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Detection of non-emetic and emetic *Bacillus cereus* by propidium monoazide multiplex PCR (PMA-mPCR) with internal amplification control

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ABSTRACT

Bacillus cereus is an etiological agent of food-borne disease that can cause a type of emesis. To develop a sensitive and reliable diagnosis technique for detecting all the species of the *B. cereus* group, specific primers were designed to target a recently discovered part of the cereulide synthetase gene (*cesB*) for emetic *B. cereus* and *16S rRNA* for non-emetic *B. cereus*. To detect PCR signals only from viable cells, propidium monoazide (PMA) was selected to eliminate the false-positive results. In addition, an internal amplification control (IAC) was applied to meet diagnostic multiplex PCR requirements that will prevent the occurrence of false-negative results. The inclusivity and exclusivity of the mPCR assay were estimated using a panel of 100 strains, including 2 emetic *B. cereus*, 77 non-emetic *B. cereus* and 21 non-*Bacillus* strains. The limit of detection (LOD) for dead *B. cereus* without PMA treatment in pure bacteria culture was 4.0×10^2 CFU/mL, as low as 7.5×10^0 CFU/mL for viable *B. cereus* without PMA treatment, and 7.5×10^1 CFU/mL for viable *B. cereus* with PMA treatment. *B. cereus* in spiked food produce was detected specifically and sensitively at 1.0×10^3 CFU/g which was the lowest concentration detected. This novel PMA-mPCR-IAC assay is rapid and reliable, providing an efficient diagnostic tool with promising application in monitoring food samples.

1. Introduction

Bacillus cereus is increasingly recognized as a human pathogen of food-borne diseases that causes food poisoning outbreak caused by the emetic toxin cereulide (Fricker, Messelhäußer, Busch, Scherer, & Ehling-Schulz, 2007). Two different clinical symptoms of food poisoning caused by *B. cereus* are distinctly distinguished as emesis and diarrhea. The emesis type is connected to a heat-stable depsipeptide toxin which is called cereulide, while the diarrhea type is a result of heat-labile enterotoxins (Granum & Lund, 2006). Since *B. cereus* can be found in soil ubiquitously and commonly, it can consequently be easily spread to various kinds of food that are of plant origin which are found in our daily diet (Dzieciol, Fricker, Wagner, Hein, & Ehling-Schulz, 2013). Moreover, *B. cereus* is a heat-resistant etiological agent that can still grow well after inexhaustive cooking which causes spore germination (Granum & Lund, 2006). Food poisoning from *B. cereus* is usually related to cooked rice, Chinese noodles, pasta, meat products, vegetables and milk products (Ehling-Schulz, Fricker, & Scherer, 2004; Shiota et al., 2010).

In recent years, several poisoning cases caused by emetic *B. cereus* that gave rise to hospitalization or death have been reported. In June 2006 in Germany, seventeen children (3–5 years old) began vomiting and collapsed in a day care center an hour after

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eating a rice dish with vegetables. Diagnosis from the left-over food showed 10^4 CFU/g *B. cereus* detection (Fricker et al., 2007). In August 2003, 5 children (7–14 years of age) of a Belgian family started respiratory distress and vomiting after eating pasta salad that was stored for three days in the fridge; the seven-year old girl died 13 h after the meal (Dierick et al., 2005). Consequently, *B. cereus* is a potential threat to public health and the food industry (Dzieciol et al., 2013).

Rapid and accurate detection of emetic and non-emetic *B. cereus* from food-borne outbreaks is difficult. Conventional detection methods for *B. cereus* generally relies on selective medium and serological studies, which may include homogenization, enrichment, enumeration, antibiotic susceptibility, and toxicity studies of flagellar antigens have been used extensively (Drobniewski, 1993). Nevertheless, these are all time-consuming and laborious processes. Moreover, conventional selective plating media often leads to underestimation of food-borne diseases and substantial misidentification when caused by *B. cereus* (Fricker, Reissbrodt, & Ehling-Schulz, 2008). Thus, a highly specific and accurate method for the detection of emetic and non-emetic *B. cereus* in food, without false-positive results, is urgently needed.

Polymerase chain reaction (PCR) is a rapid and sensitive technique for pathogenic bacterium detection and identification in food (Wang, Li, & Mustapha, 2009). However, conventional PCR cannot distinguish viable from dead cells (Wang & Levin, 2006). DNA from dead microorganisms can also serve as a template during PCR amplification (Nogva, Dromtorp, Nissen, & Rudi, 2003). The inability of discrimination between DNA from viable and dead cells has seriously hampered the general application of almost all of the diagnostic DNA-based methods (Nocker, Mazza, Masson, Camper, & Brousseau, 2009; Nogva et al., 2003; Wan et al., 2012). More recently, ethidium monoazide (EMA) and propidium monoazide (PMA) which eliminate positive signals from dead bacteria have been developed. EMA and PMA are DNA-intercalating dyes with azide group allowing cross-linking to DNA upon exposure to visible light (Nocker, Cheung, & Camper, 2006). These dyes can selectively penetrate compromised membranes of dead cells, intercalate into the DNA of heat- and chlorine-treated cells, and can be covalently cross-linked to the DNA (Chang et al., 2009; Nocker et al., 2006; Nocker, Sossa-Fernandez, Burr, & Camper, 2007; Wan et al., 2012). However, recent reports confirmed that EMA can also penetrate the membranes of viable cells of some bacterial species and induced degradation of a portion of the genomic DNA (Chang et al., 2009; Clayton, Kleanthous, Coates, Morgan, & Tabaqchali, 1992; Nocker et al., 2006). Hence, EMA is not as useful as PMA in distinguishing viable from dead organisms.

In this research, PMA was selected in combination with multiplex PCR (mPCR) for the specific detection of emetic and non-emetic *B. cereus*. The PMA-mPCR assay uses highly specific primers based on 16S rRNA gene and the specific gene *cesB* (GenBank ID: AY691650.1) which codes cereulide synthetase of *B. cereus*. In addition, an internal amplification control (IAC) was included using 16S rRNA primers to eliminate false-negative results caused by interference factors. Thus, a diagnostic mPCR combined with PMA in the presence of an IAC assay for a one-step detection and discrimination of emetic *B. cereus* and the rest of the *B. cereus group* in food was developed for the first time. This mPCR-PMA-IAC assay is developed as a diagnostic tool for emetic and non-emetic *B. cereus* in response to government requirements for implementation in food and clinical diagnostic laboratories in China.

2. Materials and methods

2.1. Bacterial strains and DNA templates

The bacterial strains used in this study for inclusivity and exclusivity testing of the PMA-mPCR-IAC assay are listed in Table 1. All bacterial strains were cultivated according to their individual requirements. *Micrococcus luteus* was cultured in Nutrient Broth at 30 °C, *B. cereus* and other bacterial strains were cultured in Luria-Bertani (LB) medium at 37 °C. Emetic *B. cereus* JDZ102Y was used to establish and optimize the mPCR assay. Non-emetic *B. cereus* NC0084LY and emetic *B. cereus* JDZ102Y were used to spike and prepare the contaminated food samples.

DNA templates for detecting the specificity of PCR were obtained using a single colony that was suspended in tubes containing 100 μL of phosphate-buffered saline (PBS) and boiled in a water bath for 10 min. The tubes were centrifuged at 9000 × *g* for 3 min, and the supernatant containing the DNA was treated as the template.

2.2. Preparation of viable and dead B. cereus cells

Fresh *B. cereus* (JDZ102Y/NC0084LY) was prepared from overnight culture in LB medium. This was pelleted, washed twice and re-suspended in PBS. To obtain dead *B. cereus* cells, the cell pellets were re-suspended in an equal volume of PBS. The suspensions were heat-treated at 100 °C for 15 min, immediately immersed in ice for 2 min and centrifuged at 12,000 × *g* for 2 min. Viable cells, diluted with sterile PBS, were confirmed by plating onto LB plates with 1.5% agarose powder (GENE Co., Ltd, Chaiwan, Hongkong).

2.3. PMA treatment and genome DNA extraction

PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide to obtain a stock solution at 1 mg/mL and stored at −20 °C in the dark. In particular, 2 μL of the PMA stock solution was added to 400 μL of sample in a light-transparent 1.5-mL microcentrifuge tube to create a final concentration of 5 μg/mL. The tubes were incubated in the dark at room temperature for 5 min with occasional mixing (Nocker et al., 2007), to allow the PMA to penetrate the dead cells and intercalate with the DNA. Next, samples were exposed to light using a 500-W halogen light source for 5 min. During exposure, the samples were placed on crushed ice, 20 cm far away from the light source (Zhu, Li, Jia, & Song, 2012), and laid horizontally to prevent excessive heating. The samples were shaken every 30 s to guarantee homogeneous light exposure.

Genomic DNA of *B. cereus* (viable or dead) was isolated from PMA treated samples using the boiling method described above. The samples were washed twice in an equal volume of bacteria-free PBS prior to DNA extraction and the extracted genomic DNA was used to mPCR assay immediately.

2.4. Primer design and internal amplification control

The primer pairs and IAC used in this study are listed in Table 2. The emetic *B. cereus*-specific primers were derived from one of the cereulide synthetase CDSs gene sequence (Ehling-Schulz, Fricker, & Scherer, 2006) targeting a region of the *cesB* genes and the non-emetic *B. cereus*-specific primers were derived from the 16S rRNA gene sequence (Dzieciol et al., 2013). The *cesB* primers were designed using Oligo 6.0 software (http://www.oligo.net/), and the specificity of the sequences was based on the results of search against NCBI's non-redundant database using the BLASTN algorithm (Fricker et al., 2007). All primers were synthetized by Shanghai Invitrogen Co., Ltd. Shanghai, China. A non-competitive internal amplification control constructed previously was added to each PCR reaction without any modifications (Chiang et al., 2006). In a PCR reaction without an IAC, a negative response can mean that there is no target sequence present, but it could also mean that the amplification has been inhibited. Conversely, with the use of an IAC, the absence of response both from the target