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Propidium monoazide combined with real-time PCR for selective detection of viable *Staphylococcus aureus* in milk powder and meat products

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ABSTRACT

Staphylococcus aureus is a spherical, gram-positive, pathogenic bacterium commonly associated with bovine mastitis and clinical infections. It is also recognized as a pathogen that causes outbreaks of food poisoning. The objective of this study was to develop and evaluate a rapid and reliable technique that combines propidium monoazide (PMA) staining with real-time quantitative (q)PCR to detect and quantify viable cells of *Staph. aureus* in milk powder and meat products. The inclusivity and exclusivity of the assay were evaluated using 58 strains belonging to 14 species. Serial dilutions of *Staph. aureus* cells were used to establish a standard curve and to confirm the effect of PMA treatment. Milk powder and meat products were used as the spiked foods, and the ability of PMA-qPCR to eliminate nonviable cells was determined in milk powder. Furthermore, meat products were inoculated with different concentrations of *Staph. aureus* and 10^5 cfu/g of *Bacillus cereus* and *Salmonella enterica* to test the interference by nontarget microorganisms. When PMA treatment was applied before DNA extraction, we were able to eliminate false-positive results with little effect on viable cells. The PMA-qPCR assay was specific and more sensitive than conventional PCR, and the level of detection was 3.0×10^2 cfu/g in spiked milk powder. Additionally, we observed no significant interference for the detection of viable *Staph. aureus* from other nontarget bacteria. The PMA-qPCR protocol is an effective and rapid method to quantify viable cells of *Staph. aureus* in food samples. The PMA-qPCR assay is specific and reliable, offering a valuable diagnostic tool for routine analysis in food and clinical diagnostic research at a reasonable cost.

Key words: *Staphylococcus aureus*, propidium monoazide, milk powder, real-time PCR

INTRODUCTION

Staphylococcus aureus is a major human pathogen. It is ubiquitous and can colonize mammalian hosts (Dinges et al., 2000). In dairy cows, *Staph. aureus* is one of the most common pathogens that causes bovine mastitis, the most economically important disease of the dairy industry (Phuektes et al., 2001; Schlotter et al., 2014). In humans, methicillin-resistant *Staph. aureus* (MRSA) is a major bacterial pathogen that causes several community and nosocomial infections, including soft tissue and bloodstream infections, osteomyelitis, and toxic shock syndrome (Liu et al., 2012; Brukner et al., 2013). Furthermore, *Staph. aureus* contaminates various foods such as salad, cheese, milk, fish and meat, and it can be associated with food poisoning (Alarcon et al., 2006; Vázquez-Sánchez et al., 2014).

A variety of heat-resistant staphylococcal enterotoxins can contribute to symptoms of food poisoning, including diarrhea. When ingested, staphylococcal enterotoxins can induce gastroenteric syndrome, which is characterized by nausea, vomiting, diarrhea, and abdominal cramps (Zouharova and Rysanek, 2008; Shylaja et al., 2010). A dose less than 1 μg of enterotoxin, which can be produced by $>10^5$ cfu/g of *Staph. aureus*, in contaminated food can cause symptoms after 1 to 6 h in susceptible persons (Alarcon et al., 2006; Shylaja et al., 2010). Therefore, accurate and easy-to-perform methods for detection of *Staph. aureus* are epidemiologically essential.

Conventional methods for detection of *Staph. aureus* are laborious and time consuming and have low specificity (Alarcon et al., 2006). For example, the assessment of *Staph. aureus* in the milk samples from cows is typically performed by microbiological culture of milk followed by biochemical tests (Botaro et al., 2013). However, the presence of leukocytes in mastitis milk samples and high SCC in milk samples may inhibit the growth of bacteria (Phuektes et al., 2001). Hence, current methods may underestimate the level of contamination. Moreover, it is difficult to identify *Staph. aureus* in samples containing high levels of other microorganisms.

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Methods based on PCR overcome these limitations to some extent and PCR is a useful tool in the detection of *Staphylococcus* spp. and, specifically *Staph. aureus*, in clinical cases and milk samples (Verhoeven et al., 2012; Rall et al., 2014; Schlotter et al., 2014). The main advantages of PCR methods are their speed and specificity; in particular, real-time quantitative PCR (**qPCR**) can quantify the number of microorganisms within a sample (Wang et al., 2009; Yáñez et al., 2011). Nevertheless, the inability of PCR to differentiate between viable and nonviable (dead) cells in such cases is one of its major limitations (Wang and Levin, 2006). More recently, DNA-intercalating dyes such as propidium monoazide (**PMA**) and ethidium monoazide (**EMA**) have been used to efficiently detect various microorganisms in food products. These dyes selectively penetrate the compromised membranes of dead cells, allowing cross-linking to DNA to reduce the amplification ability of the DNA template (Nocker et al., 2006). Both EMA and PMA have been used on several microorganisms successfully; however, some studies have reported that PMA is more useful than EMA in eliminating the PCR signal from dead organisms (Nocker et al., 2006).

The aim of the present study was to develop a novel qPCR assay coupled with a PMA staining step to detect and quantify viable *Staph. aureus* cells selectively in milk powder and meat products.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains belonging to 14 species were used in this study for inclusive and exclusive evaluations of the PMA-qPCR assay. These strains are shown in Table 1 and were cultured according to the requirements of individual organisms. *Candida albicans* was cultured in yeast extract peptone dextrose medium for 16 h, and *Staph. aureus* and other bacteria were cultured in Luria Bertani (**LB**) medium. All bacterial strains described above were cultured at 37°C in a rotary shaker (180 rpm). To determine the counts (cfu) of viable *Staph. aureus* cells, serial 10-fold dilutions in PBS and 100 μL of cell suspensions were plated onto LB agar plates and incubated at 37°C overnight before enumeration.

Preparation of Dead Cells and PMA Treatment of Staph. aureus

Fresh *Staph. aureus* (CMCC26001; China Medical Culture Collection, Beijing, China) was cultured in LB medium and 1 mL of culture was removed and washed twice with 1× PBS. At the same time, cell viability in the suspensions was confirmed by colony count on LB plates with 1.5% agarose powder (Gene Co. Ltd., Chaiwan, Hong Kong). To obtain dead cells, 1 mL of 10^8 cfu/mL pure culture of the target bacteria was transferred and centrifuged (9,000 × *g*, 2 min, 4°C) before washing and suspension in PBS. The samples were incubated in a water bath at 80°C for 0, 5, 9, 13, 15, 17, 20, and 25 min and cooled immediately; then, 100 μL of each heat-treated cell suspension was plated onto the corresponding agar plates. Plates were then incubated overnight at the appropriate temperature. All tests were carried out in triplicate.

Propidium monoazide (Biotium Inc., Hayward, CA) was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 1 mg/mL and stored at −20°C in the dark. Samples were treated as described previously with slight modifications (Zhang et al., 2014). Briefly, 400-μL aliquots of bacterial culture were incubated with 4 μL of PMA stock solution in the dark for 5 min with occasional mixing to promote selective penetration of PMA into the dead cells. Samples were exposed to a 500-W halogen light source for 5 min to cause the cross-linking of PMA with DNA and the conversion of unintercalated PMA to hydroxylamino propidium (Josefsen et al., 2010). During the exposure, the sample tubes were placed on ice horizontally with brief shaking every 30 s and 20 cm below the light source to avoid excessive heating. After the photo-induced cross-linking, the samples were centrifuged (12,000 × *g*, 2 min, 4°C) and washed twice with an equal volume of sterile water.

Standard Curve and DNA Extraction

A standard curve was established using serial 10-fold dilutions of viable *Staph. aureus* (1.55×10^7 cfu/mL to 1.55×10^1 cfu/mL) subjected to PMA treatment and plotted as cell number versus the threshold cycle (**Ct**), the point at which the amplified DNA exceeded the detection threshold during the qPCR. Genomic DNA extraction of *Staph. aureus* was carried out following treatment with 180 μL of enzymatic lysis buffer and subsequently by using the BacteriaGen DNA Kit (ComWin Inc., Beijing, CN) according to the manufacturer's instructions. All samples were kept at −20°C until further analysis.

Primers Design and Inclusivity and Exclusivity Tests

The primers were designed using Oligo 7.0 software (Molecular Biology Insights Inc., Colorado Springs, CO) for qPCR reactions (forward: 5′-CGATTGATGGTGATACGGTT-3′; and reverse: 5′-GCACTTGCTTCAGGACCAT-3′) targeted the specific *nuc* gene (GenBank ID: DQ507382.1) of *Staph. aureus* and

Table 1. Bacterial strains used for inclusivity and exclusivity test

Bacterial species	Strain ID	Source[1]	PCR result
Candida albicans	ATCC10231	ATCC	−
Listeria monocytogenes	ATCC13932	ATCC	−
Bacillus cereus	JDZ102Y	JX-CDC	−
	P20063L	JX-CDC	−
	DA0016L	JX-CDC	−
	DA0018L	JX-CDC	−
	DA0019L	JX-CDC	−
	DA0020L	JX-CDC	−
	FX0088Y	JX-CDC	−
	FX0093Y	JX-CDC	−
	JA0056	JX-CDC	−
	JA0059	JX-CDC	−
	NC0076L	JX-CDC	−
	JX0080LY	JX-CDC	−
	YS0024L	JX-CDC	−
Shigella flexneri	ATCC29903	ATCC	−
Escherichia coli O157:H7	NCTC12900	NCTC	−
Pseudomonas aeruginosa	CMCC10104	CMCC	−
Enterobacter sakazakii	CMCC45401	CMCC	−
	CMCC45402	CMCC	−
Staphylococcus aureus	CMCC26001	CMCC	+
	CMCC26002	CMCC	+
	CMCC26003	CMCC	+
	PSAV0001	JX-CDC	+
	PSAV0002	JX-CDC	+
	PSAV0007	JX-CDC	+
	PSAV0010	JX-CDC	+
	PSAV0012	JX-CDC	+
	PSAV0013	JX-CDC	+
	PSAV0021	JX-CDC	+
	PSAV0024	JX-CDC	+
	PSAV0027	JX-CDC	+
	PSAV0035	JX-CDC	+
	PSAV0040	JX-CDC	+
	PSAV0202	JX-CDC	+
	PSAV0207	JX-CDC	+
	PSAV0209	JX-CDC	+
	PSAV0210	JX-CDC	+
	PSAV0213	JX-CDC	+
	PSAV0214	JX-CDC	+
	PSAV0216	JX-CDC	+
	PSAV0218	JX-CDC	+
	PSAV0219	JX-CDC	+
	PSAV0220	JX-CDC	+
Vibrio parahaemolyticus	PVPA0155	JX-CDC	−
Salmonella Paratyphi	ATCC9150	ATCC	−
Salmonella Anatum	ATCC9270	ATCC	−
Salmonella Choleraesuis	ATCC10708	ATCC	−
Salmonella Enteritidis	ATCC13076	ATCC	−
Salmonella Typhimurium	ATCC13311	ATCC	−
Serratia marcescens	CMCC41002	CMCC	−
Proteus vulgaris	CMCC49101	CMCC	−
Micrococcus luteus	CMCC28001	CMCC	−
Listeria ivanovii	ATCC19119	ATCC	−
Listeria welshimeri	ATCC35897	ATCC	−
Listeria seeligeri	ATCC35967	ATCC	−
Listeria grayi	ATCC25401	ATCC	−
Listeria innocua	NCTC11288	NCTC	−

[1]ATCC = American Type Culture Collection, Rockville, MD; JX-CDC = Jiang Xi Province Center for Disease Control and Prevention, Nanching, China; NCTC = National Collection of Type Cultures, London, UK; CMCC = China Medical Culture Collection, Beijing, China.

yielded a 130-bp amplicon. The amplicon length has little PMA-mediated inhibition of DNA amplification and a high DNA amplification efficiency (Li and Chen, 2013). The specificity of the sequences was checked against National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi)

nonredundant database using the BLASTN algorithm, and primers were synthesized by Shanghai Invitrogen Co. Ltd. (Shanghai, China). Inclusivity and exclusivity tests for the PMA-qPCR were performed using a panel of 58 strains, including 24 *Staph. aureus* that were standard strains or clinical isolates from JiangXi Province Center for Disease Control and Prevention, and other gram-positive or gram-negative bacterial species that are known foodborne pathogens (Table 1). DNA templates were extracted by boiling method. One milliliter of culture was removed and centrifuged (9,000 × *g*, 5 min, 4°C), resuspended with 1 mL 1× PBS twice, and dissolved in an equal volume of deionized water before boiling for 10 min. The solution was centrifuged again (11,000 × *g*, 5 min, 4°C) and the supernatant was transferred to a new tube, and stored at −20°C.

Detection of Viable Staph. aureus Cells in Spiked Milk Powder and Meat Products Using PMA-qPCR

Milk powder (Wondersun Inc., Heilongjiang, China) and 3 fresh meat products (sausage, fish, and chicken) were purchased from a local supermarket for the spiking studies. All products were previously confirmed negative for *Staph. aureus* by plate count and standard PCR. Meat products were aseptically cut into small pieces, and 1 g of each sample was mixed with 9 mL of PBS to obtain a homogenate (1:10; Yang et al., 2013). Inoculation assays consisted of 3 parts. In part 1, each homogenate was inoculated with 10^5 cfu/g of viable or dead *Staph. aureus* (3.0×10^5 cfu/g for milk powder samples and 7.5×10^5 cfu/g for meat samples). In part 2, the milk powder homogenate was divided into 5 groups and inoculated with 3×10^2, 3×10^3, 3×10^4 cfu/g of viable cells, 3×10^2 cfu/g of viable cells with 3×10^3 cfu/g of dead cells, and 3×10^3 cfu/g of viable cells with 3×10^2 cfu/g of dead cells, respectively. One milliliter of each culture was collected at 0, 2, 4, 6, and 8 h after incubation at 37°C with shaking at 180 rpm. In part 3, the homogenates of 3 meat samples were inoculated with 1×10^5 cfu/g of nontarget bacteria (*Bacillus cereus* JDZ102Y, *Salmonella enterica* ATCC13076) and with 10^7, 10^6, or 10^5 cfu/g of viable *Staph. aureus*, respectively. The collected samples were centrifuged (900 × *g*, 1 min, 4°C) to remove debris and the suspensions were centrifuged (12,000 × *g*, 5 min, 4°C).The pellets were washed twice and resuspended in 1 mL of PBS, and then treated with or without PMA as described above before DNA extraction and PMA-qPCR analysis. All of the tests were repeated in triplicate.

PCR Conditions

Real-time PCR was performed in a 96-well microtiter plate using the ABI 7900HT Fast Real-time PCR System (Applied Biosystems, Waltham, MA). Each reaction mixture used a final volume of 20 μL and contained 10 μL of SYBR Primer *EX Taq*II (Takara, Dalian, China), 0.4 μL of ROX Reference Dye (50×; Takara), 2 μL of DNA template, and 0.8 μL of each of the primers (10 μ*M* each; the final concentration was 0.2 μ*M*) with 6 μL of milli-Q H_2O (Millipore Inc., Billerica, MA) added. The real-time PCR cycling parameters were optimized as follows: 1 cycle of initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, primer annealing at 58°C for 1 min. However, conventional PCR was also used and the reaction mixture contained 5 μL of 2× Taq MasterMix (Takara), 0.2 μmol of each primer, and 2 μL of DNA template, and milli-Q H_2O was added to obtain a final volume 10 μL. The PCR reaction began with 1 cycle of 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and an extra step of 10 min at 72°C. After amplification, the products were subjected to 1.5% agarose gel electrophoresis and the gels were stained with GoldView (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) before imaging with a UV transilluminator (Bio-Rad, Hercules, CA).

Statistical Analysis

Results of the presence and absence of nontarget bacteria in spiked food, expressed as Ct differences, were compared using unpaired Student's *t*-tests; *P*-values < 0.05 were considered statistically significant.

RESULTS

Inclusivity and Exclusivity

The specificity of the assay in this study was evaluated by inclusive and exclusive tests, for which 58 strains were used, including *Staph. aureus* and other common foodborne bacteria (Table 1). Table 1 also shows amplification signals obtained from all the *Staph. aureus* strains, which are standard strains, and isolates from contaminated food; no signals from nontarget bacteria were detected. These results indicated that the *Staph. aureus* was positively identified without any interference with other bacteria.

The Effect of PMA on Dead Staph. aureus

To test the efficiency of PMA treatment on dead *Staph. aureus*, pure culture cells (viable or dead) were 10-fold serially diluted and their DNA extracted after PMA treatment. Dead cells did not show any significant amplification signals, even at 10^8 cfu/mL. However, DNA extracted from viable cells after PMA treatment

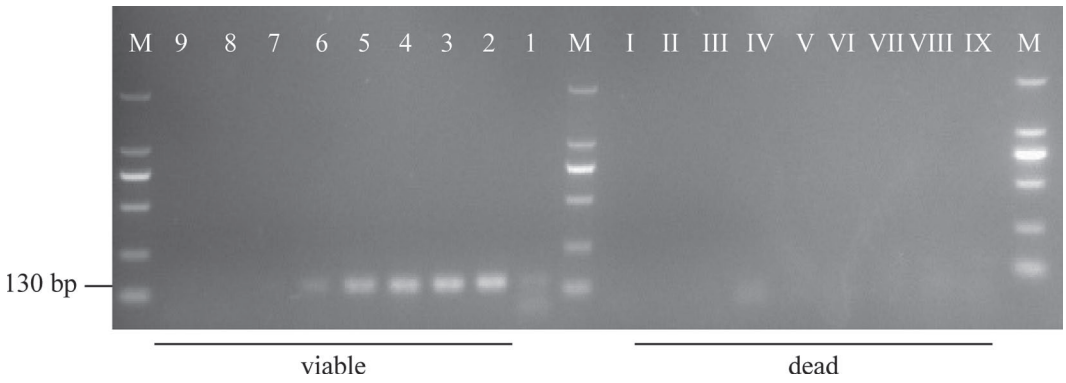

Figure 1. Agarose gel after propidium monoazide (PMA) treatment using 10-fold serial dilutions of dead and viable cells. Lanes 2 to 9 (II to IX) used DNA extracted from viable (dead) *Staphylococcus aureus* after PMA treatment of 1.5×10^8 to 1.5×10^1 cfu/mL; lane 1 (I) is the negative control; M = size marker. All tests were carried out in duplicate.

had very high signals, and the limit of detection (**LOD**) was 10^4 cfu/mL using conventional PCR (Figure 1). To obtain appropriate conditions to prepare the dead cells of *Staph. aureus*, different heat treatment conditions were evaluated. The plate counts showed 9.12 log cfu/mL in pure culture without heating; counts decreased with increased time and no colonies were observed in samples heated at 80°C for 20 min or longer. Thus, the results indicated that heat treatment at 80°C for 20 min was the optimal condition to kill the cells.

Establishment of a Standard Curve

A standard curve was generated using 10-fold serial dilutions of viable *Staph. aureus* of known concentrations and included at least 5 measurements following the procedure previously described (Alarcon et al., 2006; Kobayashi et al., 2009b). The standard curve for DNA (Figure 2) showed a strong linear correlation (R^2 = 0.9935) in the range from 10^3 to 10^7 genome equivalents per reaction, and the detection limit of the assay was 10^2 cfu (data not shown). The R^2 value >0.99 indicated that the qPCR assay was highly linear (Elizaquível et al., 2012b); a slope of −3.282 was obtained within a reasonable theoretical range and the amplification efficiency (E) was 101.7%, calculated using the formula $E = 10^{-1/\text{slope}} - 1$ (Fricker et al., 2007). This efficiency was considered acceptable because it lies between 85 and 110% (Li and Chen, 2013). Therefore, the standard curve established using the pure *Staph. aureus* DNA was used to quantify viable cells.

Detection of Viable Staph. aureus in Spiked Milk Powder

The PMA-qPCR assay was used to detect viable *Staph. aureus* in spiked milk powder. The results showed that the Ct values of milk powder were inversely correlated with the inoculated viable *Staph. aureus* concentration and duration of enrichment (Figure 3). The milk powder samples with 3×10^2, 3×10^3, and 3×10^4 cfu/g of *Staph. aureus* after 2 h of enrichment were positive; Ct values were 33.38, 31.08, and 28.81 without PMA treatment, and 33.57, 31.20, and 28.93 with PMA treatment, respectively. The sample inoculated with 3×10^2 cfu/g of *Staph. aureus* without enrichment (0 h) yielded Ct values >34 either with or without PMA treatment, and negative results were generally considered as Ct values ≥35 (Figure 3A). These results showed that *Staph. aureus* after 0 h or longer enrichment were positive either with or without PMA treatment, and the LOD was 10^2 cfu/g.

We further tested the efficiency of PMA treatment to differentiate viable cells in the presence of dead *Staph. aureus* cells in spiked milk powder samples. Combina-

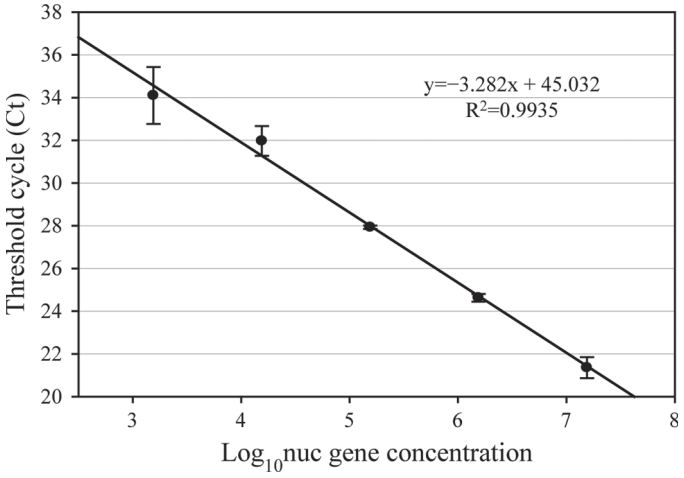

Figure 2. Standard curve of gene concentration by ABI 7900HT (Applied Biosystems, Waltham, MA) Fast real-time PCR (qPCR) targeting the thermostable nuclease encoding region (*nuc* gene) and Ct value (threshold cycle of the qPCR reaction).

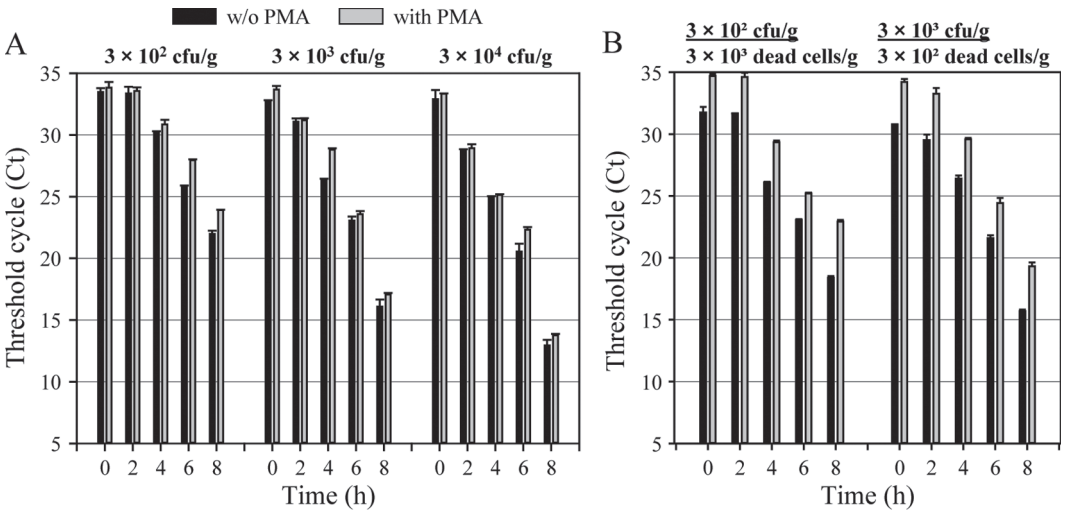

Figure 3. Detection of viable *Staphylococcus aureus* in spiked milk powder by propidium monoazide (PMA)-quantitative PCR (qPCR). The samples were incubated at 37°C, collected at 0, 2, 4, 6, and 8 h, and treated with PMA (gray bars) or not treated (black bars). (A) Milk powder samples inoculated with 3.0×10^2, 3.0×10^3, and 3.0×10^4 cfu/g of viable cells, respectively; (B) milk powder samples inoculated with 3×10^2 cfu/g of viable cells and 3×10^3 cfu/g of dead cells or 3×10^3 cfu/g of viable cells and 3×10^2 cfu/g of dead cells, respectively. Ct value = threshold cycle of the qPCR reaction.

tions of viable and dead cells were tested by PMA-qPCR. The Ct values of combinations with PMA treatment were higher than those of combinations without PMA treatment, indicating that the differences were due to the presence of dead cells (Figure 3B). Without enrichment, the milk powder with 3×10^2 cfu/g viable cells and 3×10^3 cfu/g dead cells was positive for *Staph. aureus* and the Ct value was 34.69 with PMA treatment, which was similar to the Ct value from the sample inoculated with 3×10^2 cfu/g of viable cells only (33.83 with PMA treatment; Figure 3A). Similar trends were found in the milk powder sample inoculated with 3×10^3 cfu/g of viable cells and 3×10^2 cfu/g of dead cells. These results indicated that the signals from dead cells were eliminated and PMA treatment before DNA extraction was necessary.

Detection of Staph. aureus in Meat Products in the Presence of Other Microorganisms

To determine whether the presence of other microorganisms would interfere with the proposed detection of *Staph. aureus*, the PMA-qPCR assay was performed in meat products inoculated with different concentrations of viable *Staph. aureus* and 10^5 cfu/g of nontarget bacteria (*B.* cereus JDZ102Y, *S. enterica* ATCC13076; Figure 4). The control groups (black bars) were not inoculated with nontarget bacteria and the PMA-qPCR results showed no significant differences between the experimental and control groups ($P > 0.05$). The 3 samples (sausage, fish, and chicken) with 10^5 cfu/g (5.0 log) of *Staph. aureus* generated Ct values of 29.79, 28.79, and 30.88 without nontarget bacteria, and 31.45, 31.51, and 30.64 with nontarget bacteria (Figure 4 A, B, C), respectively, indicating that nontarget bacteria slightly increased the Ct values. Similar trends were obtained in samples inoculated with 10^6 and 10^7 cfu/g. These results indicated that nontarget bacteria did not significantly affect the amplification of target DNA from viable cells using this PMA-qPCR assay.

Quantification of Staph. aureus in Spiked Food Using PMA-qPCR

These assays were confirmed by application of PMA-qPCR for the detection of viable *Staph. aureus* directly in food matrices without interference by other microorganisms (Figure 4). Then, the meat and milk powder samples in the absence of 10^5 cfu/g dead *Staph. aureus* were treated with and without PMA in parallel, and the 10^5 cfu/g viable *Staph. aureus* were treated with PMA only (Table 2). The detection of viable cells with PMA treatment was possible at 10^5 cfu/g in meat samples and milk powder, which are similar to the actual inoculated concentration. Moreover, the quantification of dead cells without PMA treatment showed a decrease of 1 log. In contrast, no significant amplification signals were obtained from dead cells treated with PMA before DNA extraction, indicating that there was no interference from dead cells in the PMA-qPCR assay.

DISCUSSION

Even though several nucleic acid-based methods are available for detection of *Staph. aureus*, there is still

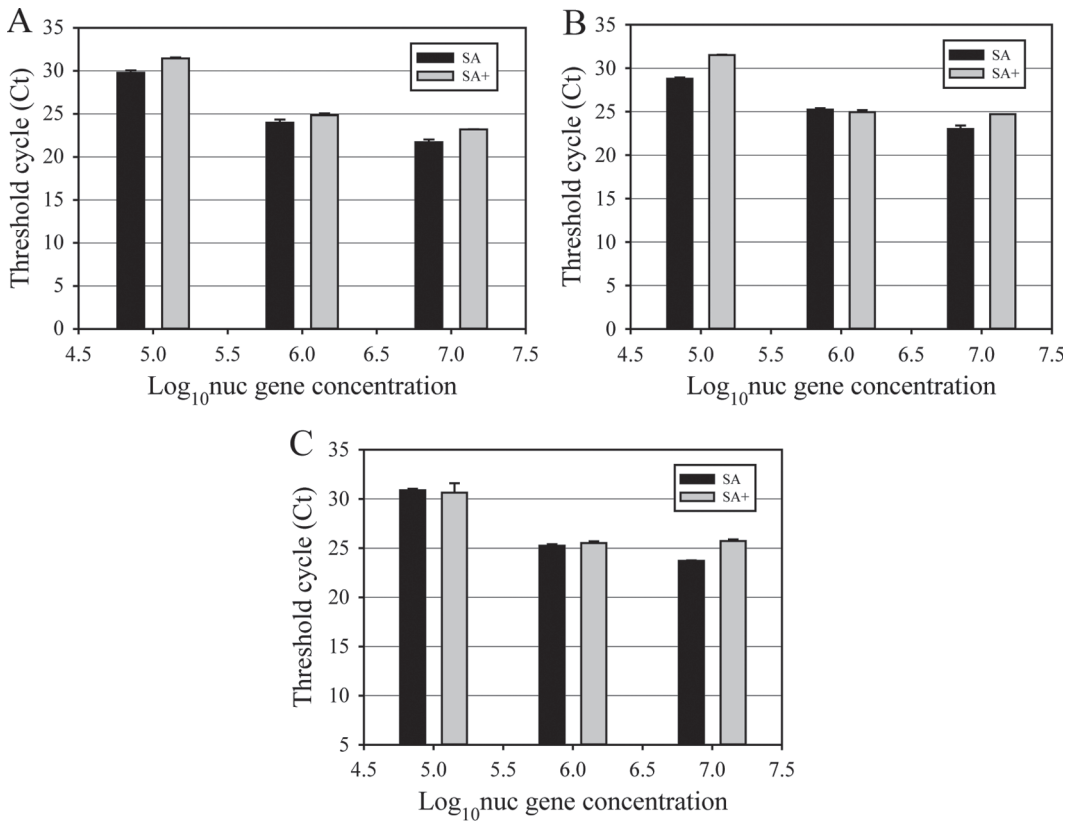

Figure 4. The effect of 1.0×10^5 cfu/g nontarget bacteria (*Bacillus cereus* JDZ102Y, *Salmonella* Enterica ATCC13076) for detection of *Staphylococcus aureus* in spiked meat samples (each sample was inoculated with viable *Staph. aureus* from 10^5 to 10^7 cfu/g): (A) sausage, (B) fish, and (C) chicken. Each bar represents the average Ct value (threshold cycle) of the quantitative PCR reaction targeting the thermostable nuclease encoding region (*nuc* gene) of a triplicate study. SA = samples inoculated with *Staph. aureus* only (black bars); SA+ = samples inoculated with *Staph. aureus* and nontarget bacteria (gray bars).

some need for improvement in qPCR tests to detect viable *Staph. aureus* in milk samples and other food samples. To our knowledge, this is the first study to use PMA-qPCR to selectively detect viable *Staph. aureus* in milk powder and meat samples. The feasibility of the method was confirmed using several organisms from our collection, and the assay was highly specific and sensitive for detection of *Staph. aureus*. Furthermore, PMA treatment eliminated the DNA amplification signals from dead cells effectively but had little effect on viable cells.

Polymerase chain reaction methods that target specific genes are appropriate for routine detection of pathogenic bacteria (Wehrle et al., 2009). However, DNA extracted from dead cells can also be amplified, which is one of the major limitations in the general application of most nucleic acid-based bacterial diagnostics (Nogva et al., 2003; Nocker et al., 2007; Zhang et al., 2014). To

Table 2. Quantification detection of viable and dead cells of *Staphylococcus aureus* in spiked food matrices using the propidium monoazide (PMA)-quantitative PCR (qPCR) assay[1]

		PMA + viable		Dead		PMA + dead
Pathogen	Food	Ct ± SD	Count	Ct ± SD	Count	Ct ± SD
Staph. aureus	Sausage	26.12 ± 0.12	5.8×10^5	29.22 ± 0.28	6.6×10^4	ND[2]
	Fish	26.89 ± 0.18	3.4×10^5	30.57 ± 0.19	2.5×10^4	ND
	Chicken	26.22 ± 0.24	5.4×10^5	30.45 ± 0.35	2.8×10^4	ND
	Milk	27.95 ± 0.08	1.6×10^5	31.44 ± 0.06	1.4×10^4	ND

[1]The food samples were inoculated with 10^5 cfu/g *Staph. aureus* and treated with PMA before DNA was extracted. Ct = threshold cycle of the qPCR assay.

[2]ND = not detected.

detect viable foodborne pathogens selectively, mRNA was evaluated using reverse transcription-PCR and was confirmed as a suitable indicator of bacterial viability (Sheridan et al., 1998). However, limitations remain when using mRNA as target molecule, because most mRNA sequences of dead bacteria have short half-lives, which are difficult to extract in cells; furthermore, reverse transcription-PCR detection is time consuming and requires high-quality mRNA (McKillip et al., 1998; Martinon et al., 2012; Soejima et al., 2012).

Propidium monoazide has been used for selective detection of viable *Escherichia coli* O157: H7, *Campylobacter*, *B. cereus* (Josefsen et al., 2010; Elizaquível et al., 2012a; Zhang et al., 2014) and to target *Staph. aureus* based on the *16S rDNA* and *tuf* genes (Kobayashi et al., 2009b). In the present study, PMA combined with real-time PCR penetrated only the compromised cell membranes of dead *Staph. aureus* and eliminated false-positive results. Our findings showed that conventional PCR signals of dead *Staph. aureus* from pure cultures with PMA treatment were completely inhibited, even at concentrations of 10^8 cfu/mL (Figure 1), and the qPCR signals of dead cells (3×10^2 or 3×10^3 cfu/g) from milk powder treated with PMA were eliminated and with little effect on viable cells (Figure 3). Ethidium monoazide has been used in the same way as PMA, but it can also penetrate some viable bacterial species, resulting in partial DNA loss (Nocker et al., 2006; Luo et al., 2010). In addition, EMA is unsuitable to distinguish between dead and viable *Staph. aureus* (Kobayashi et al., 2009a).

In this study, we chose *nuc* as the target gene; *nuc* encodes the thermostable nuclease and is thought to be present in all *Staph. aureus* (Brakstad et al., 1992). The inclusivity and exclusivity tests were performed using several species of the target bacteria as well as nontarget bacteria to show the specificity of the newly designed primers. The primer was specific and sensitive: the LOD of the PMA-qPCR assay was 1.55×10^2 cfu/mL in pure culture and 3×10^2 cfu/g in milk powder (Figure 3), indicating greater sensitivity than conventional PCR (1.5×10^4 cfu/mL; Figure 1). To our knowledge, only a few studies have tested whether the presence of nontarget microorganism can interfere with the detection of *Staph. aureus* in milk powder and meat products. In our study, the assay showed no interference from nontarget microorganisms, even at concentrations of 10^5 cfu/g (Figure 4).

CONCLUSIONS

In this study, we developed a new qPCR combined with PMA using specific primers for selective detection of viable *Staph. aureus* in milk powder and meat products. With this PMA pretreatment procedure, we completely eliminated false-positive results and quantified viable cells in spiked food samples. The method allows selective detection of 1.55×10^2 cfu/mL of *Staph. aureus* from a pure culture and 3×10^2 cfu/g in milk powder. The assay can be carried out without significant interference from nontarget microorganisms, even at a concentration of 10^5 cfu/g in meat products. Therefore, the PMA-qPCR method is a valuable diagnostic tool to assess safety of foods potentially contaminated with *Staph. aureus*.

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