Quantitative PCR coupled with sodium dodecyl sulfate and propidium monoazide for detection of viable *Staphylococcus aureus* in milk

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ABSTRACT

Conventional quantitative PCR (qPCR) are unable to differentiate DNA of viable *Staphylococcus aureus* cells from dead ones. The aim of this study was to use sodium dodecyl sulfate (SDS) and propidium monoazide (PMA) coupled with lysostaphin to detect viable *Staph. aureus*. The cell suspensions were treated with SDS and PMA before DNA extraction. The SDS is an anionic surfactant, which can increase the permeability of dead cells to PMA without compromising the viability of live cells. The lysostaphin was applied to improve the effectiveness of DNA extraction. The reliability and specificity of this method were further determined by the detection of *Staph. aureus* in spiked milk. The results showed that there were significant differences between the SDS-PMA-qPCR and qPCR when a final concentration of 200 µg/mL of lysostaphin was added in DNA extraction. The viable *Staph. aureus* could be effectively detected when SDS and PMA concentrations were 100 µg/mL and 40 µM, respectively. Compared with conventional qPCR, the SDS-PMA-qPCR assay coupled with lysostaphin was more specific and sensitive. Therefore, this method could accurately detect the number of viable *Staph. aureus* cells.

Key words: propidium monoazide, sodium dodecyl sulfate, *Staphylococcus aureus*, quantitative polymerase chain reaction, internal amplification control

INTRODUCTION

Staphylococcus aureus is a spherical, gram-positive, pathogenic bacterium that can produce enterotoxins. *Staphylococcus aureus* is a major cause of bovine mastitis in dairy herds, and it causes cellulitis and osteomyelitis (Loozen et al., 2011; Zhang et al., 2015; Cortimiglia et al., 2016). The presence of *Staph. aureus* in raw milk is also a potential source of the pathogen in the dairy food chain, with consequent risk of food contamination (Guardabassi et al., 2013). High prevalence of *Staph. aureus* has been reported in some countries, such as the United States and Italy (Heidinger et al., 2009; Cortimiglia et al., 2016).

Therefore, accurate and rapid methods for detecting *Staph. aureus* are in urgent demand. Culture-based methods for detecting *Staph. aureus* are inexpensive and reliable but laborious and time consuming (Velusamy et al., 2010). Methods based on PCR technology could overcome these drawbacks of culture-based methods because of its specificity and sensitivity. However, it is not possible to distinguish viable cells from dead ones by using quantitative PCR (qPCR), which may lead to overestimates of *Staph. aureus* numbers (Wang and Levin, 2006).

According to permeability differences between viable and dead cells, nucleic acid dyes, such as propidium monoazide (PMA), can selectively penetrate membrane-damaged cells, bind to DNA by light activation, and sequentially inhibit amplification of the bound DNA in qPCR reactions. Therefore, only DNA from viable bacteria with intact membranes can be detected in PMA-qPCR systems (Nocker et al., 2006; Gensberger et al., 2014; Xiao et al., 2015; Truchado et al., 2016). However, some inhibitors from the sample matrix may influence the effectiveness of PMA, leading to overestimation of viable cells, which limits the application of the

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methods (Seinige et al., 2014a; Truchado et al., 2016). Sodium dodecyl sulfate can increase the permeability of dead cells to PMA without compromising the viability of live cells (Takahashi et al., 2017). Therefore, SDS could be used to treat *Staph. aureus* along with PMA. Lysostaphin is an antibacterial enzyme that specifically cleaves the peptidoglycan cross-linking pentaglycine bridges of *Staph. aureus*. It was used to hydrolyze the cell wall and lyse the bacteria (Francius et al., 2008).

The aim of this study was to improve and evaluate a novel qPCR assay coupled with SDS and PMA to detect and quantify viable *Staph. aureus* cells in milk. Moreover, optimization of SDS and PMA was designed to improve the detection effect of SDS-PMA-qPCR on viable *Staph. aureus* cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

In this study, *Staph. aureus* (ATCC6538) and *Bacillus cereus* (ATCC11778) were cultured in Luria-Bertani medium (Beijing Land Bridge Technology Ltd., Beijing, China) in a rotary shaker at 37°C for 24 h until the stationary phase was achieved. Aliquots (10 mL) of the suspension were first transferred to a 50-mL centrifuge tube (Corning Inc., Corning, NY) and centrifuged at $15,000 \times g$ for 3 min at 4°C to harvest the cells. Then, the pellet was resuspended in sodium chloride solution (0.85%, Beijing Land Bridge Technology Ltd.). To determine the number of viable cells, 6 serial dilutions were prepared and 100- μ L aliquots from the serial dilutions were spread onto Luria-Bertani agar. Bacterial counts were determined by counting colonies after the plates were incubated at 37°C for 24 h.

Genomic DNA Extraction

The bacteria pellet was first resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and then lysostaphin stock solution was added to a final concentration of 200 μ g/mL. The mixture was incubated at 37°C for 1 h. Genomic DNA was extracted with the cetyl trimethyl ammonium bromide method. Rehydrated DNA was examined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to determine concentration and quality. All DNA samples were stored at -20°C until use.

Inclusivity and Exclusivity of Primer Tests

To examine inclusivity and exclusivity, tests were performed using a panel of 18 strains, including 10

Staph. aureus standard strains and other known food-borne pathogens (Table 1). The DNA templates were extracted using the cetyl trimethyl ammonium bromide method. The 25- μ L reaction volume contained 12.5 μ L of qPCR Master Mix (Sigma-Aldrich, St. Louis, MO), 2 μ L of sample templates, 1 μ L of each of the primers, and 8.5 μ L of distilled H₂O. The primers were 5'-CACCTGAAACAAAGCATCCTAAA-3' and 5'-CGCTAAGCCACGTCCATATT-3', and the probe was 5'-TGGTCCTGAAGCAAGTGCATTTACGA-3' (Li et al., 2015). The sequences of internal amplification control (IAC) used were 5'-CGCAAGGCTGAAACTCAAAG-3' and 5'-GAGGATGTCAAGACCTGGTAAG, and the probe was 5'-ACAAGCGGTGGAGCATGTGGTTTA (Li et al., 2015). The cycling protocol included an initial 10-min denaturation step at 95°C followed by 40 cycles of repeated denaturation at 95°C for 15 s and annealing and extension at 62°C for 1 min. Fluorescent data were acquired during the annealing and extension phase. A negative control with water was included in each qPCR reaction. After amplification, PCR products were subjected to 2.5% agarose gel electrophoresis and visualized with a UV transilluminator (Bio-Rad, Hercules, CA) after staining with nucleic acid dye (Qiagen, Hilden, Germany).

Optimization of SDS Treatment on Bacteria

Treatment conditions were as described by Takahashi et al. (2017) with slight modification. The SDS stock solution (20% wt/vol) was prepared by dissolving SDS in 0.1% (wt/vol) peptone water and then sterilized. The bacteria suspensions were centrifuged at $5,000 \times g$ for 10 min at 4°C. The pellets were then resuspended in 0.1% (wt/vol) peptone water with serially diluted SDS. The viability of bacteria in the SDS solutions with final concentrations of 0, 25, 50, 100, 250, 500, and 1,000 μ g/mL was examined on appropriate plates. The loss of viability of cells was tested by plating 100 μ L of cell suspension on agar plates at 37°C for 24 h. The concentration of SDS was optimized according to the results of plate counts for inhibition of amplification of dead *Staph. aureus* cells.

Preparation of Dead Cells

To prepare the dead cells, the bacteria cultures in the exponential phase were centrifuged at $5,000 \times g$ for 10 min at 4°C, and the pellets were washed and suspended in 0.1% (wt/vol) peptone water. Bacterial suspensions were adjusted to cell concentration between 10^7 and 10^8 cfu/mL. The suspension was divided into 2 aliquots—one used to prepare dead samples at 90°C for 20 min

Table 1. Bacteria strains used for specificity test of primers

Bacterial species	Strain ID	Source ¹	PCR result ²
<i>Staphylococcus aureus</i>	CICC22942	CICC	+
	CICC10787	CICC	+
	CICC21600	CICC	+
	CICC21676	CICC	+
	CICC10473	CICC	+
	CICC23699	CICC	+
	CICC21640	CICC	+
	CMCC26001	CMCC	+
	CMCC26002	CMCC	+
	CMCC26112	CMCC	+
<i>Cronobacter muytjensii</i>	ATCC51329	ATCC	–
<i>Salmonella enterica</i> ssp. <i>enterica</i>	ATCC14028	ATCC	–
<i>Enterococcus faecalis</i>	ATCC29212	ATCC	–
<i>Shigella flexneri</i>	ATCC12022	ATCC	–
<i>Escherichia coli</i>	ATCC25922	ATCC	–
<i>Bacillus cereus</i>	ATCC11778	ATCC	–
<i>Lactobacillus plantarum</i>	ATCC8014	ATCC	–
<i>Streptococcus salivarius</i> ssp. <i>thermophilus</i>	ATCC14485	ATCC	–

¹CICC = China Center of Industry Culture Collection (Beijing, China); CMCC = China Medical Culture Collection (Beijing, China); ATCC = America Type Culture Collection (Rockville, MD).

²+ = positive results; – = negative results.

(plate count was used to confirm that no bacteria had survived in the suspensions), and the other to prepare viable samples.

Optimization of PMA Treatment

The PMA (Biotium Inc., Hayward, CA) was dissolved in sterile distilled water to produce a 10-mM stock solution and then stored at -20°C in darkness until use. Before PMA treatment, the tested *Staph. aureus* mixtures were prepared by mixing 1 mL of viable cells with 1 mL of dead cells. Each 2-mL aliquot of the prepared bacterial suspension was treated with the optimized concentration of SDS; treated with different concentrations of PMA at final concentrations of 0, 10, 20, 30, 40, and 50 μM in darkness for 20 min; and then exposed to a 500-W halogen light source at a 20-cm distance for 10 min on ice (Chiao et al., 2014). The samples were centrifuged at $10,000 \times g$ for 20 min to remove free PMA and washed twice with PBS before DNA extraction. The minimal PMA concentration with threshold cycle (**Cq**) was accepted as the optimal PMA conditions. The extracted genomic DNA was subjected to TaqMan-based qPCR assays.

Validation of Lysostaphin Concentrations

To validate the lysostaphin concentrations, 3 aliquots of serial dilutions of known concentrations of viable *Staph. aureus* were prepared. Each aliquot of the prepared bacterial suspension was treated with different final concentrations of lysostaphin of 50, 100, and 200

$\mu\text{g/mL}$. The lysostaphin concentration resulting in a good linear standard curve was regarded as optimal.

SDS-PMA-qPCR Assay

The qPCR for *Staph. aureus* was performed in a 20- μL system containing 10 μL of Kapa Probe Fast qPCR Master Mix (Sigma-Aldrich), 1 μL of sample template, 1 μL of IAC template, 250 nM of each primer, and 500 nM of each probe. The primers and cycling protocol were the same as above. The DNA of *B. cereus* was used as an IAC.

Standard Curves and Sensitivity of SDS-PMA-qPCR

To establish a standard curve, we used aliquots of serial dilutions of viable known concentrations of *Staph. aureus*, which were purified to prepare 5 dilution points ranging from 1×10^3 to 1×10^7 cfu/mL as an external standard. The different bacterial concentrations (log colony-forming units for the reaction) were plotted against the corresponding Cq values. Linear relationships and slope for the curves were automatically calculated with Bio-Rad CFX Manager 3.1.

Detecting Viable *Staph. aureus* in Spiked Milk Using SDS-PMA-qPCR

The spiked milk was prepared to test the feasibility of SDS-PMA-qPCR. Ultra-high temperature milk (Mengniu, Inner Mongolia, China) was used in the spiking studies. The milk was first confirmed negative

for *Staph. aureus* by plate count. The milk homogenate was divided into 3 groups and inoculated with 3×10^5 cfu/mL of viable cells, 3×10^5 cfu/mL of viable cells with 3×10^6 cfu/mL of dead cells, and 3×10^6 cfu/mL of viable cells with 3×10^5 cfu/mL of dead cells, respectively. Then the samples were treated with or without optimal SDS and PMA before DNA extraction and analysis. All tests were repeated in triplicate.

RESULTS

Inclusivity and Exclusivity of Primers and Probe

The primers and probes in the study were evaluated individually by inclusive and exclusive tests (Table 1). Only DNA of *Staph. aureus* displayed positive signals, and no amplified signals were observed from DNA of *Cronobacter maytjensii*, *Salmonella enterica* ssp. *enterica*, *Enterococcus faecalis*, *Shigella flexneri*, *Escherichia coli*, *B. cereus*, *Lactobacillus plantarum*, and *Streptococcus salivarius* ssp. *thermophilus*. The results demonstrated that the primers were highly specific, and no interference was observed among DNA of *Staph. aureus* or other bacteria.

Optimization of SDS Treatment

The mean bacterial numbers on plates with different concentrations of SDS treatment were determined (Figure 1). For SDS concentrations of 0, 25, 50, and 100 $\mu\text{g/mL}$, the log colony-forming unit values were 6.5, 6.3, 6.2, and 6.0, respectively. For SDS concentrations of 250, 500, and 1,000 $\mu\text{g/mL}$, the log colony-forming

Figure 1. Optimization of SDS concentration. Samples of 3×10^7 cfu/mL of *Staphylococcus aureus* were treated with different SDS concentrations. Error bars indicate standard deviations. Different letters (a,b) indicate significant differences ($P < 0.05$). Color version available online.

Figure 2. Optimization of propidium monoazide (PMA) concentration that inhibits amplification of dead *Staphylococcus aureus* cells. Samples were treated with 0, 10, 20, 30, 40, or 50 μM PMA. Cq = threshold cycle. Different letters (a–d) indicate significant differences ($P < 0.05$). Color version available online.

unit values were 4, 4, and 0, respectively. There was a sharp decline of log colony-forming unit values within 100 to 250 $\mu\text{g/mL}$ of SDS ($P < 0.05$). Therefore, the concentration of 100 $\mu\text{g/mL}$ was considered optimum for inhibiting false-positive signals from dead *Staph. aureus* cells.

Optimization of PMA Treatment

After the SDS concentration was optimized, the optimal concentration of PMA was investigated. The Cq values with standard deviation are shown in Figure 2, and the optimal concentration was chosen as having the highest Cq value (Li et al., 2015). For PMA concentrations of 0, 10, 20, 30, 40, and 50 μM , the Cq values of *Staph. aureus* were 20.6, 20.8, 20.7, 21.2, 23.5, and 23.0, respectively. Significant differences were accepted at $P < 0.05$. Thus, the optimized PMA treatment concentration was 40 μM .

Evaluation of Lysostaphin Concentrations, Standard Curves, and Sensitivity of PMA-qPCR

The lysostaphin concentration was optimized by standard curve (Figure 3). For lysostaphin of final concentrations of 50, 100, and 200 $\mu\text{g/mL}$, coefficient of determination values were 0.866, 0.904, and 0.998 and amplification efficiency values were 160, 220, and 96%, respectively. Therefore, the optimal lysostaphin concentration was 200 $\mu\text{g/mL}$.

A qPCR system with efficiency of 90 to 110% is generally considered acceptable. Therefore, the standard curve with coefficient of determination >0.99 and amplification efficiency = 96% was chosen. The

standard curve for DNA (Figure 3A) showed a good linear relationship in the range of 10^3 to 10^7 cfu/mL, and the detection limit of the method was 10^3 cfu/mL. The slope of this standard curve was -2.032 (Figure 3).

Detection of Viable *Staph. aureus* in Spiked Milk

The SDS-PMA-qPCR assay was used to detect viable *Staph. aureus* in spiked milk to validate efficiency (Figure 4). The C_q values were 24.4 with SDS and PMA treatment and 24.8 without SDS and PMA treatment

Figure 3. The amplification efficiencies (E) of SDS-propidium monoazide (PMA)-quantitative PCR (qPCR) for *Staphylococcus aureus* at different concentrations of lysostaphin. Standard curve of the SDS-PMA-qPCR method for *Staph. aureus* in pure culture. The DNA templates were diluted from 1×10^3 to 1×10^7 cfu/mL. The detection limit was 10^3 cfu/mL. C_q = threshold cycle. Color version available online.

Figure 4. Detection of viable *Staphylococcus aureus* in spiked milk using SDS-propidium monoazide (PMA)-quantitative PCR (qPCR). The samples were treated with PMA (black bars) or not treated (gray bars). Milk samples inoculated with (a) 3×10^5 cfu/mL of viable cells, (b) 3×10^5 cfu/mL of viable cells and 3×10^6 cfu/mL of dead cells, and (c) 3×10^6 cfu/mL of viable cells and 3×10^5 cfu/mL of dead cells. All tests were carried out in duplicate. Methods for the preparation of dead cells and mixing viable and dead cells are described in Materials and Methods. Different letters (a,b) indicate significant differences ($P < 0.05$).

when milk samples were inoculated with 3×10^5 cfu/mL of viable cells with lysostaphin (Figure 4a).

When milk samples had 3×10^5 cfu/mL of viable *Staph. aureus* cells and 3×10^6 cfu/mL of dead cells with lysostaphin added, the C_q values were 24.7 with SDS and PMA treatment and 24.1 without SDS and PMA treatment. The C_q value of combinations with SDS and PMA treatment was higher than that of combinations without SDS and PMA treatment. There was a significant difference between the SDS-PMA-qPCR and qPCR ($P < 0.05$; Figure 4b). After SDS and PMA treatment, the C_q value (24.7) was similar to the C_q value (24.4) from the sample inoculated with only 3×10^5 cfu/mL of viable cells.

Similar trends were also found in the sample inoculated with 3×10^6 cfu/mL of viable cells and 3×10^5 cfu/mL of dead cells (Figure 4c). The C_q values were 24.0 and 22.7 with and without SDS and PMA treatment, respectively, for samples treated with lysostaphin.

DISCUSSION

Polymerase chain reaction methods are appropriate for routine pathogen detection (Wehrle et al., 2009). However, DNA extracted from dead cells can also be amplified by PCR, which is a major limitation in nucleic acid diagnosis (Nocker et al., 2007; Zhang et al., 2014). Some improvement in qPCR method is needed for detection of viable *Staph. aureus* in milk. To our knowledge, few studies have used SDS-PMA-

qPCR to selectively detect viable *Staph. aureus* in milk. Our method was specific and sensitive for detection of *Staph. aureus*.

Propidium monoazide-qPCR is a rapid, accurate, and convenient method for detecting bacteria in the reaction system (Wang et al., 2015; Xiao et al., 2015; Soejima et al., 2016; Truchado et al., 2016). Our study used 40 μ M PMA combined with dye incubation at 40°C for 20 min as described by Li et al. (2015) with slight modifications. The PMA-qPCR penetrated only *Staph. aureus* dead cells and eliminated false-positive results. Of all the intercalating dyes, PMA and ethidium monoazide (EMA) are the most commonly used, and EMA can penetrate some viable bacteria. Therefore, PMA is superior to EMA in differentiating viable cells from dead cells (Nocker et al., 2006; Krüger et al., 2014). Moreover, EMA is unsuitable for distinguishing between dead and viable *Staph. aureus* cells (Kobayashi et al., 2009). Propidium monoazide has been used for selectively detecting viable *Staph. aureus* (Li et al., 2015; Wang et al., 2015), with optimal concentrations of PMA of 15 and 10 μ g/mL to inhibit the PCR amplification of DNA in dead cells, respectively. The difference in PMA concentrations may be explained in 2 ways: there were differences in bacteria concentrations, or there were differences in primers because DNA sequence might affect the amplification signal suppression from dead cells (Fittipaldi et al., 2012). However, some dead cells retain a complete membrane that prevents PMA permeation, causing false-positive results, which could affect accuracy of the assay (Takahashi et al., 2017). Thus, general PMA-qPCR cannot completely eliminate interference from dead cells.

To solve this problem, SDS was applied to the PMA-qPCR (Figure 1). The SDS, as an anionic surfactant, was applied to enhance permeability of dead cells to PMA. It has no influence on viable cells (Takahashi et al., 2017). Our results indicated that SDS treatment inhibited the DNA amplification from live cells when the final concentration exceeded 100 μ g/mL (Figure 1), and so we chose 100 μ g/mL as the optimum. Takahashi et al. (2017) found that 250 μ g/mL was optimum for detection of *E. coli*, and it is possible that there will be differences in SDS concentrations between bacterial strains. To our knowledge, few researchers have used SDS in cell membrane destruction to improve permeability of dead cells to PMA. It was previously reported that sodium lauroyl sarcosinate (Wang et al., 2014a) and sodium deoxycholate (Yang et al., 2011) improved the permeability of dead cell membranes to PMA. However, these tested detergents were not very effective (Wang et al., 2014a).

In our study, 50, 100, and 200 μ g/mL of lysostaphin was added to the bacterial suspensions. Our results indicated that lysostaphin helped crack the cell membranes and effectively extract DNA at an optimal concentration of 200 μ g/mL. Few studies have used lysostaphin to extract DNA, and most researchers have used lysozyme (Ding et al., 2017; Yu et al., 2017). Yu et al. (2017) used 20 mg/mL of lysozyme to extract DNA, indicating that lysostaphin is more effective.

Detection of *Staph. aureus* in milk without interference from dead organisms was developed and tested in combination with IAC. The IAC was added to the PCR to eliminate possible false-negative results caused by incorrect operation; potential PCR inhibitors of the food matrix, such as infant food (Cortimiglia et al., 2016), milk (Li et al., 2015), and environment water (Chiao et al., 2014); or equipment malfunction errors caused by PCR components. In our study, we used the DNA of *B. cereus* as the IAC. Some previous studies used 16S rRNA as the IAC (Li et al., 2015; Wang et al., 2015); however, the 16S rRNA primer may compete with the specific primer for target DNA, thus affecting sensitivity of the qPCR assay. To avoid this drawback and the complex process of preparing the template of IAC, a type of nontarget DNA was chosen to eliminate the competition of specific primers in our system, the template of which was easy to prepare.

Previous studies found that bacteria concentrations higher than 10^8 cfu/mL can inhibit the efficiency of PMA entering dead cells and create false-positive results when applied to different bacterial concentrations (Løvdaal et al., 2011). Zhang et al. (2015) successfully eliminated the signals from dead cells when milk powder was inoculated with 3×10^2 cfu/g of viable cells and 3×10^3 cfu/g of dead cells, 3×10^3 cfu/g of viable cells and 3×10^2 cfu/g of dead cells, and 3×10^2 cfu/g of viable cells only. We chose a higher concentration of *Staph. aureus* to validate sensitivity of the SDS-PMA-qPCR. When the samples were inoculated with 3×10^5 cfu/mL of viable cells only, the Cq values showed significant difference between the PMA-qPCR and PCR ($P < 0.001$); this might be caused by the presence of injured cells, as they tend to appear during stress exposure (Fittipaldi et al., 2012). When the samples were inoculated with 3×10^5 cfu/mL of viable cells and 3×10^6 cfu/mL of dead cells or 3×10^6 cfu/mL of viable cells and 3×10^5 cfu/mL of dead cells, the detection of spiked bacterial samples by SDS-PMA-qPCR was similar to the enumeration of colony-forming units, suggesting that SDS-PMA-qPCR was specific and sensitive. We also validated the SDS-PMA-qPCR when milk was inoculated with a lower concentration of bacteria, but

there was no significant difference between the SDS-PMA-qPCR with qPCR (data not shown). This may be for similar reasons to a previous study in which a slight reduction of DNA amplification ($1-2 \log_{10}$) was detected when bacterial concentrations were low (10^4 cfu/mL; Loozen et al., 2011).

CONCLUSIONS

We developed a specific, sensitive, and accurate SDS-PMA-qPCR assay in combination with IAC for detection of *Staph. aureus* in milk. We concluded that false-positive results were eliminated by combining SDS and PMA treatments before DNA extraction, and false-negative results were effectively eliminated by application of IAC in each qPCR system. The combination of IAC and SDS-PMA-qPCR assay can be performed without significant interference from nontarget bacteria. Therefore, this improved SDS-PMA-qPCR assay is a useful tool for improving the safety of milk potentially contaminated with *Staph. aureus*.

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