Rapid propidium monoazide PCR assay for the exclusive detection of viable *Enterobacteriaceae* cells in pasteurized milk

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ABSTRACT

Pasteurized milk is a complex food and contains numerous PCR inhibitors and can often contain high levels of dead *Enterobacteriaceae* cells, depending on the condition of food sanitation. Usually, propidium monoazide (PMA) or ethidium monoazide PCR techniques decrease the number of dead bacteria by up to 3.5 log to the associated dead bacteria with no treatment. However, this difference could be insufficient to completely inhibit DNA amplification in the PCR from 10^6 cells of dead *Enterobacteriaceae* bacteria/mL, potentially contaminated in pasteurized milk. Actually, such potentially high levels of dead *Enterobacteriaceae* cells in milk has prevented milk researchers from applying PMA- or ethidium monoazide PCR to the assay of viable *Enterobacteriaceae* cells in milk. We, therefore, developed a rapid PMA real-time PCR whose minimum levels of detection were 1.5 log cfu/PCR for *Cronobacter muytjensii* and *Escherichia coli*, and 2.5 log cfu/PCR for *Salmonella enteritidis* without DNA purification in milk matrices. The PMA real-time PCR allowed us to specifically detect viable *Enterobacteriaceae* cells (5–10 cfu/mL) in pasteurized milk (20 mL) within 7.5 h of total testing time, following the hygienic guidelines for pasteurized milk in the United States and European Union. The long DNA amplification (mainly 2,451 bp) of the 16S-23S rRNA gene was completely suppressed in highly contaminated dead *Enterobacteriaceae* cells (7.5 log cfu of *Cronobacter muytjensii*) in 20 mL of pasteurized milk by 23- μ M PMA treatment. Although the contamination of the PCR reaction with 5% milk usually causes great inhibition, our method led to the successful elongation of PCR from viable *Enterobacteriaceae* cells still in the pasteurized milk matrices finally corresponding to 2 to 4 mL of milk PCR inhibitors without a DNA purification step. To comply with current customer demands for chilled

pasteurized milk at the most excellent possible quality, our new technique could enable laboratory persons in a factory to conduct rapid milk coliform testing before shipping from a factory.

Key words: viable *Enterobacteriaceae* cells, pasteurized milk, propidium monoazide-PCR, real-time PCR

INTRODUCTION

In Japan, the total viable coliform counts (CC) in raw milk have been established to be 3 to 6 log and 1 to 5 log cfu/mL during the summer and fall months, respectively (Higoshi et al., 1977). However, to meet current consumer demands for chilled, fresh pasteurized milk that is of excellent quality, factories must ship the milk before the absence of viable CC has been confirmed. It is only immediately before its arrival at the supermarket that the potential CC are assessed. Indeed, PCR is a promising technique that may allow for the establishment of a rapid milk coliform test that could be conducted before shipping; however, DNA amplification must be suppressed in the potentially high number (6 log cells/mL) of dead coliform/*Enterobacteriaceae* bacteria that could be found in pasteurized milk.

The discrimination between viable and dead bacteria may be achieved using a propidium monoazide (PMA) or ethidium monoazide (EMA) treatment before conducting the PCR assay (Rudi et al., 2005b; Chang et al., 2009, 2010; Nocker and Camper, 2009; Soejima et al., 2009; Luo et al. 2010). These DNA-intercalating dyes permeate the membrane-compromised cells and covalently bind to chromosomal DNA, following irradiation with visible light. The covalent binding of EMA is also associated with DNA breakage (Soejima et al., 2007, 2008). Both PMA and EMA allow for the complete suppression of PCR from 10^3 cells of dead bacteria/mL, but are likely to trigger false-positive results when over 10^3 cells of dead bacteria/mL are present (Rudi et al., 2005a,b; Nocker et al., 2006). Thus, routine PMA-PCR or EMA-PCR would not be feasible for use with pasteurized milk that is contaminated with high numbers of dead coliforms/*Enterobacteriaceae* (10^6 cells/mL).

Received January 18, 2012.

Accepted March 18, 2012.

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We have recently reported that the degree of PCR suppression by EMA is mainly correlated with the length of the targeted DNA with the same gene (Soejima et al., 2011a). Additionally, it has been recently shown that PMA penetrates dead bacteria more selectively than EMA (Nocker et al., 2006). The measurement of viable CC has been widely replaced by the direct detection of *Enterobacteriaceae* bacteria to assess milk contamination in recent years. Consequently, we have developed a PMA real-time PCR assay that targets long *Enterobacteriaceae* specific DNA to evaluate milk quality.

MATERIALS AND METHODS

Bacterial Culture and Preparation of Heat-Killed *Cronobacter mytjensii*

Pure cultures of *Cronobacter mytjensii* ATCC 51329, *Escherichia coli* DH5 α , *Salmonella enterica* serovar Enteritidis IID 604, *Citrobacter koseri* JCM 1658, *Bacillus cereus* JCM 2152, and *Staphylococcus aureus* ATCC 65388 were grown in brain heart infusion (BHI) broth (Eiken Chemical Co. Ltd., Tokyo, Japan). The broth medium was centrifuged at $3,000 \times g$ for 5 min at 4°C, unless otherwise stated. The obtained pellet was resuspended and serially diluted in saline (Eiken Chemical Co. Ltd.) or commercial pasteurized milk that was purchased from a local grocery store (henceforth referred to as milk). Plate counts were performed in duplicate on SPC agar (Kanto Chemical Co. Inc., Tokyo, Japan) after incubation at 37°C for 18 h using serially diluted bacterial suspensions. To obtain heat-killed *C. mytjensii*, a saline cell suspension was boiled for 2 min and appropriately diluted.

Linearity Between Cycle Threshold and Logarithmic Cell Number of *Enterobacteriaceae* Bacterial Suspensions as Assessed by Direct Real-Time Quantitative PCR Targeting 16S-23S Long DNA

A 5- μ L (0.1–6.9 log cfu/PCR) volume of each *Enterobacteriaceae* cell suspension (*C. mytjensii*, *E. coli*, or *Salmonella* Enteritidis) was added to 50 μ L of direct real-time quantitative PCR (DqPCR) master mix so as not to require a DNA isolation step (Soejima et al., 2011b), and 3 μ L of the forward primer (16S_2751F: 5'-CTACAATGGCGCATACAAAGAGA-AGCGACCT-3') and reverse primer (23S_5201R: 5'-CTTCTCCCGAAGTTACGGCACCA-3') targeting *Enterobacteriaceae* sequences in an available kit form (G&G Science Co. Ltd., Fukushima, Japan) was added at a final concentration of 0.6 μ M. The start and end

sequences of the targeted DNA (16S-23S rRNA gene, principally 2,451 bp in length) for *Enterobacteriaceae* bacteria are shown in Figure 1. To obtain all DNA sequences as shown in Figure 1, the sequencing primers were independently designed at the upstream to the sequences of 16S rRNA gene presented in Figure 1 and at the downstream to those of 23S rRNA gene disclosed in the same figure. Concretely, for a forward primer, the Seq_16S_2696F (5'-AAGGTGGGGATGACGTCAA-3'), targeting the conserved region in *Enterobacteriaceae* bacteria, *B. cereus*, and *Staph. aureus* was used. For reverse primers, the Seq_23S_Ent_23S_5279R (5'-TAAACAGTTGCAGCCAGCTGGTATCTTCGA-3'), Seq_BC_23S_5233 (5'-TATTCACTGCGGCTTTCCGTAA-3'), and Seq_SA_23S_5238R (5'-TTCTCTCCGAAATAGCTTTAGGGCTAG-3') were used for *Enterobacteriaceae* bacteria, *B. cereus*, and *Staph. aureus*. The PCR was performed using an iCycler iQ (Bio-Rad Laboratories Inc., Hercules, CA) as follows: 1 cycle at 94°C for 3 min, 40 cycles at 94°C for 30 s, 60°C for 40 s, and 68°C for 3 min, followed by a final cycle at 95°C for 3 min. Melting curves were obtained by heating the samples from 70 to 95°C in increments of 0.2°C/10 s. The PCR products were confirmed by gel electrophoresis on a 0.8% SeaKem GTG agarose (FMC BioProducts, Rockland, ME) gel made in 0.5 $\times$ TAE buffer (2.42 g of Tris/L, 0.57 mL of acetic acid/L, and 0.074 g/L of Na₂EDTA), as previously described (Minami et al., 2010). For the negative control and specificity tests, 5 μ L of SW and the viable *B. cereus* (2.5–6.1 log cfu/PCR) and *Staph. aureus* (2.3–7.1 log cfu/mL) saline suspensions were each combined with the DqPCR master mix.

Milk Treatment Followed by PMA Treatment

First, the absence of an initial population of live *Enterobacteriaceae* cells in 1 mL of commercial milk with no artificially introduced bacteria was confirmed by co-culturing SPC and Difco violet red bile agar (VRBA; Becton, Dickinson and Co., Sparks, MD) after growth at 37°C for 18 h. After centrifugation ($3,000 \times g$ for 5 min at 25°C) of 20 mL of the aforementioned commercial milk, the pellet was resuspended in 10 mL of BHI broth that was supplemented with 25 μ L of proteinase K (Sigma, St. Louis, MO) solution (25 U/mL). Subsequently, the suspension was incubated in a Personal 11 water bath shaker (Taitec Corp., Tokyo, Japan) at 169 rpm for 3 h at 37°C to simultaneously allow for target bacterial growth and the decomposition of PCR inhibitors, such as micellar casein. After centrifugation ($3,000 \times g$ for 5 min at 25°C), the pellet was resuspended in 1 mL of saline and transferred to a 1.5-mL microtube.

Figure 1. Start and end sequences of the gene region set with the forward (16S_2751F) and reverse (23S_5201R) primers: comparison of the 16S and 23S rRNA genes from *Enterobacteriaceae* and gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). The aforementioned primer pair principally amplified 2,451 bp DNA fragments to be targeted. The absence of an asterisk denotes differences in the amplified DNA sequences. Ent = *Enterobacteriaceae* bacteria; GP = gram-positive bacteria.

For the viability tests before and after the proteinase K treatment, the 1-mL aliquot was cocultured with SPC and VRBA. A filter-sterilized solution of 1,000 µg/mL of PMA (Biotium Inc., Hayward, CA) in deionized water was stored at −20°C in the dark until use. Then, either 12.14 µL of the PMA solution (23.5 µM final concentration) or the same volume of sterilized water (SW) was added. The PMA treatment time (10 min) and irradiation (5 min) by visible light were performed as described previously (Minami et al., 2010). After centrifugation (3,000 × *g* for 5 min at 25°C), the pellet was resuspended in 1 mL of SW. One-milliliter aliquots that were diluted 5 and 10 fold in SW were prepared (milk components corresponding to 4 and 2 mL of milk), centrifuged, resuspended in SW to a final volume of 5 µL, and added to 50 µL of the DqPCR master mix (Soejima et al., 2011b). The PCR was performed as previously mentioned in this paper. When the milk components corresponding to 4- or 2-mL milk were contaminated in the DqPCR master mix, we defined them in 4× or 2× milk matrices in later-mentioned Tables 1 to 3, respectively.

Commercial milk samples (control milk that contained an undetectable number of viable *Enterobacteriaceae* cells due to coculture with SPC and VRBA agar) were single-strain spiked with viable *Enterobacteriaceae* cells (Table 2). The inoculation levels (mean ± SD) ranged from 1.8 ± 0.0 to 2.4 ± 0.0 log cfu/20 mL of milk for *C. mytjensii*, *E. coli*, and *Salmonella* Enteritidis, taking into consideration the detection limit (5–10 cfu/mL) of viable coliform cells, following the pasteurized milk guidelines from the United States and European Union (Hillerton and Elizabeth, 2004; McLaughlin, 2006). Hereinafter, the experimental procedures followed the above-mentioned milk treatment, followed by PMA treatment.

RESULTS

Standard Curves Obtained From DqPCR Using *Enterobacteriaceae* Cells That Were Suspended in Sterile Water

For the specificity of our DqPCR assay, it revealed all negative results for the negative control (using SW) and the viable *B. cereus* and *Staph. aureus* suspensions. Furthermore, as presented in Figure 1, the elongated gene sequences significantly differed between the *Enterobacteriaceae* and gram-positive bacteria in our assay.

The range of linearity seemed to be strain dependent (Figure 2). For *C. mytjensii*, *E. coli*, and *Salmonella* Enteritidis, linearities were obtained in ranges of 2.9 to 6.0, 0.9 to 5.0, and 2.9 to 6.0 log cfu/PCR. The squares of the correlation coefficients were 0.998, 0.985, and 0.963 for *C. mytjensii*, *E. coli*, and *Salmonella* Enteritidis, respectively. Concentrations exceeding 6.9 log cfu/PCR led to the failed detection of the target bacteria due to the large quantity of bacterial cell debris.

Cycle Threshold Versus Logarithmic Cell Number for *Enterobacteriaceae* Bacteria That Were Resuspended in PMA-Treated Milk Matrices With No Artificially Introduced Bacteria

Compared with the cycle threshold (Ct) values that were observed in the *Enterobacteriaceae* cells suspended in SW, cells that were freshly suspended in a matrix of 2 × PMA-treated milk pellets (matrices) that had been procured from the procedures described above (using 20 mL of milk with no artificially introduced bacteria with a low dose of proteinase K treatment in BHI medium followed by PMA treatment, and 10-fold dilution of

Table 1. Cycle threshold (Ct) versus logarithmic cell number for *Enterobacteriaceae* bacteria that were resuspended in propidium monoazide (PMA)¹-treated milk matrices

Culture	Log cfu/PCR					
	5.5 ± 0.1	4.5 ± 0.1	3.5 ± 0.1	2.5 ± 0.1	1.5 ± 0.1	0
<i>Cronobacter muytjensii</i> ATCC 51329 ²						
In milk matrices (2×) ³						
Ct value	20.6 ± 0.2 ⁴	24.1 ± 0.3	27.6 ± 0.8	30.7 ± 1.0	33.4, ND ⁵	ND × 2
Tm (Ps, Pt) ⁶	(87.2, 88.2), 2/2 ⁷	(87.2, 88.3), 2/2	(87.1, 88.3), 2/2	(87.2, 88.4), 2/2	(87.2, 88.3), 1/2	0/2
Gel electrophoresis	2/2 ⁷	2/2	2/2	2/2	1/2	0/2
In sterile water						
Ct value	18.9 ± 0.3	22.2 ± 0.1	25.2 ± 0.6	28.0 ± 0.9	30.5, ND	ND × 2
Tm (Ps, Pt)	(87.2, 88.3), 2/2	(87.2, 88.3), 2/2	(87.2, 88.4), 2/2	(87.1, 88.3), 2/2	(87.2, 88.3), 1/2	0/2
Gel electrophoresis	2/2	2/2	2/2	2/2	1/2	0/2
<i>Escherichia coli</i> DH5α						
In milk matrices (2×)						
Ct value	20.2 ± 0.2	22.0 ± 0.4	24.8 ± 0.7	28.4 ± 1.0	31.6 ± 1.0	ND × 2
Tm (Ps, Pt)	(86.8, 88.0), 2/2	(86.8, 88.0), 2/2	(86.8, 88.1), 2/2	(86.7, 88.0), 2/2	(86.7, 87.9), 2/2	0/2
Gel electrophoresis	2/2	2/2	2/2	2/2	2/2	0/2
In sterile water						
Ct value	19.0 ± 0.1	20.1 ± 0.3	22.4 ± 0.5	25.9 ± 0.7	28.7 ± 1.1	ND × 2
Tm (Ps, Pt)	(86.8, 88.0), 2/2	(86.8, 88.0), 2/2	(86.8, 88.0), 2/2	(86.7, 88.0), 2/2	(86.7, 87.9), 2/2	0/2
Gel electrophoresis	2/2	2/2	2/2	2/2	2/2	0/2
<i>Salmonella</i> Enteritidis IID 604 ⁸						
In milk matrices (2×)						
Ct value	20.9 ± 0.4	23.3 ± 0.6	28.8 ± 0.7	31.6 ± 1.0	ND × 2	ND × 2
Tm (Ps, Pt)	(87.0, 88.2), 2/2	(87.0, 88.2), 2/2	(86.9, 88.1), 2/2	(86.8, 88.0), 2/2	ND, 0/2	0/2
Gel electrophoresis	2/2	2/2	2/2	2/2	ND, 0/2	0/2
In sterile water						
Ct value	18.8 ± 0.3	21.2 ± 0.6	26.3 ± 0.8	28.9 ± 1.2	ND × 2	ND × 2
Tm (Ps, Pt)	(87.0, 88.2), 2/2	(87.0, 88.2), 2/2	(87.0, 88.1), 2/2	(86.9, 88.1), 2/2	ND, 0/2	0/2
Gel electrophoresis	2/2	2/2	2/2	2/2	ND, 0/2	0/2

¹Biotium Inc., Hayward, CA.²ATCC = American Type Culture Collection, Rockville, MD.³A 20-mL aliquot of pasteurized milk was treated with PMA after a 3-h pre-enrichment with a low concentration of proteinase K. Following centrifugation, 1/10 amounts of milk pellets were collected in a PCR tube (2× milk matrices corresponding to pellet originating from 2 mL of milk) and the targeted bacterial inoculation was subsequently performed.⁴The direct real-time quantitative PCR (DqPCR) measurements were performed in duplicate, and the Ct values are represented as the mean ± SD.⁵ND = not detectable.⁶Tm (Ps, Pt) = the melting point analysis in duplicate. Ps indicates a peak shoulder and Pt indicates a peak.⁷i/2 (i = 0 to 2) represents the number of positive results per duplicate measurements.⁸IID = Institute of Medical Science, University of Tokyo, Tokyo, Japan.

test samples) showed higher values. The experimental results suggest that some of the components in the milk still possessed PCR-inhibitory effects (Table 1). Additionally, concentrations exceeding 1.5 log cfu/PCR of *Enterobacteriaceae* cells were necessary for the DqPCR assay to effectively assess the targeted cells.

Detection of Viable *Enterobacteriaceae* Cells Artificially Inoculated in Pasteurized Milk Using the PMA-DqPCR Assay, Melting Point Analysis, and Gel Electrophoresis

The low dose of proteinase K pretreatment at 37°C for 3 h in BHI broth caused the targeted viable cells (mean ± SD) to increase by at least 1 log unit to concentrations ranging from 3.0 ± 0.0 to 4.1 ± 0.1 log cfu/sample

(Table 2). Simultaneously, the sizes of the sample pellets markedly decreased due to the decomposition of milk proteins, such as micellar casein. As shown in Table 2, the control milk yielded no viable *Enterobacteriaceae* cells either before or after the short 3-h incubation, indicating the lack of growth of the resuscitated cells. The PMA-DqPCR targeting the 16S-23S rRNA gene led to the successful suppression of PCR amplification in the heat-killed *C. muytjensii* (7.5 log cfu/20 mL of control milk in Table 2) because at least 1 molecule of PMA may have cross-linked to the very long 2,451 bp DNA (Soejima et al., 2011a). This PMA-DqPCR assay for pasteurized milk involves targeted bacterial growth and the simultaneous decomposition of PCR inhibitors in the milk (3 h), a series of PMA treatments (1 h), and DqPCR (3.5 h).

Table 2. Detection of viable *Enterobacteriaceae* cells artificially inoculated in pasteurized milk using the propidium monoazide (PMA)¹ direct real-time quantitative PCR (DqPCR) assay, melting point (T_m) analysis, and gel electrophoresis

Culture	PMA treatment	Before incubation	After incubation (3 h)	DqPCR (2× milk matrices) ²		
		Log cfu/sample ³	Log cfu/sample ³	Ct value	T _m	Gel electrophoresis
<i>Cronobacter muytjensii</i> ATCC51329 ⁴	+	1.8 ± 0.03	3.7 ± 0.23	28.5 ± 0.92	2/2 (Ps 87.2, Pt 88.3) ⁶	2/2 ⁷
	+	2.1 ± 0.03	3.9 ± 0.20	27.8 ± 0.67	2/2 (Ps 87.2, Pt 88.3)	2/2
<i>Escherichia coli</i> DH5α	+	1.6 ± 0.05	2.8 ± 0.21	29.6 ± 0.84	2/2 (Ps 86.8, Pt 88.0)	2/2
	+	1.9 ± 0.05	3.0 ± 0.02	29.0 ± 0.57	2/2 (Ps 86.8, Pt 88.1)	2/2
<i>Salmonella</i> Enteritidis IID604 ⁸	+	2.1 ± 0.05	4.0 ± 0.05	29.0 ± 0.78	2/2 (Ps 87.0, Pt 88.2)	2/2
	+	2.4 ± 0.05	4.1 ± 0.09	28.9 ± 0.47	2/2 (Ps 87.0, Pt 88.3)	2/2
Control milk ⁹	+	ND ¹⁰	ND	ND	0/2	0/2
	– ⁵	ND	ND	24.8 ± 0.55	2/2 (Ps 87.2, Pt 88.1)	2/2
Control milk inoculated with heat-killed <i>C. muytjensii</i> ATCC51329	+	ND	ND	ND	0/2	0/2
(7.5 ± 0.06 log cfu/sample)	–	ND	ND	19.2 ± 0.43	2/2 (Ps 87.2, Pt 88.3)	2/2

¹Biotium Inc., Hayward, CA.²The 2× milk matrices corresponded to 1/10 amounts of pellets obtained from centrifugation of 20 mL of milk (following 3-h incubation and PMA treatment), which were added to a PCR tube, and then DqPCR was performed in duplicate.³Targeted viable cell count per 20 mL of milk using the culture method.⁴ATCC = American Type Culture Collection, Rockville, MD.⁵The + and – represent PMA treatment and no PMA treatment, respectively.⁶Detection by T_m analysis (number of positive results per 2 replicates); Ps indicates a peak shoulder and Pt indicates a peak.⁷Detection by gel electrophoresis (number of positive results per 2 replicates).⁸IID = Institute of Medical Science, University of Tokyo, Tokyo, Japan.⁹Control milk is the commercial milk with no artificially inoculated bacteria.¹⁰ND = not detectable.

Detection of *Enterobacteriaceae* Cells in the Commercial Pasteurized Milk With No Artificially Inoculated Bacteria Using DqPCR With or Without PMA Treatment

Instead of an internal positive control (as a positive control for each commercial pasteurized milk), to 2× and 4× milk matrices originating from each commercial pasteurized milk with no artificially introduced bacteria with or without PMA treatment in the PCR tube, the viable *C. muytjensii* (5.5 log cfu/PCR) was newly added, and then the Ct values were obtained, ranging from 18.9 to 22.6 in all positive controls. Next, the DqPCR data from the 2× and 4× milk matrices that were not treated with PMA (Table 3) revealed that 9 out of 11 tested samples developed *Enterobacteriaceae*-positive results in the former, whereas only 5 out of 11 became positive in the latter. The 4× milk matrices had a greater inhibitory effect on the DqPCR assay compared with the 2× milk matrices; thus, we mainly used the data from the 2× milk matrices to evaluate the correlation between the DqPCR without PMA, PMA-DqPCR, and culture method. Regardless of the milk sample, the PMA-DqPCR assay (2× milk matrices) was never detectable for viable *Enterobacteriaceae* cells and was strongly correlated with the culture method.

However, a significant difference ($P < 0.01$; $\Phi = 1$) was found between DqPCR without PMA and the culture method due to the McNemar test, which was performed using SPSS version 10.0 software (SPSS Inc., Chicago, IL), using a total of 22 measurement data obtained from 11 milk samples. The DqPCR without PMA was not correlated to the culture method.

DISCUSSION

Polymerase chain reaction assays have previously been applied in microbiological milk analyses that involve preliminary enrichment for 4 to 16 h (Cooray et al., 1994; Kim et al., 2001; Meiri-Bendek et al., 2002; Pillai and Jayarao, 2002; Fode-Vaughan et al., 2003; Wu and Kado, 2004; Cai et al., 2005). The PCR protocol to detect mastitis in bovine milk directly without pre-enrichment has been reported (Cressier and Bissonnette, 2011). In addition, a PCR method for assay of lactose-fermenting *Enterobacteriaceae* bacteria in the dairy food industries has already been developed (Martín et al., 2010). However, the robust discrimination method between viable and dead *Enterobacteriaceae* cells in pasteurized milk by PCR has not yet been reported, putting into consideration a routine test following the later-mentioned United States and European Union

Table 3. Detection of *Enterobacteriaceae* cells in the commercial pasteurized milk with no artificially inoculated bacteria using direct real-time quantitative PCR (DqPCR) with or without propidium monoazide (PMA)¹ treatment

Sample no.	Pasteurization conditions	Analysis ²	DqPCR					Culture method ³	
			No PMA treatment		PMA treatment				
			4× milk matrices ⁴	2× milk matrices ⁴	4× milk matrices ⁴	2× milk matrices ⁴			
Milk 1	130°C, 2 s	Ct		ND ⁵	27.4, ND		ND	ND	ND
		Tm	0/2 ⁶		1/2 (Ps 88.1; Pt 87.2) ⁷	0/2	0/2		
		Gel	0/2 ⁶		2/2	0/2	0/2		
Milk 2	120°C, 3 s	Ct		ND	28.0, ND		ND	ND	ND
		Tm	0/2		1/2 (Ps 88.3; Pt 87.1)	0/2	0/2		
		Gel	0/2		2/2	0/2	0/2		
Milk 3	130°C, 2 s	Ct		ND		ND	ND	ND	ND
		Tm	0/2		0/2	0/2	0/2		
		Gel	0/2		0/2	0/2	0/2		
Milk 4	121°C, 3 s	Ct	29.5 ± 0.42 ⁸		28.2 ± 0.18		ND	ND	ND
		Tm	2/2 (Ps 88.5; Pt 87.6)		2/2 (Ps 88.5; Pt 87.6)	0/2	0/2		
		Gel	2/2		2/2	0/2	0/2		
Milk 5	130°C, 2 s	Ct		ND		ND	ND	ND	ND
		Tm	0/2		0/2	0/2	0/2		
		Gel	0/2		0/2	0/2	0/2		
Milk 6	130°C, 2 s	Ct		ND	27.9 ± 0.1		ND	ND	ND
		Tm	0/2		2/2 (Ps 86.7; Pt 88.0)	0/2	0/2		
		Gel	0/2		2/2	0/2	0/2		
Milk 7	130°C, 2 s	Ct	30.0 ± 0.28		28.9 ± 0.21		ND	ND	ND
		Tm	2/2 (Ps 88.0; Pt 87.3)		2/2 (Ps 88.1; Pt 87.2)	0/2	0/2		
		Gel	2/2		2/2	0/2	0/2		
Milk 8	66°C, 30 min	Ct	30.2 ± 0.21		31.0 ± 0.00		ND	ND	ND
		Tm	2/2 (Ps 88.4; Pt 87.1)		2/2 (Ps 88.3; Pt 87.0)	0/2	0/2		
		Gel	2/2		2/2	0/2	0/2		
Milk 9	66°C, 30 min	Ct	29.8 ± 0.40		30.2 ± 0.40		ND	ND	ND
		Tm	2/2 (Ps 88.3; Pt 87.2)		2/2 (Ps 88.4; Pt 87.2)	0/2	0/2		
		Gel	2/2		2/2	0/2	0/2		
Milk 10	66°C, 30 min	Ct		ND	29.6 ± 0.10		ND	ND	ND
		Tm	0/2		2/2 (Ps 88.3; Pt 87.1)	0/2	0/2		
		Gel	0/2		2/2	0/2	0/2		
Milk 11	130°C, 2 s	Ct	28.2 ± 0.60		28.0 ± 0.10		ND	ND	ND
		Tm	2/2 (Ps 87.2; Pt 88.4)		2/2 (Ps 87.2; Pt 88.3)	0/2	0/2		
		Gel	2/2		2/2	0/2	0/2		

¹Biotium Inc., Hayward, CA.²Ct = cycle threshold; Tm = melting point analysis; Gel = gel electrophoresis.³Standard plate count (SPC) agar (Kanto Chemical Co. Inc., Tokyo, Japan) and Difco violet red bile agar (VRBA; Becton, Dickinson and Co., Sparks, MD) were used for coculturing and detecting the general and *Enterobacteriaceae* bacteria in 1 mL of pasteurized milk.⁴The 2× or 4 × milk matrices correspond to 1/10 or 1/5 amounts of pellets obtained after the centrifugation of 20 mL of pasteurized milk treated with proteinase K, followed by no PMA or PMA treatment before PCR. As a positive control for each commercial pasteurized milk, the viable *Cronobacter muytjensii* (5.5 log cfu/PCR) was newly added to each 2× and 4× milk matrix with or without PMA treatment in a PCR tube to confirm Ct values of all positive controls ranging from 18.9 to 22.6.⁵ND = not detectable.⁶i/2 (i = 0 to 2) represents a positive number (i) per 2 duplicates that were obtained from the Tm or gel electrophoresis of the DqPCR products.⁷Ps indicates a peak shoulder of the Tm analysis, and Pt indicates a peak of the Tm analysis.⁸Ct values by DqPCR are represented as mean ± SD (n = 2).

milk guidelines. The hygienic guidelines for pasteurized milk in the United States and European Union require that CC do not exceed 10 and 5 cfu/mL, respectively (Hillerton and Elizabeth, 2004; McLaughlin, 2006); therefore, in the present study, a 20-mL aliquot of milk to meet the sever criteria, which was either uninoculated or inoculated with viable or heat-killed *Enterobacteriaceae* cells (single-strain), was prepared for the harvest of viable *Enterobacteriaceae* cells at the highest

possible number. Polymerase chain reaction fails if the final concentration of milk in the PCR reaction mixture is higher than 5% (Bickley et al., 1996). However, we demonstrate specific detection of viable *Enterobacteriaceae* cells in pasteurized milk by DqPCR to target long DNA (16S–23S: mainly 2,451 bp) following PMA treatment even in the milk matrices corresponding to 2 mL of milk using 20 mL of milk of a great volume to harvest the target bacteria at the highest possible cells

Figure 2. Standard curves obtained from direct real-time quantitative PCR (DqPCR) using *Enterobacteriaceae* cells that were suspended in sterile water. A) *Cronobacter mydtjensii* ATCC 51329, B) *Escherichia coli* DH5α, and C) *Salmonella enterica* serovar Enteritidis IID 604. The linearity (square of correlation coefficient: R^2) between the logarithmic number of cells and the cycle threshold (C_t) values presented with the regression line. The DqPCR measurements were performed in 4 replicates at each logarithmic cell number, and C_t data are presented as the mean (♦: *C. mydtjensii* ATCC 51329; ▲: *E. coli* DH5α; ●: *Salmonella enterica* serovar Enteritidis IID 604) $\pm$ SD (error bar). As for 1.9 and 6.9 log cfu/PCR of *C. mydtjensii*, the C_t data obtained from 3 replicates were plotted owing to the nonamplification of 1 out of 4 measurements. Likewise, with regard to 0.1 and 6.0 log cfu/PCR of *E. coli* DH5α, the data obtained from replicate 2 and 3 out of 4 were plotted. For 6.9 log cfu/PCR of *Salmonella enterica* serovar Enteritidis, the data obtained from 3 out of 4 replicates were plotted on the graph of C_t (y-axis) versus logarithmic cell number. ATCC = American Type Culture Collection, Rockville, Maryland; IID = Institute of Medical Science, University of Tokyo, Tokyo, Japan.

number considering PCR inhibition. This is the first study to successfully perform PCR under strong PCR inhibitors of great milk volumes.

To meet customer demands for the highest quality chilled pasteurized milk in the freshest possible condition, factories must ship the pasteurized milk for pro-

ducing dairy products before they have confirmed the absence of viable *Enterobacteriaceae* bacteria. It is only immediately before its arrival at the supermarket that the potential viable *Enterobacteriaceae* bacteria are assessed. We should develop a promising methodology that would allow rapid milk *Enterobacteriaceae* testing

in a dairy laboratory of the processor to be conducted before shipping from a factory. Therefore, our established rapid technique may introduce the innovation for the safe distribution of chilled milk in the freshest possible condition.

Compared with EMA, PMA appeared to be highly selective in penetration into only dead bacteria with a compromised cell membrane, but not viable bacteria with an intact cell membrane and cell walls (Nocker et al., 2006). So, apart from EMA-PCR, we also report the use of PMA-DqPCR to assess commercial pasteurized milk with no artificially introduced bacteria, focusing on viable *Enterobacteriaceae* cells, as shown in Table 3. No viable *Enterobacteriaceae* cells were detected in any of the 11 commercial pasteurized milk cultures (due to coculturing with SPC and VRBA agars), as was expected for samples of Japanese origin. The 20 additional samples of pasteurized milk that were assessed also showed no targeted viable cells by the culture method (data not shown). As it is not easy for us to obtain coliform/*Enterobacteriaceae*-positive samples in commercialized pasteurized milks with no artificially inoculated bacteria in Japan, we exogenously added the viable *Enterobacteriaceae* cells to the commercial pasteurized milk (Table 2). However, in Japan, if once the presence of viable coliform/*Enterobacteriaceae* is detected in pasteurized milk, it will be a critical matter in view of the regulation of food hygiene.

The CC in raw milk range from 1 to 6 log cfu/mL throughout all of the seasons in Japan (Higoshi et al., 1977). It appears that our PMA-DqPCR assay, which targets long 16S to 23S DNA sequences, seems to completely suppress the PCR from dead (heat-killed) *Enterobacteriaceae* cells that potentially contaminate pasteurized milk (control milk with PMA treatment in Tables 2 and 3). Actually, our methodology completely inhibits the PCR amplification from the artificially introduced heat-killed *C. mytjensii* (7.5 log cfu/20 mL milk; Table 2).

A low dose of proteinase K treatment in BHI broth at 37°C for 3 h allows the increasing cell number of viable *Enterobacteriaceae* bacteria exogenously added by 1 to 2 log cfu/sample and simultaneous decomposition of potent PCR inhibitors such as micellar casein originating from 20 mL of milk (Table 2). This low dose of proteinase K treatment may accelerate PCR to be introduced into dairy milk analysis because a large milk volume can be targeted.

CONCLUSIONS

Our PMA-DqPCR assay targeting a long DNA sequence (16S-23S rRNA gene) enabled us to successfully and specifically detect low numbers (5–10 cfu/mL) of

viable *Enterobacteriaceae* cells in pasteurized milk for 7.5 h of total testing time. Moreover, we demonstrated that the presence of a high load of heat-killed *Enterobacteriaceae* cells in milk did not produce false-positive results. The use of our PMA-DqPCR enables the specific and successful amplification of viable *Enterobacteriaceae* bacteria even in the pasteurized milk matrices corresponding to 2 mL of milk PCR inhibitors, using 20 mL of a sample volume to collect the target viable cells at the highest possible level. This report is the first to demonstrate the specific and robust detection of viable *Enterobacteriaceae* cells at a low concentration, following the guidelines from the United States and European Union.

ACKNOWLEDGMENTS

We thank G&G Science Co. Ltd. (Fukushima, Japan) for the development of the primer pair designed to specifically detect *Enterobacteriaceae* cells.

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