

## Detection of enterotoxigenic *Clostridium perfringens* with a duplex PCR

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**Two sets of primers designed to detect *Clostridium perfringens* phospholipase C (*plc*) and enterotoxin (*cpe*) genes in a single PCR reaction were applied to a collection of 64 predominantly food poisoning-related *C. perfringens* isolates. In-vitro enterotoxin synthesis was tested serologically after inducing sporulation. Of the 64 isolates, 26 were clearly enterotoxigenic; 16 were classified as potentially enterotoxigenic only as serological testing did not confirm enterotoxin production. Duplex PCR for diagnosis of enterotoxigenic *C. perfringens* from vegetative cultures can be a useful tool as fresh isolates often sporulate poorly or not at all, giving rise to the possibility of false negative results by serological analysis.**

### Introduction

*Clostridium perfringens* type A produces an enterotoxin (CPE) and can cause food poisoning outbreaks with diarrhoea and severe abdominal pain related to the consumption of food [1, 2]. Several hundred cases of food poisoning associated with *C. perfringens* type A occur in the USA and Europe every year [3]. CPE induces clinical symptoms *in vivo* within 15–30 min of delivery of purified toxin [4]. In man, symptoms develop when  $>5 \times 10^8$  viable enterotoxigenic vegetative cells of *C. perfringens* reach the digestive system [1]. Detection of *C. perfringens* in faeces and consumed food, together with the identification of a common serotype, is widely applied for outbreak characterisation. However, this approach is not always sufficient to completely describe an outbreak. The classical approach to *C. perfringens* food poisoning involves the detection of  $>10^6$  bacterial cells/g in faecal samples or serological detection of CPE after inducing sporulation of an isolate, or both [5]. Characterisation of enterotoxigenic *C. perfringens* isolates can be difficult as the ability to sporulate *in vitro* can vary with different media [6]. Moreover, CPE synthesis can occur in non-sporulating cultures of *C. perfringens* [3]. Detection of enterotoxin by immunoenzymic methods, e.g., immunoelectrophoresis, latex agglutination, immunodiffusion, ELISA, Western blot or reverse passive latex agglutination (RPLA) requires relatively large amounts of CPE in a sample and has many interpretation problems [5].

Therefore, new diagnostic approaches are required for confirmation of food poisoning with *C. perfringens*. One option may be the identification by PCR or hybridisation methods [6–12] of genes encoding information on CPE and other toxins.

This study aimed to identify enterotoxigenic isolates of *C. perfringens* by amplification of fragments of genes encoding phospholipase C (*plc*) and enterotoxin (*cpe*) in a single PCR and to compare enterotoxin gene detection with CPE protein detection by serological means.

### Materials and methods

#### Bacterial strains

A set of lyophilised isolates of *C. perfringens* collected during the period 1954–1999 at the Department of Sera and Vaccines Evaluation, National Institute of Hygiene, Warsaw, Poland, was used for the study. The collection consisted of 64 type A strains associated mainly with food poisoning cases: 30 from stool samples, 10 from gastric lavage, 20 from food samples and 4 from wound biopsies.

Isolates were cultured on Merck *C. perfringens* selective agar plates and SPS agar (Merck) in anaerobic jars containing H<sub>2</sub> 95% and CO<sub>2</sub> 5%.

Seven toxin-typed reference strains of *C. perfringens*, type A strains CN3352 *cpe*<sup>-</sup> and CN3418 *cpe*<sup>+</sup>, type B strain ATCC 3626, type C strain CN5386, type D strain CN1183 and type E strain NCTC 8084, were used as controls. The specificity of the PCR was tested

with several isolates belonging to the genus *Clostridium* *C. difficile*, *C. bifermentans*, *C. novyi*, *C. histolyticum*, *C. septicum*, *C. sordelli*, *C. fallax*, *C. capitale*, *C. oedematiens* and isolates belonging to other genera (*Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Bacillus cereus*). A further 24 isolates of *C. perfringens* belonging to types B, C, D or E were analysed.

#### Identification of *C. perfringens*

*C. perfringens* isolates were identified by Gram's stain, urease and lecithinase production, and other biochemical tests as described previously [10]. Toxinotypes of *C. perfringens* isolates were determined by neutralisation of lethality in mice.

#### Enterotoxin detection by RPLA

For testing of enterotoxin production, isolates were grown at 37°C for 24 h in the sporulation medium of Ellner [13], before being heated at 75°C for 20 min. Then, 16 ml of fresh Ellner medium were inoculated with 0.8 ml of culture (taken from base of the tube) and incubated at 37°C for 24 h. After incubation, samples were centrifuged at 900 *g* for 20 min at 4°C and the supernate was used in a PET-RPLA test (Oxoid) according to the manufacturer's instructions.

#### Isolation of DNA

Overnight *C. perfringens* cultures on SPS agar were suspended in TE buffer 10 mM Tris; 1 mM EDTA then centrifuged at 6000 *g* for 10 min, the pellet was resuspended in 567  $\mu$ l of TE buffer and incubated for 1 h at 37°C. After adding 30  $\mu$ l of SDS 10% and 3  $\mu$ l of proteinase K (20 mg/ml), samples were incubated at 37°C for 1 h and then 100  $\mu$ l of 5 M NaCl and 80  $\mu$ l of CTAB-NaCl solution (cetyl trimethyl ammonium bromide 10% in 0.7 M NaCl) were added before incubation for 10 min at 65°C. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) solution and precipitated with absolute ethanol overnight at -20°C. The DNA was harvested by centrifugation at 12 000 *g* for 20 min, washed with ethanol 70% and dissolved in 100  $\mu$ l of TE buffer. The concentration and the quality of the DNA were measured spectrophotometrically at 260 and 280 nm. DNA from species other than *C. perfringens* was extracted with the QIAamp Mini Kit (Qiagen).

#### Amplification

PCR to detect *plc* and *cpe* genes was performed with primers that had been developed and validated by Fach and Popoff [9]. PL3-5' AAGTTACCTTTGCTGCAT AATCCC 3' PL7-5' ATAGATACTCCATATCATCC TGCT 3' and PL145-5' GAAAGATCTGTATCTACA ACTGCTGGTCC 3', PL146-5' GCTGGCTAAGAT TCTATATTTTGTGTC CAGT 3' yield a 283-bp frag-

ment for the *plc* gene and a 426-bp fragment for the *cpe* gene in enterotoxigenic *C. perfringens* isolates. PCR was performed in a 50- $\mu$ l reaction volume. The mixture contained 1  $\times$  buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; gelatin 0.001% 2.5 mM MgCl<sub>2</sub>; 100  $\mu$ M each dNTP (Gibco); 20 pmol of primers (synthesised by Universal DNA., USA); Taq polymerase (Biometra) 1.25 U and template DNA 100 ng. PCR was performed with the following conditions: 30 s at 94°C, followed by 30 cycles consisting of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C.

#### PCR sensitivity

Sensitivity of the PCR reaction was confirmed with a reference strain (*C. perfringens* CN3352) cultured in Reinforced Clostridial Broth (RCM, Merck). Ten-fold dilutions of culture were subjected to DNA extraction by the phenol method. Viable counts were done at the same time, and the lowest number of bacterial cells that could be detected by PCR was determined.

#### Detection of PCR products

Amplified PCR products were electrophoresed in agarose gel containing ethidium bromide 0.5  $\mu$ g/ml, observed under UV illumination and photographed with a Polaroid camera. Precise band size was determined densitometrically (Gel Doc 1000 Gel Documentation System, BioRad).

## Results

Initially, the specificity of primers designed to amplify the *plc* and *cpe* genes was investigated with several clostridial and non-clostridial species previously associated with food poisoning. Both the *plc* and *cpe* gene fragments were amplified in the duplex PCR with DNA extracted from control *cpe*<sup>+</sup> *C. perfringens* type A CN3418, a known enterotoxin producer. For CN3352, a *cpe*<sup>-</sup> known non-CPE producer, the 283-bp fragment of *plc* gene was present; however, the 426-bp fragment of the *cpe* gene was not. Similarly, in 24 *C. perfringens* isolates of B, C, D or E types, *cpe* gene fragments were not observed, while in every case the *plc* amplicon was seen. No cross-reaction with other *Clostridium* species or with other bacteria was observed. The sensitivity of PCR with both sets of primers, PL3/PL7 (*plc* gene) and PL145/PL146 (*cpe* gene), used in combination, was 10–20 bacterial cells from RCM.

Sporulation was seen in 63 of the 64 strains tested. All strains that sporulated successfully were subsequently tested for in-vitro CPE production by RPLA. A comparison of duplex PCR and RPLA results is presented in Table 1. Amplification of the *plc* gene encoding phospholipase C was successful for all 64 *C. perfringens* strains tested. In 42 (66%) of the 64 strains the 426-bp fragment of the *cpe* gene encoding

**Table 1.** A comparison of detection of *plc* and *cpe* genes by PCR and detection of enterotoxin by RPLA in *C. perfringens* strains\*

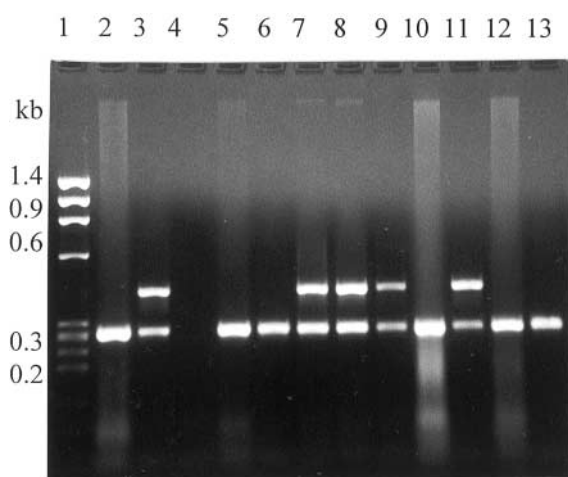
Strain source	Number of strains tested	Number (%) of strains that were		
		<i>plc</i> + <i>cpe</i> - CPE-	<i>plc</i> + <i>cpe</i> + CPE+	<i>plc</i> + <i>cpe</i> + CPE-
Stool samples	30	7	16	7
Gastric lavage	10	5	3	2
Food samples	20	8	6	6
Wounds	3	1	1	1
Total	63	21 (33%)	26 (41%)	16 (25%)

\*For a single non-sporulating strain isolated from a wound, a profile of *plc*(+)*cpe* (-) was obtained.

enterotoxin was amplified. Representative results for *plc* and *cpe* gene identification by duplex PCR are presented in Fig. 1.

## Discussion

*C. perfringens* type A strains are frequently isolated from the environment and from the digestive tract of man and animals. They can be a cause of food poisoning outbreaks when they are enterotoxin producers [1, 3]. There is a correlation between CPE synthesis and spore formation, and this is the basis for enterotoxigenic and non-enterotoxigenic strain differentiation [1]. However, there are some limitations to inducing sporulation of *C. perfringens* isolates *in vitro* on different media [3, 6, 10]. Sequencing of the genes encoding synthesis of phospholipase C and enterotoxin has enabled the introduction of a PCR for the identification and typing of *C. perfringens* strains [8–10, 14, 15]. Direct isolation of DNA from vegetative *C. perfringens* cells eliminates the need for *C. perfringens* isolates to sporulate in order to obtain toxin in quantities needed for serological tests [16].



**Fig. 1.** Results of *plc* and *cpe* gene amplification (duplex PCR) in DNA extracted from *C. perfringens* strains isolated from stool samples of patients with food poisoning. **1**,  $\phi$ X174 RFI DNA/HaeIII marker; **2**, *C. perfringens* CN3352 *cpe*- control strain; **3**, *C. perfringens* CN3418 *cpe*+ control strain; **4**, negative PCR control; **5**, **6**, **10**, **12**, **13**, *C. perfringens* *plc*+ strains; **7**, **8**, **9**, **11**, *C. perfringens* *plc*+, *cpe*+ strains.

Toxin detection and typing of *C. perfringens* by multiplex PCR in stool or food samples or culture supernates was first described by Miwa *et al.* [7] and Meer and Songer [17]. In the present study, two sets of primers applied in a duplex PCR enabled the identification of enterotoxigenic *C. perfringens*. One pair of primers designed for the phospholipase C gene (*plc*) provided specific identification of *C. perfringens* as the *plc* gene is located on the chromosome of all *C. perfringens* types [18]. A second pair of primers was derived from the *cpe* gene, which is also chromosomally located in *C. perfringens* *cpe*+ isolates from human clinical material [19]. For DNA extracted from *C. perfringens* RCM cultures, the sensitivity was 10–20 bacterial cells. Yoo *et al.* [19] were able to detect toxin genes with a sensitivity of 10 pg of DNA. Fach and Popoff [9] showed that PCR could detect the enterotoxin gene in clinical material, food and samples from the environment with a sensitivity of 10 bacterial cells, sufficient for routine food testing.

van Damme-Jongsten *et al.* [11] reported that only 6% of *C. perfringens* isolates from a wide variety of sources contained the *cpe* gene. However, the *cpe* gene was found in 59% of isolates from food samples or stools from patients with food poisoning (66% in food samples and 57% in stool samples) [6]. The present study detected the *cpe* gene by PCR in 70% of *C. perfringens* isolates from food poisoning cases. Previous serological studies have shown that only sporulating *C. perfringens* type A are capable of producing CPE [6, 20, 21]. The expression of *cpe* mRNA and CPE protein synthesis was strongly blocked in cells in the vegetative stage of growth, increasing up to 1500 times after starting the sporulation process [20, 22]. The present study could not confirm the recently published evidence of enterotoxin synthesis by *C. perfringens* types C and D [9, 19], and the *cpe* gene was seen only with *C. perfringens* type A [23].

van Damme-Jongsten *et al.* [6] showed that 19 of 20 *C. perfringens* isolates with the *cpe* gene produced CPE protein *in vitro*. In the present study, only 26 of 42 isolates containing the *cpe* gene produced CPE detectable by RPLA. These differences may reflect low levels of toxin produced or low RPLA sensitivity. However, *in-vitro* sporulation for CPE for identification

of enterotoxigenic *C. perfringens* isolates may not be reliable. Although there is evidence that 40% of even fresh *cpe*<sup>+</sup> *C. perfringens* isolates fail to sporulate in different growth conditions, only one strain of the 64 isolates in the present study did not sporulate *in vitro* in Ellner's medium. However, serological examination showed that only 35% of isolates analysed were able to synthesise CPE toxin [5]. Thus, the 26 *cpe*<sup>+</sup> CPE<sup>+</sup> *C. perfringens* isolates were clearly causes of enterotoxic infection.

The 16 *cpe*<sup>+</sup> CPE<sup>-</sup> sporulating isolates are likely to be enterotoxigenic because they were isolated from patients with food poisoning and diarrhoea, or implicated food, or both. Negative results by RPLA could be false negatives as a result of too low a level of CPE synthesis. Furthermore, not all *C. perfringens* type A isolates induce a lethal effect in mice, suggesting different enterotoxin production levels, or a lack of enterotoxin in some strains [23]. The differences in clinical presentation seen in cases of *C. perfringens* type A infection may be related to different levels of enterotoxin production [24].

It is possible that *cpe*<sup>+</sup> CPE<sup>-</sup> isolates may represent 'false positives'. The isolates may not be producing the regulatory factors required for CPE expression. Therefore, such strains should rather be referred to only as potentially enterotoxigenic, unless CPE production can be confirmed. It is also possible that *cpe*<sup>+</sup> CPE<sup>-</sup> sporulating or non-sporulating isolates are normal flora of the gut. The detection of *cpe*<sup>+</sup> CPE<sup>-</sup> isolates, especially those that are non-sporulating, should be interpreted with caution [19].

Nineteen *plc*<sup>+</sup> *cpe*<sup>-</sup> CPE<sup>-</sup> isolates were classified as a 'not enterotoxigenic' cause of food poisoning. Because *C. perfringens* is frequently distributed in the environment and present in the intestinal tract of man, food and faeces may be easily contaminated with different enterotoxin-producing strains.

In summary, compared to RPLA, PCR is a useful technique for analysis of strains suspected of being the cause of food poisoning. However, enterotoxigenicity should still be confirmed by *in-vitro* CPE detection, if possible, after gene identification. Duplex PCR is highly specific for *C. perfringens* and is able to discriminate potentially enterotoxigenic strains. Its sensitivity allows for direct application to clinical material and food samples and it can easily be applied to screen strains/clinical material before serological examinations to exclude isolates lacking the *cpe* gene which are not potentially enterotoxigenic.

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