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Comparison of enzyme-linked immunomagnetic chemiluminescence with U.S. Food and Drug Administration's Bacteriological Analytical Manual method for the detection of *Escherichia coli* O157:H7 ☆

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Abstract

Escherichia coli O157:H7, a major foodborne pathogen, has been associated with numerous cases of foodborne illnesses. Rapid methods have been developed for the screening of this pathogen in foods in order to circumvent timely plate culture techniques. Unfortunately, many rapid methods are presumptive and do not claim to confirm the presence of *E. coli* O157:H7. The previously developed method, enzyme-linked immunomagnetic chemiluminescence (ELIMCL), has been improved upon to allow for fewer incidences of false positives when used to detect *E. coli* O157:H7 in the presence of mixed cultures. The key feature of this assay is that it combines the highly selective synergism of both anti-O157 and anti-H7 antibodies in the sandwich immunoassay format. This work presents application of a newly semi-automated version of ELIMCL to the detection of *E. coli* O157:H7 in pristine buffered saline yielding detection limits of approximately 1×10^5 to 1×10^6 of live cells/mL. ELIMCL was further demonstrated to detect *E. coli* O157:H7 inoculated into artificially contaminated ground beef at ca. 400 CFU/g after a 5 h enrichment and about 1.5 h assay time for a total detection time of about 6.5 h. Finally, ELIMCL was compared with USFDA's Bacteriological Analytical Manual method for *E. coli* O157:H7 in a double-blind study. Using McNemar's treatment, the two methods were determined to be statistically similar for the detection of *E. coli* O157:H7 in ground beef inoculated with mixed cultures of select bacteria.
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Keywords: BAM; Chemiluminescence; Detection; *Escherichia coli*; Immunomagnetic beads

1. Introduction

Rapid screening tests for foodborne pathogens have been developed as alternatives to lengthy and laborious, yet selective and highly sensitive conventional culture techniques. Although most rapid methods produce presumptive results, several have been demonstrated to confirm the presence of pathogenic bacteria in foods. Typically, these tests rely on nucleic acid techniques,

Abbreviations: HRP, horseradish peroxidase; IMB, immunomagnetic beads; ELIMCL, enzyme-linked immunomagnetic chemiluminescence.

☆ Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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including polymerase chain reaction and nucleic acid hybridization, for the identification of bacteria (Feng, 1992; Fung, 1995; Hartman et al., 1992).

In this study, we have sought to develop an immunological-based rapid method that holds promise for simultaneously screening for and confirming the presence of a major foodborne pathogenic bacterium, *Escherichia coli* O157:H7. To this end, we developed a rapid method that incorporated elements of the Food and Drug Administration's Bacteriological Analytical Manual (BAM) method for *E. coli* O157:H7 (Feng and Weagant, 2002) including the same culture enrichment media and dual antibody (anti-O157 and anti-H7) discrimination. For this research, we have modified enzyme-linked immunomagnetic chemiluminescence (ELIMCL; Gehring et al., 2004) to employ a semi-automated immunoassay that now incorporates three antibodies (goat anti-O157, rabbit anti-H7, and horseradish peroxidase (HRP)-labeled mouse anti-rabbit antibody conjugate) as opposed to one antibody, in an indirect sandwich format. We have again applied this version of ELIMCL, a strictly immunological recognition-based approach, to the rapid detection and apparent confirmation of live *E. coli* O157:H7 cells in either buffered saline or artificially contaminated ground beef. We further tasked ELIMCL with the detection of *E. coli* O157:H7 contained in mixed cultures inoculated into ground beef and the subsequent test results were compared, in a double-blind study, with that obtained employing the considerably lengthier BAM method. Ground beef has again been our target sample matrix of choice due to numerous cases of association of the pathogenic bacteria, *E. coli* O157:H7, with outbreaks of food poisoning and hemolytic uremic syndrome as linked to the commodity (Besser et al., 1993; CDC, 1996, 1997).

2. Material and methods

2.1. Materials

Materials used in this research included horseradish-peroxidase (HRP)-conjugated mouse anti-rabbit IgG (H+L) antibody (HRP-Ab; 0.8 mg/mL, Pierce Biotech, Rockford, IL), DIFCO rabbit *E. coli* H antiserum H7, modified *E. coli* (mEC) medium, plate count agar (PCA), MacConkey sorbitol agar (SMAC), trypticase soy broth, blood agar base no. 2, and bile salts no. 3 (Becton Dickinson, Sparks, MD), nutrient broth (NB; Oxoid, Ogdensburg, NY), goat anti-*E. coli* O157 M-280 immunomagnetic beads (IMB) and cefixime–tellurite (CT) supplement (Dynal, Lake Success, NY), *E. coli* O157:H7 B1409 and *Salmonella typhimurium* G8430 (Centers for Disease Control, Atlanta, GA), *E. coli* O157:NM (Food Safety and Inspection Service, Omaha, NE), *Bacillus cereus* (American Type Culture Collection, Manassas, VA), cefsulodin, vancomycin, Tris, and casein (Sigma-Aldrich, St. Louis, MO), Tween 20 (Acros Organics, Fairlawn, NJ), 96-well, flat-bottomed, polystyrene microtiter plates (Corning, Inc., Corning, NY), stomacher bags (Fisher Scientific, Pittsburgh, PA), PS Atto (Lumigen, Southfield, MI), and RIM *E. coli* O157:H7 latex test kit (Remel, Lenexa, KS). Ground beef (93% lean) was obtained from a local supermarket on two separate days and the two lots were pooled prior to sampling. Other chemicals used were of reagent grade. All microbiological-related media and/or reagents were prepared as per manufacturer's directions.

2.2. Apparatus

Enumeration of bacterial cells was conducted using a Petroff–Hausser counting chamber (Hausser Scientific Partnership, Horsham, PA). All reactions with mixing were performed on a Vortex-Genie 2T (Scientific Industries, Bohemia, NY). All mixing of ground beef samples was done using a stomacher (Seward Medical Limited, London, United Kingdom). A KingFisher 96 magnetic particle processor (Thermo Electron Corporation, Vantaa, Finland) was used to conduct the immunomagnetic separations and washing steps of the ELIMCL immunoassay. Excess liquid was removed from the microtiter plates using an Elx405 plate washer (Bio-Tek Instruments, Winooski, VT). Detection of the ELIMCL assay was performed on the Victor^2 multilabel counter (Perkin Elmer Wallac, Turku, Finland).

2.3. PS Atto calibration curve

A calibration curve was prepared to observe the response of PS Atto, an HRP substrate that yields chemiluminescence upon enzymolysis by HRP, when mixed with the HRP-conjugated antibody. Fourteen two-fold serial dilutions of HRP-Ab were prepared in 25 mM Tris+150 mM NaCl+1% casein buffer (TBS+C) containing 0.05% Tween 20, (TTBS+C) starting from a 1:2000 dilution. (Note, TBS+C and TTBS+C solutions had a pH of 7.4.) Ten microliters of each dilution was reacted with 200 μL of the substrate. The substrate was automatically injected into the wells of a microtiter plate containing the HRP-Ab using the Victor^2. A 10 s shake and 2 s measurement followed injection. This procedure was done in triplicate for each HRP-Ab dilution.

2.4. Growth and enumeration of bacteria

E. coli B1409, *E. coli* O157:NM, *S. typhimurium*, and *B. cereus* were stored frozen at −70 °C in either mEC (*E. coli* serotypes) or NB (all others species) with 20% glycerol. Bacteria were cultured to stationary phase in 10 mL mEC or NB for 18 h at 37 °C (30 °C for *B. cereus*) with shaking at 160 rpm. The cultures where enumerated using a Petroff–Hausser counting chamber. In brief, the overnight cultures were diluted 1:100 in TBS+C. An aliquot (6 μL) was placed onto the counting chamber, and the center 0.2 mm×0.2 mm grid was counted. The counting chamber was extensively washed with 70% ethanol, and the counting procedure was repeated three more times for a total of four replicates. The average of the four replicates was used to determine the inoculum concentration. Subsequent dilutions were made based on this determination of total (live+dead) bacterial cells. Ten-fold serial dilutions of the stock cultures were prepared in TBS +C and 0.1 mL was spread plate cultured onto PCA that was incubated at 37 °C overnight for 16–18 h. Bacterial colonies were enumerated, CFU was determined, and CFU values were used to back-calculate the proportion of live bacteria present in total bacterial cell values as revealed using Petroff–Hausser chamber counting.

2.5. ELIMCL assay in pristine buffered saline

All reactions of this assay were performed at room temperature. Seven 10-fold serial dilutions of *E. coli* B1409 were made in TBS+C from an initial 10^8 stock dilution. One hundred microliters of each dilution was added to the wells of row A of a microtiter plate containing 10 μL of IMB. *E. coli* H antiserum H7 (100 μL) was diluted to a concentration of 1:500 in TTBS+C and added to the wells of row C. HRP-Ab (100 μL) was diluted to a concentration of 1:32 k in TTBS+C and added to row E. TTBS+C (150 μL) was added to rows B, D, and F for wash steps. TBS+C (150 μL) was added to row G for an additional wash step. TBS+C (50 μL) was added to row H for elution of assayed beads. The immunoassay was carried out in the KingFisher magnetic particle processor with a 30 min reaction for each binding step. Following completion of the immunoassay, the elution buffer was aspirated from the IMB-bacteria-antibody complex using the Elx405 plate washer outfitted with a magnetic plate holder. The finished plate was put into the Victor^2 multilabel counter and 200 μL of PS Atto was automatically injected into the wells containing the samples. The light generated from the ensuing chemiluminescent reaction was measured for 2 s and the resulting counts per second (CPS) were recorded.

2.6. Analysis of E. coli O157:H7 inoculated ground beef

E. coli B1409 was diluted to various concentrations in TBS+C, inoculated into 25 g of ground beef in a stomacher bag, and hand massaged for 5–10 s. In accordance with the FDA's BAM method protocol—to the ground beef, 225 mL of enterohemorrhagic *E. coli* (EHEC) Enrichment Broth (EEB, 30 g trypticase soy broth+1.5 g bile salts no. 3+1.5 g dipotassium phosphate+1 L deionized water) was added. The samples were mixed in a stomacher for 30 s and placed into a shaking incubator at 37 °C for 5 h at 160 rpm. After enrichment, 100 μL of sample was subjected to the ELIMCL immunoassay, as described above.

2.7. Double-blind detection of E. coli O157:H7 in ground beef study

A double-blind study was performed to test the efficacy of ELIMCL compared with an abbreviated version of the FDA's BAM method for *E. coli* O157:H7. *E. coli* B1409, *E. coli* O157 NM, *S. typhimurium*, and *B. cereus* were diluted to various concentrations and combined to form mixed cultures. A different party then took the mixed cultures and inoculated 25 g aliquots of ground beef in stomacher bags. The inoculated samples were then passed on to a third researcher who analyzed the ground beef samples as described above. After a 5 h culture enrichment, an aliquot of the sample was analyzed by ELIMCL as described above. Another aliquot was analyzed by an abbreviated version of the BAM method. In brief, 100 μL of appropriately diluted sample was spread plated onto Tellurite Cefixime Sorbitol-MacConkey agar (TC-SMAC) plates. The plates were incubated at 37 °C for 18–24 h. Up to five typical *E. coli* O157:H7 colonies were picked and tested for the presence of the O157 antigen using the RIM *E. coli* O157:H7 latex test kit. Colonies that tested positive were then streaked onto blood agar plates and incubated at 37 °C for 18–24 h. Colonies from the blood agar plates were then tested for the presence of the H7 antigen using the RIM *E. coli* O157:H7 latex test kit. Samples that tested positive for both the O157 and H7 antigens were considered positive for *E. coli* O157:H7. The final results of each method were compared for statistical similarity using McNemar's treatment.

3. Results and discussion

In the application of ELIMCL, multi-antigenic analyte (e.g., bacteria) was separated from a liquid sample mixture by IMB, the analyte was labeled with a reporter enzyme (HRP) via an enzyme-conjugated antibody in a sandwich immunoassay format, and subsequent luminescence generated after a brief reaction with a chemiluminescent enzyme substrate was measured (schematic representation of ELIMCL presented in Fig. 1). Initial experimentation sought to investigate high (putatively near saturation) levels of enzyme-antibody conjugate concentration and to test the limits of the luminometer (Victor^2) employed using HRP "alone" in pure samples of serially diluted HRP-labeled mouse anti-rabbit IgG antibody (HRP-Ab). As exhibited in Fig. 2, a linear range of detection for HRP-Ab was revealed and a concentration that saturates instrumental response, however, a decrease in luminescent response at beyond saturation HRP-Ab concentrations was observed. It was apparent that at relatively high levels of HRP-Ab, the Victor^2 instrument was believed to be marginally overwhelmed with too bright of a sample luminescence, thus yielding misleading measurements. As with the luminometer used in a previous report (Gehring et al., 2004), sample tests with the Victor^2 will require testing of original samples and dilutions thereof in order to ensure that samples results are not reported as false negatives.

PS Atto was selected as an HRP substrate for ELIMCL since, upon evaluation, it exhibited marginally superior reaction kinetics and higher production of light output relative to several other commercially available substrates including Pico (Pierce Biotechnology, Inc., Rockford, IL), Femto (Pierce Biotechnology, Inc.), and Immun-star (Bio-Rad Laboratories; Hercules, CA) at the lowest levels of HRP-Ab tested (data not shown). The relatively greater response and sensitivity to enzyme demonstrated by PS-Atto suggested that use of the substrate would elicit the best detection limit for ELIMCL assays. In the presence of a fixed level of PS Atto concentration, HRP-Ab concentration was varied in order to elicit optimal ELIMCL response at unusually high bacterial concentrations (ca. 10^8/mL) in order to maximize dynamic range and sensitivity for the assay (data not shown). A 1:32k dilution of HRP-Ab was selected for subsequent experiments reported in this study.

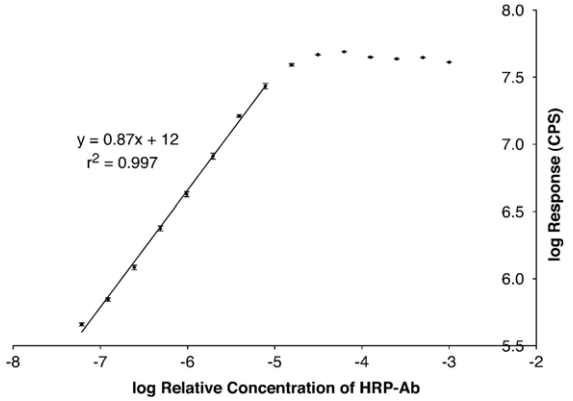

Fig. 2. Chemiluminescent response of horseradish peroxidase-labelled anti-*E. coli* O157:H7 antibody conjugate with PS Atto substrate. Serial dilutions of HRP-antibody conjugate (HRP-Ab) were reacted with PS Atto HRP chemiluminescent substrate and light production was measured as described. The plot displays the replicated (in triplicate) luminescent response for varying concentrations of HRP-Ab.

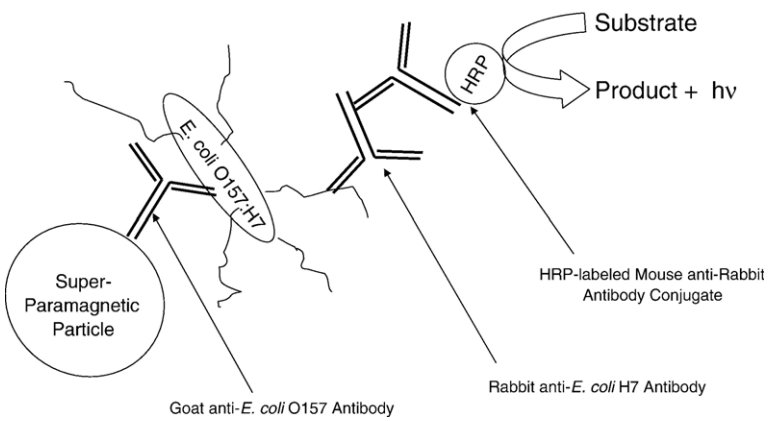

Fig. 1. Schematic representation of the enzyme-linked immunomagnetic chemiluminescent (ELIMCL) assay. *E. coli* O157:H7 analyte is sandwiched between anti-O157 antibody-coated magnetic beads (immunomagnetic beads or IMB) and anti-H7 antibody. Horseradish peroxidase (HRP)-labeled anti-antibody conjugate confers an enzymatic label that binds in proportion to captured *E. coli* O157:H7. IMB (with or without bound bacteria) are exposed to chemiluminescent substrate, and light emitted during product formation can be detected with a luminometer.

The limit of detection (LOD) of live *E. coli* O157:H7, serially diluted in pristine Tris-buffered saline containing Tween 20 detergent and 1% casein (TTBS+C) was determined for ELIMCL (Fig. 3). A lower limit of detection for the bacteria was observed to be ca. 5×10^5 CFU/mL.

Fig. 4 depicts ELIMCL detection of live *E. coli* O157:H7 serially diluted and inoculated into ground beef samples. The *E. coli* O157:H7, inoculated into ground beef that was then placed into growth enrichment media, was subjected to culture enrichment for 5 h prior to analysis with ELIMCL. Multiple ELIMCL trials involving varying inoculate levels of *E. coli* O157:H7 cells were replicated over several days of experimentation. Resultant ELIMCL responses (luminescence versus bacterial concentration) were fairly linear with some scatter attributed to variations in microbial growth observed and remarked upon in a previous study (Gehring et al., 2004). A putative detection limit for the assay, represented by the dashed line, correlates to approximately 3× the standard deviation for the replicated blank response plus the mean blank response.

In this study, an abbreviated version of the US FDA's Bacteriological Analytical Manual (BAM) method for the screening and confirmation of enterohemorrhagic *E. coli* serotype O157:H7 was employed for double-blind comparison with ELIMCL. In this comparison of the two methods with binomial results (samples were determined to be either positive or negative for *E. coli* O157:H7; positive responses for ELIMCL were those that exceeded the putative detection limit for the assay

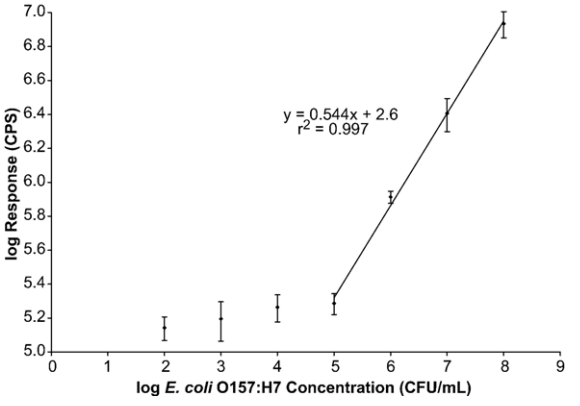

Fig. 3. ELIMCL detection of live *E. coli* O157:H7 serially diluted in buffered saline (TTBS+C). Live *E. coli* O157:H7 cells were serially diluted in TTBS+C and subjected to ELIMCL detection. The sample mixtures were subjected to ELIMCL as described and luminescent response versus cell concentration is displayed. Mean values and associated standard deviation are for triplicates acquired over at least four separate experiments.

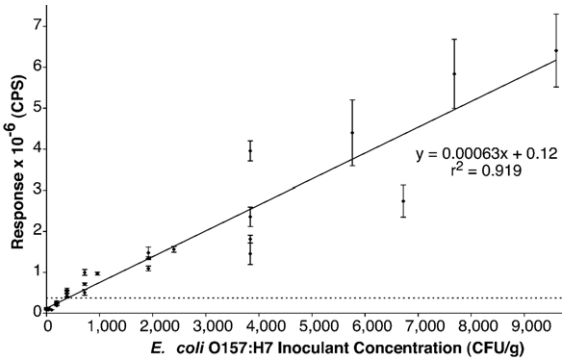

Fig. 4. ELIMCL detection of live *E. coli* O157:H7 in ground beef. Live *E. coli* O157:H7 cells were serially diluted in TTBS+C and inoculated into ground beef. The ground beef samples were culture enriched and subjected to ELIMCL as described. Luminescent response versus cell concentration (in CFU per gram of ground beef) is displayed. Mean values and associated standard deviation are for triplicates acquired over at least six separate experiments. (The dashed line represents the response derived from the line of regression at the approximate limit of detection or ca. 400 CFU/g.)

as represented by the dashed line in Fig. 4, whereas the BAM method conclusively produces either positive or negative results when carried out as directed), low and high inoculum levels of *E. coli* O157:H7, a non-motile strain of *E. coli* O157, and two potentially cross-reactive bacteria tested either in pure culture or relatively higher amounts were added to *E. coli* O157:H7 to challenge either in competitive growth of the bacteria or to elucidate potential cross-reactivity. The BAM method requires specific media for both growth enrichment and plate culture as well as several biochemical tests (including the indole and 4-methylumbelliferyl-β-D-glucuronide or MUG tests) and appearance of colonies on Levine's eosin–methylene blue or L-EMB agar for the successively selective determination of *E. coli* through confirmation of O157 and H7 serogroups of *E. coli* isolates via latex agglutination. Prior to this comparison, it was determined that the non-motile strain of *E. coli* O157 exhibited the same responses as *E. coli* O157:H7 for all of these preliminary (prior to O157 and H7 latex agglutination assays) screening tests (data not shown), and therefore, these tests were omitted for the remainder of this study. Furthermore, a shortened version of the BAM method allows for a 6 h enrichment in EEB, whereas in this study, this time was reduced to 5 h (as was used for the ELIMCL assay), a modification that appeared to have no negative effect on the final results obtained with the BAM method.

Results for a double-blind comparison between ELIMCL and BAM method for *E. coli* O157:H7 appear in Table 1. As was expected, the superior limit of detection

Table 1
Double-blind comparison of ELIMCL and US FDA's BAM for detection of live *E. coli* O157:H7 in culture-enriched ground beef

Inoculant (CFU/mL)	Co-Inoculant (CFU/mL)	BAM	ELIMCL [a]
E. coli O157:H7 (4)	(None)	+	−
E. coli O157:H7 (36)	(None)	+	+
E. coli O157 NM (1.0×10^4)	(None)	−	−
E. coli O157:H7 (36)	*E. coli* O157 NM (1.0×10^4)	−	−
E. coli O157:H7 (36)	*B. cereus* (5.8×10^3)	+	+
E. coli O157:H7 (36)	*S. typhimurium* (2×10^2)	+	+
B. cereus (5.8×10^3)	(None)	−	−
S. typhimurium (2×10^2)	(None)	−	−

[a] Results are based upon comparison of signal response to predetermined LOD.

for the BAM method, which uses plate culture-based detection, afforded it the ability to positively deduce the presence of *E. coli* O157:H7 at the lowest level (4 CFU/mL) of inoculum, whereas ELIMCL was only observed to detect the bacteria at the higher level (36 CFU/mL) of inoculation. Both methods were in agreement when tasked with *E. coli* O157 NM (non-motile), as well as either pure cultures of *B. cereus* or *S. typhimurium* or mixed cultures containing either of these latter two organisms at elevated levels, and *E. coli* O157:H7. Interestingly, both methods failed to detect *E. coli* O157:H7 in the presence of *E. coli* O157 NM. Given the assumption of identical growth rates for both *E. coli* serotypes and, hence, a culture population of *E. coli* O157 NM three orders of magnitude higher than *E. coli* O157:H7, explanations for this observation are as follows: (1) for ELIMCL, *E. coli* O157 NM out-competed *E. coli* O157:H7 for binding sites on the IMB in the sandwich immunoassay and (2) for BAM, part of the protocol requires manual removal and replicated anti-H7 latex agglutination testing of colonies from blood agar plates; with both *E. coli* serotypes producing identically similar colonies on blood agar, random selection of *E. coli* O157:H7 colonies that would otherwise elicit a positive anti-H7 result was statistically improbable.

McNemar's test was applied to the double-blind study results displayed in Table 1 and is presented in Fig. 5. Using this test, it was determined that there was no statistically significant difference between the results of ELIMCL and BAM for the selected series of experimental conditions undertaken.

4. Conclusion

The total assay time for ELIMCL detection was ca. 6.5 h. Though the immunoassay and chemiluminescent detection portion of the assay required only about 90 min, a 5 h culture enrichment was employed in order to increase the levels of bacterial analyte and, hence, improve the limit of detection for the assay. Using this technique, approximately 10^5–10^6 cells/mL of live *E. coli* O157:H7 were detected in buffered saline (TTBS +C). With culture enrichment employed to increase analyte concentration, ELIMCL was demonstrated to detect *E. coli* O157:H7 at an inoculation level of ca. 400 CFU/g in ground beef. Relative to other rapid methods that employ sensitive detection such as electrochemistry (Gehring et al., 1999), electrochemiluminescence (Yu and Bruno, 1996; Crawford et al., 2000), light-addressable potentiometric sensing (Gehring et al., 1998), and time-resolved fluorescence (Tu et al., 2004), the performance of ELIMCL for the detection of *E. coli* O157:H7 was rather poor. However, the detection limit for ELIMCL was comparable to the limit typically observed with ELISA (applied without culture enrichment) coupled with colorimetric detection for the detection of bacterial cells.

The authors of this investigation recognize that ELIMCL, as a rapid screening and potentially confirmation technique for *E. coli* O157:H7, has not been meticulously tested as the developed BAM method. This study seeks to suggest that, with future improvements including an improved limit of detection, this rapid, immunological-based method may eventually undergo rigorous testing in parallel with the BAM and other official *E. coli* O157:H7 confirmation methods (e.g., the USDA standard method for *E. coli* O157:H7) using actual field samples.

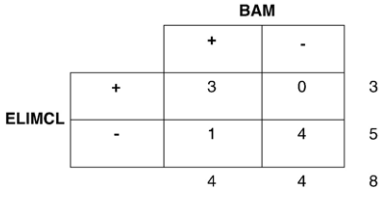

Fig. 5. McNemar's statistical analysis of the comparison of USFDAs BAM to ELIMCL for the detection of *E. coli* O157:H7 in ground beef. In a double-blind study, live *E. coli* O157:H7 cells were inoculated into ground beef containing various levels of potentially cross-reactive bacteria. The ground beef samples were culture enriched and subjected to BAM or ELIMCL as described. BAM samples were treated as specified by the published method whereas ELIMCL values were determined in triplicate. Tolerance for H_0 rejection if $\chi^2_{McNemar}$ is greater than 3.84 (critical χ^2 with one degree of freedom and the 95% confidence level).

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