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Same-Day Detection of *Escherichia coli* O157:H7 from Spinach by Using Electrochemiluminescent and Cytometric Bead Array Biosensors[▽]

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Contamination of fresh produce with *Escherichia coli* O157:H7 and other pathogens commonly causes food-borne illness and disease outbreaks. Thus, screening for pathogens is warranted, but improved testing procedures are needed to allow reproducible same-day detection of low initial contamination levels on perishable foods, and methods for detecting numerous pathogens in a single test are desired. Experimental procedures were developed to enable rapid screening of spinach for *E. coli* O157:H7 by using multiplex-capable immunological assays that are analyzed using biosensors. Detection was achieved using an automated electrochemiluminescent (ECL) assay system and a fluorescence-based cytometric bead array. Using the ECL system, less than 0.1 CFU of *E. coli* O157:H7 per gram of spinach was detected after 5 h of enrichment, corresponding to 6.5 h of total assay time. Using the cytometric bead array, less than 0.1 CFU/g was detected after 7 h of enrichment, with a total time to detection of less than 10 h. These results illustrate that both biosensor assays are useful for rapid detection of *E. coli* O157:H7 on produce in time frames that are comparable to or better than those of other testing formats. Both methods may be useful for multiplexed pathogen detection in the food industry and other testing situations.

Sensitive detection and accurate identification of bacterial pathogens are important components of many public health applications. In particular, the food industry represents a prominent field in which faster and more reliable detection methods are desirable. Food-borne illness resulting from contaminated consumer products continues to be a serious problem (reviewed in reference 41), and numerous outbreaks attributable to contaminated fresh produce have been reported in recent years (6–8, 35). One particularly well publicized outbreak was caused by spinach that was contaminated with *Escherichia coli* O157:H7, resulting in at least 199 confirmed cases of infection, 102 hospitalizations, 31 cases of hemolytic-uremic syndrome, and at least 3 deaths (9).

E. coli O157:H7 is commonly associated with food-borne disease, and the illness can range from relatively mild to fatal, depending on the patient's age and overall health (4, 15, 41, 44). Historically, this pathogen has been associated with contaminated beef, but recently other food types have been implicated, such as cookie dough and spinach (5, 9, 41). *E. coli* O157:H7 is particularly problematic due to the low infectious dose, estimated at less than 100 cells (57, 66), and the fact that it is naturally carried by cattle and other animals (28, 46, 49, 64). If these animals gain access to agricultural fields, they can contaminate large volumes of produce due to commercial processing practices.

As a result of produce-related outbreaks, many suppliers adopted testing procedures in which samples are checked for common pathogens before being made available to the general public (11, 48). For such procedures to be useful, they must be sensitive enough to detect pathogen levels that are consistent with the infectious dose and amount of product consumed. *E. coli* O157:H7 outbreaks have been traced to foods contaminated with less than 0.5 organisms per gram (57, 60), and the sensitivity standard is now often set as low as one organism per 25 g (0.04/g) (48). Testing methods must also be rapid enough to allow screening of highly perishable foods while still allowing time for shipment, purchase, and consumption. Optimally, test duration would be 1 day shift or less (11). Finally, screening methods need to be both specific and reproducible, regardless of variations in the testing matrix. This is especially true when complex food samples are screened, since they can contain high loads of nonpathogenic microorganisms, dirt, and other substances.

Current established methods for detecting *E. coli* O157:H7 in leafy greens include the FDA-Bacteriological Analytical Manual (FDA-BAM) procedure (18) and several AOAC-validated commercial kits (1), which mainly include antibody- and PCR-based methods. The culture-based FDA-BAM procedure works well, but several days are required for detection and confirmation. The available commercial kits are more rapid, but there are concerns regarding their reliability for detecting low initial contamination levels in complex samples (11, 48). Specifically, immunological methods suffer from high detection limits ($\sim 10^5$ to 10^6 organisms) that may not be overcome by a short enrichment and can also have problems with cross-reactivity, in that nontarget substances may bind nonspecifically. PCR methods are more sensitive than immunological assays but are plagued by numerous technical issues in complex matrices. These include difficulties with cell lysis and nucleic acid extraction, cross-contamination, and failed reactions due to the presence of inhibitory substances and/or competing DNA from nontarget cells (40, 43, 50, 61, 65, 67). These issues can lead to inconsistent results and reduce the appeal of PCR as a reliable detection approach (50, 51, 58, 59, 61).

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As immunoassays are frequently easier to perform, faster, and cheaper than their nucleic acid-dependent counterparts, additional research and development are needed to improve sensitivity and reduce background interference, with regard to specific sample types. Although the major limitation of immunological techniques remains the antibody quality, other assay characteristics can be manipulated to improve assay performance overall (3, 33). Method optimization for sample preparation, enrichment, and concentration in combination with stronger/brighter signaling molecules and more sensitive detectors could significantly improve pathogen detection schemes.

Biosensors are devices for pathogen detection that generally consist of at least three elements, including a biological capture molecule (e.g., probes or antibodies), a method for converting capture molecule-target interactions into a signal, and a data output system (32, 54, 59, 61). Some biosensors can be automated, many have high-throughput capacity, and some methods also have multiplex capability. Examples of such methods include electrochemiluminescent (ECL) assays performed in 96-well plates and cytometry-based assays that use antibody-coated microspheres.

ECL detection is based on electrochemical stimulation of reporter molecules which undergo electron transfer reactions resulting in the emission of light. These reporter molecules, such as ruthenium(II) trisbipyridal [$Ru(bpy)_3^{2+}$], are attached to biological conjugates (e.g., antibodies) and, when stimulated by an electrode, allow detection at very low concentrations ($\leq 10^{-11}$ M) (27, 47, 56). Instrument-specific 96-well assay plates are available for use with fully automated commercially available ECL detection systems (MesoScale Diagnostics [MSD], Gaithersburg, MD). These plates are prespotted with up to 23 different antibodies, plus positive- and negative-control spots, providing potential for multiplexed pathogen detection.

Cytometric bead arrays (CBAs) use a fluidic method in which microspheres, individually coded using different ratios of red and infrared fluorophores, serve as the solid support for a sandwich-type immunoassay (xMAP technology; Luminex Corp., Austin, TX) (20, 26, 37, 39). Capture molecules are attached to the microspheres and bind the target, while an orange-fluorescent reporter molecule (*R*-phycoerythrin [R-PE]) is used to generate the signal. The reporter for immunoassays is an R-PE-tagged antibody or a biotinylated antibody used in combination with streptavidin-conjugated R-PE. The bead/target/reporter complexes are read by a specialized flow cytometer, which determines the identity of each individual bead (based on the combined red/infrared fluorescent molecules) and the target-associated signal on each bead (based on the orange fluorescence) (62). One hundred different sets of coded beads can be distinguished (16, 62), theoretically allowing detection of up to 100 targets in one sample. Superparamagnetic fluorescent microspheres can also be used to incorporate immunomagnetic separation (IMS) procedures.

To our knowledge, the utility of the two described systems for whole-cell pathogen detection has been tested only in a limited scope. Published reports indicate that ECL labels used in combination with IMS-based antibody capture of whole *E. coli* O157:H7 cells can provide sensitive detection (52, 68), but to date, immunoassays in prespotted plates have mainly been used in combination with manual systems for detecting antibodies and cytokines (20, 37, 56). Cytometric bead arrays, though more common, have been mostly used for clinical research (10, 21, 22, 42, 45, 53). For microorganism detection using cytometric bead arrays, most reported studies used probes linked to microspheres to detect amplified pathogen nucleic acid sequences (13, 14, 19). Kim et al. and Tamborrini et al. reported whole-cell pathogen detection using antibodies in combination with cytometric bead arrays, with detection sensitivity of around 10^3 to 10^4 organisms/ml (29, 55), and Kim et al. incorporated superparamagnetic microspheres into the bead array system for pathogen detection in various food matrices (30). Their conclusions suggest that immunological bead arrays could be a promising technique for food testing, but method development for specific matrices and pathogens is needed.

Herein we present methods for detecting *E. coli* O157:H7 from artificially contaminated spinach leaves by using automated ECL assays and cytometric bead arrays. Both methods are reliable for same-day detection of low-level *E. coli* O157:H7 contamination of spinach. Our observations further suggest that both methods should be amenable to successful pathogen detection in many other testing situations.

MATERIALS AND METHODS

Cultures. *E. coli* O157:H7 ATCC 35150 was selected for spinach experiments as it has been used for AOAC validations of commercial food testing procedures (2, 31). Cells were cultured on tryptic soy agar (TSA) overnight, serially diluted into phosphate-buffered saline (PBS; pH 7.4; 2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4), and enumerated via direct microscopic counts. Direct counts were used to quantify cells for assay validation studies. Final inoculum levels for all other experiments were verified via viable counts on TSA. Viable counts were determined for all experimental samples by using the drop plate method on sorbitol MacConkey agar containing cefixime and tellurite (CTSMAC) (24, 38).

Production of spinach rinse for method validation. Fresh spinach was purchased from local wholesale markets and processed within 1 day. Spinach rinse was generated and tested for background *E. coli* O157:H7 contamination according to established FDA-BAM procedures (18). Spinach was placed into a sterile stomacher bag, mixed with an equal weight of Butterfield's phosphate buffer, and gently agitated by hand for 5 min. The rinse liquid was collected, and 125 g was used for background testing, with the remaining volume being divided into aliquots and stored at −20°C until used. For background testing, 125 g fresh rinse was mixed with 125 ml prewarmed double-strength modified buffered peptone water with pyruvate (2×mBPWp) (18). Samples were incubated for 5 h at 37°C statically, antibiotics were added (10 μg/ml acriflavine, 10 μg/ml cefsulodin, and 8 μg/ml vancomycin), and incubation was continued for an additional 18 to 24 h at 42°C statically. Enriched samples were serially diluted and plated on CTSMAC and Rainbow Agar O157 (Biolog, Hayward, CA). Plates were incubated for 18 to 24 h at 37°C and examined for the presence of colonies with characteristic *E. coli* O157:H7 morphology. Each batch of spinach rinse was confirmed by the FDA-BAM culture method to be negative for the presence of *E. coli* O157:H7 prior to initiation of experiments. Microbial background loads for each spinach batch were determined by total aerobic counts obtained via plating serially diluted samples on TSA by using the spread plate method.

Optimization of *E. coli* O157:H7 detection by using enriched spinach rinse. Experimental procedures were optimized using time course assays on enrichment cultures containing *E. coli* O157:H7 spiked into spinach rinse at either low (0.07 to 0.13 CFU/ml), medium (0.7 to 1.3 CFU/ml), or high (7 to 13 CFU/ml) concentrations. Spiked and unspiked samples (40 ml) were mixed 1:1 with prewarmed 2×mBPWp and incubated at 42°C statically without antibiotics. Enrichment at 42°C without antibiotics served as the standard operating procedure (SOP) for all assays, except where direct analyses according to the FDA-BAM procedure are specified. The SOP conditions were chosen for short-term enrichments based on published reports indicating enhanced *E. coli* O157:H7 recovery at 42°C compared to that at 37°C (23, 25). Aliquots of enrichment cultures were

taken at all indicated time points (hourly), plated on CTSMAC, and processed for analysis using the ECL or IMS-cytometric bead array (IMS-CBA) assays.

Spinach leaf inoculation. Spinach leaves were inoculated in accordance with AOAC recommendations, and contamination levels were targeted to concentrations of 0.1 CFU/g (low dose) or 1 CFU/g (high dose) (17). Inoculum concentrations were adjusted so that 100 μl of inoculum was used per 100 g of spinach. Spinach was weighed into sterile bags and inoculated with *E. coli* O157:H7 by spotting the bacteria onto numerous different randomly chosen leaves. Two bags containing 350 g spinach each were inoculated per condition. Bags were closed, mixed, and stored for 2 days (40 to 48 h) at 4°C with mixing every 24 h to allow equilibration. Unspiked controls were spotted with PBS and stored as described for spiked samples.

Detection of *E. coli* O157:H7 recovered from spinach leaves. Time course assays were performed to determine minimum time-to-positive-result for *E. coli* O157:H7 detection. Spinach rinses generated according to the FDA-BAM procedure from unspiked, low-spike, or high-spike samples (2 bags each) were mixed, split, and processed both by the SOP method and by the standard FDA-BAM procedure. One hundred twenty-five grams of fresh rinse was mixed with 125 ml prewarmed 2×mBPWp for control and each spike level for the two incubation procedures and processed accordingly. Aliquots (11 ml) were removed from all samples at all indicated time points (hourly), subdivided and plated on CTSMAC, and further processed for downstream analysis via the ECL and IMS-CBA assays. For all spiked samples, randomly selected presumptive positive colonies were verified by the latex agglutination test for the O157 antigen (RIM *E. coli* O157:H7 LT kit; Remel, Lenexa, KS). All experiments included background testing of spiked and unspiked spinach samples according to the FDA-BAM procedure (described above).

Electrochemiluminescence assays. ECL assays were performed using the Sector PR2-1500 automated assay and prespotted *E. coli* O157:H7 single-plex plates (MSD) according to the manufacturer's instructions. Initial assay sensitivities were determined by serially diluting *E. coli* cells into PBS and spinach rinse as sample matrices. Prior to analysis of spinach rinse or enriched rinses, aliquots were removed, boiled for 30 min, and analyzed immediately (rinse samples) or stored at −20°C until assayed (enriched rinse samples). All samples were run in duplicate (spinach rinse) or triplicate (PBS and enriched samples), using 250 μl sample per reaction. For enriched samples, Butterfield's phosphate buffer/mBPWp, filtered spinach rinse/mBPWp, and blank (unspiked) samples/mBPWp were included as negative controls.

Immunomagnetic separation-cytometric bead array (IMS-CBA) assay. Detection methods were developed to incorporate IMS with the cytometric bead array by using MagPlexC magnetic microbeads (fluorescence classification region 33; Luminex). For all manipulations, beads were separated from liquid solutions by using the 3-in-1 magnetic particle separator (PureBiotech, LLC, Middlesex, NJ). Microspheres were coupled to anti-*E. coli* O157:H7 polyclonal antibody (KPL, Gaithersburg, MD) by using the amine coupling kit (Bio-Rad, Hercules, CA), per the manufacturer's instructions. Beads were blocked for 1 h at room temperature (24°C) with 25% horse serum in PBS-TBN (PBS plus 0.1% bovine serum albumin [BSA], 0.05% Tween 20, 0.05% sodium azide). For target binding, 1,000 blocked, antibody-coupled microbeads were added to 1 ml of sample and incubated for 1 h at room temperature with end-over-end rotation at 24 revolutions per min. Beads were separated from the supernatant and washed twice with PBS-TBN, followed by incubation with 250 μl biotin-labeled anti-*E. coli* O157:H7 antibody (4 μg/ml; KPL) in PBS-TBN for 30 min at room temperature with rotation. The supernatant was removed, and beads were washed once with PBS-TBN. For detection, the beads were incubated with 150 μl streptavidin-conjugated R-phycoerythrin (SA-PE; emission wavelength, 575 nm; 2 μg/ml; Invitrogen, Carlsbad, CA) for 10 min at room temperature with rotation. Beads were removed from the supernatant, resuspended in 100 μl PBS-TBN, placed into 96-well round-bottom plates, and analyzed using the BioPlex 200 reader (Bio-Rad), with excitation wavelengths for bead classification and reporter detection at 635 and 532 nm, respectively. The BioPlex 200 is a flow cytometer designed to read assays performed using xMAP technology. Readings were conducted and expressed as the median fluorescence intensity of 100 beads, as suggested by the manufacturer.

To determine detection limits, heat-killed *E. coli* O157:H7 was spiked into PBS-Tween 20 (0.05% PBST) and analyzed directly. For enriched samples, aliquots were removed at indicated time points, boiled for 30 min, and stored at −20°C until assayed. All assays were run in triplicate. Butterfield's phosphate buffer/mBPWp, filtered spinach rinse/mBPWp, and blank (unspiked) samples/mBPWp were included as negative controls.

Calculations. All assays were run in duplicate or triplicate, and average values for ECL and fluorescence signals were calculated for each sample within each experiment and across experiments ± standard deviations. For signal-to-noise (S/N) ratios, signals for test samples were divided by control signals. Unspiked enrichment samples were analyzed over different preparations of spinach rinse in separate experiments and averaged to determine appropriate S/N thresholds for positive detection in enrichment cultures. Positive S/N values were set at 3 times the average value for blank samples (see Results for details). Student's *t* test was used to determine if significant differences occurred between unspiked blank samples.

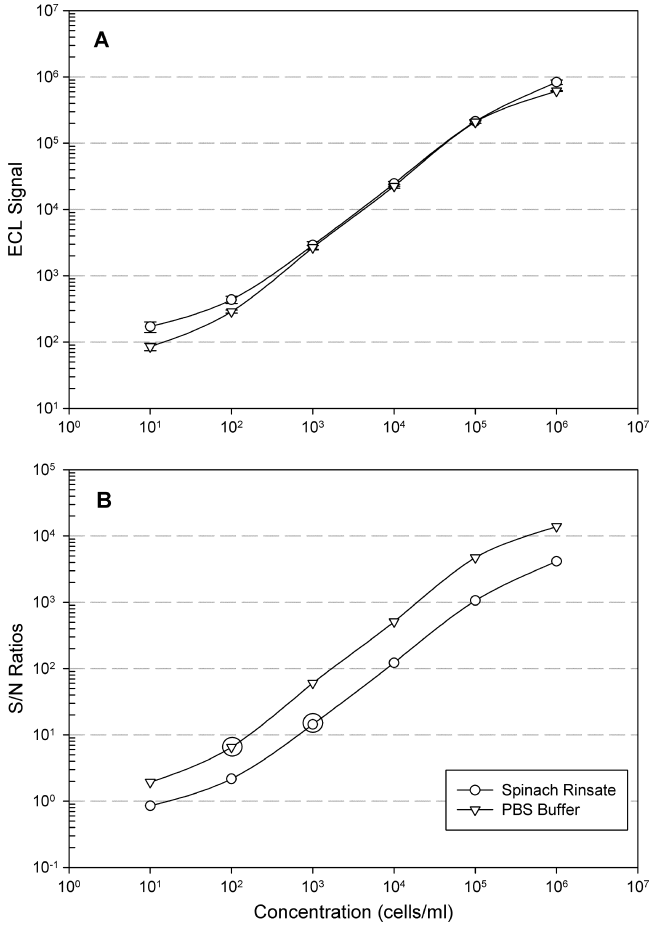

FIG. 1. *E. coli* O157:H7 detection in buffer and spinach rinse by using the automated ECL assay. *E. coli* O157:H7 was spiked into either PBS buffer or spinach rinse at increasing concentrations. (A) Raw ECL signals. *n* is 9 for PBS and 6 for spinach rinse samples. Error bars represent standard deviations but are indistinguishable from the symbols due to their small size. (B) Signal-to-noise (S/N) ratios were calculated by dividing the average signal for each concentration across all experiments by the average signal for the unspiked sample blank across all experiments. Encircled points indicate the lowest spike concentration exhibiting positive detection.

RESULTS

Detection limits for *E. coli* O157:H7 with ECL and IMS-CBA assays. Detection limits for *E. coli* O157:H7 diluted into PBS and spinach rinse were determined for the ECL assay. Samples contained increasing concentrations of *E. coli* O157:H7 ranging from 1×10^1 to 1×10^6 cells/ml, and S/N ratios were calculated in reference to the unspiked (blank) sample in each experiment. Sample ECL signals increased over blank signals starting at 10^2 cells/ml and continued to increase over the entire concentration range (Fig. 1A). A linear rela-

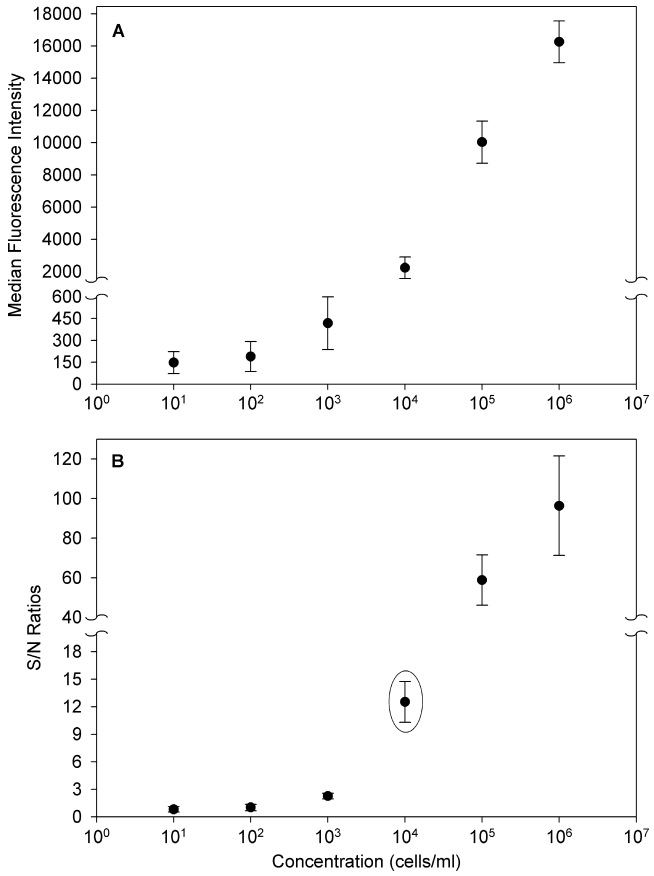

FIG. 2. *E. coli* O157:H7 detection in buffer by using the IMS-CBA assay. Heat-killed *E. coli* O157:H7 was diluted in PBST at increasing concentrations. (A) Median fluorescence intensities. (B) Signal-to-noise (S/N) ratios were calculated versus the unspiked sample. The encircled point indicates the lowest spiked concentration resulting in positive detection. *n* is 13 for blanks and 9 for all other samples. Error bars represent standard deviations between experiments.

tionship between cell concentration and signal generation was observed for both matrices ($R^2 = 0.96$ for PBS and 0.97 for spinach rinse). Assay saturation was achieved at 10^7 cells/ml (data not shown). Signal-to-noise ratios indicated that positive detection over background was achieved at 10^2 cells/ml in PBS and 10^3 cells/ml in spinach rinse (Fig. 1B).

E. coli O157:H7 detection limits for the IMS-CBA assay were determined using heat-killed cells diluted into PBST. Median fluorescence intensities increased at 10^3 cells/ml (Fig. 2A), and positive detection was noted at 10^4 cells/ml (Fig. 2B). In spinach rinse samples, excessive background noise was observed (not shown), and so samples were analyzed after one-to-one dilution in standard enrichment medium (mBPWp), and this treatment reproducibly reduced background signals. Observed *E. coli* O157:H7 detection limits in diluted spinach rinse were approximately 10^5 cells/ml, with an average S/N ratio of 15.1 (±3.4) over three experiments ($n = 9$).

Threshold settings. When analyzed using the ECL and IMS-CBA assays, detection limits for *E. coli* O157:H7 in spinach rinse are not sufficient to achieve desired detection thresholds. Thus, experiments were designed for detection in standard enrichment cultures containing spinach rinse in mBPWp. Based on preliminary assays, elevated background signals were anticipated in the spinach matrix, and so initial experiments evaluated baseline readings observed for relevant controls. Average baseline signals were determined for standard unspiked spinach rinse samples in enrichment medium (blanks), samples containing filtered spinach rinse in enrichment medium (filtered samples), and samples containing unused Butterfield's phosphate buffer in enrichment medium (buffer samples). Three different spinach rinse batches were analyzed in triplicate.

Average ECL signals were 155 (±24), 143 (±14), and 151 (±23) for buffer samples, filtered samples, and blank samples, respectively. Statistical comparisons indicated that there were no significant differences between the sample types (Student's *t* test, $P < 0.05$). In contrast, the different unspiked sample types exhibited variation in fluorescence signals when evaluated using the IMS-CBA assay. Median fluorescence intensities ranged from 306 to 1,245 for buffer samples, 1,014 to 2,146 for filtered samples, and 2,515 to 3,552 for blank samples. Variations in fluorescence signals were not due to technical error, since assay replicates within each experiment were similar, as were signals obtained from the same sample batch in multiple experiments when assayed using the same stock preparation of SA-PE. Direct comparisons of values across experiments were not possible due to different SA-PE stock preparations. However, paired statistical analyses indicated that fluorescence signals were statistically different between buffer samples, filtered samples, and blanks ($P < 10^{-5}$ for all comparisons), demonstrating the presence of matrix effects.

Matching blank samples are often not available for determining baseline signals when conducting field testing procedures. When using a completely standardized assay, it is often permissible to average baselines across different sample lots of known blanks to set a detection threshold. However, for the IMS-CBA assay, different SA-PE preparations can result in variations in raw fluorescence signals, and so individual assay controls are needed. The filtered sample reflects batch-to-batch variations in baseline signals and is also available for every experiment, and so it was selected for use as the baseline value for S/N ratio calculations in the IMS-CBA assay. For simplicity, filtered samples were also used as the baseline for ECL analyses, since all unspiked sample types were indistinguishable.

A threshold S/N ratio value for positive detection was set after evaluating signals from enrichment cultures containing six different batches of unspiked spinach rinse, in separate experiments. Average S/N ratio values for blank samples were determined, and the S/N ratio threshold was set at 3× the average. For ECL analyses, S/N ratio values averaged 1.5 (±0.7), and the threshold was set at 5. For the IMS-CBA assay, the average S/N ratio value was 2.4 (±1), and so the threshold was set at 7. These S/N ratio values were used to determine positive/negative results for all assays.

***E. coli* O157:H7 detection in enriched cultures of spiked spinach rinse.** *E. coli* O157:H7 was spiked into previously prepared batches of spinach rinse to determine minimal enrichment times required for positive detection. Three independent spinach rinse batches were analyzed in three separate experiments, with levels of endogenous background bacteria ranging from 1.3×10^5 to 2.4×10^6 CFU/ml, according to total

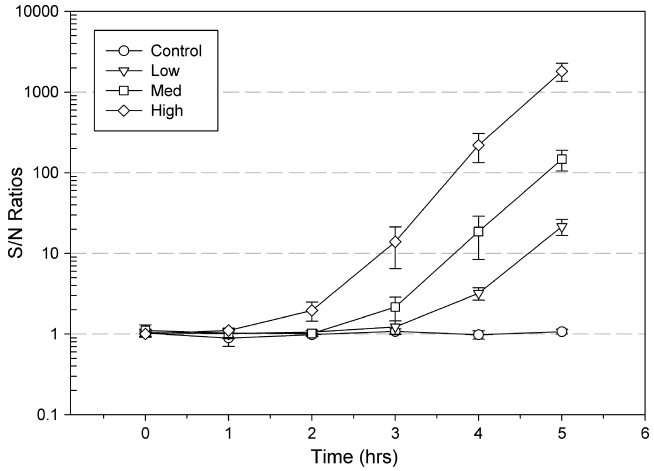

FIG. 3. *E. coli* O157:H7 detection in enriched spinach rinse by using the automated ECL assay. *E. coli* O157:H7 was spiked into spinach rinse and enriched in mBPWp. Signal-to-noise (S/N) ratios were calculated versus the filtered control. Samples were analyzed in triplicate in three independent experiments. Error bars represent standard deviations between experiments.

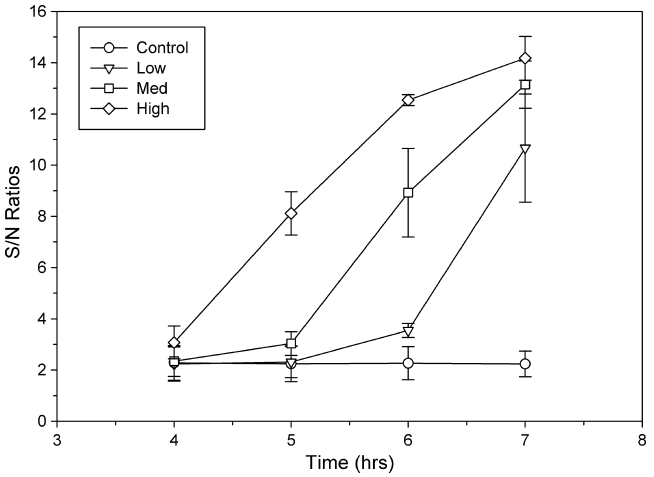

FIG. 4. *E. coli* O157:H7 detection in enriched spinach rinse by using the IMS-CBA assay. *E. coli* O157:H7 was spiked into spinach rinse and enriched in mBPWp. Signal-to-noise (S/N) ratios were calculated versus the filtered control. Samples were analyzed in triplicate in three independent experiments. Error bars represent standard deviations between experiments.

aerobic plate counts. Spinach rinses were spiked with *E. coli* O157:H7 at low (0.07 to 0.13 CFU/ml), medium (0.7 to 1.3 CFU/ml), or high (7 to 13 CFU/ml) concentrations and enriched for various times, as described above.

Viable counts indicated that *E. coli* O157:H7 exhibited an approximate 1-h lag phase and then grew by about 1 log per hour, when enriched under SOP conditions (42°C statically without antibiotics). In all three experiments, the low spike resulted in target cell concentrations in the range of 10^5 CFU/ml after 7 h of incubation, whereas the medium and high spikes resulted in 10^5 to 10^6 and 10^6 to 10^7 CFU/ml, respectively. This increase in cell number is similar to that reported for other *E. coli* O157:H7 strains when enriched under similar conditions (63). Negative-control plates containing enriched unspiked spinach samples exhibited no characteristic *E. coli* O157:H7 colonies at any time point.

ECL signals increased steadily for spiked samples starting at 2 to 3 h of enrichment and continued to increase throughout the time course (not shown). For the high, medium, and low spikes, S/N ratios versus those of filtered controls increased proportionally, with positive detection after 3, 4, and 5 h of enrichment, respectively (Fig. 3). Viable counts indicated that positive detection was achieved after target cell concentrations surpassed an average of 1.3×10^3 ($\pm 5 \times 10^2$) CFU/ml. ECL signals from all control samples remained low with stable S/N ratios (below 1.3) throughout the experiment.

The IMS-CBA assay was also successful in detecting *E. coli* O157:H7. In all three experiments, the high-, medium-, and low-spike samples produced increases in fluorescence signals resulting in positive detection after 5, 6, and 7 h of enrichment, respectively (Fig. 4). Positive detection in enriched samples occurred between 5×10^4 and 2×10^5 CFU/ml, depending on the particular batch of spinach rinse. Signals generated from unspiked controls did not vary significantly, with S/N ratios that remained at or below 3 in all experiments at all time points.

Detection of *E. coli* O157:H7 on artificially contaminated spinach leaves. Newly established protocols were further verified using artificially contaminated intact spinach leaves. In three independent analyses using three different spinach leaf batches, leaves were spiked with either low (0.046 to 0.1 CFU/g) or high (0.46 to 1.0 CFU/g) concentrations of *E. coli* O157:H7. Endogenous bacterial populations detected in wash from unspiked spinach leaves varied widely from batch to batch (8×10^4 to 7.2×10^6 CFU/ml) at time zero. At the completion of the enrichment period, these background populations ranged from 6.7×10^6 to 3.5×10^8 CFU/ml for the unspiked sample. For spiked leaves, selective viable counts indicated an approximate 6-log increase in target cell number, compared to the initial spike (7×10^4 to 1.6×10^6 CFU/ml) (Table 1). Separate duplicate rinse aliquots were processed exactly according to the FDA-BAM procedure, which confirmed that *E. coli* O157:H7 was absent from control leaves and present in spiked samples. Viable target counts obtained from FDA-BAM enrichments at the 7-h time point were 2 to 3 logs lower than those in SOP enrichments (2.4×10^2 to 4.1×10^3 CFU/ml), an observation that is consistent with previous reports (23, 25).

ECL signals and S/N ratios increased over baseline after 3 to 4 h of enrichment and continued to increase over the experimental time course (not shown). Signals for blank samples remained low and stable over the entire time course, with S/N ratios below 4 for all experiments at all time points. For spiked samples, positive detection at 3 h was sporadic and was not corroborated by viable count, as colony counts were below the statistical threshold for the drop plate method. However, for all experiments, 100% of tests resulted in positive detection after 4 and 5 h of enrichment for the high and low spikes, respectively (Table 1). The positive-detection S/N ratio threshold correlated with an average viable count of $5.1 \times 10^2 \pm 2.7 \times 10^2$ CFU/ml.

When the IMS-CBA assay was used, positive detection of

TABLE 1. Detection of *E. coli* O157:H7 on fresh spinach leaves by viable count, electrochemiluminescence, and cytometric bead array[a]

Enrichment time (h)	Low inoculation (0.046–0.1 CFU/g)			High inoculation (0.46–1.0 CFU/g)		
	Log_{10} CFU/ml (CTSMAC)	No. positive/no. tested		Log_{10} CFU/ml (CTSMAC)	No. positive/no. tested	
		ECL	IMS-CBA		ECL	IMS-CBA
3	TFTC	2/9	ND	TFTC	0/9	ND
4	1.80 ± 1.65	6/9	3/9	2.64 ± 2.45	9/9	3/9
5	2.87 ± 1.95	9/9	3/9	3.85 ± 3.47	9/9	3/9
6	3.82 ± 2.85	9/9	3/9	4.88 ± 4.61	9/9	9/9
7	5.00 ± 4.44	9/9	9/9	6.04 ± 5.80	9/9	9/9

[a] Abbreviations: ECL, electrochemiluminescent assay; IMS-CBA, immunomagnetic separation-cytometric bead array assay; TFTC, too few to count; ND, not determined.

high- and low-spike samples was achieved after a maximum 6 and 7 h of enrichment, respectively (Table 1). These time points correspond to an average viable count of $1.0 \times 10^5 \pm 1.4 \times 10^4$ CFU/ml. Although positive detection was observed at earlier time points in one experiment, it could not be corroborated by viable counts. For this individual spinach lot, increased signals for unspiked and filtered samples were also observed, with S/N ratio values for unspiked samples between 4 and 5 versus values below 2 for the other spinach batches. However, this change in baseline values was accounted for by the increased detection threshold S/N ratio value of 7, and all blank samples remained negative.

ECL signals correlate with target cell numbers. During the course of the experiments, a rough correlation between ECL signal and target cell concentration was noted. Once the target cell population achieved a threshold cell density of approximately 10^2 CFU/ml, the corresponding ECL signal maintained the same log value as the count (Fig. 5). While the exact values vary, clustering of values was observed to split based on whether or not they were above or below midlog. This trend was noted in both spiked rinse (Fig. 5A) and spiked spinach leaf (Fig. 5B) experiments. Similar trends were also found in spiked PBS samples with even less variability, due to the nonenrichment aspect of the spiked PBS (not shown).

DISCUSSION

Two new methods for *E. coli* O157:H7 detection are presented. Observed detection limits for the ECL assay were 10^2 CFU/ml in buffer and 10^3 CFU/ml in spinach rinse and enriched samples. Detection limits for the IMS-CBA assay were 10^4 cells/ml in buffer and 10^4 to 10^5 CFU/ml in enriched cultures, whereas assays for plain (undiluted) spinach rinse were not pursued due to increased background noise. It is likely that reduced background observed for enrichment cultures in the IMS-CBA assay is due to sample dilution and/or blocking effect from the enrichment medium.

The observed variability for viable counts in enrichment experiments is likely due to differences in spinach batches and variable recovery of target cells from spinach leaves by using the FDA-BAM washing method. The percent target removed from the leaves by washing is difficult to determine due to the low inoculum levels, and typical treatment, fate, or recovery studies inoculate spinach leaves with concentrations far above those used here (12, 34, 36). However, in spite of the variations in spinach batches and washing recovery, *E. coli* O157:H7 spiked onto spinach leaves at concentrations at or below 0.1 CFU/g was detected in less than 7 total hours by using the ECL assay (5 h of enrichment plus 1.5 h of assay) and less than 10 total hours by using the IMS-CBA assay (7 h of enrichment plus 2.5 h of assay) in 100% of samples.

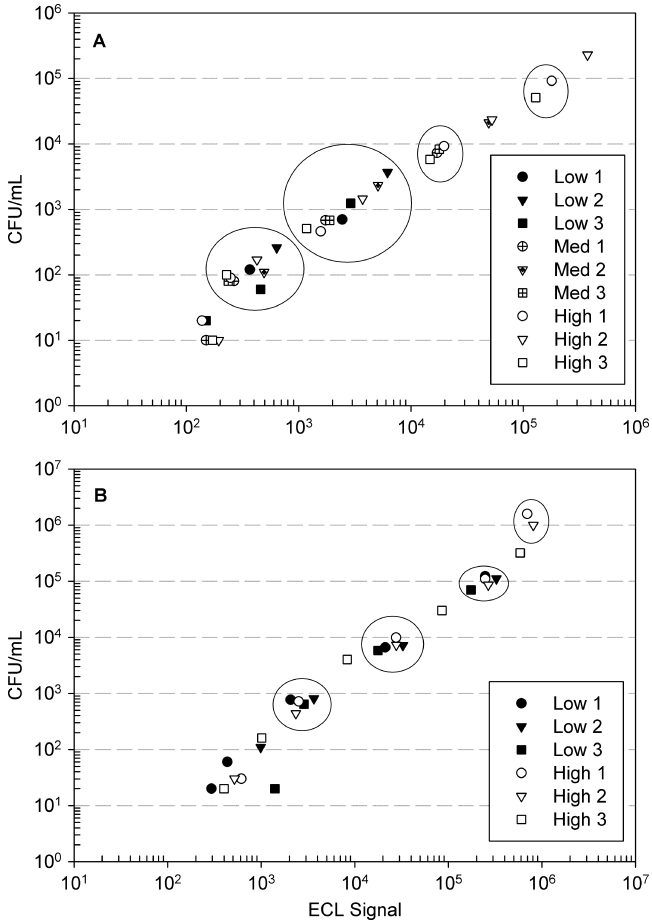

FIG. 5. Correlation of ECL signal with *E. coli* O157:H7 target cell concentration. ECL signals generated from both spiked spinach wash and spiked spinach leaves were compared against concentrations of *E. coli* O157:H7 in enrichment samples. (A) *E. coli* O157:H7 concentration versus ECL signal for spiked spinach rinse. (B) *E. coli* O157:H7 concentration versus ECL signal for spiked spinach leaves. Encircled clusters indicate correlated groupings. Data points represent the replicate average for each concentration within each experiment ($n = 3$).

D'Lima and Suslow reported a comparison of *E. coli* O157:H7 detection (0.4 CFU/g) in leafy greens by using commercially available kits (11). Four different kits were used, including two antibody and two PCR systems, with total assay times of approximately 8 to 11 h, including enrichment. Observed false-negative rates were 4 and 13% for PCR assays and 31 and 66% for the antibody tests (11). Although a direct comparison cannot be made due to different samples and pathogen strains across the two studies, our results suggest that ECL and IMS-CBA assays may represent useful alternatives to commercially available kits, as successful detection of lower starting concentrations was achieved in time frames that were comparable or shorter. Five- and seven-hour enrichments permitted complete avoidance of false-negative results for the ECL and IMS-CBA assays, respectively, and false-positive results for unspiked samples were not encountered in any assay. However, sporadic positive detection for spiked samples at early time points was observed and could represent false-positive outcomes since target cell numbers either could not be determined (due to low counts) or were below previously observed detection thresholds.

To our knowledge, detection limits for the ECL assay are superior to those of other antibody-based assays and rival detection limits obtained by commercially available PCR tests when used for complex samples. This method could significantly reduce detection times and also eliminate issues associated with PCR techniques. *E. coli* O157:H7 detection using the ECL system exhibits a wide linear signal range from approximately 10^2 to 10^3 CFU/ml to 10^7 CFU/ml, and a direct correlation between target cell concentration and ECL signal was observed, suggesting that the signal can be used as a rough indication for target concentration. This allows one to surpass the simple yes or no answers commonly encountered with pathogen detection methods and avoids lengthy culture-based enumeration schemes. The short (<1.5-h) assay duration also allows for near-real-time cell growth analysis, permitting inferences regarding cell viability via comparison of signals obtained from enrichment cultures over time.

The IMS-CBA assay was also successful for same-day *E. coli* O157:H7 detection, with detection limits comparable to those of other immunological assays. Increased detection limits in enriched spinach samples were due to increased background noise, which also necessitated identification of a suitable blanking mechanism. Filtered samples reflected batch-to-batch variations in background noise, indicating that a soluble element contributes to nonspecific signal, and so the filtered sample was used to determine baseline levels. An appropriate S/N ratio threshold was determined using different spinach lots. IMS-CBA assays performed on most batches of enriched spinach rinse (5 of 6) exhibited acceptable levels of background noise, but one spinach lot did exhibit an elevated noise level. The elevated background noise was not sufficient to result in a false-positive outcome for the unspiked spinach sample, but target cells in spiked samples were detected earlier, which may represent a false-positive result.

Observed detection limits for the IMS-CBA assay (10^4 cells/ml in buffer) are 1 log value higher than those reported by Kim et al. (30). This is due to different detection thresholds that were set. The previous report used limit-of-detection settings (the average of the blanks plus 3 standard deviations), whereas we used signal-to-noise values (sample signal divided by blank signal) and set a slightly higher cutoff, which was necessary to avoid false positives resulting from the spinach matrix. We observed more background noise than was previously reported, but this is likely due to the different means of sample preparation that were used, as we prepared spinach rinse by the standard FDA-BAM protocol and did not centrifuge samples to remove particulates. However, in spite of these slight variations, the overall results of the two studies are similar, and both conclude that cytometric arrays will be useful if matrix- and pathogen-specific protocols are developed. As suggested by Kim et al. (30), we included a short enrichment, which increased initially low pathogen concentrations to detectable levels.

The IMS-CBA assay is a useful method, but matrix-specific background levels should be determined for any given sample type by using lots shown to be devoid of the target by an established screening method (e.g., culture). If background is unacceptably high, improvements are possible by using different capture and detection antibodies, pretreating samples (diluting, centrifuging, etc.), or altering blocking and washing schemes. Subsequently, comparisons between buffer samples, filtered samples, and unspiked samples should allow identification of an appropriate S/N ratio threshold value.

Overall, the ECL assay is superior to the IMS-CBA assay in that it is more sensitive and rapid, is not subject to excessive background noise, and requires little human interaction. However, advantages to the IMS-CBA assay include more control over the experimental protocol and higher multiplex potential. In addition, the IMS-CBA assay incorporates standard IMS protocols, which allow greater flexibility and provide for further concentration of target organisms.

In summary, the ECL and IMS-CBA assays exhibit reliable same-day detection of low-level *E. coli* O157:H7 contamination on spinach. Initial inocula less than 0.1 CFU/g were detected in time frames that were shorter than or comparable to those of other commercially available detection methods. It is anticipated that other produce types, which are generally less problematic, might be easily screened for pathogens by using either method described herein. Both assays have the potential to be multiplexed for multitarget assays and should provide useful testing alternatives for a variety of situations.

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