Project Title: Rapid quantification of culturable and viable-but-nonculturable *Escherichia coli* O157:H7 in beef products using EMA-Real Time PCR

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Background

Escherichia coli O157:H7 causes an estimated 73,500 cases of infections in the United States ev ery year (Buchanan et al., 1997), with a main source of infections being undercooked ground beef and other bovine products. In order to extend beef shelf life and reduce the potential risks associated with pathogenic growth, many meat preservation methods, including heat processing, irradiation, freezing, refrigeration and addition of food grade preservation have been applied (Jay, 2000). Although these methods have been shown to be efficient at reducing pathogenic growth, outbreaks continue to occur.

Bacterial cells that have been exposed to unfavorable environments, such as those following typical meat processing steps like heating, cleaning and sanitizing, can undergo a unique physiological or metabolic change in order to adapt to these harsh conditions. These cells may enter a status known as the viable but nonculturable (VBNC) state (Rowan, 2004). This new phenomenon poses a challenge to the traditional and widely used AOAC- or FDA-approved microbial testing methods, because VBNC bacteria, which cannot grow on non-selective or selective culture media, can maintain their virulence in this state. A previous study by our group conclusively demonstrated that VBNC *E. coli* O157:H7 still retained their Shiga-toxin genes in this state (unpublished data). Therefore, the potential risk of VBNC foodborne pathogens to consumers should be a concern to the meat industry.

For the past ten years, our group has conducted extensive work on developing rapid, sensitive and selective PCR-based detection methods for *E. coli* O157:H7, *Salmonella* and *Shigella* in beef and other foods. Our most recent publication (Wang et al., 2007) showed that real-time PCR could be used to sensitively detect and quantitate pathogens in ground beef in real time. In this current work, we added an ethidium monoazide (EMA) staining step to our established real-time PCR protocol for *E. coli* O157:H7 detection and quantitation to effectively decrease false-positive results from dead cells, brought about by conventional PCR methods, and false-negative results from VBNC cells, caused by traditional cultural methods. EMA is a stain (dye) that can bind to DNA in dead cells by penetrating the disintegrated cell wall of the dead organism. With this selective binding, DNA from dead cells cannot be amplified. Thus, when a mixture of dead and viable cells are present in a sample, only DNA from viable cells will be amplified in a subsequent PCR reaction following EMA treatment. This combination of EMA-real-time PCR reaction allows for a more accurate determination of the number of viable, including VBNC, cells that may be present in beef products.

With regards to viability and pathogenicity of cells in the VBNC status, rapid identification methods, such as this developed EMA-real-time PCR, will be an effective way to detect VBNC pathogenic cells in beef before they reach retail or consumers, thus preventing the outbreaks and foodborne illnesses that might result from false negative results generated by traditional cultural techniques. At the same time, the successful development of this

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method will contribute to a better understanding of the VBNC phenomenon in *E. coli* O157:H7 and provide valuable information for further reduction of important pathogens in the beef processing line.

The stated objectives for this work were:

1. To establish an EMA-real time PCR assay for quantifying viable *E. coli* O157:H7.
2. To determine detection sensitivity and linear range of the EMA-real time PCR system for quantitating VBNC *E. coli* O157:H7.
3. To evaluate the performance of the EMA-real time PCR system for quantitating *E. coli* O157:H7 in beef.

Methodology

DNA extraction for comparison between conventional PCR and real-time PCR
Overnight grown cells of *E. coli* O157:H7 in tryptic soy broth + yeast extract (TSBY) were serially diluted to generate 1 ml cells representing 108 CFU/ml to 10 CFU/ml. The cells were centrifuged at 10,000 × g for 5 min at 5°C. Cell pellets were washed with distilled water and re-collected by centrifuging at 10,000 × g for another 5 min. The washed cell pellets were re-suspended in 100 µl of PrepMan® Ultra Sample preparation reagents (Applied Biosystem, Inc. – ABI, Foster City, CA). Cell suspensions were incubated at 56°C for 30 min, vortexed for 10 s, and boiled for 8 min (PrepMan® Ultra protocol, 1998, ABI). Upon centrifugation at 12,000×g for 2 min, the DNA extracts in the supernatants were recovered. Ten microliters of each DNA extract were used to run conventional PCR and real-time PCR separately.

Comparison between conventional PCR and real-time PCR
(i) Conventional PCR
PCR reactions were carried out using a GoTaq® PCR kit (Promega Corp., Madison, WI). Each 50 µl of PCR reaction mixture contained 10 ml of PCR buffer (2 mM Tris-HCl [pH 8.0], 10 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.05% Tween® 20 and 0.05% Nonidet®-P40), 200 µM each of deoxynucleoside triphosphates, 2.5 mM MgCl2, one pair of primers, 0.25 U of Taq DNA polymerase and 10 µl of DNA solution. With a reaction temperature of 95°C for 4 min and an additional 30 cycles at a melting temperature of 95oC for 1 min, annealing temperature of 62oC for 1 min, and extension temperature of 72°C for 1 min, multiple copies of target DNA were synthesized and maintained at 72°C for 5 min to complete the formation of all strands following the final cycle.

(ii) Real-Time PCR
Real-Time PCR was carried out in a 96-well ABI Prism® 7700 Sequence Detection System. In order to obtain the best amplification from real-time PCR, each 50 µl reaction mixture contained 25.0 µl of TaqMan™ Universal PCR Master Mix (2×), with a final concentration of 1×, 5.0 µl forward and reverse primers, with a final concentration of 50 to 900 nM, 5.0 µl TaqMan™ probe, with a final concentration of 250 nM, and sterile distilled water (ABI, Inc.).

(iii) Primers and Probe
Primers and probes used in the conventional and real-time PCR were those that have previously been designed in our lab as shown in Table 1 below. The specificities of each primer and probe have been tested and reported in our recent publication (Wang et al., 2007).

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Table 1. Primers and probes used for conventional and real-time PCR.

Primer/Probe	Sequence (5' to 3')	Target gene	Amplicon size
E. coli-primer1	TTGACCCACACTTTGCCGTAA	uidA	227 bp
E. coli-primer2	GCGAAAACTGTGGAATTGGG		
E. coli-probe	5'- VIC- TGACCGCATCGAAAC GCAGCT-TAMRA-3'		

Formation of dead cells
Fresh overnight *E. coli* O157:H7 cells were washed in distilled water and the pellets resuspended in an equal volume of distilled water. The suspension was heat treated at 95°C for 30 min to kill all cells and plated on Plate Count Agar to check their viability.

Influence of EMA on DNA amplification of viable cells
Overnight fresh *E. coli* O157:H7 cultures were washed and the pellets resuspended in distilled water. Varying amounts (0, 1, 5, 10, 25, 50, 100 µg) of EMA were added separately to seven 1-ml tubes of *E. coli* cell suspensions. Samples were covered with foil, incubated in the dark at room temperature for 5 min and exposed to a 650-W halogen light for 1, 2, 5 and 10 min to denature any excess EMA that might interfere with the subsequent PCR reaction.

Optimization of the DNA staining method
To avoid or prevent any influence of heat generated by the 650-W halogen lamp on DNA amplification of viable cells, a 1-min ice treatment step following EMA staining was applied to the samples. Specifically, the samples were incubated with EMA in the dark for 5 min, the foil cover removed from the tubes and the samples placed on ice for 1 min before being placed under a 650 W halogen light for 10 min.

Minimum concentration of EMA that inhibits DNA amplification from dead cells
Dead cells from above were pipeted at a volume of 1 ml each into seven tubes. Different amounts of EMA were added to the seven samples, to final concentrations of 0, 1, 5, 10, 25, 50, 100 µg/ml EMA per tube, respectively. Samples were covered with foil and incubated in the dark for 5 min. They were then placed on ice for 1 min and exposed for 10 min to a 650-W halogen light.

DNA extraction from EMA-treated samples
After a 10-min light exposure, samples were centrifuged at 12,000 × g for 5 min. The cell pellets were washed once in distilled water and resuspended in 100 µl of PrepMan® Ultra sample preparation reagents, followed by 56°C incubation for 30 min, vortexing for 10 s and boiling for 8 min. DNA extracts were removed by centrifugation at 12,000 × g for 2 min.

Application of EMA-real-time PCR to mixed viable and dead cells
One milliliter of dead cells was mixed with 1 ml of fresh viable cells and the mixture centrifuged at 12,000 × g for 5 min. The mixed cell pellets were re-suspended in 1 ml distilled water. Ten micrograms of EMA were added to reach a final concentration of 10 µg/ml. EMA staining and DNA extraction were conducted as described above.

Quantitation of viable cells from ground beef by EMA-real-time PCR
Ground beef, purchased from a local market, was determined free of *E. coli* O157:H7 by standard cultural methods (Bacteriological Analytical Manual, FDA, 1995). Twenty-five grams of ground beef were artificially inoculated with 2.5 ml of 108 CFU/ml fresh *E. coli* O157:H7 broth, massaged to mix and homogenized for 5 min. TSBY broth (225 ml) was added to the beef sample and the mixture stomached for 2 min. One milliliter of the suspension was transferred into a new 1.5 ml microcentrifuge tube, and centrifuged for 1 min at 600 × g to

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remove the meat tissues and fat. The pellet was resuspended in 1 ml of distilled water and the suspension vortexed for 30 s. EMA staining and DNA extraction were conducted as described above.

Determination of low viable cells counts in ground beef by established EMA-real-time PCR
Four bags of 25 g ground beef were artificially inoculated with 2.5 ml of 101 CFU/ml, 102 CFU/ml, 103 CFU/ml and 104 CFU/ml fresh *E. coli* O157:H7, respectively. Each sample was massaged to mix for 5 min. TSBY broth (225 ml) was added to each and the suspensions stomached for 2 min. The beef suspensions were enriched at 37 °C for 24 h before further sample preparations. One milliliter of enriched beef suspension from each sample was transferred into a new 1.5 ml microcentrifuge tube, followed by 1 min of centrifugation at 600 × g. Pellets were resuspended in 1 ml distilled water and vortexed for 30 s. EMA staining and DNA extraction were conducted as described above.

Findings

Comparison between conventional and real-time PCR for identification of viable (including VBN C) E. coli *O157:H7 cells*
Conventional PCR is the most common method used in EMA-related studies reported in the literature. In this project, viable, including VBNC, cells of *E. coli* O157:H7 were targeted, not dead cells. Because the infective dose of *E. coli* O157:H7 is extremely low (10-100 CFU), a highly sensitive technique is necessary in order to determine the amount of viable cells of this pathogen present in samples. The EMA concentration used in the method must be high enough to bind to the DNA of all dead cells present in order to prevent any false-positive signals, while at the same time, low enough to not influence DNA amplification of viable cells. A serial dilution series of 108 CFU/ml to 101 CFU/ml were used to extract DNA templates, and 10 µl of each DNA sample was applied to conventional PCR and real-time PCR to determine which PCR method was more reliable and sensitive. The wider the detection range, the more sensitive the method.

Figures 1, 2 and 3 below show that real-time PCR had a wider detection range and higher sensitivity than conventional PCR. As depicted in Figure 1, the lowest concentration of viable *E. coli* O157:H7 that could be detected by conventional PCR was 104 CFU/ml. Real-Time PCR, on the other hand, had a lowest detection limit of 102 CFU/ml (Figures 2 and 3). Real-Time PCR, thus, should give a more accurate determination of viable numbers of *E. coli* O157:H7.

Influence of EMA on DNA amplification from viable cells
Different concentrations of EMA were added to 1 ml of fresh viable *E. coli* O157:H7 to reach final concentrations of 1, 5, 10, 25, 50, and 100 µg/ml. These concentrations were chosen because they include almost all concentrations reported in the literature on the use of EMA and conventional PCR as a detection technique for pure cultures. Due to differences in specific methods and bacterial strains used, previous research have reported different conclusions. However, no studies to our knowledge have specifically targeted *E. coli* O157:H7 or used real-time PCR coupled with EMA staining for detection of only viable cells of this pathogen. Thus, the first step in this study was to test different EMA concentrations for staining DNA from dead cells before applying the samples to real-time PCR in order to achieve more reliable results for preventing DNA amplification from dead cells. The results are shown in Figures 4-10 below.

It was discovered that high EMA concentrations do indeed influence the detection sensitivity of the EMA-real-time PCR method. Specifically, the detection sensitivity decreased as the concentration of EMA increased. When the EMA concentration was more than 50 µg/ml, DNA amplification from viable cells did not occur (Figure 9), which leads to a false negative result. Further, the exposure time of the samples to light is very critical, in that the longer the exposure time, the less inhibition of viable cell DNA amplification after EMA staining. Because the light

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exposure step functions to denature excess unbound EMA in the sample, unsuccessful denaturation of EMA will led to inhibition of DNA amplification from viable cells in the subsequent real-time PCR step. Based on our experiments, a 10-min exposure time was found to be necessary for denaturing unbound EMA without critically influencing DNA amplification in the subsequent real-time PCR reaction (Figure 8).

Optimization of the DNA staining method

Figure 10 above shows that viable *E. coli* O157:H7 cells will generate different Ct values even without prior EMA treatment. This is due to the exposure of the samples to the 650-W halogen lamp. With its high power and energy, the heat generated from the lamp probably killed some of the cells or destroyed their DNA, leading to an increase in the Ct value, or in other words, an underestimated cell count. To avoid this occurrence, a 1-min ice cooling step was incorporated before exposing samples to the light to see if this problem could be overcome, as shown on Figure 11 below.

It was found that an increase in Ct value of DNA from viable *E. coli* O157:H7 cells occurred with increasing light exposure time. However, when a 1-min ice treatment was added before the 10-min light exposure, the Ct value decreased to almost the same value as the 1-min exposure time treatment (Figure 11). Therefore, it was decided that the optimized EMA staining procedure would include a 5-min incubation in the dark, a 1-min ice cooling treatment and a 10 min 650-W halogen light exposure prior to the real-time PCR step.

Determination of minimum EMA concentration that inhibits amplification of DNA from dead E. coli *O157:H7 cells*

108 CFU/ml heat-killed *E. coli* O157:H7 cells were stained for 5 min in the dark with 1, 5, 10, 25, 50, 100 µg/ml EMA. Stained cells were cooled on ice for 1 min and exposed to a 650 W halogen lamp for 10 min. As shown in Figure 12 below, DNA from dead cells can generate false positive signals indicated by the Ct value of 31.07, corresponding to 3 log CFU/ml of cells for the control (no EMA) sample. This is because PCR cannot distinguish between DNA from dead or live cells and although this Ct value represented DNA from dead cells, the final test result would indicate positive for *E. coli* O157H7. When these dead cells were stained with EMA, their DNA was bound by the stain, preventing its amplification by PCR. As shown in Figure 12, a minimum EMA concentration of 10 µg/ml was needed to guarantee the complete binding and inhibition of all dead cell DNA from being amplified in the real-time PCR.

Application of the EMA-real-time PCR to ground beef

Artificially inoculated ground beef with final counts of 10 CFU/g, 102 CFU/g, 103 CFU/g and 104 CFU/g viable *E. coli* O157:H7 were enriched in TSBY broth for 24 h. Each beef suspension included not only the inoculated pathogen but also other natural microflora. One milliliter of each beef suspension was subjected to EMA staining and conventional PCR as described above for identification of all viable cells as shown on Figure 13 below.

Figure 13 verified that the DNA specifically from *E. coli* O157:H7 viable cells in the ground beef was amplified, as seen by the 227 bp amplicon. This indicated that the EMA indeed did not bind the DNA from any of these viable cells and the PCR was quite specific for this pathogen despite the presence of millions of other natural microbes in the ground beef. When real-time PCR was run with these samples, Ct values ranging from 21 to 25 were generated, as shown in Figure 14 below. However, even though exact quantitation of the pathogen in ground beef could not be determined after a 24-h enrichment, as low as 10 CFU/ml could be detected. The estimated plate count number of viable *E. coli* O157:H7 cells present in the ground beef sample was 105 CFU/g after the 24-h enrichment.

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Implications

EMA, coupled with real-time PCR is demonstrated here to potentially be useful at avoiding false positive results that can come from using conventional PCR, while completely preventing any false-negative results that can be generated by traditional cultural methods. In this study, we successfully determined the optimal EMA concentration that can completely bind to DNA from a mixture of 109 CFU/ml dead and viable *E. coli* O157:H7 cell mixture, without negatively influencing the generation of specific PCR products from the viable cells. We believe that this EMA-real-time PCR technique can be a potentially powerful tool in the beef safety field, making the detection of VIABLE pathogens more accurate and reliable.

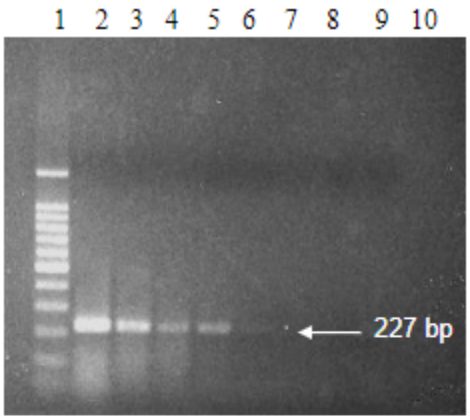

Figure 1. PCR products of different viable *E. coli* O157:H7 cell concentrations by conventional PCR. Lane 1, Marker; Lane 2, 10^8 CFU/ml; Lane 3, 10^7 CFU/ml; Lane 4, 10^6 CFU/ml; Lane 5, 10^5 CFU/ml; Lane 6, 10^4 CFU/ml; Lane 7, 10^3 CFU/ml; Lane 8, 10^2 CFU/ml; Lane 9, 10 CFU/ml; Lane 10, water control.

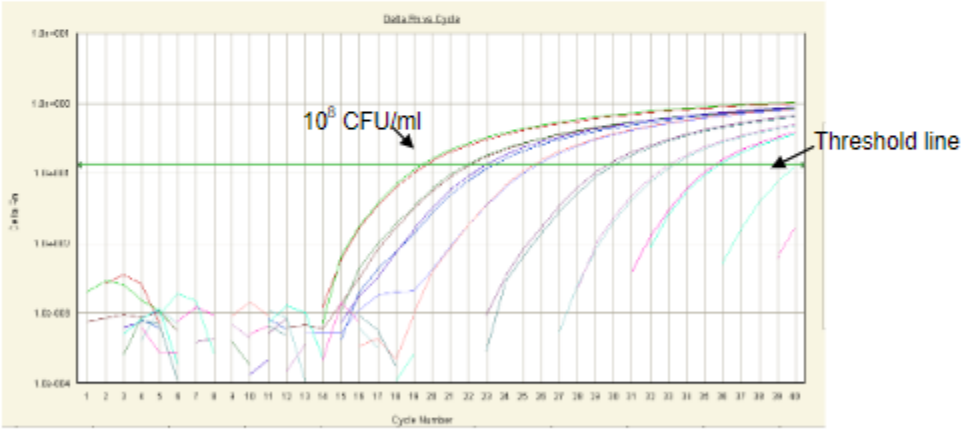

Figure 2. Real-time PCR amplification of 10^8 CFU/ml to 10^1 CFU/ml viable cells. The higher the cycle number (C_t), the lower the concentration of cells.

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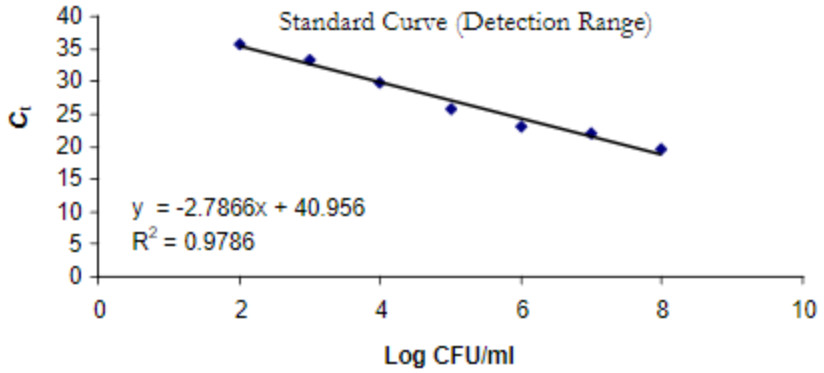

Figure 3. Standard curve of the real-time PCR. The detection range was from 10^8 CFU/ml to 10^2 CFU/ml viable *E. coli* O157:H7.

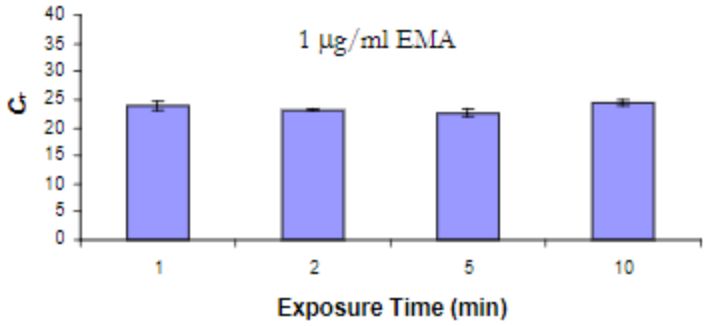

Figure 4. Influence of 1 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

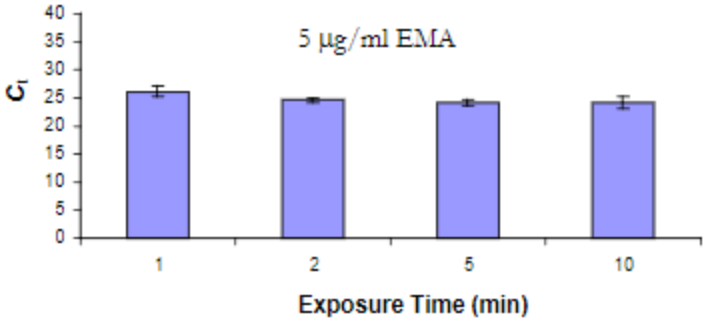

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Figure 5. Influence of 5 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

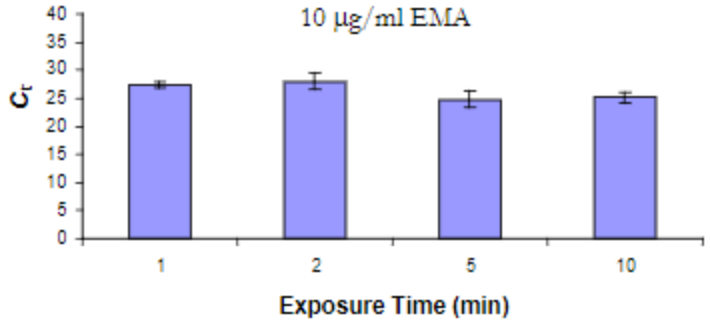

Figure 6. Influence of 10 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

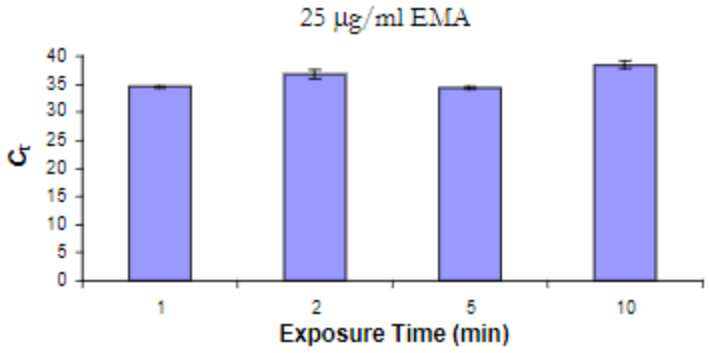

Figure 7. Influence of 25 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

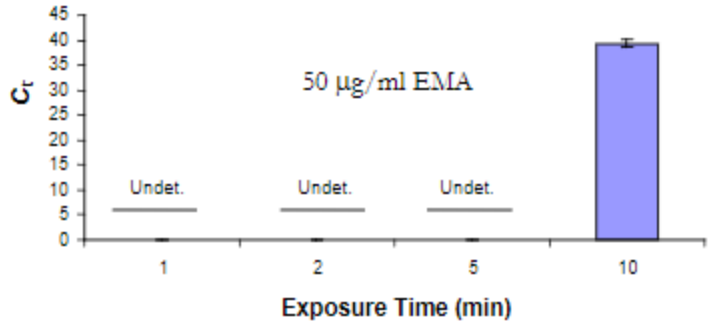

Figure 8. Influence of 50 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

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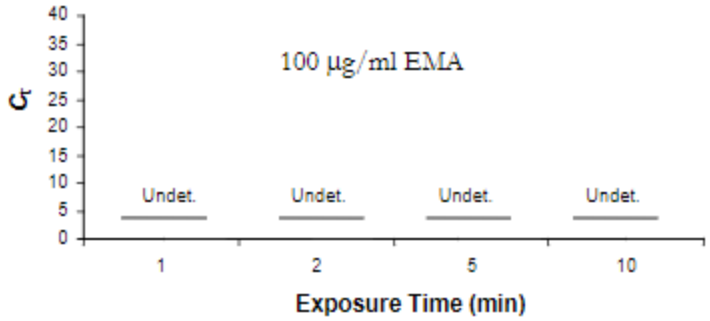

Figure 9. Influence of 100 µg/ml EMA on the amplification of viable *E. coli* O15:7H7 cells.

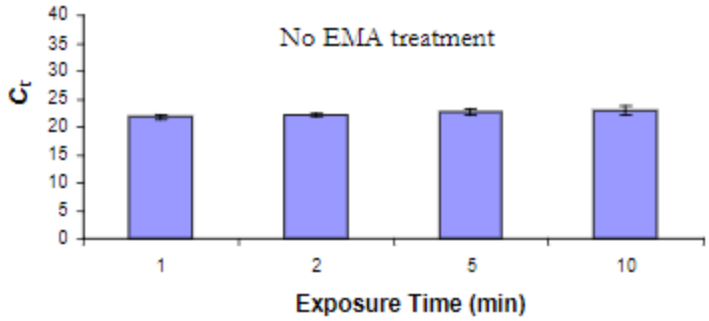

Figure 10. No EMA treatment to viable cells with different exposure times.

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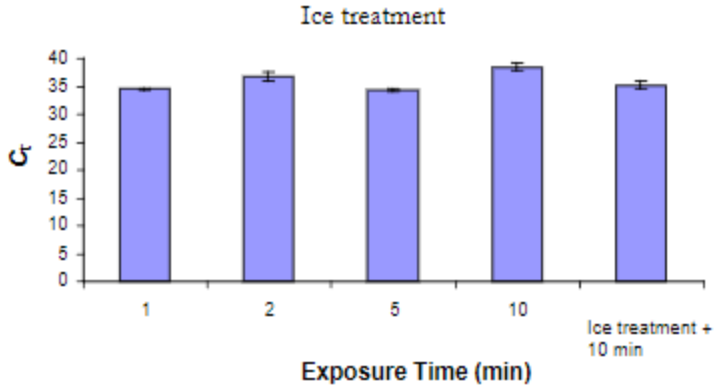

Figure 11. C_t values of DNA from viable *E. coli* O157:H7 cells stained with 25 µg/ml EMA followed by a 1-min ice treatment before halogen lamp light exposure.

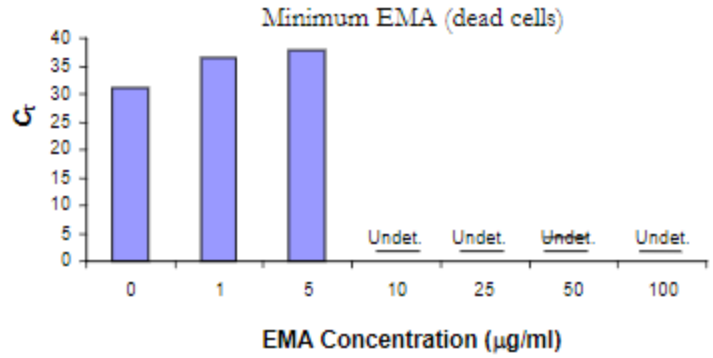

Figure 12. C_t values of DNA from dead *E. coli* O157:H7 cells stained with different EMA concentrations.

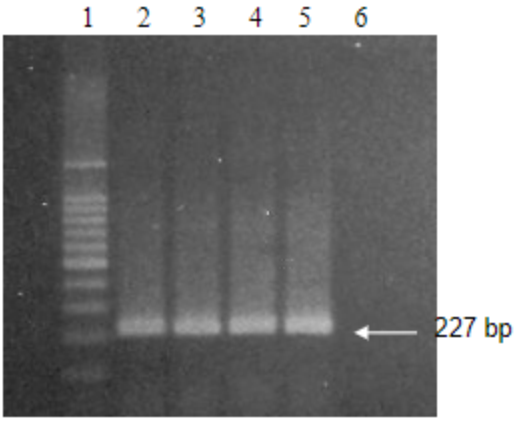

Figure 13. Conventional PCR amplification results of 24-h enriched beef samples. Lane 1, Marker; Lane 2, 10 CFU/g *E. coli* O157:H7; Lane 3, 10^2 CFU/g *E. coli* O157:H7; Lane 4, 10^3 CFU/g *E. coli* O157:H7; Lane 5, 10^4 CFU/g *E. coli* O157:H7; Lane 6, Water control.

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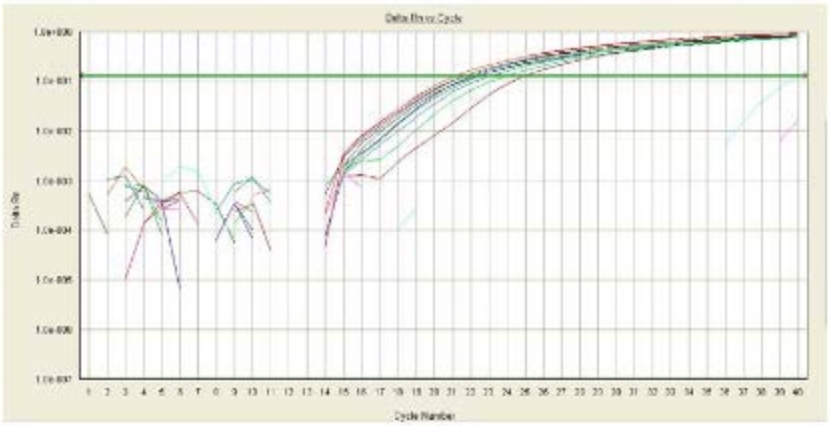

Figure 14. Real-time PCR amplification results of 24-h enriched beef samples.

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