Rapid and simultaneous quantification of viable *Escherichia coli* O157:H7 and *Salmonella* spp. in milk through multiplex real-time PCR

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ABSTRACT

Escherichia coli O157:H7 and *Salmonella* spp. in milk are 2 common pathogens that cause foodborne diseases. An accurate, rapid, specific method has been developed for the simultaneous detection of viable *E. coli* O157:H7 and *Salmonella* spp. in milk. Two specific genes, namely, *fliC* from *E. coli* O157:H7 and *invA* from *Salmonella* spp., were selected to design primers and probes. A combined treatment containing sodium deoxycholate (SDO) and propidium monoazide (PMA) was applied to detect viable *E. coli* O157:H7 and *Salmonella* spp. only. Traditional culture methods and SDO-PMA-multiplex real-time (mRT) PCR assay were applied to determine the number of viable *E. coli* O157:H7 and *Salmonella* spp. in cell suspensions with different proportions of dead cells. These methods revealed consistent findings regarding the detected viable cells. The detection limit of the SDO-PMA-mRT-PCR assay reached 10² cfu/mL for *Salmonella* spp. and 10² cfu/mL for *E. coli* O157:H7 in milk. The detection limit of SDO-PMA-mRT-PCR for *E. coli* O157:H7 and *Salmonella* spp. in milk was significantly similar even in the presence of 10⁶ cfu/mL of 2 nontarget bacteria. The proposed SDO-PMA-mRT-PCR assay is a potential approach for the accurate and sensitive detection of viable *E. coli* O157:H7 and *Salmonella* spp. in milk.

Key words: foodborne pathogen, sodium deoxycholate, propidium monoazide, multiplex real-time PCR

INTRODUCTION

In recent years, the security of milk and its derived products has faced severe challenges (Amagliani et al., 2016; Munsu et al., 2016). As vital components in diets of many countries, milk and its derived products are easily contaminated by pathogenic bacteria at any stage from production to consumption because of their rich nutrition (Angulo et al., 2009). Data from the Cen-

ters for Disease Control and Prevention showed that 87 outbreaks of foodborne illnesses were attributed to pathogen-contaminated raw milk and cheese products, causing 750 illnesses and 215 hospitalizations from 2009 to 2014 (Costard et al., 2017). Among foodborne pathogens, *Escherichia coli* O157:H7 and *Salmonella* spp. have been recognized as common disease-causing agents in milk (Kim and Bhunia, 2008; Wang et al., 2016). *Escherichia coli* O157:H7 is a highly pathogenic strain that causes serious acute illnesses, such as hemolytic uremic syndrome, hemorrhagic colitis, bloody diarrhea, and long-term sequelae at low infectious doses (Karmali, 1989; Nataro and Kaper, 1998; Paton and Paton, 1998). *Salmonella* spp. is related to typhoid fever, septicemia, gastroenteritis, and death (Andrews-Polymenis et al., 2010). Salmonellosis also accounts for 40 to 60% of foodborne diseases in China (Ran et al., 2011). Thus, a rapid, sensitive, and accurate detection method is urgently needed to identify these 2 foodborne pathogens and consequently prevent milk contamination.

The standard method used to detect these 2 pathogens is based on culturing, but this conventional approach is laborious and time consuming (Garrido et al., 2013; Ma et al., 2014). As such, a rapid method should be developed to identify these pathogens. As a replacement of traditional culture methods, PCR is increasingly applied to sensitive and rapid detection of pathogens in food (Wang et al., 1997; Elizaquível et al., 2011). Multiplex PCR (**mPCR**) can also be used to discover various pathogens simultaneously in a single reaction (Lee et al., 2014). However, mPCR results are commonly obtained through agarose gel electrophoresis that easily leads to cross-contamination or yields false-positive findings (Omiccioli et al., 2009). Multiplex real-time (**mRT**) PCR assay is a data analysis technique based on the real-time detection of a fluorescent signal integration in each reaction system, and this assay avoids time-consuming processes and laboratory cross-contamination (Mackay, 2004). Multiplex real-time PCR is designed using multiplex distinct dye-labeled probes, and this technique has been successfully applied to detect single or multiple foodborne pathogens simul-

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taneously (Wang et al., 2012; Zhang et al., 2015; Alves et al., 2016). However, general mRT-PCR assays cannot distinguish dead bacteria from viable bacteria. To determine viable bacteria distinctly from dead bacteria, Yang et al. (2011) and Li et al. (2016) proposed the use of a DNA-intercalating dye named propidium monoazide (**PMA**) that can effectively avoid the interference of DNA from dead cells. Although injured pathogenic bacteria lose their ability to infect hosts under normal conditions (Linder and Oliver, 1989), PMA treatment cannot completely inhibit DNA interference from some dead or injured cells with an integrated outer membrane during PCR amplification (Wang et al., 2014b). As a detergent, sodium deoxycholate (**SDO**) can disrupt integrated outer membranes from some dead or injured cells and change their permeability to enhance the effect of PMA treatment against injured or dead cells (Wang et al., 2015).

This study aimed to develop an mRT-PCR assay combined with SDO and PMA treatment to simultane-

ously and specifically detect viable *Salmonella* spp. and *E. coli* O157:H7. Two fluorescence probes were designed from *invA* and *fliC* for the specific detection of viable *Salmonella* spp. and *E. coli* O157:H7. The SDO-PMA treatment was administered before DNA was extracted for multiplex PCR amplification. Different proportions of viable and dead cells were selected to determine the effects of SDO and PMA. The feasibility of the SDO-PMA-mRT-PCR method was then evaluated in artificially spiked milk.

MATERIALS AND METHODS

Bacterial Strains, Culture Medium, and Growth Conditions

The strains used in this study are listed in Table 1. Target *Salmonella* spp. and *E. coli* O157:H7 strains and other nontarget strains were grown in Luria-Bertani (**LB**) medium placed in a rotary shaker at 180 rpm and

Table 1. Target and nontarget bacteria strains used in this study and the results of the PCR

Bacteria strain	Strain ID	Source ¹	mRT-PCR positive for ²	
			<i>invA</i>	<i>fliC</i>
<i>Cronobacter sakazakii</i> (n = 4)	45401	CMCC	—	—
	45402	CMCC	—	—
	21919	CICC	—	—
	21544	CICC	—	—
<i>Salmonella</i> Enteritidis	13076	ATCC	+	—
<i>Salmonella</i> Anatum	9270	ATCC	+	—
<i>Salmonella</i> Paratyphi A (n = 2)	9150	ATCC	+	—
	40004	JX-CDC	+	—
<i>Salmonella</i> Paratyphi B (n = 2)	40001	JX-CDC	+	—
	50094	JX-CDC	+	—
	40002	JX-CDC	+	—
<i>Salmonella</i> Typhi	13311	ATCC	+	—
<i>Salmonella</i> Typhimurium	10708	ATCC	+	—
<i>Salmonella</i> Choleraesuis	P20063L	JX-CDC	—	—
<i>Bacillus cereus</i> (n = 3)	JDZ102Y	JX-CDC	—	—
	JX0121LY	JX-CDC	—	—
	43888	ATCC	—	+
	44828	CMCC	—	+
<i>Escherichia coli</i> O157:H7 (n = 5)	12900	NCTC	—	+
	YC0540	/	—	+
	XY1246	/	—	+
	13932	ATCC	—	—
	54007	CMCC	—	—
<i>Listeria monocytogenes</i> (n = 2)	26001	CMCC	—	—
	26002	CMCC	—	—
<i>Staphylococcus aureus</i> (n = 3)	26003	CMCC	—	—
	PVPAO146	JX-CDC	—	—
<i>Vibrio parahaemolyticus</i>	10104	CMCC	—	—
<i>Pseudomonas aeruginosa</i>	63501	CMCC	—	—
<i>Bacillus subtilis</i>	25931	ATCC	—	—
<i>Shigella sonnei</i>				

¹CMCC = China Medical Culture Collection, China; CICC = Center of Industrial Culture Collection, China; ATCC = American Type Culture Collection, USA; JX-CDC = Jiang Xi Province Center for Disease Control and Prevention, China; NCTC = National Collection of Type Cultures, United Kingdom.

²Result (+/—) indicates positive and negative signals. mRT = multiplex real-time.

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

Target bacteria	Target gene	Primer and probe sequence (5'→3')	Amplicon length (bp)	Reference
<i>Salmonella</i> Enteritidis	<i>invA</i>	TTCCGCAACACATAGCCAAGC AATCCAACAATCCATCAGCAAGG FAM-TTTCTCCCCCTCTTCATGCGTTAC-BHQ ₁	95	This study
<i>Escherichia coli</i> O157:H7	<i>fliC</i>	TCAGAATCGGAGTTAGTCCCTGT GTAACGCCAACGACGGTATTT HEX-CACGAATACGCTGTAAGTTGTTGT-BHQ ₁	127	This study

37°C. The sterile PBS (0.01 M, pH 7.4) was applied to suspend fresh target bacteria to obtain 10 serial dilution cell suspensions. All of the cells were grown on LB agar plates at 37°C for 24 h to enumerate the number of viable cells by culture method.

Primers and Probes Used for mRT-PCR

The primers and probes (Table 2) used in this study were designed using Oligo 6.0 software (<http://www.oligo.net/>) and synthesized by Sangon Biotech (Shanghai, China).

mRT-PCR Assay

The mRT-PCR assay was conducted in a 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA) with a total reaction volume of 25 µL containing 12.5 µL of 2× TaqMan Fast qPCR Master Mix (Sangon Biotech), 0.4 µL of forward and reverse primers of 10 mM and 0.5 µL of probes from *invA* (**P-*invA***), 0.35 µL of forward and reverse primers of 10 mM and 0.5 µL of probe from *fliC* (**P-*fliC***), and 2 µL of each target genomic DNA. The thermal protocol of mRT-PCR was as follows: initial denaturation of genomic DNA at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. Sterile water was used in place of the DNA templates for negative controls to ensure the absence of contamination in the wells. These experiments were performed in triplicate and analyzed using SDS 2.3 (Applied Biosystems).

Optimization of SDO Concentration

A cell suspension with injured cells was obtained for the following operations to optimize the concentration of SDO. The cell suspensions of *Salmonella* Enteritidis ATCC 13076 (10⁸ cfu/mL) and *E. coli* O157:H7 ATCC 43888 (10⁸ cfu/mL) were heat-treated in a water bath at 60°C for 5 min and 55°C for 10 min, respectively, and immediately cooled at 4°C in an ice bath for 5 min (Arroyo et al., 2009). The number of viable cells in LB

was determined by plate counting and centrifuged at 10,000 × *g* for 3 min at room temperature. The pellet was resuspended in 500 µL of PBS for SDO treatment. A 10% (wt/vol) SDO stock solution was prepared by resolving 1 g of SDO powder with 0.1% (wt/vol) peptone water. Two groups (6- to 500-µL portions in each group) of cell suspensions, namely, refreshed viable cells and heat-treated cells with injured cells, were also prepared and treated with different quantities of 10% SDO stock solution to obtain final SDO concentrations: 0, 0.01, 0.05, 0.08, 0.1, and 1%. These suspensions were incubated in a rotary shaker at 180 rpm and 37°C for 30 min and treated with PMA (Zhou et al., 2016). The DNA was extracted from cell pellets for real-time PCR assay.

Optimization of PMA Concentration

To acquire the optimal PMA concentration, dead cells with an initial concentration of 10⁷ cfu/mL were obtained by exposure to 80°C for 10 min in a water bath and were immediately immersed in ice for 5 min. One portion of dead cells was demonstrated by plate count. The PMA (Biotium Inc., Hayward, CA) was dissolved in 20% dimethyl sulfoxide (Sun Nuclear, São Paulo, Brazil) to reach a final concentration of 1 mg/mL and was stored at -20°C in the dark. The viable and dead cell pretreatment with SDO, followed by treated PMA with final concentrations of 0, 0.5, 0.8, 1, 2, 5, 10, and 15 µg/mL, are described above. Afterward, the DNA from cell suspensions treated with PMA was extracted for PCR amplification.

Evaluation of the Limit of Detection of the SDO-PMA mRT-PCR Assay

To determine the limit of detection (LOD) of this method, the standard curve was obtained from genomic DNA extracted from pure bacterial suspension. Serial 10-fold dilutions of 2 target bacteria (10⁷ to 10¹ cfu/mL) from PBS treated earlier with SDO and PMA were obtained, after which the DNA was extracted for PCR amplification. The DNA was detected individually and

Figure 1. Overall process of sodium deoxycholate (SDO)-propidium monoazide (PMA)-multiplex real-time (RT) PCR assay for simultaneous detection of *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in milk. Color version available online.

simultaneously by a mRT-PCR assay. The standard curve was generated using corresponding linear relations between target bacteria concentration and cycle threshold (Ct) value.

To determine the feasibility for enumerating of viable bacterial cells, other standard curves were obtained from bacterial DNA extracted from artificially contaminated milk. The skim milk was purchased from the Rainbow Supermarket in Nanchang, China, and tested negative for target bacteria by traditional culture methods. One milliliter of milk was mixed with 9 mL of PBS to form 1:10 homogenate. Then, 50 μ L of 10-fold serial dilutions refresh cell suspensions were added to 450 μ L of produce homogenate to obtain serial dilution target bacteria (from 10^1 to 10^7 cfu/mL). Then the suspensions were isolated by centrifugation at $12,000 \times g$ for 5 min at room temperature and resuspended in isometric PBS. After SDO and PMA treatment, the isolated pellets were resuspended in 100 μ L of sterile water. The DNA was extracted by the

water-boiling method. The treated cell suspensions were boiled for 20 min to release the DNA, and following this the supernatant containing the genomic DNA was obtained by centrifugation at $12,000 \times g$ for 3 min at room temperature. The obtained DNA was stored at -20°C . The optimum mRT-PCR system was applied to quantify the cells from artificial samples. Figure 1 shows the overall process of SDO-PMA-mRT-PCR for detection of viable *E. coli* O157:H7 and *Salmonella* spp. in artificial samples.

Enumeration of Different Ratios of Viable Cells in Pure Culture by SDO-PMA-mRT-PCR Assay and Culture Method

To determine the accuracy of the developed SDO-PMA-mRT-PCR, different ratios of viable and dead cells were prepared. A different amount of viable and dead cells was added to 2 groups (5- to 500- μ L portions in each group) to obtain different proportions of

viable cells (100, 75, 25, 10, and 0%). One group was used for 10-fold dilutions to spread onto LB plates and incubated for 24 h before enumeration. The remaining groups were treated with SDO-PMA as described above. Afterward, the pellets were resuspended in 100 μ L of sterile water and extracted for mRT-PCR assay.

Specificity Testing in Artificial Contaminated Milk Samples

To test whether other bacteria would interfere with the SDO-PMA-mRT-PCR assay for simultaneous detection of viable *E. coli* O157:H7 and *Salmonella* spp. in milk, 10 serial dilutions of 500 μ L of *E. coli* O157:H7 and *Salmonella* spp. (10^1 to 10^7 cfu/mL) containing fresh *Staphylococcus aureus* (10^6 cfu/mL) and *Cronobacter sakazakii* (10^6 cfu/mL) were prepared. Then, the remaining operation was done as described previously.

Data Analysis

All data analyses were carried out in triplicate to prove the reproducibility of the assay. Data were analyzed for significant differences by SPSS version 19 (SPSS Inc., Chicago, IL). Results with $P < 0.05$ were considered statistically significant.

RESULTS

Optimization of mRT-PCR Assay

The concentrations of the 2 target primers, 2 probes, and annealing temperature were optimized to develop the mRT-PCR assay. These results revealed that the optimized annealing temperature of mRT-PCR was 60°C. In addition, the optimum properties of the designed primers and probes were as follows: 0.8 μ L for primers and 0.5 μ L for P-*invA* and 0.7 μ L for primers and 0.5 μ L for P-*fliC*. The optimized mRT-PCR data were obtained on the basis of Ct value (data not shown).

Optimization Concentration of SDO

The maximum SDO concentration without an effect on viable cells was confirmed to assess the ability of SDO to enhance penetrability to some dead and injured cells before PMA treatment was administered. In Figure 2a, the SDO pretreatment concentration increased from 0 to 0.25% before *Salmonella* spp. was treated with PMA, and the Ct value continuously increased. At 0.25% SDO, the Ct value reached the highest peak, and this finding indicated that the injured and some dead cells were completely inhibited in a real-time

PCR system. At 1% SDO, Ct value was significantly reduced ($P < 0.05$). Similar results were observed in *E. coli* O157:H7 (Figure 2b). These results confirmed that 0.25% SDO was the optimized concentration that can strengthen the inhibitory effect of PMA on injured and dead cells but not on viable cells (Yang et al., 2011).

Determination of the Optimal Concentration of PMA

In this work, the optimal concentration of PMA against dead cells was determined. After 2 kinds of

Figure 2. Optimization of the concentration of sodium deoxycholate (SDO). To reveal the effect of SDO, various cycle threshold (Ct) values were obtained by 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA). (a) *Salmonella* spp. and (b) *Escherichia coli* O157:H7. Each bar represents the average Ct value obtained by SDO-propidium monoazide (PMA)-multiplex real-time (mRT) PCR for enumeration of target bacteria in a triplicate study. Error bars represent SD.

target dead cells were treated with 0.25% SDO, respectively, a stepwise increase in Ct value was observed at 0 to 5 $\mu\text{g/mL}$ of PMA, whereas a constant Ct value was obtained at 10 $\mu\text{g/mL}$ of PMA (Figures 3a and 3b). These results demonstrated that a minimum of 5 $\mu\text{g/mL}$ of PMA was sufficiently effective to eliminate the interference from dead cells. Conversely, the Ct value of 2 viable cells did not significantly change at 0 to 5 $\mu\text{g/mL}$ of PMA ($P > 0.05$). The Ct values of viable

Salmonella spp. and viable *E. coli* O157:H7 evidently increased at 15 and 10 $\mu\text{g/mL}$ of PMA, respectively ($P < 0.05$). These results suggested that a maximum of 5 $\mu\text{g/mL}$ of PMA did not affect these viable cells. Considering the effect of PMA on dead and viable cells, we used 5 $\mu\text{g/mL}$ as the final optimized PMA concentration to inhibit the interference of dead cells on viable cells.

Standard Curves of Pure Culture and Artificial Contaminated Samples

The standard curve was established by the optimized SDO-PMA-mRT-PCR assay to quantify viable *Salmonella* spp. and *E. coli* O157:H7. In Figures 4a and 4b, the target bacteria were detected through mRT-PCR, and 2 standard curves showed an ideal linear correlation: $R^2 = 0.9981$ for *Salmonella* spp. and $R^2 = 0.9957$ for *E. coli* O157:H7. The LOD were 10^2 cfu/mL for *Salmonella* spp. and 10^2 cfu/mL for *E. coli* O157:H7.

The standard curve of artificial contaminated milk was also established to determine the practicability of this assay. In Figure 4a and 4b, 2 standard curves revealed an ideal linear correlation: $R^2 = 0.9689$ for *Salmonella* spp. and $R^2 = 0.9993$ for *E. coli* O157:H7. The LOD of the assay in artificial contaminated milk were 10^2 cfu/mL for *Salmonella* spp. and 10^2 cfu/mL for *E. coli* O157:H7. The LOD of pure culture did not significantly differ from that of artificial contaminated milk ($P > 0.05$). Thus, the developed assay could be applied to detect the 2 target bacterial strains without the interference of the food matrix.

Accuracy of the SDO-PMA-mRT-PCR Assay at Different Ratios of Viable Cells

Different ratios of viable cells were prepared to detect the bacterial strains through the SDO-PMA-mRT-PCR assay and to determine the accuracy of this assay. In Figure 5a, the number of viable *Salmonella* spp. obtained through the SDO-PMA-mRT-PCR assay was not significantly different from that determined through the traditional culture method ($P > 0.05$). A similar result was observed in the detected *E. coli* O157:H7 (Figure 5b). These results confirmed that SDO-PMA-mRT-PCR could accurately detect viable target bacteria.

Specificity of the SDO-PMA-mRT-PCR Assay

The specificity of the primers was initially evaluated (Table 1), and these results indicated that only target bacterial strains (*Salmonella* spp. and *E. coli* O157:H7) could display a positive signal. The specificity of the

Figure 3. Optimization of the concentration of propidium monoazide (PMA). The effect of PMA was revealed by different cycle threshold (Ct) values from 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). (a) *Salmonella* spp. and (b) *Escherichia coli* O157:H7. Each bar represents the average Ct value obtained by sodium deoxycholate-PMA-multiplex real-time (mRT) PCR for enumeration of target bacteria in a triplicate study. Error bars represent SD.

Figure 4. Establishment of standard curves for sodium deoxycholate (SDO)-propidium monoazide (PMA)-multiplex real-time (mRT)-PCR. Standard curves for *Salmonella* spp. (a) and *Escherichia coli* O157:H7 (b) in PBS (△) and the milk sample (●). Each data point represents the average cycle threshold (Ct) value of triplicate assays plotted against the number of viable cells per milliliter for each matrix. Error bars represent SD.

assay was then estimated in the presence of *S. aureus* (10^6 cfu/mL) and *C. sakazakii* (10^6 cfu/mL) in the artificial contaminated samples. In Figure 6a, the Ct value and LOD from detection of different concentrations of viable *Salmonella* spp. did not significantly differ in the presence or absence of *S. aureus* and *C. sakazakii* ($P > 0.05$). Similar results were obtained in the detection of *E. coli* O157:H7. These results implied that the developed SDO-PMA-mRT-PCR assay could be sensi-

Figure 5. Enumeration of different ratios of viable cells of 2 target bacteria by using the sodium deoxycholate (SDO)-propidium monoazide (PMA)-multiplex real-time (mRT)-PCR and plate count method in PBS. (a) *Salmonella* spp. and (b) *Escherichia coli* O157:H7. All enumerations of this assay for detection of viable cells were obtained from the standard curve in PBS. Each bar represents the average number of viable cells enumerated by SDO-PMA-mRT-PCR in a triplicate study. Error bars represent SD.

tive and could specifically detect the 2 target bacterial strains without the interference of nontarget bacteria.

DISCUSSION

Multiplex PCR has been used as an effective tool to quantify *Salmonella* spp. and *E. coli* O157:H7 because of its advantages, such as time savings, low cost, and easy operation (Truchado et al., 2016; Kibbee and Örmeci, 2017). However, mPCR assays have failed to fully distinguish among viable, dead, and injured bacteria (Nkuiipou-Kenfack et al., 2013; Zhou et al., 2016). A

Figure 6. The enumeration of 2 target bacteria by standard curves in presence of nontarget bacteria of 10^6 cfu/mL. (a) *Salmonella* spp. and (b) *Escherichia coli* O157:H7. Each bar represents the average cycle threshold (Ct) value obtained by sodium deoxycholate-propidium monoazide-multiplex real-time PCR for enumeration of target bacteria in a triplicate study. Error bars represent SD.

previous research demonstrated that an SDO pretreatment combined with PMA is an effective method to observe viable cells only (Wang et al., 2014b). In our study, an effective SDO-PMA-mRT-PCR assay was developed to detect viable *Salmonella* spp. and *E. coli* O157:H7 simultaneously in milk. This assay could efficiently eliminate the interference of dead and injured cells and could accurately and sensitively detect these bacterial cells in spiked milk.

In some reports, injured cells remain dormant under regular conditions, but their activity can be recovered under complicated and nutrient-rich conditions. Injured cells cannot infect and harm their hosts under normal conditions (Morgan et al., 1993). Thus, the inhibition of injured cells during detection is meaningful. As a DNA-binding dye, PMA is widely used to distinguish viable cells from dead and injured cells effectively in a detection system. However, PMA treatment fails to inhibit dead and injured cells completely because of the barrier properties of their integrated outer membrane. To solve this problem and enhance the effect of PMA, we applied an SDO pretreatment. The effects of SDO on dead and injured cells are based on its emulsifying properties, which can bind some proteins and lipids in the cell membrane to destroy their membrane structure. A specific amount of SDO cannot prevent the vitality of viable cells. The optimal concentrations of 0.25% SDO and 5 μ g/mL of PMA were selected. The dosage of PMA treatment was less than that in previous studies (Li et al., 2017a) mainly because an SDO pretreatment could improve the utilization rate of PMA and low initial target bacterial concentrations were selected to optimize the mRT-PCR detection system.

Primers and probes are the key point to ensure sensitive, specific, and simultaneous detection through mRT-PCR. In this study, a 127-bp fragment of primers and a 24-bp fragment of a probe from *fliC* of *E. coli* O157:H7 and a 95-bp fragment of primers and a 24-bp fragment of a probe from *invA* of *Salmonella* spp. were selected for the specific amplification. Previous studies involved the use of *fliC* of *E. coli* O157:H7 and *invA* of *Salmonella* spp. for specific detection. Conversely, the primers and probes were designed by the authors, and this study is a new application to combine these primers and probes with SDO-PMA-mRT-PCR for the detection of viable *E. coli* O157:H7 and *Salmonella* spp. in milk. The specificity of the primers was confirmed by using 30 strains.

The LOD is an important parameter in evaluating the feasibility and practicability of detection methods. Under normal conditions, pre-enrichment and immunomagnetic separation are frequently used to enrich target bacteria and improve LOD. Wang et al. (2014a) used an immunomagnetic separation assay for *E. coli* O157:H7 enrichment, and the LOD of this assay is 10^2 cfu/mL. Li et al. (2017b) applied a 12-h pre-enrichment for *Salmonella* Enteritidis, and the LOD of this assay is 10^2 cfu/mL in spiked vegetables. In our work, 2 primers and probes were demonstrated with an ideal amplification efficiency and optimized mPCR parameters, where LOD were 10^2 cfu/mL for *Salmonella* spp. and 10^2 cfu/mL for *E. coli* O157:H7 in pure culture. Similar results

could be obtained in spiked milk. Our proposed method saves more time and is more accurate than a regular mPCR assay for the detection of multiplex bacteria. In addition, this assay exhibited high specificity and exclusivity and thus could be applied to detect *Salmonella* spp. and *E. coli* O157:H7 specifically without interference from nontarget bacteria.

CONCLUSIONS

In this study, a new mRT-PCR combined with SDO and PMA was developed to detect viable *Salmonella* spp. and *E. coli* O157:H7 simultaneously and sensitively in milk. The primers and probes were designed to ensure the specificity of this assay and to avoid interference from food matrix and nontarget bacteria. This assay could quantify the detected viable cells without interference from dead and injured cells in milk. The optimized assay could reveal 10^2 cfu/mL of *Salmonella* spp. and 10^2 cfu/mL of *E. coli* O157:H7 in milk. Therefore, the developed SDO-PMA-mRT-PCR method is a valuable diagnostic tool to detect viable *E. coli* O157:H7 and *Salmonella* spp. in milk.

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