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The physiologic state of *Escherichia coli* O157:H7 does not affect its detection in two commercial real-time PCR-based tests☆

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ABSTRACT

Multiplex real-time PCR detection of *Escherichia coli* O157:H7 is an efficient molecular tool with high sensitivity and specificity for meat safety assurance. The Biocontrol GDS® and DuPont Qualicon BAX®-RT rapid detection systems are two commercial tests based on real-time PCR amplification with potential applications for quantification of specific *E. coli* O157:H7 gene targets in enriched meat samples. However, there are arguments surrounding the use of these tests to predict pre-enrichment concentrations of *E. coli* O157:H7, as well as arguments pertaining to the influence of non-viable cells causing false positive results. The present study attempts to illustrate the effects of different bacterial physiologic states and the presence of non-viable cells on the ability of these systems to accurately measure contamination levels of *E. coli* O157:H7 in ground beef. While the PCR threshold cycle (C_T) values of these assays showed a direct correlation with the number of bacteria present in pure cultures, this was not the case for ground beef samples spiked with various levels of injured or healthy cells. Furthermore, comparison of post-enrichment cell densities of bacteria did not correlate with injured or healthy cell numbers inoculated before enrichment process. Ground beef samples spiked with injured or healthy cells at different doses could not be distinguished by C_T values from either assay. In addition, the contribution of nonviable cells in generating positive real-time PCR signals was investigated using both assays on pre-enriched and post-enriched beef samples, but only if inoculated at levels of 10^6 cells/sample or higher, which are levels not typically seen in ground beef.

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1. Introduction

Escherichia coli O157:H7 is an important foodborne pathogen that poses a serious public health concern with substantial economic burden. Consumption of ground beef, dairy products, fresh produce, and water has been associated with foodborne outbreaks of illness caused by this bacterium (Hrudey et al., 2003; Kassenborg et al., 2004). In the beef processing industry, a great deal of effort has been directed at monitoring and reducing *E. coli* O157:H7 contamination in meat products to ensure food safety. Meanwhile, regulations aimed at eliminating this pathogen from the beef supply have been implemented by the U.S. Food Safety and Inspection Service (FSIS) in order to protect public health (U.S. Department of Agriculture Food Safety and Inspection Service, 1996, 2010; USDA-Food Safety and Inspection Service and Office of Public Health Science, 2010).

E. coli O157:H7 has the ability to cause severe human illness, such as hemolytic uremic syndrome (HUS) or even death, with a low infectious dose ranging from 1 to 100 cells. Since meat products are highly perishable, rapid, accurate, and reliable methods that can detect the presence of *E. coli* O157:H7 at low levels are critical for meat safety control in the industry. To date, testing methods in a variety of formats for *E. coli* O157:H7 analysis in food products have been developed and evaluated (Ahmed et al., 2009; Arthur et al., 2005; Feldsine et al., 2005; Fratamico and Bagi, 2007). Among these, the real-time quantitative PCR (RT qPCR) has become one of the most commonly used methods for pathogen nucleic acid detection and quantification. The DuPont Qualicon BAX®-RT system and the BioControl GDS® system are two molecular detection assays that utilize real time PCR detection as endpoint tests for *E. coli* O157:H7 in beef trim and ground beef. Both assays are presence/absence tests performed on enriched samples for *E. coli* O157:H7. The DuPont Qualicon BAX®-RT system uses a multiplex PCR test to detect two *E. coli* O157:H7 specific gene targets simultaneously, whereas the BioControl GDS® system

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initially concentrates the pathogen with an O157-specific immunomagnetic separation (IMS) step, then detects an *E. coli* O157:H7-specific gene target by PCR reaction.

In the beef industry, pathogens in beef trim can be present at different physiological status. For example, bacterial cells could be injured due to processing interventions during beef harvest by exposure to low pH (lactic acid or acidified sodium chlorite) and high temperatures (65 °C hot water or steam) (Lionberg et al., 2003; Fratamico and Bagi, 2007). Beef trim destined for ground beef is generally kept under refrigerated conditions but may be frozen which introduces additional stress and cell injury (Jasson et al., 2009; Fratamico and Bagi, 2007; Ternent et al., 2004; Zhao and Doyle, 2001). Further, in some grinding establishments, beef trim is exposed to an additional antimicrobial intervention before grinding (Bosilevac et al., 2004; Koohmaraie et al., 2007). Therefore, samples collected from beef trim and ground beef likely contain cells of varying degrees of injury and viability. When a contamination event occurs, starting materials are audited and often the levels of contamination are desired for HACCP reassessment plans. However, it is important to realize that even though RT-qPCR has been considered as a mature technology due to its sensitivity and specificity, inappropriate uses and unsuitable data analysis could generate results that are inaccurate and ultimately misleading, especially when this technology is applied to quantify the levels of pathogens at various physiological states.

While neither the BAX®-RT nor the GDS®-O157 test manufacturer implies enumeration capability, both systems have been used in industrial applications to not only detect but also estimate the level of *E. coli* O157:H7 contamination present in different starting materials. However, it is suspect that these systems could provide accurate quantitative results as these assays are performed on enriched samples and the growth of *E. coli* O157:H7 during the enrichment period can be highly variable due to its physiologic state and differences in background microflora populations.

Another unknown factor in the use of PCR-based detection of pathogens in complex matrices is the impact of nonviable cells of the target organism on generating positive PCR signals. When using PCR-based testing methods, a positive reaction may be obtained from nonviable bacteria which still possess DNA, but would not be capable of causing disease. Lack of data regarding this issue is one of the main reasons why PCR detection of pathogens is considered presumptive until confirmed by a culture-based method (USDA-Food Safety and Inspection Service and Office of Public Health Science, 2010).

In the present study, the detection of injured cells (freeze stressed) was compared to the detection of healthy cells (exponential growth phase) of *E. coli* O157:H7. The presence of post-enrichment *E. coli* O157:H7 bacteria in ground beef inoculated with either injured or healthy cells at low, intermediate, or high doses were examined to determine if either molecular detection systems could be used to estimate *E. coli* O157:H7 contamination levels in pre-enriched meat products. We also demonstrated that the lowest concentration of nonviable *E. coli* O157:H7 cells in ground beef resulting in a positive test result by each of these two real-time PCR-based assays requires a non-viable cell count higher than that would be expected in typical samples.

2. Materials and methods

2.1. Preparation of injured inoculation stock

The inoculation stock of *E. coli* O157:H7 was prepared by separately growing four strains of genetically diverse *E. coli* O157:H7 (Barkocy-Gallagher et al., 2001) in 5 mL Tryptone Soya Broth (TSB) for 48 h at 37 °C with orbital shaking (145 rpm) to stationary phase which contained approximately 1×10^9 CFU/mL living bacteria. The four cultures were combined, mixed, and 1 mL of the mixture was serially diluted 1:100,000 in Difco Buffered Peptone Water (BPW, Beckton–Dickinson, Sparks, MD). One milliliter of this dilution was aliquoted, 0.5 mL of 50% glycerol was added and thoroughly mixed, then frozen at −70 °C for storage. The concentration of *E. coli* O157:H7 in the frozen glycerol stocks was calculated and the proportion of stressed cells in the stock was determined by plating the culture onto selective and non-selective medium as described below (Section 2.7) and in previous study (Bosilevac et al., 2010).

2.2. Preparation of healthy inoculation stock

Healthy bacterial cells were grown to exponential phase for sample inoculation. Fifty milliliters of sterile TSB medium was inoculated with the same strains used to prepare the injured cells, and incubated at 37 °C with orbital shaking at 145 rpm. After overnight incubation (16–20 h), 100 mL of sterile TSB medium was inoculated with 10 μL of the overnight culture and incubated at 37 °C for 2.75 h with orbital shaking at 145 rpm. At the end of the incubation period, the bacterial culture was serially diluted to 10^{-5} and 10^{-6} in sterile room temperature (22–24 °C) TSB medium, and 1 mL of each dilution was plated onto PetriFilm™ AC plates (3M Microbiology, St. Paul, MN), incubated overnight (16–20 h) at 37 °C then counted to determine the number of *E. coli* O157:H7 present. The plating was repeated for a total of 5 observations. The appropriate volumes of each dilution containing low (1–3 CFU), intermediate (15–25 CFU), and high (200–250 CFU) concentrations of healthy O157:H7 cells for sample inoculation were determined based on the enumeration data.

2.3. Preparation of nonviable or dead inoculation stock

Nonviable bacterial cells were prepared by growing the above four mixed *E. coli* O157:H7 strains in TSB medium with shaking (145 rpm) overnight at 37 °C to stationary phase with a cell concentration at approximately 1×10^9 CFU/mL. The overnight culture was diluted to 10^8, 10^7, 10^6, 10^5, or 10^4 CFU/mL in sterile TSB medium, and Polymyxin B (Sigma, St. Louis, MO) was added to the diluted cultures at 50 ± 5 U/mL. After incubation with Polymyxin B for 15 min at 37 °C, all samples were frozen in a dry ice-ethanol bath for 15 min, then thawed and kept in a 65 °C water bath for 15 min. The freeze–thaw cycle was repeated an additional time, and then cell viability was determined by plating the samples onto PetriFilm™ AC plates and monitoring bacterial growth after overnight incubation at 37 °C. In addition, 1 mL and 20 μL aliquots were harvested from each sample and tested by the BioControl GDS®-O157 assay (Part No. 61007-100) and the DuPont Qualicon BAX®-RT *E. coli* O157:H7 assay (Part No. D12404903, DuPont, Wilmington, DE), respectively, to evaluate detectability of the *E. coli* O157:H7 genetic targets from the treated nonviable cells.

2.4. Growth comparison between healthy and injured E. coli O157:H7 cells

To compare growth rates between healthy and injured *E. coli* O157:H7 cells, 100 mL of pre-warmed (42 °C) TSB medium was inoculated with 10, 100, or 1000 CFU of either healthy or injured cells based on previous enumeration data. The CFU numbers in each inoculation were verified by plating onto PetriFilm™ AC plates. All samples were tested in duplicates. The inoculated TSB medium was incubated statically at 42 °C, and 1 mL aliquot was harvested from each sample at one-hour intervals over the course of the 8 h experiment. All aliquots were serially diluted in BPW, plated onto PetriFilm™ AC plates, and colonies were counted after

overnight incubation at 37 °C to determine bacterial growth at each time point. To account for the nonselective nature of PetriFilm™ AC plates, 10 colonies were removed from the underside of the PetriFilm and confirmed to be *E. coli* O157:H7 by O157 latex agglutination assay (Dry-spot, Oxoid, United Kingdom).

2.5. Detection curves and detection limits of E. coli O157:H7 in pure cultures by GDS® and BAX®-RT systems

The frozen glycerol stock of the mixture of the four *E. coli* O157:H7 strains was thawed and serially diluted in BPW. The GDS®-O157 and the BAX®-RT *E. coli* O157:H7 assays were performed following the manufacturers' instructions to detect *E. coli* O157:H7 diluted in BPW within the cell density range of 10^1–10^6 per sample. Detection curves of the GDS® and BAX®-RT systems were generated based on the PCR threshold cycle (C_T) values determined by assay software versions Rotor-Gene 6.1.93 and SW Analysis 2.7.9345, respectively, and the detection limit of *E. coli* O157:H7 diluted in BPW by each assay was determined.

2.6. Detection of nonviable E. coli O157:H7 in spiked ground beef samples by GDS® and BAX®-RT systems

Ground beef was obtained from a local retail butcher shop. Ground beef samples were placed into each of 16 Whirl-Pak filter bags (Nasco, Fort Atkinson, WI), 10 g per sample, and each inoculated with 1 mL of the nonviable *E. coli* O157:H7 at concentrations of 10^8, 10^7, 10^6, 10^5, or 10^4 cells/mL, prepared as described above. The lack of cell viability was further confirmed by diluting the bacteria 10-fold in sterile TSB medium, incubating all diluted samples statically at 42 °C for 12 h, then plating onto PetriFilm AC plates.

The above ground beef samples were inoculated in triplicates for each bacterial concentration, while the negative control sample received no inoculation. The inoculations were dispensed onto the ground beef in each bag, and 90 mL of sterile TSB medium was added to each sample. All samples were thoroughly mixed, and two aliquots of 1 mL and 20 μL were removed from each bag as pre-enrichment samples to be tested using the GDS®-O157 and the BAX®-RT *E. coli* O157:H7 methods, respectively. After the pre-enrichment samples were taken, all sample bags were sealed and incubated statically at 42 °C for 12 h in a programmable incubator that then held the samples at 4 °C (4–6 h, until further processed). After the above enrichment, another two aliquots of 1 mL and 20 μL were removed from each bag as post-enrichment samples to be tested by the GDS® and the BAX®-RT assays, respectively. In addition, another 1.0 mL aliquot was harvested from each post-enrichment sample, and any *E. coli* O157:H7 in the aliquot were isolated and concentrated with the GDS® O157:H7-specific IMS procedure, then plated onto O157 CHROMagar plates (DRG International, Inc, Mountainside, NJ) and incubated at 37 °C overnight to confirm the lack of viable *E. coli* O157:H7 in the post-enrichment samples.

2.7. Detection of healthy or injured E. coli O157:H7 in spiked ground beef samples by GDS® and BAX®-RT assays

In these experiments, ground beef was tested either fresh or stored at −20 °C for seven days then thawed and tested. To perform the tests, 65 g of ground beef was placed into each of 32 Whirl-Pak filter bags (Nasco, Fort Atkinson, WI). Inoculations of healthy *E. coli* O157:H7 cells at low, intermediate, or high doses were prepared using the 10^{-5} or 10^{-6} dilutions from the 2.75 h culture as described above. The CFU numbers in each dilution were confirmed again by plating onto PetriFilm AC plates. From the 10^{-6} dilution, 250 μL (1–3 CFU) was taken for the low inoculum, and 1.3 mL (15–20 CFU) was taken for the intermediate inoculum. The high inoculum contained 200–250 CFU of *E. coli* O157:H7 in 2 mL of the 10^{-5} dilution. Inoculations of injured cells were prepared by diluting the *E. coli* O157:H7 glycerol stock in BPW to concentrations that matched those of the healthy cell suspensions. The numbers of injured cells in each dilution were determined by comparing the CFU that grew on TSA plates to the CFU that grew on Sorbitol MacConkey Agar containing 0.05 μg/mL cefixime and 2.5 μg/mL potassium tellurite (ctSMAC) plates. In this way the total CFU of *E. coli* O157:H7 present and the portion of the population that was injured (unable to grow on ctSMAC) were determined (Bosilevac et al., 2010; Ray, 2001). The inoculations were dispensed onto the ground beef in each sample bag, and pre-warmed (42 °C) sterile TSB medium was added to the bags for enrichment, 585 mL per sample. After enrichment media was added, the bags were thoroughly mixed using a JumboMix Lab Blender (Interscience, Mountain, NJ) for 60 s at 420 rpm, sealed, and incubated statically at 42 °C for 8 h. At the end of the enrichment period, aliquots of 1 mL and 20 μL were removed from each sample bag for detection tests using either the BioControl GDS®-O157 or the DuPont Qualicon BAX®-RT *E. coli* O157:H7 methods, respectively, performed according to manufacturers' instructions. In addition, a 1 mL aliquot was harvested from each sample, diluted in sterile BPW, and plated onto PetriFilm AC plates in order to measure ultimate bacterial growth obtained at the end of the enrichment incubation period.

2.8. Statistical analysis

Analyses of variance and comparisons of C_T values for *E. coli* O157:H7 detection in each set of samples with standard deviations and 95% confidence intervals were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). A one-way analysis of variance (ANOVA) was performed using Tukey's post-hoc test. p values lower than 0.05 were considered statistically significant. The sample size for each experiment is included in the tables or the figure legends. Linear regression analysis of the detection curves was also performed using the GraphPad Prism software.

3. Results and discussion

Real time PCR has long been used as a quantitative tool to determine the concentration of DNA targets present in various sample types (Bono et al., 2004; Bosilevac and Koohmaraie, 2008; Fratamico et al., 1995; Gannon et al., 1997; Maurer, 2011; Nagano et al., 1998; Paton and Paton, 1999; Suo et al., 2010; Wang et al., 2009). However, it has become a common understanding in the research field that the wide range of protocols along with the various pre-assay conditions and poor assay design could generate data that are misleading. Thus, a set of publication guidelines that describes the minimum information necessary for evaluating qPCR results (MIQE) has been recommended to be used by journal reviewers to assure study accuracy and experimental reliability (Bustin et al., 2009; Huggett and Bustin, 2011). The purpose of MIQE guidelines is to provide a much needed standardization in order to systematize the variable qPCR methods into a more consistent format with the essential information to assure study reproduction and quality assessment. For the meat industry, unsuitable assay design and inappropriate use of qPCR methods could affect food safety control in a negative way. Even though neither the GDS® nor the BAX®-RT tests have been described for pathogen quantitative purpose, numerous industry users have been using these assays in a semi-quantitative fashion in attempts to identify more highly contaminated materials. This practice raised questions regarding

how to interpret the results of these assays that are being used outside their defined limits. Therefore in the present study, we placed our efforts on evaluating the ability of these assays to accurately identify *E. coli* O157:H7 concentrations in meat samples and illustrating the effects that cell viability can have on such tests.

3.1. Growth comparison between healthy and injured E. coli O157:H7 cells

Prior to inoculation experiments using ground beef, we defined the growth characteristics of healthy and injured *E. coli* O157:H7 and determined the number of cells necessary to generate a positive result in each test. In the growth comparison study between healthy and frozen stressed but sub-lethally injured *E. coli* O157:H7 cells in pure cultures, TSB medium was inoculated with 10, 100, or 1000 CFU doses of either healthy or injured bacteria and grown statically at 42 °C for 8 h, mimicking the sample enrichment conditions of GDS® and BAX®-RT assays. Fig. 1 shows the bacterial cell densities from each inoculation for the first 8 h of the experiment at 1-h intervals. Over the period examined, the observed $\log_{10}$ CFU/mL grew exponentially and approximately formed a linear growth curve. With each inoculation dose, the log growth was delayed in cold-stressed bacteria, and a significant ($p < 0.05$) decrease of approximately 1.0 $\log_{10}$ CFU/mL was observed in injured bacterial cultures at each time point compared to the healthy cultures. Notably, cultures inoculated with injured cells grew approximately at the same rate as samples started with healthy cells at a 10-fold lower dose, and the cell density between the two groups showed no significant difference at each time point. This observation indicates that the injured cells had a longer lag time, but then grew at the same rate in cultures compared to the healthy cells. The longer lag time was likely due to the bacterial repairing and recovery process which has been shown to be an important consideration when enriching samples of various types for *E. coli* O157:H7 (Barkocy-Gallagher et al., 2002). This confirmed that the final bacterial density at a given time in the enriched samples will not necessarily correlate with bacterial contamination levels pre-enrichment, depending on the different cell physiologic states.

3.2. Detection limits of viable E. coli O157:H7 cells in pure cultures by GDS® and BAX®-RT systems

We previously evaluated the pre-enrichment detection limits of the GDS® and BAX®-RT systems for detecting *E. coli* O157:H7 in beef trim and ground beef samples spiked with *E. coli* O157:H7 at levels as low as 1 CFU per 375 g (Arthur et al., 2005; Bosilevac et al., 2010). The previous results showed that these systems yielded more reliable results when the target concentration of *E. coli* O157:H7 in pre-enriched meat samples was 3 CFU or greater in 375 g (Bosilevac et al., 2010). However, the level of *E. coli* O157:H7 cells present after enrichment was not determined. In the present study we evaluated the detection limits for viable *E. coli* O157:H7 cells in pure cultures by these two systems. The GDS® and BAX®-RT systems perform PCR reactions for 32 and 43 cycles, respectively, and we generated detection curves based on actual cell number in each sample and the corresponding PCR threshold cycle (C_T) values. *E. coli* O157:H7 was tested within a density range from 10^1 to 10^6 cells per sample. Fig. 2 shows the C_T values of both assays and the detected cell densities of *E. coli* O157:H7 added to samples. C_T values showed an approximately linear relationship within the cell density range from 10^3 to 10^6 cells per sample, with the R^2 values of 0.940 for BAX Target *S*, 0.989 for BAX Target *W*, and 0.905 for the GDS target gene. This indicated a direct correlation between the C_T values and the numbers of bacteria present. In addition, statistical analysis

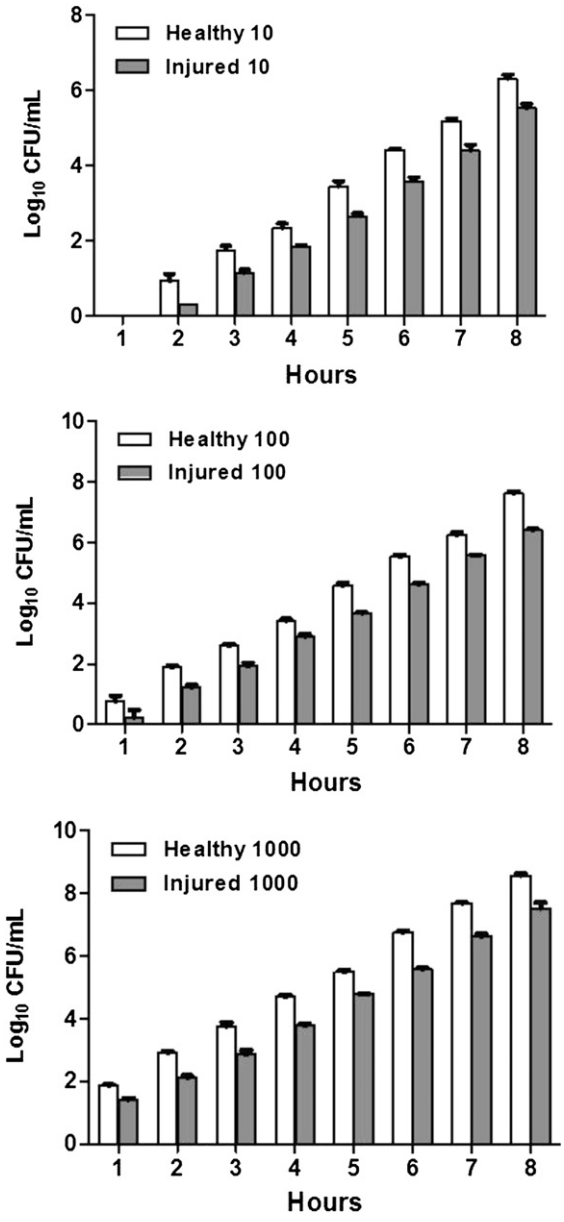

Fig. 1. Growth comparison between healthy and frozen-injured *E. coli* O157:H7 cell. Pre-warmed (42 °C) TSB medium, 100 mL per sample, was inoculated with 10 CFU (top panel), 100 CFU (middle panel), or 1000 CFU (bottom panel) of healthy or injured *E. coli* O157:H7 cells. All samples were tested in duplicates and the data are shown as mean $\log_{10}$ CFU/mL ± standard deviation vs. growth time (h).

indicated that samples containing 10^3–10^6 CFU/sample at an interval of 1.0 $\log_{10}$ CFU/sample could be distinguished by GDS® and BAX®-RT assays with statistical significance ($p < 0.05$) in C_T values. Within the density range being tested, the detection limit of cell density that yielded a valid C_T value was 1000 CFU (C_T value 29.8 ± 0.57) for the GDS® assay, and 500 CFU (C_T value 43 ± 0) for the BAX®-RT assay. However, samples containing 500 CFU or 1000 CFU could not be distinguished by the BAX®-RT system in terms of C_T value because both samples had the same C_T value of 43 ± 0. Samples containing *E. coli* O157:H7 cells lower than detection limits for either system yielded no valid C_T values. It is not immediately clear as to why the BAX®-RT assay had a lower detection limit than the GDS® assay since the GDS® assay includes an IMS concentration procedure. A possible explanation is that the *E. coli* O157:H7 cells used in this test were directly

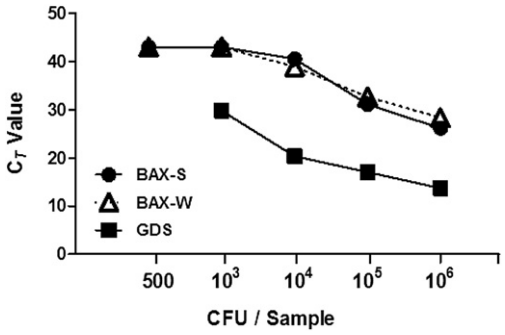

Fig. 2. Detection of *E. coli* O157:H7 cells diluted in BPW from frozen stock using BAX®-RT or GDS® systems. The BioControl GDS®-O157 assay and the DuPont Qualicon BAX®-RT *E. coli* O157:H7 assay were performed following manufacturers' instructions to detect *E. coli* O157:H7 cells diluted in BPW. CFU numbers in inoculations were confirmed by plating onto PetriFilm AC plates. Inoculums lower than 500 CFU/sample were not detected by both systems. Detection curves were generated based on the PCR threshold cycle (C_T) values determined by assay software versions Rotor-Gene 6.1.93 (GDS®) and SW analysis 2.7.9345 (BAX®-RT). Data are shown as mean C_T values ± standard deviation vs. cell densities detected in each sample. Statistical analysis using the GraphPad Prism software revealed that samples containing 10^3–10^6 CFU/sample at an interval of 1.0 $\log_{10}$ CFU/sample can be distinguished by GDS® and BAX®-RT assays with statistical significance ($p < 0.05$) in C_T values. Linear regression analysis of the detection curves was performed within cell density range between 10^3 and 10^6 cells per sample ($R^2 = 0.940$ for BAX Target *S*; $R^2 = 0.989$ for BAX Target *W*; $R^2 = 0.905$ for GDS target gene.).

diluted from stationary phase bacteria stored at −70 °C in 30% glycerol stock, which would contain a certain level of dead cells or viable bacteria with varying degrees of injury. These dead or injured cells could still provide DNA targets for the BAX-RT assay. However, these bacteria likely bear damages of cell surface structures and the antibody epitopes necessary for successful IMS, therefore, negatively affecting the antibody binding affinity and the isolation efficiency of the IMS procedure which was required for pathogen detection by the GDS assay.

3.3. Detection limits of nonviable E. coli O157:H7 cells by GDS® and BAX®-RT systems

While it has been known that DNA template from nonviable *E. coli* O157:H7 cells could lead to positive test results, it had not been determined the minimal level of nonviable target organism required in a meat sample to give a positive test result. To address this question, we measured the detection limits for nonviable bacterial cells in pure cultures and in spiked ground beef by the two assays. The effects of background microflora during enrichment and storage period on nonviable *E. coli* O157:H7 cells in ground beef also were investigated by comparing the test results between pre-enrichment and post-enrichment samples. We prepared nonviable *E. coli* O157:H7 cells by treatments with Polymyxin B and freeze–thaw cycles. Culture results demonstrated complete cell inactivation following overnight incubation at 37 °C and after IMS concentration. No turbidity of overnight cultures was visually apparent, and IMS procedure did not yield any viable cells. This treatment for generating nonviable cells targets and disrupts cell membranes. The presence of intact cells was not confirmed, therefore we used the nonviable suspension as an inoculum and refer to CFU equivalents based on the starting cell concentrations. The nonviable cell suspension may have been only a solution of DNA targets and cellular debris when used for our purposes.

The two assays were in agreement with regard to the detection ranges for the spiked nonviable cells (Table 1). After the enrichment process which included a 4 °C hold overnight, both GDS® and BAX®-RT systems detected *E. coli* O157:H7 only from samples spiked with 10^6, 10^7, or 10^8 equivalents of nonviable cells per sample. Concentrations of 10^4 or 10^5 equivalents of nonviable cells/sample could not be detected by either system. Both assays also detected *E. coli* O157:H7 specific genetic targets in pre-enrichment samples spiked with 10^6–10^8 nonviable cells, but not in the samples spiked with 10^4 and 10^5 cells. The only exception was the GDS® data from samples inoculated with 10^6 equivalents of nonviable cells. Although the assay yielded a negative result for this level of inoculated target, the detection curves showed clear positive inflections but did not cross the positive threshold (data not shown). This was possibly due to intrinsic experimental variations. It is worth noting that the consistent detection results between pre-enrichment and post-enrichment samples indicated that the outgrowth of background microflora had minimal effects on the genetic targets of *E. coli* O157:H7 during enrichment period and holding temperatures. It has generally been assumed that nonviable cells and their DNA would be entirely degraded and/or consumed by the background microflora during the hours of incubation for enrichment.

Based on these experiments, it can be stated that nonviable *E. coli* O157:H7 will not impact PCR-based detection methods for enriched ground beef except at high cell densities (10^5 CFU per gram and greater), which have not been found in commercial ground beef nor beef carcasses before process interventions have been applied. *E. coli* O157:H7 concentrations on carcasses have been measured to be as high as 189 CFU per area of 100 cm^2

Table 1
Detection of nonviable *E. coli* O157:H7 cells in ground beef[d] using the BAX®-RT or GDS® systems.

Inoculums[a] (cells/sample)	*n*	BAX®-RT[b]			GDS® [b,c]	
		Cells/reaction[e]	Target-*S*	Target-*W*	Cells/reaction[e]	
10^8	3	4545	36.17 ± 0.25	36.57 ± 0.23	571,429	17.8 ± 0.95
10^7	3	455	40.07 ± 0.12	39.77 ± 0.35	57,143	21.23 ± 0.91
10^6	3	45	43 ± 0	43 ± 0	5714	24.2 ± 0.53
10^5	3	4.5	Not detected	Not detected	571	Not detected
10^4	3	0.45	Not detected	Not detected	57	Not detected
0	1	0	Not detected	Not detected	0	Not detected

[a] Nonviable *E. coli* O157:H7 cells were prepared by Polymyxin B treatment and two freeze–thaw cycles. Cell death in inoculums was confirmed by plating onto PetriFilm AC plates.
[b] Inoculated samples were enriched for 12 h at 42 °C in TSB medium, then stored at 4 °C overnight. Values represent mean PCR threshold cycle (C_T) values ± standard deviation determined by assay software versions SW Analysis 2.7.9345 (BAX®-RT) and Rotor-Gene 6.1.93 (GDS®).
[c] IMS (immunomagnetic separation) step was performed for *E. coli* O157:H7 using Thermo 96 well head KingFisher IMS robot.
[d] Ground beef (10 g) was enriched in 90 ml TSB for 12 h before RT-PCR analysis.
[e] The number of cells per reaction was calculated using 20 μL enrichment for BAX®-RT and 1 mL enrichment for GDS®, followed by the manufacturers' protocol.

(Bosilevac et al., 2009), and within the range of 100–999 CFU per area of 100 cm^2 in other studies (Arthur et al., 2004). Furthermore, in recent studies of commercial ground beef contamination events, the greatest level of contamination measured was 20 CFU *E. coli* O157:H7 per gram of ground beef (Bosilevac, unpublished observation). Therefore, even if these levels of bacteria were fully inactivated and completely transferred to final beef products, the concentrations are still not near the level required to cause positive detection test results.

3.4. Detection of injured or healthy E. coli O157:H7 cells in ground beef

Based on the above growth experiments of injured and healthy cells and the observed detection limits of the RT-PCR assays, we performed two independent experiments using ground beef spiked with injured or healthy *E. coli* O157:H7 cells at low (1–3 CFU), intermediate (15–20 CFU), or high (200–250 CFU) doses. In the first experiment (Exp A.) fresh ground beef was used, whereas in the second experiment (Exp B.) frozen ground beef which had been thawed was used. The sample size of 65 g each was chosen for ease of use and because this is a common size used in the commercial setting for finished ground beef testing. Both experiments detected 100% of the spiked samples as positive for *E. coli* O157:H7 (Table 2) with GDS® or BAX®-RT systems, which is similar as other studies that reported detection rates near 100% by these two systems but using a smaller sample size (25 g per sample) and inoculations of 1–5 and 10–50 CFU per sample (Feldsine et al., 2005; Lionberg et al., 2003). Enumeration by PetriFilm AC plates showed exponential bacterial growth from each inoculation after the 8-h enrichment period. In Experiment B, an overall higher bacterial growth by 1.0–1.5 $\log_{10}$ CFU/mL was observed from each inoculation group compared to Experiment A, likely due to a higher concentration of background bacteria in the starting materials. In both experiments, samples spiked with injured cells reached a post-enrichment bacterial density similar to samples started with healthy cells at 10-fold lower doses, which is consistent with our observations from growth curve comparison between injured and healthy cells in pure cultures (Fig. 1). Previous studies also reported similar results (Duffy et al., 2000; Fratamico and Bagi, 2007) that a decrease of approximately 1.2–2.5 $\log_{10}$ CFU/mL was obtained from cold- or frozen-stressed *E. coli* O157:H7 or *Salmonella* cells compared to non-stressed bacteria after 8- or 20-h growth, indicating that cell physiologic state could significantly affect pathogen detection due to differences in bacterial growth during enrichment periods.

C_T values of Experiments A and B for detecting post-enrichment *E. coli* O157:H7 cells by GDS® and BAX®-RT systems are shown in Table 2. The BAX-RT assay targets two *E. coli* O157:H7 specific genes referred to as Target *S* and Target *W*, whereas the GDS O157 assay targets one O157:H7 specific gene. In Experiment A, C_T values for detecting BAX®-RT Target-*S* showed no significant difference among the four groups inoculated with low or intermediate doses of either injured or healthy cells. In addition, C_T values of samples spiked with low doses of healthy cells (C_T value 36.3 ± 1.5) were not different from those of samples spiked with intermediate doses of healthy cells (C_T value 36.4 ± 4.8) or intermediate/high doses of injured cells (C_T values 37.9 ± 4.1 or 32.6 ± 1.1). A similar pattern was observed when Target-*W* was detected. C_T values were not different among groups inoculated with low doses of injured cells (C_T value 40.2 ± 2.0) and intermediate doses of either injured (C_T value 38.1 ± 3.6) or healthy (C_T value 37.1 ± 4.1) cells. Also, there was no difference observed among the four groups spiked with low/intermediate doses of healthy cells and intermediate/high doses of injured cells. Using the GDS® system in Experiment A, C_T values showed no difference among all sample groups (Table 2).

In Experiment B, C_T values for detecting Target-*S* by BAX®-RT system showed no difference among samples within each of the following groups: the group spiked with low/intermediate doses of injured cells and low doses of healthy cells; the group spiked with intermediate/high doses of injured cells and intermediate doses of healthy cells; and the group spiked with intermediate/high doses of healthy cells and high doses of injured cells. When Target-*W* was detected, C_T values showed no difference among samples within two groups: the group of low/intermediate doses of injured cells and low doses of healthy cells; and the group of intermediate/high doses of healthy cells and high doses of injured cells. When the GDS® assay results from Experiment B were analyzed, the only statistical difference was observed between the two samples spiked with low doses of injured cells and intermediate doses of healthy cells, whereas all other groups were not statistically different in C_T values. The combination of these data indicates that the C_T values of both BAX®-RT and GDS® systems are not appropriate indications

Table 2
Detection of E. coli O157:H7 in 65 g samples of ground beef spiked with frozen-injured or healthy *E. coli* O157:H7 cells using the BAX®-RT or GDS® systems.

Inoculums	*n*	CFU inoculated[a]	BAX®-RT[b]		GDS®[b,c]	CFU recovered[d] [$\log_{10}$ CFU/mL]
			Target-*S*	Target-*W*		
Experiment A						
Low – Injured	7	1.2 ± 0.84	40.514 ± 2.47 A	40.243 ± 2.0 A	15.951 ± 3.34 A	4.71
Low – Healthy	7	3.15 ± 0.82	36.338 ± 1.48 AB	36.563 ± 1.01 B	12.163 ± 1.27 A	5.82
Mid – Injured	4	13.8 ± 2.68	37.9 ± 4.06 AB	38.1 ± 3.61 AB	16.8 ± 8.37 A	5.62
Mid – Healthy	4	16.38 ± 4.27	36.375 ± 4.83 AB	37.08 ± 4.08 AB	13.525 ± 6.18 A	6.14
High – Injured	4	103.2 ± 7.85	32.625 ± 1.11 BC	34.275 ± 0.76 BC	9.025 ± 0.79 A	6.38
High – Healthy	4	209.6 ± 11.26	27.575 ± 0.499 C	29.975 ± 0.39 C	8.725 ± 1.42 A	7.96
Experiment B						
Low – Injured	4	2.4 ± 1.82	35.4 ± 5.34 A	34.233 ± 1.35 A	11.625 ± 5.69 A	6.34
Low – Healthy	4	2.85 ± 0.91	33.625 ± 4.58 A	34.725 ± 2.87 A	9.425 ± 2.37 AB	6.9
Mid – Injured	6	13.4 ± 3.29	30.35 ± 1.87 AB	32.483 ± 1.41 AB	7.9 ± 0.67 AB	6.998
Mid – Healthy	6	14.82 ± 4.74	27.517 ± 1.03 BC	30.417 ± 0.73 BC	6.3 ± 0.4 B	7.934
High – Injured	6	76.6 ± 7.13	26.367 ± 1.65 BC	28.8 ± 1.18 C	7.75 ± 1.07 AB	8.33
High – Healthy	6	159.6 ± 19.51	25.35 ± 1.29 C	28.117 ± 0.88 C	7.467 ± 1.27 AB	9.32

[a] CFU numbers in inoculums were confirmed by plating onto PetriFilm AC plates.
[b] Values represent mean PCR threshold cycle (C_T) values ± standard deviation determined by assay software versions SW Analysis 2.7.9345 (BAX®-RT) and Rotor-Gene 6.1.93 (GDS®). C_T values of each experiment in the same column followed by the same letters are not statistically different ($p > 0.05$).
[c] IMS (immunomagnetic separation) step was performed for *E. coli* O157:H7 using Thermo 96 well head KingFisher IMS robot.
[d] Inoculated samples were enriched for 8 h at 42 °C in TSB medium then enumerated by serial dilution and spiral plating onto ChromAgar O157 plates.

for estimating pre-enrichment contamination levels by *E. coli* O157:H7.

3.5. Conclusions

These studies were initiated to examine questions surrounding the results of commercial RT-PCR assays as indicators of relative levels of *E. coli* O157:H7 contamination and whether nonviable cells could impact test results. We determined the effects of freeze-induced injury on recovery and growth of *E. coli* O157:H7 in cultures and reported that freeze-injured bacteria had a delayed log growth phase compared to healthy cells, and post-enrichment cell densities of neither injured nor healthy bacteria correlated with cell numbers inoculated before the enrichment process. Although there are several ways in which to generate injured cells, be it through acid treatment or exposure to high temperature, we chose to use the freeze stressed cells for the ease of use and repeatability in treatments as well as the ease of generating consistent inoculum concentrations. In addition, it has been known that treatments by low pH or high temperature could potentially affect test results of IMS-based assay methods (Kuhn et al., 2002; Mason and Williams, 1980), therefore these treatments were not selected for the present study.

In summary, we investigated the effects of bacterial physiologic status on the ability of GDS® and BAX®-RT systems to accurately measure pre-enrichment contamination levels by *E. coli* O157:H7 in meat products. Even though the C_T values of these assays showed a correlation with bacteria densities present in pure cultures, samples of ground beef spiked with injured or healthy cells at different doses could not be distinguished by C_T values by either commercial assay after sample enrichment. Our results indicated that the C_T values from both commercial assays performed on enriched samples cannot be used as an indication of pre-enrichment contamination levels of *E. coli* O157:H7 because the bacterial physiologic state and background microflora play critical roles during sample enrichment period and affect test results, thus, such use should be strongly discouraged. Additionally, nonviable *E. coli* O157:H7 cells are not likely to have a significant impact on PCR-based detection systems as they could be detected in pre-enriched and post-enriched samples only when present in high cell densities not typically found in ground beef and ground beef materials.

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References

Ahmed, I., Hughes, D., Jenson, I., Karalis, T., 2009. Validation of low-volume enrichment protocols for detection of *Escherichia coli* O157 in raw ground beef components, using commercial kits. J. Food Prot. 72 (3), 669–673.

Arthur, T.M., Bosilevac, J.M., Nou, X., Shackelford, S.D., Wheeler, T.L., Kent, M.P., Jaroni, D., Pauling, B., Allen, D.M., Koohmaraie, M., 2004. *Escherichia coli* O157 prevalence and enumeration of aerobic bacteria, *Enterobacteriaceae*, and *Escherichia coli* O157 at various steps in commercial beef processing plants. J. Food Prot. 67, 658–665.

Arthur, T.M., Bosilevac, J.M., Nou, X., Koohmaraie, M., 2005. Evaluation of culture- and PCR-based detection methods for *Escherichia coli* O157 in ground beef. J. Food Prot. 68, 1566–1574.

Barkocy-Gallagher, G.A., Arthur, T.M., Siragusa, G.R., Keen, J.E., Elder, R.O., Laegreid, W.W., Koohmaraie, M., 2001. Genotypic analyses of *Escherichia coli* O157:H7 and O157 nonmotile isolates recovered from beef cattle and carcasses at processing plants in the Midwestern states of the United States. Appl. Environ. Microbiol. 67, 3810–3818.

Barkocy-Gallagher, G.A., Berry, E.D., Rivera-Betancourt, M., Arthur, T.M., Nou, X., Koohmaraie, M., 2002. Development of methods for the recovery of *Escherichia coli* O157:H7 and *Salmonella* from beef carcass sponge samples and bovine fecal and hide samples. J. Food Prot. 65, 1527–1534.

Bono, J.L., Keen, J.E., Miller, L.C., Fox, J.M., Chitko-McKown, C.G., Heaton, M.P., Laegreid, W.W., 2004. Evaluation of a real-time PCR kit for detecting *Escherichia coli* O157 in bovine fecal samples. Appl. Environ. Microbiol. 70 (3), 1855–1857.

Bosilevac, J.M., Koohmaraie, M., 2008. Effects of using reduced volumes of nonselective enrichment medium in methods for the detection of *Escherichia coli* O157:H7 from raw beef. J. Food Prot. 71 (9), 1768–1773.

Bosilevac, J.M., Shackelford, S.D., Fahle, R., Biela, T., Koohmaraie, M., 2004. Decreased dosage of acidified sodium chlorite reduces microbial contamination and maintains organoleptic qualities of ground beef products. J. Food Prot. 67, 2248–2254.

Bosilevac, J.M., Arthur, T.M., Bono, J.L., Brichta-Harhay, D.M., Kalchayanand, N., King, D.A., Shackelford, S.D., Wheeler, T.L., Koohmaraie, M., 2009. Prevalence and enumeration of *Escherichia coli* O157:H7 and *Salmonella* in U.S. abattoirs that process fewer than 1000 head of cattle per day. J. Food Prot. 72, 1272–1278.

Bosilevac, J.M., Kalchayanand, N., Schmidt, J.W., Shackelford, S.D., Wheeler, T.L., Koohmaraie, M., 2010. Inoculation of beef with low concentrations of *Escherichia coli* O157:H7 and examination of factors that interfere with its detection by culture isolation and rapid methods. J. Food Prot. 73 (12), 2180–2188.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622.

Duffy, G., Kilbride, B., Sheridan, J.J., Blair, I.S., McDowell, D.A., 2000. A membrane-immunofluorescent-viability staining technique for the detection of *Salmonella* spp. From fresh and processed meat samples. J. Appl. Microbiol. 89, 587–594.

Feldsine, P.T., Green, S.T., Lienau, A.H., Stephens, J., Jucker, M.T., Kerr, D.E., 2005. Evaluation of the assurance GDS® for *E. coli* O157:H7 method and assurance GDS® for Shiga toxin genes method in selected foods: collaborative study. J. AOAC Int. 88, 1334–1348.

Fratamico, P.M., Bagi, L.K., 2007. Comparison of methods for detection and isolation of cold- and freeze-stressed *Escherichia coli* O157:H7 in raw ground beef. J. Food Prot. 70 (7), 1663–1669.

Fratamico, P.M., Sackitey, S.K., Wiedmann, M., Deng, M.Y., 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. 33, 2188–2191.

Gannon, V.P., D'Souza, S., Graham, T., King, R.K., Rahn, K., Read, S., 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol. 35, 656–662.

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., Hrudey, E.J., 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. Water Sci. Technol. 47, 7–14.

Huggett, J., Bustin, A.S., 2011. Standardisation and reporting for nucleic acid quantification. Accred Qual. Assur.. http://dx.doi.org/10.1007/s00769-011-0769-y

Jasson, V., Rajkovic, A., Baert, L., Debevere, J., Uyttendaele, M., 2009. Comparison of enrichment conditions for rapid detection of low numbers of sublethally injured *Escherichia coli* O157 in food. J. Food Prot. 72 (9), 1862–1868.

Kassenborg, H.D., Hedberg, C.W., Hoekstra, M., Evans, M.C., Chin, A.E., Marcus, R., Vugia, D.J., Smith, K., Ahuja, S.D., Slutsker, L., Griffin, P.M., the Emerging Infections Program FoodNet Working Group, 2004. Farm visits and undercooked hamburgers as major risk factors for sporadic *Escherichia coli* O157:H7 infection: data from a case-control study in 5 FoodNet sites. Clin. Infect. Dis. 38 (Suppl. 3), S271–S278.

Koohmaraie, M., Arthur, T.M., Bosilevac, J.M., Brichta-Harhay, D.M., Kalchayanand, N., Shackelford, S.D., Wheeler, T.L., 2007. Interventions to reduce/eliminate *Escherichia coli* O157:H7 in ground beef. Meat Sci. 77, 90–96.

Kuhn, R.C., Rock, C.M., Oshima, K.H., 2002. Effects of pH and magnetic material on immunomagnetic separation of *Cryptosporidium* oocysts from concentrated water samples. Appl. Environ. Microbiol. 68, 2066–2070.

Lionberg, W.C., Restaino, L., Frampton, E.W., Barbour, W.M., 2003. Efficacy of enrichment broths in the recovery of freeze-injured *Escherichia coli* O157:H7 in inoculated ground beef by PCR. J. Food Prot. 66, 1911–1915.

Mason, D.W., Williams, A.F., 1980. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. Biochem. J. 187, 1–20.

Maurer, J.J., 2011. Rapid detection and limitations of molecular techniques. Annu. Rev. Food Sci. Technol. 2, 259–279.

Nagano, I., Kunishima, M., Itoh, Y., Wu, Z., Takahashi, Y., 1998. Detection of verotoxin-producing *Escherichia coli* O157:H7 by multiplex polymerase chain reaction. Microbiol. Immunol. 42, 371–376.

Paton, A.W., Paton, J.C., 1999. Direct detection of Shiga toxigenic *Escherichia coli* strains belonging to serogroups O111, O157, and O113 by multiplex PCR. J. Clin. Microbiol. 37, 3362–3365.

Ray, Bibek, 2001. Fundamental Food Microbiology. Chapter 7: Microbial Sporulation and Sublethal Injury, second ed., ISBN 0-8493-0045-2

Suo, B., He, Y., Tu, S.I., Shi, X., 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella spp., Escherichia coli* O157, and *Listeria monocytogenes* in meat products. Foodborne Pathog. Dis. 7 (6), 619–628.

Ternent, H.E., Innocent, G.T., Filshie, L.M., Taylor, D.J., Steele, W.B., McEwen, S.A., Reilly, W.J., Gunn, G.J., Reid, S.W., Mellor, D.J., 2004. Frozen storage of *Escherichia coli* O157 in buffered peptone water and its detection on bovine carcasses. J. Food Prot. 67 (1), 40–45.

U.S. Department of Agriculture Food Safety and Inspection Service, 1996. Pathogen reduction; hazard analysis and critical control point (HACCP) systems; final rule. Fed. Regist. 61, 38805–38989.

U.S. Department of Agriculture Food Safety and Inspection Service, 2010. Testing of Raw Ground Beef and Raw Ground Beef Component Samples for *E. coli* O157:H7: Year-to-date Totals. Available at: http://www.fsis.usda.gov/Science/Ecoli_Raw_Beef_Testing_Data_YTD/ (accessed 01.07.10.).

USDA-Food Safety and Inspection Service, Office of Public Health Science, 2010. FSIS Procedure for the Use of *Escherichia coli* O157:H7 Screening Tests. Available at: http://www.fsis.usda.gov/PDF/Mlg_5A_02.pdf.

Wang, L., Li, Y., Mustapha, A., 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. J. Appl. Microbiol. 107 (5), 1719–1728.

Zhao, T., Doyle, M.P., 2001. Evaluation of universal preenrichment broth for growth of heat-injured pathogens. J. Food Prot. 64 (11), 1751–1755.