PCR Detection and Prevalence of Enterotoxin (*cpe*) Gene in *Clostridium perfringens* Isolated from Diarrhea Patients

UNCHALEE TANSUPHASIRI, M.Sc.*,
GUMPHOL WONGSUVAN, M.Sc..**,
BOONCHUAY EAMPOKALAP, M.Sc.***

Abstract

Clostridium perfringens isolated from patients with diarrhea (n=233) were analysed by a duplex PCR assay, in order to determine the prevalence of enterotoxin (*cpe*) gene and various factors involved in patients with *cpe*-positive isolates. This duplex PCR uses two sets of primers which amplify in the same reaction two different gene fragments: the phospholipase C (*plc*, alpha-toxin) and the enterotoxin (*cpe*) genes in *C. perfringens*. PCR analysis of 477 colonies of fecal spore isolates, from 159 patients who had a spore count $\geq 10^3$ cfu/g, gave positive *plc* gene detection in 436 colonies. The results were consistent with those obtained by using the standard method of *C. perfringens* species identification. 21 of 436 colonies gave positive results for both *plc* and *cpe* genes, indicating a prevalence of 4.8 per cent of *C. perfringens* that carried the *cpe* gene in cases of diarrhea. The majority of cases with *cpe*-positive isolates were women over 50 years of age (71.4%). These patients had diarrhea more than 6 times per day (71.4%) with a duration of 1-3 days (100%). Furthermore, 85.7 per cent of cases developed diarrhea after food consumption, 28.6 per cent had high spore counts of more than 10^6/g in their feces, and 71.4 per cent were co-infected with other enteric pathogens. The spore count should be interpreted with caution because not all isolates of *C. perfringens* from diarrhea patients with high fecal spore count carried the *cpe* gene, which encodes a sporulation-associated enterotoxin.

Conclusion : The duplex PCR assay can thus become a tool for *C. perfringens* species identification together with the detection of enterotoxin gene. This PCR assay is faster, less expensive and more suitable for large-scale use in epidemiological studies than conventional procedures. The authors recommend this assay to screen for enterotoxigenic *C. perfringens* isolates from primary fecal spore isolation cultures, particularly in elderly patients with food-borne diarrhea and non-food related diarrhea.

Key word : *Clostridium Perfringens,* Diarrhea Patients, Duplex PCR, Enterotoxin

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* Department of Microbiology, Faculty of Public Health,
** Wellcome Tropical Disease Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400,
*** Bamrasnaradura Hospital, Ministry of Public Health, Nonthaburi 11000, Thailand.

Clostridium perfringens is an endospore-forming, gram-positive anaerobic bacterium that is considered one of the most common human food-borne pathogens[1,2]. The pathological effects associated with *C. perfringens* food poisoning have been linked to the production of an enterotoxin[2-4]. The role of *C. perfringens* enterotoxin (CPE) in food-borne diarrhea, as well as non-food related diarrhea, i.e. antibiotic-associated diarrhea and sporadic diarrhea has been well documented[5-7]. Recent surveys [8,9] suggest that only a low percentage (~5%) of all *C. perfringens* isolates carry the *cpe* gene encoding enterotoxin. Therefore, it is necessary to distinguish the enterotoxigenic organisms from nonenterotoxigenic ones to confirm a food-borne outbreak by *C. perfringens*.

The previously described methods for detecting enterotoxigenic *C. perfringens* strains include enzyme immunoassays and reversed passive latex agglutination[10-13]. However, these techniques have produced unreliable results as CPE is only produced during sporulation, which may be difficult to attain in usual culture media[13] and may lead to false-negative results[8]. As well as low sensitivity and specificity, other problems that may occur with immunological assays are autoagglutination, lack of specific antiserum, high expense, time intensive, and difficulties associated with handling a large number of samples. These problems have limited routine application of these procedures regarding *C. perfringens* diagnosis.

Rapid and highly sensitive techniques based on PCR have been developed recently for the detection of many food-borne pathogens[14]. PCR procedures for detecting either the alpha-toxin (phospholipase C, *plc*) or enterotoxin (*cpe*) genes of *C. perfringens* from fecal isolates or directly from stools have also been described[15-18]. Recently, a duplex PCR assay using two pairs of primers, instead of just one pair, has also been developed by Tansuphasiri [19] for the simultaneous detection of *C. perfringens* strains that contain the enterotoxin gene.

In the present study, this duplex PCR was used to determine the prevalence of enterotoxin gene in *C. perfringens* fecal isolates associated with cases of diarrhea. Furthermore, the assay was used to determine possible associations between enterotoxin-positive *C. perfringens* patients and the various risk factors that may prove potentially useful as a tool in the epidemiology of pathogenic *C. perfringens*.

MATERIAL AND METHOD

Diarrhea patients

All patients had attended the Diarrhea Center, Bamrasnaradura Hospital, Nonthaburi between August and October 1999. All the patients were over 2 years old. All the patients had illness with diarrhea, three or more loose or watery stools or at least one episode of mucous-bloody stool per day. Patient details were recorded on a standard recorded form including general personal data (sex, age), clinical manifestations, and history of food consumption.

Collection of fecal specimens

Fresh fecal specimens were collected and placed in a sterile container (30 ml screw capped tubes). The samples were held at 4°C and transferred within 3 h to the bacteriological laboratory at the Department of Microbiology, Faculty of Public Health, Mahidol University for later Clostridial spore counts and enterotoxin gene analysis.

Isolation and enumeration of clostridial spores

One gram of fecal material was emulsified in 9 ml of sterile phosphate-buffered saline, pH 7.2. The emulsion was heated at 80°C for 20 min and then serially diluted. 0.1 ml volumes of the appropriate dilutions were inoculated into Tryptose-sulfite-cycloserine (TSC) agar (Merck) supplemented with 5 per cent (w/v) egg yolk by the spread-plate technique[20]. After incubation anaerobically at 37°C for 18-24 h, black colonies with a zone of turbidity around by lecithinase activity were counted.

Up to 3 presumptive colonies from each primary medium were randomly selected and each colony was suspended in 100 µl TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and then inoculated onto duplicate blood agar plates. One plate was incubated at 37°C for 24 h in an anaerobic jar, and another in aerobic conditions. The colonies grown only on blood agar under anaerobic conditions from each plating were confirmed by biochemical tests[21].

DNA extraction

The following reference *C. perfringens* strains served as controls for PCR analysis: enterotoxigenic strains *C. perfringens* ATCC 12916, NCTC 8198, NCTC 8239 and NCTC 10239 (received from Dr. A. Heikinheimo, Faculty of Veterinary Medicine,

University of Helsinki, Finland) and nonenterotoxigenic strains ATCC 3624, 3628, 3629 and 43402 (received from Dr. O. Suthienkul, Faculty of Public Health, Mahidol University). Total genomic DNAs from all reference strains were extracted by a QIAamp tissue kit (Qiagen, Germany) according to the manufacturer's protocol. DNAs from all fecal isolates were extracted by the boiling method. Briefly, the remaining part of the suspension (~50 µl) from each colony was boiled for 10 min and then placed on wet ice for 5 min. After centrifugation at 12,000 x g for 2 min, 10 µl of the supernatant was used as template DNA in the PCR.

Duplex PCR analysis

Two pairs of primers derived from the reference sequence Accession M98037 for *cpe* gene and Accession X17300 for *plc* gene (Gen Bank), designated CPE (CPE1 and CPE2) and PLC (PLC1 and PLC2), respectively were used in the duplex PCR reaction. All of these primers were described by Tansuphasiri[19] as follows : the CPE primers, CPE1 (5' GAAAGATCTGTATCTACAACTGCT 3') and CPE2 (5' TAAGATTCTATATTTTTGTCCAGT 3'); and the PLC primers, PLC1 (5' ATAGATACTCC ATATCATCCTGCT 3') and PLC2 (5' TTACCTT TGCTGCATAATCCC 3').

PCR 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, 0.6 µl (0.6 U) of *Taq* DNA polymerase (Promega), 1 ng (10 µl) of template DNA and up to 30 µl of distilled water. The amplifications were carried out in an automated thermal cycler (Perkin-Elmer Cetus) with the following program : 5 min at 94°C, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; followed by a 10-min final extension at 72°C. For detection, 10 µl of PCR product was electrophoresed on 2 per cent agarose gel, stained with ethidium bromide and viewed under UV light. To avoid possible contamination, all reactions were performed under stringent conditions[22].

RESULTS

Demographic data

During the 3-month period, a total of 233 patients had attended the Diarrhea Center, Bamrasnaradura Hospital. Of 233 diarrhea patients, 111 (47.6%) were males and 122 (52.4%) were females. The male : female ratio was 1 : 1.1. The youngest patient was 2 years old and the oldest was 85 years old. The mean age of the diarrhea patients was 33.7 years (SD = 20.98 years). The majority of the patients were in the age group 21-30 years (18.4%), followed by 31-40 years (18.0%), 2-10 years (17.6%), 11-20 years and 41-50 years (11.2%), 51-60 years (10.3%), 61-70 years (9.4%), and >70 years (3.9%), respectively. Times of diarrhea ranged from 1-20 times per day (mean = 6.69, median = 6 times per day). Most cases, 107 (45.8%) had diarrhea 4-6 times per day. Duration of diarrhea ranged from 1-30 days (mean = 1.85, median = 1 day), and most had a duration of 1-3 days in 184 cases (79%). Most patients, 108 (46.4%) developed diarrhea after 8-10 h of food consumption, while 41 (17.6%) were non-food associated and 84 (36.0%) were unsure.

Conventional culture and identification

From primary cultures on TSC-egg yolk agar, only black colonies surrounded by opalescence zone were counted and reported as cfu/g of feces. Of the 233 stool specimens examined, 74 samples (31.8%) had clostridial spore counts $<10^3$/g (including no growth) and 159 samples (68.2%) had $\geq 10^3$ cfu/g. Of these 159 samples, 52 samples (22.3%) had spore counts $\geq 10^3$-10^4 cfu/g; 10 samples (4.3%) $>10^4$-10^5/g; 45 samples (19.3%) $>10^5$-10^6/g, and 52 samples (22.3%) $>10^6$/g. A total of 477 colonies from 159 isolates (three colonies from each isolation that contained spore counts of $\geq 10^3$ cfu/g) were subjected to secondary subculture and then biochemical tests were performed. Results showed that 436 of 477 (91.4%) colonies were identified as *C. perfringens*, while 41 of 477 colonies belonged to other *Clostridium* spp. with H_2S production and lecithinase activity that were closely related to *C. perfringens*.

The same fecal specimens were also cultured by routine examination for other enteric pathogens which were performed at the Bacteriology Laboratory at Bamrasnaradura Hospital. Among the 233 diarrheal patients, 93 bacterial enteric pathogens were isolated from 69 cases (29.6%). Most isolates were *Vibrio parahaemolyticus* (32.3%), followed by *Plesiomonas shigelloides* (18.3%), *Vibrio* spp. (16.1%), *Aeromonas* spp. (10.7%), *V. cholerae* -O1 and non O-1 (9.7%), *Salmonella* spp. (8.6%), *Shigella* spp. (3.2%), and *Campylobacter jejuni* (1.1%).

Duplex PCR for simultaneous detection of *plc* and *cpe* genes in *C. perfringens*

The PCR products of *C. perfringens* amplified by both primer pairs showed bright and clearly visible bands with a molecular size of 280-bp and 420-bp for *plc* and *cpe* genes, respectively. *C. perfringens* non-enterotoxin producing strains showed the presence of 280-bp *plc* bands only, but *C. perfringens* enterotoxin producing strains showed the presence of both *plc* and *cpe* gene fragments of 280-bp and 420-bp, respectively (Fig. 1).

The PCR for *plc* gene detection showed high-levels of agreement with that of conventional identification. All 477 colonies after secondary growth on blood agar and biochemical characterization indicated 436 colonies were identified as *C. perfringens* and all of the 436 colonies from primary isolation were also positive for *plc* gene detection assay (the presence of 280-bp band). In addition, 21 of 436 colonies gave positive results for both *plc* and *cpe* genes (the presence of 2 bands of 280-bp and 420-bp). This suggested a prevalence of 4.8 per cent of *C. perfringens* that carried the *cpe* gene. The colonies with *cpe* positive result were confirmed by the second PCR with the primers tested individually and in combination. The band patterns on agarose gel were identical to those of the reference enterotoxigenic strain (data not shown). The 420-bp *cpe* amplicon was also confirmed by sequencing and by comparison of the sequence data obtained from the GenBank and identity to the sequence of *cpe* gene was found.

Some characteristics of patients with positive *cpe* gene detection

Details of patients with positive *cpe* gene of *C. perfringens* for sex, age, duration of diarrhea, diarrhea times per day, number of fecal spore counts, food association, and the presence of other enteric pathogens are shown in Table 1. Isolates with positive *cpe* gene detection by PCR were isolated from 7 patients. These patients were 8 years to 78 years of age. The duration of diarrhea was one day in 6 of 7 cases and three days in one case. Five patients had diarrhea more than 6 times per day (71.4%). Two of these patients (28.6%) had a high spore count ($>10^6$/g) while 5 patients (71.4%) had a spore count $<10^6$/g. Six of these patients (85.7%) had diarrhea after food consumption while 1 patient (14.3%) was non-food associated. Five of the seven patients were co-infected with other enteric pathogens and two patients were not infected with other enteric pathogens.

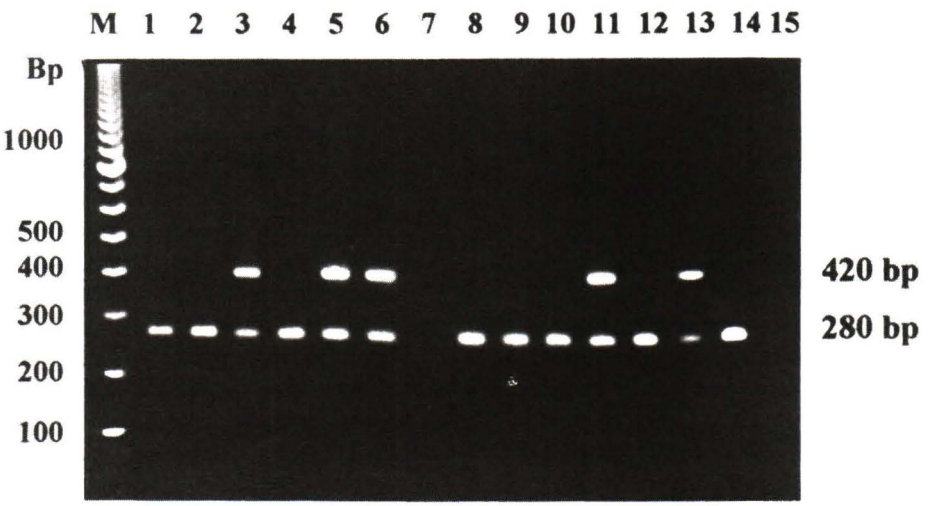

Fig. 1. PCR results for the *plc* and *cpe* genes detection of DNA extracted from primary isolation cultures. Lane M contained DNA size marker, 100 bp DNA ladder. Lanes 1, 2, 4, 8, 9, 10 and 12 were positive for *plc* gene only. Lanes 3, 5, 6 and 11 were positive for both *cpe* and *plc* genes. Lane 7 was negative for both *cpe* and *plc* genes. Lane 13 was positive control for both *cpe* and *plc* genes. Lane 14 was positive control for *plc* gene only. Lane 15 was negative reagent control.

Table 1. Distribution of positive and negative *cpe* gene detection in patients regarding to sex, age, duration of diarrhea and times per day, spore count, food association, and the presence of other enteric pathogens.

Patient characteristics	*Cpe* +ve group		*Cpe* -ve group		Total	
	No.	%	No.	%	No.	%
No. of patients	7	4.4	152	95.6	159	100.0
Sex (Male : Female)	2 : 5	0.4 : 1	69 : 83	0.8 : 1	71 : 88	0.8 : 1
Age (years)						
<10	1	14.3	24	15.8	25	15.7
10-19	0	0.0	17	11.2	17	10.7
20-29	1	14.3	32	21.0	33	20.7
30-39	0	0.0	27	17.8	27	17.0
40-49	0	0.0	17	11.1	17	10.7
≥50	5	71.4	35	23.1	40	25.2
Duration of diarrhea						
1-3 days	7	100.0	136	89.5	143	89.9
>3 days	0	0.0	16	10.5	16	10.1
Diarrhea times per day						
1-3 times	1	14.3	30	19.7	31	19.5
4-6 times	1	14.3	68	44.7	69	43.4
>6 times	5	71.4	54	35.6	59	37.1
Spore count (per g of feces)						
$\geq 10^3$-10^4	2	28.6	50	32.9	52	32.7
$>10^4$-10^5	1	14.2	9	5.9	10	6.3
$>10^5$-10^6	2	28.6	43	28.3	45	28.3
$>10^6$	2	28.6	50	32.9	52	32.7
Food association						
Yes	6	85.7	76	50.0	82	51.6
No	1	14.3	21	13.8	22	13.8
Not known	0	0.0	55	36.2	55	34.6
Other bacterial pathogens						
Presence	5	71.4	107	70.4	112	70.5
Absence	2	28.6	45	29.6	47	29.5

A total of 152 patients were negative for *cpe* gene detection. The patients ages ranged from 2 years to 87 years. Most patients (23.1%) were >50 years old. The duration of diarrhea was one to three days in 136 of 152 patients (89.5%) and >3 days in 16 patients (10.5%). 50 of 152 patients (32.9%) had spore counts $>10^6$/g. 76 cases (50.0%) had diarrhea related with food consumption and 21 cases (13.8%) had diarrhea not related with food consumption. Unfortunately, data could not be collected from 55 cases (36.2%). 107 cases were not infected with other enteric pathogens. Seventy-three bacterial enteric pathogens were isolated from 45 cases (29.6%).

DISCUSSION

Diarrhea is a major public health problem causing great morbidity and mortality in the Thai population. From epidemiological surveillance data in 1999, acute diarrheal disease was ranked first as the major cause of morbidity while its mortality was second among the top ten most common diseases (23). Most diarrheal diseases in children are of food-borne origin. It can have a direct impact on national economy. Some particular industries such as food processing, food export market or tourist industry may be affected.

At present, the conventional means for diagnosis of food-borne diarrhea in the microbiology laboratory relies on culture of the bacteria from stool samples. Because there are many food-borne bacterial pathogens, such investigation is usually concentrated only on facultative bacteria. Analysis of food-borne anaerobic bacteria are not routinely performed due to difficulties in isolation and identification. These procedures require special techniques that may be expensive, time-consuming and labor intensive. For this reason, outbreaks of these organisms often are not recognized.

C. perfringens continues to be a common cause of food-borne disease. The organism possesses several attributes that contribute to illness[2,24], including its ubiquitous nature, giving it ample opportunity to contaminate foods; the formation of heat-resistant endospores that survive normal cooking/heating temperatures; a rapid growth rate in warm food; an ability to overcome the stomach acid barrier; and the production of enterotoxin (CPE), a 35-kDa single polypeptide which is released only during sporulation of the organism in the small intestine.

There is increasing evidence that CPE is also involved in several non-food-borne human gastrointestinal illnesses, including antibiotic-associated diarrhea and sporadic diarrhea[6,7]. The disease occurs almost exclusively in the elderly[25] and following a course of antibiotic therapy[26]. Further, CPE is now also associated with some cases of sudden infant death syndrome (SIDS)[27] where it appears to be absorbed from the infant's gastrointestinal tract into the circulation. It has been hypothesized that CPE acts as a triggering agent, initiating the events associated with the development of SIDS[28].

Published criteria for implicating *C. perfringens* as the cause of food poisoning outbreaks is usually based on the finding of $>10^5$ *C. perfringens* cells per g of food or $>10^6$ spores per g of feces from an ill individuals; the presence of the same serotype in all ill patients; and the presence of the same serotype in both contaminated food and feces [29]. However, these criteria have sometimes proved inconclusive. Stringer et al[30] found that the fecal carriage of *C. perfringens* varied considerably between individuals, and some geriatric patients carried relatively high numbers of *C. perfringens* ($>10^7$/g) and the majority of these patients carried the same serotypes. Yamagishi et al[31] also found that the numbers of *C. perfringens* in the feces of some healthy adults in a Japanese House for the Aged were consistently of the order of 10^7-10^9/g.

Routine identification of *C. perfringens* also requires characterization by costly and lengthy procedures, which may take 1 week and involve the use of biochemical testing of several colonies after secondary subcultures, and final confirmation of enterotoxigenic strains by culturing in Duncan-Strong (DS) sporulation medium combined with serological analysis[20]. However, diagnosis by serological assay to demonstrate its ability to produce enterotoxin *in vitro* is quite difficult because fresh *C. perfringens* isolates often sporulate poorly or not at all in laboratory media[13].

Several studies have shown that *C. perfringens* isolates that cause human food poisoning usually carry the *cpe*-gene, which encodes a sporulation-associated enterotoxin[32]. Therefore, the *cpe* gene detection assay may represent a significant advantage over the serological assay. On the other hand, isolates positive by *cpe* gene prove assays would imply that such isolates are able to express the enterotoxin. Although, several studies of *C. perfringens* food poisoning were linked to the presence of enterotoxin in isolates that carry the *cpe* gene and progress towards defining enterotoxin structure-function relationships has been exciting, considerable questions remain unanswered.

The alpha-toxin or phospholipase C (PLC), which is common to all *C. perfringens* types, exhibits an enzyme activity that catalyses the hydrolysis of lecithin into phosphorylcholine and 1, 2-diacylglyceride[33]. The alpha-toxin gene or *plc* gene is found in all toxin types and present in all strains of *C. perfringens*[15] while the *cpe* gene is restricted to only enterotoxigenic *C. perfringens* strains and seems to be quite conserved which is favorable for genotypic diagnostics. The sequences of the genes encoding the production of PLC and CPE have been published[9,34,35].

To facilitate routine detection of *C. perfringens* and also to differentiate between the enterotoxigenic strains and non-enterotoxigenic ones, Tansuphasiri[19] combined the *plc* and *cpe* specific primers by using one annealing temperature at 55°C 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 sets produced bright clearly visible bands of corrected size without non-specific reaction. This procedure was rapid, could exhibit a result within 4 hours for both PCR amplification and gel electrophoresis analysis.

In this study, 436 of 477 colonies from primary fecal spore isolation gave positive results

for both conventional identification and the *plc* gene detection assay (the presence of 280-bp). In addition, 21 of 436 colonies gave positive results for both *plc* and *cpe* genes (the presence of 2 bands of 280-bp and 420-bp). This suggested a prevalence of 4.8 per cent of *C. perfringens* that carried the *cpe* gene. The observed prevalence of the *cpe* gene in *C. perfringens* strains isolated from fecal specimens in this study was close to those recently reported in molecular epidemiology surveys. Those surveys suggested that only a low percentage (~5%) of all *C. perfringens* isolates from various origins carried the *cpe* gene[8,9], whereas, the percentage is higher among *C. perfringens* strains isolated from confirmed outbreaks of food poisoning[17].

The 420-bp amplicons of these patients were also confirmed by sequencing and they corresponded to the *cpe* gene sequence of *C. perfringens* obtained from the GenBank. The positive *cpe* isolates were also checked for CPE production *in vitro* by using the Oxoid RPLA kit after growing in the DS medium. However, some strains showed consistent results with *cpe* gene analysis, while others were not tested since they did not sporulate in the DS medium used. There seems to be no universal medium that will encourage sporulation in all strains, and thus lead to false-negative results, if serological assay methods were used for detection.

In the present study, the authors could not determine the association between *cpe* gene-positive *C. perfringens* in the patient feces group and various factors, due to the low prevalence of *cpe* gene detection in *C. perfringens* strains isolated from these patients. From the results, most *cpe* gene-positive isolates were detected in elderly patients, at least 5 of 7 cases. Most patients (6 of 7 cases) had diarrhea after food consumption. Diarrhea in these elderly patients can be very troublesome. It may produce incontinence or may prolong hospital stay. However, in this study, diarrhea was prolonged (3 days) in only one patient aged 78 years and was not related with food consumption. Diarrhea longer than 2 days has been reported in other studies of non-food-borne human GI diseases including antibiotic associated diarrhea and sporadic diarrhea[6,7]. It is common in the elderly and the symptoms are more severe and longer in duration than those of *C. perfringens* type A food poisoning[7].

Initial studies have suggested that the *cpe* gene is located on the chromosome of food poisoning isolates but is located on a plasmid in non-food-borne GI disease isolates and in veterinary isolates [36]. Interestingly, recent studies[37] have shown that the *cpe* plasmid can be conjugatively transferred, *in vitro*, to naturally *cpe*-negative isolates, and suggested that if this conjugative transfer of the *cpe* plasmid (from a relatively few infecting *cpe*-positive strains) occurred *in vivo*, it would have the potential to convert many naturally *cpe*-negative *C. perfringens* strains already present in the normal intestinal flora into strains capable of causing CPE-associated non-food-borne GI disease.

Spore counts in most *cpe*-positive patients were $<10^6$/g indicating that detection of high numbers of *C. perfringens* was less helpful in determining the cause of diarrhea caused by *C. perfringens*. Several studies also found no correlation between *C. perfringens* spore number and the presence of CPE [5,7]. Either CPE serologic detection or *cpe* gene detection assays could be useful for evaluating isolate enterotoxigenicity. However, there is increasing evidence that feces from some healthy individuals may contain some CPE-positive *C. perfringens* isolates, thus it is important to establish the levels of specifically enterotoxigenic *C. perfringens* isolates that may be present in feces from ill individuals *versus* feces from healthy individuals[24].

In conclusion, the authors used the duplex PCR assay as a tool to determine the prevalence of enterotoxin gene, *cpe*, in *C. perfringens* isolated from patients with diarrhea; and only 4.8 per cent of all *C. perfringens* isolates carried the *cpe* gene. Due to the duplex PCR assay being faster, less expensive and more suitable for large scale use in epidemiological studies than conventional procedures, the authors recommend this assay to screen for enterotoxigenic *C. perfringens* isolates from primary fecal spore isolation cultures, particularly in elderly patients with food-borne diarrhea and non-food related diarrhea. The application of this assay to the direct detection of pathogenic *C. perfringens* in stool samples and in food is in progress. Further studies are also planned to determine the toxinotypes (A-E) of enterotoxigenic *C. perfringens* by amplifying several genes (multiplex PCR) that would be helpful for epidemiological investigation.

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การตรวจด้วยเทคนิคพีซีอาร์เพื่อหาความชุกของเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* ที่มียีนสร้างสารพิษเอ็นเทอโรท็อกซิน จากเชื้อที่แยกได้จากอุจจาระของผู้ป่วยโรคอุจจาระร่วง

อัญชลี ตัณฑ์ศุภศิริ, วท.ม.,*
*กัมพล วงศ์สุวรรณ, วท.ม.**, บุญช่วย เอี่ยมโภคลาภ, วท.ม.****

การใช้เทคนิคดูเพล็กซ์พีซีอาร์เพื่อตรวจหาเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* และหาความชุกของสายพันธุ์ที่มียีนสร้างสารพิษเอ็นเทอโรท็อกซิน ในผู้ป่วยโรคอุจจาระร่วง จำนวน 233 ตัวอย่าง รวมทั้งการศึกษาถึงปัจจัยต่าง ๆ อันเกี่ยวข้องกับผู้ป่วยที่มีเชื้อสายพันธุ์ดังกล่าว เทคนิคนี้อาศัยไพร์เมอร์ 2 คู่ เพื่อเพิ่มจำนวนยีน 2 ชนิดไปในขั้นตอนเดียวกัน ได้แก่ phospholipase C (*plc*, alpha-toxin) และ enterotoxin (*cpe*) gene เชื้อที่เพาะแยกได้ด้วยวิธีมาตรฐานจากผู้ป่วยที่มีจำนวนสปอร์ $\geq 10^3$ เซลล์/กรัม ของอุจจาระ 159 ราย จำนวน 477 โคโลนี เมื่อตรวจวิเคราะห์ด้วยดูเพล็กซ์พีซีอาร์ พบว่า 436 โคโลนี ให้ผลบวกต่อยีน *plc* ซึ่งผลสอดคล้องกับวิธีมาตรฐานการพิสูจน์หาสปีชีส์ของเชื้อนี้ และ 21 ใน 436 โคโลนี ให้ผลบวกต่อยีน *cpe* ร่วมด้วย ความชุกของเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* ที่มียีนสร้างสารพิษเอ็นเทอโรท็อกซิน จากจำนวนเชื้อที่แยกได้จากผู้ป่วยคิดเป็นร้อยละ 4.8 เชื้อที่ให้ผลบวกต่อยีนเอ็นเทอโรท็อกซินนั้น ส่วนใหญ่ (ร้อยละ 71.4) แยกได้จากผู้ป่วยเพศหญิง และมีอายุมากกว่า 50 ปี, ร้อยละ 71.4 ถ่ายอุจจาระมากกว่า 6 ครั้งต่อวัน และร้อยละ 100 มีระยะเวลาในการป่วย 1-3 วัน, ร้อยละ 85.7 มีความสัมพันธ์กับการรับประทานอาหาร, ร้อยละ 28.6 มีจำนวนสปอร์มากกว่า 10^6 เซลล์/กรัมของอุจจาระ และร้อยละ 71.4 มีการติดเชื้อในระบบทางเดินอาหารอื่น ๆ ร่วมด้วย การศึกษาพบว่า อุจจาระที่มีสปอร์จำนวนมากนั้น สปอร์บางตัวอาจไม่ได้เป็นเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* ที่มียีนสร้างสารพิษเอ็นเทอโรท็อกซิน

โดยสรุปเทคนิคดูเพล็กซ์พีซีอาร์นี้ นำมาใช้เป็นเครื่องมือในการพิสูจน์ถึงสปีชีส์ของเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* และยังใช้ตรวจหาความสามารถในการก่อโรคได้หรือไม่ในเวลาเดียวกัน มีข้อดีที่เหนือกว่าวิธีมาตรฐานในแง่ที่เป็นวิธีการที่รวดเร็วและยังลิ้นเปลืองค่าใช้จ่ายน้อยถ้าตรวจวิเคราะห์กับตัวอย่างจำนวนมาก ๆ ซึ่งนับว่าเหมาะสมกับการใช้สำรวจทางระบาดวิทยา จึงขอแนะนำให้ใช้เทคนิคนี้ในการตรวจคัดกรองโคโลนีที่เพาะแยกครั้งแรกจากสปอร์ในอุจจาระของผู้ป่วย เพื่อหาเชื้อ*คลอสตริเดียม เปอร์ฟริงเจนส์* ที่มีศักยภาพในการสร้างสารพิษเอ็นเทอโรท็อกซิน โดยเฉพาะในตัวอย่างจากกลุ่มผู้ป่วยสูงอายุด้วยโรคอุจจาระร่วงจากสาเหตุทั้งที่เกี่ยวข้องหรือไม่เกี่ยวข้องกับการรับประทานอาหาร

คำสำคัญ : *คลอสตริเดียม เปอร์ฟริงเจนส์*, ผู้ป่วยอุจจาระร่วง, ดูเพล็กซ์พีซีอาร์, เอ็นเทอโรท็อกซิน

อัญชลี ตัณฑ์ศุภศิริ, กัมพล วงศ์สุวรรณ, บุญช่วย เอี่ยมโภคลาภ
จดหมายเหตุทางแพทย์ ฯ 2545; 85: 624-633

* ภาควิชาจุลชีววิทยา, คณะสาธารณสุขศาสตร์,
** หน่วยวิจัยเวลคัม, คณะเวชศาสตร์เขตร้อน, มหาวิทยาลัยมหิดล, กรุงเทพ ฯ 10400
*** โรงพยาบาลบำราศนราดูร, กระทรวงสาธารณสุข, นนทบุรี 11000