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Development and application of a multiplex PCR assay for detection of the *Clostridium perfringens* enterotoxin-encoding genes *cpe* and *becAB*

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ABSTRACT

Clostridium perfringens causes food-borne gastroenteritis following the consumption of contaminated food by producing *C. perfringens* enterotoxin (CPE) in the intestines. Recently, we reported a novel enterotoxin, binary enterotoxin of *C. perfringens* (BEC) in *C. perfringens* isolates, which caused two disease outbreaks in Japan. Consequently, in the event of food poisoning outbreaks caused by *C. perfringens*, it is now necessary to screen for both the *cpe* and *becAB* genes by diagnostic PCR. Here, we present a simple multiplex PCR method for simultaneous detection of *cpe*, *becAB* and a *C. perfringens* control locus, phospholipase C (*plc*). Applying this method, we investigated the prevalence of *cpe*- or *becAB*-carrying *C. perfringens* strains in human stool and bovine rectum swab samples. Using a total of 169 isolates, we found that the percentage of *becAB*-carrying strains was very small (0.59%), one-tenth that of *cpe*-carrying strains. The simple method presented in this study with high specificity and sensitivity to *C. perfringens* will be a useful tool to survey the global prevalence of *becAB*-carrying *C. perfringens* strains.

1. Introduction

Clostridium perfringens is a spore-forming anaerobic rod bacterium found in the normal intestinal flora of humans and other animals, in soil, and as a component of sewage microbiota (Li et al., 2007; Mueller-Spitz et al., 2010; Miyamoto et al., 2011). *C. perfringens* causes various human diseases including gas gangrene and food-borne gastroenteritis (Kitterer et al., 2014; Stevens et al., 1987; Niilo, 1971; Duncan and Strong, 1971). Enterotoxigenic *C. perfringens* produces *C. perfringens* enterotoxin (CPE), which is an important virulence factor in food-borne gastroenteritis outbreaks (Sarker et al., 1999). After consumption of food contaminated by CPE-producing *C. perfringens*, the microbe enters the small intestine, sporulates and produces CPE. CPE forms pores in epithelial cell membranes, leading to diarrhea (Briggs et al., 2011; Smedley et al., 2007).

Recently, food-borne gastroenteritis outbreaks have occurred in Japan that have been caused by CPE-non-producing *C. perfringens*. Roast beef is suspected to have been the contaminated food. We identified a binary toxin, designated BEC (binary enterotoxin of *C. perfringens*), as the novel virulence factor causing this gastroenteritis (Yonogi et al., 2014; Stiles et al., 2014). BEC is composed of BECa and BECb, and is classified as an ADP-ribosylating binary toxin, a group that also includes *C. perfringens* iota-toxin, *Clostridium spiroforme* CST toxin (*C. spiroforme* toxin), *Clostridium difficile* CDT (*C. difficile* transferase) and *Clostridium botulinum* C2 toxin (Barth et al., 2004). In analyses of both components of the binary toxin, we found that BECb alone has fluid-accumulating activity, whereas BECa alone does not, but instead functions to enhance the activity of BECb.

Discovery of this novel enterotoxin led us to modify the diagnostic protocol for enterotoxigenic *C. perfringens* in the event of food-borne gastroenteritis outbreaks. In clinical PCR screening it is now necessary to target not only the *cpe* gene, but also the *becAB* genes. Here, we present a simple multiplex PCR method for simultaneously detecting the *cpe*, *becAB* and *plc* genes (the latter as an internal control) from isolates. The primers used in this study were already reported previously (Yonogi et al., 2014; Erol et al., 2008; Meer and Songer, 1997), but combining these primers in a single multiplex assay has not been done before. Applying this method, we investigated the prevalence of *cpe*- and *becAB*-carrying *C. perfringens* strains in human stool and bovine rectum swab samples, which are speculated to have been sources of contamination in the two aforementioned outbreaks.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

C. perfringens strains NCTC8239 (Li et al., 2007) and OS1 (Yonogi et al., 2014) were used for sensitivity tests of the multiplex PCR assay. To

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test the specificity of the assay, four groups of bacterial strains were used: Group A, comprising *C. perfringens* strains (n = 46); Group B, comprising Clostridiaceae strains except *C. perfringens* (n = 21); Group C, comprising Gram-positive bacterial strains except Clostridiaceae (n = 53); and Group D, comprising Gram-negative bacterial strains (n = 92). The strains used are summarized in Table S1.

Each strain was grown on an appropriate agar medium, either blood agar medium (BD Diagnostic Systems; Sparks, MD, USA) or trypticase soy agar medium (BD Diagnostic Systems) at 37 °C overnight. Anaerobic bacteria were cultured under anaerobic conditions with AnaeroPack-Anaero (Mitsubishi Gas Chemical CO., INC.; Tokyo, Japan). When a strain did not grow well, culture was prolonged until the strain grew sufficiently. After culture, DNA was extracted as described previously (Yamazaki et al., 2009, 2010). In brief, a loopful (1 μl) of bacterial cells was suspended in 50 μl of 25 mM NaOH. Cell suspensions were heated at 95 °C for 5 min, then neutralized with 4 μl of Tris–HCl buffer (1 mol l^{-1}, pH 7.5). After centrifugation at 15,000 × *g* for 5 min, supernatants were used as the template for PCR.

2.2. Formulation of multiplex PCR assay for enterotoxin typing

Multiplex PCR was performed using the QIAGEN Multiplex PCR *Plus* Kit (Qiagen; Hilden, Germany). Four primer pairs were used to amplify the *cpe*, *becA*, *becB* and *plc* genes (sequences listed in Table 1). PCR was performed in a 25-μl reaction mixture containing 12.5 μl of 2 × Multiplex PCR Master Mix (Qiagen), 0.4 μM primers for the *plc* gene and *cpe* genes, 0.2 μM primers for *becAB* genes, and 1 μl of template solution (at least 10 pg DNA/25 μl). The following PCR conditions were used: denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s, extension at 72 °C for 30 s, and a final extension step at 68 °C for 10 min. The PCR products were analyzed by electrophoresis on 2.5–3% agarose gels. The possession of each gene was confirmed by the presence of PCR products (233 bp for *cpe*, 499 bp for *becA*, 416 bp for *becB* and 324 bp for *plc*).

2.3. Sensitivity and specificity of multiplex PCR assay

The sensitivity of multiplex PCR was assessed as follows. The control strains (*C. perfringens* OS1 that possesses *becAB*, and *C. perfringens* NCTC8239 that possesses *cpe*) were precultured overnight with thioglycolate (TGC) medium (Nissui Pharmaceutical; Tokyo, Japan) at 37 °C under anaerobic conditions with AnaeroPack-Anaero (Mitsubishi Gas Chemical CO., INC.). An aliquot of the culture was incubated with TGY medium (3% Trypticase soy (BD), 2% D-glucose (Wako Pure Chemical Industries, Ltd.; Osaka, Japan), 1% yeast extract (BD), and 0.1% L-cysteine (Wako) (Miki et al., 2008). Ten-fold serial dilutions of the cultured medium were prepared using sterile TGY medium, and then the numbers of cells in each dilution were counted. In addition, DNA was extracted from each 1 ml of each diluted medium sample as described previously (Yamazaki et al., 2009, 2010) and multiplex PCR was performed.

The specificity of the multiplex PCR was assessed with 46 *C. perfringens* strains, 21 other *Clostridiaceae* strains and 145 other bacterial strains (strains listed in Table S1). The strains were grown by an aforementioned method and the DNA of each strain was extracted as described previously (Yamazaki et al., 2009, 2010).

Table 1
Primers used in this study.

Gene	Primer name	Primer sequence (5′–3′)	Product size (bp)	Reference
cpe	CPE F	ggagatggttggatattagg	233	Erol et al. (2008), Meer and Songer (1997)
	CPE R	ggaccagcagttgtagata		
becA	*becA* F	caatggggcgaagaaaatta	499	Yonogi et al. (2014)
	becA R	aaccatgatcaattaaaacctca		
becB	*becB* F	tgcaaatgacccttacactga	416	Yonogi et al. (2014)
	becB R	agattggagcagagccagaa		
plc	CPA F	gctaatgttactgccgttga	324	Erol et al. (2008)
	CPA R	cctctgatacatcgtgtaag		

2.4. Screening of isolates from human stools and bovine rectum swabs

In two previously reported gastroenteritis outbreaks caused by BEC-producing *C. perfringens* (Yonogi et al., 2014), roast beef was suspected as the causal agent. Therefore we investigated the prevalence of the *cpe*- and *becAB*-carrying *C. perfringens* strains, *C. perfringens* strains isolated from human stool and bovine rectum swab samples. Ninety bovine rectum swab samples were inoculated into 10 ml TGC medium (Nissui) and heated at 80 °C for 10 min. The medium was allowed to incubate anaerobically at 42 °C overnight, and then used to inoculate CW agar (Eiken, Japan) supplemented with egg yolk (Kyokuto Pharmaceutical Industrial Co., Ltd.; Tokyo, Japan). Human stool samples were obtained from 309 diarrheal patients and 130 healthy individuals, and directly inoculated on CW agar supplemented with egg yolk and 200 mg/l kanamycin (Meiji Seika Kaisha Ltd.; Tokyo, Japan). CW agar plates were anaerobically incubated at 42 °C overnight. DNA was extracted from colonies testing positive for lecithinase activity, followed by the multiplex PCR assay. The statistical analysis was performed using a McNemar's test for the harboring of *cpe* and *becAB* genes. In addition we screened total cultured enrichment medium itself without inoculating and screening colonies. For this, DNA was extracted from 1 ml of cultured TGC medium (Nissui) from a subset of bovine rectum swab samples, followed by the multiplex PCR assay.

3. Results and discussion

3.1. Specificity and sensitivity of the multiplex PCR assay

Using our multiplex PCR assay for detection of the *cpe*, *becAB*, and *plc* genes, we found that PCR amplicons for all three genes and the *cpe* control separated well on agarose gels (Fig. 1). To gauge the assay's specificity, 46 *C. perfringens* strains, 21 other *Clostridiaceae* strains and 145 other bacterial strains (Table S1) were screened by multiplex PCR. The *plc* gene was amplified from all *C. perfringens* isolates. Ten *cpe*- and 3 *becAB*-positive *C. perfringens* strains further yielded the *cpe* and *becAB* amplicons. In contrast, all except four non-*C. perfringens* strains failed to yield any amplicons whatsoever. The four strains of *Pseudomonas aeruginosa* did yield single, very weak bands of an unknown amplicon, but these clearly do not correspond to any of the target amplicons (Fig. S1). Hence, we conclude that our multiplex PCR method is highly specific to *C. perfringens*.

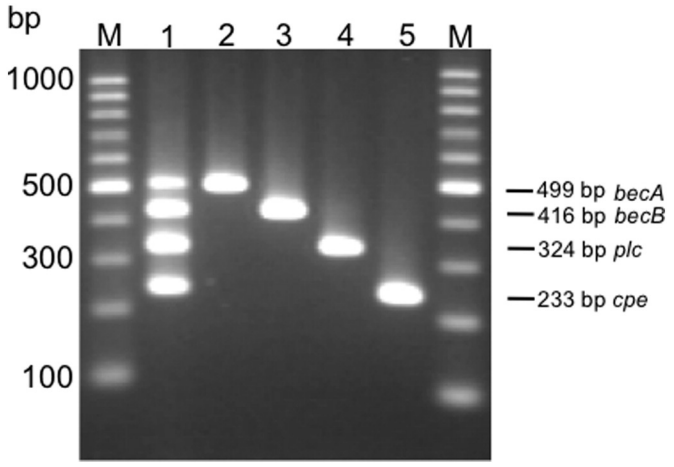

Fig. 1. Comparison of multiplex and single PCR. M, molecular weight marker; Lane 1, multiplex PCR; Lane 2, 499 bp for *becA*; Lane 3, 416 bp for *becB*; Lane 4, 324 bp for *plc*; Lane 5, 233 bp for *cpe*.