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A Multiplex PCR Assay for the Detection and Differentiation of Enterotoxin-producing and Emetic Toxin-producing *Bacillus cereus* Strains

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Abstract *Bacillus cereus* causes two different types of food poisoning syndromes: diarrhea and emesis. The diarrheal syndrome is attributed to various enterotoxins, including nonhemolytic enterotoxin, hemolytic enterotoxin, and enterotoxin-T, whereas the emetic syndrome is caused by the dodecadepsipeptide toxin cereulide. A multiplex polymerase chain reaction (PCR) assay was developed to rapidly detect and identify *B. cereus* strains. Three primer pairs specific to regions within genes encoding nonhemolytic enterotoxin (*nheA*), molecular chaperonin (*groEL*), and cereulide synthetase (*ces*) were used to identify and differentiate between the enterotoxin-producing and emetic toxin-producing *B. cereus* strains. The cereulide-producing emetic *B. cereus* showed 3 PCR products of 325, 405, and 685 bp for the *groEL*, *ces*, and *nheA* genes, respectively, whereas the enterotoxin-producing *B. cereus* showed 2 PCR products without a *ces* gene specific DNA fragment. Specific amplifications and differentiations by multiplex PCR assay were obtained using 62 *B. cereus* strains and 13 strains of other bacterial species. The detection limit of this assay for enterotoxin-producing strain and emetic toxin-producing strain from pure cultures were 2.4×10^1 and 6.0×10^2 CFU/tube, respectively. These results suggest that our multiplex PCR method may be useful for the rapid detection and differentiation of *B. cereus* strains in foods.

Keywords: *Bacillus cereus*, enterotoxin, emetic toxin, multiplex polymerase chain reaction (PCR), detection

Introduction

Bacillus cereus is a Gram-positive, spore-forming food pathogen commonly found in soil, dust, natural waters, and many kinds of foods (1,2). *B. cereus* causes two principal types of food poisoning syndromes: diarrhea and emesis. The diarrheal type is attributed to various enterotoxins, a group of heat-labile proteins causing abdominal pain and diarrhea (3). There are 3 different enterotoxins involved in food poisoning by *B. cereus* strains that have been reported: hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and enterotoxin T (*bceT*) (4-7). Among them the hemolysin BL complex is composed of 3 proteins encoded by the genes *hblC*, *hblD*, and *hblA* (8). The nonhemolytic enterotoxin also consists of 3 components encoded by *nheA*, *nheB*, and *nheC* (9). Enterotoxin T is composed of a single polypeptide (10). Another class of toxins expressed by *B. cereus*, which can induce hemolysis, includes phosphatidylcholine-specific phospholipase and sphingomyelinase, encoded by the *plc* and *sph* gene, respectively (7). The emetic type is induced by the small cyclic heat-stable dodecadepsipeptide toxin cereulide, which causes vomiting (11). Although both types of food poisoning are relatively mild, more severe cases have occasionally been reported, even involving deaths (12).

Due to the food poisoning problem associated with *B. cereus*, there is a need to develop a reliable method for detecting and differentiating enterotoxin and emetic toxin-producing *B. cereus* strains in contaminated food. Polymerase chain reaction (PCR) has been one of the most important genetic tools for detecting pathogenic bacteria (13-18). The various toxins produced by *B. cereus* mentioned above are the most popular targets for the identification of *B. cereus* strains (3,7,12). In addition, the nucleotide sequences of nonvirulence factors such as the *gyr* gene, encoding the subunit of DNA gyrase, and the *groEL* gene, which encodes molecular chaperonin, are also used in the identification of *B. cereus* strains (19,20). Occasionally, however, singleplex PCR may not give a reliable result due to sequence variations in target genes (3,21).

In order to improve the diagnostic capacity of the PCR assay, the simultaneous amplification of two or more target sequences from templates is desirable for the detection and identification of pathogenic bacteria. In this study, we developed a multiplex PCR assay, based on the nucleotide sequences of certain virulence and nonvirulence factors, for the rapid detection and differentiation of enterotoxin-producing and emetic toxin-producing *B. cereus* strains.

Materials and Methods

Bacterial strains, DNA extraction, and primers The primers and *B. cereus* strains used in this study are shown in Table 1 and 2, respectively. The non-target strains used in this study were as follows: *Bacillus subtilis* KCTC 2213, *Bacillus amyloliquefaciens* KCTC 3002, *Campylobacter jejuni* ATCC 33238, *Clostridium perfringens* ATCC 3624, *Enterobacter sakazakii* ATCC 12868, *Escherichia coli* 0157:H7 KCTC 1039, *Listeria monocytogenes* ATCC

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15313, *Salmonella enteritidis* KCCM 12021, *Salmonella typhimurium* ATCC 14028, *Shigella sonnei* KCTC 2518, *Staphylococcus aureus* ATCC 25923, *Vibrio parahemolyticus* ATCC 14547, and *Yersinia enterocolitica* KCCM 41657.

All the *Bacillus* strains were grown 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). Purified DNA was recovered in 100 μL of sterilized water. The DNA concentration was determined using a spectrophotometer (A_{260}).

PCR conditions Singleplex PCR reactions were conducted in 25 μL volumes containing 20 ng of template DNA, 20 pmol of each primer, 2.5 mM of deoxyribonucleoside triphosphates (dNTPs), 5 μL of 5× reaction buffer, 1 U of Taq polymerase, and deionized water. The optimized multiplex PCR reactions were performed in 25 μL volumes containing 20 ng of template DNA, an appropriate amount of each primer (7.5, 25, and 7.5 pmol for *groEL*, *nheA*, and *ces*, respectively), 2.5 mM of dNTPs, 5 μL of 5× reaction buffer, 1 U of Taq polymerase, and deionized water.

The PCR reactions were run on a PCR Express thermocycler (Hybaid, Waltham, MA, USA) using the following program: 1 cycle of 3 min at 95°C; 35 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, and finally 2 min at 72°C. Each reaction was conducted in triplicate. Five μL of PCR product were then loaded onto a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) illumination.

Reverse transcription (RT)-PCR The total RNAs were isolated using a SV Total RNA Isolation kit (Promega, Southampton, UK) according to the manufacturer's instructions. RT-PCR was carried out using reagents and protocols that were included in the One Step RNA PCR kit (Takara, Tokyo, Japan). The DNase-treated RNA (ca. 100 ng) was amplified with 5 U of AMV RTase XL and AMV-Optimized *Taq*, 1× buffer, 40 pmol of each forward and reverse primers, 1 mM dNTP, and 5 mM $MgCl_2$, which was made up to 50 μL with RNase-free distilled water. The RT-PCR samples were amplified in the following 2-step thermal program: cDNA was synthesized at 50°C for 30 min, and 94°C for 2 min, to inactivate the reverse transcriptase, and then amplified with 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. The RT-PCR samples (5 μL) were then electrophoresed on a 1.5% agarose gel in 1× TAE buffer.

Detection limit of multiplex PCR assay in artificially inoculated milk Twenty-five mL of sterilized whole milk purchased from a market were diluted in 225 mL of

Table 1. Primers used for amplification of *B. cereus*

Primer (forward/reverse)	Sequence (5'→3')	Target gene	Amplicon size (bp)	Reference
BTF/BTR	GAC TAC ATT CAC GAT TAC GCA GAA CTA TGC TGA CGA GCT ACA TCC ATA	*bceT*	303	This work
HAF/HAR	AAG CAA TGG AAT ACA ATG GG AGA ATC TAA ATC ATG CCA CTG C	*hblA*	1154	(14)
HCF/HCR	GAT ACY AAT GTG GCA ACT GC TTG AGA CTG CTC GYT AGT TG	*hblC*	740	(14)
HDF/HDR	ACC GGT AAC ACT ATT CAT GC GAG TCC ATA TGC TTA GAT GC	*hblD*	829	(14)
nNF[1)]/NAR	TGC GAT GAG TAG CTT AAC GAA TCA ACG AAT GTA ATT TGA GTC GC	*nheA*	685	This work/(14)
NBF/NBR	TTT AGT AGT GGA TCT GTA CGC TTA ATG TTC GTT AAT CCT GC	*nheB*	743	(14)
NCF/NCR	TGG ATT CCA AGA TGT AAC G ATT ACG ACT TCT GCT TGT GC	*nheC*	683	(14)
nCF1[1)]/CesR2	TTG TTG GAA TTG TCG CAG AG GTA AGC GAA CCT GTC TGT AAC AAC A	*ces*	405	This work/(11)
sph-F/sph-R	GAA GAA AGA ATA YCC AAA TCA AAC AGC A ARG RCT ATC AGG GAA ATT ATA TTT TGC A	*sph*	543	(7)
plc-F/plc-R	CAC TTG TAA AAC AAG ATC GAG TTG CA TAC ACC TTT TAG CAA TTT ACC TTT CAC GT	*plc*	727	(7)
BC1/BC2r	ATT GGT GAC ACC GAT CAA ACA TCA TAC GTA TGG ATG TTA TTC	*gyrB*	365	(20)
balF/nGR[1)]	TGC AAC TGT ATT AGC ACA AGC T TGC ATA TCC ACG ATC AAA TTG CA	*groEL*	325	(19)/This work
CGAF/CGAR	CTT TAC GAC AAA ACA AGG GGT T CTT GGT CTT CAT CGC CCA AC	*gyrA*	254	This work

[1)]Primers were redesigned to reduce the size of DNA products.

Table 2. Results from polymerase chain reaction (PCR) analysis of *B. cereus* reference strains

Bacterium	Strain	PCR[1)]												
		gyrA	*gyrB*	*groEL*	*bceT*	*nhe*			*hbl*			*plc*	*sph*	*ces*
						A	B	C	A	C	D			
Bacillus cereus	ATCC11778	+	-	+	+	+	-	-	+	+	+	+	+	-
	ATCC 21772	-	-	+	-	+	-	-	-	-	-	+	-	-
	ATCC 13061	-	+	+	-	+	+	+	-	-	-	+	-	-
	ATCC 14579	+	+	+	+	+	+	-	+	+	+	+	-	-
	ATCC 12480	+	+	+	+	+	-	+	+	+	+	+	-	-
	KFRI 181	+	+	+	+	+	-	-	-	-	-	+	+	-
	KCTC 1013	-	+	+	-	+	-	+	+	+	+	+	+	-
	KCTC 1014	-	+	+	-	+	-	+	-	-	-	+	+	-
	KCTC 1092	+	+	+	+	+	-	+	+	+	+	+	+	-
	KCTC 1094	+	+	+	+	+	-	+	+	+	+	+	+	-
	KCTC 1526	+	+	+	+	+	-	+	+	+	+	+	+	-
	F 4810/72	-	+	+	-	+	+	+	-	-	-	+	+	+

[1)]PCR results: (+) a PCR product of the expected size was seen; (-) no PCR product was formed.

buffered peptone water and seeded with early-stationary phase enterotoxin-producing or emetic toxin-producing *B. cereus* cells. Bacterial inocula were determined by plate count on LB medium. Artificially inoculated milk was homogenized by pulsifier for 1 min. Without previous enrichment, 1 mL aliquots were taken directly for DNA extraction using the DNA extraction kit. Appropriate amount of DNA solution was used as template for multiplex PCR. Reactions were carried out in triplicate.

Results and Discussion

Specificity of PCR assay The specificities of the 13 primer sets directed to the virulence and non-virulence factors of *B. cereus* were evaluated on 12 reference strains, 50 Korea Food & Drug Administration (KFDA) isolates, and 13 non-target strains by singleplex PCR. The specific 325, 685, and 727 bp amplicons corresponding to the molecular chaperonin (*groEL*), nonhemolytic enterotoxin (*nheA*), and phosphatidylcholine-specific phospholipase (*plc*) genes, respectively, were obtained in all *B. cereus* reference strains that were tested (Table 2). However, other primer sets also identified reference strains, but not completely. None of the tested 13 non-target strains cross-reacted with the tested primer sets (data not shown). The cereulide synthetase gene (*ces*) specific primer set that was used for detecting emetic toxin producers, amplified a 405 bp amplicon from the F4810/72 strain only, which was purchased from the National Collection of Type Cultures (NCTC) as a positive control. This result suggests that all the *B. cereus* reference strains, including the emetic toxin producer F4810/72, contained the nonhemolytic enterotoxin gene *nheA*.

The specificities of 4 chosen primer sets (*groEL*, *nheA*, *plc*, and *ces*) were further tested in 50 *B. cereus* KFDA isolates recovered from various sources (Table 3). PCR detected the *groEL* and *nheA* genes in 50 and 49 of the 50 isolated strains, respectively. The distribution of the *nhe* gene was similar in the total 62 *B. cereus* strains tested, where almost all the *B. cereus* strains from food-poisoning and food-borne ecosystems carried the *nhe* gene (14, 22).

Table 3. PCR analysis of *B. cereus* isolates

B. cereus isolates (total 50 strains)	Target genes		
	ces	*nheA*	*groEL*
KFDA[1)] 202-213	-	+	+
KFDA 219-228	-	+	+
KFDA 229	+	+	+
KFDA 230-243	-	+	+
KFDA 244	-	-	+
KFDA 245-249	-	+	+
KFDA 250	+	+	+
KFDA 251-256	-	+	+

[1)]Korea Food & Drug Administration.

However, the *plc* gene was detected in 42 of the 50 isolates (data not shown).

Analysis of emetic toxin-producing strains from food isolates of *B. cereus* PCR analysis showed that the KFDA isolate 229 and 250 were positive to the emetic *B. cereus* specific primer. DNA sequencing analysis showed that the nucleotide sequences of the cereulide synthetase genes from both isolates were exactly the same to that of the emetic toxin producer F4810/72 (data not shown). To determine whether the cereulide synthetase gene is expressed in those strains, the presence of *ces* mRNAs from both isolates as well as the emetic F4810/72 strain was examined using reverse transcription-PCR. As shown in Fig. 1, the 405 bp amplicon resulted from the amplification of *ces* mRNA, not DNA, as demonstrated by the lack of a signal when PCR was performed at the same time. Based on these results, KFDA isolates 229 and 250 are thought to be emetic toxin-producing *B. cereus* strains. Although *B. cereus* is a common bacterial isolate from soil, dust, natural waters, and many food types, emetic toxin-producing isolates are rare (2). Similarly, this study only found 2 isolates that were determined to be emetic toxin producers from 50 *B. cereus* isolates.

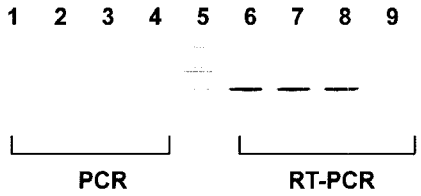

Fig. 1. Detection of *ces* mRNA (405 bp) in emetic toxin-producing *B. cereus* strains by RT-PCR. The total RNA was extracted, DNase treated, and amplified by singleplex PCR (lane 1 to 4) or RT-PCR (lane 6 to 9). Lane: 1 and 6, *B. cereus* F 4810/72; 2 and 7, KFDA 229; 3 and 8, KFDA 250; 4 and 9, no template control; 5, molecular size marker.

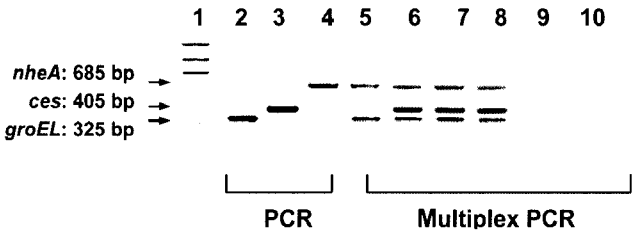

Fig. 2. Differentiation of enterotoxin-producing and emetic toxin-producing *B. cereus* strains by multiplex PCR. Lane: 1, molecular size marker; 2 and 4, simplex reactions for *groEL* and *nheA* from *B. cereus* ATCC 11778, respectively; 3, *ces* from *B. cereus* F 4810/72; 5, multiplex reaction from *B. cereus* ATCC 11778; 6, *B. cereus* F 4810/72; 7 and 8, KFDA 229 and 250, respectively; 9 and 10, *B. subtilis* KCTC 2213 and *B. amyloliquefaciens* KCTC 3002, respectively.

Multiplex PCR assay Our earlier results (Table 2 and 3) show that specific primer sets of the *groEL* and *nheA* genes can be used for detecting enterotoxin-producing and emetic toxin-producing *B. cereus* strains. Also, a *ces* gene specific primer set can be used for identifying emetic toxin-producing *B. cereus* strains (11). Using the above 3 primer sets (*groEL*, *nheA*, and *ces*), enterotoxin-producing and emetic toxin-producing *B. cereus* strains were differentiated by multiplex PCR; product profiles of the representative strains are shown in Fig. 2. The results indicated the presence of 2 and 3 distinct bands for the enterotoxin-producing strain and the emetic toxin-producing *B. cereus* strains, respectively. No PCR amplification was observed from the negative controls such as *B. subtilis* and *B. amyloliquefaciens*.

Limit of multiplex PCR detection in pure cultures and artificially inoculated milk In order to check the possibility for multiplex PCR detection of *B. cereus* in food, sensitivity assay was carried out on pure cultures and artificially inoculated milk without incubation. As shown in Table 4, the detection limits of enterotoxin-producing ATCC 21772 and emetic toxin-producing F4810/72 strains from pure cultures were 2.4×10^1 and 6.0×10^2 CFU/tube, respectively. However, the detection limits of both strains from artificially inoculated milk were found to be 10-fold lower than those of pure cultures, respectively. It is frequently reported the loss of sensitivity when the assay is applied to artificially inoculated food (23). In general, milk contains high levels of cations, proteases, nucleases, and fatty acids that may act as PCR inhibitors that bind and degrade the polymerase or the DNA template (24).

In summary, we found emetic toxin-producing *B. cereus* strains from KFDA isolates during this research. A multiplex PCR assay was established to differentiate enterotoxin-producing and emetic toxin-producing *B. cereus* strains. This multiplex PCR method was shown to be a specific and sensitive diagnostic tool for rapid detection of *B. cereus* strains in foods.

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Table 4. Detection limits of multiplex PCR in pure culture and artificially inoculated milk

B. cereus	CFU/tube	Pure culture			Artificially inoculated milk[1)]		
		groEL	*nheA*	*ces*	*groEL*	*nheA*	*ces*
ATCC 21772 (Enterotoxin-producing strain)	2.4×10^0	-	-	-	-	-	-
	2.4×10^1	+	+	-	-	-	-
	2.4×10^2	+	+	-	+	+	-
	2.4×10^3	+	+	-	+	+	-
	2.4×10^4	+	+	-	+	+	-
F4819/72 (Emetic toxin producing strain)	6.0×10^0	-	-	-	-	-	-
	6.0×10^1	-	-	-	-	-	-
	6.0×10^2	+	+	+	-	-	-
	6.0×10^3	+	+	+	+	+	+
	6.0×10^4	+	+	+	+	+	+

[1)]DNA extracted from 1 mL aliquots of artificially inoculated milk without enrichment was used as template.

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