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Vol. 8(47), pp. 3821-3829, 19 November, 2014
DOI: 10.5897/AJMR2014.7146
Article Number: 61B4F6A48994
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Improved multiplex polymerase chain reaction (PCR) detection of *Bacillus cereus* group and its toxic strains in food and environmental samples

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Received 23 September, 2014; Accepted 18 November, 2014

A multiplex PCR assay for the rapid detection of *Bacillus cereus* group, enterotoxic and emetic strains was developed. A panel of emetic and enterotoxic reference strains, *B. cereus* group members and non-target strains were used for the evaluation of the assay. Verification of PCR results on pure culture and inoculated foods successfully confirmed the specificity of approach for detection of target genes for *B. cereus* group (*groEL*), diarrheal (*cytK*, *nheA*, *hblC*, *entFM*) and emetic strains (*CER*). The sensitivity of approach was satisfying in pure culture as 20 pg of DNA per reaction tube. Artificial contamination of seven different food matrices with distinct bacterial counts revealed a minimum detection limit of 10^3 cfu/g in food samples. The detection limits were improved to approximately 10^1 cfu/g after 7 h enrichment. Natural contamination of rice and kimbab as well as environmental samples (soil, cow feces) was studied. The incidence of *B. cereus* was 63.88 and 38.88% in rice and kimbab, and 84.61 and 69.23% in soil and feces, respectively. To the best of our knowledge, this is the first time that an assay for simultaneous detection of *B. cereus* group, emetic and enterotoxic strains with such a wide range of detection target genes in food and environmental samples has been described.

Key words: *Bacillus cereus* group, multiplex polymerase chain reaction (PCR), enterotoxic strains, emetic strains, food and environmental samples.

INTRODUCTION

Bacillus cereus sensulato, the Gram-positive, rod-shaped, spore-forming opportunistic human pathogen is widely distributed in nature (Bartoszewicz et al., 2008). Therefore, it is an ever-present problem in a broad range of foods (Martínez-Blanch et al., 2010). Cereal products, rice, vegetables, pasta, meat, milk, liquid egg, spices, herbs and texturing agents are among the important food sources of *B. cereus* (Agata et al., 2002; Dierick et al., 2005; Fricker et al., 2007). The *B. cereus sensulato* group includes the species *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis* (Kim et al., 2012).

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Although *B. cereus* has been reported to be responsible for eye, wound and other systematic infections, it is widely known as a causative agent of gastrointestinal diseases (Kotiranta et al., 2000; Ghelardi et al., 2002). The symptoms of food poisoning resulting from consumption of contaminated food may be either diarrheal and/or emetic type (Sandra et al., 2012). The diarrheal food poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine. Among these protein compounds, non-haemolytic enterotoxin (NHE), enterotoxin FM (ent FM), Haemolysin BL (HBL) and cytotoxin K (cytK) are of the highest importance and therefore often used for the detection of enterotoxic strains (Kim et al., 2012). Symptoms of the diarrheal syndrome include abdominal pain and diarrhea and occur 8-16 h after ingestion of contaminated food which can be occasionally misdiagnosed with *Clostridium perfringens* food poisoning (Park et al., 2009).

The emetic food poisoning is caused by emetic toxin (cereulide), a heat and acid stable small cyclic peptide (dodecadepsipeptide) that induces swelling of mitochondria in Hep-2 cells, respiratory distress, and occasional loss of consciousness possibly leading to coma and ultimately death of individual (Ladeuze et al., 2011). The symptoms usually include nausea, vomiting and stomach pain which occur 1-5 h after food ingestion and can easily be misdiagnosed with *Staphylococcus aureus* food poisoning (Kim et al., 2011). Due to the increasing number of reports of food-borne disease and higher public demand for consumer safety, fast detection methods are required for diagnosis as well as prevention of food contamination (Toh et al., 2004; Yang et al., 2005).

The detection of *B. cereus* toxin producing strains was primarily limited to costly and labor intensive methods such as Hep-2 cell culture vacuolation assay, HPLC-MS analysis and the limited available enterotoxin detection kits. Later on, sperm bioassay for the detection of cereulide-producing strains was developed (Andersson et al., 2004; Buchanan et al., 1994). More recently, molecular diagnostic assays are being developed due to their advantages such as simplicity, high sensitivity, low cost and rapidity (Hyeon et al., 2010), especially after several marker genes of *B. cereus* were identified and characterized. Among the molecular methods, different types of polymerase chain reaction (PCR) have been established as valuable alternatives to the traditional detection methods (Elizaquível et al., 2011).

Ghelardi et al. (2002) used PCR along with other methods for identification of *B. cereus* by toxin genes. Nakano et al. (2004) performed a PCR assay based on a sequence-characterized amplified region marker for the detection of emetic *B. cereus* and Priha et al. (2004) detected *B. cereus* group on cardboard and paper using real-time PCR. In 2006, Ehling-Schulz et al. reported multiplex PCR detection of emetic and enterotoxic strains. Later on, real-time PCR assays were developed by Wehrle et al. (2010) and Martínez-Blanch et al. (2010) for detection and quantification of enterotoxigenic strains, respectively. Also Martínez-Blanch et al. (2010) evaluated a real-time PCR assay for the detection and quantification of *B. cereus* group spores in food and detection and quantification of viable *B. cereus* in food by real-time quantitative PCR (RT-qPCR) (Martínez-Blanch et al., 2011). In our laboratory, we previously developed two multiplex PCR assays, one for the simultaneous detection of *B. cereus* emetic and enterotoxic strains (Kim et al., 2012) and one for the detection of *B. cereus* emetic strains (Kim et al., 2013).

Despite the advantages of the above mentioned methods, none of them could simultaneously detect and identify *B. cereus sensulato* group non-toxic members, enterotoxic and emetic strains together. Some of them detected *B. cereus* group, some the enterotoxic and/or emetic strains and finally, some detected *B. cereus* group with either emetic or enterotoxic strains. Thus, the aims of this study were to 1) develop an efficient multiplex PCR assay to simultaneously detect *B. cereus* group, its enterotoxic and emetic strains and 2) to evaluate the assay in a wide variety of foods to ensure practical suitability. An advantage of this method would be its ability to detect members of *B. mycoides* and/or *B. thuringiensis* which may not produce any of the known toxins resulting in foodborne illness (Priha et al., 2004; Yang et al., 2005). Hence, this approach could be a useful tool not only for the detection of *B. cereus* group toxic strains but also for the large scale monitoring, genotyping and preliminary studies in search of *B. cereus sensulato* in foods and other samples.

MATERIALS AND METHODS

Bacterial strains

A total of 35 reference strains including 11 emetic *B. cereus* reference strains, 7 enterotoxic *B. cereus* reference strains, 5 members of *B. cereus* group and finally 12 other foodborne pathogens were used in the study (Table 1). All strains were obtained from the Department of Food Science and Biotechnology, Kangwon National University, South Korea.

DNA isolation

All strains were grown on tryptic soy agar (TSA; Difco, Detroit, MI, U.S.A.) plates at 35°C for 24 h. A single colony was inoculated into Luria-Bertani (LB broth; Difco) broth and incubated at 35°C for 8 h. One milliliter of the pure culture was centrifuged at 5000× g for 10 min at 4°C and the DNA was extracted using a DNeasy Tissue Kit (Qiagen AB, Uppsala, Sweden) according to the manufacturer's instructions. The purified DNA was recovered in 100 µL of sterilized distilled water and DNA concentration was determined by using a NanoDrop ND-2000 UV/VIS spectrophotometer (NanoDrop Technologies) at 260 nm. The purified DNA was diluted to reach the concentration of approximately 15-20 ng/µL and stored at -20°C for the following experiments.

Primers

Table 2 shows the primers used in this study. The four primer pairs

Table 1. List of reference strains used for the evaluation of multiplex PCR assay.

Emetic reference strains	Enterotoxic reference strains	*B. cereus* group members	Non-target strains
B. cereus F4810/72	*B. cereus* ATCC13061	*B. thuringiensis*KCTC1508	*Escherichia coli* KCCM32396
B. cereus JNHE36	*B. cereus* ATCC12480	*B. thuringiensis*824	*Escherichia coli* O157:H7 ATCC 43895
B. cereus JNHE78	*B. cereus* KCTC1013	*B. mycoides*KCTC 3453	*Salmonella* typhimuriumATCC14028
B. cereus KUGH164	*B. cereus* KCTC1014	*B. weihenstephanensis*KACC12001	*Listeria monocytogenes*ATCC19119
B. cereus KNIHuls1	*B. cereus* KCTC1092	*B. pseudomycoides*KACC12098	*Listeria monocytogenes*ATCC19115
B. cereus KNIHuls3	*B. cereus* KCTC1094		*Staphylococcus aureus* ATCC12500
B. cereus KNIHuls4	*B. cereus* KCTC1526		*Staphylococcus aureus* ATCC27729
B. cereus KNIHuls5			*Clustridiumperfringens*KCTC5101
B. cereus KNIHuls7			*B. subtilis* KCCM11316
B. cereus KNIHuls8			*B. subtilis* KCTC3135
B. cereus KFDA250			*Sseudomonasputida*KCCM35479
			*Escherichia coli*ATCC3565

Table 2. Primers used in this study for the multiplex PCR assay.

Target gene	Sequence (5´→ 3´)	Product size (bp)	Design source	Reference
cytK	TGCTAGTAGTGCTGTAACTC CGTTGTTTCCAACCCAGT	881	DQ019311	Kim et al., 2012
nheA	GGAGGGGCAAACAGAAGTGAA CGAAGAGCTGCTTCTCTCGT	750	DQ019312	Kim et al., 2012
CER	GCGTACCAAATCACCCGTTC TGCAGGTGGCACACTTGTTA	546	AY576054	Kim et al., 2012
hblC	CGCAACGACAAATCAATGAA ATTGCTTCACGAGCTGCTTT	421	AY786407	Kim et al., 2012
entFM	AGGCCCAGCTACATACAACG CCACTGCAGTCAAAACCAGC	327	AY789084	Kim et al., 2012
groEL	AGCTATGATTCGTGAAGGT AAGTAATAACGCCGTCGT	236	AB077143	Kim et al., 2013

for the detection of enterotoxin genes (*cytK, nheA, hblC, entFM*) and *CER* primers for the detection of emetic strains were previously used in a multiplex PCR assay to detect enterotoxic and emetic strains simultaneously (Kim et al., 2012). The *groEL* primer pair targeting *B. cereus sensulato* group using gene sequences encoding molecular chaperonins was also successfully evaluated in our previous studies (Kim et al., 2013) as well as others (Chang et al., 2003) for the detection of *B. cereus* group. All primers were commercially produced by Bioneer (Daejeon, South Korea) using *AccuOligo*® technology (http://eng.bioneer.com/products/Oligo/CustomOligonucleotides-technical.aspx).

Optimization of multiplex PCR assay

One emetic reference strain (F4810/72) and one enterotoxic reference strain (ATCC12480) were used for optimization of the multiplex PCR assay. In brief, ability of primers to be used in a multiplex assay was checked and primer concentrations were optimized in singleplex, duplex and multiplex PCRas well as optimum PCR conditions by trial and error. All PCR assays were conducted using Mygenie32 Thermal Block (Bioneer) thermal cycler. PCR products were checked on 2% agarose gel (Sigma-Aldrich; St. Louis, MO, USA) at 100 V for 30 min in × 0.5 Tris-borate EDTA (TBE) buffer using electrophoresis machine (Mupid-exU, Mupid, Tokyo, Japan). The gels were stained with SafeView™ (ABM, Richmond, BC, Canada) and visualized using a UV transilluminator (Gel Doc 2000; Bio-Rad, Hercules, CA, USA). A 100-bp DNA ladder (Solgent, Daejeon, South Korea) was used as molecular weight marker.

Evaluation of multiplex PCR assay

The specificity of the primers as well as their ability to perform in a multiplex PCR assay was confirmed by assessing 35 bacterial strains. These included a panel of 11 emetic reference strains, 7 enterotoxic reference strains and 5 strains of *B. cereus* group members. Furthermore 12 non-target Gram-positive and Gram-negative bacterial species were tested (Table 1). Gene profiles of the reference strains resulted in this study were compared with

previous data to confirm the specificity of the developed multiplex PCR assay.The specificity of *CER* primer was evaluated using emetic reference strains and non-target strains and enterotoxin gene primers were evaluated using the enterotoxic reference strains as well as non-target strains. Finally, the specificity of *groEL* primer set was evaluated using the emetic and enterotoxic strains as well as *B. cereus sensulato* group members.

Detection limit in pure culture

Detection limit of the developed assay in pure culture was determined using 4 enterotoxic (ATCC13061, ATCC12480, KCTC1013, KCTC1094) and one emetic (F4810/72) *B. cereus* reference strain. After preparing tenfold dilution series of pure DNA from each strain ranging from 20 ng to 200 fg, each dilution was subjected to multiplex PCR assay.

Artificially inoculated food assays

Baby cereal, kimbab (Korean food containing rice and other ingredients rolled in seaweed), pasteurized milk, pasta, rice, sunflower oil and tteok (Korean rice cake made with glutinous rice flour) were purchased from local food stores in Chuncheon, South Korea. If necessary, food samples were autoclaved and tested for the absence of any naturally occurring contamination with *B. cereus* by the standard reference culture methods (Fricker et al., 2007). In brief, 25 g of sample was homogenized in a filter stomacher bag (Nasco Whirl-Pak, Janesville,WI) using a Seward stomacher (400 Circulator,Seward, London, UK) with 225 mL of brain heart broth (BHIG; Merck, Darmstadt, Germany) supplemented with 0.1% glucose. The homogenate was incubated at 37°C for 24 h without shaking. Subsequently, 100 µL of each dilution was spread onto mannitol-eggyolk-polymixin agar (MYP; Difco) to check presence of *B. cereus* cells. Samples confirmed for the absence of *B. cereus* were used for artificial contamination.

For inoculation, one emetic (F4810/72) and three enterotoxic reference strains (ATCC12480, ATCC13061 and KCTC1014) were separately seeded into 25 g of food and homogenized for 2 min with 225 mL of 0.1% buffered peptone water (BPW; Difco) or tryptic soy broth (TSB; Difco) as mentioned above resulting in a final *B. cereus* cell concentration of approximately 1.8×10^6, 4.2×10^6, and 2.1×10^6 and 2.7×10^6 cfu/g of *B. cereus* F4810/72, ATCC 13061, ATCC12480 and KCTC 1014, respectively. Serial dilutions covering a range of 10^6 to 10^1 cfu/g were prepared and DNA was immediately extracted from 1 mL of BPW diluted homogenates using NucleoSpin food kit (Macherey-Nagel, Germany) according to the manufacturer's guidelines. TSB diluted homogenates were incubated at 37°C for 7 h with 200 rpm agitation and DNA was extracted as mentioned above. DNA extracts of each dilution were used for multiplex PCR assay while in parallel 0.1 and 1 mL of each dilution was spread plated onto MYP agar to compare the developed approach efficiency with conventional culture method.

Analysis of naturally contaminated food

Rice and kimbab (36 samples, each) were purchased from local markets, wholesales and restaurants in Kangwon-do province, South Korea and immediately transferred to laboratory to test the natural contamination. In brief, 25 g of each sample was homogenized for 2 min with 225 mL of 0.1% BPW (Difco). 1 mL of the homogenate was used for DNA extraction as mentioned above and subjected to the developed multiplex PCR assay. All experiments, including PCR and conventional culture methods were conducted in duplicate.

Analysis of soil and feces

Soil samples (13 samples) were collected in Kangwon National University campus. Samples were taken with a sterilized spoon from a depth of 10 to 20 cm into a stomacher bag. All samples were treated on the same day of collection. Cow feces (13 samples) were collected into sterile containers from cattle at Kangwon National University farm from individual animals and analyzed within the same day.

Briefly, 10 g of each sample was homogenized for 5 min with 90 mL of 0.1% BPW or TSB as mentioned above. The sample was left to settle for 2 min and 1 mL of the liquid phase was used for DNA extraction using the DNeasy Tissue Kit (Qiagen) as mentioned above. In parallel, 0.1 mL aliquots were used for spread plating on MYP agar (Difco).

RESULTS

Optimization of the multiplex PCR assay

The optimum PCR mixture (25 µL) contained 800 nM of *cytK* and *nheA* primers, 500 nM of *CER*, *hblC* and *groEL* primers and 400 nM of *entFM* primers, approximately 15-20 ng of DNA template, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 40 mM KCl, 250 µM dNTP mixture and 1 U Taq polymerase (Takara Taq™, Otsu, Japan). The optimum amplification conditions were as follows: initial denaturation at 95°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min.

Evaluation of multiplex PCR assay

Gene profiles of the *B. cereus* reference strains, obtained from the multiplex PCR assay were in agreement with the previous reports, confirming the specificity of the approach (Table 3). Gel electrophoresis of the multiplex PCR results on the 18 *B. cereus* reference strains used in this study confirmed the presence of expected 881, 750, 546, 421, 327 and 236 bp amplicons for *cytK*, *nheA*, *CER*, *hblC*, *entFM* and *groEL*. Also no amplicons were observed for the non-target strains. Figure 1 shows the agarose gel electrophoresis for 6 of the reference strains used in this study. The ability of *groEL* primer to specifically detect *B. cereus* group members was further confirmed by using 5 members of *B. cereus* group members in addition to the reference strains. The *groEL* primer successfully detected all the 5 *B. cereus* group member strains.

Detection limits in pure culture and artificially contaminated foods

The results obtained from multiplex PCR assay of 5 *B. cereus* reference strains used for sensitivity study in pure culture were positive for all strains up to 20 pg of DNA/reaction tube which corresponds to approximately 3×10^3 cfu/mL of bacterial cells.

Figure 2 shows the detection of the four reference strains

Table 3. Gene profiles of emetic and enterotoxic reference strains of *B. cereus* using multiplex PCR assay.

Strain	Type	Multiplex PCR assay					
		CER	*groEL*	*Cyt K*	*entFM*	*hblC*	*nheA*
B. cereus ATCC13061	Enterotoxic	-	+	-	+	-	+
B. cereus ATCC12480	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1013	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1014	Enterotoxic	-	+	-	+	-	+
B. cereus KCTC1092	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1094	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1526	Enterotoxic	-	+	+	+	+	+
B. cereus F4810/72	Emetic	+	+	-	+	-	+
B. cereus JNHE36	Emetic	+	+	+	+	+	+
B. cereus JNHE78	Emetic	+	+	-	-	-	+
B. cereus KUGH164	Emetic	+	+	-	-	-	+
B. cereus KNIHuls1	Emetic	+	+	-	+	-	+
B. cereus KNIHuls3	Emetic	+	+	-	+	-	+
B. cereus KNIHuls4	Emetic	+	+	-	+	-	+
B. cereus KNIHuls5	Emetic	+	+	-	+	-	+
B. cereus KNIH7uls7	Emetic	+	+	-	+	-	+
B. cereus KNIHuls8	Emetic	+	+	-	+	-	+
B. cereus KFDA250	Emetic	+	+	-	+	-	+

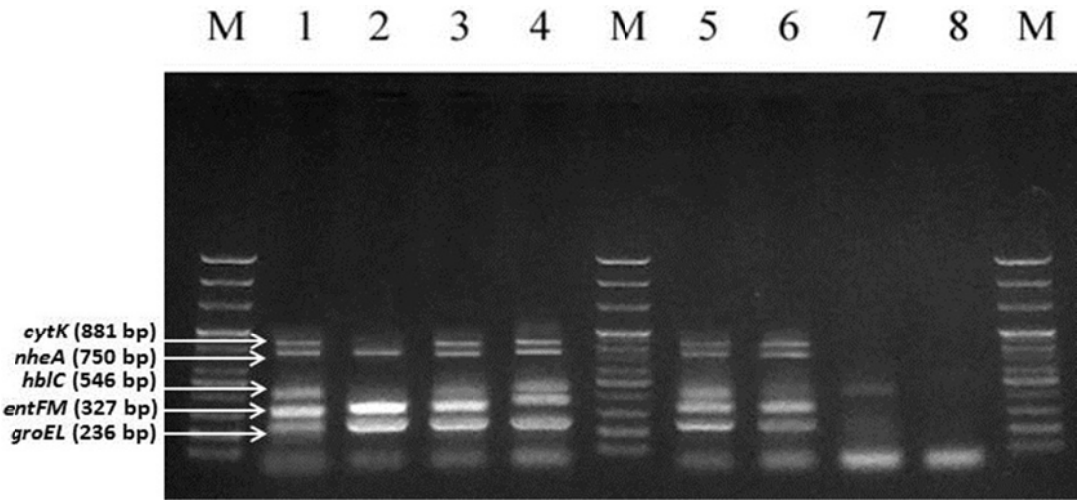

Figure 1. Gel electrophoresis results of 6 enterotoxic reference strains and 1 non-target strain. M, 100-bp DNA size marker; Lane 1, *B. cereus* KCTC1013; Lane 2, *B. cereus* KCTC1014; Lane 3, *B. cereus* KCTC1092; Lane 4, *B. cereus* ATCC12480; Lane 5, *B. cereus* KCTC1526; Lane 6, *B. cereus* KCTC1508; Lane 7, *Pseudomonas putida* KCCM35479; Lane 8, no template control (NTC).

in artificially inoculated food (kimbab). Detection limits of the multiplex PCR assay in food were evaluated using 7 foods (Baby cereal, kimbab, pasteurized milk, pasta, rice, sunflower oil and tteok). Results showed detection limits of 1.8×10^3, 4.2×10^3, 2.1×10^3 and 2.7×10^3 cfu/mL for *B. cereus* F4810/72, ATCC 13061 ATCC12480 and KCTC1014, respectively, for all foods without enrichment. These limits were similar or tenfold higher than the spread cultures performed in parallel. The sensitivity of the approach improved to approximately 10^1 cfu/mL after enrichment (37°C for 7 h with 200 rpm agitation in TSB).

Analysis of naturally contaminated food

Table 4 shows the summary of toxin gene profiles obtained

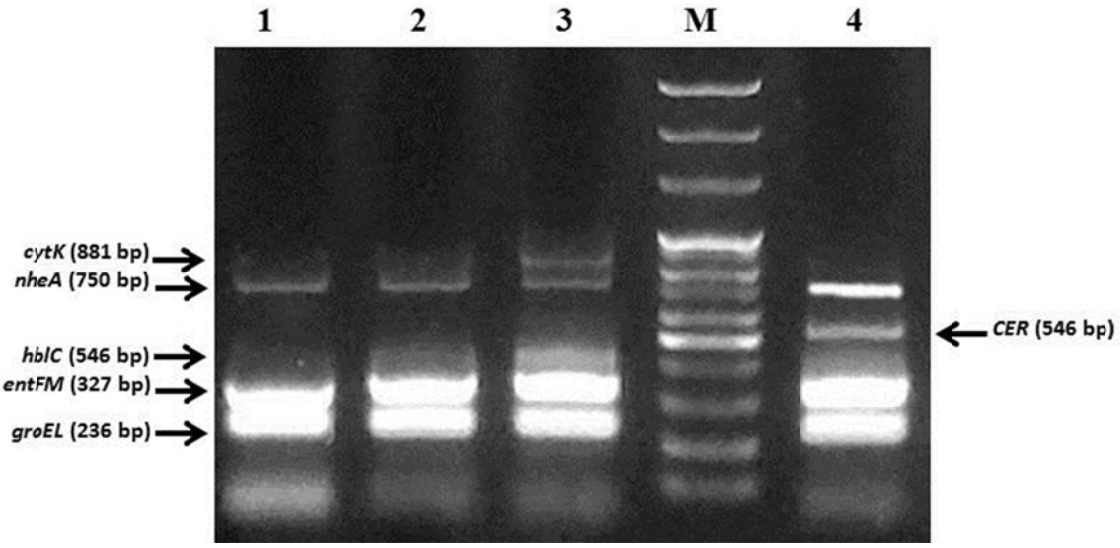

Figure 2. Gel electrophoresis results of 4 *B. cereus* reference strains detection by multiplex PCR approach in artificially inoculated food (kimbab). M, 100-bp DNA size marker; Lane 1, *B. cereus* ATCC 13061; Lane 2, *B. cereus* KCTC 1014; Lane 3, *B. cereus* ATCC 12480; Lane 4, *B. cereus* F4810/72.

Table 4. Summary of toxin genes distribution in positive samples of naturally contaminated rice, kimbab, soil and feces.

Toxin gene	Kimbab (n = 12)	Rice (n = 23)	Soil (n = 11)	Feces (n = 9)
cytK	4 (33.33%)	12 (52.17%)	6 (54.54%)	6 (66.66%)
nheA	11 (91.66%)	20 (86.95%)	11 (100%)	8 (88.88%)
hblC	7 (58.33%)	16 (69.56%)	7 (63.63%)	5 (55.55%)
entFM	9 (75%)	18 (78.26%)	7 (63.63%)	5 (55.55%)
CER	ND	2 (8.69%)	ND	ND

ND: not detected.

in naturally contaminated foods, soil and feces. Among the 36 rice samples analyzed, contamination with *B. cereus* was detected in 23 of them (63.88 %). Among the contaminated samples only two (8.69%) were positive for *CER* primer meaning the presence of emetic strains. Other toxin genes showed different frequencies with *NHE* being the most frequent gene detected (86.95% of positive samples). In kimbab, 12 out of the total 36 (33.33%) samples analyzed were contaminated by *B. cereus* group while no contamination by emetic strains was detected and *NHE* showed the highest frequency as well as in rice while the frequency orders of other toxingenes were different from rice.

Analysis of *B. cereus* presence in soil and feces

In soil samples (Table 4), 11 out of 13 (84.61%) were found positive for the presence of *B. cereus* group. This result was in comparison with direct plating on MYP agar. In case of feces samples, 9 out of 13 (69.23%) were found positive for *B. cereus* presence while direct spread plating was positive for 7 samples (53.84%). This might be due to the lower detection limits or that *B. cereus* cells in one samples did not grow by culturing due to some reasons (death or incapability to adjust to the new medium) but still they were detected by the multiplex PCR approach.

DISCUSSION

B. cereus has been increasingly involved in several foodborne outbreaks (Wehrle et al., 2010). Therefore, there has been increasing interest in the development of appropriate methods for its detection in food. Recently, PCR procedures have been found suitable for pathogen detection in food products since they are rapid and

simple to use (Elizaquível et al., 2011). These advantages were further extended by the application of multiplex PCR allowing amplification of all sequences of interest simultaneously in a "multiple" reaction (Wu et al., 2007). Therefore, in this study four primer pairs (*cytK, nheA, hblC* and *entFM*) for the detection of four commonly present enterotoxin genes in *B. cereus* group as well as *CER* primer for the detection of emetic strains were used, resulting in a wide range of detection to maximize the chance for the detection of *B. cereus* group in food and environmental samples since usually each enteropathogenic strain contains at least one of these genes.

Based on the literature (Priha et al., 2004; Yang et al., 2005), it is possible for some of the *B. cereus* group members, especially *B. thuringiensis* and *B. mycoides* to not contain any of the toxin genes. This information becomes more important knowing that a big portion of *B. cereus* strains isolated from environmental as well as food samples consists of *B. thuringiensis* and *B. mycoides* (Rosenquist et al., 2005). Wehrle et al. (2010) developed a multiplexreal-time PCR based on SYBR green I for the detection of enteropathogenic *B. cereus*. This approach enabled fast and reliable detection of enteropathogenic *B. cereus.* However, it lacked the ability to detect enterotoxin FM gene (*entFM*), which prevalence studies have detected in most *B. cereus* outbreak-associated strains (Kim et al., 2009; Tran et al., 2010).

The first approach to detect and quantify viable *B. cereus* in food was developed by Martínez-Blanch et al. (2011). This approach detected viable *B. cereus* group cells by targeting the phosphatidylcholine-specific phospholipase C (*pc-plc*) mRNA. However, it only detected *B. cereus* group and could not differentiate between toxigenic and non-toxigenic strains. In the present study, we added the *groEL* primer set to the multiplex approach. Therefore, the present approach is able to detect and differentiate *B. cereus* toxigenic group members and also the strains that do not contain any of the known toxin genes.

Usually, foods with *B. cereus* contamination levels below 10^3 cfu/g are considered safe for the consumers (Martínez-Blanch et al., 2011). However, different legal limits have occasionally been set in some countries (Häggblom et al., 2002). In order to further confirm the practical applicability of the approach, 7 foods prone to *B. cereus* contamination were artificially inoculated and analyzed. The developed multiplex PCR approach showed a detection limit of approximately 3×10^2 to 10^3 cfu/mL in pure culture. This result was similar to our previous studies (Kim et al., 2012, 2013). However, interestingly, the detection limits in inoculated foods were 1.8×10^3 and 2.1×10^3 cfu/mL for *B. cereus* F4810/72 and ATCC12480, respectively, for all foods without enrichment which did not always show a tenfold lower limit as compared to the pure culture. The improved sensitivity of the approach in food as compared to the previous studies (Alarcon et al., 2005) might be due to the different DNA extraction method used in this study. As it is well known, foods have a complex matrix containing nucleases, cations, proteases, fatty acids and other PCR inhibitors. Therefore, application of an efficient DNA extraction/purification method may eventually improve the detection limit of PCR approach. Hence, the sensitivity (10^3 cfu/g) of the approach was enough to detect minimum levels of contamination considered as legal limit in food samples, more sensitive than the assay developed by Martı´nez-Blanchet al. (2011) and similar to some other previous results (Wehrle et al., 2010; Kim et al., 2012, 2013) and more sensitive than the work of Nakano et al. (2004), in which the developed approach was not sensitive enough for the direct detection without a 7 h enrichment step. However, typical cell counts in food samples connected to food poisoning are usually much higher in the range of 10^5 to 10^8 cfu/g of *B. cereus* (Fricker et al., 2007). The detection limit was improved to approximately 10^1 cfu/gof *B. cereus* after enrichment. Nakano et al. (2004) also reported the efficiency of a similar enrichment. In this study, TSB medium was used instead of BHI and 37°C instead of 35°C. On the other hand, the conventional culture method not only takes at least 24 h to get the results but also practically is not as sensitive as expected. Although, using 1 mL aliquots may improve sensitivity but such a high volume makes handling of plates harder, needs more time and finally is hard to perform.

Foods containing rice are the main source of *B. cereus* contamination. Cooked or fried rice is involved in approximately 95% of *B. cereus* emetic food poisonings. Despite the lower prevalence of emetic food poisoning as compared to the enterotoxic syndromes, it is still of quite importance as it also can have negative health effects and may occasionally even lead to liver failure or death (Ladeuze et al., 2011; Martinez-Blanch et al., 2009). Therefore, rice and kimbab, as two main foods in the daily diet of Koreans were selected to evaluate the developed approach in naturally contaminated foods and also to perform a preliminary test on the level of *B. cereus* contamination in these foods in Chuncheon city/Kangwon-do province.

Only 2 rice samples (8.69% of total contaminated samples) were positive for *CER* primer while no emetic strains were detected in kimbab. These results are in agreement with the works of Ehling-Schulzet al. (2006), reporting a low incidence for emetic strains and Park et al. (2009), reporting a low incidence for emetic strains in cereals. In general, 63.88 and 33.33% of rice and kimbab samples, respectively, were contaminated by *B. cereus*. Kim et al. (2009) reported an average contamination of 41.7% by *B. cereus* without enrichment in rice samples in South Korea. In the same study, contamination was detected in 95% of the samples after enrichment. In another study, a 40% contamination level was reported (Jang et al., 2006). In kimbab, Cho et al. (2008) reported a 43.3% *B. cereus* contamination level. An important point to be mentioned here might be the application of

only one target gene in above mentioned studies for the detection of *B. cereus* contamination in food samples. Therefore, the reported incidences might be lower than the actual numbers since *B. cereus* strains have a high diversity especially in case of toxigenic profiles. A study performed in Malaysia reported contamination levels of 100, 76.2, 70.4 and 50% in different types of raw rice (Sandra et al., 2012). The results obtained in this study show that there is a need for further study on the contamination levels of products such as rice and kimbab and also to be more careful about performing appropriate actions such as good washing of raw rice prior to cooking, keeping hygiene and preventing cross contamination during handling and storage of cooked products in appropriate temperatures. Also following good agricultural practices (GAP) is necessary for the production of rice as well as similar products since results of the soil and cow feces analysis revealed a high incidence of *B. cereus* in these sources which can easily be transferred to the crop on the field or during the process chain.

In conclusion, the developed multiplex PCR approach meets requirements as a useful tool for the rapid detection of *B. cereus* group in food and environmental samples, as well as identification of enteropathogenic strains. Future studies will have to reveal the capability of the present approach to be used as a combined assay such as with propidiummonoazide DNA intercalating dye for the rapid detection of viable strains, for the toxigenic profiling of *B. cereus* or in combination with most probable number to obtain a MPN-PCR method with quantitative and qualitative detection capability.

Conflict of Interest

Authors have no conflict of interest.

ACKNOWLEDGEMENT

This research was supported by Bio-industry Technology Development Program (112137-3) Ministry of Agriculture, Food and Rural Affairs.

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