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A novel pentaplex real time (RT)- PCR high resolution melt curve assay for simultaneous detection of emetic and enterotoxin producing *Bacillus cereus* in food

Fereidoun Forghani [a], Prashant Singh [b], Kun-Ho Seo [c], Deog-Hwan Oh [d, *]

[a] Department of Microbiology, Immunology and Biochemistry, The University of Tennessee Health Science Center, Memphis, TN 38163, USA
[b] Department of Food Science & Technology, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, USA
[c] KU Center for Food Safety, College of Veterinary Medicine, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul, 143-701, Republic of Korea
[d] Department of Bioconvergence Science and Technology, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea

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ABSTRACT

Bacillus cereus causing emetic and diarrheal type of food poisoning is widely distributed in nature and is therefore considered a major foodborne pathogen. There is a growing demand for fast, accurate, reliable and economic detection of potentially toxigenic *B. cereus*. To improve differential diagnosis of toxigenic *B. cereus*, a highly sensitive pentaplex RT- PCR high resolution melt curve assay was developed for simultaneous detection of 4 major enterotoxim genes (*cytK*, *entFM*, *hblD*, *nheA*) and emetic toxin gene (*ces*). The average melting temperatures (T_m) of PCR products were 72.2 °C (*ces*), 74.23 °C (*cytK*), 76.55 °C (*nheA*), 78.42 °C (*entFM*) and 81.90 (*hblD*). The multiplex assay was evaluated using 71 bacterial strains including 17 emetic *B. cereus* reference strains, 9 enterotoxic *B. cereus* reference strains, 4 *B. cereus* group members, 23 wild *B. cereus* strains, 18 non-target strains, and was further tested on artificially inoculated foods. The detection limit in food samples was approximately 10^3 CFU/g without enrichment and 10^1 CFU/g was observed following 7 h enrichment. The DNA intercalating dye SYTO9 used in this study generated high resolution melt curve peaks for the target strains and genes in which the peaks were sharp and easily distinguishable from each other. Thus, the developed multiplex real-time (RT) PCR approach can be a reliable tool for the identification of emetic and enterotoxic strains of *B. cereus* present in food and food-related samples.

1. Introduction

Bacillus species are ubiquitous and diverse both in the marine and terrestrial ecosystems (Oguntoyinbo, 2007). Accordingly, the "*Bacillus cereus* group" (*B. cereus sensu lato*) are Gram-positive, spore-forming, rod-shaped, opportunistic human pathogens and are ever-present threat in food (Abdou, Awny & Abozeid, 2011; Agata et al., 1994; Dierick et al., 2005; Fricker, Messelhäußer, Busch, Scherer, & Ehling-Schulz, 2007; Priha, Hallamaa, Saarela, & Raaska, 2004). *B. cereus* group comprises eight closely related species: *B. cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus cytotoxicus* and *Bacillus toyonensis*, among which many of them are of great medical and economic importance (Jiménez et al., 2013). It is widely distributed in the environment and is a causative agent of foodborne illness (Helgason et al., 2000; Jiménez et al., 2013). Foodborne illness resulting from consumption of *B. cereus*-contaminated food may result in emetic or diarrheal type syndromes (Kim et al., 2010). Although *B. cereus* has been implicated in several foodborne outbreaks around the world, resulting in occasional hospitalization or even death (Dierick et al., 2005; Mahler et al., 1997), its true incidence is usually underestimated mainly due to similar symptoms to other types of food poisoning (Fricker et al., 2007).

The diarrheal type of food poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Ehling-Schulz, Fricker, & Scherer, 2004). The

* Corresponding author. No. 414, 309 #, Department of Bioconvergence Science and Technology, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea.
E-mail address: deoghwa@kangwon.ac.kr (D.-H. Oh).

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nonhemolytic enterotoxin (NHE), enterotoxin FM (EntFM), hemolysin BL (HBL) and cytotoxin K (CytK) are the main enterotoxins produced by *B. cereus* (Forghani, Kim, & Oh, 2014). Abdominal pain and diarrhea usually occur 8–16 h after ingestion of contaminated food and may be misdiagnosed with *Clostridium perfringens* food poisoning (Park et al., 2009). The NHE complex consists of three subunits which are encoded by *nheA*, *nheB* and *nheC* in one operon (Granum, O'Sullivan & Lund, 1999). Enterotoxin FM is a *B. cereus* cell wall peptidase implicated in virulence (Untergrasser et al., 2012). The HBL complex consists of three components L1, L2 and B which are encoded in one operon by the genes *hblD*, *hblC* and *hblA*, respectively, and has haemolytic and dermonecrotic activity (Corona, Fois, Mazzette, & De Santis, 2003), making it an important causative agent of the diarrheal syndrome (Beecher & Wong, 2000). Finally, the single-component protein toxin CytK is a β-barrel pore-forming toxin with dermonecrotic, hemolytic and cytotoxic activities (Lund, DeBuyser, & Granum, 2000). The emetic syndrome is caused by emetic toxin (cereulide), a small cyclic peptide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val$_3$) belonging to dodecadepsipeptide molecules family. It is a heat-stable toxin causing nausea and vomiting approximately 1–5 h after consumption of contaminated food (Agata et al., 1994), with symptoms which resemble to *Staphylococcus aureus* food poisoning (Forghani, Kim & Oh, 2014).

Conventional methods for the detection of *B. cereus* are time consuming, laborious and occasionally not precise. Thus, several molecular methods have been developed for the detection of *B. cereus* (Fernández-No et al., 2011; Fricker, Reissbrodt, & Ehling-Schulz, 2008; Martínez-Blanch, Sánchez, Garay, & Aznar, 2009). These tools either targeted a limited number of toxin producing genes or species-specific genes. However, assessment of multiple toxin genes which will help to reveal the full pathogenic potential of the strains by multiplex PCR assays might be better than only species detection for outbreak investigations (Ehling-schulz & Messelhäusser, 2013; Oh, Ham, & Cox, 2012).

Several conventional PCR assays for the detection of *B. cereus* group as well as its emetic and/or enterotoxin producing strains have been developed (Ehling Schulz, Fricker, & Scherer, 2004; Ehling-Schulz et al., 2006; Forghani, Seo, & Oh, 2014; Ghelardi et al., 2002; Kim et al., 2012; Nakano et al., 2004). However, a major drawback of conventional PCR is the requirement for post-PCR analysis which is time consuming and bears the risk of false-positive results due to laboratory contamination (Fan, Hamilton, Webster-Sesay, Nikolich, & Lindler, 2007; Fricker et al., 2007). On the other hand, real-time PCR allows sensitive high-throughput results with easy automation and does not require post-PCR detection procedures (Zhang et al., 2014). There are two main technologies applied in real-time PCR for the detection purpose. One is the application of fluorescent probes which will specifically attach to the target DNA sequence. The other procedure uses fluorescent dyes such as SYBR® Green I or SYTO9 intercalating with double-stranded (ds) DNA, binding to all amplicons generated in PCR reaction (Singh & Mustapha, 2014). The latter is less expensive, does not require probe design and PCR products can be differentiated by melting temperature (T_m) analysis which is also capable of multiplexing (Arya et al., 2005; Zhang et al., 2014).

Thus far, some real-time assays for the detection of *B. cereus* group, enterotoxin producing strains, emetic toxin producing strains, quantification, viable cells detection and detection of *B. cereus* spores in food have been reported (Dzieciol, Fricker, Wagner, Hein, & Ehling-schulz, 2013; Fernández-No et al., 2011; Martínez-Blanch et al., 2009, 2010, 2011). However, they either used dual-labeled probes such as TaqMan® which are expensive and require separate probe design and/or did not detect the toxin genes. Hence, the aim of this study was to develop a novel pentaplex RT- PCR high resolution melt curve assay for the wide range detection of emetic and enterotoxin producing *B. cereus* in food.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All 71 bacterial strains used in this study were obtained from the Dept. of Food Science and Biotechnology, Kangwon Natl. Univ. (Table 1). In brief, they consisted of 17 *B. cereus* emetic reference strains, 9 *B. cereus* enterotoxic reference strains, 4 members of *B. cereus* group, 23 wild *B. cereus* strains as well as 18 non-target strains. All strains were grown on nutrient agar (Difco, Detroit, Mich., USA) plates at 35 °C for 24 h. A single colony was inoculated in Luria–Bertani broth (Difco) and incubated at 35 °C for 12 h for further experiments. The emetic (F4810/72) and enterotoxic (ATCC 12480) *B. cereus* reference strains were used for the optimization and primary evaluation of the approach.

2.2. Bacterial DNA extraction

For the DNA extraction, 1 ml of the overnight culture was centrifuged (15000 × g; 2 min) and the pellet was resuspended in 100 μl of the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). The DNA was extracted according to the manufacturer's instructions. The concentration of DNA was adjusted to 10 ng/μL using a NanoDrop 2000 UV/VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 nm and also its purity was checked. The DNA was stored at −20 °C for the following experiments.

2.3. Primer design

Table 2 shows the primers used in this study along with their design source and product size. Specific primers for the amplification of enterotoxin producing genes (*cytK*, *entFM*, *hblD*, *nheA*) and emetic toxin gene (*ces*) were designed using the Primer3 software (Untergrasser et al., 2012) and commercially synthesized using *AccuOligo*® technology (Bioneer, Daejeon, Korea) (http://eng.bioneer.com/products/Oligo/CustomOligonucleotides-technical.aspx).

To further maximize the detection range of the primers, the International Union of Pure and Applied Chemistry (IUPAC) standards were taken into account if necessary. The primers were designed to keep the melting temperature (T_m) of the PCR amplicons between 70 °C and 85 °C and each amplicon T_m was separated by approximately 2 °C. The specificity of the designed primers was tested using the Primer BLAST tool and the T_m of all amplicons was predicted using the BioEdit software (Hall, 1999).

2.4. Pentaplex RT- PCR high resolution melt curve assay

In this study SYTO9-based 2 × MeltDoctor™ HRM master mix (Applied Biosystems, Foster City, CA, USA) was selected over the SYBR® Green I-based master mix according to the literature reporting several advantages for SYTO9 dye in comparison with SYBR® Green. PCR amplification was performed in duplicate in a 20 μl reaction volume with 20 ng of DNA. The primer concentrations were 50, 150, 100, 50 and 100 nM for *ces*, *cytK*, *nheA*, *entFM*, and *hblD*, respectively. A StepOne™ real-time PCR (Applied Biosystems, Foster City, CA, USA) instrument with StepOne™ Software (Version 2.2.2; Applied Biosystems, Foster City, CA, USA) was used for the standardization of the real-time multiplex PCR assay.

StepOne™ Software (Version 2.2.2; Applied Biosystems, Foster