DEVELOPMENT OF DUPLEX PCR ASSAY FOR RAPID DETECTION OF ENTEROTOXIGENIC ISOLATES OF *CLOSTRIDIUM PERFRINGENS*

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Abstract. A duplex PCR assay was developed for the rapid and specific amplification of the alpha-toxin (phospholipase C, *plc*) gene and the enterotoxin (*cpe*) gene from *Clostridium perfringens*. Two pairs of primers were newly designed for the species identification and also for the differentiation between enterotoxin-positive and enterotoxin-negative *C. perfringens* strains in a single reaction. The detection by agarose gel electrophoresis yielded 2 bands of 280-bp of *plc* and 420-bp of *cpe* for all four enterotoxin-positive reference strains tested without the need for further hybridization, and one band of 280-bp of *plc* for all seven enterotoxin-negative reference strains. While 50 strains of other *Clostridium* species and other bacteria tested by PCR were negative for both genes. The detection limit, as measured with purified DNA was 10 fg or as few as 4 organisms could be detected. This assay was used to identify primary fecal spore isolates from 244 fecal specimens of patients with diarrhea. Of total 432 colonies from 144 positive growth cultures determined, 21 revealed both *plc* and *cpe* genes and 411 were positive for *plc* gene only. This suggested a prevalent of 5% of all *C. perfringens* strains that carry the enterotoxin gene. The results indicate the duplex PCR as a simple, sensitive, specific, cost-effective and time saving assay for detection of potentially enterotoxigenic isolates of *C. perfringens*, and has potential application for epidemiological investigations of food poisoning outbreaks and quality control of food products for humans and animal feeds.

INTRODUCTION

Clostridium perfringens is one of the most common causes of food-borne diseases. This species is divided into five types (A-E) according to the major virulence factors. Type A is the agent of classic *C. perfringens* food poisoning, and one of the leading causes of human food-poisoning (Stringer *et al*, 1980). Almost all strains of this type isolated from outbreaks produce alpha-toxin (phospholipase C, PLC) and a unique enterotoxin (CPE) which is responsible for the characteristic disease symptoms (diarrhea and abdominal cramps) of *C. perfringens* food poisoning. Only isolates that cause human food poisoning carry the *cpe* gene, which encodes a sporulation-associated enterotoxin (Skjelvale and Uemura, 1977). The role of *C. perfringens* and its enterotoxin in foodborne diarrhea as well as non-food related diarrhea, sporadic diarrhea, infectious diarrhea and antibiotic-associated diarrhea have already well documented (Birkhead *et al*, 1988; Borriello *et al*, 1984; Mpamugo *et al*, 1995).

Laboratory identification of *C. perfringens* food poisoning outbreaks is complicated by its presence as normal fecal flora and by its ubiquitous distribution in the environments. Since its enterotoxin is found only in feces from symptomatic or infected persons and is absent from those of convalescent or healthy subjects (APHA, 1985). For determining whether a food-borne outbreak is due to *C. perfringens*, several studies have suggested the detection of enterotoxin in fecal specimens as a marker for *C. perfringens* diarrhea in epidemiological investigations. Several biological and serological tests are available for differentiating entero-toxigenic strains: enzyme-linked immuno-sorbent assay (ELISA), reversed passive latex agglutination (RPLA), and Vero cell assays are the most commonly used. However, these

phenotypic methods have produced unreliable results since *C. perfringens* sporulation, which is a prerequisite for enterotoxin production, is limited in the usual culture media. Many strains of *C. perfringens* do not sporulate well in these media (Uemura *et al*, 1992). This sporulation problem can lead to the false-negative results (Kokai-Kun *et al*, 1994).

Recently, potentially enterotoxigenic strains have been identified successfully with genotypic methods based on hybridization or polymerase chain reaction (PCR) amplification of the *cpe* gene (Baez *et al*, 1996; Fach and Popoff, 1997; Ridell *et al*, 1998; Saito *et al*, 1992). The sequences of the genes encoding the production of PLC and CPE have been published (Czeczulin *et al*, 1993; Titball *et al*, 1989; Tso and Siebel, 1989; Van Damme-Jongsten *et al*, 1989). The *plc* gene is found in all toxin types and present in all strains of *C. perfringens* (Canard *et al*, 1992; Fach and Guillou, 1993) while the *cpe* gene is restricted to enterotoxigenic *C. perfringens* strains and seems to be quite conserved which is favorable for genotypic diagnostics. PCR has been highlighted as a rapid and accurate method for the detection of low copy numbers of genes and can circumvent the disadvantages of serologic assays. It can be used to distinguish the enterotoxigenic strains from the non-enterotoxigenic ones to identify the true agent of food poisoning that should be helpful for epidemiological investigations. In this study, we developed a duplex PCR for rapid detection of enterotoxigenic *C. perfringens* isolates, both well-characterized reference strains and fresh fecal isolates using two sets of newly design primers which amplify in the same reaction two different DNA fragments of *C. perfringens* simultaneously : the *plc* and the *cpe* gene fragments.

MATERIALS AND METHODS

Bacterial strains

Reference strains of *C. perfringens* were used to set up unique conditions for the duplex PCR, including enterotoxigenic strains, *ie C. perfringens* ATCC 12916, NCTC 8198, NCTC 8239 and NCTC 10239 which were kindly provided by A. Heikinheimo, Faculty of Veterinary Medicine, University of Helsinki, Finland; and nonenterotoxigenic strains, *ie* ATCC 3624, 3628, 3629, 3631, 27324, 29348 and 43402 were a kind gift of Dr O Suthienkul, Faculty of Public Health, Mahidol University, Thailand. One hundred and forty-four isolates of *C. perfringens* were fecal isolates from diarrheal cases at Bamrasnaradura Hospital, they were used to investigate the prevalent of enterotoxigenic *C. perfringens* isolates in fecal samples. Other *Clostridium* species including 4 *C. sporogenes*, 3 *C. difficile*, 3 *C. bifermentans*, 3 *C. septicum*, 2 *C. botulinum* as well as other bacteria including 10 *Staphylococcus aureus*, 10 *Salmonella typhimurium*, 5 *Escherichia coli*, 5 *Pseudomonas aeruginosa* and 5 *Bacillus* species were used to confirm the specificity of our duplex PCR.

Identification of *C. perfringens*

The *Clostridium* strains were grown on Tryptose-sulfite-cycloserine (TSC) agar (Merck) supplemented with 5% egg yolk or on sheep blood agar plates and incubated anaerobically at 37ºC for 24 hours. With TSC-egg yolk agar plates, a zone of turbidity occurred around black colonies, whereas dual hemolytic zones appeared around colonies when blood agar was used. The colonies were identified biochemically by using lactose, glucose, and inositol fermentation, motility test, nitrate reduction and gelatinase production. Other bacterial organisms were also re-identified by biochemical tests as described (Cato *et al*, 1986). To test the enterotoxigenicity of *C. perfringens* strains, stock cultures were incubated in cooked meat medium (Oxoid) for 18 hours at 37ºC followed by a heat-shocked at 70ºC for 20 minutes and then 1.0-ml sample from cooled tubes was transferred to 20 ml of Duncan-Strong (DS) medium (Duncan *et al*, 1972) and incubated for 24 hours at 37ºC. Sporulation of the cultures was confirmed microscopically. After centrifugation at 1,500*g* for 10 minutes, the supernatant was assayed for enterotoxin by reverse passive latex agglutination (RPLA) with PET-RPLA (Oxoid, Hampshire, England).

Isolation of genomic DNA

In order to develop unique conditions for the duplex PCR, *C. perfringens* reference strains *ie* NCTC 8239 and ATCC 3624 were used as a positive and a negative control DNA in all PCR amplification. Total genomic DNAs from all reference strains and other *Clostridium* species and bacteria were extracted by a QIAamp commercial kit (Qiagen, Germany) according to the manufacturer's protocol and used to investigate the sensitivity and specificity of the duplex PCR. The DNA concentration was measured at OD 260 nm with a spectrophotometer. The DNA solution was kept at -20ºC until used. Total DNAs from all fecal isolates were extracted by the boiling method. Briefly, few colonies of bacteria grown on a blood agar plate were suspended in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) to make a suspension of McFarland No. 0.5 (estimated 10^8 cfu/ml) and diluted 1:1,000 with TE buffer (estimated 10^5 cfu/ml) and the suspension was boiled for 10 minutes and then placed on wet ice for at least 5 minutes. The pellets were removed by centrifugation at 12,000*g* for 10 minutes. Ten µl of the supernatant was used as template DNA in the PCR.

Primers

Specific primers corresponding to the phospholipase C (alpha-toxin) and enterotoxin genes were designed by using the sequence data obtained from GenBank accession no. X17300 and M98037, respectively. Optimal upstream and downstream primer sequences were checked for internal and 3′ end complementarity by using the oligonucleotide primer design and analysis software. The specificities of these primers were checked against the GenBank and EMBL data banks. The primers were synthesized commercially (Gibco BRL, Life Technologies). The oligonucleotide primers, sequences and locations are given in Table 1.

Duplex PCR

Optimal conditions for PCR using either primer set 1 (PLC1 and PLC2) or set 2 (CPE1 and CPE2), or both sets combined in a duplex PCR reaction, were determined with the use of DNA templates extracted from *C. perfringens* reference strains both enterotoxin positive and negative at a concentration of 100 pg/µl. The PCR procedure was performed in a total volume of 30 µl. The PCR mixture contained 3 µl of 10X PCR buffer (1X buffer includes 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin), 0.6 µl of 10 mM deoxynucleoside triphosphate mixture, 0.5 µM each of primer, 1 ng (10 µl) of template DNA, 0.6 µl (0.6 U) of *Taq* DNA polymerase (Promega), and up to 30 µl of distilled water. The reaction mixtures were processed in a programmable thermal cycler (480, Perkin-Elmer Cetus). The following program was used in this experiment: 5 minutes at 94ºC, followed by 35 cycles consisting of 1 minute at 94ºC, 1 minute at 55ºC, 1 minute at 72ºC; followed by a 10-

Table 1
Nucleotide sequences of primers used in duplex PCR.

Primer region and map position	Primer designation	Sequence (5′ - 3′)	Product length (bp)
Phospholipase C gene[a]			
Forward 1801-1824	PLC1	5′ ATA GAT ACT CCA TAT CAT CCT GCT 3′	280
Reverse 2059-2080	PLC2	5′ TTA CCT TTG CTG CAT AAT CCC 3′	
Enterotoxin gene (CPE)[b]			
Forward 643-666	CPE1	5′ GAA AGA TCT GTA TCT ACA ACT GCT 3′	420
Reverse 1039-1062	CPE2	5′ TAA GAT TCT ATA TTT TTG TCC AGT 3′	

[a]Map position and sequence refer to *C. perfringens* phospholipase C (α-toxin) gene, Accession X 17300.
[b]Map position and sequence refer to *C. perfringens* enterotoxin gene, complete genome. Accession M 98037.

minutes final extension at 72ºC. A 10-µl aliquot of amplified sample from each PCR tube was electrophoresed through a 2% agarose gel in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.2) for 1 hour at 100 V. Amplification products were visualized and photographed under a UV light transilluminator (Fotodyne, Hartlands, Wis) after ethidium bromide (0.5 µg/ml) staining. The presence of band of the expected size (280-bp for primer PLC1-PLC2 and 420-bp for primer CPE1-CPE2) was assessed by comparison with a molecular size marker (100 bp DNA Ladder, New England Biolabs, Inc, USA).

Detection sensitivity of duplex PCR

A 10-fold dilution of purified DNA from *C. perfringens* (NCTC 8239) were prepared in TE buffer, with the concentrations ranging from 100 ng to 0.01 fg per 10 µl, and 10 µl were used in the PCR. Serial 10-fold dilutions were also made from the same culture isolate. The number of CFU of the original suspension per ml was estimated by routine growing in a standard spread plate technique using duplicate blood agar plates and colonies were counted after anaerobically growth at 37ºC for 24 hours. Identical quantities from various dilutions were used for DNA extraction by heat treatment as described and 10 µl from each tube was used as the template for 35 amplification cycles and analyzed by gel electrophoresis. The detection limit for each detection assay was determined by the number of colonies showing characteristic dual hemolytic zones grown on blood agar medium of the corresponding dilution. The highest dilution yielding an amplicon with 2 bands of corrected sizes (280-bp and 420-bp) from a 10-µl sample volume was taken as the end-point.

Detection of *cpe* gene from fecal spore isolates

Fecal specimens were obtained from 244 patients with diarrhea at Bamrasnaradura Hospital, Nonthaburi. They were cultured for clostridia after heat treatment at 80ºC for 20 minutes to select for clostridial spores. Serial 10-fold dilutions of fecal suspension were plated onto TSC egg-yolk agar plates. After incubation overnight at 37ºC in an anaerobic jar, up to 3 lecithinase-positive colonies were subcultured to blood agar to evaluate for double zone hemolysis. Then colonies with dual hemolysis were tested for biochemical tests (Cato *et al*, 1986). The same 3 colonies with *C. perfringens* morphology from primary fecal spore isolation were used directly for analysis by the duplex PCR as described above.

Sequencing analysis of amplified products

The PCR products in the size of either 280-bp or 420-bp was excised from agarose gel following electrophoresis, and purified by using the Qiaex DNA Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's recommendations. Nucleotide sequencing was carried out by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli *Taq* DNA polymerase (Perkin Elmer) and the results were checked with an ABI PRISM 377 automated sequencer (Perkin Elmer). The identities of the products were confirmed by comparison of the sequence with previous reports obtained from the GenBank and the EMBL database.

RESULTS

To assess the quality of the isolated DNA by a QIAamp kit, samples were resolved by gel electrophoresis on 0.8% agarose gel in TBE buffer. A typical pattern was obtained for the DNA samples tested, indicative of undegraded DNA by this method (data not shown). The isolated DNA had an A260/280 ratio of 1.8. The average yield of DNA extracted from 1 ml-volume of 10^8 organisms/ml was 1.5 µg.

Specificity of duplex PCR

Fig 1 shows the results of the PCR products test with two sets of primers obtained with purified DNA extracted from some representative *C. perfringens* reference strains and some other bacterial strains at 1 ng per reaction. The detection by agarose gel electrophoresis yielded 2 bands of 280-bp of *plc* and 420-bp of *cpe* for all enterotoxin-positive strains tested,

one band of 280-bp for all enterotoxin-negative strains and none for bacteria other than *C. perfringens* tested. Fig 2 shows the electrophoretic patterns of both positive and negative reference strains using single primer pairs or both primer pairs were combined in a duplex PCR reaction. For enterotoxin-positive reference strain, a product consistent in size with that predicted is shown, *ie* 280-bp of *plc* when using PLC1 and PLC2 primers, or 420-bp of *cpe* when using CPE1 and CPE2 primers, or both 280-bp and 420-bp when all primers were combined in a duplex reaction. The same result was also shown when using DNA extracted from other enterotoxin-positive reference strains. For enterotoxin-negative strain, no 420-bp band was noted when using either single primer pairs or both primer pairs. On the other hand, no band was shown when using CPE1-CPE2 primers singly. The specificity of the duplex PCR with the two sets of primers was also assessed with different *Clostridium* species (15 strains) and other bacteria (35 strains) frequently associated with food or known to produce lecithinase (alpha toxin), and these strains tested yielded negative results for both *plc* and *cpe* genes.

Detection sensitivity of duplex PCR

To determine the sensitivity of duplex PCR, the templates for amplification were either purified DNA or heat treatment of the same bacterial culture suspension obtained from enterotoxigenic *C. perfringens* NCTC 8239. The sensitivity of duplex PCR for detection of both *plc* and *cpe* genes as determined by agarose gel electrophoresis was 10 fg (Fig 3), or as few as 4 organisms per reaction yielded a positive result (data not shown).

Detection of *cpe* gene of *C. perfringens* from primary fecal spore isolates

Of the 244 fecal specimens examined between August and October 1999, 144 samples were shown to contain high numbers of clostridial spores, *ie* $>10^3$ cfu/g. Three colonies with *C. perfringens* morphology from each isolation were selected, and each was identified by conventional biochemical tests after secondary

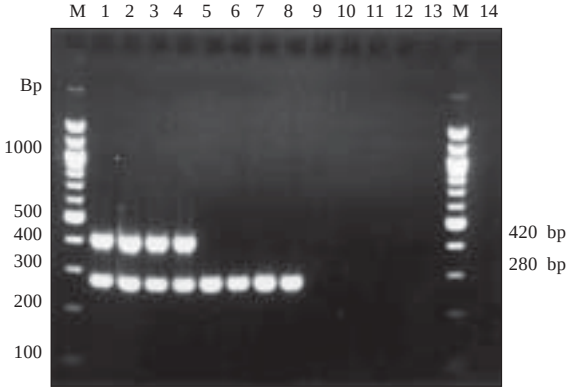

Fig 1–Agarose gel electrophoresis of the PCR products tested with two primer pairs using purified genomic DNA extracted from some representative *C. perfringens* reference strains and bacteria other than *C. perfringens* as the templates. Lanes 1 to 4 are enterotoxigenic *C. perfringens* strains. Lanes 5 to 8 are nonenterotoxigenic *C. perfringens* strains. Lanes 9 to 13 are *C. botulinum, C. bifermentans, S. aureus, S. typhimurium* and *B. cereus*, respectively. Lane M, molecular size marker (100-bp DNA Ladder). Lane 14, negative reagent control.

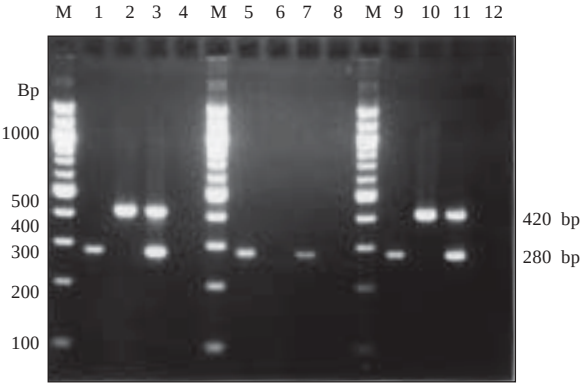

Fig 2–Band patterns on agarose gel observed with PCR products of two *C. perfringens* reference strains: one was positive for enterotoxin gene (lanes 1 to 3), another was negative for enterotoxin gene (lanes 5 to 7) compared to PCR product of one *cpe* positive sample isolated from fecal specimen of patient with diarrhea (lanes 9 to 11). The primers were tested singly and in combination with the primer pairs PLC1-PLC2 (lanes 1, 5 and 9), primer pairs CPE1-CPE2 (lanes 2, 6 and 10), and combination of both primer pairs in the duplex PCR (lanes 3, 7 and 11). Lane M, molecular size marker (100-bp DNA Ladder). Lanes 4, 8 and 12; negative reagent control (no DNA) tested with both primer pairs.

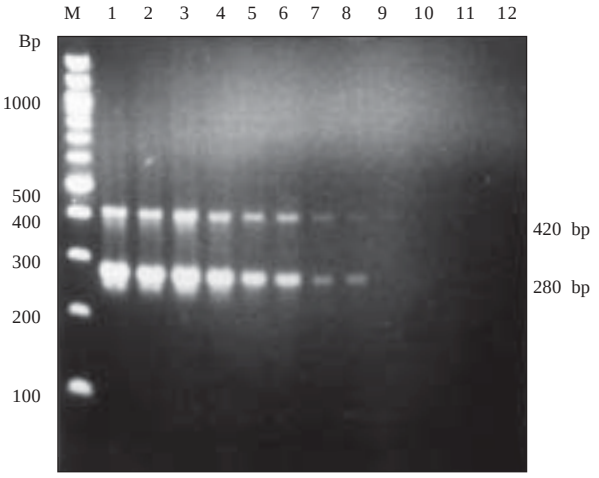

Fig 3–Agarose gel showing sensitivity of the duplex PCR for the detection of both *plc* and *cpe* genes of *C. perfringens*. Genomic DNA was isolated from *C. perfringens* NCTC 8239, and the DNA was diluted 10-fold. The highest dilution yielding an amplicon with 2 bands of corrected sizes (280-bp and 420-bp) from a 10-µl sample volume was taken as the end-point. Lane M, molecular size marker (100-bp DNA Ladder). Lanes 1 to 11, 10-fold serial dilutions of DNA; 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg per reaction, respectively. Lane 12, negative reagent control.

subcultures. The characteristics of the isolates were positive for fermentation of glucose, lactose, inositol; nitrate reduction; hydrolysis of gelatin; nonmotility; and a production of lecithinase. The same 3 colonies from primary fecal spore isolates were used directly for analysis by duplex PCR. Of total 432 colonies determined, 411 gave positive results for *plc* gene only (1 band of 280-bp), while 21 revealed both *plc* and *cpe* genes (2 bands of 280-bp and 420-bp). This suggested a prevalence of 4.9% of *C. perfringens* that carry the *cpe* gene. These colonies with *cpe* positive results were confirmed by the second PCR with the primers tested singly and in combination. The band patterns on agarose gel were identical to those of the reference enterotoxigenic strain (Fig 2). The specificity of the 420-bp *cpe* amplicon was also confirmed by sequencing with either primer CPE1 or CPE2 and by comparison of the sequence data obtained from the GenBank and identity to the sequence of *C. perfringens* enterotoxin gene was found.

DISCUSSION

Diarrhea is one of indicator of public health and public welfare, and a direct impact on national economy. At present, the laboratory investigation of bacterial diarrhea is mostly relied on aerobic or facultative anaerobic bacteria; while the detection of anaerobes that cause foodborne illness is limited due to some certain degree of difficulties and high expenses. However, the role of *C. perfringens* in foodborne or non-foodborne diarrheas is well known. *C. perfringens* type A is responsible for several outbreaks of food poisoning in many countries due to ubiquitous nature, ability to contaminate carcass intravitally, heat-resistance of spores, relatively short generation time at high temperature, survival in chilled and frozen meat and ability to overcome stomach acid barrier (McClane, 1996). Although the illness is typically mild, fatalities may occasionally occur in debilitated patients, especially the elderly.

Traditionally, a spore count of $>10^6$/g in the feces of ill patients or $>10^5$/g from epidemiologically incriminated foods; and the presence of the same serological type in most of the ill patients or in the food and feces have been regarded as the criteria for diagnosis (APHA, 1985). However, the carriage of *C. perfringens* in the feces varied considerably between individuals (Stringer *et al*, 1985) where spore counts of $>10^6$/g may be found in fecal samples taken from institutionalized well patients or healthy people (Sutton,1966; Yamagishi *et al*, 1976). The quantitative fecal spore counts and biochemical testing of several colonies after secondary subcultures also lead to labor intensive, high cost and time-consuming where the results may take 1 week to be known. Serotyping has also been less successful in some countries, where many strains are untypeable. Other problems of serotyping involve autoagglutination, no commercially available,

and high expense.

It is well documented that foodborne diarrhea as well as non-food related diarrhea associated with *C. perfringens* are caused by enterotoxin (CPE), a 35-kDa polypeptide which is expressed during sporulation (McClane, 1996). Thus determining the enterotoxigenicity of food or fecal *C. perfringens* isolates by serologic assay to demonstrate its ability to produce CPE *in vitro* is helpful in epidemiological investigations of *C. perfringens* food poisoning outbreaks. But obtaining CPE production *in vitro* constantly is quite difficult. Many media have been developed to enhance sporulation of *C. perfringens*, *ie* DS medium. Until now there seem to be no universal medium that will encourage sporulation in all strains; and thus lead to false-negative results if detection by serologic assay methods. However, these false-negative results in the identification of enterotoxigenic *C. perfringens* caused by sporulation problems can be avoided by the use of genotypic methods to detect the *cpe* gene.

In an effort to facilitate routine detection of *C. perfringens* and also to differentiate between the enterotoxigenic strains and the non-enterotoxigenic ones, we combined the *plc* primers (PLC1-PLC2) and the *cpe* specific primers (CPE1-CPE2) by using one annealing temperature at 55ºC in a duplex PCR. These primer pairs were chosen so that they can give rise to amplicons of different sizes (280-bp and 420-bp) which could be easily resolved from each other by gel electrophoresis. All reference strains with previously known enterotoxigenicity produced the expected results. The positive PCR products with any primer set produced bright clearly visible bands of corrected size without non-specific reaction. Positive results were not observed in the reagent blanks included in these experiments. On the other hand, neither *Clostridium* species other than *C. perfringens* nor other bacteria tested revealed the 280-bp and 420-bp fragments, confirming the great specificity of both primer pairs.

Since a set of amplicon with 2 bands of corrected sizes will be expected if a sample contains the enterotoxin-positive strain, this obviates the need for hybridization confirmation. The result can be made by simple agarose gel electrophoresis thus rendered the test simple, less expensive and rapid. The results will be obtained within 4 hours after primary growth isolation. While conventional identification requires secondary subcultures, biochemical tests and then serologic assay for CPE production which all procedures take at least 3 days. In addition, heat treatment by boiling provided an effective method of liberating *C. perfringens* DNA for PCR detection. This heat treatment is simple, economic, convenient, and rapid for extraction of genomic DNA from culture isolates. The expense of this duplex PCR assay is also reduced by using small reaction volumes of 30 μl. Also, this premixed PCR reaction solution can be prepared in advance and kept at -20ºC for many weeks. When tested, it was only necessary to use a sterile wooden applicator (or a toothpick) to transfer a small amount of a single colony into microcentrifuge tube containing a premixed solution; this made it an easy and convenient tool for rapid screening of large numbers of suspected colonies from primary cultures without secondary subcultures.

With this system, the sensitivity of our duplex PCR as measured with purified DNA isolated from *C. perfringens* reference strains (based on 35 cycles) was 10 fg or at least 4 organisms could be detected. When duplex PCR was used directly to identify primary fecal spore isolates with *C. perfringens* morphology (black colonies with zone of turbidity), only a low percentage (5%) of all *C. perfringens* isolates carry the *cpe* gene. Comparison of the sequence data of 420-bp amplicons with those obtained from the GenBank, they are identical to the *cpe* gene sequence of *C. perfringens*. We also confirmed these positive isolates for CPE production *in vitro* by using the Oxoid RPLA kit after growing in sporulation medium. However some strains showed consistent result with duplex PCR; while the others were not tested since they did not sporulate in the DS medium used. The observed prevalence of *cpe* gene in *C. perfringens* strains isolated from fecal specimens in our study was close to those recently reported in molecular epi-

demiologic surveys where enterotoxigenic isolates represent only a small (< 6%) fraction of the global *C. perfringens* population (Kokai-Kun *et al*, 1994; Van Damme-Jongsten *et al*, 1990).

In conclusion, this study suggests that the duplex PCR assay using two sets of newly design primers which amplify in the same reaction two different DNA fragments of *C. perfringens* simultaneously, the *plc* and the *cpe* gene fragments, offers a sensitive and specific means for the detection of *C. perfringens* from primary growth cultures and it also allows the differentiation between enterotoxigenic and nonenterotoxigenic *C. perfringens* strains which is helpful for epidemiologic investigations of agents of foodborne illness. The method is found to be simple, rapid, cost-effective and convenient. Studies to further evaluate the use of this method directly with stool specimens and foods are in progress.

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