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

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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 caused 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)], and 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

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diagnostics and monitoring of food hygiene in the food industry (Garrido et al., 2013). Up-to-date 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 nonemetic *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 according 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 × *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 μL 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 μL in the mPCR system, including 3 μL of the genome DNA, 10 μL of 2 × 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 μL, 2 μL 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 0.2