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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 *Escherichia 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.

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, althought 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

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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 non-magnetic 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_2O_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; Widjojoatmodjo 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