Research paper

Enzyme-linked immunomagnetic chemiluminescent detection of Escherichia coli O157:H7

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Abstract

E. coli O157:H7 is a pathogenic microorganism that has been implicated in numerous cases of foodborne illnesses. A variety of rapid methods exist that show promise for the presumptive detection of this pathogen without the immediate need for incubating test samples for hours to days in microbial enrichment and culture media. In recent years, highly sensitive chemiluminescence has become a more affordable and portable detection method. Chemiluminescent detection has been coupled with the selectivity of antibodies, magnetic microparticle separation/isolation, and enzymatic signal amplification in order to develop a rapid method, termed enzyme-linked immunomagnetic chemiluminescence (ELIMCL). This work presents the application of ELIMCL to the detection of E. coli O157:H7 in pristine buffered saline with a detection limit of \(7.6 \times 10^3\) for live cells in approx. 75 min assay time. The blocking agent casein and the surfactant Tween 20 were used to lower background luminescence and thus maximize signal-to-noise ratios. After 5.5 h of enrichment culture, ELIMCL was demonstrated to detect E. coli O157:H7 inoculated in ground beef at 10 CFU/g in a total assay time of about 7 h.

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1. Introduction

Rapid bacterial detection methods have been developed as alternative food screening tests to lengthy and laborious, yet selective and highly sensitive conventional culture techniques (Feng, 1992; Hartman et al., 1992; Fung, 1995; Seo et al., 1998). Several biosensor-based methods have com-
bined the selectivity, potentially high surface area-mediated capture capability, and simplistic magnetic separation property of antibody-coated superparamagnetic microparticles or immunomagnetic beads (IMB) with highly sensitive instrumentation for the detection of molecular analytes or bacteria and include immunomagnetic-electrochemiluminescence (Crawford et al., 2000), light-addressable potentiometric sensing (Tu et al., 2002b), polymerase chain reaction (Sharma, 2002), and time-resolved fluoroimmunoassay (Tu et al., 2002a). In addition, some of these methods have combined the flexibility of IMB with both the rapidity and the sensitivity of chemiluminescence or bioluminescence for the specific detection of multi-antigenic analytes including mouse immunoglobulin G (Matsunaga et al., 1996) and bacteria (Tu et al., 1999).

In this study, we have applied enzyme-linked immunomagnetic chemiluminescence (ELIMCL) to the rapid detection of live *Escherichia coli* (*E. coli*) O157:H7 cells in either buffer or artificially contaminated ground beef. The ELIMCL methodology involved “sandwiching” of *E. coli* O157:H7 cells between IMB and alkaline phosphatase (AP)-conjugated antibody. Application of an external magnetic field was used to retain IMB (with or without bound bacteria) in polypropylene reaction tubes, chemiluminescent enzyme substrate (APS-5) then was added, and conversion to a light-emitting product was quantified using a luminometer. Ground beef has been selected as a target sample matrix due to numerous cases of association of the pathogenic bacteria, *E. coli* O157:H7, with outbreaks of food poisoning and/or hemolytic uremic syndrome cases linked to the commodity (Anonymous, 1997; Tuttle et al., 1999; Macdonald et al., 2000; Uhitil et al., 2001; Proctor et al., 2002).

### 2. Material and methods

#### 2.1. Materials

Materials used in this research included alkaline phosphatase (AP)-conjugated goat anti-*Escherichia coli* O157:H7 antibody conjugate (AP-Ab; 1 mg/ml) and heat-killed *E. coli* O157:H7 (positive control antigen) from Kirkegaard and Perry Laboratories (Gaithersburg, MD), *E. coli* O157:H7 B1409 (Centers for Disease Control, Atlanta, GA), goat anti-*E. coli* O157 M-280 immunomagnetic beads (IMB; Dynal, Lake Success, NY), Brilliant Green Bile broth (2%), plate count agar, and EC Medium (DIFCO Laboratories, Detroit, MI), BCM O157:H7(+) Plating Medium (Biosynth, IL, USA), novobiocin, Tris, and casein (Sigma-Aldrich, St. Louis, MO), Tween 20 (Acros Organics, Fairlawn, NJ), Stomacher Bags (Fisher Scientific; Pittsburgh, PA), and APS-5 (AP substrate 5; Lumigen, Southfield, MI). Other chemicals used were of reagent grade.

#### 2.2. Apparatus

All reactions with shaking were performed on a Vortex-Genie 2™ (Scientific Industries, Bohemia, NY). Luminescent measurements were conducted using an FB-12 single-tube luminometer (Zylux, Oak Ridge, TN). Immunomagnetic separations were performed with a Magnetic Particle Concentrator (MPC-S; Dynal). All reactions with rocking were done on a Speci-Mix (Barnstead/Thermolyne, Dubuque, IA). Enumeration of bacterial cells was conducted using a Petroff-Hausser counting chamber (Thomas Scientific, Swedesboro, NJ).

#### 2.3. Growth and enumeration of *E. coli* O157:H7

One milliliter of frozen *E. coli* O157:H7 stationary phase culture was freshly thawed and inoculated into 10 ml of EC broth and incubated at 37°C for 18 h with shaking at 160 rpm. A portion of the cells were pelleted by centrifugation at 10,000 rpm for 5 min and the pellet was resuspended in TBS (25 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 1% casein (hereinafter referred to as TTBS+casein) to 10−6 dilutions. A portion (100 μl) of the resuspension was spread plated onto plate count agar in triplicate, incubated at 37°C for 18 h, resultant colonies were enumerated, and the bacterial culture concentration was back calculated.

For generation of the growth curve, *E. coli* O157:H7 was grown overnight in EC broth for 18 h at 37°C as above, cell cultures were enumerated (Gehring et al., 1998) with a Petroff-Hausser counting chamber, serially diluted in TBS containing 1% casein to 350 CFU/ml, 1 ml was placed in a Stomacher Bag,
100 ml of Brilliant Green Bile broth was added, the bag, folded over, was incubated as above, and aliquots for time 0 and every 30 min thereafter were removed and plated onto plate count agar for enumeration as described.

2.4. ELIMCL detection of bacteria

All of the following reactions were conducted at room temperature. One milliliter of heat-killed or live *E. coli* O157:H7 bacteria, serially diluted in TTBS+casein to indicated concentrations, was placed in 1.5-ml polypropylene microcentrifuge reaction tubes that contained 20 μl of IMB. The mixture was rocked (minimal agitation sufficient to prevent settling of the IMB) for 30 min. Immunomagnetic separation of the IMB (and IMB bound cells) was performed by placing the tubes in the MPC-S for 3 min with rocking in order to trap the IMB (a portion containing bound bacteria when present) against the walls of the tubes and the liquid was removed by aspiration with a pipette and discarded. The IMB were resuspended by gentle vortexing with 1 ml of AP-Ab (diluted to indicated concentrations in TTBS+casein) and reacted with rocking for 30 min. The IMB again were separated using the MPC-S for 3 min and the liquid was removed as before. The IMB then were resuspended/washed once with 1 ml of TTBS+casein followed by magnetic separation for 3 min as above and then again using 1 ml TBS containing 1% casein.

Finally, the IMB were resuspended in 250 μl of APS-5 (equilibrated at room temperature) by gentle vortexing and the reaction tube (capped) was placed in the reading chamber of the FB-12. Sample generated light was measured using a single kinetic assay measurement mode for the FB-12 software with data points collected every 10 s over a 3-min total assay. (A schematic representation of ELIMCL is presented in Fig. 1.)

For detection of *E. coli* O157:H7 in ground beef, 1 ml of stationary phase cells (presumed to be 1×10⁹ cells/ml, the actual concentration was later confirmed via plate culturing on plate count agar and reported values/CFUs with associated standard deviations reflect adjustment from back calculation) diluted in TTBS+casein to 25 or 250 cells/25 g was added to 25 g of ground beef in a stomacher bag, hand massaged (5–10 s) to mix, 100 ml of selected, in part, for its ability to solubilize fat, Brilliant Green Bile broth was added and the bag was placed into a shaking incubator at 37 °C for 5.5 h. Inoculated Brilliant Green Bile reference samples were prepared as above but did not contain ground beef. A “matrix standard” sample was prepared by adding 50 ml of enriched 10 CFU/g reference sample to 12.5 g of ground beef in a Stomacher Bag and hand massaging (5–10 s) to mix. The matrix standard was not enriched any further. Aliquots were drawn from the filtrate side of the bags and either 1 ml was subjected to ELIMCL or 0.1 ml was spread plated onto both plate count agar and

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**Fig. 1.** Schematic representation of the enzyme-linked immunomagnetic chemiluminescent (ELIMCL) assay. Multiply immunogenic analyte (bacteria, for example) is sandwiched between antibody-coated superparamagnetic microparticles (immunomagnetic beads or IMB) and antibody–enzyme conjugate. IMB (with or without bound bacteria) are exposed to chemiluminescent substrate, and light emitted during product formation can be detected with a luminometer.
BCM agar for culture enumeration and confirmation of growth of *E. coli* O157:H7.

3. Results and discussion

The ELIMCL (Fig. 1) procedure entails the capture of multi-antigenic analyte (e.g., bacteria) by IMB, labeling with enzyme (e.g., AP)-conjugated antibody in a sandwich immunoassay format, and subsequent luminescent analysis after a brief reaction with a chemiluminescent enzyme substrate. Preliminary research revealed that combinations of IMB, bacteria, and AP-Ab led to high levels of background luminescence attributed to the non-specific binding of AP-Ab to the walls of the polypropylene reaction/measurement tubes. Control of non-specific binding was affected through the usage of the blocking agent casein and the detergent Tween 20 (data not shown). These initial experiments also revealed that IMB, unlike other ELIMCL reaction components with the exception of AP-Ab, contributed to the production of a significant amount of luminescent response, possibly due to substrate oxidation by exposed or released iron at the core of the polystyrene-coated IMB. Further investigation revealed that older lots of IMB, not used beyond their reported expiration date, appeared to contain a liquid phase constituent (present in residual wash of washed and magnetically separated IMB) that reacted with the AP substrate, APS-5 (data not shown). Both older and newer lots of IMB were commercially acquired and came already suspended in liquid that included 0.1% bovine serum albumin. Perhaps less pure bovine serum albumin, containing contaminant AP, was used in older lots. Regardless, the multiple separations/washes used in ELIMCL were sufficient to eliminate this potential contribution to background luminescence.

In order to maximize the dynamic response of ELIMCL for bacterial analyte, further preliminary experimentation saw the optimization of ELIMCL component amounts; especially required was determining the saturating concentration of AP-Ab. Optimal AP-Ab concentration studies were initially performed with APS-5 reacted with TBS-diluted AP-Ab. The chemiluminescent reaction produced a stable response for the time period in which data was acquired (Fig. 2A). Although better exhibited by Fig. 2B, both Fig. 2A and B not only show a linear range of detection for AP-Ab and a concentration that saturates response for the selected reaction conditions, but an unexpected decrease in luminescent response at beyond saturation levels of AP-Ab concentration. It was determined that the FB-12 instrument used, when marginally overwhelmed with too bright of a sample, reported misleading, yet surprisingly reproducible luminescent readings. This translates to applications of the FB-12 for sample testing will necessitate dilution of samples to ensure that measurements are not reported erroneously low as false negatives.

Using heat-killed *E. coli* O157:H7 cells, the ideal ELIMCL assay concentration of AP-Ab was empirically determined. Fig. 3A and B exhibits two graphical representations for determining the widest dynamic range for detection of the heat-killed cells at varying levels of AP-Ab concentration. Best levels of AP-Ab were found to be 1:85,000 and 1:65,000 dilutions or ±12 to 15 ng/ml, respectively. The former value, 1:85,000, was chosen for further ELIMCL assays.

The limit of detection (LOD) for ELIMCL with live *E. coli* O157:H7, serially diluted in pristine buffer (TTBS+casein), was determined (Fig. 4). The bacteria were detected over a linear range of 0 to approx. 5×10^5 CFU/ml. Though higher amounts could be detected, it is unlikely that such levels of contamination by these harmful pathogens would occur without notice. Regardless, unknown samples could be out of detection range and thus will require dilution (10- to 100-fold) for confirmation before assignment as a presumptive positive result. Using previously published concepts (Irwin et al., 2000), a conservative LOD for the data presented in Fig. 4 was determined to be 7.6×10^3 CFU/ml.

As with the pristine buffer study, an analysis of LOD was conducted for ELIMCL of live *E. coli* O157:H7 inoculated into ground beef that was enriched in Brilliant Green Bile broth for 5.5 h at 37 °C. In these experiments, *E. coli* O157:H7 inoculated Brilliant Green Bile broth (containing no ground beef) was used as a reference whereas fresh ground beef mixed with a portion of the 5.5-h enriched/incubated reference sample, yet not enriched any further, was used as a control or “matrix standard”. Using an analysis of variance (randomized complete-block design) (Steel and Torrie, 1960), ELIMCL responses...
Fig. 2. Chemiluminescent response of alkaline-phosphatase-labeled anti-\textit{E. coli} O157:H7 antibody conjugate reaction with APS-5 substrate. Serial dilutions of AP-antibody conjugate (AP-Ab; 1 mg/ml stock) were reacted with APS-5 chemiluminescent substrate. (A) displays the varying magnitude and stability of light production as luminescent response (relative light units per second or RLU/s) for different dilutions of AP-Ab in a kinetic assay up to 3 min. The legend indicates relative concentration of AP-Ab. (B) displays the luminescent response (at 1 min) for varying dilutions of AP-Ab.

\[ y = (1.0 \times 10^{15})x + 260,000 \]
\[ r^2 = 0.992 \]
Fig. 3. ELIMCL response for varying concentrations of heat-killed *E. coli* O157:H7 cells and AP-Ab. Heat-killed *E. coli* O157:H7 cells were serially diluted in TTBS+casein and subjected to ELIMCL as described in Materials and methods. (A) displays the luminescent response versus bacteria concentration reacted with varying amounts of AP-Ab and (B) displays luminescent response versus AP-Ab concentration at different levels of bacteria concentration. The legend for (A) indicates relative concentration of AP-Ab whereas the legend for (B) indicates *E. coli* O157:H7 cell concentration in CFU/ml.
for ground beef inoculated with 0 and 10 CFU/ml of *E. coli* O157:H7 were determined to be distinguishable thus demonstrating that *E. coli* O157:H7 could be detected in ground beef that minimally contained 10 CFU/g of the pathogen (Fig. 5). The matrix standard response indicated negligible contribution of the ground beef homogenate to the overall assay signal strength; this observation was further supported in that the reference and ground beef sample curves were not parallel. Therefore, the elevated response for the inoculated ground beef sample may be a result of the added nutrient (i.e. ground beef) stimulating growth of the *E. coli* O157:H7. Taken together, these results also indicated that there was negligible non-specific cross-reactivity of the antibodies and/or IMB used in ELIMCL with enriched non-*E. coli* O157:H7 contaminant microorganisms. Enriched ground beef samples were confirmed, via selective plate culture on BCM agar, to contain *E. coli* O157:H7 in levels that correlated with ELIMCL response (data not shown).

The results presented in Fig. 5 had an averaged correlation of variance of 62%. Upon further investigation, it was determined that a major contribution to this lack of reproducibility resided in the variability of the growth rate of the bacteria that occurred during culture enrichment. Under virtually identical conditions (starting cultures, reagents, technique, apparatus, time, temperature, etc.), growth curves for *E. coli*
O157:H7 were generated, in triplicate, in Brilliant Green Bile broth (no ground beef added) for each of six separate daily experiments (Fig. 6). The plots in Fig. 6 show that when samples were collected for analysis at 5.5 h of incubation, it can be construed that growth rate alone, even in the well-defined growth medium, contributed largely to variation in concentrations of bacterial analyte.

4. Conclusion

The total assay time, based on a single sample, was ~75 min for ELIMCL. However, since the samples may be concurrently reacted in multiple reaction tubes, only ~3 min is required for the analysis of additional samples. Using this technique, approximately 7.6×10^3 cells/ml of live E. coli O157:H7 were detected in TTBS+casein. This result is comparable to findings with other reported biosensor-based rapid methods including light-addressable potentiometric sensing (2.5×10^4 cells/ml; 30 min) (Gehring et al., 1998), enzyme-linked immunomagnetic electrochemistry (~4.3×10^3 cells/ml; ~80 min) (Gehring et al., 1999), multiplex polymerase chain reaction/agarose gel electrophoresis (1.3×10^4 CFU/ml; 2.75 h) (Deng and Fratamico, 1996), and immunomagnetic-electrochemiluminescence (~1×10^2 bacteria/ml; 1 h) (Yu and Bruno, 1996), as applied to the detection of E. coli.
O157:H7 in buffer and/or media. With culture enrichment, live E. coli O157:H7 was detected at an inoculation level of 10 CFU/g in ground beef samples in approx. 7 h using ELIMCL. The performance of ELIMCL for the detection of E. coli O157:H7 in culture-enriched ground beef samples again favorably compares with other reported methods including enzyme-linked immunomagnetic colorimetry (<1 CFU/g; 4–6 h) (Tu et al., 2001), fluorescent bacteriophage assay (2.2 CFU/g; 6 h) (Goodridge et al., 1999), flow cytometry/immunomagnetic bead separation (4 cells/g; 7 h) (Seo et al., 1998), and polymerase chain reaction (1 CFU/g; 12 h) (Cui et al., 2003).

One of the more notable results of this report derives from the practice of using growth enrichment to increase the concentration of analyte, namely, bacteria. Not only does this practice improve LOD (though, with the drawback of increasing overall assay time), but also it can be used to discern if a suspect contaminated sample contains live, targeted microorganisms. However, a major disadvantage of using enrichment is that the assay results are no longer quantitative and become qualitative, binomial (i.e., contamination is either present or not), or perhaps semi-quantitative at best. This is partly due to the adverse affects on growth rate (e.g., a major contribution to error reported in Figs. 5 and 6) that varying enrichment conditions (temperature, time, sample matrix, enrichment broth composition, likely existence of potentially competitive flora in typically higher concentrations than targeted bacteria in “field” samples, etc.) may yield. Consequently, relatively poor precision (within day) and reproducibility (day-to-day) was observed for the results exhibited by the relatively controlled conditions of the experiment presented in Fig. 5. Nevertheless, imposed “zero tolerance” limits for the presence of E. coli O157:H7 in foods precludes quantitation; hence, screened food samples that elicit outlying responses above background levels would be considered suspect.

Fig. 6. Growth curve for E. coli O157:H7 enriched in Brilliant Green Bile (BGB) broth. Live E. coli O157:H7 grown overnight in EC broth was serially diluted in TBS+casein to 350 CFU/ml. One milliliter was removed and culture enriched in BGB broth for 5.5 h. At time 0 and every 30 min thereafter, aliquots were removed, plated onto plate count agar, the culture plates were incubated overnight, and resultant colonies were enumerated. Each curve represents the mean and standard deviation of triplicate observations (bacterial counts in CFU/ml per enrichment time) for each of six daily experiments. (Inset is a plot of the main plot in Fig. 6 in which the bacteria concentration was log transformed.)
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