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Development of Real-Time PCR for the Detection of *Clostridium perfringens* in Meats and Vegetables

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A real-time PCR assay was developed and validated in-house specifically for the detection of *Clostridium perfringens* (*Cl. perfringens*) in meats and vegetables by comparing with the culture method. The detection limit of the real-time PCR assay in phosphate-buffered saline was 10^2 CFU/ml. When the two methods were compared in food samples inoculated with *Cl. perfringens*, the culture method detected 52 positives, whereas real-time PCR detected 51 positives out of 160 samples. The difference was without statistical significance ($p>0.05$). Real-time PCR assay is an option for quality assurance laboratories to perform standard diagnostic tests, considering its detection ability and time-saving efficiency.

Keywords: *Clostridium perfringens*, real-time PCR, culture method, foods, detection

Clostridium perfringens (*Cl. perfringens*) can cause necrotic enteritis, enterotoxemia, and hamorrhagic gastroenteritis in animals, while the bacterium is also a food-borne pathogen that causes diarrhea and abdominal pain in humans [5, 13]. The bacterium is commonly found in vegetables and crops as well as meat products [2, 9]. When cooked foods are gradually cooled, heat-stable spores of *Cl. perfringens* may germinate and proliferate [14].

Although the culture method is regarded as a standard method for the detection of *Cl. perfringens* from clinical samples and foods because of its reliability, it is time and labor intensive [4, 8]. Polymerase chain reaction (PCR) is one of the most commonly used methods for the detection of food-borne bacteria [10]. Real-time PCR using fluorescent reaction of amplified products is known to have high sensitivity and efficiency among different types of PCR assays that detects organisms within 24 h, including the enrichment step [4, 6].

Although detection of *Cl. perfringens* by real-time PCR has been applied in various studies, particularly in feces or animal gut [1, 7, 16], few studies have been conducted on food samples. In this study, one primers/probe set was designed to target α toxin, which is produced by all five types of *Cl. perfringens*, for the development of a real-time PCR assay [13]. The real-time PCR was evaluated using the primers/probe set in sensitivity and specificity tests. Real-time PCR was also compared with the standard culture method in the detection of *Cl. perfringens* in various food types composed of different matrix and background microflora, meats and vegetables.

Five of *Cl. perfringens* and 11 of non-*Cl. perfringens* strains were used in this study (Table 1). All non-*Clostridium* spp. were streaked onto nutrient agar (NA; Difco, Becton Dickinson, Sparks, MD, USA) for two passages and incubated in tryptic soy broth (Difco), whereas *Clostridium* spp. strains were streaked onto 5% horse blood agar (Oxoid, Hampshire, UK) for two passages and incubated in a broth, a cooked meat medium (CMM; Oxoid) at 37°C for 24 h. Culturable *Cl. perfringens* counts were obtained by plating 100 µl of the inocula onto 5% horse blood agar and incubated at 37°C for 24 h under anaerobic condition. Whenever necessary, cultures in CMM were serially diluted

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Table 1. Sensitivity and specificity of primers/probe.

Reference strain	Results
Clostridium perfringens ATCC 3624	+
Clostridium perfringens ATCC 13124	+
Clostridium perfringens PH1	+
Clostridium perfringens PH2	+
Clostridium perfringens PH3	+
Clostridium difficile ATCC 9689	-
Clostridium butyricum ATCC 19398	-
Clostridium ramosum ATCC 25582	-
Clostridium paraputrificum ATCC 25780	-
Escherichia coli O157:H7 ATCC 43888	-
Salmonella Enteritidis ATCC 10708	-
Bacillus cereus ATCC 11778	-
Yersinia enterocolitica ATCC 23715	-
Staphylococcus aureus ATCC 25923	-
Listeria monocytogenes ATCC 19111	-
Vibrio parahaemolyticus ATCC 33844	-

in phosphate-buffered saline (PBS; pH 7.4; Sigma, St. Louis, MS, USA) as needed, and *Cl. perfringens* numbers were enumerated as above.

The *cpa* gene, an α toxin producing gene, was targeted for the design of primers and probe. The sequence of the gene was provided by GenBank (http://www.ncbi.nlm.nih.gov/Genbank/; Accession Number: X13608) and one primers/probe set was designed using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). The set was validated at the NCBI Web site (http://www.ncbi.nlm.nih.gov/blast/) using BLAST (Basic Local Alignment Search Tool). The *Cl. perfringens* probe was labeled with 6-carboxyfluorescein (FAM, the reporter dye) and 6-carboxytetramethylrhodamine (TAMRA, the quencher dye). The sequences were as follows: *cpa* (amplicon size 85 bases); forward primer: 5'-AAA AGA AAG ATT TGT AAG GCG CTT AT-3'; reverse primer: 5'-CCC AAG CGT AGA CTT TAG TTG ATG-3'; probe: 5'-FAM TGC CGC GCT AGC AAC TAG CCT ATG G -3'TAMRA. The oligonucleotides and all reagents for PCR reaction used in this study were synthesized and purchased from Bioneer Corporation (Daejeon, Korea).

DNA templates of the bacteria were extracted with reference to Seo and Brackett [15]. One milliliter of sample from pure cultures in PBS or food samples in enrichment broth was centrifuged at 14,000 rpm for 3 min. The pellets were resuspended in 200 µl of PrepMan Ultra Reagent (Applied Biosystems) and boiled for 10 min. The samples were centrifuged at 14,000 rpm for 3 min. The supernatant fluids were used for real-time PCR assay.

The extracted DNA fluids (5 µl) were transferred into 20 µl of PCR mix consisted of TaqMan Universal PCR Master Mix (Applied Biosystems, 12.5 µl), forward primer (2.5 µl, 900 nM), reverse primer (2.5 µl, 900 nM), and TaqMan probe (2.5 µl, 250 nM). The 96-microwell plate sealed with optical adhesive covers (Applied Biosystems) was placed in an ABI-Prism 7500 sequence detector (Applied Biosystems). The reaction was run at 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

For sensitivity and specificity test of the designed sequences, the reaction of real-time PCR was examined in 16 strains, 5 of *Cl. perfringens* and 11 of non-*Cl. perfringens*. To determine the detection limit of real-time PCR in PBS, genomic DNA was extracted as described above from diluted overnight culture containing 10^5 CFU per milliliter. The extracted DNA was then serially diluted (10-fold) in PBS, and the cycle threshold value (Ct value) of the dilutions was measured by real-time PCR. The detection limit of real-time PCR using the primers/probe set was also measured in various foods. One strain producing α toxin, ATCC 3624, was used. Inocula (1 ml each) containing 1.8×10^2~1.8×10^8 CFU of ATCC 3624 were serially spiked into 10 g of foods, making the final concentrations of *Cl. perfringens* 1.8×10^1 to 1.8×10^7 CFU/g. Each inoculated sample was put into 90 ml of 0.85% saline water, followed by homogenizing for 30 s using a BagMixer stomacher (Interscience, St. Nom, France) and real-time PCR was performed with genomic DNA extracted from 1 ml of each diluted sample (1.8×10^0 to 1.8×10^6 CFU/ml) as described above. The lowest number of bacteria showing positive reaction was determined as the detection limit of real-time PCR.

Foods with different matrix and background microflora, meats and vegetables, were used to determine the difference in detection ability of the real-time PCR and culture method. Meat samples included steamed pork and ground beef, and vegetable samples included canned bean and vegetable mix composed of cabbage, cucumber, carrot, and lettuce. All samples were purchased from a local retail market in Seoul, Korea. A mesophilic aerobic plate count was performed on uninoculated food samples according to a previously published study [4].

One milliliter of the inoculum prepared by serial dilution of the overnight culture of CMM was spiked into 200 g of bulk samples evenly by pipetting to generate partial positives and partial negatives for statistical comparison when divided into 20 subsamples. The concentrations of the inoculums ranged 820~3,600 CFU/ml and 1,140~15,140 CFU/ml of *Cl. perfringens* for bulk samples of meats and vegetables, respectively. The inoculated bulk sample was stabilized at 4°C for 24 h, and subsequently divided into 20 subsamples of 10 g each. Two additional food samples (10 g each) were used as positive and negative controls. A positive control was prepared by spiking 10 g of the sample with approximately 10^7 CFU/ml of *Cl. perfringens* ATCC 3624. For negative control, uninoculated food (10 g) and sterilized PBS (1 ml) were also prepared. All experiments were

Table 2. Detection limits of *Cl. perfringens* ATCC 3624 using designed primers/probe set in pure culture.

Strain	CFU/ml	Results
Cl. perfringens ATCC 3624	10^5	+
	10^4	+
	10^3	+
	10^2	+
	10^1	-

conducted twice per one sample type at different inoculation levels. Samples were put into 90 ml of 0.85% saline water and homogenized for 30 s. After stomaching, 1 ml of sample was incubated in 9 ml of CMM at 37°C for 24 h. The incubated broth was streaked onto tryptose sulfite cycloserine (TSC; Oxoid) agar with 5% of egg yolk emulsion (Oxoid), followed by incubation at 37°C for 24 h under anaerobic condition. Presumptive identification was based on colony morphology and aerobic/anaerobic growth. Suspicious colonies were finally confirmed by API 20A strips (BioMérieux, Marcy l'Etoile, France). One milliliter of enrichment broth was collected and used for real-time PCR assay. Extraction of DNA templates and performance of real-time PCR was conducted as described above.

The number of positives identified out of the total number of samples was analyzed statistically by using the GraphPad Instat Software (GraphPad Software, San Diego, CA, USA). Differences between two methods were examined for level of significance by using Fisher's exact test.

Data providing the sensitivity and specificity of the designated primers/probe set are presented in Table 1. With the exception of *Cl. perfringens*, there was no positive reaction with any unrelated bacteria. It provided good sensitivity and specificity for the detection of *Cl. perfringens* at the species level.

The detection limits of the primers/probe set in PBS are listed in Table 2. In PBS, the detection limit of real-time PCR assay was approximately 10^2 CFU/ml. The detection limits were also determined in artificially inoculated food samples with different levels of background microflora.

The number of background microflora and detection limit of the real-time PCR in each food sample are presented in Table 3. As determined by aerobic plate counts, the number of background microflora was 5.98 log CFU/g, 4.48 log CFU/g, and 3.44 log CFU/g in vegetable mix, ground beef, and steamed pork, respectively. From canned bean, less than 2 log CFU/g of background microflora was detected. For the detection limit test, greater than 1.8×10^3 CFU of bacteria was required for positive reaction with real-time PCR in all tested foods. Lee *et al.* [11] found that the detection limit of real-time PCR was higher in vegetable than other types of foods for the detection of *S. aureus*. They reported that the detection limit of real-time PCR could be influenced by the matrix or background microflora of foods. In this study, however, the detection limit of *Cl. perfringens* by real-time PCR was identical in all types of foods (Table 3). It appears that the primers/probe sequences used in this study could be applied in various food samples with different matrixes and background microflora levels.

The performance of the real-time PCR and culture method in food samples was compared in Table 4. No positive reactions were obtained in the negative controls with the culture method and real-time PCR, whereas all positive controls were shown as positives with the two detection methods. Therefore, it was concluded that samples used in the experiments were not naturally contaminated by *Cl. perfringens*, thereby excluding possible false positives and negatives. In all food samples, the culture method detected 52 positives, whereas real-time PCR detected 51 positives, out of 160 samples. There was no statistical difference between the two methods ($p>0.05$). It appears that the culture method and real-time PCR have similar capability in the detection of *Cl. perfringens* in foods regardless of the matrix and number of background microflora. Malorny *et al.* [12] detected *Salmonella* spp. using the culture method and real-time PCR in various foods. Real-time PCR showed 100% sensitivity and specificity, compared with the culture method. Cai *et al.* [3] detected *V. parahaemolyticus* using the culture method

Table 3. Detection limit of *Cl. perfringens* ATCC 3624 using a designed primers/probe set in experimentally inoculated food samples without enrichment.

Strain	Number of cells (CFU/ml)	Food samples (No. of background microflora, log CFU/g)			
		Steamed pork (3.44)	Ground beef (4.48)	Canned bean (< 2)	Vegetable mix (5.98)
Cl. perfringens ATCC 3624	1.8×10^6	+	+	+	+
	1.8×10^5	+	+	+	+
	1.8×10^4	+	+	+	+
	1.8×10^3	+	+	+	+
	1.8×10^2	-	-	-	-
	1.8×10^1	-	-	-	-
	1.8×10^0	-	-	-	-

Table 4. Comparison between the culture method and real-time PCR for the detection of *Cl. perfringens* in artificially inoculated foods.

Sample		Trial	Inoculation level (CFU/g)	Number of positive samples/total number of samples		p value[a]
				Culture method	Real-time PCR	
Meat	Steamed pork	1	6.3	9/20	9/20	1.2488
		2	18.0	14/20	14/20	1.2689
	Ground beef	1	4.1	3/20	3/20	1.3386
		2	8.2	5/20	5/20	1.2836
Vegetables	Canned bean	1	5.7	3/20	3/20	1.3386
		2	11.4	7/20	7/20	1.2589
	Vegetable mix	1	27.0	1/20	0/20	1.0000
		2	75.7	10/20	10/20	1.2476
Total			**-**	**52/160**	**51/160**	**1.0000**

[a]There is statistical difference between the two methods if the p value is under 0.05.

and real-time PCR in various seafoods. Those outcomes correspond with that of the present study, suggesting real-time PCR's superiority.

Previous studies reported that the cultured method's detection ability was profoundly diminished in fresh vegetable samples, compared with real-time PCR, owing to high levels of competing background microflora in fresh vegetable that could hinder the selective growth of target organism in traditional culture media [8, 11]. In the present study, however, there was no difference in the isolation rate between the two detection methods, regardless of types of food. It suggests that the selectivity of TSC agar, the standard selective medium for *Cl. perfringens*, was excellent for selectively detecting the bacteria in various types of food samples while excluding any possible interference from normal background microflora.

In this study, real-time PCR showed excellent performance that generated more positives, albeit without statistical difference. Moreover, real-time PCR significantly saves assay time and labor, compared with the standard culture method [4, 8]. It appears that real-time PCR is an effective and sensitive presumptive screening tool for *Cl. perfringens* in various types of food, complementing the standard culture method.

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