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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†

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ABSTRACT

Currently used industry testing programs require the ability to detect *Escherichia coli* O157:H7 in samples of beef trim or ground beef at levels as low as 1 CFU/375 g. We present a reliable protocol for generating a control inoculum for verification testing at this low concentration and evaluate its use. Results show that half of all samples received no cells when 1 CFU was the target concentration and that targets greater than 3 CFU were much more reliable. Detection by culture isolation and two commercial assays, Qualicon BAX-MP and BioControl GDS, detected 94% ± 11%, 92% ± 10%, and 92% ± 7% of samples inoculated with 5.4 CFU (range 1 to 9 CFU), respectively. We also examined the effect of background aerobic plate count (APC) bacteria and fat content effects on the detection of *E. coli* O157:H7. At APC concentrations below 6 log CFU/g, the rapid methods detected all beef trim samples inoculated with 26 CFU of *E. coli* O157:H7 per 65 g. At an APC of 6.7 log CFU/g, culture, BAX-MP, and GDS detected 100, 75, and 13%, respectively, of inoculated samples. Neither commercial method detected *E. coli* O157:H7 in the samples when APC was 7.7 log CFU/g, whereas culture was able to detect 63% of *E. coli* O157:H7 in the samples when APC was at this concentration. Increased fat content correlated with decreasing recovery of immunomagnetic separation beads, but this was not observed to interfere with detection of *E. coli* O157:H7.

Escherichia coli O157:H7 was identified as a foodborne hazard during outbreaks associated with ground beef in the 1980s and early 1990s *(32, 36)*. Since then this pathogen has been associated with disease outbreaks involving meat, produce, and water *(23, 25, 35)*. The U.S. Department of Agriculture, Food Safety and Inspection Service (FSIS) implemented several regulations aimed at eliminating this pathogen from red meat *(37)*. At the same time, the public and private research sectors worked to help the beef processing industry to implement antimicrobial interventions that reduced *E. coli* O157:H7 contamination *(28)*. Moreover, the industry is systematically testing and holding beef trimmings before shipment; they consider this an extra measure to help them reduce recalls of product batches that yield *E. coli* O157:H7–positive samples *(39)*. Limitations of test-and-hold programs include the low prevalence of the pathogen, its nonhomogeneous distribution in the product, and difficulties in obtaining representative samples *(9)*. In addition, the results of any sampling and testing program are more reliable if the detection limit of the testing method is 1 CFU of *E. coli* O157:H7 per sample tested (or a 375-g sample tested by the meat industry in its test-and-hold programs). To assess this in practice requires the generation of diluted control stock cultures of *E. coli* O157:H7 to use as an inoculum to reliably place 1 CFU into each control sample. No standardized procedure has been described to generate a one-cell inoculum, and the range of results achieved by such a low inoculum is not known. Further, the reproducibility of dispensing 1 CFU needs to be examined in order for this sort of control to be accurately used.

Numerous procedures have been evaluated as endpoint tests for *E. coli* O157:H7 in beef trim and ground beef *(2, 15, 17)*. Among the molecular detection systems currently in use are the Qualicon BAX system and the BioControl Genetic Detection System (GDS). The Qualicon system test, known as BAX-MP, uses a multiplex PCR to detect multiple *E. coli* O157:H7–specific targets in an enriched sample. The BioControl system uses an O157-specific immunomagnetic separation (IMS) step to concentrate the pathogen for detection by PCR for an *E. coli* O157:H7–specific target. In the past we have evaluated premarket versions of these tests *(2)* but not at the very low concentrations now being considered by industry and regulatory officials. The work presented here shows the

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† Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

results of these two methods compared with IMS-based culture isolation, which in these studies was set to be the ''gold standard'' for detecting very low concentrations of *E. coli* O157:H7 *(22, 31)*.

During the initial phase of these studies we noted two factors that may contribute to inconsistent *E. coli* O157:H7 detection results. One was the concentration of background organisms present, and the other was the lean-to-fat ratio of the trim or ground beef being tested. The concentration of background organisms in trim may vary due to the effectiveness of sanitary hide removal, processing interventions, and the temperature control of the trim or ground beef before it is tested. The lean-to-fat ratio of ground beef is varied by producers to meet customer requirements. Test methods suspend samples in media and generate fat droplets that may clog lateral flow detection systems or interfere with the binding of magnetic particles to targets in an IMS concentration step *(16, 34)*. Therefore, although these experiments were initiated to examine the detection of 1 CFU of *E. coli* O157:H7 in beef trim and ground beef, they were expanded to examine the effects of background bacteria and fat composition on the accuracy of the detection systems as well.

MATERIALS AND METHODS

Experiment 1: low-concentration inoculation studies. These studies were performed in three parts. First, a protocol to prepare a consistent 1 CFU inoculum was developed. Then the repeatability of achieving the targeted inocula was examined. Finally, multiple samples of beef trim were inoculated at the lowest concentration possible for consistent delivery of at least 1 CFU/sample and tested using culture isolation and two commercial molecular detection methods.

Experiment 1: preparation of low-inoculation stock. The low-inoculation stock of *E. coli* O157:H7 was prepared by growing four strains of genetically diverse *E. coli* O157:H7 *(3)* to stationary phase in 5 ml of tryptic soy broth (TSB) (37°C, 48 h) to yield 1×10^9 CFU/ml. The four cultures were combined and mixed, and then 1 ml was serially diluted 1:100,000 in buffered peptone water (BPW; Difco, BD, Sparks, MD). A 1-ml aliquot of this 5-log dilution was thoroughly mixed with 0.5 ml of 50% glycerol and then frozen at −70°C. The 1.5-ml glycerol–*E. coli* O157 stocks were thawed, subaliquoted to 100 μl, and stored at −70°C for 10 weeks. Once each week the colony count of a 100-μl vial was determined. Each vial was thawed and serially diluted 10-fold three times. One hundred microliters was spread plated onto tryptic soy agar (TSA; Difco, BD), and another 1 ml was plated to PetriFilm aerobic count plates (3M Microbiology, St. Paul, MN) in triplicate. The TSA plates and PetriFilm were incubated overnight (16 to 20 h) at 37°C, and the colonies were counted. Using this protocol, the concentration of *E. coli* O157:H7 in the frozen glycerol stocks was calculated to be 6,700 ± 100 CFU/ml. The proportion of stressed cells present in the control inoculum was determined by repeating the above dilutions and plating onto (i) CT-SMAC medium, i.e., sorbitol MacConkey agar (Difco, BD) supplemented with 0.05 mg/liter of cefixime and 2.5 mg/liter of potassium tellurite (Dynal, Lake Success, NY) and (ii) ntCHROMagar, i.e., CHROMagar O157 (DRG International, Mountainside, NJ) containing 5 mg/liter novobiocin and 2.5 mg/liter potassium tellurite. The difference in colony counts between these two selective media and the nonselective TSA provided the relative percentage of stressed cells in the inoculum.

Experiment 1: repeatability of low-concentration dilutions. Based on the concentration determined above, 50 ml of BPW were generated with expected concentrations of 5, 3, and 1 CFU of *E. coli* O157:H7 per milliliter. An appropriate volume (74.6 μl) of the 6,700 CFU/ml stock was diluted into 100 ml of BPW to generate a 5 CFU/ml stock; 30 ml of the 5 CFU/ml stock was removed and added to 20 ml of BPW to generate a 3 CFU/ml stock, and 10 ml of the 5 CFU/ml stock was removed and added to 40 ml of BPW to generate a 1 CFU/ml stock. All diluted stock inocula were maintained on ice, thoroughly mixed by inversion, and used immediately. One milliliter was removed from each tube and plated onto PetriFilm aerobic count plates to determine the number of *E. coli* present. The plating was replicated for a total of 48 observations. PetriFilm plates were incubated overnight (16 to 20 h) at 37°C and then counted. Colonies on PetriFilm plates were confirmed to be *E. coli* O157:H7 by performing PCR for the rfb_{O157}, $fliC_{H7}$, and *stx* genes as described previously *(24)*.

Experiment 1: low-concentration inoculation of beef trim and detection of *E. coli* O157:H7. Beef trimmings (approximately 60% lean) were obtained from a local retail butcher shop. The outer surfaces of the beef trimmings were cut away with a knife and collected to form a sample similar to that used in the ''N60'' method of testing beef trimmings destined for ground beef *(26)*. Three hundred seventy-five grams of trim was placed into each of 48 Whirl-Pak filter bags (Nasco, Fort Atkinson, WI). A low inoculum was prepared as described above for the 5 CFU/ml target range. The mean concentration of *E. coli* O157:H7 in the inoculum was 5.4 CFU/ml, and the concentration ranged from 1 to 9 CFU/ml. One milliliter of this inoculum was dispensed onto the beef trimmings in each sample bag, and then the bag was thoroughly mixed using a JumboMix Lab Blender (Interscience, Mountain, NJ) for 30 s at 420 rpm. Samples were held at room temperature (21 to 23°C) for 30 to 40 min to allow the inocula to bind and interact with the beef trim surface. The bags were then divided into two sets of 24 and enriched by addition of either 1 liter of prewarmed (42°C) TSB or mEHEC broth (part no. 65012-5K, BioControl Systems, Bellevue, WA). After enrichment media were added, each sample was again mixed in the lab blender as described above, sealed, and incubated at 42°C. After 8 h of incubation, 1 ml and 5 μl were removed from each enrichment for detection tests using either the BioControl GDS-O157 (part no. 61007-100) or the DuPont Qualicon BAX-MP O157 (part no. D12404903, DuPont, Wilmington, DE) methods, respectively, performed according to their manufacturers' recommendations. The samples were returned to the incubator, and incubation was continued for an additional 4 h. After 12 h of incubation, 1 ml of enrichment was removed from each sample bag for culture isolation as described previously *(4, 7)* using a KingFisher robot (Thermo Fisher Scientific, Waltham, MA) to perform the IMS steps. The final step of IMS suspends the recovered beads in 100 μl of buffer in a 96-well plate. Culture results were considered the indicator of whether a sample was inoculated with at least 1 CFU of *E. coli* O157:H7 *(4)*. No sample found to be culture negative was positive by any other test method.

Experiment 2: effects of increasing background organisms on detection of *E. coli* O157:H7. The effects of background bacteria on the detection of *E. coli* O157:H7 were determined by diluting increasing amounts of natural ground beef background bacteria into samples that were each inoculated with either a low or

a high concentration of *E. coli* O157:H7. The background bacteria used were recovered from 80/20 chub ground beef that had been opened and then stored in a Whirl-Pak bag at 10°C overnight to approximate temperature abuse. This ground beef was suspended in an equal volume of cold (4°C) BPW and stomached in a laboratory blender as described above. The supernatant was removed and two 10-fold serial dilutions were prepared such that there were three background inocula: a low, a medium, and a high inoculum. Sixty-four samples of fresh (80/20) retail ground beef, 65 g each, were prepared; half were inoculated with a low (0.46 ± 0.07 CFU/g) concentration and half with a high (4.2 ± 1.0 CFU/g) concentration of *E. coli* O157:H7 that had been prepared from stocks as described above. The *E. coli* O157:H7 inoculum was hand massaged into the samples and then thoroughly mixed into the samples using a lab blender as described above. Five milliliters of each of the three background dilutions was dispensed into eight low- and eight high-inoculated ground beef samples. The remaining eight samples of the low and high inocula, which received no background bacteria, did receive 5 ml of sterile BPW and served as a control. The ground beef samples were thoroughly hand massaged for 10 s to incorporate the background inoculum or control BPW; then they were combined with 585 ml of prewarmed (42°C) TSB and placed in the lab blender to generate a uniform suspension. One milliliter of each suspension was removed for aerobic plate counts (APCs) and *Enterobacteriaceae* counts (EBCs) as described below. Finally, the samples were incubated at 42°C for 8 and 12 h and then subjected to the *E. coli* O157:H7 detection methods as described above. In addition to the *E. coli* O157:H7 detection methods, portions of the enrichments were enumerated as previously described *(7),* using spiral plating to determine the concentrations of *E. coli* O157:H7 compared with background bacteria growing on ntCHROMagar.

Experiment 3: effects of fat composition on detection of *E. coli* O157:H7. Ground beef samples of varying lean/fat ratios were generated from fat beef trim (determined to be 60% lean) and lean beef trim (determined to be 95% lean) that had been obtained from a local abattoir. The APC of each percentage of lean ground beef was confirmed to be less than 2.0 log CFU/g, and the percentage of fat in the ground beef was determined using a Hobart fat percentage measuring kit (Hobart Corporation, Troy, OH). Thirty-two 65-g portions of each lean/fat ratio (60/40, 68/32, 78/22, and 88/12) and starting material (fat trim and lean trim) were inoculated with *E. coli* O157:H7 as described above. The number of *E. coli* O157:H7 inoculated into each sample was determined by colony counting to be 5.7 ± 1.5 CFU. The 32 inoculated samples of each lean percentage or starting material were divided into two groups of 16 and then enriched in prewarmed (42°C) TSB. One group of 16 was enriched in three volumes (195 ml; 1:4) of medium and the other group was enriched in nine volumes (585 ml; 1:10) of medium. All samples were incubated at 42°C for 8 and 12 h and then subjected to the *E. coli* O157:H7 detection methods.

Experiment 3: quantitation of IMS bead recovery. Magnetic beads were recovered at the conclusion of IMS in 96-well plates as described above. Before beads were removed for spread plating, the 96-well plate was photographed on a light box (Kodak Gel Logic 200 Imager, Carestream Health, Rochester, NY). Kodak Molecular Imaging Software version 4.0 (Carestream Health) was used to determine the relative density of each well. The volume of IMS beads recovered from samples of varying lean/fat ratios (described above) was determined by linear regression based on a standard curve of IMS bead densities generated from 1 to 20 μl of IMS beads. The volume of beads recovered was equal to (measured density − 40.237)/2.2339. The R^2 of the linear regression was 0.9937. All measurements were performed in triplicate and expressed as a percentage of beads recovered.

APC and EBC determinations. APCs and EBCs were estimated by impedance measurements obtained with a bioMérieux Bactometer (Hazelwood, MO). One hundred microliters was removed from the 1 ml of suspension described above and then placed into 900 μl of bioMérieux General Purpose Medium–Plus supplemented with 18 g/liter of dextrose for APC or 900 μl of bioMérieux enteromedium for EBC. The values for APC and EBC were based on values generated from a standard curve that had been determined using the appropriate PetriFilm plate counts as described previously *(8).*

Statistics. Analyses of variance and comparisons of frequencies of *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). Specifically, the nonparametric data were analyzed using Kruskal-Wallis one-way analysis of variance by ranks with Dunn's multiple comparison test. P values of less than 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Experiment 1. In response to recent recalls of ground beef due to contamination with *E. coli* O157:H7, detection procedures and tests have been rigorously reexamined, and some processors have established a desired detection proficiency of 1 CFU of *E. coli* O157:H7 in a 375-g sample at a rate of 95% or greater. All of the currently used tests have been validated through AOAC International and shown to detect one cell in 25-g samples under optimal conditions. However, test-and-hold samples are often 150 g or 375 g in size, and they vary in percent lean as well as in background organisms. Proficiency and validation studies have been reported that used samples ranging from 65 to 375 g, but with concentrations of inoculated *E. coli* O157:H7 that were 10 to 100 CFU per sample *(2, 7).* We initiated these studies to determine if it was feasible to generate a control sample with 1 CFU/375 g and then to determine the abilities of the test methods to detect an inoculum at this low level.

Inoculation with a control suspension that reliably contains 1 CFU/ml appears to be an unrealistic goal. When 5, 3, and 1 CFU were targeted, average values of 4.8, 2.9, and 0.8 CFU were observed (Fig. 1). At the 1 CFU target, 46% of the colony counts were zero, illustrating the binomial nature of the distribution of *E. coli* cells in the suspension. Attempts to use such a dilution as a source of *E. coli* O157:H7 for a positive control would result in an unacceptably high number of negative test results. A minimum target concentration of 3 CFU was necessary for the distribution of bacterial cells to repeatedly be at least 1 CFU or greater per 1 ml. The range of bacteria per milliliter of solution at the 3 CFU target was 1 to 8 CFU. For this reason the low-inoculation studies described below did not use *E. coli* O157:H7 CFU at the proposed target of 1 CFU in 375 g of beef. Instead, 5 CFU (an equivalent of about 1 CFU in a 65-g sample and 0.4 CFU in a 25-g sample) were

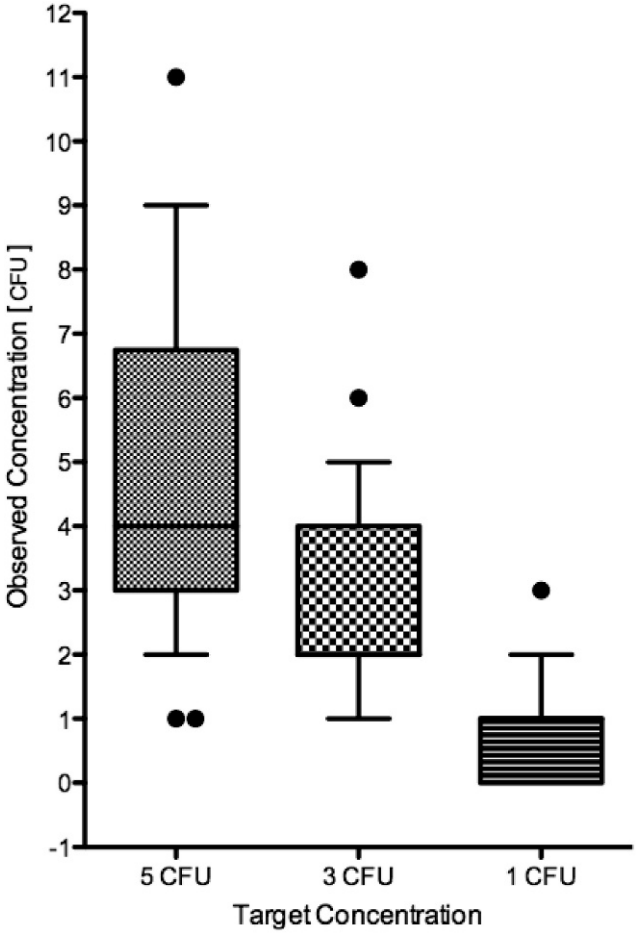

FIGURE 1. *Distribution of observed CFU per milliliter of* E. coli *O157:H7 at target concentrations. The mean CFU per milliliter observed at a target concentration of 5 CFU/ml (*n = *48) was 4.8 ± 2.5 with a 95% confidence interval (CI) of 4.0 to 5.5. At the 3 CFU/ml target concentration (*n = *48), mean CFU per milliliter observed was 2.9 ± 1.5 with 95% CI of 2.4 to 3.3. At the 1 CFU/ml target concentration (*n = *48), mean CFU per milliliter observed was 0.8 ± 0.9 with a 95% CI of 0.5 to 1.0.*

used to measure the sensitivity of the most commonly used molecular detection tests and culture isolation.

The difficulties of maintaining consistent low-concentration inoculations have also been described in other reports. In the AOAC International collaborative study of the GDS *E. coli* O157 detection test, 31 of 72 samples inoculated at a low concentration (1 to 5 CFU/25 g) and 3 of 72 samples inoculated at a high concentration (10 to 50 CFU/25 g) were found to be negative *(15),* indicating that those samples did not receive the desired inoculum. In other studies examining reduced volumes of enrichment media, Ahmed et al. *(1)* found that 13 and 14 of 20 inoculated 25-g beef trim samples did not receive their targeted inoculum of about 2 CFU.

In other inoculation studies on ground beef and beef trim, the inoculated beef has often been stored for 48 h at 4°C to more closely model the ways in which boneless beef trim is handled before grinding *(2, 4, 29).* However, we wished to model the occurrence of very low numbers of *E. coli* O157:H7 in a sample. In order to maintain the low cell count, we only allowed the inoculum to interact with the meat surface for 30 to 40 min before enrichment. This is because we have observed that *E. coli* O157:H7 counts increase up to 0.5 log with 24 h of storage at 4°C (data not shown), which would have raised the low-level inoculum considerably above our desired target range.

TABLE 1. *Effect of detection method and enrichment medium on mean percentage and confidence interval of samples identified as positive for* E. coli *O157:H7 following low inoculation of beef trim*[a]

Enrichment medium[b]	Culture	BAX-MP	GDS
TSB			
Mean % ± SD	94 ± 11	92 ± 10	92 ± 7
95% CI	77–112	76–109	80–103
mEHEC			
Mean % ± SD	71 ± 38	92 ± 10	92 ± 19
95% CI	10–131	76–109	77–107

[a] Inoculum was 5.4 CFU (range 1 to 9 CFU) per 375-g sample of beef trim ($n = 48$).

[b] One liter of enrichment medium, tryptic soy broth (TSB) or mEHEC broth, was used.

We have recently demonstrated the benefits of using nonselective TSB to enrich beef samples for the isolation and detection of *E. coli* O157:H7 *(20).* However, various beef industry laboratories and reference laboratories use a modified or selective media rather than TSB in their *E. coli* O157:H7 detection tests *(12, 18).* Therefore, we elected to use mEHEC broth as a representative selective medium for our studies using 375-g samples. The FSIS *Microbiology Laboratory Guidebook (38)* recommends diluting samples 1:10 in enrichment medium (1 part sample to 9 parts medium); however, due to issues regarding capacity, sample size, and economics, our laboratory and others enrich 375-g samples in 1 liter of medium instead of 3.4 liters *(1, 20).* This results in a final dilution of about 1:4 (1 part sample to 2.7 parts medium).

The results of experiment 1 (Table 1) show that at low-concentration inoculation the molecular detection tests performed equally at detecting *E. coli* O157:H7. However, mEHEC broth was an impediment to successful detection using culture isolation. All of the samples in Table 1 tested positive by at least one of the methods. No detection method detected all of the positive samples at a rate of 95% or greater. However, with the exception of culture isolation using mEHEC media, all had a mean detection rate very near the desired detection rate of 95% or greater.

BAX-MP uses a multiplex PCR to detect two *E. coli* O157:H7–specific targets in an enrichment. The final realized volume of enrichment tested in the reaction is about 1.25 μl. Therefore, greater than 10^3 CFU/ml must be present in the enrichment for detection. This is the equivalent of 21 doublings of 1 CFU in a 1-liter sample *(20),* a reasonable expectation if one assumes rapid log phase growth for 7 to 8 h and population doublings every 20 min. The BioControl GDS uses about 60% of an O157-IMS concentration from 1 ml of enrichment, followed by a PCR that detects an *E. coli* O157:H7–specific target. Therefore, in theory, the detection limit for GDS is

TABLE 2. *Effects of increasing concentrations of background bacteria on the detection of* E. coli *O157:H7 in 65-g samples of ground beef*

Mean concn of bacteria[a]			Detection method[b]		
E. coli O157:H7 (CFU/g)[c]	APC (log CFU/g)	EBC (log CFU/g)	Culture (% positive)	BAX-MP (% positive)	GDS (% positive)
0.4	4.3	0.4	100 A[d]	100 A	100 A
0.4	5.9	3.8	100 A	100 A	100 A
0.4	6.7	4.5	100 A	75 A	13 B
0.4	7.9	5.9	63 A	0 B	0 B
4.3	4.3	0.0	100 A	100 A	100 A
4.3	6.0	3.6	100 A	100 A	100 A
4.3	6.8	4.8	100 A	100 A	63 A
4.3	7.7	6.3	100 A	50 AB	13 B

[a] Mean concentrations of *E. coli* O157:H7 inoculum, aerobic plate count (APC) bacteria, and *Enterobacteriaceae* count (EBC) before enrichment, resulting from no background inoculation, and the use of serially diluted background organisms collected from temperature-abused ground beef.

[b] For each observation, $n = 8$.

[c] Two concentrations of *E. coli* O157:H7 were inoculated into samples.

[d] Percentages in the same column for a given *E. coli* O157:H7 concentration followed by the same letters are not different ($P > 0.05$).

considerably lower than for BAX-MP. Enrichment time was extended to 12 h for culture isolation, which also used IMS to concentrate *E. coli* O157:H7 from 1 ml of enrichment broth. Culture isolation is considered the gold standard of *E. coli* O157:H7 detection and is often relied upon to confirm positive molecular screening tests *(22, 31)*. However, variable culture detection results of low-concentration *E. coli* O157:H7 were observed between the use of TSB and mEHEC broths. Therefore, unsuccessful culture confirmation of positive molecular tests from enrichments using media other than TSB may require additional examination and should not be immediately considered a false positive.

In comparison studies of *E. coli* O157:H7 detection tests, Arthur et al. *(2)* reported that GDS detected 73% and BAX-MP detected 66% of positive samples inoculated at a concentration of 1.7 CFU/65 g (equivalent to 10 CFU/375 g). That group compared the molecular methods to culture isolation, which detected 91% of the positive samples. However, these reported detection rates cannot be directly compared with those reported here. The previously reported detection rates were determined using separate sets of samples enriched in each manufacturer's specified medium, whereas the detection rates described herein compare all three detection methods used on the same enrichments.

Other studies that examined the GDS and BAX-MP detection methods reported detection rates near 100%, but these all used 25-g samples and inoculations of 1 to 5 and 10 to 50 CFU per sample *(15, 29)*. The variations observed between studies raised the question as to the role of sample composition on the detection assays. One possible explanation could have been the residual effects of antimicrobials used during processing. Applications of organic acid to meats have been shown to have residual effects *(13, 21, 40)*, and other commonly used compounds such as acidified sodium chlorate and peroxyacetic acid also have been shown to have residual effects *(19)*. We do not know what compounds may have been used on the test materials before we obtained them, but the possibility exists that there were residual compounds present that could have reduced the overall microbial growth or injured the cells when initially inoculated into the sample. However, during preliminary studies we noted that the most consistent results were obtained when fresh beef trim was used. Inconsistent results were observed when the samples of trim were 7 to 10 days old or when they had been shipped to the laboratory at less than optimal temperatures. Both are situations where the concentrations of background organisms were likely increased. We hypothesized that the inconsistent detection results were due to increased concentrations of background bacteria present in the samples at the time of testing instead of any residual antimicrobial effects.

Experiment 2. Previous observations from our laboratory had noted that excessive background bacterial growth could interfere with the results of culture isolation *(4, 7)*. Experiment 2 was performed to determine if increasing concentrations of background bacteria affected the performance of the detection methods. As the amount of background bacteria present before enrichment increased, the likelihood of detecting the inoculated *E. coli* O157:H7 decreased (Table 2). At background concentrations below 6 log CFU/g APC and 4 log CFU/g EBC, *E. coli* O157:H7 was readily detected by all methods. However, the greatest concentrations of background bacteria, APC nearing 8 log CFU/g and EBC of about 6 log CFU/g, completely prevented the detection of *E. coli* O157:H7 inoculated at 0.4 CFU/g (26 CFU/65 g sample) by the molecular methods and prevented 37% from being detected by culture isolation. These high APC and EBC concentrations are similar to those that can occur in temperature-abused samples or those of ground beef near the end of its shelf life *(6)*. Even when the inoculum of *E. coli* O157:H7 was increased 10-fold to 4.3 CFU/g (280 CFU/65 g sample), the molecular methods had difficulty detecting the *E. coli* O157:H7 when background concentrations exceeded 6 log CFU/g APC and 4 log CFU/g EBC. The concentration of the background

TABLE 3. *Effect of increasing concentrations of preenrichment background bacteria on the concentrations of* E. coli *O157:H7 and nonsuspect background bacteria in 65-g samples of ground beef*

Concn of bacteria preenrichment[a]			Mean concn of bacteria postenrichment[b]	
E. coli O157:H7 (CFU/g)	APC (log CFU/g)	EBC (log CFU/g)	*E. coli* O157:H7 (log CFU/g)[c]	Nonsuspect background bacteria (log CFU/g)[d]
0.7	4.1	−0.4	4.7 A[e]	6.2 A
0.7	5.5	2.7	4.4 A	7.2 AB
0.7	5.9	3.8	1.8 B	7.2 AB
0.7	6.6	4.9	≤1.3 B	7.7 B

[a] Values represent the concentration of inoculated *E. coli* O157:H7 (CFU per gram) and the mean log CFU per gram of aerobic plate count (APC) bacteria and *Enterobacteriaceae* count (EBC) before enrichment.
[b] Inoculated samples were enriched for 8 h and then enumerated by direct spiral plating onto ChromAgar O157.
[c] Suspect *E. coli* O157:H7 (mauve colonies) were identified and confirmed by latex agglutination.
[d] Nonsuspect bacteria were blue or white colonies.
[e] Percentages in the same column for a given postenrichment concentration followed by the same letters are not different ($P > 0.05$). For each observation, $n = 8$.

organisms had a greater effect on detection by GDS than by BAX-MP, even though the GDS method relies upon IMS to concentrate *E. coli* O157:H7 cells and remove them from background organisms. The background organisms used in these experiments were generated from chub-packaged ground beef. Had ground beef from modified atmosphere packaging of ground beef collected directly from the grinding head been used, a different population of background organisms may have altered the results.

In experiment 1 it was observed that culture isolation performed poorly when mEHEC broth was used as enrichment medium. We suspect that since culture isolation was shown to be affected by increasing background organisms in experiment 2, this selective medium may have allowed the growth of organisms that interfered with culture plate interpretation and suspect colony identification. Alternately, the target population of *E. coli* O157:H7 may not have been present at a sufficient number for detection because the mEHEC was too harsh of a medium and the enrichment period was too short for injured *E. coli* O157:H7 to recover and multiply to a detectable concentration. Sixty to ninety percent of the population in the inoculation used in these experiments was determined to be injured based on lack of growth on selective media (data not shown).

It should be noted that, under typical conditions, the levels of background bacteria in fresh beef trim and ground beef are usually at lower concentrations than the concentrations we found to cause failure of the methods examined here *(6, 7)*. If samples of ground beef or trim are temperature abused prior to enrichment, the concentration of background bacteria may exceed a point that interferes with detection. The ability to use culture confirmation of positive screening tests from such samples decreases as well. Therefore rigorous monitoring of test-and-hold sample temperature is encouraged during storage and transport to the laboratory for analysis.

Others have previously described the inhibitory effects of background bacteria on *E. coli* and *E. coli* O157:H7. During testing of municipal drinking water for coliforms and *E. coli,* an apparent inhibition effect was observed when there were high numbers of background bacteria in the samples *(10)*. Further examination showed that there was no direct inhibitory effect of the background bacteria on the target organisms; differences in detection methods and growth media were cited as explanations. In studies that examined ground beef inoculated with 10^3 CFU of *E. coli* O157:H7 per gram, varying results were observed depending on the stock of background bacteria used *(41)*. It was concluded that certain lactic acid bacteria may inhibit the outgrowth of *E. coli* O157:H7 over many days of storage. One possible explanation for this inhibition is quorum sensing *(11)*. Although our observations could be taken to agree in various ways with these previous studies, the more likely explanation in experiment 2 is that the background bacteria outcompeted or outgrew the *E. coli* O157:H7.

In an effort to determine if growth of background bacteria inhibited the growth of *E. coli* O157:H7, the concentrations of *E. coli* O157:H7 and nonsuspect background organisms were enumerated postenrichment (Table 3). The concentration of *E. coli* O157:H7 decreased as the starting concentrations of APC and EBC reached and passed 6 and 4 log CFU/g, respectively (data not shown). This correlated with the enumerable postenrichment *E. coli* O157:H7 decreasing significantly ($P < 0.05$) by about 2.5 log CFU, while nonsuspect bacteria remained about the same at 7.2 to 7.7 log CFU/g. At the lowest concentrations of background, the number of *E. coli* O157:H7 present postenrichment were significantly higher and represented a much greater proportion of the enumerable bacteria present, thus providing optimal targets for detection and isolation.

Even though detection of *E. coli* O157:H7 by culture isolation was able to overcome the effects of increasing background bacteria, the ability to successfully identify suspect colonies as *E. coli* O157:H7 required increased technical effort. Our protocol calls for up to three colonies of suspect *E. coli* O157:H7 to be picked for identification by latex agglutination before being put forward to PCR confirmation testing. When background organisms were at a high concentration it was necessary to examine up to 10 suspect colonies to identify presumptive *E. coli* O157:H7. Had the original guideline of testing only three colonies been followed, an increased number of culture results would

TABLE 4. *Effects of fat composition of beef trim and ground beef on the percent recovery of immunomagnetic particles during IMS*[a]

	Enrichment medium ratio[b]:	
	1:4	1:10
Beef trim (%)[c]		
50	53 ± 8 A[d]	53 ± 8 A
95	78 ± 11 A	86 ± 9 A
Ground beef (%)		
61	52 ± 8 A	64 ± 12 A
68	57 ± 16 A	61 ± 8 A
78	54 ± 14 A	68 ± 10 B
88	71 ± 7 A	80 ± 13 A

[a] Values represent mean percentage ± standard deviation of beads recovered by immunomagnetic separation (IMS) ($n = 16$) determined by densitometric analysis and linear regression. IMS was performed for *E. coli* O157:H7 using a Thermo 96-well head KingFisher IMS robot.

[b] Two ratios of sample to enrichment medium, tryptic soy broth (TSB), were used: 1:4 (65 g of beef and 195 ml of TSB) and 1:10 (65 g of beef and 585 ml of TSB).

[c] Lean percentage of beef trim or ground beef. The designated beef trim was the starting materials used to blend the ground beef of varying lean percentages shown.

[d] Percentages in the same column followed by the same letters are not different ($P > 0.05$).

have been considered negative when background organism concentrations were increased.

Experiment 3. During the IMS steps in experiment 1 it was observed that inoculated beef trim samples that had a higher fat proportion appeared to have a lower amount of IMS beads recovered in the final elution plate. This decreased IMS bead recovery may have contributed to the inconsistent test results of the preliminary tests (data not shown). The fat content of ground beef samples may have an effect on detection tests, because fat, collagen, polysaccharides, and myoglobin have been reported to interfere with PCR-based assays *(5, 18, 27, 30)*. Also, at least one *E. coli* O157:H7 detection protocol instructs the user to ensure that no fat is included when removing aliquots of enriched beef to IMS tubes, because fat would inhibit the binding of IMS beads *(15)*. However, other protocols do not address this issue. We have not been able to find any published data that addresses the effect of fat content on the recovery of IMS beads examined. Therefore, experiment 3 was performed to examine the potential problems fat content may introduce to detection assays and IMS procedures.

Since the volume of medium used alters the final fat concentration in the enriched sample, we examined two commonly used sample-to-medium ratios in this experiment. Freshly blended ground beef ranging from 61 to 88% lean and the starting materials (95/5 beef trim and 50/50 beef trim) were diluted in enrichment medium at ratios of 1:4 and 1:10, enriched, and subjected to IMS. When the amount of recovered IMS beads in the elution plate was measured by densitometry, it was observed that leaner samples had greater bead recovery (Table 4). The recovery of IMS beads also was greater in samples diluted 1:10 compared with samples diluted 1:4. In 78% lean ground beef the ratio effect was significant ($P < 0.05$).

Even though samples with the greatest levels of fat distributed through the enrichment had lower recoveries of IMS beads, the differences did not affect the detection of *E. coli* O157:H7 (Table 5). BAX-MP was not affected by the levels of fat in the samples, as would be expected since this method does not depend on IMS. GDS, which is IMS based, was not affected by fat content of the sample. The culture

TABLE 5. *Effects of fat composition on the detection of* E. coli *O157:H7 inoculated at a low concentration in beef trim and ground beef*[a]

	Detection method[b]:					
	Culture		BAX-MP		GDS	
Enrichment medium ratio[c]:	1:4	1:10	1:4	1:10	1:4	1:10
Beef trim (%)[d]						
50	88 B[e]	81 B	100 A	100 A	100 A	100 A
95	100 A	100 A	100 A	100 A	88 B	94 A
Ground beef (%)						
61	81 B	69 B	100 A	100 A	100 A	100 A
68	94 AB	69 B	100 A	100 A	100 A	94 A
78	75 B	94 AB	100 A	100 A	100 A	100 A
88	88 B	75 B	100 A	94 A	100 A	94 A

[a] Trim and ground beef of varying fat compositions also had varying APC before enrichment of 3.4, 1.2, 2.7, 2.9, 2.8, and 2.5 log CFU/g, respectively, for fat trim, lean trim, and 61, 68, 78, and 88% lean ground beef. *E. coli* O157:H7 inoculum was 5.7 ± 1.5 CFU per 65-g sample.

[b] Values represent percent positive ($n = 16$).

[c] Two ratios of sample to enrichment medium, tryptic soy broth (TSB), were used: 1:4 (65 g of beef and 195 ml of TSB) and 1:10 (65 g of beef and 585 ml of TSB).

[d] Lean percentage of beef trim or ground beef. The designated beef trim was the starting materials used to blend the ground beef of varying lean percentages shown.

[e] Percentages in a column followed by the same letters are not different ($P > 0.05$).

detection of *E. coli* O157:H7, however, was variably affected by increasing fat content, possibly due to decreasing IMS bead recovery. Since many laboratories screen with lateral-flow, antibody-based tests before performing a molecular test, we also examined the effects of fat composition on a lateral-flow test in this comparison and observed no differences (data not shown).

The concentrations of background bacteria were examined to determine if these helped to explain the variable culture detection rates in experiment 3. It is known that the types and levels of background bacteria on fat surfaces are different than those on lean surfaces *(14, 33)*. We confirmed that the 50/50 trim had increased background APC concentrations (3.4 log CFU/g) compared with the lean trim (1.2 log CFU/g) and that the APCs of the ground beef blended from these two starting materials ranged between these two input concentrations (2.5 to 2.9 log CFU/g). However, based on the results of experiment 2, these concentrations should not have interfered with culture detection.

In summary, these studies were initiated to determine if the 1 CFU/375 g sample size was a reasonable limit to expect under typical laboratory conditions. It was noted that no readily available procedure exists that outlines the necessary steps to generate a 1 CFU inoculum; therefore, one has been described and its use evaluated in control samples for *E. coli* O157:H7 detection. Due to the nonuniform distribution of cells in a 1 CFU/ml suspension, it is suggested that a minimum of 3 CFU/ml be used in order to avoid a large number of noninoculated samples. However, even when 5 CFU of *E. coli* O157:H7 was inoculated into 375 g of ground beef, no detection method was 100% effective. These experiments also determined the effects of interference by background organisms and fat content on the detection of *E. coli* O157:H7 in ground beef. The results provide caveats for situations where temperature abuse may have occurred or when the samples being tested are of varying fat percentages.

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