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ARTICLE

Real Time Reverse Transcriptase-PCR to Detect Viable Enterobacteriaceae in Milk

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Abstract

This study was conducted to develop a real time reverse transcriptase-PCR (RT-PCR) method for the detection of viable Enterobacteriaceae in milk using primers based on the genes of ribosomal proteins S11 and S13 and to determine effects of heating and subsequent treatments on the threshold cycle (Ct) of the real time RT-PCR. Total RNA was isolated from 17 strains of bacteria including 11 strains of Enterobacteriaceae suspended in milk using a modified Tri reagent method. SYBR Green Master Mix was added to the RNA and the mixture was subjected to the real time RT-PCR. The Cts of eleven type strains of the Enterobacteriaceae in milk (10^7 cells) in the real time RT-PCR ranged from 21.5 to 24.6. However, the Cts of *Pseudomonas fluorescens*, *Acinetobacter calcoaceticus*, and three gram-positive bacteria were more than 40. The real time RT-PCR detected as low as 10^3 cells in agarose gel electrophoresis. The Cts increased from 22.0 to 34.2 when milk samples contaminated with *Escherichia coli* (10^7 cells/mL) were heated at 65°C for 30 min. In addition, subsequent incubation at 37°C for 6 and 24 h increased the Cts further up to 36.2 and 37.2, respectively. Addition of RNase A to the bacterial suspension obtained from the heated milk and subsequent incubation at 37°C for 1 h increased the Cts to more than 40. The results of this study suggests that pretreatment of bacterial cells heated in milk with RNase A before RNA extraction might enhance the ability to differentiate between viable and dead bacteria using real time RT-PCR.

Key words: reverse transcriptase-polymerase chain reaction, real time, Enterobacteriaceae, milk

Introduction

Pasteurization of raw milk destroys infectious microorganisms, such as *Mycobacterium*, *Brucella*, and Q fever rickettiae, and other pathogenic bacteria related with food-borne illness, such as *Escherichia coli* O157:H7, *Salmonella*, *Yersinia enterocolitica*, etc. Aerobic spore-forming bacteria, such as *Bacillus*, and thermoduric bacteria such as *Microbacterium*, *Microbacteria*, *Streptococcus thermophilus* may survive pasteurization (Muir, 1990; Varnam and Sutherland, 1994). However, all the gram-negative bacteria except *Alcaligenes tolerans* are destroyed by the heat treatment (International Dairy Federation, 1993).

Thus presence of gram-negative bacteria in market milk should be due to either improper pasteurization procedure or post-pasteurization contamination, which results in loss of shelf life of market milk during storage and distribution and causes even food-borne illness among consumers. Culture methods using selective nutrient media for enrichment and enumeration of either coliform bacteria, Enterobacteriaceae, or gram-negative bacteria have been suggested as tools for quality control of market milk and for estimation of potential shelf life of pasteurized milk (Bishop and White, 1986; White, 1993). Since fresh market milk produced in a sanitary environment of normal milk processing may be contaminated with the psychrotrophic gram-negative bacteria at very low level, enrichment culture of milk at 21-30°C for 18-24 h is required before colony enumeration on selective agar, ATP bioluminescence assay, electrical impedance assay, and dye reduction method (Baustista, *et al*., 1992; Bishop *et al*., 1984; Choi *et al*., 1999; White *et al*., 1993).

Developments of diagnostic assays based on nucleic acids to detect viable bacteria in dairy products will augment ability of the dairy industry to maintain safety of the products (Batt, 1997). Reverse transcriptase-PCR (RT-PCR) in which DNA is amplified using mRNA template has been reported to detect selectively viable bacteria including *Listeria monocytogenes* (Klein and Juneja, 1997), *Salmonella* serotype Enteritidis (Szabo and Mackey,

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1999) and *Escherichia coli* O157:H7 (McIngvale *et al.,* 2002; Sharma, 2006; Yaron and Matthew, 2002). Viable bacteria, molds, and yeasts are directly detected by reverse transcriptase PCR in contaminated milk after heat treatment (Vaitilingom *et al.,* 1998). RT-PCR signal may persist for a long period of time depending on the treatment to kill the bacteria and post-treatment holding conditions (Sheridan *et al.,* 1999). Residual mRNA may survive pasteurization of milk at 65°C for 30 min, which results in amplification of DNA in RT-PCR. It has been suggested that treatment of the pasteurized milk with RNase A may remove the residual mRNA (Choi and Lee, 2011).

The objectives of this study were to develop a real time RT-PCR method to detect viable Enterobacteriaceae in contaminated milk and to evaluate various pretreatment procedures.

Materials and Methods

Bacteria culture

Seventeen type strains of ATCC and KCTC were purchased from American Type Culture Collection in USA and Biological Resources Center in Korea, respectively. The bacterial strains which belonged to the family of Enterobacteriacease consisted of *E. coli* ATCC 43890, *E. coli* KCTC 2441, *Salmonella choleraesuis* KCTC 2932, *Salmonella typhimurium* KCTC 2514, *Shigella sonnei* KCTC 2009, *Yersinia enterocolitica* ATCC 23715, *Cronobacter sakazakii* KCTC 2949, *Klebsiella pneumoniae* KCTC 2208, *Citrobacter freundii* KCTC 2006, *Serratia marcescens* KCTC 2355, and *Proteus mirabilis* KCTC 2566. Other gram-negative bacteria included *Pseudomonas fluorescens* KCTC 2344 and *Acinetobacter calcoaceticus* KCTC 2357. Gram positive bacteria included *Enterococcus faecalis* KCTC 3512, *Listeria monocytogenes* KCTC 3569, and *Bacillus coagulans* KCTC 1015. The gram-positive bacteria and Gram-negative bacteria were cultured in brain heart infusion broth (Difco, USA) and tryptic soy broth (Difco, USA) at 35°C, respectively, except *Pseudomonas fluorescens* which was cultured at 26°C. Gram-negative bacteria and *Bacillus* were cultured at 100 rpm in a shaking incubator. The other Gram-positive bacteria were cultured statically. The cultures used to isolate RNA extract was incubated for a short period of time until the bacterial number reached approximately 10^9 cells/mL. The cultures were cooled in ice and then diluted tenfold sequentially to appropriate cell numbers with phosphate-buffered saline using McFarland nephelometer (Balows *et al.,* 1991).

Heating and subsequent incubation of milk

Aliquots (0.1 mL) of *E. coli* ATCC 43890 (10^8 cells/mL) were added to milk samples (0.9 mL) and vortexed briefly. The milk samples were heated at 65°C for 30 min, 100°C for 10 min, or 121°C for 15 min. Each heated milk samples were then incubated at 37°C for 6 and 24 h, and then subjected to isolation of RNA extract and the real time RT-PCR.

Treatment of heated milk with RNase

Aliquots (0.1 mL) of *E. coli* ATCC 43890 (10^8 cells/mL) were added to milk samples (1 mL) and then heated at 65°C for 30 min. The heated milk samples (1 mL) were then centrifuged at 5,000 g for 15 min. After removing supernatant, either phosphate-buffered saline (1 mL) or tryptic soy broth (1 mL) which contained RNase A (6 mg/mL) was added to pellet and the mixture was dispersed by vortex, incubated at 37°C for 1 h, and then subjected to RNA isolation and the real time RT-PCR.

Preparation of RNA extract

The cell number of the bacteria added to a commercial UHT-sterilized milk (1 mL) was either 10^7 or 10^8. The contaminated milk (1 mL) and 25% sodium citrate (60 μL) were added into a tube. The mixture was agitated on a shaker for 5 min and centrifuged at 5,000 g for 5 min. After the cream layer and supernatant were removed, Tri reagent (Sigma Aldrich, USA) was used to isolate RNA extract from the cell pellet following the manufacturer's instruction, which was modified as shown as follows. Yeast tRNA (50 μg) (Sigma Aldrich) was added with isopropanol as a carrier. RNA pellet finally precipitated using ethanol was dissolved in RNase-free RQ DNase (1 unit) (Promega, USA) and 1×RQ DNase reaction buffer (20 μL). The dissolved RNA extract was incubated at 37°C for 60 min, the stop solution was added, and the mixture was heated at 67°C for 30 min. The RNA extract (2 μL) was used for real time RT-PCR.

Real time RT-PCR

The real time RT-PCR method consisted of the first step of reverse transcriptase reaction and the second step of real time PCR. The forward primer 5'-CCGTATAGCAGGCATTAACATTCC-3' and reverse primer 5'-GGAACCACCG/TGCTGTTGC-3' were designed based on the nucleotide sequences of conserved regions in alpha ribosomal protein operon encoding ribosomal proteins

S11 and S13, respectively, by comparing nucleotide sequences among *Escherichia coli* (X02543), *Salmonella enterica* serovar Typhi (AL627282), and *Yersinia pestis* (AJ414141) and by using Beacon Designer (Premier Biosoft International, USA). The expected size of amplified DNA was 518 bp. The mixtures in duplicate for reverse transcriptase (RT) reaction consisted of RNA extract (2 μL) and TaqMan RT buffer (23 μL) containing 1.0 mM $MgCl_2$, 0.5 mM dNTP. Ten U RNase inhibitor, 25 U Multiscribe reverse transcriptase, and 0.5 μM reverse primer. A PCR thermal cycler (Applied Biosystem, USA) was used to heat the RT reaction mixture at 48°C for 5 min for reverse transcriptase reaction. For subsequent real time PCR, the RT reaction mixture (2 μL), 375 nM forward primer, and 375 nM reverse primer were added into SYBR Green Master Mix (12.5 μL) (Applied Biosystem), and distilled water was added to make up to 25 μL. The real time PCR was done using iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, USA). The reaction condition consisted of an initial step of 95°C for 10 min, then 40 cycles of 95°C for 15 s, 58°C for 1 min, and 72°C for 15 s, and a final step of 72°C for 7 min. The fluorescence emissions from PCR samples were automatically measured at 58°C in every cycle. The threshold cycle (Ct) was registered as the cycle number where fluorescence emission was first detected.

The average and standard deviation of the Cts in duplicate were calculated.

Agarose gel electrophoresis

After the real time RT-PCR was terminated, the reaction mixture was subjected to agarose gel electrophoresis using 2.5% agarose gel in 1× Tris-acetate buffer at 50 V. The gel was then stained with 0.5 μg/mL ethidium bromide and illuminated with ultraviolet light to visualize stained DNA (Sambrook and Russell, 2001). DNA marker used to estimate size of amplified DNA was 100 bp DNA ladder (Bioneer, Korea).

Table 1. The threshold cycles (Cts) in the real time RT-PCR of RNA extracts from various bacteria (10^7 cells) in milk (1 mL)

Bacteria	Cts	Bacteria	Cts
E. coli ATCC 43890	22.3±0.5	*C. freundii*	22.0±0.0
E. coli KCTC 2441	21.5±0.0	*S. marcescens*	23.1±0.2
S. choleraesuis	24.5±0.0	*P. mirabilis*	22.3±0.0
S. typhimurium	22.1±0.2	*P. fluorescens*	>40
S. sonnei	22.1±0.0	*A. calcoaceticus*	>40
Y. enterocolitica	22.3±0.2	*E. faecalis*	>40
C. sakazakii	24.6±0.1	*L. monocytogenes*	>40
K. pneumoniae	23.1±0.1	*B. coagulans*	>40

Results and Discussion

Specificity of real time RT-PCR

In order to determine specificity of the real time RT-PCR in detection of *Enterobactericeae*, various bacteria cultures were added into a commercial UHT-sterilized milk. The cell number of the bacteria added to the milk (1 mL) was 10^7 cells. RNA extract was obtained from the contaminated milk and subjected to the real time RT-PCR (Table 1) and the resultant reaction samples were examined in the agarose gel electrophoresis (Fig. 1). The range of Cts obtained in the real time RT-PCR method of the type strains of family Enterobacteriaceae including *E. coli* ATCC 43890, *E. coli* KCTC 2441, *Salmonella choleraesuis* KCTC 2932, *Salmonella typhimurium* KCTC 2514, *Shigella sonnei* KCTC 2009, *Yersinia enterocolitica* ATCC 23715, *Cronobacter sakazakii* KCTC 2949, *Klebsiella pneumoniae* KCTC 2208, *Citrobacter freundii* KCTC 2006, *Serratia marcescens* KCTC 2355, and *Proteus mirabilis* KCTC 2566 were 21.5-24.6 (Table 1). The average and standard deviation of Cts of the type strains

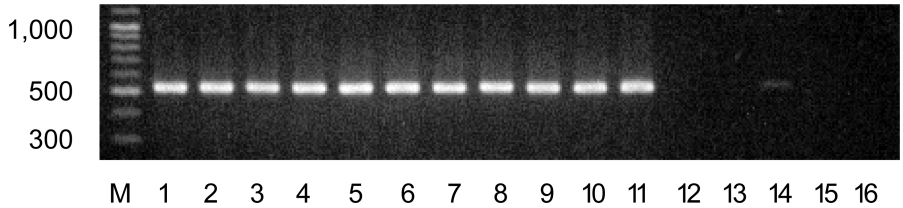

Fig. 1. Agarose gel electrophoresis of DNA amplified in the real time RT-PCR as described in Table 1. Lane 1. *E. coli* ATCC 43890; lane 2. *E. coli* KCTC 2441; lane 3. *Salmonella choleraesuis* KCTC 2932; lane 4. *Salmonella typhimurium* KCTC 2514; lane 5. *Shigella sonnei* KCTC 2009; lane 6. *Yersinia enterocolitica* ATCC 23715; lane 7. *Cronobacter sakazakii* KCTC 2949; lane 8. *Klebsiella pneumoniae* KCTC 2208; lane 9. *Citrobacter freundii* KCTC 2006; lane 10. *Serratia marcescens* KCTC 2355; lane 11. *Proteus mirabilis* KCTC 2566; lane 12. *Pseudomonas fluorescens* KCTC 2344; lane 13. *Acinetobacter calcoaceticus* KCTC 2357; lane 14. *Enterococcus faecalis* KCTC 3512; lane 15. *Listeria monocytogenes* KCTC 3569; lane 16. *Bacillus coagulans* KCTC; lane M. 100 bp DNA ladder marker (Bioneer, Korea)

of family Enterobacteriaceae were 22.7 and 0.97, respectively. The Cts of *Pseudomonas fluorescens* KCTC 2344, *Acinetobacter calcoaceticus* KCTC 2357, *Enterococcus faecalis* KCTC 3512, *Listeria monocytogenes* KCTC 3569, and *Bacillus coagulans* KCTC 1015 were more than 40. Agarose gel electrophoresis also showed strong DNA amplification in the real time RT-PCR of the eleven type strains of family Enterobacteriaceae and the size of amplified DNA was about 520 bp, as expected from the amplicon size derived from the primers (lanes 1-11 in Fig. 1). However, there was no amplified DNA of *Pseudomonas fluorescens* KCTC 2344, *Acinetobacter calcoaceticus* KCTC 2357, *Listeria monocytogenes* KCTC 3569, and *Bacillus coagulans* KCTC 1015 as shown in agarose gel electrophoresis (lanes 12, 13, 15, and 16 in Fig. 1). Though the Ct of *Enterococcus faecalis* KCTC 3512 was more than 40, there was weakly amplified DNA shown in agarose gel electrophoresis (lane 14 in Fig. 1). These results showed that the real time RT-PCR method detected specifically bacteria belonging to the Enterobacteriaceae.

Sensitivity of the real time RT-PCR

Threshold cycles (Cts) in the real time RT-PCR of 10^8 - 10^2 cells of *E. coli* ATCC 43890 in milk were investigated in order to determine its sensitivity (Table 2, Fig. 2). The Cts increased relatively in linear scale, as the cell number decreased exponentially. The Ct at the cell number of 10^2 was more than 40 (Table 2). The agarose gel electrophoresis (Fig. 2) also showed that the intensity of amplified DNA band was at a maximum at the cell number of 10^8 and gradually decreased at lower cell numbers. There was no DNA detected at the cell number of 10^2. The detection limit of the real time RT-PCR method was not sensitive, which might due to large size, 520 bp, of the amplicon. Smaller amplicon gave more consistent results, because PCR was more efficient and more tolerant of reaction conditions. Amplicon size of PCR should range between 50 and 150 bp and should not exceed 400 bp (Apte and Daniel, 2003). However, our previous study showed that the amplicon of 134 bp was more recalcitrant to degradation by heating than the amplicons of 450 and 565 bp in the RT-PCR to detect viable *Escherchia coli* O157:H7 in milk (Choi and Lee, 2011). That was why the primers for the relatively large amplicon were designed to discern between viable and dead bacteria in this study.

Table 2. The threshold cycles (Cts) of the real time RT-PCR of RNA extracts from various cell numbers of *Escherichia coli* ATCC 43890 in milk (1 mL)

Cell numbers	Cts	Cell numbers	Cts
10^8	20.2±0.2	10^4	36.3±0.5
10^7	23.9±0.1	10^3	35.2±2.4
10^6	26.6±0.1	10^2	>40
10^5	31.8±0.8		

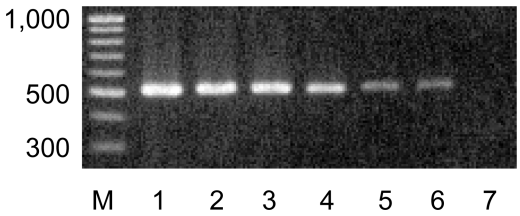

Fig. 2. Agarose gel electrophoresis of DNA amplified in the real time RT-PCR as described in Table 2. Lanes 1, 2, 3, 4, 5, 6, and 7 were from 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cells, respectively. Lane M, 100 bp DNA ladder marker (Bioneer, Korea)

Difference of Cts between 10^3 and 10^8 cells of *E. coli* ATCC 43890 was 15 (Table 2). If DNA mass increased theoretically twice during every PCR cycle, increase of DNA during the 15 PCR cycles should be 2^{15} which is approximately 3.3×10^4. The amplification efficiency of the 15 PCR cycles was calculated to be 33%, which showed that further improvements of PCR condition by adjusting amplicon size, primer sequence, annealing temperature, etc. were required.

Effects of heating and subsequent incubation on the real time RT-PCR

Effects of heating and subsequent incubation on degradation of mRNA was determined in the real time RT-PCR (Table 3, Fig. 3). As described in Material and Methods, milk samples containing *E. coli* ATCC 43890 were heated

Table 3. Effects of heating and incubation at 37°C on the threshold cycles (Cts) of the real time RT-PCR of RNA extract from *E. coli* ATCC 43890 (10^7 cells) in milk (1 mL)

Heating	Incubation at 37°C (h)	Cts
Control (no heating)		22.0±1.6
65°C for 30 min	0	32.9±0.4
	6	34.2±0.3
	24	35.2±0.5
100°C for 10 min	0	37.2±0.7
	6	> 40
	24	> 40
121°C for 15 min	0	> 40
	6	> 40
	24	> 40

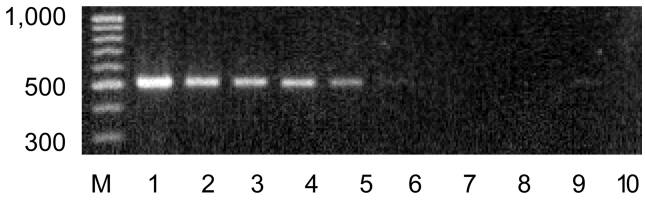

Fig. 3. Agarose gel electrophoresis of DNA amplified in the real time RT-PCR as described in Table 3. Lane 1. no heating; lanes 2-4, 65°C for 30 min; lanes 5-7, 100°C for 10 min; lanes 8-10, 121°C for 15 min; lanes 2, 5, 7, no incubation; lanes 3, 6, 9, 37°C for 6 h; lanes 4, 7, 10, 37°C for 24 h; lane M, 100 bp DNA ladder marker (Bioneer, Korea)

at 65°C for 30 min, 100°C for 10 min, or 121°C for 15 min. Each heated milk samples were then incubated at 37°C for 6 h and 24 h. Table 3 showed that the Cts of the control milk without heat treatment and of the milk which was heated at 65°C for 30 min were 22.0 and 32.9, respectively, which indicated that the amount of mRNA template was reduced to about one-two thousandth. The subsequent incubation at 37°C for 6 h and 24 h increased the Ct of the heated milk samples to 34.2 and 35.2, respectively, which suggested that there were additional reductions of mRNA template to half and one-fifth, respectively. Heat treatment at 100°C for 10 min increased Ct to 37.2 and subsequent incubations at 37°C for 6 h increased Ct to more than 40. The milk samples with Cts of which were more than 40 did not show DNA band in agarose gel electrophoresis (Fig. 3).

These results showed that heating at higher temperature destroyed mRNA more efficiently. There was still residual RNA left after heat treatment at 65 and 100°C. Subsequent incubation at 37°C after heating at 100°C degraded residual mRNA further not to be detectable in the real time RT-PCR. However, there was still detectable mRNA after the incubation of the milk samples which had been heated at 65°C. These results indicated that the incubation of heated milk samples at 37°C after heating might induce further degradation of mRNA but could not reach complete degradation of mRNA.

Effects of treatment with RNase A on the real time RT-PCR

Effects of treatments with RNase A on Cts of the real time RT-PCR were investigated (Table 4, Fig. 4). As described in Materials and Methods, milk samples containing *E. coli* ATCC 43890 were heated at 65°C for 30 min. After centrifuging the milk samples and removing supernatant, either phosphate-buffered saline or tryptic soy broth which contained RNase A was added to pellets. The mixtures were incubated at 37°C for 1 h. Table 4 showed that there was increase in Ct from 24.2 to 37.3 after heating of the contaminated milk. Treatments of the heated bacteria with RNase A in both phosphate buffered saline and tryptic soy broth increased Ct further to more than 40. However, when the bacteria without heating was treated with RNase A in tryptic soy broth, there was decrease in Cts from 24.2 to 22.8, suggesting that mRNA template was synthesized during the treatment (Table 4). Agarose gel electrophoresis (Fig. 4) also showed strong DNA band of the milks without heating regardless RNase treatment. However, it showed no DNA band of the heated milk which was then treated with RNase A. These results suggested that treatment of heated bacteria with RNase A might degrade further mRNA templates which left intact after heating at 65°C for 30 min and that tryptic soy broth stimulated mRNA synthesis of viable bacteria.

It has been known that mRNA molecules in bacteria degrade quickly after their death because of short half-life and their presence in bacterial cell may therefore indicates viability. It was suggested that RT-PCR to detect mRNA should be a rapid sensitive molecular method to detect viable bacteria (Klein and Juneja, 1997; McIngvale *et al*., 2002; Sheridan *et al*., 1999). Klein and Juneja (1997) reported that after bacteria was heated at 121°C,

Table 4. Effects of heating of milk at 65°C for 30 min and RNase A treatment of bacterial suspension at 37°C for 1 h obtained from *E. coli* ATCC 43890 (10^7 cells) in milk (1 mL) on the threshold cycles (Cts) of the real time RT-PCR of RNA extract

RNase A treatment	Cts	
	No heating	Heating at 65°C for 30 min
Control	24.2±0.2	37.3±0.2
RNase A in phosphate buffered saline	24.9±0.3	>40
RNase A in tryptic soy broth	22.8±0.1	>40

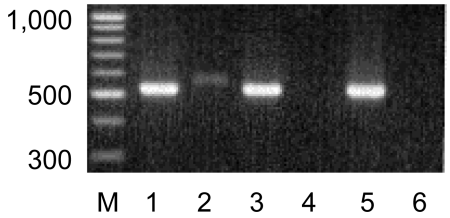

Fig. 4. Agarose gel electrophoresis of DNA amplified in the real time RT-PCR as described in Table 4. Lanes 1-2. no RNase A treatment; lanes 3-4, RNase A in phosphate buffered saline; lanes 5-6, RNase A in Tryptic soy broth; lanes 1, 3, 5, no heating; lanes 2, 4, 6, heating 65°C 30 min; lane M, 100 bp DNA ladder marker (Bioneer, Korea).

there was no DNA amplification in RT-PCR of specific regions of the genes, indicating that mRNA was destroyed. However, Sheridan *et al.* (1999) reported that intact mRNA still left immediately in ethanol-killed cells gave signal in RT-PCR, but the subsequent incubation at 37°C of the killed cells resulted in no DNA amplification in RT-PCR. They suggested that RT-PCR thus would have potential as an indicator of viability of cells, if samples were pre-incubated under appropriate conditions that would ensure decay of any residual mRNA in dead cells.

The present study showed that the treatment of dead cells with RNase A might further increase specificity to detect viable bacteria in the real time RT-PCR, since pretreatment of the milk samples with RNase in tryptic soy broth increased Cts of the heated milk but decreased Cts of the milk without heat treatment (Table 4). The real time RT-PCR enabled us to estimate reduction of the intact mRNA template after the heating at various temperatures and the subsequent treatment. The subsequent incubation at 37°C of the contaminated milk which was heated at 65°C for 30 min was not enough to degrade residual mRNA template. The treatment with RNase A of heated bacteria might enhance degradation of residual mRNA template. This study suggested that the real time RT-PCR method would be an efficient tool to detect viable *Enterobacteriacea* in pasteurized milk without false positive signal, provided that the heated milk were treated with RNase A for a short period of time before RNA extraction to degrade residual mRNA of dead bacteria and to activate mRNA synthesis of viable bacteria. Further studies are necessary to increase sensitivity of the real time RT-PCR to detect viable bacteria by designing primers for an amplicon of 50-100 bp. It was concluded that the real time RT-PCR methods designed in this study could provide an improved molecular method to detect viable Enterobacteriaceae specifically in heated milk.

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References

1. Apte, A. and Daniel, S. (2003) PCR primer design. In: PCR primer: A laboratory manual. 2nd ed, Dieffenbach, C. W. and Dveksler, G. S. (eds) Cold Spring Harbor Laboratory Press, NY, pp. 61-74.
2. Balows, A., Hausler, Jr. W. J., Herrmann, K. L., Isenberg, H. D., and Shadomy, H. J. (1991) Manual of clinical microbiology. 5th ed, American Society of Microbiology, Washington, DC.
3. Batt, C. A. (1997) Molecular diagnostics for dairy-borne pathogens. *J. Dairy Sci*. **80**, 220-229.
4. Baustista, D. A., McIntyre, L., Laleye, L., and Griffiths, M. W. (1992) The application of ATP bioluminescence for the assessment of milk quality and factory hygiene. *J. Rapid Methods Autom. Microbiol*. **1**, 179-193.
5. Bishop, J. R., White, C. H., and Firstenberg-Eden, R. (1984) Rapid impedimetric method for determining the potential shelf-life of pasteurized whole milk. *J. Food Prot*. **47**, 471-475.
6. Bishop, J. R. and White, C. H. (1986) Assessment of dairy product quality and potential shelf – a review. *J. Food Prot*. **49**, 739-753.
7. Choi, S. H., Choi, J. J., and Lee, S. B. (1999) Gram-negative bacteria selective medium to detect post-pasteurization contamination of market milk by using dye reduction test. *Korean J. Dairy Sci*. **21**, 231-240.
8. Choi, S. H. and Lee, S.-B. (2011) Improved detection of viable *Escherichia coli* O157:H7 in milk by using reverse transcriptase-PCR. *Korean J. Food Sci. Ani. Resour.* **31**, 158-165.
9. International Dairy Federation (1993) Catalogue of tests for the detection of post-pasteurization contamination of milk. *IDF Bulletin*. **281**, 13-34.
10. Klein, P. G. and Juneja V. K. (1997) Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Appl. Environ. Microbiol*. **63**, 4441-4448.
11. McIngvale, S. C., Elhanafi, E., and Drake, M. A. (2002) Optimization of reverse transcriptase PCR to detect viable Shiga-toxin producing *Escherichia coli*. *Appl. Environ. Microbiol*. **68**, 799-806.
12. Muir, D. D. (1990) The microbiology of heat-treated fluid milk products. In: Dairy microbiology, vol. 1, the microbiology of milk. 2nd ed, Robinson, R.K. (ed) Elsevier Applied Science, NY, pp. 209-243.
13. Sambrook, J. and Russell, D. W. (2001) Molecular cloning - a laboratory manual. 3rd ed, Cold Spring Harbor Laboratory Press, NY.
14. Sharma, V. J. (2006) Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfbE* and *eae* of genes of *Escherichia coli* O157:H7. *Mol. Cell. Probes* **20**, 298-306.
15. Sheridan, G. E., Szabo, E. A., and Mackey, B.M. (1999) Effect of post-treatment holding conditions on detection of *tufA* mRNA in ethanol-treated *Escherichia coli*: implications for RT-PCR-based indirect viability tests. *Lett. Appl. Microbiol*. **29**, 375-379.
16. Szabo, E. A. and Mackey, B. M. (1999) Detection of *Salmonella enteritidis* by reverse transcriptase-polymerase chain reaction (PCR). *Intl. J. Food Microbiol.* **51**, 113-122.
17. Vaitilingom, M., Gendre, F., and Brignon, P. (1998) Direct detection of viable bacteria, molds, and yeasts by reverse

transcrpitase PCR in contaminated milk samples after heat treatment. *Appl. Environ. Microbiol*. **64**, 1157-1160.
18. Varnam, A. H. and Sutherland, J. P. (1994) Milk and milk products – Technology, chemistry, and microbiology. Chapman & Hall, London, UK.
19. White, C. H. (1993) Rapid methods for estimation and prediction of shelf-life of milk and dairy products. *J. Dairy Sci*. **76**, 3126-3132.
20. White, C. H., Bishop, J. R., and Morgan, D. M. (1993) Microbiological methods for dairy products. In: Standard methods for the examination of dairy products. Marshall, R. T. (ed) American Public Health Association, Washington, DC.
21. Yaron, S. and Matthew, K. R. (2002) A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. *J. Appl. Microbiol.* **92**, 633-640.

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