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Full Length Research Paper

Rapid detection of emetic toxin producing *Bacillus cereus* strains using triple-primer polymerase chain reaction (PCR) assay

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Emetic toxin producing *Bacillus cereus* can cause emetic food poisoning. In this study, an improved triple-primer polymerase chain reaction (PCR) assay was developed as a reliable and rapid identification method for the detection of emetic toxin producing *B. cereus* strains based on the unique gene sequences of the *CER*, *ces* and *groEL*. Specificity and sensitivity of the primers was confirmed using conventional PCR and gel electrophoresis. In addition, detection limit was determined in pure culture, rice and milk. In brief, sensitivity in pure culture was 10 fold or more higher than artificially inoculated food samples in improved triple-primer PCR detection limit assay. The primers did not react with genes from enterotoxin producing *B. cereus* or other non-target strains. The presented PCR assay is a useful tool for the rapid screening and diagnosis of emetic toxin producing *B. cereus* strains in food.

Key words: Triple-primer polymerase chain reaction (PCR), *Bacillus cereus*, emetic toxin, food poisoning.

INTRODUCTION

Bacillus cereus, the gram-positive, spore-forming opportunistic human pathogen is found in a wide range of sources including foods (Kotiranta et al., 2000; Seong et al., 2008). It is widely known that *B. cereus* causes food poisoning syndromes of two distinct types, the diarrheal and emetic type and that both types have serious effect on human health (Ehling-Schulz et al., 2006a). Diarrheal type syndrome is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Granum, 1994; Ehling-Schulz et al., 2006b), while emetic type syndrome (emesis) is caused by the consumption of food contaminated by heat resistant emetic toxin. Emetic toxin is a circular dodecadepsipeptide (D-O-Leu-D-Ala-L-O-val-L-val$_3$) known as cereulide, which is closely related to the potassium ionophorevalinomycin. The small size of cereulide and its non-antigenicity have resulted in difficulties in producing reliable detection methods (Horwood et al., 2004). Cereulide does not lose its activity even at 121°C and has tolerance for a wide pH range of 2 to 11 (Kotiranta et al., 2000; Ehling-Schulz et al., 2005a; Seong et al., 2008). According to its chemical structure, cereulide is synthesized enzymatically via genomic non-ribosomal peptide synthetase (NRPS) sequences (Dommel et al., 2010; Ehling-Schulz et al., 2005b). Emesis symptoms include heavy nausea, vomiting and abdominal pain which occur 1 to 5 h after ingestion of contaminated food (Rajkovic et al., 2006a, b; Taylor et al., 2005; Makarasen et al., 2009). Emetic toxin is a dangerous factor in food. However, due to the lack of suitable assays, it is not easy to detect the gene responsible for emetic toxin production. Recently, it has been shown that the toxin is encoded by NRPS sequences and specifically by *ces* gene (Ehling-Schulz et al., 2006b; Andersson et al., 1998; Horwood et al., 2004). Usually, fried or cooked rice is involved in approximately 95% of *B. cereus* food poisoning cases with emetic

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symptoms (Jenson and Moir, 2003). Korea Food and Drug Administration (KFDA; http://www.kfda.go.kr) reported 33 outbreaks and 861 patients associated with *B. cereus* from 2002 to 2010 in Korea. However, emetic toxin type has not been specifically studied.

According to the importance of emetic toxin poisoning and limited detection methods, we developed an improved triple-primer polymerase chain reaction (PCR) assay based on conventional PCR systems for the specific detection of emetic *B. cereus* strains. Although, it has been previously described to detect the toxin gene and identify the emetic toxin producing strains using PCR (Ehling-Schulz et al., 2006a; Kim et al., 2010a), in this study we used a mixed set of primers in order to maximize the sensitivity and specificity.

The multiplex PCR (mPCR) is a variation of polymerase chain reaction, which involves more than one pair of primers allowing a single reaction to detect more than one type of microorganism/gene resulting in the reduction of detection time and expenses (Malkawi and Gharaibeh, 2003). The presented mPCR greatly increases the sensitivity and rapidity for the detection of emetic *B. cereus* strains. This method will enable food microbiology laboratories to take a preventative approach to detect emetic food poisoning (Jenson and Moir, 2003). Furthermore, it can be advantageous because of its economic and efficient primers.

MATERIALS AND METHODS

Bacterial strains

A total of 41 *B. cereus* emetic strains from different sources were used in this study. The emetic toxin producing reference strain *B. cereus* F4810/72 was used as the positive control and 40 emetic toxin producing *B. cereus* strains were tested for the evaluation of approach (Table 1). The emetic toxin producing strains used in our study were previously confirmed (Kim et al., 2010a). All strains were stored at -80°C and routinely grown in trypticase soy broth (TSB; BD, Detroit, MI, USA) or on trypticase soy agar (TSA; Detroit, MI, USA) at 30°C for 24 to 48 h. A total of 40 emetic toxin producing *B. cereus* strains previously identified were used. Strains obtained from Jeollabuk-do Research Institute of Health and Environment, Jeonju, Korea (JNHE; 20 strains) and Korea National Institute of Health, Seoul, Korea (KNIH; 12 strains) were identified using Hep-2 cell culture vacuolation assay. Strains obtained from Gyeonggi-do Research Institute of Health and Environment, Suwon, Korea (KUGH; 6 strains), were identified using HPLC/MS. And finally, 2 strains identified as emetic toxin producing *B. cereus* using reverse transcription-PCR were provided by KFDA (Kim et al., 2010a, b). The non-target strains tested in this study were as follows: enterotoxin producing *B. cereus* strains JUHE 28, JUHE 72, KUGH 13, ICHE 1 and KCPC1092 58 (Kim et al., 2010 b,c), *Staphylococcus aureus* (ATCC 25823, ATCC 12500, ATCC 13565), *Escherichia coli* (KCCM 32395, ATCC 35150), *Listeria monocytogenes* (ATCC 19118), *Salmonella typhimurium* (ATCC 14028) and *Bacillus subtilis* (KCCM 11315).

DNA extraction

The DNA for molecular experiments was extracted from overnight cultures. All bacterial strains were cultured in TSB at 35°C for 24 h. Subsequently, 1 ml of the cell suspension was harvested and centrifuged at 4000 ×g for 10 min at 4°C. Total DNA of each strain was extracted using DNA extraction kit (Wizard Genomic DNA purification kit, Promega, Madison, WI, USA) in accordance with the manufacturer's guidelines. The purified DNA was suspended in 100 µl of sterilized distilled water and DNA concentration was determined using a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) at 260 nm. This DNA was diluted to reach the concentration of approximately 1 µg/ml and stored at -20°C for PCR amplification.

Primer design and PCR reaction conditions

The developed triple-primer in this study were synthesized using the *CER* and *ces* genes for the detection of emetic toxin producing strains and another specific primer for the *B. cereus* group (*groEL* gene). The oligonucleotide primers targeting specific genes were designed using GeneFisher 2 web-based program (http://bibiserv.techfak.uni97bielefeld.de/genefisher2/). The *CER*, *ces* and *groEL* gene sequences with Genebank accession numbers AY331260, AY691650 and AB077143 respectively, were used for the primer design (Lee et al., 2008; Ehling-Schulz et al., 2006a). Finally, the primers with closest annealing temperature, melting point and the least chance of dimer production were selected among the primary designed sequences and synthesized by Bioneer Corp (Seoul, Korea). The GC% rate of primers were designed as 40 to 60% in order to maintain strong adhesion. The resulting primers are presented in Table 2. The PCR conditions were as follows: predenaturation at 94°C for 10 min, 35 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension of 5 min at 72°C. The PCR mixtures (20 µl) contained 30 pM of each primer, 20 ng of DNA template, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 30 mM KCl, 250 µM dNTP mixture and 1 U Top DNA polymerase (Bioneer, Daejeon, Korea). The amplification results were resolved using electrophoresis machine (Mupid-exU, Mupid, Tokyo, Japan) on 1% agarose gel (Bioneer, Daejeon, Korea). Also, the concentration of the extracted DNA was measured using a UV-spectrophotometer (model UV-1700, Shimadzu, Tokyo, Japan).

Detection limits of PCR assay

B. cereus F4810/72 used as emetic type reference strain and JNHE 88 were inoculated in TSB and incubated at 35°C for 24 h. 1 ml of cultured broth was seeded into 25 ml of TSB and 25 ml of sterilized whole milk or rice diluted in 225 ml of peptone water and homogenized for 2 min using a stomacher (400 Circulator, Seward, London, UK). Subsequently, serial dilutions were prepared and DNA from each dilution tube without previous enrichment, were extracted using DNA extraction kit. All dilutions were analyzed by the mPCR assay. Also, 100 µl aliquots of each dilution were spread onto TSA plate to count the cell numbers after incubation at 35°C for 24 to 48 h.

RESULTS

Evaluation of triple-primer PCR assay

In order to check the specificity and sensitivity of the presented triple-primer PCR assay for the emetic toxin producing *B. cereus* detection, a total of 16 strains: 6 emetic toxin producing *B. cereus* strains (JNHE 41, JNHE 53, JNHE 88, JNHE 95, KCDC uls 1, KCDC

Table 1. List of bacterial strains used in this study.

Species	Bacterial strains	No. of strains	PCR with CER, *ces* and *gro EL*
B. cereus (reference)	F4810/72	1	Positive
B. cereus (emetic type)	JNHE 6; JNHE 7; JNHE 13; JNHE 15; JNHE 21; JNHE 22; JNHE 23; JNHE 24; JNHE 36; JNHE 41; JNHE 53; JNHE 54; JNHE 56; JNHE 60; JNHE 61; JNHE 78; JNHE 80; JNHE 82; JNHE 88; JNHE95; KNIH 20; KNIH 24, KNIH 25; KNIH 28; KNIH unlsan1; KNIH unlsan2; KNIH unlsan3; KNIH unlsan4; KNIH unlsan5; KNIH unlsan6; KNIH unlsan7; KNIH unlsan8; KUGH 164; KUGH 10; KUGH 11; KUGH 12; KUGH 27; KUGH 85; KFDA 229; KFDA250	40	Positive
B. cereus (diarrheal type)	JUHE 28, JUHE 72, KUGH 13,ICHE 1; KCPC1092 58	5	Positive (only *groEL*)*
S. aureus	ATCC 25823; ATCC 12500; ATCC13565	3	Negative
E. coli	ATCC 35150; KCCM 32395	2	Negative
L. monocytogenes	ATCC 19118	1	Negative
S. typhimurium	ATCC 14028	1	Negative
B. subtilis	KCCM 11315	1	Negative

*Positive (only *groEL*); negative on CER, *ces*.

Table 2. Sequence of oligonucleotide primers and amplicon size.

Primer name	Primer sequence (5'-3')	Amplicon size (bp)
cer F-5	CAAGTCAAGATAAGAGGCTTC	370
cer R-5	AAAGCTCTTGCCAAATAACC	
ces F-10	GCATTTCGTGAAGCAGAGGT	699
ces R-10	CCCTTTATCCCCTTCGATGT	
gro EL F-1	AGCTATGATTCGTGAAGGT	236
gro EL R-1	AAGTAATAACGCCGTCGT	

24), 2 enterotoxin producing *B. cereus* strains (ICHE 1, KCPC 1092 58) and 8 non-target strains including *S. aureus* (ATCC 25823, ATCC 12500, ATCC 13565), *E. coli* (KCCM 32395, ATCC 35150), *L. monocytogenes* (ATCC 19118), *S. typhimurium* (ATCC 14028), *B. subtilis* (KCCM 11315) were used. The triple-primer PCR assay was capable of amplifying three DNA fragments of 236, 370 and 699 bp from the *groEL*, *ces* and *CER* genes of emetic toxin producing *B. cereus* F4810/72 as reference strain, respectively. The *CER* and *ce*s primers for the detection of emetic toxin producing *B. cereus* were designed based on the NRPS genes responsible for emetic toxin synthesis. The *groEL* specific 236 bp amplicon for the detection of *B. cereus* group was obtained in all emetic toxin and enterotoxin producing *B. cereus* strains and not in any of the non-*Bacillus* strains. But, *CER* and *ces* amplicons were obtained only in emetic toxin producing *B. cereus* strains. As shown in Figure 1, the 236, 370 and 699 bp amplicons were obtained for the *groEL*, *CER* and *ces*, respectively, without non-specific target bands. The 2 enterotoxin producing *B. cereus* strains of lanes 8 and 9 in Figure 1 had no amplified DNA fragment target bands of *ces* and *CER* primer. But *groEL* band was present. Furthermore, none of the 8 non-target strains showed any band for the three primers. This proves that *CER*, *ces* and *groEL* primers of the triple-primer kit can identify *B. cereus* group with high sensitivity and specificity and would not show false positive results by the presence of non-target strains.

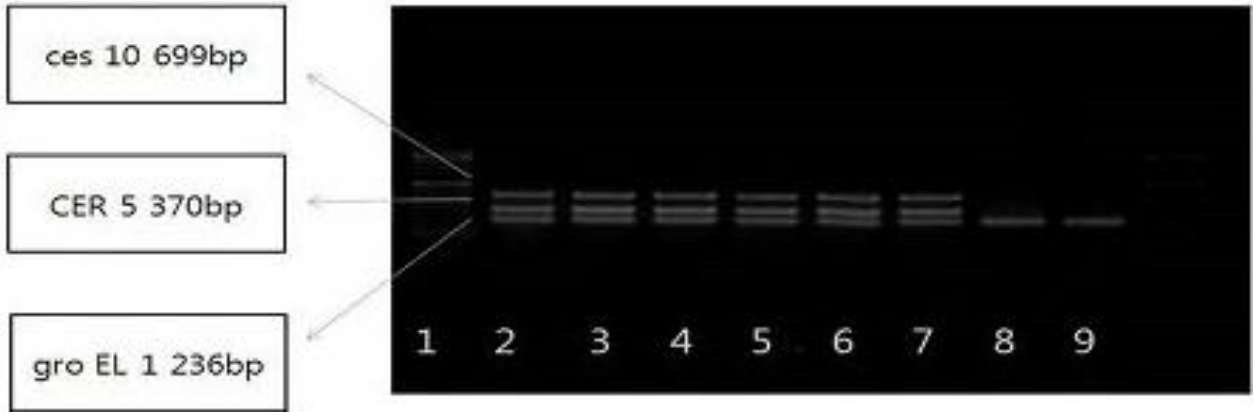

Figure 1. Gel electrophoresis of PCR products amplified with triple-primer on emetic toxin producing *B. cereus* strains. Lane 1: size marker, Lane 2: *B. cereus* F4810/72 as positive control, Lane 3: *B. cereus* JNHE 53, Lane 4: *B. cereus* JNHE 88, Lane 5: *B. cereus* JNHE 95, Lane 6: *B. cereus* KCDC uls 1, Lane 7: *B. cereus* KCDC 24, Lane 8: *B. cereus* ICHE 1 as negative control, Lane 9: *B. cereus* KCPC 1092 58 as negative control.

Table 3. Detection limits of triple-primer PCR assay in pure culture and artificially inoculated milk.

Strains	CFU/tube	Pure culture	Rice	Milk
F4810/72	$7.6x10^0$	-	-	-
	$7.6x10^1$	-	-	-
	$7.6x10^2$	+	-	-
	$7.6x10^3$	+	+	+
	$7.6x10^4$	+	+	+
JNHE88	$6.8x10^0$	-	-	-
	$6.8x10^1$	-	-	-
	$6.8x10^2$	+	-	-
	$6.8x10^3$	+	+	+
	$6.8x10^4$	+	+	+

Detection limits of triple-primer PCR assay

In order to check the sensitivity of the presented approach, it was applied in pure culture, artificially inoculated milk and rice, using *B. cereus* F4810/72 as reference strain and *B. cereus* JNHE 88 emetic toxin producing strain. As shown in Table 3, the detection limits of triple-primer for emetic toxin producing strain *B. cereus* F4810/72 in pure culture, artificially inoculated milk and rice were 7.6×10^2, 7.6×10^3 and 7.6×10^3 CFU/ml, respectively. Also, detection limits for JNHE 88 emetic toxin producing strain in pure culture, artificially inoculated milk and rice were 7.6×10^2, 7.6×10^3 and 7.6×10^3 CFU/ml, respectively. As the results show, the detection limits of triple-primer PCR assay in artificially inoculated milk and rice were found to be 10-fold lower than pure culture in both strains.

DISCUSSION

Identification of conditions favorable for cereulide or cereulide synthesis genes in foods has been difficult due to the lack of suitable assays for accurately measuring cereulide concentrations (Häggblom et al., 2002). Although, a number of mPCR assays capable of detecting genes inducing emetic toxin have been developed so far, it is still not easy to evaluate these genes specifically and rapidly. Hence, in this study, we designed a triple-primer mPCR assay based on the *CER*, *ces* and *groEL* genes to improve the rapid detection of emetic toxin producing *B. cereus* strains with high specificity and sensitivity.

Like the 16S rRNA gene, *groEL* which encodes molecular chaperonin is a valuable tool for phylogenetic studies to detect the *B. cereus* group (Chang et al., 2003)

and has already been used in PCR assay to detect the *B. cereus* group (Taylor et al., 2005; Chang et al., 2003). The *CER* and *ces* genes have illustrated high flexibility in the module organization of bacterial NRPS (Jenson and Moir, 2003). Cereulide synthetase genes and their flanking regions known to be the main virulence factors of emetic toxin producing *B. cereus* are located in the extra-chromosomal location on a plasmid with a pXO1-like back-bone (Ehling-Schulz et al., 2006b). Thus, all *B. cereus* strains may be potentially toxigenic, and the detection of these organisms in foods is important to prevent food poisoning (Park et al., 2007).

The presented primers successfully amplified the target genes in the triple-primer PCR assay without any non-specific or additional band for all strains (Figure 1). Also, the primers did not interact with genes from other non-target organisms under the prescribed PCR conditions. According to our previous results (data not shown), specific primers of the *CER*, *ces* and *groEL* genes can be used to detect emetic toxin producing *B. cereus* strains among different strains such as *S. aureus*, *E. coli*, *L. monocytogenes*, *S. typhimurium* and *B. subtilis*. This is in good agreement with the results of this study and Ehling-Schulz et al. (2005b) report.

Kim et al. (2009) reported one case of emetic outbreak caused by *B. cereus* in Korea. However, emesis risk might be underestimated due to misdiagnosis of the illness, which is symptomatically similar to staphyloenterotoxicosis (Schoeni and Wong, 2005). The presented approach can precisely distinguish between emetic toxin producing *B. cereus* and *S. aureus* and therefore solve this problem.

B. cereus has been incriminated in several foodborne outbreaks involving cereal products (including rice), pasta, meat, vegetables and milk (Martinez-Blanch et al., 2009). The developed triple-primer PCR assay capable of detecting small amounts of 7.6×10^2, 7.6×10^3 and 7.6×10^3CFU/ml of *B. cereus* F4810/72 bacterial cells in the broth medium, rice and milk, respectively, can be effectively used for the detection of *B. cereus* and particularly emetic strains in food samples. The detection limits in artificially inoculated milk and rice were found to be 10-fold lower than pure culture regardless of the bacterial strain. This was in agreement with previous studies (Kim et al., 2010b; Lin and Tsen, 1995; Lee and Fairchild, 2006; Alarcon et al., 2005). Milk and rice contain high levels of cations, proteases, nucleases and fatty acids that may act as PCR inhibitors, binding and degrading the polymerase or the DNA template (Lee et al., 2008). This might be the cause of low detection limit levels in milk and rice. Therefore, further studies should be conducted with the view of improving the methods of DNA isolation from bacterial cells in milk and rice or other methods that can increase the sensitivity of the approach. Agata et al. (1994) and Häggblom et al. (2002) reported cereulide concentrations of 5 µg/ml in 10% skim milk and 1 µg/ml in TSB cultures of *B. cereus* strain NC7401 grown at 30°C.

In conclusion, the present triple-primer PCR assay can be useful for rapid detection of *B. cereus* and specifically emetic toxin strains in food samples and for preventing the misdiagnosis of *B. cereus* emetic strains.

REFERENCES

Agata N, Mori M, Ohta M, Suwan S, Ohtani I, Isobe M (1994). A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. FEMS Microbiol. Lett. 121:31-34.

Alarcon B, Vicedo B, Aznar R (2005). PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. J. Appl. Microbiol. 100:352-364.

Andersson MA, Mikkola R, Helin J, Andersson MC, Salkinoja-Salonen M (1998). A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptideionophores. Appl. Environ. Microbiol. 64(4):1338-1343.

Chang YH, Shangkuan YH, Lin HC, Liu HW (2003). PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. Appl. Environ. Microbiol. 69(8):4502-4510.

Dommel MK, Frenzel E, Strasser B, Blőchinger C, Scherer S, Ehling-Schulz M (2010). Identification of the main promoter directing cereulide biosynthesis in emetic *Bacillus cereus* and its application for real-time monitoring of *ces* gene expression in foods. Appl. Environ. Microbiol. 76(4):1232-1210.

Ehling-Schulz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S (2006b). Cereulidesynthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. BMC Microbiol. 6(1):20-31.

Ehling-Schulz M, Guinebretiere MH, Monthán A, Berge O, Fricker M, Svensson B (2006a). Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol. Lett. 260(2):232-240.

Ehling-Schulz M, Svensson B, Guinebretiere MH, Lindbäck T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Märtlbauer E (2005b). Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology 151(1):183-197.

Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E, Scherer S (2005a). Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production if emetic *Bacillus cereus.* Appl. Environ. Microbiol. 71(1):105-113.

Granum PE (1994). *Bacillus cereus* and its toxins. J. Appl. Microbiol. 76:61-66.

Häggblom MM, Apetroaie C, Andersson MA, Salkinoja-Salonen MS (2002). Quantitative analysis cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. Appl. Environ. Microbiol. 68(5):2479-2483.

Horwood PF, Burgess GW, Jane Oakey H (2004). Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. FEMS Microbiol. Lett. 236(2):319-324.

Jenson I, Moir CJ (2003). *Bacillus cereus* and other *Bacillus* species. In Hocking (eds) Foodborne microorganisms of public health significance, 6th Edition, AIFST Inc, Australia, pp. 445-478.

Kim JB, Kim JM, Kim CH, Seo KS, Park YB, Choi NJ, Oh DH (2010b). Emetic toxin producing *Bacillus cereus* Korean isolated contain genes encoding diarrheal-related enterotoxins. Int. J. Food Microbiol. 144(1):182-186.

Kim JB, Kim JM, Kim SY, Kim JH, Park YB, Choi NJ, Oh DH (2010c). Comparison of enterotoxin production and phenotypic characteristics between emetic and enterotoxic *Bacillus cereus*. J. Food Prot. 73(7):1219-1224.

Kim JB, Kim JM, Park YB, Han JA, Lee SH, Kwak HS, Hwang IG, YoonMH, Lee JB, Oh DH (2010a). Evaluation of various PCR assays for detection of emetic-toxin-producing *Bacillus cereus*. J. Microbiol. Biotechnol. 20(7):1107-1113.

Kim JB, Lim EG, Jang HC, Park JY, Lee SJ, Park MS, Choi GB, Lee BK (2009). A case of emetic toxin producing *Bacillus cereus* strains isolated from outbreak. Korean J. Clin. Microbiol. 12(1):48-52.

Kotiranta A, Lounatmaa K, Haapasalo M (2000). Epidemiology and

pathogenesis of *Bacillus cereus* infections. Microb. Infect. 2(2):189-198.
Lee DS, Kim KS, Kwon KS, Hong KW (2008). A multiplex PCR assay for the detection and differentiation of enterotoxin-producing and emetic toxin-producing *Bacillus cereus* strains. Food Sci. Biotechnol. 17(4):761-765.
Lee MD, Fairchild A (2006). Sample preparation for PCR. PCR methods in foods, (Maurer JJ ed), Springer, New York, NY, USA, pp. 41-50.
Lin CK, Tsen HY (1995). Development and evaluation of two novel oligonucleotide probes based on 16S rRNA sequence for the identification of Salmonella in foods. J. Appl. Microbiol. 78(5):507-520.
Makarasen A, Yoza K, Isobe M (2009). Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus.* Chem. Asian J. 4(5):688-698.
Malkawi HI, Gharaibeh R (2003). Multiplex PCR for the direct of *Salmonella enteric* from chicken, lamb and beef food products. J. Basic Microbiol. 43(4):328-336.
Martinez-Blanch JF, SánchezG, Garay E, Aznar R (2009). Development of a real-time PCR assay for detection and quantification of enterotoxigenic members of *Bacillus cereus* group in food samples. Int. J. Food Microbiol. 135(1):15-21.
Park SH, Kim HJ, Kim JH, Kim TW, Kim HY (2007). Simultaneous detection and identification of *Bacillus cereus* group bacteria using multiplex PCR. J. Microbiol. Biotechnol. 17(7):1177-1182.
Rajkovic A, Uyttendaele M, Deley W, VanSoom A, Rijsselaere T, Debevere J (2006a). Dynamic of boar semen motility inhibition as a semi-quantitative measurement of *Bacillus cereus* emetic toxin (Cereulide). J. Microbiol. Methods 65(3):525-534.
Rajkovic A, Uyttendaele M, Ombregt SA, Jaaskelainen E, Salkinoja-Salonen M, Debevere J (2006b). Influence of type of food on the kinetics and overall production of *Bacillus cereus* emetic toxin. J. Food Prot. 69(4):847-852.
Schoeni JL, Wong AC (2005). *Bacillus cereus* food poisoning and its toxins. J. Food Prot. 68(3):636-648.
Seong SJ, Lim JS, Lee KG, Lee SJ, Hong KW (2008). Toxin Gene profiling of *Bacillus cereus* food isolates by PCR. J. Korean Soc. Appl. Biol. Chem. 51(4):263-268.
Taylor JMW, Sutherland AD, Aidoo KE, Logan NA (2005). Heat-stable toxin production by strains of *Bacillus cereus, Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus licheniformis*. FEMS Microbiol. Lett. 242(2):313-317.