

# AN ENRICHMENT BROTH CULTURE-DUPLEX PCR COMBINATION ASSAY FOR THE RAPID DETECTION OF ENTEROTOXIGENIC *CLOSTRIDIUM PERFRINGENS* IN FECAL SPECIMENS

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**Abstract.** An enrichment broth culture-duplex PCR combination assay was devised to identify *Clostridium perfringens* directly from fecal samples. The method consists of a combination of short enrichment of samples in selective media, DNA isolation, and performing duplex PCR using two pairs of primers which identify *C. perfringens* strains that harbor the virulence enterotoxin gene. Comparison of two selective enrichment media and two incubation temperatures showed that the reinforced clostridial medium with neomycin was better than the fluid thioglycollate medium with neomycin ( $p < 0.001$ ); and incubation at 37°C vs 45°C showed no statistically significant difference ( $p = 0.238$ ). The optimal short time for pre-enrichment culture was 4 hours. The developed assay was applied to detect phospholipase C (*plc*) and enterotoxin (*cpe*) genes for *C. perfringens* in feces inoculated artificially with enterotoxigenic *C. perfringens*. The method could detect both gene products in samples inoculated with a minimum of  $10^4$  CFU per ml. When the method was applied to detect enterotoxigenic *C. perfringens* in 198 diarrhea patients, *C. perfringens* was found in 121 samples; 7 out of 121 samples were positive for both *plc* and *cpe* (prevalence of 5.8%). These results indicate that the developed assay was a suitable method for the rapid and specific detection of enterotoxigenic *C. perfringens* directly in fecal specimens of diarrhea patients, which will assist epidemiological investigations of food poisoning outbreaks and quality control of food products.

## INTRODUCTION

*Clostridium perfringens* is an important cause of foodborne diarrheal disease, particularly in association with spore-contaminated food. Presently, the detection method for *C. perfringens* from fecal or food samples is the use of selective culture followed by biochemical tests and final confirmation of enterotoxigenic strains by serological analysis, enzyme-linked immunosorbent assay (ELISA), latex agglutination or vero cell assay, for differentiating the enterotoxigenic strains from the non-enterotoxigenic ones (McClane and Strouse, 1984; Berry *et al*, 1988). However, these approaches require a long time to provide positive identification. Some 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 which can lead to false-negative results (Kokai-Kun *et al*, 1994).

We previously developed a duplex PCR to identify enterotoxigenic strains of fecal isolates directly from primary culture plates (Tansuphasiri, 2001; Tansuphasiri *et al*, 2002) by using two sets of primers which amplify in the same reaction two different DNA fragments for *C. perfringens* simultaneously: alpha toxin (phospholipase C, *plc*) and enterotoxin (*cpe*) genes. However, this method has yet to be perfected because PCR is sensitive to inhibition by factors present in the crude clinical samples (Widjojatmodjo *et al*, 1992).

Recently, an enrichment culture-PCR combination assay was reported for the detection of *Salmonella* directly from fecal samples (Chiu and Ou, 1996). Prior to the PCR assay, a short enrichment procedure was used to circumvent the effect of inhibitory compounds as well as selec-

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tive cultivation of the bacteria. In this study we applied enrichment broth culture with a slight modification that is suitable for the enrichment of *C. perfringens* in fecal specimens of diarrhea patients. The assay included a short pre-enrichment period of the fecal specimen in selective enrichment media, DNA isolation, and then analysis by a duplex PCR.

## MATERIALS AND METHODS

### Bacterial strains and fecal samples

Enterotoxigenic *C. perfringens* ATCC 12916, NCTC 8198, and NCTC 8239 were obtained from A Heikinheimo, Faculty of Veterinary Medicine, University of Helsinki, Finland; nonenterotoxigenic strains, ATCC 3624, 3629, 27324, 29348 and 43402, were obtained from Prof Dr O Suthienkul, Faculty of Public Health, Mahidol University. The non-*C. perfringens* which served as negative controls were *E. coli* ATCC 25922, clinical isolates of *C. sporogenes*, *C. difficile*, food isolates of *C. botulinum* and *Bacillus cereus*.

A total of 193 fecal samples were obtained from diarrhea patients at two hospitals, Siriraj Hospital, Bangkok; and Nakhon Nayok Hospital, Nakhon Nayok. These patients were over 2 years old and all had acute diarrhea. Fresh fecal specimens were collected in sterile containers, held at 4°C, and then sent within 3 hours to a laboratory for culture and enterotoxin analysis by duplex PCR.

### Conventional culture and identification

Fecal specimens were serially diluted in 0.1% (w/v) peptone-water and plated onto tryptose-sulfite-cycloserine (TSC)-egg yolk agar (Merck) overlaid with an additional 10 ml of TSC agar. The plates were incubated overnight at 37°C in an anaerobic jar with Gaspak (Difco), and the black colonies by sulfite reduction with a zone of turbidity due to lecithinase activity were counted and presumptively identified as *C. perfringens*. Up to five presumptive colonies were randomly selected and each colony was sub-cultured on duplicate blood agar. One plate was incubated overnight at 37°C in an anaerobic jar and another was incubated overnight at 37°C in aerobic conditions. The colonies grown only on

blood agar with dual hemolytic zones under anaerobic conditions were confirmed as *C. perfringens* based on the following tests: Gram stain and morphology, motility, nitrate reduction, gelatin liquefaction and lactose fermentation (FDA, 1998). To test the enterotoxigenicity of *C. perfringens*, these colonies were used as templates for the duplex PCR assay (Tansuphasiri, 2001).

### Determination of optimal conditions for pre-enrichment before PCR analysis

**Selective enrichment broth cultivation.** Two selective enrichment broth, including reinforced clostridial medium (RCM) (Scharlau, Spain) and fluid thioglycollate (FTG) (Difco), with neomycin (100 mg/l) and without neomycin, were used to compare the growth and recovery rate of *C. perfringens* and other bacteria after overnight incubation at either 37°C or 45°C. All 13 bacterial strains were serially diluted 10-fold in 0.1% peptone water, and 1 ml of each dilution (ranged from  $10^8$  to  $10^3$  /ml) was inoculated into 9 ml of the enrichment broth. Two sets of each selective medium (with and without neomycin) were prepared. After incubation, the first set at 37°C and the second set at 45°C, for 24 hours, the number of colony forming units (CFU) of the original suspension per ml was estimated by growing on TSC agar. Briefly, the broth cultures were 10-fold serially diluted in 0.1% peptone-water (from  $10^{-1}$  to  $10^{-6}$ ) and appropriate dilution (0.1 ml) was inoculated onto duplicate TSC agar by the spread plate. After overnight incubation in an anaerobic jar, the number of colonies were counted and reported as CFU/ml of enrichment culture. Three replicates were done for each experiment.

**Optimal time for pre-enrichment.** Seeded fecal samples from healthy individuals were used to determine the optimal short time for pre-enrichment before PCR analysis. An enterotoxigenic strain (*C. perfringens* ATCC 12916) was used as the seed. One ml of bacteria ( $10^8$  CFU/ml) was serially diluted 10-fold with 0.1% peptone-water, and 1 ml of each dilution (ranged from  $10^8$  to  $10^3$  CFU/ml) was seeded into 9 ml of fecal suspension (1:10 in 0.1% peptone-water). The 6 concentrations of *C. perfringens* in the spiked feces corresponded to approximately  $10^2$

to  $10^7$  CFU/ml of fecal suspension, respectively. A same aliquot of fecal specimen without *C. perfringens* was used as an original control.

One ml of spiked feces with 6 concentrations of *C. perfringens* that ranged from  $10^2$  to  $10^7$  CFU/ml was inoculated into 9 ml of predetermined suitable enrichment broth. Each concentration was subjected to 4 different pre-enrichment times: 0 hour, 4 hours, 8 hours, and 24 hours before PCR analysis. After aerobic incubation at 37°C for each time duration, all the fecal specimens (both spiked and nonspiked with *C. perfringens*) were taken for DNA isolation and PCR analysis. The number of CFU of the original suspension per ml was also estimated by growing onto duplicate TSC agar; and colonies were counted as mentioned before.

#### DNA isolation from enrichment broth culture.

The pre-enrichment broth culture of spiked feces was processed to remove particulate fecal substances by low-speed centrifugation at 300g for 3 minutes, then high-speed centrifugation at 17,000g for 3 minutes. Following three more cycles of washing with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8), the final pellet was suspended in 400  $\mu$ l of TE buffer and divided into 2 aliquots for DNA isolation by the boiling method and the QIAamp DNA Stool Mini Kit (QIAGEN Pty Ltd, Australia). For boiling, 200  $\mu$ l of concentrated broth culture was boiled in a water bath at 95°C for 10 minutes, then placed on wet ice. The pellet was removed by centrifugation at 12,000g for 2 minutes, and 10  $\mu$ l of the supernatant was used in the PCR reaction. The second aliquot was extracted using the QIAamp DNA Stool Mini Kit, following the manufacturer protocol. Ten microliters of the eluate was used in the PCR reaction.

#### Duplex-PCR assay

Duplex PCR, to amplify *plc* and *cpe*, was performed using two pairs of primers (Tansuphasiri, 2001). PLC1 and PLC2 were 5'-ATAGATACTCCATATCATCCTGCT-3' and 5'-TTACCTTTGCTGCATAATCCC-3', which anneal to a sequence in the *plc*; and CPE1 and CPE2 were 5'-GAAAGATCTGTATCTACAAGTCTGCT-3' and 5'-TAAGATTCTATATTTTTGTCCAGT-3', which anneal to a sequence in the *cpe*. The re-

action mixture, the amplification time, and temperature followed the optimized condition as described previously with slight modification. The PCR mixture (30  $\mu$ l) consisted of 3  $\mu$ l of 10X PCR buffer (1X buffer included 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.01% gelatin), 0.6  $\mu$ l of 10 mM dNTP mix, 0.6  $\mu$ l (25 pmoles) each of CPE1 and CPE2; 0.3  $\mu$ l (12.5 pmoles) each of PLC1 and PLC2, 1  $\mu$ l (1 U) of *Taq* polymerase (Promega), 10  $\mu$ l of DNA template, and distilled water to make a total volume of 30  $\mu$ l. PCR was performed in an automated thermal cycler (Perkin-Elmer Cetus) under the following conditions: 94°C for 10 minutes, followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C, and 1 cycle at 72°C for 10 minutes. Amplified products were electrophoresed in 2% agarose gel and stained with ethidium bromide and viewed under UV light. The presence of a band of the expected size (280-bp for PLC1-PLC2 and 420-bp for CPE1-CPE2) was assessed by comparison with 100-bp DNA molecular size markers (New England Biolabs, Inc, USA).

#### Direct detection of enterotoxigenic *C. perfringens* in fecal specimens

According to the results of predetermined selective broth and conditions, all 198 fecal specimens collected from diarrhea patients were subjected to pre-enrichment in the RCM (with neomycin) by inoculating 1 ml of fecal specimen into 9 ml of the enrichment broth and incubated at 37°C for 4 hours. After incubation, 1 ml of pre-enrichment broth was taken for conventional culture on TSC agar by the streak plate technique and incubated at 37°C in an anaerobic jar for 24 hours and five presumptive colonies from each primary plating media were randomly selected and confirmed for *C. perfringens* by biochemical tests and PCR analysis. The 9-ml aliquot of 4 h-pre-enrichment broth culture was taken for DNA isolation by using the QIAamp DNA Stool Mini Kit and then the eluent from the spin column was analyzed by duplex PCR and for product detection by agarose gel electrophoresis.

#### Statistical analysis

An agreement rate between the two methods for the species identification of *C. perfringens*

(conventional culture-biochemical tests, and enrichment broth culture-PCR for *plc* detection) from all the fecal specimens was determined using Kappa analysis. Statistical comparison was performed using the McNemar chi-square test. A p-value of <0.05 was considered statistically significant.

## RESULTS

### Optimal condition for pre-enrichment of fecal specimens

Two selective enrichment media, with and without antibiotic, and at two temperatures of incubation, were optimized for the enrichment of *C. perfringens* before PCR analysis. Colony counts in (CFU/ml) all 13 strains including the reference *C. perfringens* of both enterotoxigenic (n=3) and nonenterotoxigenic (n= 5) and other bacteria (n= 5) were determined after culturing 1 ml of bacteria ( $\sim 10^8$  CFU/ml) in 9 ml of either FTG or RCM with or without neomycin and incubating at either 37°C or 45°C for 24 hours. The geometric mean (GM) concentrations of the three replicates for each bacteria tested were calculated and the results are shown in Table 1, which is reported as the difference between the GM upon start ( $\sim 10^7$  CFU/ml) before incubation and the GM upon finish, after incubation for 24 hours.

Comparing FTG with RCM for supporting the growth of all the bacteria, the growth (CFU/ml) in RCM was much better than in the FTG at both 37°C and 45°C (p-value = 0.000). For each enrichment medium with or without neomycin, the mean concentrations of all the bacteria tested were statistically different (p-value = 0.000). The addition of an antibiotic to each medium seemed to inhibit the growth of bacteria other than *C. perfringens* with a statistically significant difference (p-value = 0.000). Neither of the incubation temperatures (37°C and 45°C) used for pre-enrichment of the bacteria tested were statistically different (p-value = 0.238); for FTG with neomycin, GM  $\pm$  SD at 37°C was  $3.4 \times 10^2 \pm 1.2 \times 10^2$  and at 45°C was  $3.9 \times 10^2 \pm 2.5 \times 10^2$ ; and without neomycin at 37°C was  $4.4 \times 10^2 \pm 1.5 \times 10^2$  and at 45°C was  $3.8 \times 10^2 \pm 1.4 \times 10^2$ ; for RCM with neomycin at 37°C was  $4.8 \times 10^2 \pm 1.2 \times 10^2$  and

at 45°C was  $4.5 \times 10^2 \pm 1.0 \times 10^2$ ; without neomycin at 37°C was  $6.1 \times 10^2 \pm 1.4 \times 10^2$  and at 45°C was  $5.6 \times 10^2 \pm 1.5 \times 10^2$ .

### Optimal short time for pre-enrichment and DNA isolation

To determine the optimal short time for pre-enrichment, the fecal specimens from healthy individuals were spiked with various concentrations of enterotoxigenic *C. perfringens*. The result of determining the CFU of the original suspension by growing on TSC agar showed the *C. perfringens* concentrations corresponded to  $2.5 \times 10^2$ - $2.5 \times 10^7$  CFU per ml of fecal suspension. Four sets of each concentration of spiked feces were prepared for used with 4 different pre-enrichment times. Duplex PCR analyses were performed after processing and DNA isolation of spiked feces, and the result for the detection of PCR products by agarose gel electrophoresis is shown in Fig 1. The sensitivities of duplex PCR for detecting the minimum amount of the enterotoxigenic strain spiked in fecal samples for each time set, 0, 4, 8 and 24 hours was corresponded to  $10^5$ ,  $10^4$ ,  $10^6$ , and  $10^5$  CFU of *C. perfringens* spiked in one ml of fecal suspension, respectively. From the result, 4 hours was the optimal time for pre-enrichment in this study due to the highest sensitivity and the shortest time of duration for pre-enrichment culture before PCR analysis.

For DNA isolation from pre-enrichment broth culture of spiked feces, the templates prepared by boiling had a negative PCR result while those prepared by the QIAamp DNA Stool Mini Kit showed positive results for both the *plc* and *cpe* (data not shown). Therefore, this commercial kit test was suitable for isolation of DNA from pre-enrichment broth.

### Detection of enterotoxigenic *C. perfringens* in fecal specimens of diarrhea patients

Of the 198 stool specimens analyzed by the developed protocol for duplex PCR, 121 samples gave positive results for *plc*. In addition, 7 of 121 samples gave positive results for both *plc* and *cpe*. After the first PCR products of these 7 samples were diluted and then confirmed by the second PCR with the CPE1 and CPE2 primers, the second PCR products of

these 7 samples were positive for *cpe* only (Fig 2). The specificity of the 420-bp *cpe* amplicon of some samples 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. This suggests that 5.8% of *C. perfringens* carry the *cpe* gene when detected using the devel-

oped combination assay.

As shown in Table 2, the result of direct detection of only the *p/c* in fecal specimens by the developed assay showed high-levels of agreement (0.87 by Kappa analysis) with that of the conventional identification (secondary sub-culture and biochemical tests). All of the 198 stool specimens from diarrhea patients after pre-enrichment culture and incubated at 37°C for 4

Table 1  
Comparison of two selective enrichment media (fluid thioglycollate medium and reinforced clostridial medium) with and without neomycin and two temperatures for incubation (37°C and 45°C) for supporting the growth of 13 bacterial strains.

Bacterial strains	(GM ± SD)* of bacteria with the following media and incubations			
	Medium with neomycin		Medium without neomycin	
	37°C	45°C	37°C	45°C
<b>A. Fluid thioglycollate medium (FTG)</b>				
<i>C. perfringens</i> NCTC 8198	2.9x10 <sup>2</sup> ± 4.3x10 <sup>3</sup>	2.5x10 <sup>2</sup> ± 9.3x10 <sup>3</sup>	4.2x10 <sup>2</sup> ± 3.2x10 <sup>3</sup>	4.4x10 <sup>2</sup> ± 6.1x10 <sup>3</sup>
<i>C. perfringens</i> NCTC 8239	4.2x10 <sup>2</sup> ± 1.8x10 <sup>3</sup>	3.4x10 <sup>2</sup> ± 7.2x10 <sup>3</sup>	4.4x10 <sup>2</sup> ± 6.2x10 <sup>3</sup>	3.2x10 <sup>2</sup> ± 2.4x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 12916	2.9x10 <sup>2</sup> ± 7.1x10 <sup>3</sup>	4.6x10 <sup>2</sup> ± 2.6x10 <sup>3</sup>	4.1x10 <sup>2</sup> ± 2.1x10 <sup>3</sup>	2.6x10 <sup>2</sup> ± 7.5x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 3624	3.3x10 <sup>2</sup> ± 1.1x10 <sup>2</sup>	3.6x10 <sup>2</sup> ± 8.0x10 <sup>2</sup>	2.7x10 <sup>2</sup> ± 5.1x10 <sup>3</sup>	2.6x10 <sup>2</sup> ± 8.4x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 3629	4.6x10 <sup>2</sup> ± 6.4x10 <sup>3</sup>	3.3x10 <sup>2</sup> ± 4.7x10 <sup>3</sup>	4.8x10 <sup>2</sup> ± 3.4x10 <sup>3</sup>	4.5x10 <sup>2</sup> ± 2.2x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 27324	3.0x10 <sup>2</sup> ± 4.3x10 <sup>3</sup>	2.1x10 <sup>2</sup> ± 5.3x10 <sup>3</sup>	4.3x10 <sup>2</sup> ± 1.6x10 <sup>3</sup>	3.4x10 <sup>2</sup> ± 1.4x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 29348	4.2x10 <sup>2</sup> ± 7.0x10 <sup>3</sup>	3.2x10 <sup>2</sup> ± 5.9x10 <sup>3</sup>	6.6x10 <sup>2</sup> ± 5.0x10 <sup>3</sup>	4.8x10 <sup>2</sup> ± 4.5x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 43402	4.3x10 <sup>2</sup> ± 5.3x10 <sup>3</sup>	4.2x10 <sup>2</sup> ± 5.9x10 <sup>3</sup>	5.2x10 <sup>2</sup> ± 2.8x10 <sup>3</sup>	5.0x10 <sup>2</sup> ± 3.5x10 <sup>3</sup>
<i>C. sporogenes</i>	2.4x10 <sup>2</sup> ± 2.3x10 <sup>3</sup>	2.4x10 <sup>2</sup> ± 3.0x10 <sup>3</sup>	2.4x10 <sup>2</sup> ± 6.2x10 <sup>3</sup>	2.8x10 <sup>2</sup> ± 2.7x10 <sup>3</sup>
<i>C. botulinum</i>	1.7x10 <sup>2</sup> ± 1.9x10 <sup>3</sup>	1.8x10 <sup>2</sup> ± 2.3x10 <sup>3</sup>	2.5x10 <sup>2</sup> ± 8.6x10 <sup>3</sup>	2.0x10 <sup>2</sup> ± 2.2x10 <sup>3</sup>
<i>C. difficile</i>	1.2x10 <sup>2</sup> ± 2.5x10 <sup>3</sup>	1.5x10 <sup>2</sup> ± 4.7x10 <sup>3</sup>	2.5x10 <sup>2</sup> ± 3.6x10 <sup>3</sup>	1.9x10 <sup>2</sup> ± 8.9x10 <sup>4</sup>
<i>E. coli</i> ATCC 25922	4.4x10 <sup>2</sup> ± 1.7x10 <sup>3</sup>	5.6x10 <sup>2</sup> ± 3.3x10 <sup>3</sup>	6.3x10 <sup>2</sup> ± 2.3x10 <sup>3</sup>	6.8x10 <sup>2</sup> ± 3.5x10 <sup>3</sup>
<i>B. cereus</i>	5.3x10 <sup>2</sup> ± 3.6x10 <sup>3</sup>	4.9x10 <sup>2</sup> ± 6.3x10 <sup>3</sup>	6.8x10 <sup>2</sup> ± 1.0x10 <sup>2</sup>	5.3x10 <sup>2</sup> ± 6.1x10 <sup>3</sup>
Mean Total	3.4x10 <sup>2</sup> ± 1.2x10 <sup>2</sup>	3.9x10 <sup>2</sup> ± 2.5x10 <sup>2</sup>	4.4x10 <sup>2</sup> ± 1.5x10 <sup>2</sup>	3.8x10 <sup>2</sup> ± 1.4x10 <sup>2</sup>
<b>B. Reinforced clostridial medium (RCM)</b>				
<i>C. perfringens</i> NCTC 8198	4.4x10 <sup>2</sup> ± 4.5x10 <sup>3</sup>	4.3x10 <sup>2</sup> ± 7.6x10 <sup>3</sup>	7.5x10 <sup>2</sup> ± 4.5x10 <sup>3</sup>	5.3x10 <sup>2</sup> ± 9.0x10 <sup>3</sup>
<i>C. perfringens</i> NCTC 8239	5.2x10 <sup>2</sup> ± 3.7x10 <sup>3</sup>	4.1x10 <sup>2</sup> ± 3.5x10 <sup>3</sup>	7.1x10 <sup>2</sup> ± 4.2x10 <sup>3</sup>	5.0x10 <sup>2</sup> ± 4.1x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 12916	4.5x10 <sup>2</sup> ± 6.4x10 <sup>3</sup>	3.7x10 <sup>2</sup> ± 1.4x10 <sup>3</sup>	4.6x10 <sup>2</sup> ± 3.3x10 <sup>3</sup>	4.6x10 <sup>2</sup> ± 2.6x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 3624	4.6x10 <sup>2</sup> ± 3.4x10 <sup>3</sup>	2.8x10 <sup>2</sup> ± 5.4x10 <sup>2</sup>	4.9x10 <sup>2</sup> ± 5.4x10 <sup>3</sup>	3.9x10 <sup>2</sup> ± 1.0x10 <sup>2</sup>
<i>C. perfringens</i> ATCC 3629	4.7x10 <sup>2</sup> ± 6.5x10 <sup>3</sup>	4.5x10 <sup>2</sup> ± 5.2x10 <sup>3</sup>	5.0x10 <sup>2</sup> ± 5.4x10 <sup>3</sup>	5.0x10 <sup>2</sup> ± 2.1x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 27324	5.3x10 <sup>2</sup> ± 6.7x10 <sup>3</sup>	5.0x10 <sup>2</sup> ± 8.6x10 <sup>4</sup>	6.2x10 <sup>2</sup> ± 6.7x10 <sup>3</sup>	5.8x10 <sup>2</sup> ± 6.8x10 <sup>3</sup>
<i>C. perfringens</i> ATCC 29348	5.6x10 <sup>2</sup> ± 7.5x10 <sup>3</sup>	5.1x10 <sup>2</sup> ± 6.4x10 <sup>2</sup>	8.1x10 <sup>2</sup> ± 6.7x10 <sup>3</sup>	7.8x10 <sup>2</sup> ± 1.0x10 <sup>2</sup>
<i>C. perfringens</i> ATCC 43402	6.6x10 <sup>2</sup> ± 1.0x10 <sup>2</sup>	6.0x10 <sup>2</sup> ± 5.3x10 <sup>3</sup>	7.6x10 <sup>2</sup> ± 2.8x10 <sup>3</sup>	7.4x10 <sup>2</sup> ± 7.8x10 <sup>3</sup>
<i>C. sporogenes</i>	3.4x10 <sup>2</sup> ± 6.3x10 <sup>4</sup>	4.3x10 <sup>2</sup> ± 7.5x10 <sup>3</sup>	4.8x10 <sup>2</sup> ± 8.1x10 <sup>4</sup>	4.2x10 <sup>2</sup> ± 4.9x10 <sup>3</sup>
<i>C. botulinum</i>	2.9x10 <sup>2</sup> ± 1.0x10 <sup>3</sup>	3.5x10 <sup>2</sup> ± 4.4x10 <sup>3</sup>	4.1x10 <sup>2</sup> ± 6.7x10 <sup>3</sup>	4.4x10 <sup>2</sup> ± 6.7x10 <sup>3</sup>
<i>C. difficile</i>	3.6x10 <sup>2</sup> ± 0.0	3.6x10 <sup>2</sup> ± 4.0x10 <sup>3</sup>	4.5x10 <sup>2</sup> ± 7.9x10 <sup>3</sup>	4.2x10 <sup>2</sup> ± 2.4x10 <sup>3</sup>
<i>E. coli</i> ATCC 25922	6.0x10 <sup>2</sup> ± 3.1x10 <sup>3</sup>	6.2x10 <sup>2</sup> ± 3.7x10 <sup>3</sup>	7.3x10 <sup>2</sup> ± 6.1x10 <sup>3</sup>	8.3x10 <sup>2</sup> ± 2.5x10 <sup>3</sup>
<i>B. cereus</i>	5.8x10 <sup>2</sup> ± 1.1x10 <sup>2</sup>	4.6x10 <sup>2</sup> ± 6.7x10 <sup>2</sup>	7.0x10 <sup>2</sup> ± 5.9x10 <sup>3</sup>	6.5x10 <sup>2</sup> ± 7.5x10 <sup>3</sup>
Mean Total	4.8x10 <sup>2</sup> ± 1.2x10 <sup>2</sup>	4.5x10 <sup>2</sup> ± 1.0x10 <sup>2</sup>	6.1x10 <sup>2</sup> ± 1.4x10 <sup>2</sup>	5.6x10 <sup>2</sup> ± 1.5x10 <sup>2</sup>

\*Geometric mean (GM) concentrations ± standard deviation of three replicates for each bacteria tested which is reported as the difference between the GM at the start (~10<sup>7</sup> CFU/ml) before incubation and GM at the finish, after incubation for 24 hours.

hours indicated 121 samples were positive for *plc* detection assay and 106 of 121 samples were identified as *C. perfringens* by the conventional method.

A total of 530 colonies from 106 isolates (5 colonies from each primary isolation on TSC-egg yolk agar) were also confirmed by PCR for *plc* detection, and the PCR result showed high agreement (1.0 by Kappa analysis) with that of conventional identification (Table 3). All 530 colonies after secondary growth on blood agar and biochemical identification indicated 520 colonies were *C. perfringens* and all 520 colonies from primary fecal isolation were also positive for the *plc* detection assay. The other 10 colonies might have been *Clostridium* spp, which also showed positive lecithinase and H<sub>2</sub>S production on primary fecal isolation. These colonies were also negative for the *plc* and *cpe* detection by duplex PCR. Of the 520 colonies from primary fecal isolation which were positive for *plc* detection assay, 496 colonies showed positive results for *plc* only. In addition, 24 out of 520 colonies were positive for both *plc* and *cpe* or a prevalence of 4.6% of *C. perfringens* isolates that carry the *cpe* when detected from culture isolation with duplex PCR confirmation.

## DISCUSSION

A PCR-based detection system 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 enterotoxigenic *C. perfringens* strains from non-enterotoxigenic ones to identify the true agent of food poisoning, which should be helpful for epidemiological investigations. However, a variety of food components, heme and its metabolic products, acidic polysaccharides, humic substances, and collagen, have been shown to inhibit PCR amplification (Kim *et al*, 2000). Feces also contain many compounds, such as bilirubin and bile salts, which can inhibit PCR analysis (Widjojatmodjo *et al*, 1992).

Not only several potent inhibitors found in feces but also a high number of intestinal bacterial flora might interfere with PCR analysis if

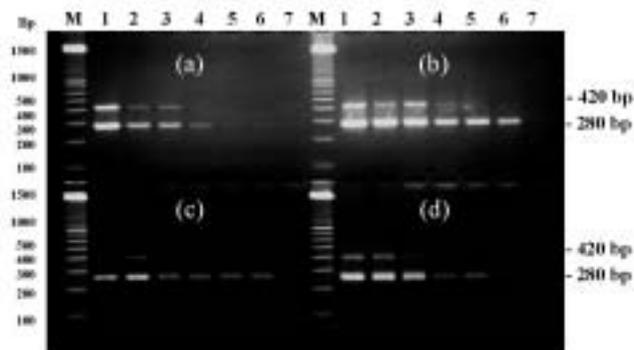


Fig 1—Agarose gel showing both *plc* (280-bp) and *cpe* (420-bp) products of duplex PCR assay for the detection of the minimum amount of enterotoxigenic *C. perfringens* reference strain spiked in feces at 6 concentrations, 10<sup>2</sup> - 10<sup>7</sup> CFU per ml of fecal suspension, and at 4 different pre-enrichment times: (a) 0 hour, (b) 4 hours, (c) 8 hours, and (d) 24 hours before PCR analysis. Lane M, DNA size marker, 100 bp DNA ladder; lanes 1-6, PCR products of spiked feces corresponding to *C. perfringens* concentrations of 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> CFU per one ml of fecal suspension, respectively; and lane 7, a negative reagent control.

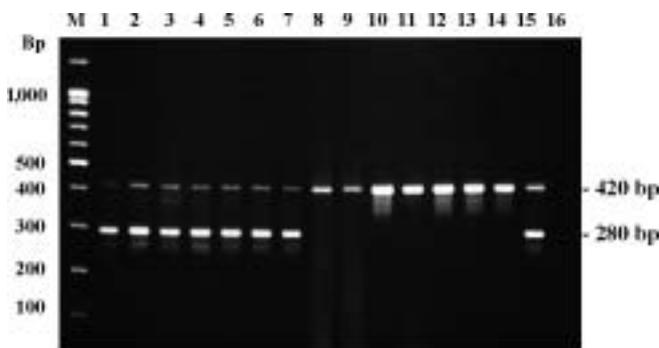


Fig 2—PCR results for the specific detection of the both *plc* and *cpe* for enterotoxigenic *C. perfringens* by enrichment broth culture-duplex PCR combination assay for the direct detection of enterotoxigenic *C. perfringens* in fecal specimens obtained from diarrhea patients. Lane M; DNA size marker (100 bp DNA ladder); lanes 1-7, samples No.1-7 which were positive for both *plc* and *cpe* when used as both PLC and CPE primers; lanes 8-14, samples No.1-7 which were positive in the second PCR for *cpe* only when used for only CPE primers; lane 15, *C. perfringens* ATCC 12916 as a positive control for both *plc* and *cpe* PCR products; and lane 16, deionized distilled water as a negative reagent control.

Table 2

Comparison between the *plc* detection by the developed enrichment broth culture-duplex PCR combination assay for direct detection of fecal specimens and the conventional method for identification of *C. perfringens* (secondary subculture and biochemical tests).

<i>plc</i> detection by the developed combination assay	No. of samples with the conventional method		Total
	<i>C. perfringens</i> positive	<i>C. perfringens</i> negative	
Positive	106	15	121
Negative	0	77	77
Total	106	92	198

Table 3

Comparison of the *plc* detection assay for direct detection of fecal specimens with the conventional (secondary subculture and biochemical tests) for species identification of *C. perfringens*.

<i>plc</i> detection by PCR from colonies of primary culture	No. of colonies with biochemical confirmation by the conventional method		Total
	<i>C. perfringens</i> positive	<i>C. perfringens</i> negative	
Positive	520	0	520
Negative	0	10	10
Total	520	10	530

the assay is performed directly from stool samples. In addition, low PCR sensitivity may happen due to low numbers of enterotoxigenic strains compared to higher numbers of non-pathogenic *C. perfringens* intestinal flora. To overcome these difficulties, some studies have developed a two-step PCR, based on the use of two pairs of primers in a nested configuration, together with the concentration and purification of the target bacteria from crude fecal samples. However, some fecal specimens probably failed to yield *cpe*-positive nested PCR results because of low organism loads, which was aided by the enrichment of enterotoxigenic *C. perfringens* (Asha and Wilcox, 2002).

Several broth media have been used for the enrichment of *C. perfringens* in routine culture isolation from clinical specimens. In our study, the growth of *C. perfringens* in RCM was much better than those in FTG at both 37°C and 45°C. The formulae of both media was different in some components, especially soluble starch, which was present in RCM, but not in FTG. The presence of carbohydrate, especially starch, in several media for the cultivation *C. perfringens* is

necessary for abundant growth and high spore levels of this organism (Labbe and Duncan, 1975). Sporulation and enterotoxin formation occur in the initial phase of growth in a starch-based medium, presumably, as a result of the slow release of reducing sugars by the action of amylase (Shih and Labbe, 1995). *C. perfringens* has an optimal growth temperature of 43° to 45°C (Labbe, 1989). However, several authors found only very low spore production at elevated temperatures, at which little amylase is excreted at these temperatures by most enterotoxin-positive (Ent+) strains, and they reported a temperature of 37°C and a near-neutral pH are optimal for sporulation and toxin production of *C. perfringens* (Labbe and Duncan, 1974).

The report by Garcia-Alvarado *et al* (1992) contradicted an earlier report that Ent+ strains could not sporulate well near their optimal growth temperature. They determined the effect of temperature at 37° and 46°C on starch hydrolysis, growth, and sporulation of Ent+ and Ent- strains in Duncan-Strong sporulation medium containing starch. At 37°C, all strains degraded starch and sporulated well. However, only Ent- strains

could hydrolyze starch, grow extensively, and sporulate at 46°C. Growth, sporulation, and starch hydrolysis by Ent+ strains at 46°C were equivalent to those obtained at 37°C when alpha-amylase was added to the cultures during growth. They suggested that the synthesis of alpha-amylase in Ent+ strains was regulated by temperature.

Due to the low numbers of the target enterotoxigenic *C. perfringens* in the intestinal content compared to the high numbers of several bacterial intestinal flora, a short enrichment procedure of the fecal specimen prior to PCR assay might be helpful, since the number of the target bacteria was substantially increased when selective enrichment medium was used. Several enrichment broth media, which are relatively inexpensive and easy to prepare, can be made for this selective purpose by the addition of antibiotics. Neomycin is a generally used antibiotic, added to several media because of its wide antibiotic spectrum to both gram-positive and gram-negative organisms. It is also inexpensive and readily available, and stable at the high temperatures that can be incorporated in culture media for sterilization by autoclaving.

From the results tested with pure culture isolation for all bacterial strains, RCM with neomycin and incubated at 37°C was found to be suitable for the enrichment of *C. perfringens*, because this medium and condition can inhibit other bacteria other than *C. perfringens*, thus this medium and condition were used for pre-enrichment of the fecal specimens before PCR analysis. We also attempted to eliminate any potentially interfering materials derived from host tissue. The cost-effectiveness for processing the specimen was also of concern. The centrifugation process is simple and cheap. Both the particulate and soluble substances were mostly removed by low- and high-speed centrifugation. However, some target bacteria may be lost in the processing steps and some interfering substances may remain in the final DNA supernatant, leading to lower PCR sensitivity (Kongmuang *et al*, 1994).

However, the centrifugation and washing steps can only remove some PCR inhibitors from feces. PCR detection still requires DNA purification as a means to eliminate inhibitory substances

from feces (Wang *et al*, 1996). In this study, the QIAamp DNA Stool Mini Kit was suitable for isolation of DNA from preenrichment broth. Guilbault *et al* (2002) also reported the usefulness of this kit, which provided fast and easy purification of total DNA from stool samples. Although the PCR itself is a very sensitive technique, significant losses of target molecules usually occur during DNA extraction. Therefore, relatively high numbers of microbes are needed for detection. The minimum amount of enterotoxigenic *C. perfringens* spiked in fecal samples that could be detected by our developed combination assay was 10<sup>4</sup> CFU per ml of fecal suspension.

The enrichment broth culture-duplex PCR assay performed as well as, or even better than, the culture technique. Of the 198 samples, 106 were culture positive for *C. perfringens* by the conventional method and 121 were positive for *C. perfringens* by the developed combination assay. Although primary isolation on TSC agar takes 24 hours, the standard method of confirmation for presumptive *C. perfringens* can take up to 72 hours (FDA, 1998). Presumptive sulfite-reducing clostridia, including *C. perfringens*, produce black colonies on TSC agar. Several colonies on TSC agar should be subcultured onto blood agar, for aerotolerance testing, purity checking, and Gram staining before confirmation as *C. perfringens* by biochemical tests. Confirmation of isolates is labor intensive, requires significant anaerobic workspace, and is prone to misreporting of results due to the selection of mixed cultures upon subculturing from TSC agar (Adcock and Saint, 2001). Biochemical testing of several colonies from a large number of samples is labor-intensive, costly 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, not commercially available, and high cost.

The observed prevalence of the *cpe* gene in *C. perfringens* in this study was 4.6% when detected by culture isolation with duplex PCR confirmation, or 5.8% when detected by the developed combination assay; which are close to those reported in several surveys (Kokai-Kun *et*

al, 1994; Songer and Meer, 1996). Some studies indicate that in most *C. perfringens* isolates from food poisoning, the *cpe* has a chromosomal location, whereas in non-food-borne gastrointestinal illnesses the *cpe* is located on a plasmid (Cornillot *et al*, 1995; Collie and McClane, 1998). Furthermore, the *cpe* plasmid of at least some non-food-borne gastrointestinal disease isolates, can transfer via conjugation, and the *cpe* chromosome has been proposed to be located on a transposon (Brynestad *et al*, 2001). Thus, both chromosomal *cpe* and plasmid *cpe* may be present on mobile genetic elements. Therefore, further studies on those *cpe*-positive *C. perfringens* isolates will be done in an attempt to determine whether those *cpe*-positive isolates carry a chromosomal or a plasmid-borne *cpe*, and also to determine their antimicrobial susceptibility patterns.

In conclusion, the developed enrichment broth culture-duplex PCR combination assay was a suitable method for the rapid and specific detection of enterotoxigenic *C. perfringens* directly in feces of diarrhea patients. The whole process took less than 8 hours and thus the results could be obtained within one day or on the same day as specimen collection. The time saved compared to conventional method requiring up to one week, is marked, which is helpful for epidemiological investigations of food-borne outbreaks or non-food-borne GI disease caused by enterotoxin producing strains. Moreover, the bacteria obtained in the enrichment media may be used for other studies, antibiotic susceptibility testing and serotyping. Further study on genotyping of these *cpe* positive isolates is required.

#### ACKNOWLEDGEMENTS

The authors are grateful to all the contributors of the reference strains and fecal specimens, with special thanks to two hospitals (Siriraj Hospital, Bangkok; and Nakhon Nayok Hospital, Nakhon Nayok).

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