that could amplify a product (of ~2.1 kb) from type A isolates carrying a chromosomal *cpe* gene but not from type A isolates carrying a plasmid-borne *cpe* gene.

This hypothesis was tested by using purified DNA extracted from the same 18 well-characterized *cpe*-positive type A isolates and a single *cpe*-negative type A control isolate in the *dcm-cpe* PCR assay (Fig. 2A). As indicated by the representative results shown in Fig. 2B, the single *cpe*-IS*1470* PCR amplified the expected ~2.1-kb PCR product when purified DNA extracted from all five type A isolates whose chromosomal *cpe* locus had previously been investigated were used (17). However, this PCR failed to amplify a product when purified DNA extracted from any of the 13 previously examined type A isolates carrying a plasmid-borne *cpe* gene or the *cpe*-negative type A isolate, ATCC 297442, was used.

Development of a duplex PCR assay for genotyping *cpe*-positive *C. perfringens* type A isolates. The success of the individual *dcm-cpe* and *cpe*-IS*1470* PCR assays in identifying *C. perfringens* type A isolates carrying a plasmid-borne or a chromosomal *cpe* gene, respectively, suggested that those two PCRs could be combined in a duplex PCR assay capable of genotyping *cpe*-positive type A isolates, i.e., determining whether those isolates carry a plasmid-borne or a chromosomal *cpe* gene.

This hypothesis was first tested by using as a PCR template purified DNA extracted from the same 18 *cpe*-positive and 1 *cpe*-negative *C. perfringens* type A isolates (Fig. 2A and B). As indicated by the results shown in Fig. 3A, the duplex PCR assay correctly amplified an ~3.3-kb product from all 13 initially tested type A isolates carrying a plasmid-borne *cpe* gene, an ~2.1-kb product from all 5 initially tested type A isolates carrying a chromosomal *cpe* gene, and no product from the 1 initially tested *cpe*-negative type A isolate.

Given these promising results, further testing of the duplex PCR *cpe* genotyping assay was performed to confirm its reliability. For this purpose, purified DNA was extracted from 1 additional *cpe*-negative type A isolate and 66 additional *cpe*-positive type A isolates. Those 66 additional *cpe*-positive type A isolates had all been previously genotyped by traditional Southern blot analysis-based assays to determine whether they carry a chromosomal or plasmid-borne *cpe* gene (see Materials and Methods). However, the organization of the *cpe* locus in these 66 isolates had not yet been examined; i.e., it was unknown whether the isolates in this additional isolate group carrying plasmid-borne *cpe* genes had *dcm* sequences upstream of their *cpe* genes or whether the isolates in this additional isolate group with a chromosomal *cpe* gene had IS*1470* sequences, in the same orientation as shown in Fig. 1, downstream of their *cpe* genes. Among these 66 additional *cpe*-positive type A isolates were 19 more food-poisoning isolates carrying a chromosomal *cpe* gene and 47 more human non-food-borne GI disease isolates or veterinary isolates carrying a plasmid-borne *cpe* gene.

In this testing of additional isolates, the duplex PCR assay amplified (data not shown) an ~3.3-kb product from all 47 of the additional type A isolates carrying a plasmid-borne *cpe* gene, an ~2.1-kb product from all 19 of the additional food-poisoning isolates carrying a chromosomal *cpe* gene tested, and no product from ATCC 3624, the additional *cpe*-negative type A isolate.

Finally, when the duplex PCR was tested by using a mock sample spiked with both the plasmid-borne and chromosomal copies of *cpe*, no PCR product was obtained. However, this
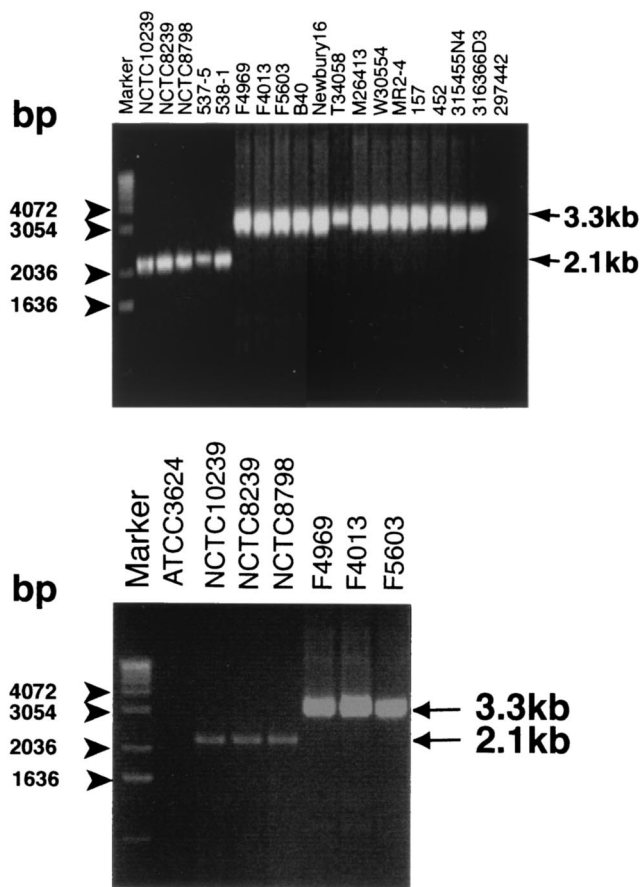
FIG. 3. Development of a duplex PCR *cpe* genotyping assay for distinguishing *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene from isolates carrying a chromosomal *cpe* gene. (A) Representative duplex PCR results obtained by use of purified DNA extracted from isolates with chromosomal *cpe* genes (first 5 lanes from the left) or isolates carrying plasmid-borne *cpe* genes (the next 13 lanes). Also shown are the results of duplex PCR with purified DNA from *cpe*-negative isolate ATCC 297442. (B) Representative duplex PCR results obtained with colony lysates as a DNA template source. Results shown are for *cpe*-negative isolate ATCC 3624 (second lane from the left), three type A isolates with chromosomal *cpe* genes (next three lanes), and three type A isolates carrying plasmid-borne *cpe* genes (three lanes at the right). Arrows on the right in both panels depict the migration of the expected *dcm-cpe* PCR products (3.3 kb) and the expected *cpe*-IS*1470* PCR products (2.1 kb). The numbers on the left of each gel indicate the migration of molecular size markers.

failure is not a significant limitation of the assay, as none of the >84 natural CPE-positive type A isolates previously genotyped by RFLP or PFGE techniques had been found to simultaneously carry both chromosomal and plasmid *cpe* genes (8, 9, 20).

Use of the duplex PCR *cpe* genotyping assay with colony lysates. The final experiment performed in this study involved testing of whether the duplex PCR *cpe* genotyping assay can use colony lysates as a DNA template source. For this analysis, 33 isolates carrying plasmid-borne *cpe* genes and 25 isolates with chromosomal *cpe* genes were randomly selected from our collection of 84 *cpe*-positive isolates whose purified DNA had been successfully genotyped by the duplex PCR in the studies whose results are shown in Fig. 3A. Representative results with

colony lysates as a DNA template source are shown in Fig. 3B, which demonstrates that colony lysates from all three isolates with chromosomal *cpe* genes and all three isolates carrying plasmid-borne *cpe* genes, but not from the one *cpe*-negative isolate (ATCC 3624), supported the successful amplification of the appropriate PCR product in the duplex PCR *cpe* genotyping assay. Similarly, colony lysates from 49 other *cpe*-positive isolates tested also proved to be adequate template sources for this duplex PCR *cpe* genotyping assay. However, despite repeated attempts, no PCR product could be obtained with colony lysates from two other isolates carrying plasmid-borne *cpe* genes or one other isolate with a chromosomal *cpe* gene, even though those isolates had been successfully genotyped by the duplex PCR assay using their purified DNA as the template.

DISCUSSION

Prior to the current study, *cpe*-positive type A isolates of *C. perfringens* could be *cpe* genotyped only by Southern blotting of their DNA after PFGE or RFLP analysis. The successful development of the duplex PCR assay reported in this study represents a substantial improvement upon those existing *cpe* genotyping assays. For example, while Southern blots of RFLP or PFGE gels require 1 to 2 weeks for genotyping of *cpe*-positive type A isolates, our new PCR-based assay can determine whether a *cpe*-positive type A isolate carries a chromosomal or a plasmid-borne *cpe* gene in 1 to 2 days by use of purified DNA. By use of colony lysates as the template DNA source, the duplex PCR assay generates genotyping results within 6 h. Furthermore, our survey results with 55 *cpe*-positive type A isolates indicate that colony lysates suffice as a duplex PCR DNA template source for ~95% of all *cpe*-positive type A isolates. In addition, the new duplex PCR assay more easily accommodates genotyping of large numbers of samples than Southern blotting of *cpe* on RFLP and PFGE gels does. Finally, this new genotyping assay requires no more sophisticated or costly equipment than a PCR thermocycler and agarose electrophoresis capability and uses PCR expertise already familiar to most laboratories.

The availability of this new PCR-based *cpe* genotyping assay provides a powerful tool for the study of the epidemiology of CPE-associated food-borne and non-food-borne human or veterinary GI diseases. Although CPE-positive type A isolates have been linked to enteric illness for many years, little is known about the natural reservoirs for the type A isolates carrying either plasmid-borne or chromosomal *cpe* genes. Similarly, it is unclear how or when type A isolates with chromosomal *cpe* genes enter the food chain to contaminate foods.

In addition, the new duplex PCR genotyping assay developed in this study holds considerable diagnostic promise. It should be useful to clinical microbiologists for differentiating human cases of CPE-associated food poisoning (which involve type A isolates with chromosomal *cpe* genes) and cases of CPE-associated non-food-borne human GI disease (which involve type A isolates carrying plasmid-borne *cpe* genes). It should also make routine testing for the involvement of isolates carrying plasmid-borne *cpe* genes in antibiotic-associated diarrhea cases much more feasible.

Finally, our results validating this duplex PCR assay are also informative regarding the diversity of *cpe*-positive type A iso-

lates. Recent studies that have used Southern blotting, PCR, and sequencing approaches (17) demonstrated differences between the organization of the plasmid-borne *cpe* locus and the chromosomal *cpe* locus in 18 *cpe*-positive type A isolates. In the present study, our new duplex PCR assay assessed the *cpe* locus organization in approximately fourfold more *cpe*-positive type A isolates than had previously been examined. It is notable that these 66 additional *cpe*-positive type A isolates studied came from various geographic, host, and disease sources and had isolation dates ranging from the 1950s to the late 1990s (see Materials and Methods). Duplex PCR results with these 66 additional isolates provide important data confirming that the organizational differences noted between the plasmid-borne and chromosomal *cpe* loci in a previous study (17) are highly conserved in most (if not all) *cpe*-positive type A isolates. Specifically, the new duplex PCR assay's amplification of an ~3.3-kb PCR product from all 47 additionally tested type A isolates carrying a plasmid-borne *cpe* gene supports previous suggestions (17) that the region upstream of the *cpe* gene is conserved in most (or all) type A isolates carrying plasmid-borne *cpe* genes. However, it should be noted that a previous study (17) did identify some diversity in the region downstream of the *cpe* locus among type A isolates carrying plasmid-borne *cp*e genes, where either IS*1470*-like or IS*1151*-like sequences can be situated; that diversity downstream of the plasmid-borne *cpe* gene in type A isolates explains why we designed primers to amplify the upstream *dcm-cpe* region for identifying type A isolates carrying plasmid-borne *cpe* genes by a duplex PCR assay.

Similarly, the ability of the duplex PCR to amplify the expected ~2.1-kb PCR product from all 19 additionally tested type A isolates carrying a chromosomal *cpe* gene supports previous suggestions (17) that the region downstream of the *cpe* gene is conserved among most (if not all) type A isolates carrying chromosomal *cpe* genes. Whether sequence variations occur downstream of the chromosomal *cpe* locus of type A isolates requires further study.

REFERENCES

1. **Abrahao, C., R. Carman, H. Hahn, and O. Liesenfeld.** 2001. Similar frequency of detection of *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxins in patients with antibiotic-associated diarrhea. Eur. J. Clin. Microbiol. Infect. Dis. **20:**676–677.
2. **Asha, N., and M. Wilcox.** 2002. Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhea. J. Med. Microbiol. **51:**891–894.
3. **Borriello, S. P.** 1995. Clostridial diseases of the gut. Clin. Infect. Dis. **20**(Suppl. 2):S242–S250.
4. **Borriello, S. P., F. E. Barclay, A. R. Welch, M. F. Stringer, G. N. Watson, R. K. T. Williams, D. V. Seal, and K. Sullens.** 1985. Epidemiology of diarrhea caused by enterotoxigenic *Clostridium perfringens*. J. Med. Microbiol. **20:**363–372.
5. **Borriello, S. P., A. R. Welch, H. E. Larson, F. Barclay, M. F. Stringer, and B. A. Bartholomew.** 1984. Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhea. Lancet **i:**305–307.
6. **Brett, M. M., J. C. Rodhouse, T. J. Donovan, G. M. Tebbut, and D. N. Hutchinson.** 1992. Detection of *Clostridium perfringens* and its enterotoxin in cases of sporadic diarrhea. J. Clin. Pathol. **45:**609–611.
7. **Brynestad, S., B. Synstad, and P. E. Granum.** 1997. The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. Microbiology **143:**2109–2115.
8. **Collie, R. E., and B. A. McClane.** 1998. Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. J. Clin. Microbiol. **36:**30–36.
9. **Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Carnard, and S. T. Cole.** 1995. The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Mol. Microbiol. **15:**639–647.
10. **Czeczulin, J. R., R. E. Collie, and B. A. McClane.** 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. **64:**3301–3309.
11. **Daube, G., P. Simon, B. Limbourg, C. Manteca, J. Mainil, and A. Kaeckenbeeck.** 1996. Hybridization of 2,659 *Clostridium perfringens* isolates with gene probes for seven toxins (α, β, ε, ι, θ, μ and enterotoxin) and for sialidase. Am. J. Vet. Res. **57:**496–501.
12. **Jackson, S., D. Yip-Chuck, J. Clark, and M. Brodsky.** 1986. Diagnostic importance of *Clostridium perfringens* enterotoxin analysis in recurring enteritis among the elderly, chronic care psychiatric patients. J. Clin. Microbiol. **23:**748–751.
13. **Kokai-Kun, J. F., J. G. Songer, J. R. Czeczulin, F. Chen, and B. A. McClane.** 1994. Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J. Clin. Microbiol. **32:**2533–2539.
14. **Marks, S., E. Kather, P. Kass, and A. Melli.** 2002. Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. J. Vet. Intern. Med. **16:**533–540.
15. **McClane, B. A.** 2001. *Clostridium perfringens*, p. 351–372. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers, 2nd ed. ASM Press, Washington, D.C.
16. **McClane, B. A., and J. I. Rood.** 2001. Clostridial toxins involved in human enteric and histotoxic infections, p. 169–209. *In* H. Bahl and P. Duerre (ed.), Clostridia: bio/technology and medical applications. Wiley-VCH, Weinheim, Germany.
17. **Miyamoto, K., G. Chakrabarti, Y. Morino, and B. A. McClane.** 2002. Organization of the plasmid *cpe* locus of *Clostridium perfringens* type A isolates. Infect. Immun. **70:**4261–4272.
18. **Mpamugo, O., T. Donovan, and M. M. Brett.** 1995. Enterotoxigenic *Clostridium perfringens* as a cause of sporadic cases of diarrhoea. J. Med. Microbiol. **43:**442–445.
19. **Songer, J. G.** 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. **9:**216–234.
20. **Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane.** 2001. Genotyping of enterotoxigenic *Clostridium perfringens* isolates associated with gastrointestinal disease in North America. J. Clin. Microbiol. **39:**883–888.