A rapid and highly specific immunofluorescence method to detect *Escherichia coli* O157:H7 in infected meat samples

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Abstract

Developing rapid and sensitive methods for the detection of pathogenic *E. coli* O157:H7 remains a major challenge in food safety. The present study attempts to develop an immunofluorescence technique that uses Protein-A-coated, magnetic beads as the platform. The immunofluorescence technique described here is a direct detection method in which *E. coli* O157:H7 cells are labeled with tetramethylrhodamine (TRITC) fluorescent dye. TRITC-labeled bacteria are captured by the desired antibody (Ab), which is immobilized on the Protein-A magnetic beads. Fluorescence of the captured cells is recorded in a fluorescence spectrophotometer, where the fluorescence values are shown to be directly proportional to the number of bacteria captured on the immunobead. The formation of an immunocomplex is evidenced by the fluorescence of the beads under microscopy. The Ab immobilization procedure is also evidenced by microscopy using fluorescein isothiocyanate (FITC)-labeled Ab. The total experimental time, including preparation of the sample, is just 1 h. The minimum bacterial concentration detected by this method is $1.2 \pm 0.06 \times 10^3$ CFU ml⁻¹. The high specificity of this method was proved by using the specific monoclonal Ab (MAb) in the test. The proposed protocol was successfully validated with *E. coli* O157:H7-infected meat samples. This approach also opens the door for the detection of other bacterial pathogens using Protein-A magnetic beads as a detection platform.

Keywords: *E. coli* O157:H7; immunofluorescence; food safety; magnetic bead; Protein-A; TRITC

1. Introduction

Escherichia coli (*E. coli*) is a dominant species found in the human gut as a facultative anaerobe, and this bacterium is generally harmless to humans. However, some groups of *E. coli* strains are pathogenic and can cause diarrheal diseases. Pathogenic *E. coli* are commonly classified into the following six groups, although there might be others that have yet to be characterized: (1) diffusely adherent *E. coli* (DAEC), (2) enteroaggregative *E. coli* (EAEC), (3) enterohemorrhagic *E. coli* (EHEC), (4) enteroinvasive *E. coli* (EIEC), (5) enteropathogenic *E. coli* (EPEC), and (6) enterotoxigenic *E. coli* (ETEC) (Clements et al., 2012; Croxen et al., 2013; Huang et al., 2011; Kaper et al., 2004). *E. coli* O157:H7 is one of the most important EHEC pathogens because it is capable of causing bloody diarrhea (hemorrhagic colitis), which could then progress into the potentially fatal hemolytic uremic syndrome (HUS). The serotype O157:H7 is one of the Shiga-toxin-producing *E. coli* (STEC) and causes infections worldwide. In addition to the serotype O157, there are many other STEC serotypes called non-O157 STEC members that are capable of producing the same symptoms. However, non-O157 STEC infections are rare or only of local interest (Amani et al., 2015; Buchanan, 1997; Lim et al., 2010). In detection methods, it is also considered that the cross-reactions of non-O157 STEC with O157 STEC strains or vice versa could affect the outcome (Quinones et al., 2011). According to the Centers for Disease Control and Prevention (CDC), *E. coli* O157:H7 causes 60 deaths per year in the United States, with a total of 73,000 cases of the illness on record annually. Though the rate of infection has decreased from its peak in 1999, economic loss and death continue to occur as a result of periodic outbreaks (Lim et al., 2010).

Isolation and detection of pathogenic *E. coli* remain a significant challenge in food safety, especially with outbreak investigations for differentiating the pathogenic strains from non-pathogenic *E. coli*. Though *E. coli* O157:H7 has three decades of infection history, researchers are still refining the detection process through a variety of advanced techniques. Current research targets reducing the detection time and lowering the cell detection limit. Advanced methods, such as biosensors, chip-based nucleic acid methods, microfluidic device-based impedance analysis, and immunoassays are being used to achieve single cell and rapid detection (Wu et al., 2015).

Immunomagnetic separation (IMS) is a novel laboratory technique that isolates specific cells or molecules out of body fluid or cultured cells, which results in purification. The IMS tool uses paramagnetic beads or polystyrene beads that are coated with antibodies (Abs) that allow for the specific isolation of viable pathogenic cells present in the culture media (Philippova et al., 2011). Through the application of a magnetic field, the beads with the captured pathogen are attracted to the magnet, which allows the rest of the liquid solution to wash out of the tube. Therefore, the captured antigens/pathogens can be detected. Magnetic beads are magnetic nano- or micro-crystals enclosed in a nonmagnetic matrix, such as biocompatible polymers or silicon-based materials (Gijs, 2004). These crystals are mostly made up of iron oxides (Fe_2O_3 or Fe_3O_4). Also, alloys of metals (e.g., Ni, Fe, Co, Mg, and Zn) or rare earth compounds (e.g., NdFeB and SmCo) can be used to make magnetic particles. Analytic materials are protected from direct contact with the metal oxides by a process of embedding the metallic crystals of the beads in a polymer matrix (Horak et al., 2007; Philippova et al., 2011). To capture target particles/cells, the magnetic beads have to be coated with a particular ligand that binds to the target. Abs are the

most common ligands used, and the binding of that Ab with the magnetic beads needs some special linker molecules. Immunoglobulin (Ig)-binding proteins (Protein-A, Protein-G) are important linker molecules that can be coated on the surface of the magnetic beads (Borlido et al., 2013; Widjojatmodjo et al., 1993). These proteins specifically bind with immunoglobulin G (IgG) Abs and allow for easier and more precise purification of the pathogen.

Magnetic bead-based immunoassays are an emerging type of diagnostic method that uses magnetic beads as the platform for immobilizing the desired Ab or antigen (Ag) (Wei et al., 2012). Using Protein-A on the beads increases the speed and strength of the Ab binding. This coating technique is commonly employed in the purification of desired IgG Abs from serum. However, researchers have recently discovered another way of implementing these protein-coated beads in immunoassays for the detection of pathogenic microorganisms (Barizuddin et al., 2015).

Immunofluorescence is a very common technique that is used for visualizing cells and cellular components. Recently this method has attracted increased interest as a means to improve pathogen detection methods for foodborne, environmental, and clinical pathogens. In this technique, either the Ab or Ag of interest will be stained with fluorescent dyes (Kourkine et al., 2003). The stained molecules specifically will be bound to their counterparts, which will allow the pathogen of interest to be detected by use of a fluorescence-based technique similar to that of fluorescence microscopy and fluorescence spectrophotometry. Emissions of fluorescent molecules have a thousandfold superior sensitivity than other staining molecules in spectrophotometry. In fluorescent dyes, specific wavelengths excite the fluorophore, and it emits light in specific wavelengths. This property leads to increased sensitivity for detection of the desired analytes (Li et al., 2013; Yu et al., 2014). Using fluorescent dye minimizes the time, makes the labeling process easier, gives the possibility of multiplexed assays with the use of different dyes, and does not disturb the properties of functional groups that are involved in the detection process (Li et al., 2013; Waggoner, 2006). Fluorescent dyes, such as alexa fluor, cyanine, fluorescein, rhodamine, and sulforhodamine are commonly used in fluorescence assay techniques (Panchuk-Voloshina et al., 1999; Waggoner, 2006). Fluorescein was the first compound used as a fluorescent tag and is widely used in biomolecular assays. This dye has the possibility of yielding a higher quantum of emission values. Among all fluorescent dyes, fluorescein is one of the most commonly used fluorescent biomarker (Li et al., 2004, 2013). In addition to fluorescein, rhodamine is widely used as a fluorescent tag. TRITC is the prevailing rhodamine dye, which has advantages over other fluorescent dyes in its resistance to pH and photodegradation.

Taking all of this information into consideration, the present study seeks to develop a magnetic bead-based immunofluorescence technique for the detection of pathogenic *E. coli* O157:H7. The method presented here is highly specific because it uses a specific MAb for the targeted bacteria. The method is made rapid by use of magnetic beads that make washing and handling procedures easy. Using Protein-A on the beads increases the speed and specificity of the immobilization process. The use of fluorescent dyes and fluorescence spectroscopy enhances the accuracy of the test when compared to absorption methods such as ELISA. In the present study, *E. coli* O157:H7 cells were labeled with TRITC dye. Using labeled bacteria in the detection process enables direct detection and also increases the accuracy of the detection

process. As studies have reported, direct methods are better for quantification of bacteria than indirect methods. Presently, most of the reported fluorescence methods are indirect techniques and use fluorescently labeled secondary Abs in the detection process (Kourkine et al., 2003). The specific MAb is immobilized on the Protein-A magnetic bead. When the immobilized Ab comes into contact with the TRITC-labeled bacteria, the entire bacterial cell can be captured. While subjecting this immunocomplex to emission fluorescence spectrophotometry, the emission values will be directly proportional to the quantity of the bacteria captured. Fluorescence microscopy can effectively confirm the formation of an immunocomplex, and fluorescence microscopy can also be used as an efficient and effective detection method. Though some previously reported methods used direct bacterial labeling, the detection process was limited to microscopy or flow cytometry (Hara-Kaonga and Pistole, 2007). Using fluorescent spectroscopy for the assay of labeled bacteria is unique to the present study.

2. Materials and Methods

2.1. Magnetic Beads

The Protein-A magnetic beads were purchased from Lab on a Bead AB, Sweden (Protein A UltraRapid Agarose™). The bead size is between 45 µm and 165 µm.

2.2. Bacteria and inoculum preparation

The bacteria used in the study, *E. coli* O157:H7 (ATCC 700728) and *E. coli* O104:H4 (ATCC BAA-2326) were obtained from American Type Culture Collection (ATCC, USA). *E. coli* O104:H4 was used as a negative control throughout the study. *E. coli* (EC) broth (Fluka Analytical, USA) was used for culturing the *E. coli* bacteria in the study, and culture conditions were maintained with 18 h of incubation time at 37° C.

2.3. Immobilization of Ab on the bead

For this part of the experiment, 10 µl of Protein-A magnetic beads were mixed with 10 µg of mouse anti-*E. coli* O157 MAb (Meridian Life Science, Inc., USA). The total reaction volume was maintained at 250 µl using phosphate-buffered saline (PBS) (15 mM phosphate in 150 mM of NaCl, pH 7.4). This content was incubated for 20 min at room temperature and 40 rpm using a tube rotator (Fisher Scientific, USA). After incubation, the mixture was washed with PBS, during which the beads were retained using a magnet.

2.3.1. Validating the Ab immobilization process

To validate the Ab immobilization process, FITC-labeled mouse anti-*E. coli* O157 MAb (Meridian Life Science, Inc., USA) was used in the immobilization procedure, as mentioned above. After immobilization, the Ab-immobilized beads were subjected to spectrofluorometric emission scanning (at λ_{Ex} 494 nm) and fluorescence microscopic analysis. A standard graph, using the emission results of various known quantities of FITC-labeled Ab, was used to find the maximum quantity of the Ab that became bound to the Protein-A magnetic beads.

2.4. TRITC labeling of bacteria

To produce desired amount of cells, the culture was allowed for 18 h incubation; then, 10 ml of the culture were centrifuged. The pellet was washed twice with PBS and resuspended in 1 ml of 2 mM TRITC (Thermo Scientific™, USA) in PBS. The content was mixed by pipetting and was then incubated for 10 min (4° C) before the extra TRITC was centrifuged out. The resultant pellet was mixed with 0.5 ml of PBS and transferred to the Dye Removal Column (Thermo Scientific™, USA) for the removal of excess dye. Excess dye was removed per the manufacturer's instructions. The recovered TRITC-labeled bacteria were used for the detection process.

2.5. Capturing Ag onto the beads

In this step, 50 µl of FITC-labeled bacteria were added to the Ab-immobilized bead solution and mixed briefly (total reaction volume maintained at 250 µl using PBS). The content was incubated for 30 min at room temperature at 40 rpm using the tube rotator (Fisher Scientific, USA). After incubation, the excess bacteria were washed out using PBS, while the beads in the tube were held back with a magnet. After washing, the beads were resuspended in PBS and an emission scan was performed at an excitation wavelength of $\lambda_{Ex}541$ nm. One set of samples was taken for microscopic analysis.

2.6. Quantification of bacteria

To quantify the bacteria in this test, a standard graph was prepared using 10 different dilutions of TRITC-labeled bacteria. Various dilutions were subjected to an emission scan at $\lambda_{Ex}541$ nm. The peak values of the emission scan were taken for standard graph preparation in addition to the bacterial concentrations of the respective dilutions. The bacterial concentration of each dilution was identified using colony forming unit (CFU) analysis.

2.7. Saturation limit for the detection

To find the maximum bacterial population that would bind with 10 µl of Ab-immobilized beads, saturation point analysis was performed. Different dilutions of TRITC-labeled bacteria were prepared and subjected to the Ag capturing process as described above. The emission values of the each dilution were used in calculations with the standard curve to determine the bacterial concentrations.

2.8. CFU analysis

Each diluted sample was subjected to CFU analysis to obtain the bacterial concentrations. For serial dilutions, sterile deionized water was used; subsequently, the dilutions were plated on tryptic soy agar (Fluka Analytical, USA). Inoculated plates were incubated for 24 h at 37° C. After incubation, the plates were observed and the recorded values used in calculations for the number of CFU present in each dilution. To enumerate the mixed culture of both *E. coli* strains, dilutions were plated on MacConkey-sorbitol agar (SMAC, Fluka Analytical, USA).

2.9. Fluorescence Spectrophotometer

Fluorescence spectrophotometer analysis was accomplished using the QuantaMaster™ 400 Intensity Based Spectrofluorometer (Photon Technology International, USA). The

instrument set-up was programmed using **Flex GX** software. Slit sizes on the device were fixed at 6 mm and 8 mm for excitation, and 8 mm and 6 mm for emission (Barizuddin et al., 2015). For FITC, an excitation wavelength of λ_{Ex} 494 nm was set, and the emission range was designated between 400-500 nm. For TRITC, an excitation wavelength of λ_{Ex} 541 nm was fixed, and the emission range was assigned between 550-600 nm. The integration step size was chosen as 1 nm for high resolution. The observed fluorescence value was expressed in **counts per second** (CPS).

2.10. Microscopic analysis

The **fluorescence** and light microscopic analysis was carried out using **an** inverted microscope (Leica Microsystems – Leica DMI6000 B; **camara** – Leica DFC365 FX; **fluorescence imaging systems** Leica LAS AF6000; **light source** – Leica CTR6000; **fluorescence** illumination system – PRIOR, Lumen 200) for possible green (FITC) and red (TRITC) fluorescence on the **beads**. Captured images were processed using LAS AF6000 (2.5.0.6735) software. The presence of green fluorescence on the **Protein-A beads** confirmed the immobilization of Ab (FITC), and red fluorescence **confirmed** the presence of whole cell *E. coli* O157:H7 Ag (TRITC).

2.11. Specificity and sensitivity of the experiment

To find the specificity **and sensitivity** of the test, the following combinations of the different samples were prepared and tested with **fluorescence** spectrophotometry and **fluorescence** microscopy. **Details of the samples used are listed in Table 1.**

Table 1

Description of the samples used to determine the specificity and sensitivity of the test.

Sample	Description
1	Anti- <i>E. coli</i> O157 MAb-immobilized beads in PBS buffer
2	Rabbit anti- <i>E. coli</i> O104:H4 polyclonal Ab (PAb) (Abraxis, USA) immobilized beads in PBS buffer
3	<i>E. coli</i> O104:H4 bacteria with Protein-A magnetic beads, without Ab
4	<i>E. coli</i> O157:H7 bacteria with Protein-A magnetic beads, without Ab
5	<i>E. coli</i> O104:H4 bacteria with Protein-A magnetic beads immobilized using mouse anti- <i>E. coli</i> O157 MAb
6	<i>E. coli</i> O157:H7 bacteria with Protein-A magnetic beads immobilized using rabbit anti- <i>E. coli</i> O104:H4 PAb
7	<i>E. coli</i> O104:H4 bacteria with Protein-A magnetic beads immobilized using goat anti- <i>E. coli</i> O157:H7 PAb (Meridian Life Science, Inc., USA)
8	<i>E. coli</i> O104:H4 bacteria with Protein-A magnetic beads immobilized using rabbit anti- <i>E. coli</i> O104:H4 PAb
9	<i>E. coli</i> O157:H7 bacteria with Protein-A magnetic beads immobilized using mouse anti- <i>E. coli</i> O157 MAb
10	<i>E. coli</i> O157:H7 bacteria with Protein-A magnetic beads immobilized using goat anti- <i>E. coli</i> O157:H7 PAb

2.12. Testing with the meat sample

The proposed method for detection of *E. coli* O157:H7 was tested with food samples to prove its practical utility. Ground beef was bought from the local supermarket, and 100 g of the ground meat was sterilized by autoclaving (at 15 psi and 121° C for 15 min). In a stomacher bag, 25 g of sterilized meat was taken, and 50 ml of EC broth was added to it. This sample was inoculated with 132 ± 2 CFU of *E. coli* O157:H7 (calculated by plating the inoculum) and homogenized using a Stomacher® 400 Circulator (Seward Laboratory Systems Inc., USA). In the same way, the second set of samples was prepared by inoculating both *E. coli* O157:H7 (132 ± 2) and *E. coli* O104:H4 (131.5 ± 1) cells as a mixed culture. The third set of samples was prepared only with *E. coli* O104:H4 (with 131.5 ± 1 CFU) and used as a negative control. The same inoculation and homogenization were performed with raw ground beef samples as well. To identify whether the purchased ground beef was naturally infected with *E. coli* O157:H7, a set of both sterile and raw samples were processed without inoculating *E. coli* O157:H7. After homogenization, the bag was incubated at 37° C for 12 h. After incubation, the sample was briefly homogenized again with the stomacher machine. Then, 10 ml of solution was collected and centrifuged to retrieve the cell pellet. The pellet was then washed and suspended in 1 ml PBS. This prepared sample was subjected to the Protein-A magnetic bead-based immunoassays as described earlier. Positive control was maintained with a pure culture of *E. coli* O157:H7 inoculated (132 ± 2 CFU) in the EC broth. Triplicates were maintained for all preparations in the study.

3. Results and Discussion

3.1. Immobilization of Ab

Assessing the immobilization of the antibody is a highly important step in all immunoassays. The quality and quantity of the immobilized Ab is directly related to the efficient capture of bacteria and also determines the optimal test concentrations necessary to reduce the cost of detection. Fluorescence values of different concentrations of the FITC-labeled mouse anti-*E. coli* O157 MAb linearly varied according to the concentration of the Ab in the solution. This confirms that the Ab concentration is directly proportional to the fluorescence value detected by the instrument. A standard graph was prepared based on the fluorescence values obtained from the different concentrations of the Ab. Based on the standard graph, the amount of immobilized Ab was calculated by interpolating the fluorescence of the Ab-immobilized beads. It was found that 10 µl of the Protein-A magnetic beads can immobilize 5.03 ± 0.02 µg of the Ab (while the manufacturers claim that 10 µl can bind up to 30 µg of human IgG Ab). However, the test concentration of the Ab was maintained at 10 µg per 10 µl of the beads. This concentration was continuously used throughout the study. To avoid experimental error, the experimental Ab concentration was nearly doubled from the maximum binding concentration. The immobilization process of the Ab was also confirmed by fluorescence microscopy, where the presence of fluorescence on the beads served as confirmation of the immobilization of the Ab (Fig. 1). The method used here was affinity-based immobilization, which is the most specific method for IgG Abs, which eliminates interference of other proteins or molecules present in the sample. For these reasons, this approach is considered an efficient method for the maximum purification of the Ab from serum (Yu et al., 2014).

Fig. 1. Fluorescence microscopic imaging of Protein-A magnetic beads immobilized by FITC-labeled anti-*E. coli* O157 MAb (5x magnification): (a) bright field microscopy of the Ab-immobilized beads; (b) fluorescence microscopy of the Ab-immobilized beads; all the beads in the test showed fluorescence; (c) bright field microscopy of the control sample; and (d) fluorescence microscopy of the control sample showing no fluorescence.

3.2. Microscopic detection of *E. coli* O157:H7

After the Ab was immobilized on the Protein-A magnetic beads, the TRITC-labeled bacteria was added to the immobilized Ab beads. The entire cell of the bacterium was attracted by the specific MAb, and the binding was firm and reversible. As the bacteria were labeled with red fluorescent dye (TRITC), the immunocomplex formed on the bead could be visually observed under the fluorescence microscope (Fig. 2). This method is a means for qualitative detection of the pathogen, and the entire detection process can be completed within 1 hour. This is a direct detection method that increases efficiency by significantly reducing testing times relative to indirect methods. The microscopic observation in Fig. 2 is depicted in low magnification (5x) to illustrate that all of the beads were successfully involved in the detection.

Fig. 2. Fluorescence microscopic imaging of immunomagnetic beads after capturing an entire TRITC-labeled cell Ag of *E. coli* O157:H7 (5x magnification): (a) bright field microscopy of the immunobeads with the captured Ag; (b) fluorescence microscopy of the immunobeads with the captured Ag; all the beads showed fluorescence; (c) bright field microscopy of the control sample; and (d) fluorescence microscopy of the control sample showing no fluorescence.

3.3. Spectrofluorometric detection of *E. coli* O157:H7

To determine the concentration of the bacteria that can be detected by this method, a standard graph was prepared with TRITC-labeled bacteria. Fluorescence scanning of different dilutions showed that each time the bacterial concentration was reduced, the fluorescence values decreased linearly. Based on the dilution values (i.e., the bacterial concentration in the CFU), the graph was plotted against the fluorescence value in the standard graph. The results show that the 10 µl of the Ab-immobilized beads can hold up to $5.04 \pm 0.04 \times 10^5$ CFU ml⁻¹ of *E. coli* O157:H7 (Fig. 3). The minimum concentration of bacteria that could be detected with this method was $1.2 \pm 0.06 \times 10^3$ CFU ml⁻¹. In general, for any rapid detection methods of *E. coli* O157:H7, there is a minimum concentration necessary; the range is about 10³ CFU or cells ml⁻¹. A few rare methods, such as impedance-based biosensors, have shown that they can detect lower levels of bacteria. However, those techniques have more practical limitations and have yet to prove their practicality. Additionally, the idea that some methods are so sensitive that they are capable of detecting a single cell of this bacteria is purely based on theoretical calculations. Also, these methods did not use a sample preparation technique that can be applied in the field (Wang et al., 2011; Wang and Salazar, 2016; Waswa et al., 2007; Wu et al., 2015).

All the bacteria in the reaction mixture were not involved in the immunocomplex formation (**Table 2**), but the increased concentration of bacteria allowed for an increased percentage of bacterial binding with the desired Ab. This phenomenon can be witnessed by observing a low value of fluorescence with the dilution that contains a nearly maximum binding concentration of bacteria (**Table 2**). This is a common phenomenon in all immunoassays. Only a fraction of the included bacteria participate in the formation of the immunocomplex due to a number of factors: competitive binding between the Ag, masking of the Ag-binding site on the Ab by the size of the Ag, affinity, avidity, and the law of mass action involved between the Ag and the Ab.

Fig. 3. Saturation curve of an *E. coli* cell captured on 10 µl of an immunomagnetic bead.

Table 2

Results of the test bacterial concentration for 10 µl of immunobeads and the final concentration of bacteria captured by the beads with the observed fluorescence value. The fluorescence values represent the difference from the threshold limit (5000 CPS).

Dilution	Test bacterial concentration (CFU)	Bacteria captured on the beads (CFU)	Fluorescence observed (CPS)
1	9.70×10^5	5.04×10^5	29117.16
2	8.50×10^5	5.12×10^5	29648.95
3	8.25×10^5	5.01×10^5	28860.71
4	7.40×10^5	4.97×10^5	28583.23
5	6.65×10^5	4.30×10^5	23596.05
6	4.30×10^5	2.83×10^4	12596.13
7	3.75×10^5	2.52×10^4	10260.31
8	3.40×10^5	2.48×10^4	09992.37
9	3.05×10^5	2.36×10^4	09109.05
10	1.10×10^5	1.20×10^3	00437.48

3.4. Specificity and sensitivity of the detection method

The specificity test showed positive results for the samples containing bacteria with their respective Ab. These results are illustrated in **Fig. 4**. The *E. coli* O157:H7 sample showed the highest fluorescence value with anti-*E. coli* O157:H7 PAb. The second highest fluorescence value resulted from the combination of *E. coli* O157:H7 with the respective anti-*E. coli* O157 MAb. It is obvious that the PAb can capture more antigens than their monoclonal counterparts because of the presence of multiple Ag binding sites (Lipman et al., 2005). The combination of *E. coli* O104:H4 with its respective anti-*E. coli* O104:H4 PAb also gave a positive result, but the fluorescence value was less in comparison to the positive outcomes given by the *E. coli* O157:H7 samples. In all other samples, there was a negligible amount of fluorescence. It is possible that this mild fluorescence value was due to the residual effect of the fluorescent dye that was retained on the beads or the negligible cross-reactivity of the Ab. The fluorescence value is low enough to be considered insignificant. Moreover, this low value can be used as a control or baseline value to validate positive sample values in the calculation, and it will nullify the experimental error in the results. In contrast, this residual effect was not observed with the fluorescence microscopy. It was concluded that the test results would be considered positive if the fluorescence value was at least 5000 CPS (threshold limit) in the spectrofluorometric analysis. This value is approximately twice the maximum residual effect observed throughout the study.

The defining strength of immunoassays is specificity, which is a major concern in other bacterial assays (Skottrup et al., 2008). The present method also proved its high level of specificity with the use of the MAb for the test. The research of Westerman et al. (1997) indicates that the use of PAb results in the false-positive identification of *E. coli* strains. The specificity of this new method makes it applicable in field studies with a simple sample processing method. The study suggests that the quality of the Ab used should be considered as a factor in the results. The use of an affinity-purified MAb is recommended because the specificity

366 and repeatability of any good immunoassay depends on the quality of the antibody (Shen et al.,
367 2014).

368

369 **Fig. 4.** Specificity of the proposed method. Significant fluorescence was observed only with
370 bacteria captured using the respective Ab. Maximal capture of *E. coli* O157:H7 cells occurred
371 with the PAb, which also had the highest fluorescence value.

372 3.5. Testing the method with infected meat sample

373 Significant fluorescence from the test of *E. coli* O157:H7-infected meat samples proves
374 that the method was able to capture the target bacteria from the infected food sample (**Fig. 5**).
375 There was no significant variation observed between the sterile and unsterile meat samples. The
376 control (i.e., uninoculated meat samples) did not show any significant fluorescence. This means
377 the locally purchased meat sample was not naturally infected with the *E. coli* O157:H7 pathogen.
378 This food sample was enriched for 12 h; this time period was determined based on a separate
379 experiment carried out using different time intervals to achieve a bacterial concentration that
380 resulted in the maximum detection limit, as discussed previously. The bacteria infecting the meat
381 (*E. coli* O157:H7) were able to grow in the enrichment broth to a concentration of $8.55 \pm 0.21 \times 10^6$ CFU ml⁻¹. This concentration was achieved when the initially infected concentration of *E.*
382 *coli* O157:H7 was only 2.6 CFU ml⁻¹. This showed that the enrichment process allows high
383 sensitivity detection in food samples. This enrichment process also substantially reduced the time
384 when compared to the methods for *E. coli* O157:H7 recommended by the US Food and Drug
385

Administration (FDA) in their Bacteriological Analytical Manual (BAM) (Feng et al., 2011). Incubation of the infected meat sample was performed to mimic the natural contamination process, whereby the bacteria alive in the meat sample would begin to multiply in an enrichment media to reach a desirable bacterial concentration for the detection. If there were no bacteria in the meat sample, there would not be any significant fluorescence in the assay. The proposed technique worked equally well for the meat sample and the pure bacterial culture. The mixed culture test showed a lower fluorescence value than the *E. coli* O157:H7 samples. This might be due to *E. coli* O104:H4 interference with the binding of the *E. coli* O157:H7 to the Ab and the inadequate concentration of bacteria compared to that of the pure culture. The competitive growth of *E. coli* O104:H4 might reduce the growth concentration of *E. coli* O157:H7. The enriched mixed culture grew pink-colored colonies of *E. coli* O104:H4 ($6.05 \pm 0.21 \times 10^6$) cells and colorless colonies of *E. coli* O157:H7 ($6.05 \pm 0.21 \times 10^6$). The sample with only *E. coli* O104:H4 did not show any significant fluorescence. These results were also confirmed with fluorescence microscopy as illustrated in Fig. 6.

The present study suggested the use of a minimum *E. coli* O157:H7 concentration of $1.2 \pm 0.06 \times 10^3$ CFU ml⁻¹ for an optimal result. However, some methods using magnetic particle-based immunoassays reported lower bacterial concentrations for detection (about 10^2 CFU ml⁻¹), but that limit only applies to milk, beverage, and vegetable samples. The same methods required approximately 10^3 CFU ml⁻¹ for any meat samples. This difference is due to components of meat that interfere with the detection process (Shen et al., 2014). Some sample processing steps, such as homogenization or blending of food samples, can release enzymes, antimicrobial chemicals, or other metabolic components that interfere with downstream analysis and detection (Balakrishnan et al., 2014; Bhunia, 2008; Wang and Salazar, 2016). These interferences hugely affect any detection process, especially the PCR-based detection methods, which are considered to be the most advanced technique in foodborne pathogen detection (Skottrup et al., 2008; Wang and Salazar, 2016). The success of PCR methods depends on nucleic acid purity, and it is time-consuming and expensive to achieve that type of purity. These drawbacks prevent the PCR methods from being useful in on-site detection in food processing units. However, the method discussed in this study directly targets the epitopes that are present on the live bacteria. Column filtration for the preparation of TRITC-labeled bacteria and the use of specific MAb will eliminate interference from the food matrix compounds, which is an extremely important parameter in detection methods because it reduces the sensitivity and applicability of the test (Wang and Salazar, 2016; Wu et al., 2015). This direct detection method increases the affinity strength of the antibody by not labeling the antibody of interest. Using a labeled antibody in the detection process will decrease the sensitivity because a labeled Ab considerably affects the binding affinity of that antibody (Liu et al., 2010; Takai et al., 2011). However, it has been proven that fluorescent labeling of the entire bacteria cell will not affect any pathogenic or immunological properties (Agerer et al., 2004; Drevets and Elliott, 1995). As a whole, the present method, with its high specificity, showed a high level of practical applicability in the food industries.

Fig. 5. Validation of the proposed technique with the infected meat samples. Positive results were observed with all samples containing *E. coli* O157:H7 cells.

Fig. 6. Fluorescence microscopic imaging of immunomagnetic beads after capturing TRITC-labeled *E. coli* O157:H7 cells from infected and enriched meat: (a) bright field microscopy in lower magnification (5x); (b) fluorescence microscopy in lower magnification (5x); (c) bright field microscopy in higher magnification (20x); and (d) fluorescence microscopy in higher magnification (20x). Threadlike structures around the bead are bacterial aggregation.

4. Conclusion

The present study described a novel immunofluorescence method for detecting pathogenic *E. coli* O157:H7 from meat samples. The technique discussed here is highly specific to the pathogen by using specific MAb. This method reduced the detection time when compared to the available detection methods in the FDA (2015) guidelines. This protocol can also enable the detection of other antigens or pathogens when using a respective Ab, but the specificity of the particular Ab needs to be considered along with the characteristics of the bacteria to be tested. This technique can also be used for any food or environmental samples when optimizing their individual sample processing and enrichment procedures. The proposed method showed sufficient repeatability, such that anyone trained in this method could easily accomplish it. Additionally, it has been suggested that the use of different kinds of immunoglobulin-binding proteins and magnetic beads is possible, but each material would need to be evaluated and optimized for its individual specific conditions. Future research will focus on using this immunomagnetic bead as a platform for various detection techniques, ELISA, real-time PCR, and impedance-based biosensors.

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