Korean J. Food Sci. Ani. Resour.
Vol. 31, No. 2, pp. 158~165(2011)

ARTICLE

Improved Detection of Viable *Escherichia coli* O157:H7 in Milk by Using Reverse Transcriptase-PCR

Suk Ho Choi* and Seung-Bae Lee

Division of Animal Resources and Life Science, Sangji University, Wonju 220-702, Korea

Abstract

A sensitive reverse transcriptase-PCR (RT-PCR) method to detect viable *Escherichia coli* O157:H7 in milk was established. The primer sets were designed based on the nucleotide sequences of the *rfbE* (*per*) and *wbdN* genes in the O157 antigen gene cluster of *E. coli* O157:H7. RT-PCR using five different primer sets yielded DNA with sizes of 655, 518, 450, and 149-bp, respectively. All five of the *E. coli* O157:H7 strains were detected by RT-PCR, but 11 other bacterial species were not. The sensitivity of RT-PCR was improved by adding yeast tRNA as a carrier to the crude RNA extract. The RT-PCR amplifying the 149-bp DNA fragment was the most sensitive for detecting *E. coli* O157:H7 and the most refractory to the bactericidal treatments. Heat treatment at 65°C for 30 min was the least inhibitory of all bactericidal treatments. Treatment with RNase A strongly inhibited the RT-PCR of heated milk but not unheated milk. This study described RT-PCR methods that are specific and sensitive with a detection limit of 10 *E. coli* O157:H7 cells, and showed that pre-treating milk samples with RNase A improved the specificity to detect viable bacteria by RT-PCR.

Key words: reverse transcriptase-polymerase chain reaction, *Escherichia coli* O157:H7, milk, heat treatment, bactericidal treatment

Introduction

Escherichia coli O157:H7 is an important pathogen that has been associated with severe gastrointestinal and systemic diseases, such as hemorrhagic colitis and hemolytic-uremic syndrome (Karmali, 1989). The major virulence attributes of *E. coli* O157:H7 include production of Shiga toxins 1 and 2, which are responsible for development of systemic symptoms of the diseases, which have been reported following consumption of raw or undercooked ground beef (Doyle, *et al*., 1997). Cattle are generally considered major reservoir for *E. coli* O157:H7 and these animals excrete the bacteria in feces. Fecal contamination of foods, such as meats and dairy products, as well as water is the major mode of spread of this pathogen to humans (Griffin *et al*., 1991; Hancock *et al*., 1994). Although most commonly associated with foods of animal origin, *E. coli* O157:H7 may also be isolated from contaminated drinking water, salad dressing, and buttermilk (Dineen *et al*., 1998; McIngvale *et al*., 2000; Szabo *et al*., 1986).

The conventional culture method to detect *E. coli* O157:H7 is laborious and requires 2-3 d for completion. The molecular methods including PCR have been developed for rapid sensitive detection of low level of the bacteria in bovine feces, foods, and water (Fratamico *et al*., 1995; Paton and Paton, 1998). Genes, such as *sltI* and *sltII* encoding Shiga toxin 1 and Shiga toxin 2, respectively, (Gooding and Choudary, 1997; Witham *et al*., 1996), *rfbE* encoding for O antigen O157 serotype (Bilge *et al*., 1996), and *eae* encoding intimin (Yu and Kaper, 1992) were investigated as the target genes for PCR to detect *E. coli* O157:H7. However, major shortcoming of the PCR in detection of a pathogen is that the assay synthesizes DNA as far as there is sufficient template DNA, which is not degraded in the bacteria killed by heating, drying, and starvation (Dupray *et al*., 1997; Masters *et al*. 1994).

There are several types of RNA in bacterial cells, including mRNA and rRNA. rRNA is an constituent of ribosomes and present in high copy numbers. However, rRNA was reported to remain intact for extended period of time in dead bacteria (McKillip *et al*., 1998). mRNA is considered to have a short half-life of only a few minutes (Kushner, 1996). RT-PCR in which DNA is amplified

*Corresponding author: Suk Ho Choi, Division of Animal Resources and Life Science, Sangji University, Wonju 220-702, Korea. Tel: 82-33-730-0543, Fax: 82-33-730-0503, E-mail: shchoi@sangji.ac.kr

using mRNA template has been reported to detect selectively viable bacteria including *Listeria monocytogenes* (Klein and Juneja, 1997), *Salmonella* serotype Enteritidis (Szabo and Mackey, 1999) and *E. coli* O157:H7 (McIngvale *et al*., 2002; Sharma, 2006; Yaron and Matthew, 2002). These methods had problems with low efficiency of nucleic acid purification, which resulted in increased detection limit and required extended preenrichment culture of food sample before RT-PCR assay (McIngvale *et al*., 2002; Sharma, 2006; Yaron and Matthews, 2002). 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).

The objectives of this study were to establish sensitive specific RT-PCR methods using primer sets derived from the O157 antigen gene cluster and to determine effects of various bactericidal treatments and RNase A treatment of heated milk on specific detection of viable *E. coli* O157:H7.

Materials and Methods

Microorganisms

E. coli O157:H7 ATCC 43834, 43888, 43889, 43890, and 43895, *E. coli* KCTC 2441, *Salmonella* Typhimurium KCTC 2541, *Shigella sonnei* KCTC 2009, *Enterobacter sakazakii* KCTC 2949, *Yersinia enterocolitica* ATCC 23715, *Klebsiella pneumoniae* KCTC 2208, *Citrobacter freundii* KCTC 2006, *Pseudomonas fluorescens* KCTC 2344, *Enterococcus faecalis* KCTC 3512, *Listeria monocytogenes* KCTC 1945, and *Bacillus coagulans* KCTC 1015 were used in this study. *K. pneumoniae* KCTC 2208 and *C. freundii* KCTC 2006 were cultured at 32°C. *P. fluorescens* KCTC 2344 was cultured at 26°C. The rest of the bacteria were cultured at 37°C. ATCC and KCTC strains were purchased from American Type Culture Collection and Biological Resources Center in Korea, respectively.

Preparation of culture and milk samples

Gram-positive bacteria and Gram-negative bacteria were cultured in brain heart infusion broth (Difco, USA) and tryptic soy broth (Difco, USA), respectively. Gram-negative bacteria and *Bacillus* were cultured in a shaking incubator at 100 rpm. The other Gram-positive bacteria were cultured statically. The culture used to isolate RNA extract was incubated for a short period of time until the bacterial number reached approximately 10^9 CFU/mL which was estimated using McFarland nephelometer. The culture was cooled in iced water and diluted to obtain the bacterial number from 10^2 to 10^9 CFU/mL with sterile phosphate-buffered saline (PBS) containing 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4. The bacterial suspension (1 mL) was added to commercial UHT-sterilized milk (9 mL). The milk sample was used to isolate RNA extract.

Preparation of RNA extract

The milk sample (1 mL) and 25% sodium citrate (60 μL) were added into a tube. The mixture was vortexed for 5 min and centrifuged at 5,000 g for 5 min. The cream layer and supernatant were discarded. Tri reagent (1 mL) (Sigma and Aldrich, USA) was added to the pellet. The mixture of Tri reagent and the pellet was vortexed for 30 s and left at room temperature for 5 min. 1-Bromo-2-chloropropanol (Sigma Aldrich, USA) (100 μL) was added, then vortexed for 30 s, and left at room for 10 min. The mixture was centrifuged at 10,000 g for 15 min. Colorless aqueous upper phase, crude RNA extract, was transferred to a new tube. Yeast tRNA (10 mg/mL, 5 μL) and isopropanol (500 μL) were added to the crude RNA extract. The mixture was vortexed for 15 s, left for 10 min, and then centrifuged at 10,000 g for 10 min. After discarding the supernatant 75% ethanol was added, vortexed, and then centrifuged at 10,000 g for 10 min. After drying RNA pellet, 1×RQ DNase reaction buffer (20 μL) and RNase-free RQ DNase (1 unit) (Promega, USA) were added. The dissolved RNA extract was transferred to a new tube and incubated at 37°C for 60 min and then stop solution was added. The RNA extract was heated at 67°C for 30 min and stored frozen at –20°C.

RT-PCR and PCR

The primer sets used in RT-PCR and the expected sizes of amplified DNA are listed in Table 1. The nucleotide sequences of the primers of RFB and OAG are based on *rfbE* (*per*) and *wbdN* genes, respectively, in the O157 antigen gene cluster. The NCBI's website with accession number of AE005429 was referred to obtain the nucleotide sequences of the genes. Beacon Designer (Premier Biosoft International, USA) was used to optimize the nucletide sequences of primer sets for efficient DNA synthesis. The composition of Access RT-PCR (Promega, USA) used in this study was 1×AMV/*Tfl* reaction buffer, 0.7 mM $MgSO_4$, 0.2 mM dNTP, 2.5 U AMV reverse transcriptase, 2.5 U *Tfl* DNA polymerase, 1 μM forward primer, 1 μM reverse primer, and RNA extract (5 μL).

Table 1. The primers used in RT-PCR and PCR

Forward primer Reverse primer	Nucleotide sequences	Expected size of amplified DNA (bp)
RFB1	5'-CGGACATCCATGTGATATGG-3'	655
RFB4	5'-CTTGCTCATTCGATAGGCTG-3'	
RFB16	5'-ACTACAGGTGAAGGTGGAATGG-3'	450
RFB17	5'-ACGCCAACCAAGATCCTCAG-3'	
REB10	5'-GGACCGCAGAGGAAAGAGAG-3'	149
RFB11	5'-TCCACGCCAACCAAGATCC-3'	
OAG5	5'-CGTTTCAATAATTATGCCCGTTTAC-3'	518
OAG6	5'-TCCAGCCACATCAAATAATCCTC-3'	

RNase-free water was added to make up to 25 μL. The reaction steps of RT-PCR using thermal cycler (Applied Biosystem, USA) consisted of one cycle of 48°C for 45 min, one cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, and a final cycle of 68°C for 7 min.

PCR was used to detect residual DNA in the RNA extract. The PCR mixture (Promega, USA) contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, 0.5 U *Taq* polymerase, and RNA extract (5 μM). The total volume was made up to 25 μM with sterile distilled water. The reaction steps of PCR consisted of one cycle of 94°C for 5 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final cycle of 72°C for 4 min.

DNA synthesized in RT-PCR and PCR was separated in 2.5% agarose gel or 4.0% Nusieve 3:1 agarose (Lonza, USA) with 1×Tris-acetate buffer. DNA was visualized by staining with 0.5 μg/mL ethidium bromide. A 100-bp-ladder DNA marker (Bioneer, Korea) was used to estimate DNA size.

Bactericidal treatments

Three aliquots (5 mL) of milk samples containing *E. coli* O157:H7 ATCC 43890 (10^8 CFU/mL) were heated at 65°C for 30 min in water bath, at 100°C for 10 min in boiling water, and at 121°C for 15 min in autoclave, respectively.

Two aliquots (0.1 mL) of bacterial suspensions of *E. coli* O157:H7 ATCC 43890 (10^9 CFU/mL) in PBS were added to 75% ethanol (0.9 mL) and 1.33% Bactdown (Danon Labs, Inc., USA) (0.9 mL), respectively. The mixtures were left at room temperature for 30 min and centrifuged at 10,000 g for 5 min. The resultant pellets were suspended in UHT-sterilized milk (1 mL).

An aliquot (5 mL) of bacterial suspension of *E. coli* O157:H7 ATCC 43890 (10^9 CFU/mL) in PBS was placed on petri dish. The petri dish with lid open was placed under ultraviolet light lamp in clean bench for 30 min. An aliquote (1 mL) of the bacterial suspension was added to UHT-sterilized milk (9 mL).

RNase A treatment of milk sample

The milk sample (1 mL) containing *E. coli* O157:H7 ATCC 43890 (10^8 cells) and 25% sodium citrate (60 μL) were added into a tube. The mixture was vortexed for 5 min and centrifuged at 5,000 g for 5 min. The cream layer and supernatant were discarded. PBS (1 mL) and RNase A (6 μg/mL, 25 μL) were added to the pellet and the mixture was agitated into vortex, incubated at 37°C for 1 h, and then centrifuged at 5,000 g for 5 min. Tri reagent (1 mL) was added to the pellet and the RNA extract was isolated as described above.

Results and Discussion

Specificity of RT-PCR to detect *E. coli* O157:H7 in the milk samples was investigated. Fig. 1 showed that DNA was amplified in RT-PCR of the RNA extracts only from the five strains of *E. coli* O157:H7, but not from *E. coli* KCTC 2441, the other Enterobacteriaceae, and Gram positive bacteria examined in this study. The RT-PCR with the primer sets of RFB1 and RFB4, RFB16 and RFB17, RFB10 and RFB11, and OAG 5 and OAG6 produced DNA with the size of 655, 450, 149, and 518 bp, respectively, as expected in Table 1. These results showed that all the RT-PCR methods showed high specificity to detect *E. coli* O157:H7.

Yaron and Matthews (2002) suggested that *rfbE* (*per*) gene was the most appropriate target for detection of viable *E. coli* O157:H7 in RT-PCR, since the gene was

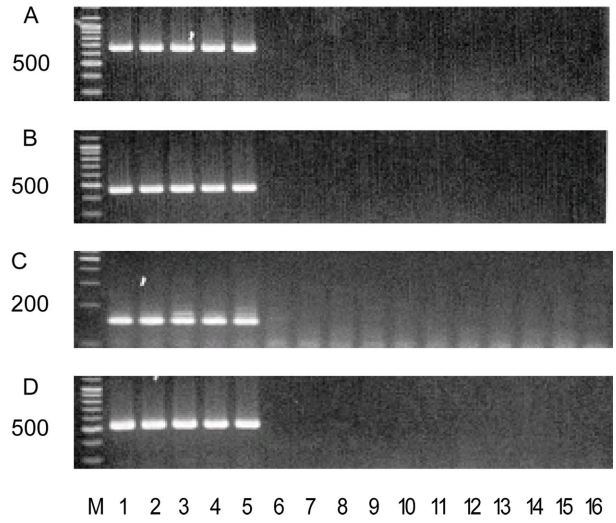

Fig. 1. Specificity of RT-PCR methods using the primer sets of RFB1 and RFB4 (A), RFB16 and RFB17 (B), RFB10 and RFB11 (C), and OAG5 and OAG6 (D). Lane M, DNA marker; lanes 1, 2, 3, 4, and 5, *E. coli* O157:H7 ATCC 43890, 43895, 43889, 43884, and 43888, respectively; lane 6, *E. coli* KCTC 2441; lane 7, *S. typhimurium*; lane 8, *S. sonnei*; lane 9, *E. sakazakii*; lane 10, *Y. enterocolitica*; lane 11, *K. pneumoniae*; lane 12, *C. freundii*; lane 13, *P. fluorescens*; lane 14, *E. faecalis*; lane 15, *L. monocytogenes*; lane 16, *B. coagulans.*

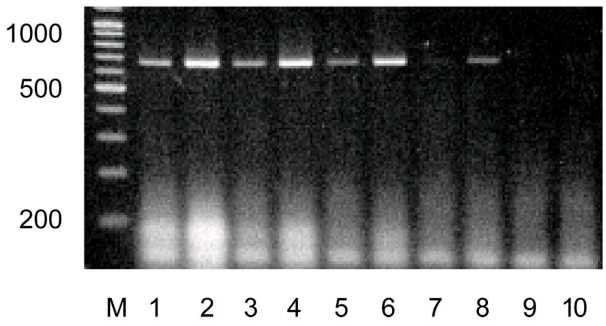

Fig. 2. Effects of yeast tRNA addition to the crude RNA extract on RT-PCR methods using the primer set of RFB1 and RFB4 to detect *Escherichia coli* O157:H7 (10^5 cells). Lane M, DNA marker; lanes 1 and 2, 50 μg tRNA; lanes 3 and 4, 20 μg tRNA; lanes 5 and 6, 10 μg tRNA; lanes 7 and 8, 4 μg tRNA; lanes 9 and 10, 0 μg tRNA; lanes 1, 3, 5, 6, 7, and 9, 2 μL RNA extract; lanes 2, 4, 6, 8, and 10, 5 μL RNA extract.

expressed continuously during growth for 24 h. However, their RT-PCR method required 10^7 CFU of the target organisms for detection of viable cells without enrichment. McIngvale *et al*. (2002) evaluated the primers targeting a specific region to the *slt-II* operon of shiga-toxin-producing *E. coli* O157:H7 in RT-PCR. However, enrichment in cooked ground beef of *E. coli* O157:H7 for 12 h with initial inoculum of 1 CFU/g was necessary for detection of viable bacteria by using RT-PCR in their study.

Sensitivity of RT-PCR in detecting bacteria tends to be low, because of susceptibility of bacterial mRNA to degradation and inhibitory substances in foods. Thus, RT-PCR method requires presence of great number of target organisms in the food sample or enrichment of food sample for extended times before giving a detectable signal. The probable inhibitory substances in food should be removed from RNA extract using appropriate extraction methods and recovery of RNA should be optimized, if low level of pathogenic bacteria should be detected using RT-PCR (Bickley *et al*., 1996; Weeratna and Doyle, 1991). de Wet *et al* (2008) reported that addition of a high number of nontarget cells *E. coli* K12 to the sample improved yield of RNA extraction from low abundance of *E. coli* O157:H7. Addition of non-target bacterial cells to the sample prior to RNA extraction seemed to facilitate co-precipitation of target RNA along with non-target RNA and thus enhance recovery of target RNA and sensitivity of RT-PCR.

Several measures have been taken in this study to improve sensitivity and specificity of RT-PCR to detect viable bacteria. Sodium citrate was added to milk sample to prevent co-precipitation of calcium and milk proteins with bacterial cells. The effects of yeast tRNA on sensitivity of RT-PCR were examined by adding it to crude RNA extract at the level of 50, 20, 10, 4, and 0 μg per tube before precipitation of RNA with isopropanol. Fig. 2 showed no DNA synthesis in RT-PCR from RNA extracts prepared from 10^5 cells of *E. coli* O157:H7 ATCC 43890 without addition of yeast tRNA. As the amount of yeast tRNA increased up to 50 μg, DNA amplification in RT-PCR increased. Yeast tRNA was used for extraction of RNA from the milk in the following study, as described in Materials and Methods. Addition of 5 μL of the RNA extract to the RT-PCR mixture, which gave better DNA amplification than that of 2 μL, was applied to the RT-PCR in the following study.

The RNA extract which was treated with RNase-free RQ DNase to remove residual DNA was subjected to PCR as well as RT-PCR (Fig. 2). The PCR did not amplify DNA from the RNA extract treated with RNase-free RQ DNase, but amplify DNA from the RNA extract not treat with RNase-free RQ DNase. These results indicated that there was significant amount of residual DNA in the RNA extract. The RNA extract was treated with RNase-free RQ DNase as described in Materials and Methods.

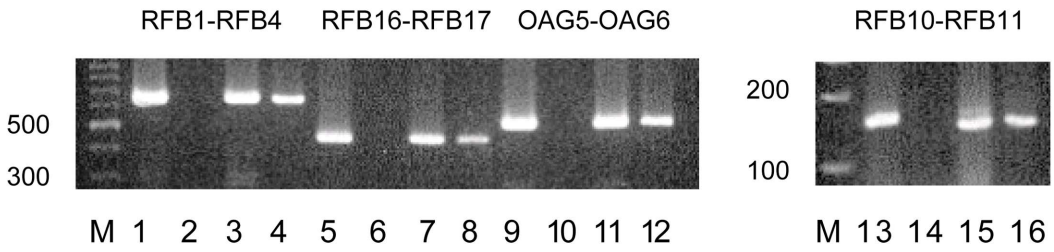

Fig. 3. Effects of RNase-free RQ DNase treatment of the RNA extract isolated from *Escherichia coli* O157:H7 ATCC 43890 on PCR and RT-PCR methods using the primer sets of RFB1 and RFB4 (lanes 1-4), RFB16 and RFB17 (lanes 5-8), RFB10 and RFB11 (lanes 13-16), and OAG5 and OAG6 (lanes 9-12). Lane M, DNA markers; lanes 1, 5, 9, and 13, RT-PCR of DNase-treated RNA extract; lanes 2, 6, 10, and 14, PCR of DNase-treated RNA extract; lanes 3, 7, 11, and 15, RT-PCR of RNA extract without DNase treatment; lanes 4, 8, 12, and 16, PCR of RNA extract without DNase treatment.

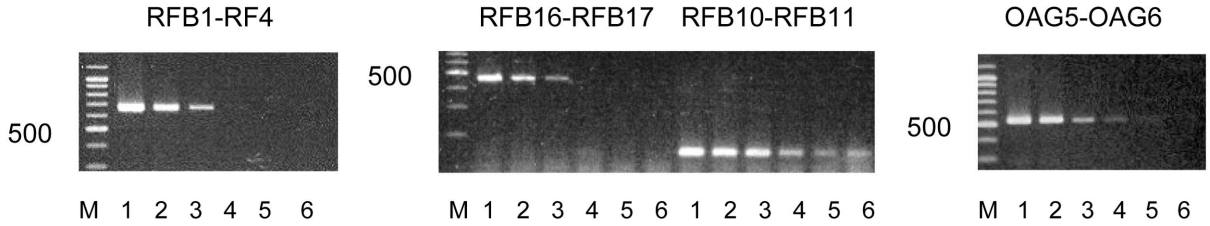

Fig. 4. Sensitivity of RT-PCR methods to detect *Escherichia coli* O157:H7 ATCC 43890. Lane M, DNA marker; lane 1, 10^6; lane 2, 10^5; lane 3, 10^4; lane 4, 10^3; lane 5, 10^2; lane 6, 10 in milk.

Sensitivity of RT-PCR methods to detect *E. coli* O157:H7 ATCC 43890 was determined by adding 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 cells into milk (1 mL). Fig. 4 showed that the minimum cell numbers of the bacteria which the RT-PCR methods detected using the primer sets of RFB1 and RFB4, RFB 16 and RFB17, RFB10 and RFB11, and OAG5 and OAG6 were 10^4, 10^4, 10, and 10^3, respectively. Even though the detection limit of 10^4 was not practical for detection of low number of the bacteria in the milk, it was better than those in the previous report (Yaron and Matthews, 2002; McIngvale *et al*., 2002). In addition to this, the RT-PCR with the primer set of RFB10 and RFB11 could detect 10 cells of the bacteria, which may be sensitive enough to detect low abundance of the bacteria without pre-enrichment culture of milk samples.

Milk samples containing *E. coli* O157:H7 (10^8 CFU/mL) were heated as described in bactericidal treatments of Materials and Methods, and then either cooled in ice or incubated at 37°C for 2, 4, and 6 h. Fig. 5 shows that heat treatment at 121°C for 15 min was the most effective of all the heating treatments in disturbing RT-PCR to detect *E. coli* O157:H7. Heat treatment at 100°C for 10 min inhibited partially the RT-PCR using the primer set of RFB1-RFB4 and subsequent incubation at 37°C for 2-6 h inhibit completely it. Heat treatment at 100°C for 10 min

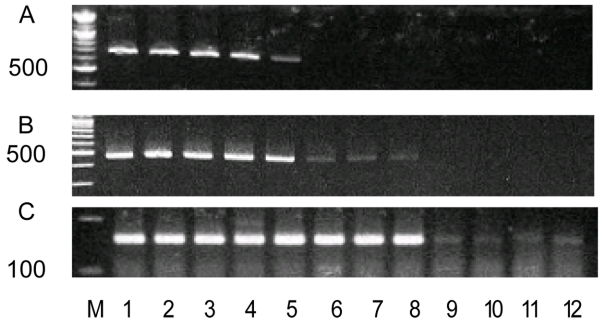

Fig. 5. Effects of heat treatment and subsequent incubation at 37°C on RT-PCR methods to detect *E. coli* O157:H7 ATCC 43890 in the milk which was heated at 65°C for 30 min (lanes 1-4), 100°C for 10 min (lanes 5-8), and 121°C for 15 min (lanes 9-12). A, RFB1 and RFB4; B, RFB16 and RFB17; C, RFB10 and RFB11; lanes 1, 5, and 9, without incubation; lanes 2, 6, and 10, incubation for 2 h; lanes 3, 7, and 11, incubation for 4 h; lanes 4, 8, and 12, incubation for 6 h; lane M, DNA marker.

did not inhibit completely RT-PCR using RFB16 and RFB17 but subsequent incubation inhibited significantly. Heat treatment at 65°C for 30 min and subsequent incubation did not inhibit completely all the RT-PCRs using the three RFB primer sets.

Effects on RT-PCR of treatments using Bactdown, ultraviolet light, and 75% ethanol and subsequent incuba-

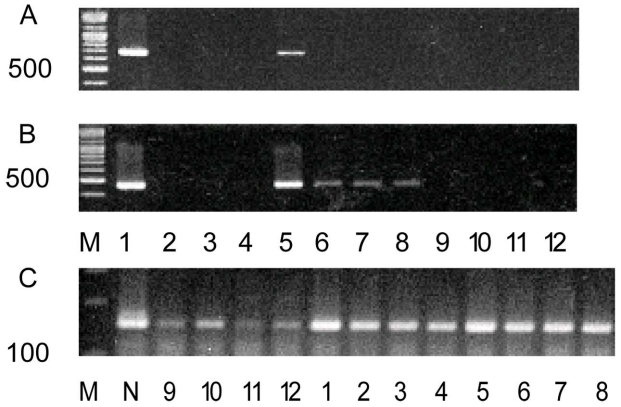

Fig. 6. Effects of bactericidal treatments and subsequent incubations at 37°C on RT-PCR method to detect *E. coli* O157:H7 ATCC 43890 which was treated with Bactdown (lanes 1-4), ultraviolet light (lanes 5-8), and 75% ethanol (lanes 9-12). A, RFB1 and RFB4; B, RFB16 and RFB17; C, RFB10 and RFB11; lanes 1, 5, and 9, without incubation; lanes 2, 6, and 10, incubation for 2 h; lanes 3, 7, and 11, incubation for 4 h; lanes 4, 8, and 12, incubation for 6 h; lanes M, DNA marker; lane N, no bactericidal treatment.

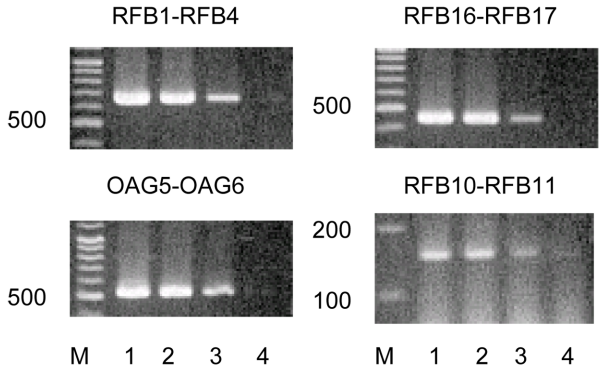

Fig. 7. Effects of RNase A treatment on RT-PCR method to detect *E. coli* O157:H7 ATCC 43890 in the contaminated milk which was heated at 65°C for 30 min. Lane M: DNA marker, lane 1: milk without RNase treatment, lane 2: milk with RNase treatment, lane 3: heated milk without RNase treatment, lane 4: heated milk with RNase treatment.

tion at 37°C were investigated as shown in Fig. 6. Treatment with 75% ethanol inhibited completely the RT-PCR using the primer set of RFB1 and RFB4 and the primer set of RFB16 and RFB17 and inhibited partially the RT-PCR using the primer set of RFB10 and RFB11. Treatments with Bactdown or ultraviolet light inhibited partially the RT-PCRs using the primer sets of RFB1 and RFB4, RFB16 and RFB17, and RFB10 and RFB11. Subsequent incubation at 37°C completely inhibited the RT-PCR using the primer set of RFB1 and RFB4, but did not inhibit the RT-PCR using the primer set of RFB10 and RFB11. These results suggested that the effects of the bactericidal treatments depended on the length of amplified DNA in RT-PCR and the subsequent incubation at 37°C increased degradation of RNA in dead bacteria.

The milk samples without the heat treatment at 65°C for 30 min showed DNA amplification in RT-PCR regardless the RNase A treatment (Fig. 7). The heat-treated milk samples showed relatively weak DNA amplification in the RT-PCR. The RNase treatment after the heat treatment completely inhibited DNA amplification in RT-PCR using the primer sets of RFB1 and RFB4, RFB16 and RFB17, and OAG5 and OAG6. There was weak DNA amplication in RT-PCR using the primer set of RFB10 and RFB11 after the RNase treatment, which might be due to the high cell number (10^8 CFU/mL) used in this study as well as the sensitivity of the RT-PCR which detected down to 10 CFU/mL of *E. coli* O157:H7. These results suggested that the RNase A treatment of the milk samples should promote degradation of intact RNA template in dead bacteria and enable specific detection of viable bacteria in RT-PCR.

DNA in dead bacteria does not degrade rapidly and remains intact for extended period of time after heating, nutrient depletion, and drying (Dupray *et al*., 1997; Masters *et al*., 1994; McKillip *et al*., 1998). However, mRNA in dead cell is known to degrade within hours. It was suggested that RT-PCR should be a rapid sensitive molecular method to detect viable bacteria (Herman, 1997; Klein and Juneja, 1997; McKillip *et al*., 1999; Sheridan *et al*., 1999). Klein and Juneja (1997) reported RT-PCR methods using the primers to amplify the genes of *iap*, *hly*, and *prfA* of *Listeria monocytogenes*. When the bacteria was heated at 121°C, there was no amplification in RT-PCR. Herman (1997) reported that there was no DNA amplication in RT-PCR of RNA extract from *Listeria monocytogenes* heated at 65°C for 30 min. Vatilingom *et al*. (1998) reported no DNA amplication signal in RT-PCR of genes of EF-Tu and EF-1α was obtained, when bacteria, yeast, and mold was killed by heating at 65°C for 30 min and 120°C for 30 min. However, according to Sails *et al*. (1998), *Campylobacter jejuni* heated at 75°C for 5 min was shown to still contain mRNA which was amplifiable in RT-PCR immediately after heat treatment but not after 5 h.

In this study, we concluded that the RT-PCR method with the primer sets of RFB10 and RFB 11 enabled sensitive detection down to 10 cells of *E. coli* O157:H7 in

the milk samples and that the pre-treatment of milk samples with RNase A promoted further degradation of intact RNA template of dead cells, but not viable cells, and thus improved specificity to detect viable cells in the RT-PCR.

Acknowledgement

The present research was supported in 2000 by Agricultural Research and Development Promotion Center in Republic of Korea.

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(Received 2010.6.16/Revised 2010.9.2/Accepted 2010.12.7)