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Detection and Differentiation of Non-Emetic and Emetic *Bacillus cereus* Strains in Food by Real-Time PCR

Ji Su Lim, Mi Ra Kim, Won Kim, and Kwang Won Hong*

Department of Food Science and Technology, Dongguk University, Seoul 100-715, Republic of Korea

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Duplex real-time polymerase chain reaction (PCR) method was developed for direct detection and identification of non-emetic and emetic *Bacillus cereus* strains in foods without enrichment. Primers and TaqMan probes were designed for molecular chaperonin gene *groEL* and cereulide synthetase gene *ces*. A total of 62 *B. cereus* strains, of which 59 were non-emetic and 3 emetic, were found positive by *groEL*-specific conventional PCR. Three emetic strains were found by *ces*-specific PCR to be positive, whereas 59 non-emetic strains were negative. Ten strains other than *B. cereus* were all negative by both *groEL*-specific and *ces*-specific PCR assays. The limits of detection of the duplex PCR assays for both non-emetic and emetic strains from their pure cultures were 3× 10^0 CFU/reaction. A total of 10 *doenjang* (traditional Korean fermented soybean paste) samples were analyzed simultaneously by real-time PCR assay and analytical profile index (API) test kits. All tested samples were positive for *B. cereus* contamination except two samples. The result of the real-time PCR assay was consistent with that of the API test. Among the eight positive samples, six and two samples were contaminated with non-emetic and emetic strains, respectively. The results suggest that the real-time PCR method developed in the present study may be useful for the direct detection and differentiation of *B. cereus* strains in foods.

Key words: *Bacillus cereus*, direct detection, emetic, non-emetic, real-time PCR

B. cereus is a Gram-positive and spore-forming bacterium responsible for diarrheal and emetic types of food poisoning, which are caused by enterotoxins and emetic toxin, respectively [Lund and Granum 1996; Guinebretière *et al*., 2002]. Specifically, the occurrence of diarrhea is attributed to various enterotoxins such as nonhemolytic enterotoxin (NHE), haemolysin (HBL), cytotoxin-K, enterotoxin-T and enterotoxin FM, a group of heat-labile proteins that cause abdominal pain and diarrhea [Sergeev *et al*., 2006; Stenfors-Arnesen *et al*., 2008]. The emetic type of food poisoning is induced by the small cyclic heat-stable toxin cereulide, which causes vomiting and nausea [Agata *et al*., 2002; Altayar and Sutherland, 2006]. Based on food poisoning types, *B. cereus* strains can be divided into a non-emetic *B. cereus* strain producing various enterotoxins and an emetic *B. cereus* strain producing emetic toxin [Ehling-Schulz *et al*., 2005; Hoton *et al*., 2005; Yang *et al*., 2005]. Although the food poisoning symptoms caused by *B. cereus* strains are relatively mild, some severe cases have resulted in death [Dierick *et al*., 2005]. *B. cereus* is commonly found in food production environments due to the resistance of its endospores to various stresses, thereby contaminating many kinds of foods during food production [Pirttijärvi *et al*., 2000; Ghelardi *et al*., 2002]. It is known that ingestion of more than 10^5 CFU of *B. cereus* per gram of food may cause food poisoning [Kotiranta *et al*., 2000].

Generally, many foodborne pathogens are analyzed by biochemical and microbiological culture methods. In the case of *B. cereus*, individual colonies on selective Mannitol-Egg Yolk-Polymyxin (MYP) medium are further characterized by various biochemical methods such as API tests. However, these techniques are both time- and labor-intensive and cannot differentiate the non-emetic strain and emetic toxin-producing strain. Polymerase chain reaction (PCR), based on the amplification of species-specific DNA fragments, is one of the most important genetic tools for detecting foodborne pathogens [Mäntynen and Lindström 1998; Chang *et al*., 2003; Alarcón *et al*., 2006]. Recently, real-time PCR assays using fluorescent probes have been increasingly exploited for more sensitive, specific and rapid identification of pathogens [Thisted-Lambertz *et*

*Corresponding author
Phone: +82-2-2260-3369; Fax: +82-2-2260-3369
E-mail: hkwon@dongguk.edu

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al., 2008; Wehrle *et al*., 2010]. This technique does not require gel electrophoresis for identification of amplified PCR products, thus saving time and reducing the risk of contamination.

PCR methods generally target the various enterotoxin genes of *B. cereus* such as *nheABC*, *hblCDA*, and *cytK* for the detection of *B. cereus* [Mäntynen and Lindström 1998; Hansen and Hendriksen 2001; Ngamwongsatit *et al*., 2008]. However, *ces*, which encodes cereulide synthetase, is restricted to emetic toxin-producing *B. cereus* [Ehling-Schulz *et al*., 2006]. Although *nheABC* genes are the most widely distributed toxin genes in *B. cereus* strains, these genes are not easily detectable [Hansen and Hendriksen 2001; Martínez-Blanch *et al*., 2009; Wehrle *et al*., 2010]. The *groEL*-encoding molecular chaperonins are ubiquitous in both prokaryotes and eukaryotes, and *groEL* sequences are used for species identification and taxonomic classification of *Staphylococcus* [Gob *et al*., 1997]. Nucleotide sequence similarities in *groEL* among *B. cereus* group strains, including closely related species such as *B. cereus, B. thuringiensis, B. anthracis, B. mycoides, B. pseudomycoides*, and *B. weihenstephanensis*, have been used for detection and differentiation using *groEL*-specific PCR [Chang *et al*., 2003].

In the present study, therefore, a duplex real-time PCR assay capable of simultaneously targeting *groEL* and *ces* genes was developed for the detection and differentiation of non-emetic and emetic *B. cereus* strains in food samples. This method was used to detect and discriminate *B. cereus* strains from *doenjang*, a Korean fermented food commonly found to harbor this pathogen, without enrichment, and the results were compared with those of the API test.

Materials and Methods

Bacterial strains and DNA extraction. The 62 *B. cereus* strains used in the present study are shown in Table 1. Ten other bacterial strains were used to test the specificity of the PCR assay, including *Bacillus subtilis* KCTC (Korean Collection for Type Culture) 2213, *Bacillus amyloliquefaciens* KCTC 3002, *Escherichia coli* O157:H7 ATCC (American Type Culture Collection) 4931, *Enterobacter sakazakii* ATCC 51329, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* ATCC 4931, *Shigella dysenteriae* ATCC 13313, *Staphylococcus aureus* ATCC 6538, *Vibrio parahaemolyticus* ATCC 17802, and *Yersinia enterocolitica* ATCC 23715. *Vibrio* cells were grown in tryptic soy broth containing 2.5% NaCl. For culture of *Listeria* strains, brain heart infusion broth was used. For other bacteria, cells were cultured in Luria-Bertani (LB) broth at 37°C. The bacterial DNA was extracted and purified from 1 mL of overnight culture using a Power Prep™ DNA Extraction Kit (Kogenebiotech, Seoul, Korea) according to the manufacturer's instructions. Purified DNA was recovered in 100 μL of sterilized water. The DNA concentration was determined using a spectrophotometer.

Primers and TaqMan probes. The primers and TaqMan probes used in the present study are shown in Table 2. The molecular chaperonine gene *groEL* (GenBank accession no. AE016877) was used to design a PCR primer set and a probe specific for *B. cereus*. The cereulide synthetase *ces* (GenBank accession no. AY691650) was used to design primers and a probe specific for emetic toxin-producing *B. cereus*. The primers and TaqMan probes were purchased from Bioneer (Seoul, Korea). The 5' ends of the *groEL*- and *ces*-specific probes

Table 1. Conventional PCR results of different strains of *B. cereus* using specific primers for *groEL*, *nheA*, and *ces*

Bacteria	Strains	*groEL*	*nheA*	*ces*
B. cereus (total 62 strains)	ATCC 11778, 12480	+	+	-
	ATCC 13061, 14579, 21772	+	+	-
	KCTC 1013, 1014	+	+	-
	KCTC 1092, 1094, 1526	+	+	-
	KFDA 202-213, 219-228, 230-243	+	+	-
	KFDA 244	+	-	-
	KFDA 245-249, 251-256	+	+	-
	KFDA 229, 250 (emetic type)	+	+	+
	KFRI 181	+	+	-
	F4810/72 (emetic type)	+	+	+

ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Culture; KFDA, Korea Food & Drug Administration; KFRI, Korea Food Research Institute

+: PCR product of the expected size was observed.

-: No PCR product was observed.

Table 2. Primers and TaqMan probe sequences

Primer & probe	sequence (5'→3')	Amplicon (bp)	Target gene	Reference
NA-F1	ATT ACA GGG TTA TTG GTT ACA GCA GT	475	*nheA*	Yang *et al.,* 2005
NA-R1	AAT CTT GCT CCA TACT CT CTT GGA TGC T			
RbalF	TGCAACTGTATTAGCACAAGCT	238	*groEL*	Chang *et al.,* 2003
RbalR	TTACCAACGCGCTCCATTGCTT			this study
Probe 1	**FAM**-GCTGCTATTTCTGCTGCTGACGAAGA-**BHQ2**			this study
RCesF1	GGTGACACATTATCATATAAGGTG	120	*ces*	this study
RCesR2	ATTCAACATAATATTATACGCCGT			
Probe 2	**JOE**-AGCGTCATAAACATCGTTACACCATTT-**BHQ2**			

were labeled with 6-carboxyfluorescein (FAM) and dichlorodimethoxylfluorescin (JOE), respectively. The 3' ends of both probes were labeled with black hole quencher-2 (BHQ2).

PCR conditions. Conventional PCR reactions were conducted in a total volume of 25 μL containing 50-100 ng of template DNA, 10 pmol of each primer, 5 μL of 5× reaction buffer, 1 U of Taq polymerase, and deionized water. The PCR reactions were run by a PCR Express thermocycler (Hybaid, Waltham, MA) using the following program: 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C, followed by 5 min at 72°C. Each reaction was conducted in triplicate. A total of 5 μL of PCR product was then loaded onto an agarose gel containing ethidium bromide and visualized under UV illumination.

The real-time PCR reactions were performed in 20 μL volumes containing 1 μL of purified DNA, an appropriate amount of each primer set (10 and 5 pmol for *groEL* and *ces*, respectively) and probe (2.5 pmol for each *groEL* and *ces*), 10 μL of 2×TaqMan master mix (Applied Biosystems, Foster City, CA) and deionized water. The real-time PCR reactions were run on an MX 3000P™ system (Stratagene, La Jolla, CA) using the following program: 1 cycle for 2 min at 50°C and 10 min at 95°C, 40 cycles for 15 s at 95°C and 1 min at 60°C. All PCR reactions were conducted in triplicate. The standard curve for *B. cereus* ATCC 14579 was constructed using DNAs recovered from the logarithmic culture broth samples with varying cell numbers. Simultaneously, the number of *B. cereus* cells in each of the 10-fold dilutions was determined by plate counting. The threshold cycle (Ct) values were plotted against the colony forming units (CFU).

Isolation and identification of *B. cereus* in fermented soybean sources. A total of 10 *doenjang* samples were purchased from grocery markets in Seoul. Twenty-five grams of each sample was individually added to 225 mL of sterile phosphate-buffered saline (PBS) in a sterile plastic bag containing a lateral filter and then homogenized with a pulsifier (Microgen Bioproducts, Surrey, UK) for 1 min. The resulting mixture was collected from the filter. Aliquots of 1 mL were used for enumeration of cells and extraction of DNA prior to duplex real-time PCR assay. *B. cereus* cells in 10-fold serial dilutions of homogenates were recovered from MYP (Merck, Darmstadt, Germany) agar plates at 30°C for 24-48 h. The five suspected colonies from each sample that appeared pink surrounded by a white precipitate were toothpicked and further cultivated on Nutrient Agar plates (Difco, Detroit, Michigan) at 30°C for 24 h. All colonies grown on the plate were confirmed to be *B. cereus* by API 50CH and API 20E systems (bioMerieux, Inc., Marcy l'Etoile, France). API 50CH and API 20E strips were inoculated with each colony and incubated according to the manufacturer's instructions.

Results and Discussion

Specificity of PCR detection. To investigate the specificity of the primers for *groEL*, *nheA* and *ces* in different *B. cereus* and other bacterial strains, conventional PCR assays were performed. Amplification of the 238-bp DNA fragment of *groEL* was observed in all *B. cereus* strains tested (Table 1). The specific 475-bp amplicon corresponding to *nheA* was obtained in all *B. cereus* strains tested except the KFDA 244 strain; *groEL* appeared to be a more desirable target than the *nheA* enterotoxin gene for the detection of *B. cereus* strains in food. However, *groEL*-specific PCR was still unable to discriminate between emetic and non-emetic *B. cereus* strains.

Before the nucleotide sequence of *ces* encoding cereulide synthetase was available, emetic strains were identified by the analysis of cereulide formation using bioassay [Andersson *et al*., 1998] or chemical assay [Häggblom *et al*., 2002]. Recently, emetic strains were identified by the detection of *ces* using PCR [Ehling-Schulz *et al*., 2006]. In our study, the *ces*-specific 120 bp amplicon was only amplified from the three previously

known emetic strains: *B. cereus* F4810/72 [Andersson *et al*., 1998], and KFDA 229 and 250 strains [Lee *et al*., 2008].

PCR amplifications of the *groEL, nheA* and *ces* were not found in any of the 10 non-target bacteria species tested in the present study (data not shown). *groEL* was common in all *B. cereus* strains tested, whereas *ces* was specific only in the emetic toxin-producing *B. cereus*. Therefore, simultaneous amplification of the *groEL* and *ces* could be desirable for the rapid detection and differentiation of non-emetic and emetic *B. cereus* strains in food.

Sensitivity of real-time PCR detection. Two TaqMan probes specific to regions within the *groEL* and *ces* amplicons were designed for a duplex real-time PCR assay (Table 2). Because *ces* is specific to the emetic *B. cereus* strain, a positive signal for *groEL* in the duplex real-time PCR demonstrates the non-emetic *B. cereus* strain that does not produce emetic toxin. However, two positive signals for *groEL* and *ces* indicate the presence of the emetic toxin-producing *B. cereus* strain.

The limits of sensitivity of duplex real-time PCR assay, which consists of two primer sets and two probes within a single reaction mixture, were determined in the purified DNA of non-emetic *B. cereus* ATCC 14579 and emetic F4810/72. Linear values for duplex real-time PCR amplification were achieved for dilutions of purified DNA at concentrations ranging from 3×10^5 CFU/reaction to 3×10^0 CFU/reaction (Fig. 1). The Ct values decreased as the number of CFU/reaction increased in the reaction tubes. The Ct values for the purified DNA of the non-emetic ATCC 14579 strain at 3×10^5, 3×10^4, 3×10^3, 3×10^2, 3×10^1, and 3×10^0 CFU/reaction were 24.17±0.37, 27.65±0.41, 31.13±0.43, 34.94±0.55, and 37.97±0.46, respectively, for *groEL*. The coefficient of correlation of the standard curve generated for *groEL* of the ATCC 14579 strain was 0.99 (Fig. 1-a). The Ct values for *ces* of the ATCC 14579 strain could not be determined under the same conditions.

The Ct values of the purified DNA of the emetic F4810/72 strain at 3×10^5, 3×10^4, 3×10^3, 3×10^2, 3×10^1, and 3×10^0 CFU/reaction were 23.58±0.46, 26.79±0.41, 30.33 ±0.44, 33.53±0.47, and 36.75±0.55 for *groEL* and 23.36 ±0.44, 26.76±0.66, 30.01±0.61, 33.43±0.36, and 36.61± 0.33 for *ces*, respectively (Fig. 1-b). A good linear correlation was observed between the Ct values and the concentrations of target DNA (*groEL*, r^2=0.99 and *ces*, r^2=0.99). Therefore, our duplex real-time PCR assay was able to detect at as small as 3×10^0 CFU per assay for both strains.

Direct detection of indigenous *B. cereus* in *doenjang* samples. *Doenjang* is a one of the most common

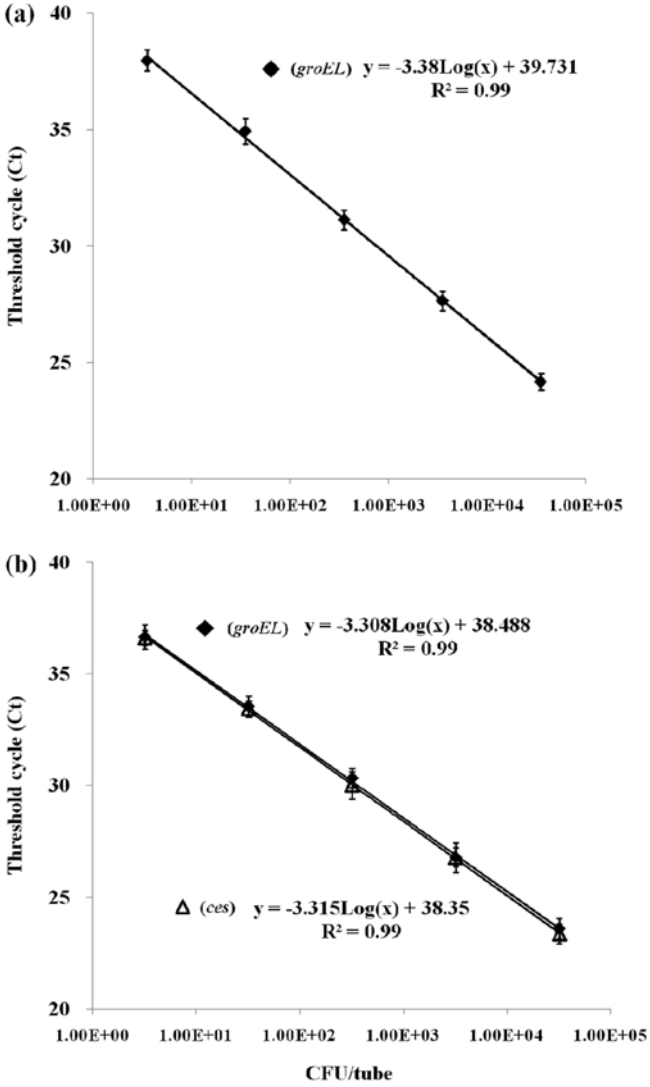

Fig. 1. Standard curves for non-emetic *B. cereus* strain ATCC 14579 (a) and emetic *B. cereus strain* F4810/72 (b).

fermented foods in Korea and contain many micro-organisms such as *Bacillus* sp. and *Aspergillus* sp. involved in the fermentation [Park *et al*., 2003; Kwon *et al*., 2010]. Additionally, the possibility of natural contamination of *doenjang* with *B. cereus* strains during fermentation process remains.

Therefore, contamination due to the presence of *B. cereus*, cells in a total of ten *doenjang* samples were analyzed by duplex real-time PCR assay and culture methods, followed by API tests. DNA was extracted from the homogenate of each *doenjang* sample without enrichment and then used as a template for duplex real-time PCR. A total of eight *doenjang* samples were positive and two negative as determined by duplex real-time PCR assay (Table 3). Among the eight positive samples, six and two of the samples were contaminated

Table 3. Naturally contaminated *doenjang* samples analyzed for the presence of *B. cereus* by MYP selective medium, real-time PCR, and API tests

Source	Culture method (CFU±SD/g)[a]	Duplex rt-PCR *groEL*:*ces*	API tests
doenjang-1	$(1.3\pm0.2)\times10^4$	+ -	+
doenjang-2	$(4.7\pm0.3)\times10^3$	+ -	+
doenjang-3	$(7.0\pm0.2)\times10^2$	+ -	+
doenjang-4	0	- -	nd
doenjang-5	$(6.1\pm0.1)\times10^6$	+ -	+
doenjang-6	0	- -	nd
doenjang-7	$(2.4\pm0.3)\times10^3$	+ -	+
doenjang-8	$(8.2\pm0.2)\times10^5$	+ +	+
doenjang-9	$(5.1\pm0.1)\times10^4$	+ +	+
doenjang-10	$(3.0\pm0.3)\times10^3$	+ -	+

nd: not determined

[a]The cell numbers are means±SDs of triplicate experiments.

with the non-emetic and emetic strains, respectively. Previous studies indicated that *B. cereus* is ubiquitous in the environment, but the incidence of emetic strain is not common [Agata *et al*., 1996; Altayar and Sutherland 2006]. Similarly, out of a total of eight *B. cereus* isolates, the present study found two isolates that were thought to be emetic strains.

To determine the number of *B. cereus* cells present in the *doenjang* samples, a 1-mL aliquot of each homogenate was smeared onto MYP selective medium, which is generally used for the enumeration, detection, and isolation of *B. cereus* in food [Mossel *et al*., 1967]. The numbers of *B. cereus* cells from eight positive samples ranged from $(7.0\pm0.2)\times10^2$ to $(6.1\pm0.1)\times10^6$ CFU/g (Table 3). Under these conditions, our duplex real-time assay could detect a minimum of $(7.0\pm0.2)\times10^2$ viable cells per gram of *doenjang* sample within 3 h without enrichment. The two *doenjang* samples, which were negative by duplex real-time PCR assay, showed no pink colonies on MYP medium.

Comparison of real-time PCR assay and API test. Accuracy of our real-time PCR assay for the detection of *B. cereus* strains in food samples needed to be confirmed by other standard methods. API 50CH and API 20E kits, which examine various biochemical reactions, are traditional methods for the identification of *Bacillus* [Valero *et al*., 2002; Torkar and Matijašiè, 2003]. However, this API *Bacillus* identification system can identify the *B. cereus* strain, but is not able to differentiate between emetic and non-emetic *B. cereus* strains.

To check whether or not the pink colonies grown on MYP selective medium were *B. cereus*, five candidate colonies from each of the eight positive samples were tested by real-time PCR assay using *groEL* primers and probe, and all 40 colonies were found to be positive (Table 4). These colonies were further evaluated biochemically using API 50CH and API 20E kits, and all 40 colonies were confirmed as *B. cereus* by API tests (Table 4). No true negatives, false positives or false negatives were observed. Therefore, a complete agreement between real-time PCR assay and API tests was obtained, which indicates that the real-time PCR assay method developed in the present study can provide rapid and accurate diagnosis of *B. cereus* isolates obtained through culture on selective MYP medium.

Several PCR and real-time PCR methods have been developed for the detection of *B. cereus* or *B. cereus* group strains in food samples. Many of them focus on the detection of enterotoxignic *B. cereus* strains causing diarrheal type of food poisoning [Mäntynen and Lindström, 1998; Hansen and Hendriksen, 2001; Martínez-Blanch *et al*., 2009; Gracias and McKillip, 2010; Wehrle *et al*., 2010]. The distribution of the emetic *B. cereus* strain in the environment is not known well, and relatively less attention has been given to the emetic type of food poisoning. Recently, several studies indicated that the incidence of emetic type food poisoning including some fetal cases has increased [Dierick *et al*., 2005; Duc *et al*., 2005; Yabutani *et al*., 2009]. In this regard, development of a rapid detection and differentiation method for non-emetic and emetic *B. cereus* strains in food samples is required.

In summary, our real-time PCR assay using TaqMan

Table 4. Comparison of *groEL*-specific real-time PCR and API tests for identification of *B. cereus*

No. of samples with result/total samples				Overall agreement[e]
(PCR^+, API^+)[a]	(PCR^+, API^-)[b]	(PCR^-, API^+)[c]	(PCR^-, API^-)[d]	
40/40	0/40	0/40	0/40	40/40 (1.00)

[a]True positive: Colony was identified as *B. cereus* by both rt-PCR assay and API test.
[b]False positive: Colony was identified as *B. cereus* by rt-PCR assay but not by API test.
[c]False negative: Colony was identified as *B. cereus* by API test but not by rt-PCR assay.
[d]True negative: Colony was not identified as *B. cereus* by either rt-PCR assay or API test.
[e]Overall agreement represents (true positive+true negative)/total sample.

probe was rapid and accurate for the identification of *B. cereus* and could detect 7.0×10^2 viable cells per gram of food sample within 3 h without enrichment. Furthermore, this method targeting the *groEL* and *ces* genes could be used to detect and differentiate between non-emetic and emetic *B. cereus* strains in food samples.

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