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

Shuang Yu,* Leina Yan,*† Xin Wu,‡ Fan Li,* Dong Wang,‡¹ and Hengyi Xu*¹

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

†Jiangxi Food and Drug Administration, Nanchang, 330047, PR China

‡Jiang Xi Institute for Food Control, Nanchang, 330047, PR China

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

Received May 3, 2017.

Accepted May 27, 2017.

¹Corresponding authors: 2580663544@qq.com and kidyxu@163.com or HengyiXu@ncu.edu.cn

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 mM Tris, 2 mM 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

Table 1. Bacteria strains used in this study and the results of the multiplex PCR assay

Bacteria strain	Strain ID	Source	Multiplex PCR-positive			
			<i>OmpA</i>	<i>invA</i>	<i>cesB</i>	<i>16S rRNA</i>
<i>Cronobacter sakazakii</i> (n = 6)	45401	CMCC ¹	+	—	—	+
	45402	CMCC	+	—	—	+
	21544	CICC ²	+	—	—	+
	21570	CICC	+	—	—	+
	21564	CICC	+	—	—	+
	21545	CICC	+	—	—	+
<i>Salmonella</i> Enteritidis	13076	ATCC ³	—	+	—	+
<i>Salmonella</i> Anatum	9270	ATCC	—	+	—	+
<i>Salmonella</i> Paratyphi A (n = 2)	9150	ATCC	—	+	—	+
	40004	JX-CDC ⁴	—	+	—	+
<i>Salmonella</i> Paratyphi B (n = 2)	40001	JX-CDC	—	+	—	+
	50094	JX-CDC	—	+	—	+
<i>Salmonella</i> Typhi	40002	JX-CDC	—	+	—	+
<i>Salmonella</i> Typhimurium	13311	ATCC	—	+	—	+
<i>Salmonella</i> Choleraesuis	10708	ATCC	—	+	—	+
<i>Bacillus cereus</i> (n = 10)	P20063L	JX-CDC	—	—	+	+
	JDZ102Y	JX-CDC	—	—	+	+
	JX0111LY	JX-CDC	—	—	—	+
	JX0121LY	JX-CDC	—	—	—	+
	PX0031L5	JX-CDC	—	—	—	+
	PX0053L16	JX-CDC	—	—	—	+
	PX0054L17	JX-CDC	—	—	—	+
	DA0096L	JX-CDC	—	—	—	+
	DA0050L	JX-CDC	—	—	—	+
	DA0036L	JX-CDC	—	—	—	+
<i>Escherichia coli</i> O157:H7	43888	CMCC	—	—	—	+
<i>Listeria monocytogenes</i> (n = 2)	13932	ATCC	—	—	—	+
	54007	CMCC	—	—	—	+
<i>Staphylococcus aureus</i> (n = 2)	26002	CMCC	—	—	—	+
	26003	CMCC	—	—	—	+
<i>Vibrio parahaemolyticus</i>	PVPAO146	JX-CDC	—	—	—	+
<i>Pseudomonas aeruginosa</i>	10104	CMCC	—	—	—	+
<i>Bacillus subtilis</i>	63501	CMCC	—	—	—	+
<i>Shigella sonnei</i>	25931	ATCC	—	—	—	+

¹China Medical Culture Collection, Beijing, China.²Center of Industrial Culture Collection, Beijing, China.³American Type Culture Collection, Rockville, MD.⁴Jiang Xi Province Center for Disease Control and Prevention, Nanchang, Jiangxi, China.

in water bath, followed by the addition of ethanol. This mixture was transferred into a DNeasy Mini spin column and washed with AW1 and AW2 buffer. Finally, cells were transferred from the spin column to a new 1.5-mL microcentrifuge tube, and DNA were eluted by adding 50 μ L of AE buffer.

mPCR Conditions

The 25 μ L volume of the PCR reaction contained 12.5 μ L of 2 $\times$ Taqmix (Tiangen Biotech Co., Ltd., Beijing, China), 2 μ L of each target DNA template, 1 μ L of *Listeria monocytogenes* DNA template, 0.2 μ M

Table 2. Primers used in the multiplex PCR

Target bacteria	Target gene	Primer sequence (5'→3')	Amplicon length (bp)	Reference
<i>Bacillus cereus</i>	<i>cesB</i>	Forward (F): ACCCATCTTGGCGTCATT Reverse (R): CAGCCAAGTGAAGAATACC	154	Zhang et al., 2014
<i>Salmonella</i>	<i>invA</i>	F: CGCCCCATATTATCGCTATCG R: AGCTTTACGGTTCCTTTGACG	262	Current study
<i>Cronobacter sakazakii</i>	<i>OmpA</i>	F: CATTTGGTGACGCGCAGACT R: TGGACGGGATACCTTTGG	380	Li et al., 2016
Bacterial DNA	<i>16S rRNA</i>	F: CCTACGGGAGGAGCAGT R: CGTTTACGGCGTGGACTAC	475	Chiang et al., 2006

each forward and reverse *cesB* primer, 0.16 μM each forward and reverse *invA* primer, 0.24 μM each forward and reverse *OmpA* primer, and 0.1 μM each forward and reverse 16S rRNA primer. The PCR program used was predenaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55.3°C for 30 s and elongation at 72°C for 30 s, with a final elongation at 72°C for 10 min. The amplified DNA was separated at 2.5% agarose gel and stained with 0.5 $\mu\text{g/mL}$ of ethidium bromide. Finally the DNA bands were visualized with the UV transilluminator (Bio-Rad, Hercules, CA).

Specificity and Sensitivity of the PMA-mPCR Assay in Pure Culture

To examine the specificity of the primers, a large number of nontarget cultures were tested by mPCR: 17 target strains and 17 nontarget strains belonging to other 6 species of pathogens. To test the limit of detection (LOD) of the PMA-mPCR assay, a 10-fold series of viable bacterial resuspensions ranging from 10^7 to 10^1 cfu/mL were prepared, PMA treatment and DNA extraction were applied as above, and followed by mPCR assay. All of the tests were repeated triplicates.

Sensitivity of the PMA-mPCR Assay in Artificially Contaminated Milk Products

The sensitivity of the method was further evaluated in pure milk, walnut peanut milk, and whole-wheat milk from a local supermarket. The samples were verified for the absence of 3 target pathogens by traditional culture method, then 1 mL of sample was mixed with 9 mL of LB to obtain the sample mixture; afterward, viable target bacteria was added to achieve a final concentration of 10^0 cfu/mL. After enrichment at 37°C in a rotary shaker at 180 rpm for 5, 7, 9, and 11 h, 1 mL of culture was obtained at every time point. The culture was centrifuged at $800 \times g$ for 2 min at room temperature to remove larger debris. The pellets were then obtained by centrifugation at $12,000 \times g$ for 5 min at room temperature, washed twice with PBS, and resuspended with 1 mL of PBS; PMA treatment followed and genomic DNA was extracted for mPCR using DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All of the tests were repeated in triplicate.

Investigation of Milk and Milk Product Samples

The PMA-mPCR method was applied to detect the 3 pathogens in milk and milk products. A total of 1 mL or 1 g of sample was mixed with 9 mL of LB and

incubated at 37°C in a rotary shaker at 180 rpm for 7 h; a 1-mL aliquot of the culture was then used for the PMA-mPCR assay. In addition, pathogens were detected using culture-based methods in accordance with the National Food Safety Standards of China (GB 4789.4–2010, GB 4789.40–2010, and GB 4789.14–2014; China, 2010a,b, 2014). All of the tests were repeated in triplicate.

RESULTS

Optimization and Validation of mPCR

The concentrations and annealing temperature of primers were optimized to ensure high amplification efficiency for each amplicon. The annealing temperature was selected at 55.3°C because the 4 target bands were more intense at this temperature. The concentration of each primer pair was optimized by orthogonal test, the optimum conditions were 0.2 μM *cesB* primers, 0.16 μM *invA* primers, 0.24 μM *OmpA* primers, and 0.1 μM 16S rRNA primers. Finally, DNA bands were visualized with an UV transilluminator. Amplification products with lengths of 154 bp for *B. cereus*, 262 bp for *Salmonella* Enteritidis, 380 bp for *C. sakazakii*, and 475 bp for IAC were obtained (Figure 1). In addition, the results showed that the mPCR could detect not only each bacterial species individually, but also simultaneously detect the 3 bacterial species within the same reaction.

Effect of PMA Treatment on Viable and Dead Cells

After optimizing the mPCR assay, the effects of PMA treatment on dead and viable cells were investigated with 7 mixtures of the 3 target bacterial species. In accordance with a previous work (Li et al., 2016), bacterial cells were treated with 10 $\mu\text{g/mL}$ of PMA. Bands from PMA-treated viable cells were similar to those from PMA-untreated cells. The PMA-treated dead cells showed no bands on the gel, whereas PMA-untreated dead cells expressed bright bands (Figure 2). These results indicated that pretreatment with 10 $\mu\text{g/mL}$ of PMA effectively eliminated interference from dead cells without influencing viable cells.

Specificity and Sensitivity of the PMA-mPCR Assay in Pure Culture

The specificity of the assay was tested using 17 target strains and 17 nontarget strains of 6 other kinds of pathogens (Table 1). Among 10 strains of *B. cereus*, only *B. cereus* PZ0063L and *B. cereus* JDZ102Y had *cesB*, which was consistent with our previous investiga-

Figure 1. Results of optimized multiplex PCR and PCR assays for the detection of *Bacillus cereus*, *Salmonella* Enteritidis, and *Cronobacter sakazakii*. Lane M = DL 2000 marker (Takara, Dalian, China); lane 1 = *B. cereus*, *Salmonella* Enteritidis, and *C. sakazakii* combined with internal amplification control (IAC); lane 2 = *B. cereus* and *Salmonella* Enteritidis combined with IAC; lane 3 = *B. cereus* and *C. sakazakii* combined with IAC; lane 4 = *Salmonella* Enteritidis and *C. sakazakii* combined with IAC; lane 5 = *B. cereus* combined with IAC; lane 6 = *Salmonella* Enteritidis combined with IAC; lane 7 = *C. sakazakii* combined with IAC; lane 8 = *B. cereus* only; lane 9 = *Salmonella* Enteritidis only; lane 10 = *C. sakazakii* only; lane 11 = negative control.

tion (Zhang et al., 2014). The results showed that the assay could generate 2 bands with the target strains. By contrast, only 1 band for *16S rRNA* was found when the mPCR assay was applied to nontarget strains.

These results demonstrated that the primers were specific for the target strains. Furthermore, the LOD of the PMA-mPCR detection method for viable cells in pure culture was 10^4 cfu/mL for *C. sakazakii*, 10^2 cfu/

A

Mixtures							
	1/I	2/II	3/III	4/IV	5/V	6/VI	7/VII
<i>Cronobacter sakazakii</i>	+	-	+	+	-	-	+
<i>Salmonella</i> Enteritidis	+	+	-	+	-	+	-
<i>Bacillus cereus</i>	+	+	+	-	+	-	-

(+/-) indicate viable cells and dead cells respectively.

B

Figure 2. Effects of propidium monoazide (PMA) treatment on multiplex PCR assay in the detection of viable and dead bacterial cells. (A) Scheme for 7 mixture of the 3 target bacteria. The concentration of each target bacteria was 10^7 cfu/mL. (B) The results of multiplex PCR assay with (+) and without (-) PMA treatment. Lane M = DL 2000 marker (Takara, Dalian, China); lane C = negative control. All of the tests were repeated triplicates.

Figure 3. Limit of detection of the propidium monoazide-multiplex PCR assay with internal amplification control (IAC) using a 10-fold serial dilution of *Cronobacter sakazakii*, *Salmonella* Enteritidis, and *Bacillus cereus* in pure culture. (A) *C. sakazakii*, lanes 1 to 7 from 9.5×10^7 to 9.5×10^1 cfu/mL; (B) *Salmonella* Enteritidis, lanes 1 to 7 from 7.4×10^7 to 7.4×10^1 cfu/mL; (C) *B. cereus*, lanes 1 to 7 from 7.5×10^7 to 7.5×10^1 cfu/mL; lane M = DL 2000 marker (Takara, Dalian, China); lane C = negative control. All of the tests were repeated triplicates.

mL for *Salmonella* Enteritidis and 10^2 cfu/mL for *B. cereus* (Figure 3).

Sensitivity of the PMA-mPCR Assay in Artificial Milk Products

Pure milk, walnut peanut milk, and whole-wheat milk samples were spiked with low concentrations (9 cfu/mL for *C. sakazakii*, 7 cfu/mL for *Salmonella* Enteritidis, and 8 cfu/mL for *B. cereus*) of viable bacteria. Samples

were subjected to the PMA-mPCR assay after specific enrichment times. As shown in Table 3, 5 h of enrichment time was required to detect *B. cereus* in the 3 milk products, and 7 h of enrichment time was required to detect *C. sakazakii* and *Salmonella* Enteritidis.

Investigation of Milk and Milk Product Samples

The antimatrix interference ability of the PMA-mPCR assay was further evaluated in 1,165 milk product

Table 3. Results of propidium monoazide-multiplex PCR assay with internal amplification control (IAC) for the detection of *Cronobacter sakazakii*, *Bacillus cereus*, and *Salmonella* Enteritidis in 3 kinds of milk products after different enrichment time

Enrichment time (h)	Product type	Results of multiplex PCR			
		<i>cesB</i>	<i>invA</i>	<i>OmpA</i>	IAC
5	Pure milk	+	—	—	+
	Walnut peanut milk	+	—	—	+
	Whole wheat milk	+	—	—	+
7	Pure milk	+	+	+	+
	Walnut peanut milk	+	+	+	+
	Whole wheat milk	+	+	+	+
9	Pure milk	+	+	+	+
	Walnut peanut milk	+	+	+	+
	Whole wheat milk	+	+	+	+
11	Pure milk	+	+	+	+
	Walnut peanut milk	+	+	+	+
	Whole wheat milk	+	+	+	+

samples. The results of the PMA-mPCR assay showed that 2 powdered infant milk samples from the manufacturer were contaminated with *C. sakazakii* and no target pathogens were found in other samples (Table 4), which was consistent with the traditional culture method. These results demonstrated that the developed PMA-mPCR assay can reliably detect viable *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* in milk products. Thus, the PMA-mPCR assay is a potential tool for monitoring and preventing pathogen contamination in milk products.

DISCUSSION

The PCR assay is efficient and specific in the detection of pathogens in food samples (Kong et al., 2002; Chiu et al., 2005; Chen et al., 2012). Moreover, mPCR has further strengthened the advantages of traditional PCR by allowing the amplification of different products in a multiplex reaction (Wu et al., 2007). In the present study, the PMA-mPCR assay combined with IAC was

developed for the simultaneous detection of viable *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* cells in milk and milk products.

In our study, the results of the effects of PMA showed less suppression for viable cells and an efficient suppression of dead cell signals, which was consistent with a previous report (Yang et al., 2013). According to Fitipaldi et al. (2012), PMA showed less suppression for viable cells and more homogeneous performance with different species; however, the generation of false-positive signals due to incomplete signal suppression was a disadvantage of PMA. Therefore, sodium deoxycholate has been combined with PMA because it is effective for destabilizing membranes of dead cells that have not accumulated sufficient membrane damage (Zhou et al., 2016). Furthermore, the addition of IAC in the mPCR eliminated the possible false-negative results, which may be caused by incorrect operation, malfunction equipment, and inhibitors of the food matrix. However, the *16S rRNA* primer may compete with the specific primer for available target DNA, thus affecting the sensitivity of the mPCR assay. Therefore, we added 1 type of nontarget DNA to eliminate the competition between *16S rRNA* and specific primers in our experimental mPCR system. The results of the specificity tests demonstrated that only the *16S rRNA* primer could amplify the genomic DNA of *L. monocytogenes* to produce a band. Thus, we selected *L. monocytogenes* DNA as the additional DNA, and *L. monocytogenes* DNA was confirmed to be a superior nonspecific DNA indicator for the PCR procedure.

Considering the fact that milk may be contaminated with a low level of pathogens (Kandhai et al., 2010), an enrichment step was applied to improve the sensitivity. In our study, 10^0 cfu/mL of viable pathogens were detected after 7 h of enrichment. A study by Chen et al. (2012) developed an mPCR assay with the lower sensitivity at 10 to 17 cfu/g of spiked food samples of *Staphylococcus aureus*, *L. monocytogenes*, *Escherichia*

Table 4. Detection of 3 food-borne pathogens in 1,165 samples via the propidium monoazide-multiplex PCR assay

Sample types (number of samples)	Origin	Number of samples	Positive/negative		
			<i>Bacillus cereus</i>	<i>Salmonella</i>	<i>Cronobacter sakazakii</i>
Pasteurized milk	Supermarket	264	0/264	0/264	0/264
(376)	Manufacturer	112	0/112	0/112	0/112
Fresh milk	Supermarket	138	0/138	0/138	0/138
(284)	Manufacturer	146	0/146	0/146	0/146
Powdered skim milk	Supermarket	164	0/164	0/164	0/164
(237)	Manufacturer	73	0/73	0/73	0/73
Powdered infant milk	Supermarket	179	0/179	0/179	0/179
(268)	Manufacturer	89	0/89	0/89	2/87

coli O157:H7, *Salmonella* Enteritidis, and *Shigella flexneri* after 24 h of enrichment. Wang et al. (2015) reported using a sodium deoxycholate-PMA-mPCR assay to detect *Salmonella* Typhimurium, *Shigella sonnei*, and *Staph. aureus*, with the LOD of 10^1 cfu/mL in milk after 15 h of enrichment. Compared with these reports, the PMA-mPCR assay with IAC developed in the present study is faster than the methods reported previously with approximately equal sensitivity.

To verify the anti-interference capability of the PMA-mPCR assay in real samples, the PMA-mPCR assay with IAC was applied to detect the 3 target bacterial species in 1,165 milk product samples. The results of the PMA-mPCR assay and the culture-based method both showed that 2 powdered infant milk samples from manufacturer were contaminated with *C. sakazakii*, whereas no target pathogens were found in other samples. The consistent results of the 2 different methods validated the reliability of the PMA-mPCR assay. The traditional culture-based method, however, required more than 4 d to detect the 3 target pathogens. By contrast, the PMA-mPCR method required only approximately 12 h for pathogen detection. Therefore, the PMA-mPCR assay combined with IAC is a rapid, specific, and facile tool for the detection of viable foodborne pathogens in milk and milk products without the interference of false-positive and false-negative results.

CONCLUSIONS

We developed a sensitive, specific, and convenient PMA-mPCR assay with IAC to detect viable *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* in milk and milk products. The LOD of the assay for *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* in pure culture were 9.5×10^4 , 7.4×10^2 , and 7.5×10^2 cfu/mL, respectively. Moreover, 9 cfu/mL of viable *C. sakazakii* cells, 7 cfu/mL of viable *Salmonella* Enteritidis cells, and 8 cfu/mL of viable *B. cereus* cells were detected in milk and milk products after 7 h of enrichment. The PMA-mPCR method was then used to detect the 3 target bacterial strains in 1,165 milk product samples. The results of the PMA-mPCR method were consistent with those of the traditional culture-based method, thus confirming the validity of the proposed method. Therefore, the PMA-mPCR assay developed in this study is an accurate, sensitive, and cost-effective method for detecting viable *C. sakazakii*, *Salmonella* Enteritidis, and *B. cereus* cells in milk and milk products.

ACKNOWLEDGMENTS

The work was supported by the Research Foundation for Young Scientists of State Key Laboratory of Food

Science and Technology, Nanchang University, China (No. SKLF-QN-201504), Training Plan for the Young Scientist (Jinggang Star) of Jiangxi Province, Nanchang, Jiangxi, China (20142BCB23004), and Science and Technology Planning Project of Jiangxi Provincial Food and Drug Administration, Nanchang, Jiangxi, China (2016SP14).

REFERENCES

- Aarestrup, F. M., R. S. Hendriksen, J. Lockett, K. Gay, K. Teates, P. F. McDermott, D. G. White, H. Hasman, G. Sørensen, and A. Bangtrakulnonth. 2007. International spread of multidrug-resistant *Salmonella* Schwarzengrund in food products. *Emerg. Infect. Dis.* 13:726.
- Ahmed, A. A., M. K. Moustafa, and E. H. Marth. 1983. Incidence of *Bacillus cereus* in milk and some milk products. *J. Food Prot.* 46:126–128.
- Bennett, S. D., K. A. Walsh, and L. H. Gould. 2013. Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*—United States, 1998–2008. *Clin. Infect. Dis.* 57:425–433.
- Cai, X.-Q., H.-Q. Yu, Z.-X. Ruan, L.-L. Yang, J.-S. Bai, D.-Y. Qiu, Z.-H. Jian, Y.-Q. Xiao, J.-Y. Yang, and T. H. Le. 2013. Rapid detection and simultaneous genotyping of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) in powdered infant formula using real-time PCR and high resolution melting (HRM) analysis. *PLoS One* 8:e67082.
- Chen, J., J. Tang, J. Liu, Z. Cai, and X. Bai. 2012. Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *J. Appl. Microbiol.* 112:823–830.
- Chiang, Y.-C., C.-Y. Yang, C. Li, Y.-C. Ho, C.-K. Lin, and H.-Y. Tsen. 2006. Identification of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S ribosomal DNA-based oligonucleotide array hybridization. *Int. J. Food Microbiol.* 107:131–137.
- China. 2010a. National Food Safety Standard—Food microbiological examination: *Salmonella*. Accepted Mar. 26, 2010. <http://bz.cfsa.net.cn/staticPages/322C876C-077B-4917-A0FC-28C692671183.html>.
- China. 2010b. National Food Safety Standard—Food microbiological examination: *Cronobacter sakazakii*. Accepted Mar. 26, 2010. <http://bz.cfsa.net.cn/staticPages/682F5D90-674F-4DC6-974D-19932C387917.html>.
- China. 2014. National Food Safety Standard—Food microbiological examination: *Bacillus cereus*. Accepted Dec. 1, 2014. <http://bz.cfsa.net.cn/staticPages/C684DEB2-911E-49B3-9E1E-57765212FA68.html>.
- Chiu, T.-H., T.-R. Chen, W.-Z. Hwang, and H.-Y. Tsen. 2005. Sequencing of an internal transcribed spacer region of 16S–23S rRNA gene and designing of PCR primers for the detection of *Salmonella* spp. in food. *Int. J. Food Microbiol.* 97:259–265.
- Dewey-Mattia, D., S. D. Bennett, E. Mungai, and L. H. Gould. 2015. Surveillance for Foodborne Disease Outbreaks, United States, 2013, Annual Report. Atlanta, Georgia: US Department of Health and Human Services. <https://www.cdc.gov/foodsafety/pdfs/foodborne-disease-outbreaks-annual-report-2013-508c.pdf>.
- Crump, J. A., S. P. Luby, and E. D. Mintz. 2004. The global burden of typhoid fever. *Bull. World Health Organ.* 82:346–353.
- de Freitas, C. G., Á. P. Santana, P. H. C. da Silva, V. S. P. Gonçalves, M. A. F. Barros, F. A. G. Torres, L. S. Murata, and S. Perelman. 2010. PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat. *Int. J. Food Microbiol.* 139:15–22.
- Evelyn, E., and F. V. Silva. 2015. Thermosonication versus thermal processing of skim milk and beef slurry: modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. *Food Res. Int.* 67:67–74.

- Journal of Dairy Science Vol. 100 No. 10, 2017