Multiplex PCR coupled with propidium monoazide for the detection of viable *Cronobacter sakazakii*, *Bacillus cereus*, and *Salmonella* spp. in milk and milk products

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ABSTRACT

Cronobacter sakazakii, *Bacillus cereus*, and *Salmonella* spp. are common food-borne pathogens. The aim of this study was to develop a sensitive, specific, and rapid method for the simultaneous detection of these 3 pathogens in milk and milk products. Three specific primers were designed based on *ompA*, *invA*, and *cesB* of *C. sakazakii*, *Salmonella* spp. and *B. cereus*, respectively, for use in a multiplex PCR (mPCR). To eliminate false-positive results, cells were pretreated with propidium monoazide (PMA) for the selective elimination of the genomic DNA of dead cells. An internal amplification control was applied as an indicator of false-negative results from the interference of inhibitors in the food matrix. Results showed that, in pure culture, the limits of detection of the assay for *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* were 9.5×10^4, 7.4×10^2, and 7.5×10^2 cfu/mL, respectively. Moreover, 8 cfu/mL of viable *B. cereus* cells were detected after 5 h of enrichment, and 9 cfu/mL of viable *C. sakazakii* and 7 cfu/mL of *Salmonella* Enteritidis were detected after 7 h of enrichment in spiked pure milk, walnut peanut milk, and whole-wheat milk. To validate the PMA-mPCR assay, the PMA-mPCR assay and the traditional culture method were performed to detect the 3 bacterial strains in 1,165 milk product samples. The PMA-mPCR assay obtained the same results as the culture-based method. Results demonstrated that the PMA-mPCR assay has excellent sensitivity and specificity for the simultaneous detection of viable *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* in milk and milk products.

Key words: food-borne pathogens, propidium monoazide, multiplex PCR, internal amplification control

INTRODUCTION

Cronobacter sakazakii, *Bacillus cereus*, and *Salmonella* spp., common causative agents of food poisoning outbreaks, are present in milk and milk products (Ahmed et al., 1983; Crump et al., 2004). *Cronobacter sakazakii* is commonly found in powdered infant formula and is related to outbreaks of infant meningitis and necrotizing enterocolitis. Infections caused by *C. sakazakii* result in a 40 to 80% mortality rate among newborns, children, and even adults (FAO, 2008; Cai et al., 2013; Ye et al., 2015). *Bacillus cereus*, which is present in pastas, cheeses, vegetables, cakes, meats, and milk products, may cause emesis and diarrhea (Evelyn and Silva, 2015). Among the 13,405 food-borne disease outbreaks in the United States from 1998 to 2008, 235 outbreaks were caused by *B. cereus* (Bennett et al., 2013). *Salmonella* spp. are the second most commonly reported pathogen among food-borne disease outbreaks with confirmed etiologies and of high public concern given their ubiquity in a wide range of food (Aarestrup et al., 2007; Gould et al., 2013). In 2013, of the 818 food-borne disease outbreaks in the United States, 157 were caused by *Salmonella* spp. (Dewey-Mattia et al., 2015). Thus, establishing a rapid and accurate method for the simultaneous detection of these food-borne pathogens is urgently needed.

Culture-based methods are the present gold standards for food-borne pathogen detection. Traditional culture-based methods include enrichment, selection, biochemical identification, and serological studies. Nevertheless, these methods are laborious and time-consuming (Murakami, 2012). In the past few years, PCR has been widely used to detect pathogens because of its celerity, sensitivity, specificity, and simplicity. Compared with traditional PCR, multiplex PCR (**mPCR**) effectively reduces the use of reagents and the number of operating steps by simultaneously detecting 2 or more pathogens in a single analysis (de Freitas et al., 2010).

Normal mPCR, however, is unable to distinguish DNA signals from dead or viable cells, thus leading

to false results. (Wang and Levin, 2006; Zhang et al., 2014). Ethidium monoazide (**EMA**) and propidium monoazide (**PMA**) are 2 kinds of nucleic intercalating dyes that could penetrate the membranes of dead cells and form covalent bonds with genomic DNA (Nocker et al., 2007; Liu and Mustapha, 2014). Nocker et al. (2006) explored the effects of EMA and PMA on interference signals from dead cells. Their results showed that the 2 dyes could effectively remove the interferences of dead cells; EMA, however, could penetrate the membranes of viable cells and form covalent bonds with a portion of the genomic DNA. Recently, PMA has been successfully applied in detecting viable *B. cereus* (Forghani et al., 2015), *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Listeria monocytogenes* (Yang et al., 2013), *C. sakazakii*, *Staphylococcus aureus*, and *B. cereus* (Li et al., 2016) in food samples.

The aim of our study was to establish a PMA-mPCR assay for the simultaneous detection of viable *C. sakazakii*, *B. cereus*, and *Salmonella* spp. in milk and milk products. The specific primers of the PMA-mPCR assay were designed based on 3 specific genes, *ompA* of *C. sakazakii*, *cesB* of *B. cereus*, and *invA* of *Salmonella* spp. Furthermore, a pair of universal primers based on the conserved region of *16S rRNA* were added as the internal amplification control (**IAC**) to indicate false-negative results that may be caused by problematic PCR equipment, the wrong reagent, or inhibitors in food samples. The PMA-mPCR assay combined with IAC was then successfully used to analyze milk product samples from supermarkets and manufacturers.

MATERIALS AND METHODS

Preparation of Bacterial Strains

The bacterial strains used in our study for specificity testing of the primer are listed in Table 1. *Salmonella* Enteritidis ATCC 13076, *C. sakazakii* CMCC 45401, and *B. cereus* PZ0063L were used as reference strains to establish the PMA-mPCR assay. All bacterial strains were cultured in Luria Bertani (**LB**) medium (Solarbio, Beijing, China) at 37°C in a rotary shaker at 180 rpm overnight.

Primer Design

Information for the primer and IAC used in this study are listed in Table 2. The primer of *invA* was designed using Oligo 7.0 software (http://www.oligo.net/), and the specificity of the sequences was verified by the results of searches against the NCBI nonredundant database using the BLASTN algorithm (Fricker et

al., 2007). The other 3 pairs of primers for IAC, *ompA*, and *cesB* were obtained from previous reports (Chiang et al., 2006; Zhang et al., 2014; Li et al., 2016). All primers were synthesized by GenScript (Nanjing) Co. Ltd. (Nanjing, China).

Preparation of Viable and Dead Cells

One-milliliter aliquots of overnight cultures of fresh cells were pelleted, washed twice, and resuspended in PBS (pH 7.4). To obtain dead cells, the suspended cells were exposed to 80°C for 10 min (*Salmonella* Enteritidis and *C. sakazakii*) and 100°C for 20 min (*B. cereus*) in a water bath. Finally, viable and dead cells were confirmed by plating onto LB plate with 2.5% agarose powder and cultured at 37°C for 24 h before enumeration.

PMA Treatment

The PMA (Biotium, Inc. Hayward, CA) was dissolved in 20% dimethyl sulfoxide to obtain a stock solution of 1 mg/mL, which was stored at -20°C in the dark. Samples were prepared according to Nocker et al. (2006) with a few modifications. Briefly, a 5-µL aliquot of the PMA solution was added to 500 µL of sample solution in a light-transparent 1.5-mL microcentrifuge tube to obtain the final concentration of 10 µg/mL. Afterward, the samples were incubated in the dark with occasional mixing for 5 min to promote PMA entering the dead cells, following a light exposure period of 5 min on ice using a 500-W halogen light source to ingrain the combination of PMA and DNA from dead cells. During light exposure period, the samples were placed at ~20 cm from the light source, shaking the tubes every 30 s guarantee homogeneous light exposure. After the light exposure, the samples were centrifuged at 12,000 × *g* for 5 min at room temperature and washed twice with equal volumes of PBS to remove the free PMA, and then resuspended in 100 µL of sterile water.

DNA Extraction

The DNA extraction of cells was prepared by resuspending the bacterial pellets in 200 µL of enzyme incubation buffer [20 m*M* Tris, 2 m*M* Na2-EDTA, 1.2% TritonX-100 (Solarbio), and 20 mg/mL of lysozyme], followed by an incubation period of 30 min at 37°C (Schnaitman, 1971). Afterward, bacterial DNA was extracted using DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, proteinase K and AL buffer were added to the mixture and incubated at 56°C for 30 min