Simultaneous quantitative detection of viable *Escherichia coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. using sodium deoxycholate-propidium monoazide with multiplex real-time PCR

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ABSTRACT

Escherichia coli O157:H7, *Cronobacter* spp., and *Salmonella* spp. are common food-borne pathogens in milk that may cause serious diseases. In the present study, we established a simple, rapid, and specific method to simultaneously detect viable *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in milk. Three specific genes, *fliC* from *E. coli* O157:H7, *cgcA* from *Cronobacter* spp., and *invA* from *Salmonella* spp., were selected and used to design primers and probes. False-positive results were eliminated with the use of a combined sodium deoxycholate (SD) and propidium monoazide (PMA) treatment. Using the optimized parameters, this SD-PMA treatment combined with multiplex real-time PCR (SD-PMA-mRT-PCR) detected *E. coli* O157:H7, *Cronobacter* spp. and *Salmonella* spp. respectively, at 10² cfu/mL in pure culture or artificially spiked skim milk samples. A reasonable recovery rate (from 100 to 107%) for detection of viable bacteria using the SD-PMA-mRT-PCR assay was obtained in the presence of dead bacteria at 10⁷ cfu/mL. The SD-PMA-mRT-PCR method developed in this study can accurately detect and monitor combined contamination with *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in milk and milk products.

Key words: multiplex real-time PCR, sodium deoxycholate, propidium monoazide, viable bacteria detection

INTRODUCTION

Food-borne diseases have become a major public health issue worldwide, and significant food contamination

incidents have been confirmed, resulting in 7,890 illness, 83 deaths, and 9 miscarriages in the United States between 2000 and 2016 (Bhunia, 2018). A survey indicated that milk, the second most-consumed food product, is easily contaminated by food-borne pathogens (Painter et al., 2013). Among food-borne pathogens, *Escherichia coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. have been frequently reported as common disease-causing agents in milk (Kumar et al., 2013; Yu et al., 2017). *Escherichia coli* O157:H7 is a typical food-borne pathogen that causes serious human illness such as hemorrhagic colitis and hemolytic uremic syndrome at a low infectious dose, particularly in children under 5 yr of age and adults over 60 yr, with a death rate of 5 to 10% (Amani et al., 2015). *Cronobacter* spp. can cause severe meningitis, bacteremia, and necrotizing enterocolitis in neonates, with high mortality rates ranging between 40 and 80% (Sulovská et al., 2017). In adults, most *Cronobacter* spp. infections occur in the elderly, especially those who have serious illnesses. *Salmonella* spp. have been involved in severe enteritis, typhoid fever, and even death (Coburn et al., 2007). Food-borne diarrheal disease agents, particularly nontyphoidal *Salmonella enterica*, caused 230,000 deaths in 2010, according to the World Health Organization (Havelaar et al., 2015). Therefore, there is an urgent need to establish a rapid, sensitive, and specific method for detecting these 3 pathogenic bacteria specifically in milk.

At present, accurate detection of food-borne pathogens relies mainly on specific biochemical and immunological identification methods that are time-consuming and laborious (Russell et al., 1997; Gracias and McKillip, 2004). Molecular biology techniques such as PCR-based methods are widely used for the detection of food-borne pathogens because of their high specificity and sensitivity and short detection time (Mandal et al., 2011). Particularly, multiplex TaqMan real-time PCR (**mRT-PCR**) is used to monitor multiple fluo-

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rescent signals to avoid laboratory cross-contamination and time-consuming processes (Kawasaki et al., 2010; Kumar et al., 2013). It achieves simultaneous detection of multiple targets by using different fluorophores and quenchers (Botezatu et al., 2017). The specificity of the 5' nuclease hydrolysis TaqMan probe has been shown to be higher than that of the SYBR Green dye method (Botezatu et al., 2017). However, mRT-PCR alone cannot distinguish between dead and viable bacteria, which can lead to false-positive results. To eliminate the interference of DNA from dead cells, a strategy that relies on sodium deoxycholate (SD) combined with propidium monoazide (PMA) pretreatment had been reported (Chang and Lin, 2018; Huang et al., 2018). Sodium deoxycholate is a bile salt that promotes membrane rupture of dead cells; PMA is a nucleic acid cross-linking agent that can selectively penetrate bacteria with injured cell membranes to sequester the DNA, preventing its amplification during PCR (Liu and Mustapha, 2014; Erkus et al., 2016). Wang et al. (2014a) used immunomagnetic separation combined with SD-PMA and RT-PCR to detect viable *E. coli* O157:H7 in milk with a limit of detection (LOD) of 10^2 cfu/mL.

In this paper, we applied SD-PMA pretreatment in combination with mRT-PCR for the specific and quantitative detection of viable *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in milk and milk products. Three fluorescence probes and 3 pairs of primers were designed for the specific multiplex detection of *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. We hypothesized that the combination of SD and PMA in the pretreatment process would eliminate amplification of DNA from dead cells. Detection of results for the viable cells was recorded through fluorescent emission spectra and confocal fluorescence microscopy (CFM) imaging.

MATERIALS AND METHODS

Bacterial Strains and Cultural Conditions

The bacterial strains used in this paper are listed in Table 1. All strains were cultured in Luria-Bertani medium overnight at 37°C with shaking at 180 rpm. The target bacteria were prepared in sterile PBS (0.01 M, pH 7.4) to obtain a serial 10-fold dilution of the cell suspensions. To obtain suspensions of dead and injured cells of *E. coli* O157:H7 ATCC 43888 (10^8 cfu/mL) and *Cronobacter* spp. CICC 21654 (10^8 cfu/mL), these were heat treated at 60°C for 5 min; *Salmonella* spp. ATCC 13076 (10^8 cfu/mL) was heated at 55°C for 10 min. Immediately after heat treatment, suspensions were cooled at 4°C in an ice bath for 5 min. Combined viable

and dead cells were spread on Luria-Bertani plates that were incubated at 37°C for 24 h before enumeration.

Primers and TaqMan Probes Design

The primers and TaqMan probes used in this study were designed using Oligo 6.7 software (<http://www.oligo.net/>) and Beacon Designer 7 software (<http://www.premierbiosoft.com/>). The design of primers and probes was based on the specific genes *fliC* (*E. coli* O157:H7), *cgcA* (*Cronobacter* spp.), and *invA* (*Salmonella* spp.). Specificity comparison among the primers and probes was performed using BLAST analysis (National Center for Biotechnology Information; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All primers and probes were synthesized by Sangon Biotech (Shanghai, China). The oligonucleotide sequences of primers and probes are listed in Table 2. All experimental results were performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

DNA Extraction and mRT-PCR Assay

Bacterial DNA was extracted using the boiling water method. The samples were incubated in a boiling water bath for 15 min and then immediately placed on ice at 4°C for 5 min. Next, the treated samples were centrifuged at $12,000 \times g$ for 5 min at room temperature to obtain the supernatant, which contained the genomic DNA. The supernatant was stored at -20°C before use.

The mRT-PCR assay was conducted in a CFX96 Touch real-time PCR detection system, with a final volume of 25 μ L containing 12.5 μ L of premixed Ex Taq probe (TaKaRa Biotech Co. Ltd., Dalian, China), 1 μ L of each primer (10 μ M) for *fliC*, *cgcA*, and *invA*, 0.5 μ L of each probe (10 μ M) for *fliC*, *cgcA*, and *invA*, and 2 μ L of each target bacteria DNA. The mRT-PCR conditions were as follows: preincubation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1.5 min. The mRT-PCR assay was performed in triplicate parallel analysis in 8-tube strips. Sterile water replaced the DNA templates for negative controls to ensure the absence of contamination in the tubes. All data and results were analyzed with Bio-Rad CFX Manager software (Bio-Rad Laboratories).

Optimization of SD Concentration

The treatment steps were based on a previous report (Wang et al., 2014b). The SD stock solution (10%, wt/vol) was prepared by dissolving SD in 0.1% (wt/vol)

Table 1. Target and nontarget bacterial strains and results of the PCR

No.	Bacterial strain	Strain ID	Source ¹	PCR ²		
				<i>invA</i>	<i>fliC</i>	<i>cgcA</i>
1	<i>Cronobacter sakazakii</i>	29544	ATCC	—	—	+
2	<i>Cronobacter muytjensii</i>	51329	ATCC	—	—	+
3	<i>Cronobacter turicensis</i>	24178	CICC	—	—	+
4	<i>Cronobacter condimenti</i>	24184	CICC	—	—	+
5	<i>Cronobacter dublinensis</i>	24179	CICC	—	—	+
6	<i>Cronobacter dublinensis</i>	21564	CICC	—	—	+
7	<i>Cronobacter malonaticus</i>	45402	CMCC	—	—	+
8	<i>Cronobacter</i> spp.	21590	CICC	—	—	+
9	<i>Cronobacter</i> spp.	21654	CICC	—	—	+
10	<i>Cronobacter</i> spp.	45401	CMCC	—	—	+
11	<i>Cronobacter</i> spp.	YC633B	—	—	—	+
12	<i>Salmonella enteritidis</i>	13076	ATCC	+	—	—
13	<i>Salmonella typhimurium</i>	13311	ATCC	+	—	—
14	<i>Salmonella typhimurium</i>	50115	CMCC	+	—	—
15	<i>Salmonella typhi</i>	40002	JX-CDC	+	—	—
16	<i>Salmonella paratyphi</i>	50094	CMCC	+	—	—
17	<i>Escherichia coli</i> O157:H7	43888	ATCC	—	+	—
18	<i>Escherichia coli</i> O157:H7	44828	CMCC	—	+	—
19	<i>Escherichia coli</i> O157:H7	2502	—	—	+	—
20	<i>Staphylococcus aureus</i>	26001	CMCC	—	—	—
21	<i>Staphylococcus aureus</i>	26002	CMCC	—	—	—
22	<i>Staphylococcus aureus</i>	26003	CMCC	—	—	—
23	<i>Staphylococcus aureus</i>	25923	ATCC	—	—	—
24	<i>Staphylococcus aureus</i>	7907	—	—	—	—
25	<i>Bacillus cereus</i>	14579	ATCC	—	—	—
26	<i>Pseudomonas aeruginosa</i>	10104	CMCC	—	—	—
27	<i>Listeria monocytogenes</i>	13932	ATCC	—	—	—
28	<i>Listeria monocytogenes</i>	54007	CMCC	—	—	—
29	<i>Listeria seeligeri</i>	35967	ATCC	—	—	—

¹ATCC = American Type Culture Collection (Manassas, VA); CICC = Center of Industrial Culture Collection (Beijing, China); CMCC = China Medical Culture Collection (Beijing, China); — = laboratory separation and identification; JX-CDC = Jiang Xi Province Center for Disease Control and Prevention (Nanchang, China).

²Result (+/–) indicates positive and negative signals for the *invA*, *fliC*, and *cgcA* genes specific for *Salmonella* spp., *E. coli* O157:H7, and *Cronobacter* spp.

peptone water. The suspensions of dead and injured bacteria in the exponential phase were centrifuged at $12,000 \times g$ for 3 min at room temperature and the resulting pellets were suspended in sterile PBS (0.01 M, pH 7.4) at different concentrations of SD: 0, 0.05, 0.10, 0.25, and 0.50%. The suspensions were mixed by vortexing at 180 rpm and incubated at 37°C for 30 min before the PMA treatment (described below). At

the end of these processes, the bacterial DNA was extracted for RT-PCR analysis.

Verification and Optimization of PMA Treatment

A 1 mg/mL PMA stock solution was prepared by dissolving 1 mg of PMA in 20% dimethyl sulfoxide (DMSO) and stored at –20°C in the dark. Suspen-

Table 2. Primers and probes used in the real-time PCR

Target bacteria	Target gene	Primer and probe sequence (5'→3')	GenBank accession no.
<i>Escherichia coli</i> O157:H7	<i>fliC</i>	TCAGAATCGGAGTTAGTCCCTGT GTAACGCCAACGACGGTATTT HEX–CACGAATACGCTGTAAGTTGTTGT–BHQ1	JX847136.1
<i>Cronobacter</i> spp.	<i>cgcA</i>	TCTGGCTTATGGCTATCTTCCC TCCGCAATGTTGTCTCAGC TexasRed–AACGCATCGCTTGCCGAGCTGGA–BHQ2	CP012253.1
<i>Salmonella</i> spp.	<i>invA</i>	TTCCGCAACACATAGCCAAGC AATCCAACAATCCATCAGCAAGG FAM–TTTCTCCCCCTCTTCATGCGTTAC–BHQ1	DQ644631.1

sions of viable and dead bacteria (10^8 cfu/mL) were subjected to SD pretreatment as described above, and the PMA stock solution was added to obtain final concentrations of 0, 5, 10, 15, and 20 $\mu\text{g/mL}$, respectively. After incubating the samples for 5 min in the dark with occasional mixing, they were placed on ice and exposed to a 500-W halogen source for 5 min. During the exposure period, the tubes containing the samples were placed approximately 20 cm from the light source and shaken every 30 s to ensure uniform illumination. To eliminate free PMA after PMA cross-linking, the sample tubes were centrifuged at $12,000 \times g$ for 5 min and the pelleted cells were washed with PBS twice. The genomic DNA of these SD-PMA-treated cell suspensions was extracted for the RT-PCR assay.

Propidium monoazide is a high-affinity photo-reactive DNA binding dye with very weak fluorescence regardless of the source of the DNA. In a bacterial assay, only the viable cells are of interest because these cause diseases, unlike dead cells. Therefore, the dead cells that absorb the PMA must be eliminated. A microplate reader (Varioskan LUX, Thermo Fisher Scientific, Waltham, MA) was used to read the fluorescence intensity after the PMA treatment. For optimization purposes, fluorescent DNA dyes were used to visually verify the effect of PMA treatment. For this experiment, mixed solutions containing 1:1 dead and viable bacteria were treated with SD and PMA accordingly. These were treated with the fluorescent DNA dye Hoechst 33342 (Keygen Biotech, Nanjing, China), washed, and fixed in 4% paraformaldehyde for 30 min at room temperature and mounted into glass slides containing anti-quenching reagents. Confocal fluorescence microscopy images were collected using an inverted Leica confocal laser scanning microscope (Leica TCS SP8 CARS, Leica Microsystems, Wetzlar, Germany) with a $100\times$ oil objective and $3\times$ zoom condition. Fluorescent images of bacteria stained with PMA and Hoechst 33342 were obtained at absorbance/emission wavelengths of 510/610 nm and 425/475 nm, respectively.

Standard Curves and LOD of the SD-PMA-mRT-PCR

Three overnight cultures of fresh target bacteria were prepared to obtain a 10-fold serial dilution (10^7 to 10^1 cfu/mL) followed by SD and PMA treatment. Each reaction was amplified in triplicate, and sterile water was used instead of the template DNA to obtain the negative control. The cycle threshold (C_t) values corresponding to different bacterial concentrations were used to establish the curve.

To verify the effect of the SD-PMA-mRT-PCR method in a sample matrix, we established another standard curve using bacterial DNA extracted from artificially

spiked skim milk. The skim milk was purchased from a local supermarket (Rainbow Supermarket, Nanchang, China). For this, 1 g of skim milk powder was mixed with 9 mL of PBS to form a milk homogenate. The absence of the 3 target pathogens in the homogenate was analyzed by culture methods. The milk homogenate was inoculated with *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. at final concentrations ranging from 10^1 to 10^7 cfu/mL. After treatment with SD and PMA, the isolated bacteria pellets were resuspended in 100 μL of ultrapure water. Genomic DNA was extracted by the boiling water method as described above. Optimized SD-PMA-mRT-PCR conditions were applied for detection of viable target cells in artificially contaminated (spiked) samples. All experiments were conducted in triplicate. Figure 1 shows the process and principle behind the mRT-PCR detection of viable *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in artificially contaminated milk.

Recovery Assessment of Viable Target Bacteria by SD-PMA-mRT-PCR

To determine the accuracy of the proposed assay, a mixed bacterial suspension containing a known number of dead and viable bacteria was prepared. The experiments were divided into 2 groups, one of which contained 10^6 cfu/mL viable bacteria and the other a mixture of 10^7 cfu/mL dead bacteria (identified using the culture method) and 10^6 cfu/mL viable bacteria. The 2 groups were treated with SD and PMA as described above, and DNA samples from each group were extracted for mRT-PCR assay. The C_t values were used for recovery assessment.

Statistical Analysis

All data are expressed as means $\pm$ standard deviations. Data from the optimized SD-PMA-mRT-PCR assays were analyzed by one-way ANOVA. Statistical analysis and standard deviation calculations were carried out by using Excel software (Microsoft Office 2010 for Windows, Microsoft Corp., Redmond, WA). Drawings were done with GraphPad Prism 6 (GraphPad-Prism Software Inc., San Diego, CA) and SigmaPlot 12.5 software (Systat Software GmbH, Erkrath, Germany).

RESULTS

Verification of Primers and Probes

To evaluate the specificity and accuracy of primers and probes used in this study, mRT-PCR was used to perform melting curve analysis of the amplicons. As

shown in Figure 2A, all 3 pair of primers generated separate single melting peaks in the mRT-PCR reaction. Furthermore, 3 strong fluorescence signals from the TaqMan-based mRT-PCR reaction indicated that all primers and probes achieved simultaneous, efficient, and accurate amplifications (Figure 2B).

Optimization of mRT-PCR Assay and SD Treatment of Bacteria

The concentration and annealing temperature of each pair of primers and probes was optimized. The results showed that optimum conditions were 0.4 μM for *fliC*, *cgcA*, and *invA* primers and 0.2 μM for P-*fliC*, P-*cgcA* and P-*invA* (probes) at an annealing temperature at 60°C (data not shown). The mRT-PCR measurement was performed using a Ct value.

Before PMA treatment was administered, we determined the most suitable SD concentration to assess the ability of SD to enhance membrane penetrability on dead and membrane-injured cells without affecting the viable cells. As shown in Figure 3A, the Ct value obtained from *E. coli* O157:H7 detection first increased and then decreased with increasing SD concentration from 0 to 0.50%. The Ct value reached its peak at a SD concentration of 0.1%, indicating that a 0.1%

SD concentration was the optimum concentration for the detection of *E. coli* O157:H7 without interference from injured and dead cells before the PMA treatment. Similarly, a 0.25% SD treatment for *Cronobacter* spp. (Figure 3B) and 0.1% SD treatment for *Salmonella* spp. (Figure 3C) were obtained. To inhibit the detection of injured and dead cells without a negative effect on viable cells, a 0.1% SD concentration was chosen as the optimum condition for later studies.

Optimization of PMA Treatment on Bacteria

We established the most appropriate PMA concentration that did not affect viable cells to assess the ability of PMA to inhibit the detection of dead bacteria DNA after SD treatment. As shown in Figure 4A, after the PMA treatment of dead *E. coli* O157:H7 cells, we observed an increase in Ct value at 0 to 10 $\mu\text{g}/\text{mL}$, which reached a plateau at 10 to 20 $\mu\text{g}/\text{mL}$ PMA. Thus, a PMA concentration of 10 $\mu\text{g}/\text{mL}$ allowed detection of viable *E. coli* O157:H7 without interference from injured and dead cells after SD treatment. The same concentration of PMA was optimal for *Cronobacter* spp. (Figure 4B) and *Salmonella* spp. (Figure 4C); thus, a PMA concentration of 10 $\mu\text{g}/\text{mL}$ was used in the subsequent studies.

Figure 1. Working principle of the sodium deoxycholate (SD)-propidium monoazide (PMA)-multiplex real-time (mRT)-PCR assay for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157:H7, and *Cronobacter* spp. in milk.

Figure 2. Verification of primers and probes: (A) melting peaks of the 3 pairs of primers (Table 2) during PCR amplification; and (B) amplification curves (relative fluorescence units, RFU) of the 3 pairs of probes for detection of *Salmonella* spp., *Escherichia coli* O157:H7, and *Cronobacter* spp. in milk. $-d(RFU)/dT$ = the negative first derivative of the RFU. Horizontal lines in panel B are baseline thresholds.

Verification of PMA Treatment Effect

According to the spectral properties of PMA, the absorption and emission wavelengths after binding to DNA/RNA are 510 and 610 nm, respectively. As shown in Figure 5, fluorescence intensity reached a maximum at 610 nm in dead bacteria and was close to zero in viable bacteria. Imaging was obtained using CFM to

validate the effects of the PMA treatment. In Figure 6, each bacterium tagged with PMA or Hoescht 33342 emitted the corresponding fluorescence, with red indicating dead bacteria (Figure 6B) and blue indicating viable bacteria (Figure 6C), which were merged in Figure 6D. These results indicated that PMA successfully cross-linked DNA from dead bacteria and did not affect the signals for viable bacteria.

Figure 3. Optimization of concentration of sodium deoxycholate (SD) for detection of (A) *Escherichia coli* O157:H7, (B) *Cronobacter* spp., and (C) *Salmonella* spp. Each bar represents the average cycle threshold (Ct) value of the real-time PCR in triplicates; error bars represent SD.

Figure 4. Optimization of concentration of propidium monoazide (PMA) for detection of (A) *Escherichia coli* O157:H7, (B) *Cronobacter* spp., and (C) *Salmonella* spp. Each bar represents the average cycle threshold (Ct) value of the real-time PCR in triplicates; error bars represent SD.

Figure 5. Verification of the effect of propidium monoazide (PMA) treatment. Fluorescent emission spectra (excited by a 510-nm UV lamp) for detection of (A) *Escherichia coli* O157:H7, (B) *Cronobacter* spp., and (C) *Salmonella* spp.

Standard Curves and LOD of the mRT-PCR

Standard curves were established by using the optimized SD-PMA-mRT-PCR assay to determine viable *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. concentrations. As shown in Figure 7, the standard curves gave linear correlations: $R^2 = 0.9935$ for *E. coli* O157:H7, $R^2 = 0.9914$ for *Cronobacter* spp., and $R^2 = 0.9919$ for *Salmonella* spp. The LOD were 10^2 cfu/mL for *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in pure culture. To verify the feasibility of this method to test actual samples, standard curves were established for detection of target bacteria in artificially contaminated milk. In Figure 7, standard curves for each of the bacteria showed linear correlations: $R^2 = 0.9895$ for *E. coli* O157:H7, $R^2 = 0.9948$ for *Cronobacter* spp., and $R^2 = 0.9856$ for *Salmonella* spp. A similar LOD of 10^2 cfu/mL was determined for *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. There was no significant difference in signals obtained using the SD-PMA-mRT-PCR detection of the 3 pathogens in pure culture and in artificially contaminated milk. Therefore, the method can be used to simultaneously detect these 3 pathogenic bacteria without interference from the milk components.

Recovery Assessment of Viable Bacteria by mRT-PCR

To determine the dependability of this SD-PMA-mRT-PCR method, we analyzed mixed bacteria at high concentrations (10^7 cfu/mL dead bacteria and 10^6 cfu/mL viable bacteria). Mean Ct values obtained from the mixture and the pure viable cells were used to calculate the recovery. As shown in Figure 8, the recovery of the 3 target bacteria was between 100 and 107%. Even in the presence of high concentrations of dead bacteria, the viable bacteria could be detected accurately using the method developed in this study.

Specificity of the mRT-PCR

The specificity of the mRT-PCR assay was evaluated using 29 strains including 19 target bacteria, as listed in Table 1. Among the 19 target bacteria, 11 strains were *Cronobacter* spp., 5 strains were *Salmonella* spp., and 3 strains were *E. coli* O157:H7. The amplification results indicated that *cgcA* can be used to specifically detect strains of *Cronobacter* spp., and similar results were found for *invA* and *fliC*; none of the non-target strains yielded positive results. The results revealed that the SD-PMA-mRT-PCR assay developed in this study was specific to *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp.

Figure 6. Confocal fluorescence microscopy images of double-stained *Escherichia coli* O157:H7: (A) with light microscopy with no stain, (B) stained with Hoechst 33342, (C) stained with propidium monoazide (PMA), and (D) merged image (Hoechst 33342 and PMA).

DISCUSSION

The mRT-PCR assay has been widely used as an effective technology in the quantitative detection of pathogenic bacteria (Deora et al., 2015; Li et al., 2015) because of several advantages, including high sensitivity, wide detection linear range, and good detection accuracy and repeatability. However, RT-PCR analysis fails to distinguish between surviving, dead, and injured bacteria, and has not been shown to detect multiple

bacteria in one reaction tube at the same time. Previous studies have shown that SD pretreatment combined with PMA was an effective method for detecting live cells (Wang et al., 2014b; Zhou et al., 2017). Combining SD and PMA with the mRT-PCR platform as described here shows potential for the simultaneous detection of 3 viable target bacterial pathogens.

It has been reported that, under normal conditions, injured cells are dormant and do not cause infection and harm to their hosts (Morgan et al., 1993). Previous

Figure 7. Establishment of standard curves for sodium deoxycholate and propidium monoazide multiplex real-time PCR. (A) *Escherichia coli* O157:H7; (B) *Cronobacter* spp.; and (C) *Salmonella* spp. The DNA templates were from a 10-fold serial dilution of bacteria. Each point represents the average cycle threshold (Ct) value from triplicate analysis. Error bars represent SD.

Figure 8. Recovery of viable *Escherichia coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. by sodium deoxycholate (SD)-propidium monoazide (PMA)-multiplex real-time (mRT)-PCR assay. Each bar represents the average cycle threshold (Ct) value from triplicate analysis; error bars represent SD.

research had successfully used PMA pretreatment with RT-PCR to detect viable food-borne pathogens in milk (Wang et al., 2014b; Dong et al., 2018). However, PMA treatment does not completely inhibit DNA amplification from dead and injured bacteria due to the ineffective barrier function of injured cell membranes. To eliminate PMA sequestration of DNA from dead cells, we used the SD pretreatment to enhance the PMA method (Zhou et al., 2016). The optimum concentrations of 0.10% SD and 10 $\mu\text{g/mL}$ PMA effectively inhibited DNA amplification of dead and membrane-injured cells but did not affect the signal from intact cells.

Propidium monoazide can be used not only to distinguish between dead and viable bacteria, but also as a fluorescent dye that binds to DNA to verify the effect of treatment. However, fluorescence intensity significantly increases after binding the nucleic acid (Nocker et al., 2006; Fittipaldi et al., 2012) from both dead and viable cells. Previous reports only studied optimization of PMA treatment without verification of how it affects the bacteria (Wang et al., 2014b). In this study, we applied this feature of PMA to determine the ability to distinguish between viable and dead bacteria. Compared with viable bacteria, the DNA of dead bacteria that was cross-linked to PMA showed significant fluorescence intensity at 610 nm. In addition, we used double fluorescent markers to successfully distinguish between dead and viable bacteria by CFM

imaging. This technique confirmed visually that PMA was able to penetrate dead bacteria but not the viable bacteria, which allowed for specific detection of target viable bacteria.

Design of primers and probes is crucial for the development of sensitive, specific mRT-PCR. In the present report, we selected *fliC* (encoding flagellin protein of *E. coli* O157:H7), *cgcA* (the diguanylate cyclase gene of *Cronobacter* spp.), and *invA* (type III secretion system protein InvA of *Salmonella* spp.) genes for the PCR (Collazo and Galán, 1997; McNeilly et al., 2008; Hu, 2018). According to the literature, *fliC* is specific to *E. coli* O157:H7 and does not exist in other serotypes (Narang et al., 2009). The *invA* gene is a virulence gene encoding an invading protein that has been shown to be unique to *Salmonella* spp. (Rahn et al., 1992). The *cgcA* gene can be used to detect all 7 recognized species within the *Cronobacter* genus (Carter et al., 2013; Yan and Fanning, 2015). Three pairs of primers were designed to have similar melting temperature values (~60°C), whereas that of the probes was about 10°C higher to ensure efficient hybridization with the template (Thornton and Basu, 2011). The size of the amplified fragment was between 80 and 200 bp to ensure efficient amplification. Li et al. (2015) reported that amplicon sizes of 80, 123, and 149 bp had good detection efficiency. These primers and probes did not cross-react as indicated by a software simulation that was confirmed through the RT-PCR verification. Therefore, the 3 pairs of primers and probes chosen for the SD-PMA-mRT-PCR assay developed in this study could be used for simultaneous and specific detection of the 3 viable target bacterial pathogens in milk.

CONCLUSIONS

We established a rapid and accurate multiplex RT-PCR combined with SD and PMA pretreatment platform (SD-PMA-mRT-PCR) for simultaneous detection of 3 viable organisms: *E. coli* O157:H7, *Cronobacter* spp., and *Salmonella* spp. in milk. The reaction conditions were optimized to specifically detect 10² cfu/mL of *E. coli* O157:H7, 10² cfu/mL of *Cronobacter* spp., and 10² cfu/mL of *Salmonella* spp. in milk. The specific and rapid SD-PMA-mRT-PCR method developed in this study holds promise for the simultaneous quantitative detection of food-borne pathogen contamination in milk.

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