Development of a Flow Cytometry-Based Method for Rapid Detection of *Escherichia coli* and *Shigella* Spp. Using an Oligonucleotide Probe

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Abstract

Standard methods to detect *Escherichia coli* contamination in food use the polymerase chain reaction (PCR) and agar culture plates. These methods require multiple incubation steps and take a long time to results. An improved rapid flow-cytometry based detection method was developed, using a fluorescence-labeled oligonucleotide probe specifically binding a 16S rRNA sequence. The method positively detected 51 *E. coli* isolates as well as 4 *Shigella* species. All 27 non-*E. coli* strains tested gave negative results. Comparison of the new genetic assay with a total plate count (TPC) assay and agar plate counting indicated similar sensitivity, agreement between cytometry cell and colony counts. This method can detect a small number of *E. coli* cells in the presence of large numbers of other bacteria. This method can be used for rapid, economical, and stable detection of *E. coli* and *Shigella* contamination in the food industry and other contexts.

Introduction

Pathogenic strains of *Escherichia coli* have been reported to be responsible for human food-borne diseases and death [1]. Cattle are major carriers of enterohemorrhagic *E. coli* (EHEC) and contamination of beef by EHEC has caused massive recalls and economic loss to the food industry [2, 3]. Also, EHEC contamination has been implicated in foods other than beef, such as cookie dough, unpasteurized apple juice [4], and spinach [5–7]. There were 5 outbreaks of *E. coli* O157:H7, the most important EHEC strain, associated with beef products in 29 states reported by the Centers for Disease Control and Prevention from 2006–2011 [8]. Most recently 1.8 million pounds of ground beef were recalled because of *E. coli* O157:H7 contamination [9]. Furthermore, additional shigatoxigenic *E. coli* (STEC) non-O157 strains have been associated with outbreaks involving sausages, beef, milk, apple cider, lettuce, and ice cream.
review. Rapid and sensitive tests capable of detecting both pathogenic and non-pathogenic *E. coli* are potentially useful as screens for general contamination. Products producing negative results can be released into the food distribution system while those yielding positive results can be studied more closely to detect pathogens among the general *E. coli* or *Shigella* spp. Furthermore, a quantitative generic *E. coli/Shigella* assay is also potentially useful to assess the efficacy of disinfection and plant cleaning procedures.

To identify *E. coli* in foods, USDA and FDA have established standard methods based on conventional culture incubation and PCR [10, 11]. Plate-culture methods have lower detection limits and lower cost, while the PCR method has greater specificity. However, because both methods stipulate enrichment of the target organism to quantifiable levels, they require a long time to results (TTR): 48 to 56 hours [12].

Our group recently developed an integrated flow cytometer based system (RAPID-B) to detect microbial contamination rapidly with high sensitivity [13, 14]. Using fluorescent dye-conjugated antibodies specific to *E. coli*, the system is able to provide quantitative results in minutes, or semi-quantitative results in a few hours if very few cells are present in the sample so that a brief enrichment is required.

Flow cytometry employs a photoelectric method for cell counting, which was developed by Moldavan in 1934 and successfully used to detect airborne bacterial spores by Gucker et al. in 1940 [15]. Historically, flow cytometers analyze cells flowing through the center of a channel in a quartz chamber, arrayed in single file, as a laser beam illuminates each cell and signals are recorded as forward scatter and side scatter. If cells are also labeled with a fluorescent dye, emitted fluorescence passes through a series of filters to establish the wavelength of the emission which is then detected by photomultiplier tubes (PMTs). The collection of information from coincident light signals from the various detectors can be used to analyze characteristics such as size, granularity, response to probe, etc., for each cell individually. Fluorescence *in situ* hybridization (FISH) is a robust and sensitive molecular method to detect intact, permeabilized bacterial cells using sequence-specific rRNA-targeted fluorescently-labeled oligonucleotide probes [4]. FISH can be coupled with flow cytometry to accomplish rapid and specific detection of bacteria [16, 17].

Although RAPID-B antibody-based assays have advantages like short assay time, high sensitivity, and the ability to perform analysis directly in food and other difficult matrices, there are still more advantages to be gained from the development of a genetic based assay. Development of highly specific antibody reagents for emerging pathogens is costly and time-intensive. By contrast, genetic-based reagents can be developed quickly at much lower cost, reducing barriers for the rapid development of assays for novel pathogens. Specificity toward a target organism may not be achieved with antibodies in every case. For these reasons it is advantageous to develop genetic-based assays that are compatible with this instrumental platform. In this study, we developed a rapid flow cytometric-based detection method for generic *E. coli* using an oligonucleotide probe targeting a 16SrRNA sequence selective for the species (Flow-FISH) [18]. For inclusivity/exclusivity testing, 51 *E. coli* strains, 4 *Shigella* strains and 23 non-*E. coli* strains were analyzed. Optimal hybridization conditions, assay sensitivity, and linearity were also studied.

**Materials and Methods**

**Strains and media**

*E. coli* serotype O157: H7 strain (ATCC 43895) was used to develop an oligonucleotide probe based *E. coli* assay and optimize Flow-FISH conditions. This strain expresses stx1 and stx2 genes, which encode Shiga-like toxins I and II proteins, respectively. For inclusivity tests, 5...
E. coli strains from our stock, 45 E. coli isolates from Dr. Rajesh Nayak in the Division of Microbiology, NCTR, and 4 Shigella species from Dr. Fatemeh Rafii in the Division of Microbiology, NCTR were studied. Shigella spp. are very similar to E. coli and were regarded as appropriate for inclusivity in a generic E. coli assay. E. coli spp. included both pathogenic and non-pathogenic strains; all Shigella spp. were pathogenic. Strain names and sources are listed in Table 1. All E. coli strains were incubated in Trypticase Soy Broth (TSB) liquid medium (Becton Dickinson and Company, Washington, DC, USA) at 37°C. For the exclusivity study, 23 non-E. coli bacteria from our inventory were tested. Strain information and growth media are listed in Table 2. BHI: Brain heart infusion medium (Becton Dickinson and Company, Washington, DC, USA); NB: Nutrient broth medium (Becton Dickinson and Company, Washington, DC, USA); TSB: Trypticase soy broth medium (Becton Dickinson and Company, Washington, DC, USA).

Sample preparation
A generic E. coli oligonucleotide probe ES445 [19] with sequence: 5’ CTT TAC TCC CTT CCT CCC 3’ was produced and Alexa Fluor® 488 was linked to its 5’ end by Integrated DNA Technologies, Inc., Coralville, IA, USA. Bacterial colonies were picked up from agar plates, inoculated into 5 mL liquid media and cultured in an incubator overnight. The next morning 1 mL from each of the cell cultures was centrifuged at 5000×g for 5 min and the supernatants were removed. Cell pellets were washed with 1 mL phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged again at 5000×g for 5 min. Cell pellets were suspended in 1 mL 10% buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) and fixed at room temperature for 30 min with slow shaking. After centrifugation at 5000×g for 5 min, pellets were washed with 1 mL PBS and centrifuged at 5000×g for 5 min. Pellets could be stored in 1 mL of 50% ethanol/PBS at -20°C for several weeks prior to analysis. Fixed cells were resuspended in 150 μL hybridization buffer (20 mM Tris, pH 7.5, 0.01% SDS, 3 M NaCl) containing 1 μL of 200 ng/μL DNA probe, and incubated at 55°C for 30 min in a Thermomixer (Eppendorf, Hauppage, NY, USA). After centrifugation at 5000×g for 5 min, cell pellets were resuspended in 1 mL PBS and used for flow cytometer analysis.

Flow cytometry analysis
The RAPID-B model 9013 flow cytometer from Vivione Biosciences (Vivione Biosciences, LLC, Pine Bluff, AR, USA) was used for the flow-FISH assay. The excitation source is a solid state 20 mW laser (488nm). It has standard cytometry scatter channels but it has been optimized for detection of small cells by virtue of its low angle scatter detector (LS2) and its high angle scatter detector (LS1). Fluorescence emission can be detected and distinguished within wavelength channels using 3 filters: FL1: green channel collecting light with 515–525 nm; FL2: yellow channel collecting light with 575–600 nm; FL3: red channel collecting light with > 615 nm. All light scatters events and emissions are detected by PMTs. Fluorescence signals from all three channels are collected in order to reduce confusing background noise from autofluorescence [20]. The spectral resolution of light scatter events in the instrument is 130 nm, so it is optimal for detecting and distinguishing bacterial cells.

After the FISH hybridization and wash, cell samples were suspended in 1 mL PBS and analyzed by flow cytometry. The E. coli O157 antibody-based flow cytometry protocol created by our group [12] was modified and used for the genetic probe assay used in this study. Flow cytometry data were further analyzed and exported using FCS Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA, USA).
### Table 1. Inclusivity panel result.

| Organism | Strain/Source | Detected cells | Result of hybridization |
|----------|--------------|----------------|-------------------------|
| *E. coli* | ATCC 43888   | 76965          | +                       |
| *E. coli* | ATCC 35421   | 84129          | +                       |
| *E. coli* | ATCC 4157    | 58194          | +                       |
| *E. coli* | ATCC 35218   | 53094          | +                       |
| *E. coli* | ATCC 11775   | 72990          | +                       |
| *E. coli* | ATCC 51446   | 65731          | +                       |
| *E. coli* | Avian        | 194122         | +                       |
| *E. coli* | Canine       | 187535         | +                       |
| *E. coli* | Lemur        | 122905         | +                       |
| *E. coli* | Bovine       | 101752         | +                       |
| *E. coli* | Equine       | 108881         | +                       |
| *E. coli* | Ferret       | 78513          | +                       |
| *E. coli* | Bovine       | 77660          | +                       |
| *E. coli* | Avian        | 46326          | +                       |
| *E. coli* | Equine       | 82070          | +                       |
| *E. coli* | Feline       | 27856          | +                       |
| *E. coli* | Canine       | 25296          | +                       |
| *E. coli* | Equine       | 27318          | +                       |
| *E. coli* | Bovine       | 25119          | +                       |
| *E. coli* | Bovine       | 40767          | +                       |
| *E. coli* | Canine       | 153532         | +                       |
| *E. coli* | Caprine      | 33980          | +                       |
| *E. coli* | Canine       | 64695          | +                       |
| *E. coli* | Bovine       | 155137         | +                       |
| *E. coli* | Canine       | 130137         | +                       |
| *E. coli* | Chicken      | 113101         | +                       |
| *E. coli* | Canine       | 81565          | +                       |
| *E. coli* | Chicken      | 125021         | +                       |
| *E. coli* | Canine       | 102032         | +                       |
| *E. coli* | Quail        | 139283         | +                       |
| *E. coli* | Canine       | 112575         | +                       |
| *E. coli* | Bovine       | 128700         | +                       |
| *E. coli* | Bovine       | 11576          | +                       |
| *E. coli* | Equine       | 16978          | +                       |
| *E. coli* | Equine       | 23954          | +                       |
| *E. coli* | Canine       | 57014          | +                       |
| *E. coli* | Canine       | 47425          | +                       |
| *E. coli* | Canine       | 17924          | +                       |
| *E. coli* | Leopard      | 53547          | +                       |
| *E. coli* | Canine       | 37519          | +                       |
| *E. coli* | Humans       | 45267          | +                       |
| *E. coli* | Humans       | 34892          | +                       |
| *E. coli* | Humans       | 75944          | +                       |
| *E. coli* | Humans       | 40540          | +                       |
| *E. coli* | Humans       | 63323          | +                       |
| *E. coli* | Humans       | 133479         | +                       |

(Continued)
TPC assay

TPC reagent (Vivione Biosciences, LLC, Pine Bluff, AR, USA) containing thiazole orange (TO) and propidium iodide (PI) is used for cell counting. DNA of live cells could be intercalated by TO, a permanent dye, generating green light fluorescence (FL1 channel). PI can only penetrate dead cells whose membranes are compromised; it quenches the TO, and emits red fluorescence.

Table 1. (Continued)

| Organism    | Strain/Source | Detected cells | Result of hybridization |
|-------------|---------------|----------------|-------------------------|
| *E. coli*   | Humans        | 80980          | +                       |
| *E. coli*   | Humans        | 169752         | +                       |
| *E. coli*   | Humans        | 92031          | +                       |
| *E. coli*   | Humans        | 133193         | +                       |
| *Shigella sonnei* | ATCC 25931 | 118644         | +                       |
| *Shigella flexneri* | ATCC 15931 | 65544          | +                       |
| *Shigella boydii* | ATCC 8700 | 72190          | +                       |
| *Shigella dysenteria* | ATCC 11835 | 22742          | +                       |

Table 2. Exclusivity panel result.

| Organism                        | Strain/ Source | Growth medium | Detected cells¹ | Result of hybridization |
|---------------------------------|----------------|---------------|-----------------|-------------------------|
| *Listeria monocytogenes*        | ATCC19115      | BHI           | 0               | –                       |
| *Listeria grayi*                | ATCC 19119     | BHI           | 0               | –                       |
| *Listeria innocua*              | ATCC 33090     | BHI           | 0               | –                       |
| *Salmonella enterica* serovar paratyphi A | ATCC 11511 | NB            | 0               | –                       |
| *Salmonella enterica* serovar Paratyphi B | ATCC 8759 | NB            | 0               | –                       |
| *Salmonella enterica* serovar anatum* | ATCC 9270 | NB            | 0               | –                       |
| *Salmonella enterica* serovar newport* | ATCC 6962 | NB            | 0               | –                       |
| *Salmonella enterica* serovar typhi* | ATCC 6539 | NB            | 0               | –                       |
| *Salmonella enterica* serovar choleraesuis* | ATCC 10708 | NB            | 0               | –                       |
| *Salmonella enterica* serovar montevideo* | ATCC 8378 | NB            | 0               | –                       |
| *Staphylococcus aureus*         | ATCC 25923     | TSB           | 0               | –                       |
| *Citrobacter diversus*          | KM11012        | NB            | 0               | –                       |
| *Pseudomonas aeruginosa*        | ATCC 9027      | NB            | 0               | –                       |
| *Enterococcus durans* Collins et al.* | ATCC 11576 | TSB           | 0               | –                       |
| *Enterobacter cloacae* subsp. cloacae (Jordan) Hormaechae and Edwards, subsp. nov.* | ATCC 35030 | NB            | 0               | –                       |
| *Enterobacter aerogenes* Hormaechae and Edwards* | ATCC 35028 | NB            | 0               | –                       |
| *Pseudomonas aeruginosa* (Schroeter) Migula* | ATCC 9027 | NB            | 0               | –                       |
| *Staphylococcus aureus* subsp. aureus Rosenbach* | ATCC 6538 | TSB           | 0               | –                       |
| *Bacillus cereus* Frankland and Frankland* | ATCC 14579 | NB            | 0               | –                       |
| *Staphylococcus capitis* subsp. capitis Kloos and Schleifer* | ATCC 35661 | TSB           | 0               | –                       |
| *Aerococcus viridans* Williams et al.* | ATCC 11563 | BHI           | 0               | –                       |
| *Acinetobacter sp.*              | ATCC 19139     | NB            | 0               | –                       |

BHI: Brain heart infusion; NB: Nutrient broth; TSB: Trypticase soy broth

¹ The number of fluorescent events detected were reduced by a threshold determined empirically from multiple experiments.

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Thus, a combination of these two dyes provides a rapid method for total cell quantification, as well as discriminating live and dead cells [21].

The TPC assay was used for cell counting in the linearity study as a control and was performed before formalin fixation. For TPC assay, 1 mL cell culture were centrifuged at 5000×g for 5 min. Cell pellets were suspended in 1 mL PBS, mixed with 333 μL TPC reagent, and incubated at room temperature with slow shaking for 6 min. The samples were then analyzed by the RAPID-B flow cytometer using the TPC-specific protocol.

**Results**

**Flow-FISH analysis of *E. coli***

Flow cytometry-based detection assay was developed using a 5′-Alexa Fluor 488 dye conjugated-oligonucleotide probe that is specific for 16S rRNA *E. coli*. As shown in Fig 1, fluorescently labeled *E. coli* cells (black plot) were clearly distinguished from the unlabeled *E. coli* cells (red plot), and from *Salmonella* cells (green plot) that were treated with the same fluorochrome probe.

Repeating hybridization experiments were performed with the Alexa-Fluor labeled probe in order to optimize detection of *E. coli*. The assay was optimized to determine the ideal probe concentration, NaCl concentration in hybridization buffer, hybridization temperature, and time. The optimal hybridization conditions were determined to be: hybridization buffer contains 3 M NaCl and 2 ng/μl DNA probe; hybridization is performed at 55°C for 30 min.

**Inclusivity study**

After overnight incubation, 1 mL of each *E. coli* culture was prepared for inclusivity testing. Flow cytometer assays positively identified all 51 *E. coli* strains (Table 1). To get accurate cell counting, a serial gates strategy was developed [12] and used for all inclusivity and exclusivity studies (Figs 2 and 3). Because of the relatively broad emission spectral bandwidth (500–600 nm) of the fluorescent Alexa Fluor 488 labeled probe (Fig 1), the signal of probe-labeled bacteria was present in both FL1 and FL2 channels. The serial gates selection of light scatter parameters and fluorescent intensity includes most target signals and also significantly reduces irrelevant noise. In each plot, a gate indicating the region of interest was determined based on populations (clusters of events with similar combinations of light scatter and emission profiles) from all tested isolates (*E. coli* strains and non-*E. coli* strains) using FCS Express 4 software.

While testing four *Shigella* strains as part of an exclusivity study, we found that the *E. coli* probe could bind all *Shigella* strains and the fluorescence signal appeared within the same set of flow cytometry gates (Fig 4c) as *E. coli*. The positive result caused by *Shigella* spp. strains was predicted because of the close genetic relationship between *E. coli* and *Shigella* [22].

**Exclusivity study**

A total of 27 non-*E. coli* strains were tested for specificity by flow cytometry. Due to off-target particulate fluorescence background and chemical noise, a threshold was established to exclude background signals. This was accomplished by performing comprehensive comparisons of negative control samples. A threshold of 100 counts was determined for the exclusivity assay. After subtraction of the threshold from reported counts, remaining positive numbers were recorded as “positive” results.

Except for 4 *Shigella* species, the assay gave negative results for all the other microorganisms (Table 2). Fig 4d shows the flow cytometer density plot of a *Salmonella enterica* strain, one of
the negative samples. The Alexa Fluor fluorescence intensity of Salmonella in FL1 is much lower than that of E. coli (Fig 4a).

**Linearity study**

Serial dilutions of $10^{-1}$ to $10^{-6}$ of an E. coli O157 overnight culture were compared using the genetic E. coli probe assay, agar plate counting, and the TPC assay. The sensitivity of the R-squared value between genetic probe assay and TPC data is 0.9986, and that between genetic probe assay and agar plate counting is 0.9996 (Fig 5). The sensitivity of generic E. coli probe assay is comparable to that of the TPC assay and agar plate counting for both low cell density ($10^5$–$10^6$ cells/mL) and high cell density ($10^4$–$10^6$ cells/mL).

**Challenge tests**

We performed challenge tests to determine whether the genetic E. coli probe assay can distinguish E. coli from other bacteria, because multiple microbial populations are commonly present in food such as fermented meat and dairy products. E. coli O157 overnight culture was...
Fig 2. Flow cytometer analysis of *E. coli* O157 through serial gates. Signal information from the scatter plot (a) pass through a combination of scatter and fluorescence (b, c, d and e) to the final fluorescence plot (f). Gate logic is indicated by arrows.

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Fig 3. Flow cytometer analysis of Salmonella enterica serovar paratyphi A (ATCC 11511) through serial gates. The same gate logic is used as in Fig 2.

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diluted to $10^{-1}$ to $10^{-6}$ and each dilution was mixed with high density of *Salmonella enterica* serovar Choleraesuis ($\sim 3 \times 10^6$ cells/mL). As shown in Fig 6, as few as 30–40 cells/mL *E. coli* cells can be detected by the genetic *E. coli* probe assay in the presence of large number of *Salmonella* cells.

**Discussion**

Our study demonstrates that a RAPID-B based method using an oligonucleotide probe can be used for rapid detection and quantification of *E. coli* and *Shigella* species. It has potential
application in quality control in food industry, environmental, and clinical contexts. It serves as a model for similar RAPID-B assays based on genetic sequences for which the target (here 16S rRNA) has multiple copies present in each cell. This method was tested to have good specificity (exclusivity study) and sensitivity (challenge study). A small number of *E. coli* can be detected in the presence of a large number of other bacteria (*Salmonella* is tested here).

Bacterial cells were fixed by formalin treatment and permeabilized to improve accessibility for the oligonucleotides. After the fluorochrome-tagged probe enters permeabilized cells and binds to the target region of 16S rRNA, it can be detected by its green fluorescent emission signal in the FL1 channel. Fixing the cells means that cell viability cannot be determined, unlike the case for antibody-based RAPID-B tests. Also, the sample preparation required for genetic-based detection is more involved and takes more time than detection using antibodies (2 h versus 5 min). However, the resulting fluorescence-tagged cells are stable and therefore more amenable to automated or batch analysis, which reduces TTR and labor when 20 or more analyses are done.

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**Fig 5. Linearity of generic *E. coli* probe assay.** Cell culture was diluted over 6 orders of magnitude and equal volume of each dilution were assessed by generic *E. coli* probe assay, TPC assay and agar plate counting. The sensitivity of the R-squared value between genetic probe assay and TPC assay (■) is 0.9986, and the R-squared value between genetic probe assay and agar plate counting (▲) is 0.9996. Each data point is an average of 3 repeats.

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**Fig 6. Challenge test of generic *E. coli* probe assay.** *E. coli* O157 culture was diluted over 6 orders of magnitude, mixed with equal volume of *Salmonella enterica* overnight culture, and assessed by generic *E. coli* probe assay, TPC assay and agar plate counting. The sensitivity of the R-squared value between genetic probe assay and TPC assay (■) is 1, and the R-squared value between genetic probe assay and agar plate counting (▲) is 0.9999. Each data point is an average of 3 repeats.

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In the study, Shigella strains were positively detected by E. coli generic probe, which is due to the close relationship between Shigella and E. coli. Although Shigella was classified as a separate genus according to their medical significance in the 1940s [23], it is more appropriately treated as E. coli in the phylogenetic perspective [22]. The binding of E. coli generic probe with Shigella isolates was also verified by NCBI BLAST search. 100% identical sequences were found in Shigella 16S rRNA.

Although sample preparation takes approximately 2 h for the RAPID-B genetic based assays (versus a few minutes for the antibody-based assays), many samples can be processed in parallel during the same time frame. For example, our laboratory currently has the capability to process up to 24 genetic assay samples in a 2 h period using one Eppendorf Thermomixer R module. Additional centrifuges and Thermomixers would allow even more samples to be prepared in parallel over the same 2 h period. Because of this feature, RAPID-B genetic based assays can be used in food production and safety/regulatory contexts to analyze many samples in meaningful time frames. We are currently developing RAPID-B genetic-based methods for other pathogens including Listeria monocytogenes, an organism that is notoriously difficult to label with antibodies.

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The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

Author Contributions
Conceived and designed the experiments: YX JW DB. Performed the experiments: YX. Analyzed the data: YX JW TM DB. Contributed reagents/materials/analysis tools: AW WC RN FR. Wrote the paper: YX JW DB.

References
1. Besser RE, Griffin PM, Slutsker L. Escherichia coli O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. Annu Rev Med. 1999; 50:355–67. doi:10.1146/annurev.med.50.1.355 PMID: 10073293.
2. Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL. Epidemiology of Escherichia coli O157: H7 outbreaks, United States, 1982–2002. Emerging infectious diseases. 2005; 11(4).
3. Vogt RL, Dippold L. Escherichia coli O157: H7 outbreak associated with consumption of ground beef, June–July 2002. Public Health Reports. 2005; 120(2):174. PMID: 15842119.
4. CDC. Outbreak of Escherichia coli O157: H7 infections associated with drinking unpasteurized commercial apple juice—British Columbia, California, Colorado, and Washington, October 1996. MMWR Morbidity and mortality weekly report: Centers for Disease Control and Prevention (CDC); 1996. p. 975.
5. Neil KP, Biggerstaff G, MacDonald JK, Trees E, Medus C, Musser KA, et al. A novel vehicle for transmission of Escherichia coli O157: H7 to humans: multistate outbreak of E. coli O157: H7 infections associated with consumption of ready-to-bake commercial prepackaged cookie dough—United States, 2009. Clinical infectious diseases. 2012; 54(4):511–8. doi: 10.1093/cid/cir831 PMID: 22157169.
6. Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, Monson T, et al. Multistate outbreak of Escherichia coli O157: H7 infection associated with consumption of packaged spinach, August–September 2006: the Wisconsin investigation. Clinical infectious diseases. 2009; 48(8):1079–86. doi: 10. 1086/597399 PMID: 19265476.
7. Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, et al. Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. International journal of food microbiology. 2010; 139:S3–S15. doi: 10.1016/j.ijfoodmicro.2010.01.021 PMID: 20153070.
8. CDC. E. coli Outbreak Investigations. Available: http://www.cdc.gov/ecoli/outbreaks.html: Centers for Disease Control and Prevention (CDC); 2011.
9. Mathusa EC, Chen Y, Enache E, Hontz L. Non-O157 Shiga toxin–producing Escherichia coli in foods. Journal of Food Protection® 2010; 73(9):1721–8.
10. FSIS. Procedure for the Use of *Escherichia coli* O157:H7 Screening Tests. Available: http://www.fsis.usda.gov/PDF/Mlg_5A_02.pdf; Food Safety and Inspection Service (FSIS); 2010.

11. Feng P, Weagant SD, Jinneman K. BAM: Diarrheagenic *Escherichia coli*. Available: http://www.fda.gov/food/foodscience/ucm070080.htm2013.

12. Buzatu DA, Moskal TJ, Williams AJ, Cooper WM, Mattes WB, Wilkes JG. An Integrated Flow Cytometry-Based System for Real-Time, High Sensitivity Bacterial Detection and Identification. PloS one. 2014; 9(4):e94254. doi: 10.1371/journal.pone.0094254 PMID: 24718659

13. Buzatu DA, Cooper WM, Summage-West C, Sutherland JB, Williams AJ, Bass DA, et al. 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 microbiology. 2013; 36(2):416–25. doi: 10.1016/j.fm.2013.07.007 PMID: 24010624

14. Wilkes JG, Tucker RK, Montgomery JA, Cooper WM, Sutherland JB, Buzatu DA. 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 microbiology. 2012; 30(1):281–8. doi: 10.1016/j.fm.2011.11.002 PMID: 22265313

15. Davey HM, Kell DB. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiological reviews. 1996; 60(4):641–96. PMID: 8987359

16. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and environmental microbiology. 1990; 56(6):1919–25. PMID: 2200342

17. Joachimsthal E, Ivanov V, Tay S, Tay J. Quantification of whole-cell in situ hybridization with oligonucleotide probes by flow cytometry of *Escherichia coli* cells. World Journal of Microbiology and Biotechnology. 2003; 19(5):527–33.

18. Rufer N, Dragowska W, Thombury G, Roosnek E, Lansdorp PM. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. Nature biotechnology. 1998; 16(8):743–7. PMID: 9702772

19. Kenzaka T, Yamauchi N, Prapagdee B, Mikami E, Nasu M. Bacterial Community Composition and Activity in Urban Rivers in Thailand and Malaysia. Journal of Health Science. 2001; 47(4):353–61.

20. Bumann D. Examination of Salmonella gene expression in an infected mammalian host using the green fluorescent protein and two-colour flow cytometry. Molecular microbiology. 2002; 43(5):1269–83. PMID: 11918812

21. Doherty S, Wang L, Ross R, Stanton C, Fitzgerald G, Brodkorb A. Use of viability staining in combination with flow cytometry for rapid viability assessment of *Lactobacillus rhamnosus* GG in complex protein matrices. Journal of microbiological methods. 2010; 82(3):301–10. doi: 10.1016/j.mimet.2010.07.003 PMID: 20638420

22. Lan R, Reeves PR. *Escherichia coli* in disguise: molecular origins of Shigella. Microbes and infection. 2002; 4(11):1125–32. PMID: 12361912

23. Ewing WH. Shigella nomenclature. Journal of bacteriology. 1949; 57(6):633. PMID: 16561743