EVALUATION OF SYNTHETIC DNA PROBES FOR CONFIRMATION OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN GENE PCR PRODUCTS

Unchalee Tansuphasiri[1], Taniya Muadcheingka[2] and Suwan Choonharuangdej[2]

[1]Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok;
[2]Department of Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Abstract. A new diagnostic reagent was developed that is capable of detecting the presence of *Clostridium perfringens* rapidly and accurately compared to the conventional methods. *C. perfringens* enterotoxin (*cpe*) gene is the gene of interest since it encodes the enterotoxin responsible for food poisoning. Two new *cpe*-specific labeled DNA probes were evaluated using Southern and dot blot hybridization. Bacterial DNA was amplified by a duplex PCR procedure. The results showed that 40 enterotoxin producing *C. perfringens* strains generated two bands of amplicons with sizes of 420 and 280 bp, whereas 40 non-enterotoxin producing strains produced a single band of 280 bp on agarose gel-electrophoresis. No bands were observed from 32 strains of *Clostridium* spp and other bacteria. Southern blot analysis using either *cpe*-specific DNA or oligonucleotide probe showed hybridization specifically to the 420 bp band in enterotoxin-positive *C. perfringens*. On the dot blot membrane, both *cpe*-specific DNA and oligonucleotide probes were able to hybridize specifically with the corresponding DNA templates but with different efficacy (100% *vs* 91.1%).

INTRODUCTION

Clostridium perfringens is ubiquitously present throughout the natural environment. This organism is commonly encountered in soil, food, dust, intestinal tract and feces of man and domestic animals (Hobbs, 1979). Consequently, merely demonstrating the presence of this organism in food or feces is insufficient to establish *C. perfringens* as a cause of food-borne diseases (Granum, 1990; Fach and Popoff, 1997; Sparks *et al*, 2001). Laboratory identification of this organism has traditionally relied on bacteriological criteria, whose procedures are laborious and time-consuming. Immunological methods such as enzyme-linked immunosorbent assay (ELISA) and reversed passive-latex agglutination (RPLA) have subsequently been applied to quantitate such organisms (McClane and Strouse, 1984; Berry *et al*, 1988). However, these relatively more sensitive and specific techniques still have their difficulties. Firstly, antibodies specific for *C. perfringens* are not commercially available. Secondly, the reagent kit for *C. perfringens* determination is quite expensive. Thirdly, the immunological methods have an unacceptably high percentages of false-negative and false-positive results.

Recently, simpler, quicker, more sensitive and specific diagnostic techniques employing polymerase chain reaction (PCR) and DNA-hybridization have been developed. As with other studies corresponding to many fastidious bacteria, PCR and DNA-hybridization methods have been applied to investigate *C. perfringens* present in feces and food to indicate infection and food contamination (van Damme-Jongsten *et al*, 1990; Saito *et al*, 1992; Wang *et al*, 1994; Baez and Juneja, 1995; Tansuphasiri, 2001; Augustynowicz *et al*, 2002). Two genes designated as *plc* and *cpe* encoding for phospholipase C (PLC) and *C. perfringens* enterotoxin (CPE) synthesis have been studied extensively because they are responsible for the microbial pathogenesis (Rood, 1998; Sparks *et al*, 2001). Either simple or duplex PCR has been used to

Correspondence: Unchalee Tansuphasiri, Department of Microbiology, Faculty of Public Health, Mahidol University, 420/1 Rajvithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354 8528; Fax: 66 (0) 2354 8538
E-mail: unchalee@loxley.co.th

investigate these two genes derived from isolates, clinical specimens (feces) and food samples (Saito *et al*, 1992; Fach and Guillou, 1993; Augustynowicz *et al*, 2002). DNA and oligonucleotide probes have also been used successfully to detect the presence of *C. perfringens* containing both *plc* and *cpe* (van Damme-Jongsten *et al*, 1990; Baez and Juneja, 1995; Schlapp *et al*, 1995).

In this study, two DNA probes specific to *cpe*, one probe synthesized by duplex PCR from the enterotoxin-producing *C. perfringens* ATCC 12916 reference strain and a commercially synthesized oligonucleotide probe, were evaluated using two hybridization techniques, Southern blot and dot blot hybridization. These DNA probes were also used to demonstrate the presence of the virulence *C. perfringens* strains in both stock bacterial cultures and in fecal isolates from diarrhea patients.

MATERIALS AND METHODS

Bacterial strains

A total of 112 strains of bacteria comprising enterotoxigenic *C. perfringens* (n = 40), non-enterotoxigenic *C. perfringens* (n = 40), other *Clostridium* spp (n = 8) and other bacterial species (n = 24) used in this study are listed in Table 1. The reference enterotoxin producing strains were provided by Dr A Heikinheimo, Faculty of Veterinary Medicine, University of Helsinki; and some enterotoxigenic strains had previously been isolated from primary fecal cultures from diarrhea patients (Tansuphasiri *et al*, 2002). *C. perfringens* ATCC 12916 was used as a positive control for *cpe* (enterotoxin positive) and *C. perfringens* ATCC 27324 was used as a negative control (enterotoxin negative) in PCR and hybridization analysis. Other *Clostridium* species and other bacteria frequently associated with food or known to produce lecithinase (alpha toxin), of either standard strains or clinical isolates were stock cultures and were used as negative control strains for phospholipase C (PLC) or lecithinase activity. The identification of *C. perfringens* grown on tryptose-sulfite-cycloserine (TSC) agar (Merck) supplemented with 5% (w/v) egg yolk under anaerobic condition was confirmed by biochemical tests (Allen *et al*, 1999), and enterotoxin detection by RPLA with a commercial kit, PET-RPLA (Oxoid, Hampshire, England).

Isolation of total DNA

Overnight cultures of all bacterial strains were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8) to obtain cell concentration of approximately 10^8 cfu/ml by standardizing with McFarland 0.5 suspension. The bacterial suspension was centrifuged at 12,000*g* for 10 minutes before bacterial DNA was extracted using a silica membrane-based spin column (Tansuphasiri *et al*, 2004). Briefly, after resuspending the pellet in 180 μl of lysis buffer (20 mg/ml lysozyme, 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.2% Triton X) at 37ºC for 30 minutes, the sample was digested with proteinase K (final concentration of 5 mg/ml) at 56ºC for 30 minutes. Then, 200 μl of guanidinium buffer (4 M guanidine isothiocyanate, 50 mM Tris HCl pH 7.0, 20 mM EDTA) were added before incubation for 10 minutes at 70ºC, and then 210 μl of absolute ethanol were added and the solution mixed by vortexing. All the sample was applied to the silica spin column and centrifuged for 1 minute at 10,000*g*. The column was washed twice with 500 μl of washing buffer (50% ethanol containing 200 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4). DNA was eluted with 200 μl of prewarmed (70ºC) 2.5 mM Tris-HCl, pH 9. The concentration and the quality of the DNA were measured spectrometrically at 260 and 280 nm. One OD unit is equivalent to 50 μg of double stranded DNA per ml. Additionally, DNA was sized by electrophoresis through 0.8% agarose gel. The DNA solution was stored at -20ºC until used.

Duplex PCR

Duplex PCR to amplify *plc* and *cpe* was performed with two pairs of primers that had been developed and validated by Tansuphasiri (2001). PLC1 (5′-ATAGATACTCCATATCATCC TGCT-3′) and PLC2 (5′-TTACCTTTGCTGCAT AATCCC-3′), and CPE1 (5′-GAAAGATCTG TATCTACAACTGCT-3′) and CPE2 (5′-TAAGA TTCTATATTTTTGTCCAGT-3′) yield a 280-bp fragment for *plc* and a 420 bp fragment for *cpe* from enterotoxigenic *C. perfringens* isolates.

Table 1
Bacterial strains used in this study.

Organism and description	No.	Source or reference
Clostridium perfringens (n = 80)		
Enterotoxigenic strains (n = 40)		
C. perfringens ATCC 12916	1	American Type Culture Collection
C. perfringens NCTC 8198	1	National Culture Type Collection
C. perfringens NCTC 8239	1	National Culture Type Collection
C. perfringens clinical isolates	37	Clinical isolates from diarrhea patients at Bamrasnaradura Hospital
Non-enterotoxigenic strains (n = 40)		
C. perfringens ATCC 3624	1	American Type Culture Collection
C. perfringens ATCC 3628	1	American Type Culture Collection
C. perfringens ATCC 3629	1	American Type Culture Collection
C. perfringens ATCC 3631	1	American Type Culture Collection
C. perfringens ATCC 27324	1	American Type Culture Collection
C. perfringens ATCC 29348	1	American Type Culture Collection
C. perfringens ATCC 43402	1	American Type Culture Collection
C. perfringens clinical isolates	33	Clinical isolates from diarrhea patients
Other *Clostridium* spp (n = 8)		
C. botulinum	1	Food isolate
C. difficile	2	ATCC and clinical isolated strains
C. bifermentans	1	Clinical isolate
C. sporogenes	1	Clinical isolate
Clostridium spp (unidentified)	3	Isolates from environmental water
Other bacterial strains (n = 24)		
Staphylococcus aureus	4	ATCC 25923 and 3 clinical isolated strains
Salmonella spp	7	Clinical isolates from diarrhea patients
Escherichia coli	4	ATCC 25922 and 3 clinical isolated strains
Pseudomonas aeruginosa	3	ATCC 27853 and 2 clinical isolated strains
Bacillus cereus	1	ATCC strain
Bacillus thuringiensis	1	ATCC strain
Bacillus subtilis	1	ATCC strain
Proteus spp	1	Clinical isolate
Yersinia enterocolitica	1	Clinical isolate
Listeria monocytogenes	1	Food isolate

PCR was performed in a volume of 30 μl with 3 μl of 10X PCR buffer (1X buffer included 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 dNTP mix, 0.6 μl (25 pmoles) of each primer, 0.6 μl (0.6 U) of *Taq* polymerase (Promega), 10 μl of DNA template (1 ng), and distilled water to make a total volume of 30 μl. PCR was performed in an automated thermal cycler under the following conditions: 94ºC for 10 minutes, followed by 35 cycles consisting of 1 minute at 94ºC, 1 minute at 55ºC, 1 minute at 72ºC, and 1 cycle at 72ºC for 10 minutes. Amplified products were electrophoresed in a 2% agarose gel and stained with ethidium bromide and viewed under UV light. The size of each DNA band present in the gel was estimated by comparing with 100-bp DNA molecular size markers (Gibco, BRL).

Confirmation of enterotoxin gene PCR products by hybridization

Both DNA and oligonucleotide probes specific to *cpe* were employed in Southern and dot blot hybridization. The 20-bp oligonucleotide probe, CTTTCTGTAGCAGCAGCTAA was syn-

thesized by Biobasic, Canada. The CPE DNA probe was synthesized from the reference *C. perfringens* ATCC 12916 strain using duplex PCR with two sets of primers (PLC1/PLC2 and CPE1/CPE2). The amplicons of interest were then analyzed by agarose gel-electrophoresis (AGE). Only the 420-bp amplicon was isolated from the gel and purified by NucleoSpin® Extract column (Macherey-Nagel, Germany) and then used as the template for a second PCR with only CPE1/CPE2 primers. The second PCR product was then purified and labeled by random priming with fluorescein-dUTP, using the random Primer Fluorescein Labeling Kit (Renaissance®, Perkin Elmer Life Sciences, USA).

Southern blot hybridization (SBH)

After AGE, PCR products in the gel were transferred to a nylon membrane (Hybond N^+; Amersham) using downward capillary transfer (Southern, 1975). The protocol for hybridization followed the instructions in the Primer Fluorescein Labeling Kit (see above) using 1-hour prehybridization and a further 2-hour hybridization with fluorescein-labeled probe (final concentration, 20 ng/ml) at 55ºC. The membrane was washed successively with wash buffer 1 (2XSSC, 1.0% w/v SDS) at 55ºC for 15 minutes, followed with wash buffer 2 (0.2XSSC, 0.1% w/v SDS) at 55ºC for 15 minutes, and finally in rinsing buffer (1.5 M NaCl, 0.1 M Tris-HCl pH 7.5) for 5 minutes at room temperature. Immunological detection with antifluorescein-alkaline phosphatase conjugate and chemiluminescent detection with CDP Star® (Tropix, USA) substrate were performed as recommended by the manufacturer. Briefly, the membrane was incubated in blocking buffer [0.15 M NaCl, 0.10 M Tris-HCl pH 7.5, 0.5% (w/v) blocking reagent] at room temperature for 1 hour. Anti-fluorescein-alkaline phosphatase conjugate was then added and incubated for a further 1 hour and then washed with wash buffer (1.5 M NaCl, 0.10 M Tris-HCl pH 7.5) four times for 5 minutes each, followed by twice washing with washing buffer (0.1 M NaCl, 0.1 M Tris-HCl pH 9.5) for 5 minutes each. The membrane subsequently reacted with chemiluminescent substrate (CDP Star®, Tropix, USA). The reaction was observed after exposure to Kodak XAR5 photographic film for 1 to 4 minutes.

Dot blot hybridization (DBH)

To prepare the dot blots, 10 μl of amplified product was denatured by boiling in 240 μl of 6XSSC for 10 minutes and the denatured product was spotted onto a nylon membrane using a manifold system (Hybridot Manifold, Life Technologies; BRL). The membrane filter was air dried and fixed by UV cross-linker. The filter was then prehybridized, hybridized, washed, and immunologically detected with chemilunescent substrate as described above for the Southern procedure.

Sensitivity of detection

Ten-fold dilutions of purified *C. perfringens* DNA from both reference strain (ATCC 12916) and culture isolates were prepared in TE buffer to obtain the concentrations ranging from 0.01 fg to 100 ng per 10 μl, and used in the PCR. Serial 10-fold dilutions were also made from the same culture isolates. Colonies from blood agar plate were adjusted with TE buffer to equal the density of a 1 McFarland standard (~10^8 organisms per ml). The CFU of the original suspension per ml was estimated by growing on duplicate TSC-egg yolk agar, and colonies were counted after 24 hours of growth under anaerobic condition. Tubes containing ~10^8 to ~10^2 organisms per ml were placed in boiling water bath for 10 minutes, and quickly chilled on ice for 5 minutes. After centrifugation at 10,000*g* for 3 minutes, 10 μl of supernatant from each tube were used as template for 35 PCR amplification cycles and analyzed by gel- electrophoresis and hybridization. The highest dilution yielding an amplicon with 2 bands of 280 and 420 bp corresponding to *plc* and *cpe*, respectively, was accepted as an end-point for AGE analysis. Consequently, the number of colonies corresponding to such dilution was established as a detection limit of the assay.

Statistical analysis

Sensitivity and specificity of DNA hybridization methods to investigate the presence of enterotoxigenic strains of *C. perfringens* were compared with the one derived from PCR analysis. Statistical comparison was performed by using paired Student's *t* test and p-value of <0.05 is considered as significant.

RESULTS

The PCR products of *C. perfringens* amplified by both primer pairs (PLC and CPE primers) produced clearly visible bands with molecular size of 280 bp and 420 bp for *plc* and *cpe*, respectively (Fig 1). All *C. perfringens* non-enterotoxin producing strains showed the presence of 280 bp band only, whereas all *C. perfringens* enterotoxin producing strains showed the presence of both 280 bp and 420 bp bands. Other bacteria, including other *Clostridium* spp, showed no band. As shown in Table 2, all 40 enterotoxin-positive *C. perfringens* strains were positive by PCR (100% sensitivity), whereas all 72 strains of other bacteria, 40 strains of enterotoxin-negative *C. perfringens* strains and 32 strains of bacteria other than *C. perfringens*, showed no 420-bp band of *cpe* (100% specificity).

The PCR products from all 112 bacterial strains were denatured and dotted on nylon membrane and detected with either fluorescein-labeled oligonucleotide probe or synthetic DNA probe. The films from DBH showed clearly visible dark spots for all *C. perfringens* enterotoxin producing strains with any nonspecific reaction when using CPE DNA probe (Fig 2). Some nonspecific reactions occurred when using the oligonucleotide probe tested with some bacterial strains including *C. perfringens* enterotoxin-negative and bacteria other than *C. perfringens* (data not shown). Hybridization with CPE DNA probe also showed nonspecific faint dark spots with those strains if undiluted PCR products were dotted. However, if diluted PCR products (1:10) were dotted on nylon membrane, these nonspecific spots were not seen. As shown in Table 2, all 40 enterotoxin-positive *C. perfringens* strains were positive by PCR with detection by DBH with either oligonucleotide probe or CPE DNA probe (100% sensitivity); however 10 of 72 enterotoxin-negative strains, including enterotoxin-negative *C. perfringens* strains (n = 3) and other bacterial species (n = 7), showed positive spots when hybridized with oligonucleotide probe (86.1% specificity), but none of enterotoxin-negative bacteria showed positive spots with CPE DNA probe (100% specificity).

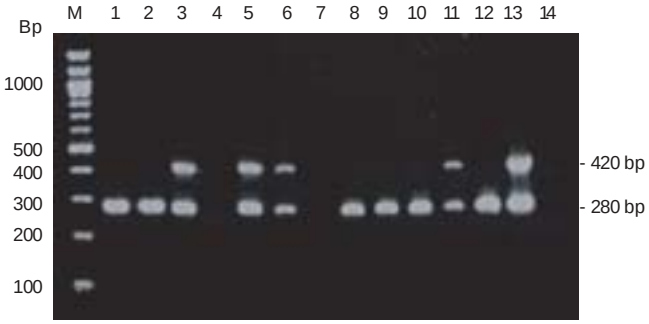

Fig 1–Agarose gel electrophosresis of duplex PCR amplification of enterotoxin and non-enterotoxin producing *C. perfringens.* Lane M contained DNA size marker, 100 bp DNA ladder. Lanes 3, 5, 6, and 11 contained DNA from enterotoxin producing *C. perfringens* strains. Lanes 1, 2, 8, 9 and 10 contained DNA from non-enterotoxin producing *C. perfringens* strains. Lanes 4 and 7 contained DNA from *Clostridium botulinum* and *E. coli*, respectively. Lanes 12 and 13 contained DNA of *C. perfringens* ATCC 27324 and ATCC 12916 as positive control for enterotoxin-negative and enterotoxin-positive, respectively. Lane 14 contained nuclease free water.

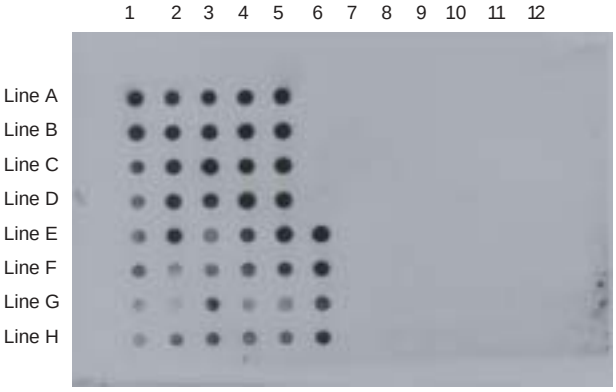

Fig 2–Dot blot hybridization with the fluorescein-labeled 420-bp amplicon of *C. perfringens* ATCC 12916 *cpe*. Lane 6 shows 2 *C. perfringens* positive controls in duplicate (E6 and F6: ATCC 12916; G6 and H6: NCTC 8239). All the other positive signals represent *C. perfringens* enterotoxin producing isolates. In contrast, no signals were visible with any of the negative controls (positions A6 and B6: *C. botulinum;* C6 and D6: *E. coli* ATCC 25922) other bacterial strains: position A7 to A12, B7 to B12, C7 to C12, D7 to D12, E7 to E12, F7 to F12, G7 to G12, and H7 to H12.

Table 2
Detection of enterotoxin (*cpe*) gene by PCR and analysis of PCR products by AGE, DBH and SBH using either oligonucleotide probe or synthetic DNA probe, compared with conventional identification of enterotoxin producing *C. perfringens.*

PCR product detection	No. of strains with conventional identification result [a] for enterotoxin detection :		Sensitivity (%)	Specificity (%)	Efficiency (%)
	Positive	Negative			
PCR-AGE[b]			100	100	100
Positive	40	0			
Negative	0	72			
PCR-DBH[c]					
using oligonucleotide probe			100	86.1	91.1
Positive	40	10			
Negative	0	62			
using synthetic DNA probe			100	100	100
Positive	40	0			
Negative	0	72			
PCR-SBH [d]					
using oligonucleotide probe			100	100	100
Positive	40	0			
Negative	0	72			
using synthetic DNA probe			100	100	100
Positive	40	0			
Negative	0	72			

[a] Using conventional culture, biochemical identification, and RPLA test for enterotoxin producing strains, as the "gold standard".
[b]Duplex PCR with amplicon detection by agarose gel electrophoresis (AGE).
[c]Duplex PCR with amplicon detection by dot blot hybridization (DBH).
[d]Duplex PCR with amplicon detection by Southern blot hybridization (SBH).

The PCR products on agarose gel were Southern transfered onto nylon membrane and detected with fluorescein labeled oligonucleotide and CPE DNA probes. The films obtained from DNA hybridization with both probes showed clearly bands at the positions corresponding to 420-bp *cpe* product only, without bands at 280-bp *plc* product (Fig 3). As shown in Table 2, all 40 enterotoxin positive *C. perfringens* strains were positive by PCR with detection by SBH with either oligonucleotide probe or CPE DNA probe (100% sensitivity). No signals were visible with any of the enterotoxin negative bacteria when tested with either probe, thus yielding 100% specificity.

The limits of detection by the three methods were compared to the number of colony growth on TSC-egg yolk agar, and the highest dilution yielding an amplicon with 2 bands of 280 and 420 bp corresponding to *plc* and *cpe*, respectively, was accepted as an end-point for AGE analysis. The sensitivity of PCR with detection by AGE, DBH, and SBH was 40, 0.4 and 0.4 cfu/10 μl, respectively (Fig 4). In addition, the smallest amount of purified *C. perfringens* DNA detectable by PCR with AGE was 1 pg and by hybridization (both DBH and SBH) was 10 fg (data not shown).

DISCUSSION

C. perfringens possesses several characteristics that significantly contribute to its pathogenicity as a food-borne disease. Firstly, *C.*

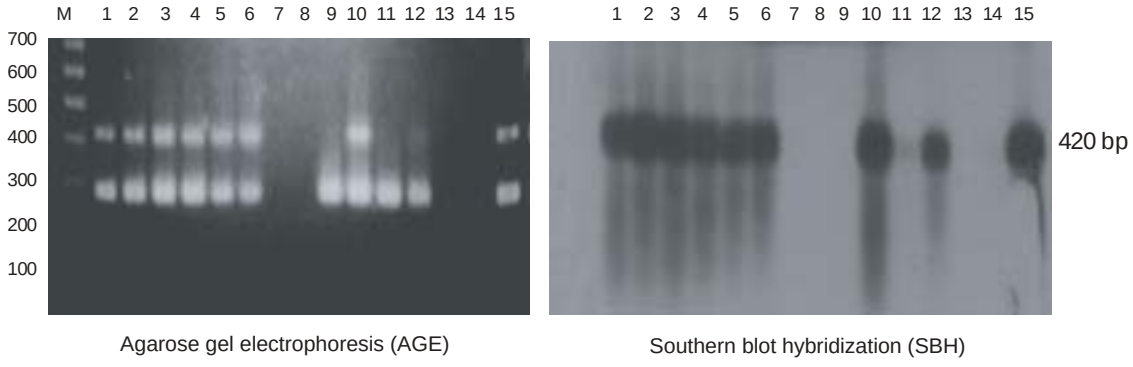

Fig 3–AGE and SBH of duplex PCR products from enterotoxigenic *C. perfringens.* The PCR products were analyzed on agarose gel (left), and by Southern blot hybridization (right). The PCR products from the agarose gel were immobilized on the nylon membrane and probed with CPE DNA probe, then detected by CDP Star® chemiluminescence detection reagent. Lane M, DNA size marker, 100-bp DNA ladder; lanes 1-6, 10, and 12, enterotoxin producing *C. perfringens* strains; lanes 9 and 11, non-enterotoxin producing *C. perfringens* strains; lanes 7, 8 and 13, other *Clostridium* spp and bacteria; lane 14, deionized water as negative reagent control; lane 15, *C. perfringens* ATCC 12916 as *cpe* control.

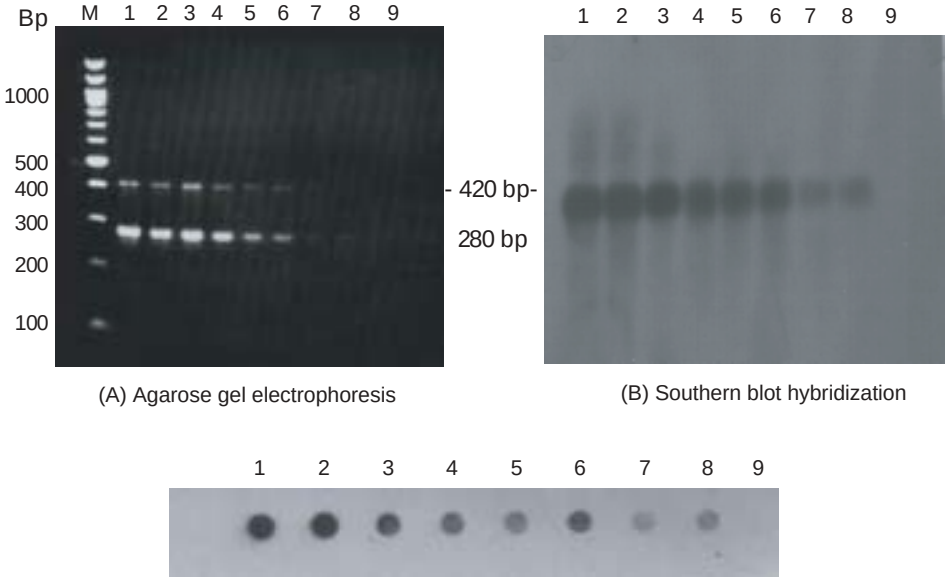

Fig 4–Limit of PCR detection by agarose gel electrophoresis (A), Southern blot hybridization (B), and dot blot hybridization (C). Ten-fold dilutions were made from *C. perfringens* ATCC 12916 colonies and the number of organisms from each dilution determined by growing on TSC-egg yolk agar. Lane M, molecular size marker, 100-bp ladder. Lanes 1 to 8; DNA extracted from $4x10^6$, $4x10^5$, $4x10^4$, $4x10^3$, $4x10^2$, 40, 4, 0.4 cfu/10 μl/ lane, respectively. Lane 9, negative reagent control.

perfirngens vegetative cells can double in such a short time as 10 minutes allowing the organism to multiply rapidly in food. Secondly, spore formation renders the organism highly resistant to environmental stresses including radiation, desiccation and heat. Thirdly, the higher tolerance against air exposure is observed, compared to other anaerobes. These allow the organism

to survive even in any inappropriate conditions (McClane, 2001).

Besides those characteristics, *C. perfringens* contains the ability to generate several pathogenic toxins such as α-toxin (phospholipase C, PLC) and *C. perfringens* enterotoxin (CPE) (Rood, 1998). PLC, the common protein-toxin decoded from *plc* and produced in all types of *C. perfringens* (Fach and Guillou, 1993), exhibits enzymatic, lethal, hemolytic and necrotic activities (Rood and Cole, 1991). Therefore, PLC is considered as a species-specific signal indicating the exposure to *C. perfringens*. The other toxin known as CPE, a single 35-kDa polypeptide product from *cpe* mainly found during sporulation of type A strain, is believed to play a key role in its pathogenesis (Czeczulin *et al*, 1993). This heat-stable enterotoxin can cause many pathological effects including water and electrolyte losses (diarrhea) and tissue damage in gastrointestinal tract of a number of mammalian species (Songer, 1996).

As with previous studies on microorganisms, traditional bacteriological method of cultivation and identification was employed primarily to identify all stock bacterial strains used in this study. Both enterotoxin and non-enterotoxin producing strains of *C. perfringens* were re-investigated for their identities before being used as origins for DNA extraction. The virulent *C. perfringens* strains was distinctive from the non-virulent ones by the ability to produce *C. perfringens* enterotoxin (CPE) as tested by latex agglutination using specific antibody (RPLA) (data not shown).

The DNA probe specific for *cpe* of enterotoxin producing strains of *C. perfringens* (420 bp in length) used in this study was established by Tansuphasiri (2001). Both CPE DNA and commercially synthesized oligonucleotide probes specific to *cpe* were evaluated using Southern and dot blot hybridization methods. To increase the sensitivity of DNA hybridization technique, the amount of bacterial DNA was amplified using duplex PCR procedure with dual set of primers of CPE1/CPE2 and PLC1/PLC2 corresponding to *cpe* and *plc*, respectively. The results of amplicon detection by AGE agreed with those reported previously (Tansuphasiri, 2001).

Both *cpe*-specific probes clearly recognized the 420-bp amplicon derived from the DNA extracts of enterotoxin-positive *C. perfringens* and showed no cross-reactivity with the extracts from enterotoxin-negative *C. perfringens* strains and the other bacteria. This result illustrates that the *cpe*-specific DNA and oligonucleotide probes could be employed successfully to differentiate virulent strains of *C. perfringens* from the non-virulent and the other bacterial strains through SBH. SBH analysis using either *cpe*-specific DNA or oligonucleotide probe has provided excellent sensitivity (100%) and specificity (100%) in comparison to the conventional method employed in most microbiology studies. Nevertheless, the SBH is relatively complicated and time consuming since it is comprised of several processes such as AGE, transferring, blotting and detecting. Thus, the simpler DNA dot blot hybridization was developed to render the identification of enterotoxin-positive *C. perfringens* strains easier and more rapid.

Interestingly, both *cpe*-specific DNA and oligonucleotide probes were able to hybridize specifically with corresponding DNA templates contained on the dot blot membrane but with different efficacy. Using the *cpe*-specific DNA probe, the DBH technique has provided an efficacy as good as SBH analysis. Moreover, no reactivity was found when enterotoxin-negative *Clostridium* species and other bacteria were tested. On the other hand, when the amplicon was probed with *cpe*-specific oligonucleotide, in addition to the strong hybridization reactivity obtained from all undiluted amplicons of enterotoxin-positive *C. perfringens*, some weak reactivity were visualized from amplicons of enterotoxin-negative *C. perfringens* and other bacteria tested. Such cross-reactivity seems to be the consequence of cross binding between this rather short (20 bp) synthetic oligonucleotide probe and partial complementary nucleotide sequences possibly present among the bacterial strains. Nevertheless, such cross-reactivity was reduced or eliminated completely when the diluted test-samples were used. As a result, *cpe*-specific oligonucleotide in conjunction with DBH differentiate virulent strains of *C. perfringens* from the others, including the non-virulent strains and

other bacteria, with sensitivity and specificity of 100% and 86%, respectively.

The lowest amount of DNA from enterotoxin-positive *C. perfringens* strains that could be detected by *cpe*-specific DNA probe analysis via SBH and DBH was 10 fg per 10 μl whereas a level of 1 pg per 10 μl was detected for simple AGE technique, equivalent to 0.4 and 40 organisms per reaction, results similar to previous report (Tansuphasiri, 2001). It illustrates that the *cpe*-specific DNA probe employed in both SBH and DBH methods should become a confirmatory method for determination of enterotoxin-positive *C. perfringens* strains, compared to the simple-AGE method. Theoretically, DNA probe analysis provides higher sensitivity and specificity than AGE. This may be the consequence of (a) interfering substances contained in samples and/or (b) DNA loaded on the gel may be lower than the detection limit of ethidium bromide staining. However, among these three molecular-based methods, no statistical difference in terms of sensitivity and specificity was found in this study. This may be that the amount of bacterial DNA (420 bp) applied in these methods was much higher than the detection limits of these assays. For diagnostic aspect, besides sensitivity and specificity, efficacy of the test is another important factor to be taken into consideration. Both DNA probe analysis and AGE have provided an excellent efficacy compared to the gold standard method. Consequently, this CPE DNA probe will render the investigation for the presence of virulent *C. perfringens* strains simpler, faster, and highly accurate.

This synthetic DNA probe was also used to confirm the specificity of the 420-bp *cpe* amplicon in enterotoxigenic *C. perfringens* obtained directly from fecal specimens of diarrhea patients (Tansuphasiri *et al*, unpublished). In this study, 7 of 121 samples gave positive PCR results for both *plc* and *cpe* on agarose gel. After Southern transfer and hybridized with this CPE DNA probe, all PCR products of these 7 samples showed positive signals (clearly visible dark bands) on nylon membrane at the position corresponding to 420 bp of *cpe* product.

In summary, this *cpe*-specific DNA probe was utilized successfully via the highly sensitive and specific technique of SBH and DBH for identification of enterotoxin producing *C. perfringens* from pure cultures. In the future, this CPE DNA probe will be tested for its ability to indicate the presence of virulent *C. perfringens* in clinical specimens and in contaminated food samples directly with or without DNA amplification. However, in clinical investigations, the appearance of enterotoxin-positive *C. perfringens* is not the only criterion to rule out pathology in patients caused by this organism. The quantity detected is much more important and useful in terms of interpretation, and consequently, besides qualitative investigation, quantitative assessment of this saprophytic organism is necessary and required.

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