Detection of viable enterotoxin-producing *Bacillus cereus* and analysis of toxigenicity from ready-to-eat foods and infant formula milk powder by multiplex PCR

Zhihong Zhang,* Lixia Feng,* Hengyi Xu,* Chengwei Liu,† Nagendra P. Shah,‡ and Hua Wei*¹

*State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

†Institute of Nutrition and Food Safety, Jiangxi Provincial Centre for Disease Control, Nanchang 330046, P. R. China

‡Food and Nutritional Science, School of Biological Science, The University of Hong Kong, Pokfulam Road, Hong Kong, China

ABSTRACT

Bacillus cereus is responsible for several outbreaks of foodborne diseases due to its emetic toxin and enterotoxin. Enterotoxins, cytotoxin K (CytK), nonhemolytic enterotoxin (Nhe), and hemolysin BL (Hbl), have been recorded in several diarrheal cases due to food poisoning from *B. cereus*. The objective of this study was to develop a rapid and accurate method that combines multiplex PCR with propidium monoazide to selectively detect viable cells of enterotoxin-producing *B. cereus* in milk powder, noodles, and rice, and investigate the distribution of enterotoxins in 62 strains of *B. cereus* in Jiangxi province, China. The specificity of primers of 3 enterotoxins (i.e., *cytK*, *nheA*, and *hblD*) of *B. cereus* was verified by inclusivity and exclusivity tests using single PCR. Upon optimization of multiplex PCR conditions, it was found that the detection limit of viable cells was 10^2 cfu/mL of *B. cereus* in pure culture. By enrichment for 3 or 4 h and propidium monoazide pretreatment, a protocol for detection of viable cells as low as 2.2×10^1 cfu/g in spiked food (e.g., milk powder, noodles, and rice) was established and proved valid even under the interference of non-*Bacillus cereus* at as high as 10^5 cfu/g. Moreover, the protocol based on multiplex PCR for detection was applied for the analysis of distribution of toxin gene of *B. cereus*, and the results showed a regional feature for toxin gene distribution, indicating that potential toxigenicity of *B. cereus* should be evaluated further.

Key words: *Bacillus cereus*, enterotoxin-producing, multiplex PCR, propidium monoazide, toxigenicity

INTRODUCTION

Bacillus cereus is a ubiquitously distributed soil microorganism associated with reheated or inadequately

cooked foods [e.g., fried rice, noodles, milk, and other dairy products (Stenfors Arnesen et al., 2008; Hwang and Park, 2015)]; it could colonize the gut of humans and invertebrates as an opportunistic pathogen (Ghosh, 1978; Jensen et al., 2003) resulting in bacteremia, meningitis, endophthalmitis, and pneumonia in hospitals (Bottone, 2010). It also gives rise to white patch disease on *Litopenaeus vannamei*, leading to gradual mortality (Velmurugan et al., 2015).

For 6 decades, foodborne diseases from *B. cereus* occurred mostly in European countries and North America (Beattie, 1999; Hwang and Park, 2015) since its first outbreak in Norway during 1947 to 1949 (Hauge, 1950, 1955). Due to strong thermotolerance of spores against high pasteurization temperature, *B. cereus* has become a frequent foodborne disease worldwide (Singh and Chaturvedi, 2015). For instance, a healthy 39-yr-old woman started vomiting and suffered from watery diarrhea after eating microwave-heated chicken (López et al., 2015). It was demonstrated that toxin led to the typical symptoms of emesis and diarrhea due to consumption of food contaminated with *B. cereus* ($>10^5$ cfu/g; Hwang and Park, 2015). Emetic toxin was attributed to peptide cereulide, a thermostable and acid-resistant depsipeptide toxin. Among those toxins causing diarrhea, 5 enterotoxins have been commonly studied. Nonhemolytic enterotoxin (Nhe) and hemolysin BL (Hbl) are both composed of 3 proteins, whereas cytotoxin K (CytK), enterotoxin FM (EntFM), and BceT are composed of 1 protein only (Hwang and Park, 2015). The Nhe complex is encoded by *nheA*, *nheB*, and *nheC*, and the Hbl complex by *hblA*, *hblC*, and *hblD* (Bonerba et al., 2010). The CytK toxin induces diarrhea similar to Nhe and Hbl toxins (Lund and Granum, 1997). In those 5 toxins, only Nhe, Hbl, and CytK were demonstrated to be responsible for food poisoning in the gastrointestinal tract (Fagerlund et al., 2010; Berthold-Pluta et al., 2015; Hwang and Park, 2015).

Generally, rapid, accurate, and internationally accepted methods for determining the causes of foodborne pathogens are of utmost importance for the clinical

Received July 21, 2015.

Accepted September 27, 2015.

¹Corresponding author: weihua114@live.cn

diagnostics and monitoring of food hygiene in the food industry (Garrido et al., 2013). The current protocol for detection of *B. cereus* by traditional selective plating and biochemical studies is time consuming and laborious (Zhang et al., 2014). Moreover, conventional media often lead to substantial misidentification and underestimation of *B. cereus* (Fricker et al., 2008) due to interference by nontarget microorganisms in food samples. Alternatively, PCR is regarded as a promising method for the detection and characterization of bacteria in a wide range of samples (Garrido et al., 2013); multiplex PCR (mPCR) can be used for simultaneous amplification of various specific DNA sequences in a single reaction, with the advantage of saving time and costs (Park and Ricke, 2015). However, the lack of differentiation between DNA from viable and dead cells seriously restricts routine applications of detection methods based on DNA (Nocker et al., 2009; Seinige et al., 2014). Targeting at differentiation of viable and dead cells, pretreatment with DNA-intercalating dyes [e.g., propidium monoazide (PMA) or ethidium monoazide] has been applied to efficiently eliminate false-positive results due to dead cells. In our previous study, a combination of PMA treatment with mPCR has been successfully used for the detection of emetic and non-emetic *B. cereus* (Zhang et al., 2014). Moreover, the universal applicability of that protocol for enterotoxin detection of *B. cereus* needs to be studied.

Therefore, in this study, we developed a PMA combined with mPCR method for simultaneous and selective detection of 3 enterotoxin genes in one type strain of *B. cereus* (ATCC14579), and assessed the general suitability for the toxigenicity characterization of *B. cereus* isolated from ready-to-eat foods and infant formula milk powder. To our knowledge, this is the first study of using PMA-mPCR to set up simultaneous detection of relevant enterotoxin genes (*cytK*, *nheA*, and *hblD*) of *B. cereus* involved in several food poisoning cases in humans. This PMA-mPCR protocol might provide a model to set up an efficient detection tool for monitoring food contaminants from pathogens besides *B. cereus*, and thus ensure food safety in ready-to-eat foods or in food processing.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacillus cereus ATCC14579 was used as a type strain of enterotoxin-positive organism for 3 enterotoxin genes (i.e., *cytK*, *nheA*, and *hblD*; Ngamwongsatit et al., 2008). All bacterial strains belonging to 13 species were listed in Table 1 and used for inclusivity and exclusivity testing. All strains were cultivated accord-

ing to their individual protocols. *Candida albicans* was cultured in yeast extract peptone dextrose medium and *Micrococcus luteus* in nutrient broth at 30°C. However, *B. cereus* and other bacterial strains were cultured in Luria-Bertani medium at 37°C. All strains were cultured overnight and DNA templates were extracted by boiling method for the inclusivity and exclusivity assay (Zhang et al., 2014).

PMA Pretreatment and Genome DNA Extraction

The schematic of PMA-mPCR and process of PMA cross-link to DNA are shown in Figure 1 (<http://biotium.com/product/pmatm-dye-propidium-monoazide/>; van Frankenhuyzen et al., 2011). The PMA only cross-links to DNA of dead cells and eliminate the amplification signal. Dead cell preparation and PMA treatment were carried out as described previously (Zhang et al., 2014). The genome DNA was extracted as follows: 1 mL of the sample was centrifuged ($12,000 \times g$, 2 min, 4°C) and suspended in sterilized water using DNeasy Blood & Tissue Kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. Particularly, the cell pellet was treated with 180 μ L of enzymatic lysis buffer, and the debris was removed with the aim to enhance DNA purity. The purified DNA was stored at -20°C for further use.

Primer Design

The primer pairs used for the amplification of enterotoxin genes *cytK* (GenBank: DQ885233.1), *nheA* (GenBank: Y19005.2), and *hblD* (GenBank: U63928.1) are listed in Table 2. The primer was designed using Oligo 7.0 software (Molecular Biology Insights Inc., Colorado Springs, CO), and the specificity of the sequences was tested with the help of the National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). GenScript Co. Ltd. (Nanjing, China) offers a commercial service for providing primer.

PCR Conditions

The PMA-mPCR was performed using a final volume of 20 μ L in the mPCR system, including 3 μ L of the genome DNA, 10 μ L of $2 \times$ Taq mix (Novoprotein Scientific Inc., Shanghai, China), 0.175 μ M of each forward and reverse *cytK* primer, 0.25 μ M of each forward and reverse *nheA* primer, and 0.2 μ M of each forward and reverse *hblD* primer. Sterilized distilled water was supplemented to a final volume of 20 μ L, and 2 μ L of paroline was added last for sealing. For single and duplex PCR, all the parameters (the volume of genome DNA and Taq mix) were the same as mPCR except for

Table 1. Bacterial strains used for inclusivity and exclusivity study of single PCR testing

Bacteria strains	Strain ID	Source ¹	Single PCR result ²		
			<i>cytK</i>	<i>nheA</i>	<i>hblD</i>
<i>Bacillus cereus</i>	ATCC14579	ATCC	+	+	+
<i>Listeria monocytogenes</i>	ATCC13932	ATCC	—	—	—
<i>Candida albicans</i>	ATCC10231	ATCC	—	—	—
<i>Salmonella</i> Paratyphi	ATCC9150	ATCC	—	—	—
<i>Salmonella</i> Anatum	ATCC9270	ATCC	—	—	—
<i>Salmonella</i> Choleraesuis	ATCC10708	ATCC	—	—	—
<i>Salmonella enteritidis</i>	ATCC13076	ATCC	—	—	—
<i>Salmonella</i> Typhimurium	ATCC13311	ATCC	—	—	—
<i>Shigella flexner</i>	ATCC29903	ATCC	—	—	—
<i>Staphylococcus aureus</i>	CMCC26001	CMCC	—	—	—
	CMCC26002	CMCC	—	—	—
<i>Serratia marcescens</i>	CMCC41002	CMCC	—	—	—
<i>Enterobacter sakazakii</i>	CMCC45401	CMCC	—	—	—
	CMCC45402	CMCC	—	—	—
<i>Micrococcus luteus</i>	CMCC28001	CMCC	—	—	—
<i>Pseudomonas aeruginosa</i>	CMCC10104	CMCC	—	—	—
<i>Proteus vulgaris</i>	CMCC49101	CMCC	—	—	—
<i>Escherichia coli</i> O157:H7	NCTC12900	NCTC	—	—	—
<i>Vibrio parahaemolyticus</i>	PVPA0155	JX-CDC	—	—	—

¹ATCC = American Type Culture Collection, Rockville, Maryland; CMCC = China Medical Culture Collection, Beijing, China; NCTC = National Collection of Type Cultures, London, UK; JX-CDC = Jiang Xi Province Center for Disease Control and Prevention, Nanchang, China.

²+ and — indicate positive and negative signal, respectively.

Figure 1. Schematic of selective detection of viable cells using propidium monoazide (PMA)-multiplex PCR (A) and proposed mechanism of PMA covalently cross-linked to DNA (B). Color version available online.

Table 2. Oligonucleotide primers used in this study (F = forward; R = reverse)

Target	Primer sequence (5' → 3')	Amplicon length (bp)	Reference
<i>cytK</i>	cytK-F: ATCGGTCAAAATGCAAAAACAC cytK-R: ACCCAGTTACCAGTTCCGAATG	800	This study
<i>nheA</i>	nheA-F: AGGTAAATGCGATGAGTAG nheA-R: TTGTTGAATGCCAAGAG	617	This study
<i>hblD</i>	hblD-F: GTTAGATACAGCGAAGCCAC hblD-R: CCGCCAGTTACAACAATA	465	This study

0.2 μ M of each primer. The PCR reaction began with 1 cycle of 10 min at 95°C, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 52.2°C for 30 s, and elongation at 72°C for 30 s, and then an extra elongation step of 10 min at 72°C. After PCR amplification, the products were removed and subjected to 2% agarose gel electrophoresis and the gels were stained with GoldView (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) before imaging under the UV transilluminator (Bio-Rad, Hercules, CA).

Detection of Viable *B. cereus* Cells in Spiked Rice, Noodle, and Milk Powder Using PMA-mPCR

Fresh rice, noodles, and milk powder (Wondersun Inc., Heilongjiang, China) purchased from a local supermarket were used for spiking studies. All the food samples were previously confirmed negative for *B. cereus* by plate count and standard PCR. Each sample (25 g) was inoculated with 2.2×10^1 cfu/g *B. cereus* ATCC14579 and mixed with 225 mL of Luria-Bertani and homogenized for 2 min (Li and Chen, 2013). One milliliter of each enriched cultures was collected at 2, 3, 4, and 5 h after incubation at 37°C with shaking at 180 rpm. Additionally, to determine whether the presence of non-*Bacillus cereus* interferes with the detection of viable *B. cereus* in foods, 10^5 cfu/g of *Staphylococcus aureus* CMCC26001 and *Salmonella enteritidis* ATCC 13076 were added after the 3 spiked foods (inoculated with 2.5×10^1 cfu/g) pre-incubation for 4, 3, and 4 h, respectively. The collected samples were centrifuged at $900 \times g$ for 1 min at 4°C to remove large debris, and the suspensions were centrifuged at $12,000 \times g$ for 5 min at 4°C (Zhang et al., 2015). The pellets were washed twice and suspended in sterilized water, genome DNA was extracted after PMA pretreatment, and then analyzed by using PMA-mPCR. All of the tests were repeated in triplicate.

RESULTS

Inclusivity and Exclusivity of PCR Primers

The specificity of the primers was assessed by inclusivity and exclusivity test using single PCR on 13 spe-

cies, including 9 types of common foodborne pathogens (e.g., *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp.), and other pathogens (e.g., *Candida albicans* and *Pseudomonas aeruginosa*; Table 1). Only *B. cereus* ATCC 14579 was proven to possess 3 toxic genes (i.e., *cytK*, *nheA*, and *hblD*), whereas other microorganisms did not possess these toxic genes.

To avoid false-positive results during PCR processing, a sequencing of the PCR products was performed. The sequence of 3 PCR products was compared in the GenBank database using BLAST (available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The results showed that the reliability of *cytK*, *nheA*, and *hblD* was 96, 97, and 97%, respectively, corresponding to type strain of *B. cereus* ATCC14579. Thus, the primers were reliable for identification of the 3 enterotoxins of *B. cereus*.

Optimization of Multiplex PCR for Detection of 3 Enterotoxin Genes

To set up the mPCR method for simultaneous detection of 3 toxic genes of *cytK*, *nheA*, and *hblD*, a single PCR and mPCR were applied. As shown in Figure 2, all the genes were simultaneously detected, the primers for *cytK*, *nheA*, and *hblD* produced amplicons of 800, 617, and 465 bp, respectively.

Considering primer concentration, annealing temperature and cycles might affect the efficiency of mPCR, so several tests were performed by varying annealing temperatures from 49.9 to 60.1°C, primer concentrations from 0.1 to 0.4 μ M, and cycles from 30 to 35. Thereafter, the optimal parameters for mPCR were the combination of concentration of primer for *cytK*, *nheA*, and *hblD* of 0.175, 0.25, and 0.2 μ M, respectively, and optimum annealing temperature at 52.2°C as well as cycles of 35 (data not shown).

The Limit of Detection of Cells in Pure Culture with PMA Pretreatment

To differentiate whether the contamination with *B. cereus* was in state of viable cells or dead cells, we introduced PMA pretreatment for the detection of *B. cereus*

Figure 2. Detection of the 3 enterotoxin genes using single and multiplex PCR. M = DL2000 DNA marker; the 800-, 617-, and 465-bp PCR products were produced by the primers for *cytK*, *nheA*, and *hblD*, respectively.

in pure culture by mPCR using optimal conditions as above. As shown in Figure 3, PCR products were performed in lanes by viable cell counts from 5.5×10^8 to 5.5×10^1 cfu/mL, whereas in lane 8 ($< 5.5 \times 10^2$ cfu/mL) no signal was found. In contrast, all the lanes corresponding to 5.5×10^8 to 5.5×10^1 cfu/mL dead cells had no signals. The results showed that the limit of detection (LOD) of viable cells was 5.5×10^2 cfu/mL in pure culture, which indicated that PMA treatment was efficient and essential before DNA extraction.

Selective Detection of Viable *B. cereus* in Spiked Foods

To verify the validity of PMA-mPCR protocol for detection of viable cell count of *B. cereus* in foods, 3

types of spiked foods (milk powder, noodles, and rice) with 2.2×10^1 cfu/g of *B. cereus* were investigated. As shown in Figure 4, the protocol determined in pure culture was also suitable for all test foods; namely, below the LOD in pure culture (5.5×10^2 cfu/mL), the viable cell counts of *B. cereus* could be detected in spiked foods (milk powder, noodle, and rice samples, with an initial inoculation of 2.2×10^1 cfu/g) after enrichment for 4, 3, and 4 h, respectively.

Detection of Viable *B. cereus* in the Presence of Non-*B. cereus* and Heat-Killed *B. cereus* in the Absence of Non-*B. cereus* in Spiked Foods

Although the protocol for detection of viable cells was verified in spiked foods, whether the presence of non-*B. cereus* interferes with the protocol is unknown. Therefore, a further verification test was performed on the former assay by additional inoculation of 10^5 cfu/g of non-*B. cereus*; namely, viable cells of *Staphylococcus aureus* and *Salmonella enterica* were added after the 3 spiked food pre-incubation for 4, 3, and 4 h, respectively. As shown in Figure 5, positive detection of *B. cereus* in all samples was achieved, confirming the suitability of our protocol as described in Figure 4, and also indicating that non-*Bacillus cereus* indeed did not affect an accurate detection of this organism.

Moreover, to verify that our protocol could detect viable cells, but not dead cells of *B. cereus* in foods in general, after the same enrichment time with 2.5×10^1 cfu/g of *B. cereus*, all samples were heated to kill the bacteria and performed with PMA treatment and mPCR assay. Also, shown in Figure 5, negative results were presented, indicating that the method was valid

Figure 3. The limit of detection of pure culture *Bacillus cereus* (viable or dead) using 10-fold serially diluted cultures. Lanes 1 to 8 (or a to h), from 5.5×10^8 to 5.5×10^1 cfu/mL. Dead cells of *B. cereus* were prepared by water bath at 100°C for 15 min. The DNA was extracted after propidium monoazide pretreatment. M = DL2000 DNA marker.

Figure 4. Detection of viable *Bacillus cereus* spiked in foods by propidium monoazide (PMA)-multiplex PCR. Milk powder, noodles, and rice were inoculated with 2.2×10^1 cfu/g of viable cells and incubated at 37°C up to 5 h. Samples were collected at different enrichment time points and then treated with PMA before DNA extraction.

to eliminate the amplification signal of dead cells in spiked foods.

Toxigenicity Characterization of *B. cereus* Isolated from Ready-to-Eat Foods and Infant Formula Milk Powder

To verify the feasibility of the protocol for detection of epidemic strains of *B. cereus*, an investigation on the distribution of enterotoxin of *cytK*, *nheA*, and *hblD* in 62 isolates of *B. cereus* was carried out. As shown in Table 3, generally, among all the isolates from noodles, fried rice with egg, fried rice, rice, tikoy, apple butter, pork floss, cooked corn flour, and infant formula milk powder, 34 (55%) harbored the 3 toxic genes of *cytK*, *nheA*, and *hblD*, but only 3 (4.8%) did not. From noodles, more than 64% isolates possessed the 3 genes, whereas from cooked corn flour isolates possessing the 3 genes were 17%. The toxic genes, *cytK*, *nheA*, and *hblD*, were harbored in 79, 87, and 61% of isolates, respectively.

DISCUSSION

Several DNA-based methods are available for detection of *B. cereus*; however, most of them targeted qualitative detection of whole cells in the state of dead, or alive and unculturable or culturable. To our knowledge, very few protocols were reported for detecting viable *B. cereus* cells from foods or environmental samples. In this study, a protocol of mPCR was invented for simultaneous detection of enterotoxin genes of viable *B. cereus*, and therewith toxigenicity of *B. cereus* isolates was characterized. Our protocol was found to be

highly specific and sensitive for selective detection of viable cells of *B. cereus*, and PMA pretreatment was able to efficiently eliminate interference of dead cells of *B. cereus*.

Specific enterotoxin genes (i.e., *cytK*, *nheA*, and *hblD*) of *B. cereus* were chosen in the mPCR assay due

Figure 5. The detection of viable *Bacillus cereus* in the presence of non-*Bacillus cereus* and heat-killed *B. cereus* in the absence of non-*B. cereus* in spiked foods by propidium monoazide (PMA)-multiplex PCR. Lanes 1 and a represent contaminated milk powder, lanes 2 and b represent contaminated noodles, and lanes 3 and c represent contaminated rice. All the samples were inoculated with 2.5×10^1 cfu/g viable *B. cereus*. Non-*B. cereus*: spiked samples (1–3) enriched for 4, 3, and 4 h, respectively, and then inoculated with 10^5 cfu/g non-*B. cereus* before PMA treatment and DNA extraction. Dead bacteria: spiked samples (a–c) enriched for 4, 3, and 4 h, respectively, and then heat treatment before PMA treatment and DNA extraction.

to frequent outbreaks of foodborne diseases (Lund and Granum, 1997; Lund et al., 2000). Those primers were proven to be specific, and the LOD on pure culture of *B. cereus* was 5.5×10^2 cfu/mL for viable cells; in contrast, Forghani et al. (2015) proposed a protocol for detection of *B. cereus* at 10^2 cfu/mL. In their study, the cells might contain dead cells because no treatment by PMA was included. Recently, an improvement in sensitivity of detection for microorganisms was demonstrated (Settanni and Corsetti, 2007); nevertheless, for practical purposes, a direct detection of very low density of viable microbial cells is still unclear. In fact, pathogens (e.g., *B. cereus*, *Staphylococcus aureus*, and *Salmonella enteritidis*) are often present in very low numbers in food (Omiccioli et al., 2009). Thus, an enrichment step is still necessary not only to increase the density of target bacteria in samples but also to recover the physiologically stressed or injured cells (Garrido et al., 2013).

In this study, the spiked food (e.g., milk powder, noodles, and rice) with inoculation as low as $2.2 \times$

10^1 cfu/g was enriched and subsequently detected by PMA-mPCR, and a rapid detection of viable cells of *B. cereus* was completed in 3 or 4 h, even under the interference of non-*Bacillus cereus* at as high as 10^5 cfu/g in foods. The PMA treatment eliminated the false-positive results from dead cells and maintained the validity of mPCR for the detection of viable cells of *B. cereus*. This is accordance with our previous study (Zhang et al., 2014).

To further expand the application of the protocol, the toxigenicity of 62 *B. cereus* isolates from ready-to-eat foods and infant formula milk powder was characterized. *Bacillus cereus* produces at least 5 different enterotoxins, including CytK, Nhe, Hbl, EntFM, and BceT. The first 3 toxins are proved to be available in food contaminants, their distribution in food intoxication due to *B. cereus* is unclear (Hansen and Hendriksen, 2001; Ngamwongsatit et al., 2008; Hwang and Park, 2015), and the previous reports hypothesized that some isolates might cause food poisoning with only 1 or 2 genes among Nhe component or Hbl component,

Table 3. Toxigenicity characterization of *Bacillus cereus* isolated from ready-to-eat foods and infant formula milk powder

Source of isolation ¹ (no. of isolates)	Code of isolates ²		Multiplex PCR results ³		
			<i>cytK</i>	<i>nheA</i>	<i>hblD</i>
Infant formula milk powder (5)	JX0060LY	JX0067LY	+	+	+
	FX0130Y		+	–	+
	JX0061LY		–	+	–
	JX0074LY		–	–	–
Noodle (39)	DA0090L	JA0089	+	+	+
	JX0121LY	NC0071L	+	+	+
	NC0013L	JA0062	+	+	+
	DA0097L	JJ0398	+	+	+
	NC0076L	JX0119LY	+	+	+
	FX0088Y	NC0078L	+	+	+
	JA0064	DA0096L	+	+	+
	DA0092L	DA0094L	+	+	+
	DA0036L		+	+	+
	JA0056	NC0001L	+	+	–
	JA0061	JDZ0050Y	+	+	–
	JDZ0139Y	FX0093Y	+	–	+
	JX0076LY	NC0084LY	–	+	–
	YS0024L	JDZ0102Y	–	–	–
	DA0098L	DA0088L	+	+	+
Fried rice with egg (6)	DA0019L		+	+	+
	DA0020L	PZ0063L	+	+	–
	JX0125LY		+	+	–
Fried rice (1)	JJ0457LY		+	+	–
Rice (2)	JX0080LY		–	+	–
Tikoy (1)	JDZ0138Y		+	+	+
Apple butter (1)	JA0095		+	–	+
Pork floss (1)	JA0091		–	+	–
Cooked corn flour (6)	PX0053L16		+	+	+
	PX0040L8		+	+	–
	PX0057L19	PX0031L5	–	+	–
	PX0043L10		–	+	–

¹All the samples collected by JX-CDC (Nanchang, China).

²All the *B. cereus* isolated from the food samples.

³+ and – indicate positive and negative signal, respectively.

though evidence for their contribution to poisoning is unclear (Hansen and Hendriksen, 2001; Ngamwongsatit et al., 2008; Hwang and Park, 2015). In our study, 79% strains harbored *cytK* and more than 61% strains possessed *hblD*, which are consistent with previous studies that reported 48 to 77% and 48.7 to 89%, respectively, of strains harboring *cytK* and *hblD* (Ankolekar et al., 2009; Chaves et al., 2012; Chon et al., 2012; Lee et al., 2012; Hwang and Park, 2015). Additionally, more than 87% of 62 isolates possessed *nheA*, which is lower than the 100% of 39 strains of *B. cereus* reported earlier (Lee et al., 2012). Interestingly, 8 strains of 62 isolates possessed only *nheA*, and no strains possessed only *cytK* or *hblD*.

In conclusion, a specific, rapid, and sensitive protocol for detection of viable cells of *B. cereus* in milk powder, noodles, and rice was developed. By combination of PMA pretreatment and mPCR, a selective detection of *B. cereus* at 10^1 cfu/g from spiked food samples was completed upon 3 or 4 h of enrichment, and even under the interference from non-*Bacillus cereus* at a high concentration. Moreover, the toxin gene distribution of *B. cereus* was analyzed with the aid of this protocol, which should be helpful to understand the potential toxigenicity of *B. cereus* in food poisoning cases.

ACKNOWLEDGMENTS

This work was supported by Academic and Technical Leaders Training Program for Major Subjects of Jiangxi Province (2009), Ganpo Talent 555 Engineering Project of Jiangxi Province, and Graduate Student Innovation Special Funds of Jiangxi Province (YC2015-B013), China.

REFERENCES

- Ankolekar, C., T. Rahmati, and R. G. Labbé. 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in US rice. *Int. J. Food Microbiol.* 128:460–466.
- Beattie, S. H. 1999. Detection of toxins. *Int. J. Food Sci. Technol.* 45:141–148.
- Berthold-Pluta, A., A. Pluta, and M. Garbowska. 2015. The effect of selected factors on the survival of *Bacillus cereus* in the human gastrointestinal tract. *Microb. Pathog.* 82:7–14.
- Bonerba, E., A. Di Pinto, L. Novello, F. Montemurro, V. Terio, V. Colao, G. Ciccarese, and G. Tantillo. 2010. Detection of potentially enterotoxigenic food-related *Bacillus cereus* by PCR analysis. *Int. J. Food Sci. Technol.* 45:1310–1315.
- Bottone, E. J. 2010. *Bacillus cereus*, a volatile human pathogen. *Clin. Microbiol. Rev.* 23:382–398.
- Chaves, J. Q., C. F. G. Cavados, and A. M. Vivoni. 2012. Molecular and toxigenic characterization of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from commercial ground roasted coffee. *J. Food Prot.* 75:518–522.
- Chon, J., J. Kim, S. Lee, J. Hyeon, and K. Seo. 2012. Toxin profile, antibiotic resistance, and phenotypic and molecular characterization of *Bacillus cereus* in Sunsik. *Food Microbiol.* 32:217–222.
- Fagerlund, A., T. Lindbäck, and P. E. Granum. 2010. *Bacillus cereus* cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. *BMC Microbiol.* 10:304–312.
- Forghani, F., T. Langae, M. Eskandari, K. Seo, M. Chung, and D. Oh. 2015. Rapid detection of viable *Bacillus cereus* emetic and enterotoxic strains in food by coupling propidium monoazide and multiplex PCR (PMA-mPCR). *Food Contr.* 55:151–157.
- Fricker, M., R. Reissbrodt, and M. Ehling-Schulz. 2008. Evaluation of standard and new chromogenic selective plating media for isolation and identification of *Bacillus cereus*. *Int. J. Food Microbiol.* 121:27–34.
- Garrido, A., M.-J. Chapela, B. Román, P. Fajardo, J. Lago, J. M. Vieites, and A. G. Cabado. 2013. A new multiplex real-time PCR developed method for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Contr.* 30:76–85.
- Ghosh, A. 1978. Prevalence of *Bacillus cereus* in the faeces of healthy adults. *J. Hyg. (Lond.)* 80:233–236.
- Hansen, B. M., and N. B. Hendriksen. 2001. Detection of enterotoxigenic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl. Environ. Microbiol.* 67:185–189.
- Hauge, S. 1950. Food poisoning caused by *Bacillus cereus*. *Nord. Hyg. Tidskr.* 31:189–206.
- Hauge, S. 1955. Food poisoning caused by aerobic spore-forming bacilli. *J. Appl. Bacteriol.* 18:591–595.
- Hwang, J., and J. Park. 2015. Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. *J. Dairy Sci.* 98:1652–1660.
- Jensen, G., B. Hansen, J. Eilenberg, and J. Mahillon. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. *Environ. Microbiol.* 5:631–640.
- Lee, N., J. M. Sun, K. Y. Kwon, H. J. Kim, M. Koo, and H. S. Chun. 2012. Genetic diversity, antimicrobial resistance, and toxigenic profiles of *Bacillus cereus* strains isolated from Sunsik. *J. Food Prot.* 75:225–230.
- Li, B., and J.-Q. Chen. 2013. Development of a sensitive and specific qPCR assay in conjunction with propidium monoazide for enhanced detection of live *Salmonella* spp. in food. *BMC Microbiol.* 13:273.
- López, A. C., J. Minnaard, P. F. Pérez, and A. M. Alippi. 2015. A case of intoxication due to a highly cytotoxic *Bacillus cereus* strain isolated from cooked chicken. *Food Microbiol.* 46:195–199.
- Lund, T., M. L. De Buyser, and P. E. Granum. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol.* 38:254–261.
- Lund, T., and P. E. Granum. 1997. Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. *Microbiology* 143:3329–3336.
- Ngamwongsatit, P., W. Buasri, P. Pianariyanon, C. Pulsrikarn, M. Ohba, A. Assavanig, and W. Panbangred. 2008. Broad distribution of enterotoxin genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. *Int. J. Food Microbiol.* 121:352–356.
- Nocker, A., A. Mazza, L. Masson, A. K. Camper, and R. Brousseau. 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. *J. Microbiol. Methods* 76:253–261.
- Omiccioli, E., G. Amagliani, G. Brandi, and M. Magnani. 2009. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiol.* 26:615–622.
- Park, S. H., and S. C. Ricke. 2015. Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm.* Enteritidis, *Salm.* Heidelberg and *Salm.* Typhimurium. *J. Appl. Microbiol.* 118:152–160.
- Seinige, D., M. von Köckritz-Blickwede, C. Krschek, G. Klein, and C. Kehrenberg. 2014. Influencing factors and applicability of the viability EMA-qPCR for a detection and quantification of *Campylobacter* cells from water samples. *PLoS ONE* 9:e113812.

- Settanni, L., and A. Corsetti. 2007. The use of multiplex PCR to detect and differentiate food-and beverage-associated microorganisms: A review. *J. Microbiol. Methods* 69:1–22.
- Singh, V. K., and S. S. A. Chaturvedi. 2015. Study the incidence of *Bacillus cereus* isolates from dairy foods. *Pharma Innovation J.* 3:41–43.
- Stenfors Arnesen, L. P., A. Fagerlund, and P. E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32:579–606.
- van Frankenhuyzen, J. K., J. T. Trevors, H. Lee, C. A. Flemming, and M. B. Habash. 2011. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. *J. Microbiol. Methods* 87:263–272.
- Velmurugan, S., P. Palanikumar, P. Velayuthani, M. Donio, M. M. Babu, C. Lelin, S. Sudhakar, and T. Citarasu. 2015. Bacterial White Patch Disease caused by *Bacillus cereus*, a new emerging disease in semi intensive culture of *Litopenaeus vannamei*. *Aquaculture* 44:49–54.
- Zhang, Z., W. Liu, H. Xu, Z. P. Aguilar, N. P. Shah, and H. Wei. 2015. Propidium monoazide combined with real-time PCR for selective detection of viable *Staphylococcus aureus* in milk powder and meat products. *J. Dairy Sci.* 98:1625–1633.
- Zhang, Z., L. Wang, H. Xu, Z. P. Aguilar, C. Liu, B. Gan, Y. Xiong, W. Lai, F. Xu, and H. Wei. 2014. Detection of non-emetic and emetic *Bacillus cereus* by propidium monoazide multiplex PCR (PMA-mPCR) with internal amplification control. *Food Contr.* 35:401–406.