International Journal of Food Microbiology 215 (2015) 1–6

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Level 2 validation of a flow cytometric method for detection of *Escherichia coli* O157:H7 in raw spinach

Anna J. Williams [a,*], Willie M. Cooper [a], Christine V. Summage-West [b], Lillie M. Sims [b], Robert Woodruff [c], Jessica Christman [c], Ted J. Moskal [d], Shawn Ramsaroop [e], John B. Sutherland [f], Pierre Alusta [a], Jon G. Wilkes [a], Dan A. Buzatu [a]

[a] U.S. Food and Drug Administration, National Center for Toxicological Research, Division of Systems Biology, Jefferson, AR 72079, USA
[b] U.S. Food and Drug Administration, National Center for Toxicological Research, Office of Scientific Coordination, Jefferson, AR 72079, USA
[c] U.S. Food and Drug Administration, Arkansas Regional Laboratory, Division of Microbiology, Jefferson, AR 72079, USA
[d] Ted Moskal, Life Sciences Consultant, 515 W. Matthews Ave., Jonesboro, AR 72401, USA
[e] Vivione Biosciences, LLC, Pine Bluff, AR 71602, USA
[f] U.S. Food and Drug Administration, National Center for Toxicological Research, Division of Microbiology, Jefferson, AR 72079, USA

ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 10 August 2015
Accepted 14 August 2015
Available online 21 August 2015

Keywords:
Flow cytometry
Microorganisms
Food pathogens
Public health
Escherichia coli O157

ABSTRACT

The Bacteriological Analytical Manual (BAM) method currently used by the United States Food and Drug Administration (FDA) to detect *Escherichia coli* O157:H7 in spinach was systematically compared to a new flow cytometry based method. This Food and Drug Administration (FDA) level 2 external laboratory validation study was designed to determine the latter method's sensitivity and speed for analysis of this pathogen in raw spinach. Detection of target cell inoculations with a low cell count is critical, since enterohemorrhagic strains of *E. coli* require an infective dose of as few as 10 cells (Schmid-Hempel and Frank, 2007). Although, according to the FDA, the infectious dose is unknown (Food and Drug Administration, 1993). Therefore, the inoculation level into the spinach, a total of 2.0 ± 2.6 viable *E. coli* O157 cells, was specified to yield between 25% and 75% detection by the new method, out of 20 samples (10 positives and 10 negatives). This criterion was met in that the new method detected 60% of the nominally positive samples; the corresponding sensitivity of the reference method was 50%. For both methods the most likely explanation for false negatives was that no viable cells were actually introduced into the sample. In this validation study, the flow cytometry method was equal to the BAM in sensitivity and far superior in speed.

1. Introduction

Many foodborne outbreaks due to *Escherichia coli* O157:H7 are associated with vegetables and fruits (including spinach, lettuce, coleslaw, salad) as a result of fecal contamination from domestic or wild animals at some phase during cultivation or handling (World Health Organization (WHO), 2011). Other modes of transmission to humans are through contaminated undercooked ground meat and raw milk; hand-to-mouth contact through the oral and fecal route; and contaminated water (World Health Organization (WHO), 2011). Food safety regulators, food producers, distributors, and retailers need effective microbiological methods with improved sensitivity, specificity, and speed for quality control purposes to eliminate or reduce product contamination by foodborne pathogens (American Type Culture Collection (ATCC), 2012).

E. coli strains that produce Shiga-like toxins cause diarrheal disease in humans (Paton et al., 1996). *E. coli* O157:H7, which produces Shiga-like toxins, is a serotype that is commonly associated with major pathogenicity and is implicated in many cases of foodborne illness in the United States (Chin, 2000). This organism can cause as high as 50% mortality in the elderly (Anon., 2009) and kidney failure in children (Reilly, 1998). Accordingly, sensitive and rapid detection of its presence in the food chain would be of great benefit to public health.

A variety of rapid methods for detecting *E. coli* O157:H7 in food have been developed to augment or enhance conventional methods (López-Campos et al., 2012). Some of these are based on bioluminescence, flow cytometry, immunology (e.g., enzyme-linked immunosorbent assay ELISA), fluorescent in situ hybridization (FISH), and quantitative real-time polymerase chain reaction (Q-PCR) (López-Campos et al., 2012). The latest U.S. Food and Drug Administration (FDA) Bacteriological

* Corresponding author.
E-mail addresses: anna.williams@fda.hhs.gov (A.J. Williams), willie.cooper@fda.hhs.gov (W.M. Cooper), christine.summage-west@fda.hhs.gov (C.V. Summage-West), lillie.sims@fda.hhs.gov (L.M. Sims), robert.woodruff@fda.hhs.gov (R. Woodruff), jessica.christman@fda.hhs.gov (J. Christman), tmoskal62@gmail.com (T.J. Moskal), sramsaroop@vivionebiosciences.com (S. Ramsaroop), john.sutherland@fda.hhs.gov (J.B. Sutherland), pierre.alusta@fda.hhs.gov (P. Alusta), jon.wilkes@fda.hhs.gov (J.G. Wilkes), dan.buzatu@fda.hhs.gov (D.A. Buzatu).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.08.011

Analytical Manual (BAM) chapter for detection of diarrheagenic *E. coli* in food, BAM 4a, specifies the use of PCR, although earlier methods based on selective media and colony morphology are also allowed when PCR instrumentation is not available (Feng et al., 2011). In all situations, the aim is to detect the offending pathogen as quickly and specifically as possible. Screening large sample volumes from a diverse assortment of fresh and processed foods necessitates that pre- and postharvest food safety procedures be sensitive, dynamic, specific, versatile, cost-effective, and easy to use (Gracias and McKillip, 2004).

New methods need to be compared and validated relative to a reference method (FDA, 2011). Under validation, the new method must exhibit equivalent or superior sensitivity and selectivity compared to the reference method. In a validation study, representative food matrix samples should be assessed in a double-blind experimental design (Feng et al., 2011).

Flow cytometry has been used in research since 1975 for bacterial and yeast cell analysis (Frelat et al., 1990). However, flow cytometry was not used routinely in industrial microbiology until 1988 (Laplace-Builhé et al., 1993). Flow cytometry combines sensitivity, specificity, and speed for high-throughput detection. It detects individual particles, distinguishing target cells from miscellaneous particles or non-target bacteria based on the combination of light signals scattered from or emitted by each cell. Detection of light scatter from a particle above a user-defined threshold is known as an event (Van der Vlist et al., 2012). The ability to detect a single cell enables direct examination of samples without involving a lengthy enrichment process, thus eliminating the need for overnight growth (Manti et al., 2010). It has been used in combination with fluorescent probe technology for rapid and specific detection and enumeration of bacteria in medical, veterinary and environmental microbiology (Manti et al., 2010).

The flow cytometer used in this study can quickly detect and enumerate a few cells of *E. coli* O157, differentiating them from other serotypes. Instrumental analysis takes 1 min with approximately 2 additional minutes required for an automated rinse procedure and loading of the subsequent sample.

Four personnel that were inexperienced in using the flow cytometer, only required 2 days to learn how to use the instrument. This also assisted in allowing faster sample processing than the standard regulatory method. The purpose of this study was to characterize and validate a new flow cytometry (FC) method developed by the FDA National Center for Toxicological Center (NCTR) for detection of *E. coli* O157:H7 in 25 g samples of spinach with comparison to the reference Q-PCR method described in the BAM Chapter 4a. Dr. Snyder (1998) cites references from a compilation of outbreak data, that *E. coli* O157:H7 has an infective dose ranging from 1–10 colony forming units (Paton and Paton, 1998; Schmid-Hempel and Frank, 2007; Food and Drug Administration, 1993). Therefore, the goal here was to demonstrate detection of only a few *E. coli* O157 cells in raw spinach, a perishable food for which minimal time-to-result (TTR) is required. This was accomplished by performing an external laboratory FDA level 2 validation. Results of this validation are detailed herein.

2. Materials and methods

2.1. Materials and methods of analysis

E. coli serotype O157:H7 isolate (ATCC 43895), originally isolated from an outbreak, which produces both Shiga-like toxins I and II (Feng et al., 2001), was used as a target strain. The experimental food source was raw spinach obtained as two pound bags (West Creek, Richmond, VA). The stock culture of *E. coli* was grown to stationary phase in Tryptic Soy Broth (Becton Dickinson and Company, DIFCO™) and used for preparation of dilutions in 1× Phosphate Buffered Saline (PBS) from 10^{-1} through 10^{-8}. The number of cells in the 10^{-8} dilution was later verified by plate count to be 2 ± 2.6 cells per 100 μL. This volume was used to inoculate 25 g of spinach for the validation before the plate count number was available, but the concentration of viable cells in the inoculum had been estimated as 1–4 per 100 μL using the flow cytometry method.

2.2. Validation experimental design

Based on the FDA Methods Validation Guidelines for Microbial Pathogens (pg. 8, FDA Foods Program and Science and Research Steering Committee), external laboratory level 2 validation requires 20 samples each for the standard and experimental methods, one inoculated and one non-inoculated, for a total of 40. The validation also calls for aging of the food matrix being validated, fresh spinach in this case. After inoculation, with either the bacterial dilution or phosphate buffered saline (PBS), 40 sealed bags, containing spinach were aged for 17.5 h in the refrigerator (3–4 °C). Twenty blank spinach samples and twenty low-level positive inoculations of spinach were analyzed by the flow cytometry method and the BAM 4a method. The most recent BAM 4a method uses a target-specific genetic probe for PCR amplification in both the initial detection and the confirmation steps (Feng et al., 2011).

Because the low inoculation was intended to demonstrate the detection limit for the novel flow cytometric method, range finding (published earlier in Buzatu et al., 2013) established the nominal failure of it at inoculation amounts so low that the most likely explanation was failure to introduce any target cells. Since the rapid and regulatory methods were being compared based on parallel samples rather than samples split after enrichment, comparison of sensitivity was possible only by recovery statistics; that is, of 10 or 20 nominally positive samples, what percentage was actually deemed positive by the two methods. The required sensitivity for the rapid method was equivalent or better recovery than either PCR or the alternative plate culture regulatory standard.

Samples resulting in a questionable population of bacteria, in the final gate (FL1–FL3), and since we were doing method validation based on blind sampling, were reprocessed to confirm accurate cell counts (data not shown) and avoid false positives/negatives. Reprocessing included an additional 48-hour incubation at room temperature and a 10^{-4} serial dilution of the sample before addition of the reagents, for detection of the bacteria.

2.3. Sample preparation details for the validation

Forty samples of spinach (25 g each) were weighed out and placed into sterile Whirl-Pak® filter bags (Nasco Whirl-Pak®). Twenty of the samples were inoculated with 100 μL of the 10^{-8} dilution of *E. coli* O157:H7 cells and 20 were controls, blanks inoculated using 100 μL of sterile 1× phosphate buffered saline (PBS) instead of target positive inoculum. The liquids were gently massaged into the spinach leaves of each sample. Simultaneous with spinach inoculation, triplicate plates of tryptic soy agar (TSA) were inoculated with the same volume of the 10^{-8} dilution and incubated overnight to confirm cell numbers by colony count. The 40 samples were placed inside a refrigerator set at 3 °C and aged overnight. The next morning, 20 of the samples, including 10 inoculated samples and 10 controls, were arbitrarily chosen, blind labeled, and assigned for processing using the reference method (BAM Chapter 4a, PCR method). The other 20, 10 of each kind, were assigned for processing using the FC method.

2.4. The flow cytometer

The flow cytometer was an Apogee Model A40 (Apogee Flow Systems, Hemel Hempstead, England, UK). This flow cytometer has 170 nm optical resolution, particularly useful in detecting small particles of the size of bacteria (FDA, 2011). The excitation source is a solid state 20 mW 488 nm laser. Fluorescence emission is detected at the standard FL1 = 525 nm, FL2 = 575 nm and FL3 > 610 nm wavelengths. Photomultiplier tubes are used for all light scatter and emission

detection. The electronic gains and voltages are pre-calibrated so that, for any specific flow cytometric assay (i.e., *E. coli* O157 in this case), the transmitted and excluded events are the same. This permits the sharing of gate definitions for these cytometry assays among model A40 instruments (Wilkes et al., 2012).

The appropriate gates for efficient detection were defined empirically based on the scatter or emission responses of a variety of *E. coli* O157 strains used as calibration standards, wherein samples were cultured and handled by the methods indicated in the 21 step protocol. Each gate had dimensions that would include all of the calibration samples but would exclude any events falling outside this range. The result of using so many gates so defined and used in series was the exclusion of non-qualified signals with an efficiency of about 99.999%, a specification necessary when analyzing for pathogens in complex matrices like food. By serial qualification, the first gate of a negative sample can show many signals from the background signal and the last one, used for counting true events, can show only zero or one qualified signal.

2.5. Bacteriological Analytical Manual sample processing

BAM 4a samples were processed using a modification of the standard regulatory procedure. Since only a 25 g sample of spinach, per bag, was used instead of the 200 g composite sample amount specified in the manual, a proportionally smaller volume of sterile PBS was added to each sample (i.e., 25 mL rather than 200 mL) before they were placed in a 37 °C incubator for 5 min. The rationale for this deviation from the prescribed regulatory standard was to avoid disadvantaging the sensitivity of the regulatory method by inoculating such a small number of cells and then diluting them into a large volume of PBS. The likelihood of recovery for such a dilute cell suspension would have been reduced relative to the rapid method because extreme dilution of cells provides extra stress and reduces recovery (Feng et al., 2011). Modification of the FC method for 200 g food samples would have been possible but would have compromised one of the rapid method's strongest features: e.g., low cost per analysis that allows individual 25 g samples rather than 200 g composite samples to be run. The decision to modify the BAM 4a was approved by FDA new regulatory method development authorities. All other processing using BAM 4a was the same as outlined in the manual (Feng et al., 2011). A few salient features are summarized in the following two paragraphs.

The 5-min incubation with shaking was executed so bacteria were dislodged into suspension from the spinach leaves. After this time, a 20 mL volume of 2× modified buffered peptone water pyruvate (mBPWP) (Remel, Labsource, Romeoville, IL) was added to each, before placing them back into the 37 °C incubator for 5 h. Volumes for the three antibiotics/inhibitors — acriflavine, celfsulodin, and vancomycin (MP Biomedicals, LLC, Solon, OH), which are all ineffective against *E. coli*, were also adjusted proportionally to produce a 45 mL total volume of each sample. At the 5-hour point, the inhibitors/antibiotics were added to depress competitive microflora and the samples were returned to the incubator for overnight selective enrichment.

BAM 4a sample analysis included real-time polymerase chain reaction (RT-PCR) using a SmartCycler® Real-Time PCR Thermal cycler, Cepheid™, Sunnyvale, CA, USA (Feng et al., 2011).

2.6. Flow cytometric processing

The spinach samples went through several processing steps before being analyzed on the flow cytometer. These processing steps were necessary to reduce or remove background fluorescence associated with concentrated spinach samples (Buzatu et al., 2013). In this validation, concentration of the samples was required and multiple steps were included so that incubation time could be reduced to 5 h and thus allow detection of the target bacteria at the earliest possible time.

Processing included pulsification to suspend the bacteria in liquid media, photobleaching, large volume centrifugation for concentration of cells with spinach debris, filtration and density gradient centrifugation for spinach debris elimination followed by addition of two reagents, Rapid-B™ Reagent A *E. coli* O157 for specific target cell detection and Rapid-B™ Reagent B (Vivione Biosciences, Pine Bluff, AR), which provides a mixture of accessory reagents (Buzatu et al., 2013, 2014). Reagent A contains fluorophore-labeled antibodies that bind selectively to cell surface epitopes associated with *E. coli* O157. Reagent B contains a red DNA dye, propidium iodide, that is only absorbed by bacteria if their cell membranes are compromised. When the red color appears, it shows that the cells are dead. Reagent B also contains cell surface conditioners and surfactants, is of general utility, serves for all flow cytometric pathogen-specific assays and allows for improved epitope access (Buzatu et al., 2014).

There were 22 steps involved in the validation setup and sample preparation (Buzatu et al., 2013). Step 13 in this validation, however, used a 20 μm Nylon vacuum filter (Millipore Corporation, Billerica, MA), before filtration with the 5 μm Polyvinylidene Fluoride (PVDF) syringe filter (Merck Millipore Ltd., Billerica, MA). A virulent strain of *E. coli* O157:H7 ATCC 43895 was used in this validation, as opposed to the avirulent strain in Buzatu et al.'s study.

3. Results

3.1. External laboratory level 2 validation, flow cytometry data acquisition protocol

Two employees with the Arkansas Regional Laboratory (ARL in Jefferson, Arkansas), performed the NCTR flow cytometry and BAM assays. The NCTR Laboratory staff and the ARL analysts were blinded to the identity of the samples (Figs. 1 and 2).

Triplicate plate counts later confirmed an average of 2.0 ± 2.6 colony-forming units/100 μL. Neither BAM 4a nor the flow cytometry methods reported false positives. BAM 4a reported 5 positives as negatives, whereas the FC method reported 4 positives as negatives (Table 1). In both BAM 4a and the FC method, false negative results are potentially explained by the low inoculation level: i.e., 100 μL of the nominally positive spiking solutions actually contained no target cells, resulting in a negative result.

The low level positive inoculation was chosen to demonstrate the detection limit for the flow cytometry method, in which 2.0 ± 2.6 viable cells/25 g of spinach could be detected in 25–75% of the inoculated samples. Range finding established the nominal failure of the FC method at inoculation concentrations so low that the most likely explanation was failure to introduce any target cells. (i.e., if one inoculates at an estimated concentration of only 2.0 ± 2.6 viable cells, there is an almost equal probability that any particular inoculum contains zero *E. coli* O157 cells as it is that one or more were successfully applied to the spinach). The low level inoculation indicates an average inoculation of 2 cells plus a calculated standard deviation of 2.6 cells. This indicates an inoculation range of −0.6 to 4.6, which indicates that some of the inoculated samples, may not have actually been inoculated with any *E. coli* O157 cells at all.

Optimal growth media and enrichment temperatures for the FC method differed from those of the regulatory method. This affected experimental design eliminating possible use of samples split after enrichment, necessitating the use of parallel samples. Since the NCTR and regulatory method were being compared based on parallel samples, comparison of sensitivity was possible only by recovery statistics. That is, the number of samples correctly identified as positive by the two methods were compared out of 10 positive samples per method, with each sample likely to contain at least one CFU *E. coli* O157:H7. The required sensitivity for the FC method was equivalent or better recovery than the regulatory standard.

A single cell successfully inoculated might have suffered an extended lag phase, potentially leading to a lack of recovery and growth during the short 5-hour enrichment period. For example, NCTR sample 1 included at least one *E. coli* O157 cell, because a later reprocessing (data

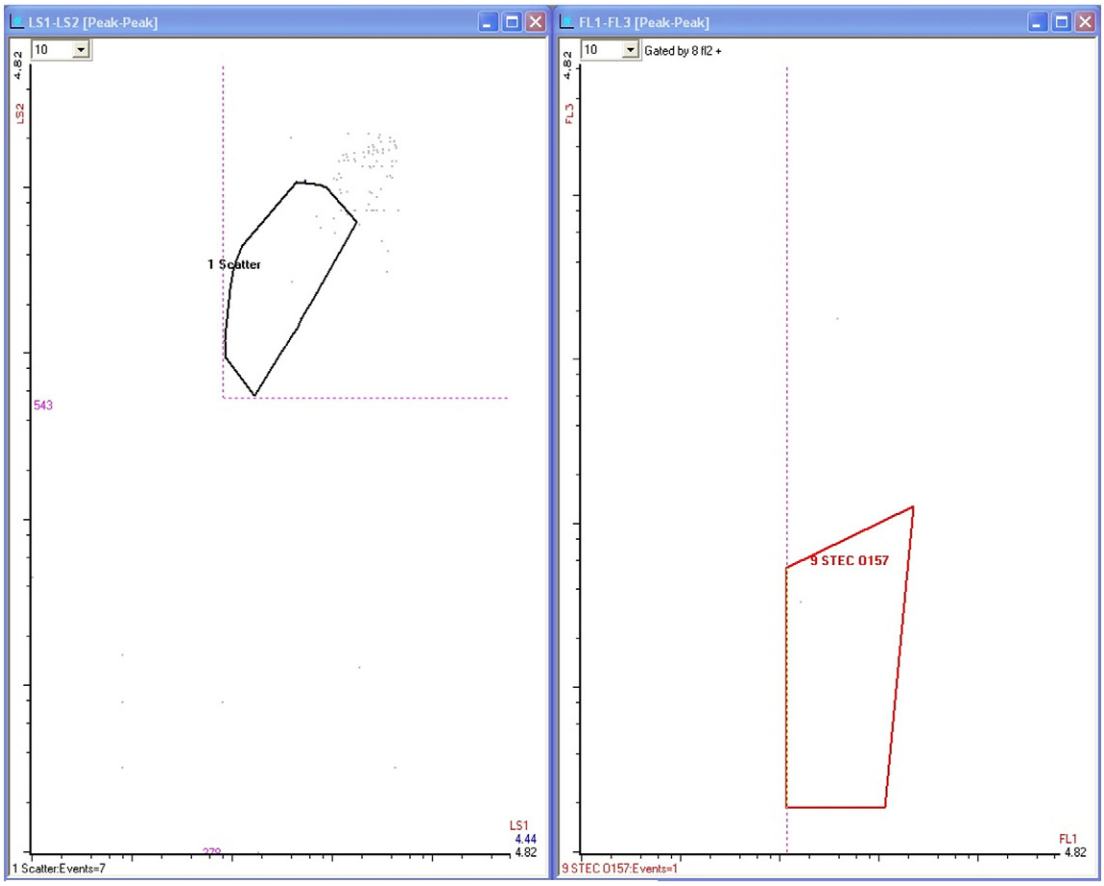

Fig. 1. This figure illustrates the appearance of two dimensional light scatter and fluorescence dot plots typical for an *E. coli* O157 negative sample in this validation experiment.

not shown) after further growth, indicated that this sample was actually positive. This likely happened because there was only one cell initially and it was slow in the beginning to multiply. This interpretation argues for lengthening enrichment time and consequently, a 6.5-hour enrichment was incorporated into further validation studies (data not shown).

In summary, BAM 4a reported five of ten and the FC method, four of ten nominal false negatives. These results are shown in Table 1.

4. Discussion

4.1. Fast flow cytometric method using antibody specific reagents

Developing rapid assays for food pathogen detection is needed to decrease the time required to get accurate results and to increase surveillance throughput. Traditional or conventional bacteriological methods typically take several days to determine the presence of a pathogen or toxin in a particular food (López-Campos et al., 2012) and this can be problematic to all concerned. Various strategies, such as antibody-based as well as chemical- or physical-based methods, have been developed to detect pathogens from numerous sample matrices (Bhunia, 2008; Stevens and Jaykus, 2004). In this study, we used an antibody-based method, in conjunction with flow cytometry, to detect the presence or absence of the food pathogen *E. coli* O157:H7 in spinach. This technique proved to be fast and very specific using reagents specifically developed for the target bacteria.

The validation showed that the FC method was as sensitive as BAM 4a with PCR detection at an inoculation level of 2.0 ± 2.6 cells per 100 μL but much faster than the BAM 4 method (9 h TTR for the FC method vs 51 h for BAM). The flow cytometry method incorrectly identified 4 of 10 nominally positive samples as negatives. Only one of the 4 was a true positive, based on the reprocessing of the samples after an additional 48 h incubation. BAM incorrectly identified 5 of 10 nominally positive samples as negatives. The BAM method had no way of reprocessing suspect samples that may have been incorrectly identified. These results indicate that the flow cytometry based method is at least as sensitive as the reference method.

For any sample that the FC method determined as positive, there were at least 14 viable cells, but typically many more, average 695 ± 544, n = 6. Each sample determined as negative had from 0 to 4 counts, 1.2 ± 1.5, n = 13. Based on the sample key, these low single-digit counts were correctly classified as negatives. The averages 695 and 1.2 generate a ratio of 579 for distinguishing positive from negative samples. This signal to noise assumes that all the positive samples were able to grow successfully in the 5 h enrichment period, which did not happen for one flow cytometry sample (i.e., sample 1).

4.2. Advantages using the flow cytometric method

Compared to the BAM method to determine the presence of *E. coli* O157 in spinach, the advantages of the FC method are that it (1) takes much less time, (2) is less labor intensive, and (3) is as accurate as BAM. This 22-step, 9-hour flow cytometry method/protocol is useful as a screening tool to detect pathogens in foods (Buzatu et al., 2013; Wilkes et al., 2012). One might use this method in cases where the food matrix consisted of high concentrations of fats or particulate matter, because it is effective for eliminating or decreasing the high numbers of non-target fluorescent food particles detected by the flow cytometer. The addition of phloxine B followed by light exposure bleaches color from foods (Buzatu et al., 2013). Elimination of food background fluorescence makes it easier to detect a small number of target organisms tagged with a fluorophore.

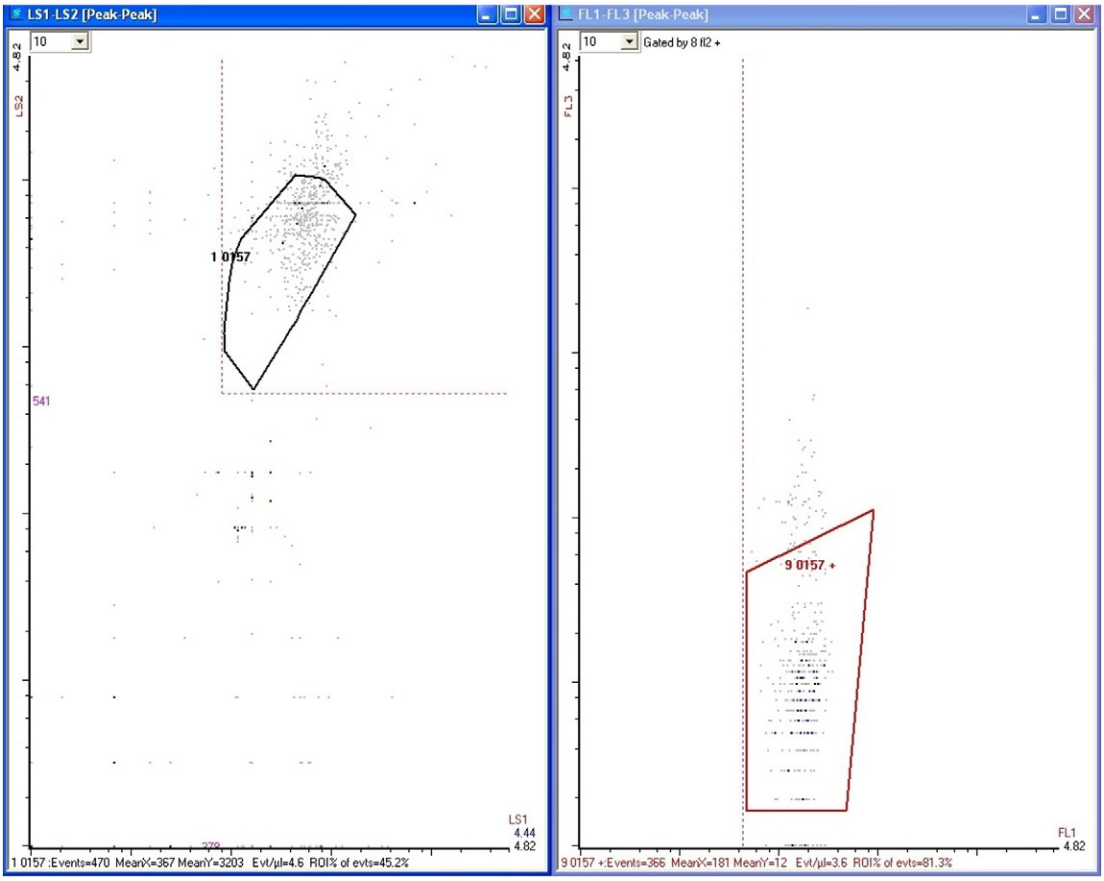

Fig. 2. This figure displays the same gates as in Fig. 1 for a typical *E. coli* O157 positive analysis.

The NCTR flow cytometry-based system was able to detect as few as 1 cell per 100 μL in a 25 g amount of spinach and process 20 samples in about 9 h. These times suggest use of the system in factory/shift work schedules that would allow for screening of many food samples in a two shift, 16-hour period. The time savings and higher sample monitoring throughput would help to decrease the number of cases of illness resulting from ingestion of contaminated spinach by the food pathogen *E. coli* O157:H7.

Table 1
External laboratory level 2 *E. coli* O157 in spinach validation flow cytometer vs. Bacteriological Analytical Manual results.

Sample number	Flow cytometer counts	Flow cytometer results*	BAM results	†Nominal sample identification
1	1	–	+	+
2	3	–	–	–
3	0	–	–	–
4	14	+	–	+
5	55	+	+	+
6	1	–	+	+
7	0	–	–	–
8	1	–	–	–
9	120	+	+	+
10	3	–	–	–
11	146	+	–	+
12	4	–	–	–
13	32	+	+	+
14	0	–	–	–
15	0	–	–	+
16	0	–	–	–
17	59	+	–	+
18	3	–	–	–
19	1	–	–	–
20	0	–	–	+

Bold red "−" symbols indicate a false negative (i.e., the result obtained after processing of the sample was incorrect compared to that of the nominal sample identification).
*Results indicate the presence (+) or absence (−) of *E. coli* O157.
†Indicates nominal identity, see text under Validation experimental design and Discussion sections.

5. Conclusions

The flow cytometry method (FC) provided results in a fraction of the time required for the BAM procedure (9 h total time-to-results for the FC method, compared to 51 h total time-to-results for BAM). It produced no false positives among the sample blanks. In regard to the validation, when compared to the PCR regulatory method, among ten nominal positives (containing *E. coli* O157) inoculated with 2.0 ± 2.6 viable cells per sample, the FC method reported 6 of 10 as positive whereas on parallel samples the PCR method reported 5 of 10 as positive. Both of these results on very low level positive inoculations could be reasonably explained, in all but the first FC method sample, by the likelihood that the "false negatives" actually never contained any viable *E. coli* O157 cells. These results do not demonstrate a statistically significant difference, so no claim is made based on them that the flow cytometry based method showed greater sensitivity than the BAM 4a with PCR. It did, however, reduce detection time.

Disclaimers

Use of trade names in this publication does not imply endorsement by the U.S. Food and Drug Administration of Vivione Biosciences, LLC products named nor criticism of similar products not mentioned. The views presented do not necessarily reflect those of the Food and Drug Administration.

Acknowledgments

We wish to thank Ms. Gwendolyn Anderson, Ms. Joanna Deck, and Ms. Deborah Bass for providing supplies and advice. We also thank Drs. Fatemeh Rafii, William (Woody) Tolleson, and Mark Hart for their time in reviewing the manuscript.

References

American Type Culture Collection (ATCC), 2012. Setting the standard with "Big Six" non-O157 STEC reference materials October/November, Food Saf. Mag. 42 (Retrieved August 1, 2013 from www.foodsafetymagazine.com).

Anon., 2009. The Center for Food Security and Public Health. Enterohemorrhagic *Escherichia coli* Infections. Iowa State University, pp. 1–10 (Retrieved April 14, 2014 from www.cfsph.iastate.edu/Factsheets/pdfs/e_coli.pdf).

Bhunia, A.K., 2008. Biosensors and bio-based methods for the separation and detection of foodborne pathogens. Adv. Food Nutr. Res. 54, 1–44.

Buzatu, D., Cooper, W.M., Summage-West, C., Sutherland, J.B., Williams, A.J., Bass, D.A., Smith, L.L., Woodruff, R.S., Christman, J.M., Reid, S., Tucker, R.K., Haney, C.J., Ahmed, A., Rafii, F., Wilkes, J., 2013. Photobleaching with phloxine B sensitizer to reduce food matrix interference for detection of *Escherichia coli* serotype O157:H7 in fresh spinach by flow cytometry. Food Microbiol. 36, 416–425.

Buzatu, D.A., Moskal, T.J., Williams, A.J., Cooper, W.M., Mattes, W.B., Wilkes, J.G., 2014. An integrated flow cytometry-based system for real time, high sensitivity bacterial detection and identification. PLoS ONE 9 (4), e94254. http://dx.doi.org/10.1371/journal.pone.0094254.

Chin, J. (Ed.), 2000. Control of Communicable Diseases Manual, 17th ed. American Public Health Association, Washington (624 pp. (selected excerpts). www.mtpinnacle.com/pdfs/ccdm.pdf (accessed Aug. 2015)).

Feng, P., Dey, M., Abe, A., Takeda, T., 2001. Isogenic strain of *Escherichia coli* O157:H7 that has lost both Shiga toxin 1 and 2 genes. Clin. Diagn. Lab. Immunol. 8, 711–717.

Feng, P., Weagant, S.D., Jinneman, K., 2011. Bacteriological Analytical Manual, Chapter 4A: Diarrheagenic *Escherichia coli*. (Retrieved August 1,2013 from www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm).

Food and Drug Administration, 1993. HACCP. Regulatory Food Applications in Retail Establishments. Department of Health and Human Services. Division of Human Resource Development, HFC-60, Rockville, MD.

Food and Drug Administration (FDA), Department of Health and Human Services, 2011. FDA Methods Validation Guidelines for Microbial Pathogens [Memorandum] September, Foods Program Science and Research Steering Committee (www.fda.gov/downloads/ScienceResearch/FieldScience/UCM273418.pdf (accessed Aug. 2015)).

Frelat, G., Laplace-Builhé, C., Grunwald, D., 1990. Microbial analysis of flow cytometry: present and future. Flow Cytometry: Advanced Research and Clinical Applications vol. 2. CRC Press, Inc., Boca Raton, FL, pp. 225–279.

Gracias, K.S., McKillip, J.L., 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. Can. J. Microbiol. 50, 883–890.

Laplace-Builhé, C., Hahne, K., Hunger, W., Tirilly, Y., Drocourt, J.L., 1993. Application of flow cytometry to rapid microbial analysis in food and drinks industries. Biol. Cell. 78, 123–128.

López-Campos, G., Martínez-Suárez, J.V., Aguado-Urda, M., López-Alonso, V., 2012. Microarray Detection and Characterization of Bacterial Foodborne Pathogens: Springer Briefs in Food, Health, and Nutrition. Springer Science and Business Media, LLC, New York, NY.

Manti, A., Falcioni, T., Campana, R., Medina, V., Dominici, S., Papa, S., Baffone, W., 2010. Detection of environmental *Vibrio parahaemolyticus* using a polyclonal antibody by flow cytometry. Environ. Microbiol. Rep. 2, 158–165.

Paton, J.C., Paton, A.W., 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11 (3), 450–479.

Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A., Paton, J.C., 1996. Molecular microbiological investigation of an outbreak of hemolytic–uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. J. Clin. Microbiol. 34, 1622–1627.

Reilly, A., 1998. Prevention and control of enterohaemorrhagic *Escherichia coli* (EHEC) infections: memorandum from a WHO meeting. Bull. World Health Organ. 76 (3), 245–255.

Schmid-Hempel, P., Frank, S.A., 2007. Pathogenesis, virulence, and infective dose. PLoS Pathog. 3 (10), e147 (http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.0030147).

Snyder, O.P., 1998. *Escherichia coli* O157:H7 and Other Pathogenic Strains of *E. coli*. Hospitality Institute of Technology and Management (Retrieved July 15, 2015 from www.hi-tm.com/Documents/Ecoli98.html).

Stevens, K.A., Jaykus, L.A., 2004. Bacterial separation and concentration from complex sample matrices: a review. Crit. Rev. Microbiol. 30, 7–24.

Van der Vlist, E.J., Nolte-'t Hoen, E.N.M., Stoorvogel, W., Arkesteijn, G.J.A., Wauben, M.H.M., 2012. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. Nat. Protoc. 7, 1311–1326.

Wilkes, J.G., Tucker, R.K., Montgomery, J.A., Cooper, W.M., Sutherland, J.B., Buzatu, D.A., 2012. Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. Food Microbiol. 30, 281–288.

World Health Organization (WHO), 2011. Enterohaemorrhagic *Escherichia coli* (EHEC) Fact Sheet Retrieved April 14, 2014 from www.who.int/mediacentre/factsheets/fs125/enl.