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Detection of immunomagnetically captured *Escherichia coli* O157:H7 by antibody-conjugated alkaline phosphatase

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A rapid and sensitive detection process for *Escherichia coli* O157:H7 was developed using alkaline phosphatase (APase)-labeled anti-*E. coli* O157 antibodies to tag the targeted bacteria. Immunomagnetic beads or antibody-labeled streptavidin-coated magnetic beads were then used to capture the APase-tagged *E. coli*. Immunomagnetically captured bacteria were washed and distributed into microplates or optical cuvettes. The enzyme-catalyzed hydrolysis of *p*-nitro-phenol phosphate in alkaline solutions was then followed. Less than 1000 cfu/ml of *E. coli* O157:H7 could be detected. This approach was applied to detect the bacteria artificially spiked in beef hamburgers. Less than 1 cfu/g of *E. coli* O157:H7 produced a significant response after cultural enrichment for 4–6 h at 37°C.
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Introduction

The demand for safer foods by the general public has attracted the attention of government and industry. To minimize the possibility of exposing consumers to pathogenic bacteria, their presence must be quickly determined and proper intervention applied. Many standard microbiological methods possess the necessary sensitivity for pathogenic bacteria detection. However, the requirements of long culture time and specialized facilities limit their application.

The need to develop alternative methods for selective pathogens has been summarized [4,9]. Efforts along this direction include ATP bioluminescence [14], antibody-directed fluorescent microscopy [15], polymerase chain reaction [1], quartz crystal microbalance [10] and electrochemical enzyme immunoassays [3,7]. Culture enrichment increases cell density of targeted pathogens and thus offers a better chance of detection by the alternatives mentioned above. Alternatively, cell density may be sufficiently increased by an effective concentration process [2]. One attractive approach [11] in rapidly and effectively separating and concentrating cells from complex media is the use of immunomagnetic beads. Immunomagnetic beads have been applied in several rapid methods for the capture of bacteria prior to analysis [5,12]. Recently, the advantage of concentrating targeted bacteria by immunomagnetic beads during signal detection steps has also been demonstrated [6,20]. In the past few years, we have incorporated immunomagnetic beads separation and concentration steps in the efforts of developing a few biosensor-based detection processes for *Escherichia coli* O157:H7 including digital fluorescence microscopy [19], ATP bioluminescence [17] and light addressable potentiometric sensor [18]. When applied to beef hamburger systems, these immunomagnetic-bead-utilizing biosensor methods all are capable of detecting about 1 cfu *E. coli* O157:H7/g of spiked hamburger meat after enrichment for 5–6 h at 37°C. With the exception of the ATP bioluminescence approach, adoption of other developed processes by small business may be hindered by the costs of requisite instrumentation.

In this report, a simple process that involves the use of either a spectrophotometer or 96-well plate reader commonly found in microbiological laboratories was developed. *E. coli* O157:H7 bacteria were tagged by alkaline phosphatase (APase)-conjugated anti-*E. coli* O157 antibodies and the immunomagnetic beads coated with anti-*E. coli* O157 antibodies. The target cells were then captured and a magnetic field was applied to separate and concentrate the targeted bacteria. The beads were suspended in small volumes of aqueous media containing *p*-nitro phenyl phosphate (*p*-NPP). The catalyzed hydrolysis of p-NPP by the conjugated APase was then used to determine the presence of targeted *E. coli*. The approach demonstrated that the presence of less than 1 cfu of *E. coli* O157:H7/g of spiked hamburger could be detected after a 4- to 6-h culture enrichment at 37°C.

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Materials and methods

Growth and enumeration of bacteria

E. coli O157:H7 (strain B1409; CDC, Atlanta, GA) and other bacterial samples used were inoculated from slants into 25 ml of brain–heart infusion (BHI) broth (Difco, Detroit, MI) and incubated at 37°C for 18 h with 160 rpm shaking. Bacterial suspensions were then placed on ice to halt growth and diluted 100-fold, and 6 μl of diluted sample was placed on a Petroff–Hauser bacteria-counting slide (Hausser Scientific, Horsham, PA). Bacteria were counted and the values were averaged to determine total direct cell count. Diluted bacterial suspensions in sterile Tris-buffered saline were also plated either on BHI agar or *E. coli* O157:H7 restrictive (Fluorocult *E. coli* O157:H7 Agar; EM Science, Gibbstown, NJ) agar plates to determine colony-forming units by plate counting after an 18-h incubation at 37°C.

Preparation of immunomagnetic beads and streptavidin-coated magnetic beads for bacteria capture

Commercially available immunomagnetic beads (10 mg in 1 ml of phosphate-buffered saline, pH 7.4 containing 0.1% human serum albumin and 0.02% NaN_3) coated with about 110 μg of anti-*E. coli* O157 antibodies (Dynal, Oslo, Norway) were used as stock. Thus, approximately 1.1 μg of the antibodies was present in 10 μl of immunomagnetic beads — the quantity normally applied in this work.

Streptavidin-coated magnetic beads from Dynal with a biotin binding capacity of 650–900 pmol/mg beads (about 162–225 pmol of avidin) were further treated with biotinylated anti-*E. coli* O157 antibodies according to the following procedure. Suspensions of 1.0 ml of purchased stock streptavidin-coated magnetic beads were washed 2× with phosphate-buffered saline *via* a Dynal magnetic concentrator to remove azide. The washed streptavidin-coated magnetic beads were suspended in 1 ml of phosphate-buffered saline buffer, and 10 μg of biotinylated anti-*E. coli* O157 antibodies was added and allowed to react for 30 min at 22°C. Non-conjugated biotinylated anti-*E. coli* O157 antibodies were removed from the suspension by washing the beads four to five times with phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin. The biotinylated anti-*E. coli* O157 antibodies labeled streptavidin-coated magnetic beads were then suspended and stored in 1.0 ml of phosphate-buffered saline buffer and used as stock. Thus, the biotinylated anti-*E. coli* O157 antibodies content of biotinylated anti-*E. coli* O157 antibodies streptavidin-coated magnetic beads was about 1.0 μg in 10 μl of the stock suspension.

Capture of E. coli from beef hamburger

To 225 ml of novobiocin-containing (4 μg/ml) modified EC broth (20 g tryptone, 5.0 g lactose, 4.0 g K_2HPO_4, 1.5 g KH_2PO_4, 5.0 g NaCl and 1.5 g bile salt no. 3 dissolved in 1 l of distilled water), 25 g of beef hamburger spiked with *E. coli* (1 cfu/g) was shaken at 160 rpm and 37°C. Samples were removed at the end of 4–6 h and were filtered through a disposable filter column with a pore size of 200–300 μm (Fisher Scientific, Pittsburgh, PA) to remove fat globules and meat particles and placed on ice. To 1 ml of filtered sample, 10 μl (about 6×10^6 beads) of stock immunomagnetic beads or streptavidin-coated magnetic beads, 10 μl of 10% bovine serum albumin and 20 μl (1 mg/ml) of APase-labeled goat anti-*E. coli* O157 antibodies were added and placed on a rocker for 30 min. The supernatant was discarded and the bacteria bead pellets were washed 2× and suspended in 1 ml of phosphate-buffered saline, pH 8, using a magnetic particle concentrator.

Determination of APase activity

The APase activity associated with labeled bacteria was determined spectroscopically at 400 nm using either a spectrophotometer or a plate reader. For spectrophotometer measurements, to the final bacterial suspensions in plastic centrifuge tubes, 0.4 mM *p*-NPP was added. After 30–60 min incubation with gentle mixing at 22°C, the absorbance increase due to production of *p*-nitro phenol was measured with a Beckman DU-70 spectrometer at 405 nm wavelength. For plate reader measurements, the same procedure as for the spectrophotometer was followed. After color development, volumes of 0.3 ml of the solution were rapidly transferred to each well of a transparent 96-well microplate. The absorbance (OD 405 nm) of sample wells was quickly determined with a Perkin-Elmer HTS 7000-plus Bio Assay Reader. Alternatively, volumes of 0.3 ml of the bacterial suspensions were added to wells followed by the addition of 0.4 mM *p*-NPP. After color development for 60 min, the absorbance at 405 nm was determined by the plate reader.

Results

Capture of E. coli O157:H7

To capture targeted *E. coli* O157:H7 bacteria in aqueous media, either commercially available anti-*E. coli* O157 antibodies coated immunomagnetic beads from Dynal [19] or beads obtained by conjugating streptavidin-coated magnetic beads with biotin-labeled anti-*E. coli* O157 antibodies were utilized. In current work, both approaches were chosen for bacterial capture.

Cells of freshly cultured *E. coli* O157:H7 were serially diluted from 10^8 to 10^1 cfu/ml in buffer solution. APase-labeled anti-*E. coli* O157 antibodies and immunomagnetic beads or biotinylated anti-*E. coli* O157 antibodies labeled streptavidin-coated magnetic beads were then introduced to interact with the bacteria for 60 min with gentle mixing at room temperature. The adsorbed bacteria were then separated from other solution components by the use of magnets. After washing with buffer several times, the beads were suspended in APase assaying media for enzyme activity determination. Both approaches were effective in capturing the bacteria in buffer solutions with a similar detection limit between 10^2 and 10^3 cfu/ml (Figure 1). The average sizes of magnetic beads used in both approaches were about the same (2.8 μm in diameter) and the amounts of the antibodies on the beads were similar Yet, slightly higher APase activity values were obtained by the use of streptavidin-coated magnetic beads. This observation suggests

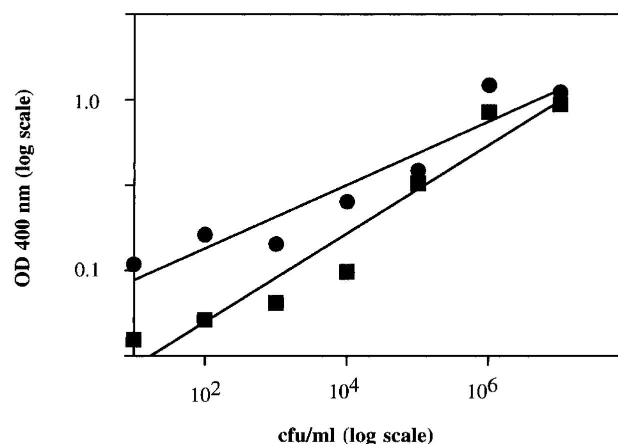

Figure 1 Comparison between immunomagnetic beads coated with anti-*E. coli* O157 antibodies and streptavidin-coated magnetic beads conjugated with biotin-labeled anti-*E. coli* O157 antibodies. Serially diluted strain B1409 of *E. coli* O157:H7 from 10^8 to 10^1 cfu/ml in 1 ml total volume. Bacterial suspensions in 1 ml were treated with 20 μl of APase-labeled goat anti-*E. coli* O157 antibodies and captured with 10 μl of immunomagnetic beads (filled squares) or freshly prepared streptavidin-coated magnetic beads conjugated with biotin-labeled anti-*E. coli* O157 antibodies (filled circles). Captured bacteria were washed with TBS, pH 8, using a magnetic concentrator. The bead pellet was suspended in *p*-NPP-containing TBS and incubated for color development as described. The absorbance at 405 nm was determined by a plate reader.

that freshly prepared streptavidin-coated magnetic beads may be more efficient in capturing *E. coli* than commercially available immunomagnetic beads.

Measurement of the enzyme activity

Hydrolysis of *p*-NPP generates the yellow colored-product, *p*-nitro phenol, with an absorption peak at about 400 nm and an extinction coefficient of 8.71/mM/cm at pH 8.0 [16]. The absorption increase can be followed accurately by a spectrophotometer. However, an ordinary spectrophotometer cannot handle a large number of samples rapidly. On the other hand, plate readers can handle 96, or more, samples in a short time. Thus, we investigated the possibility of using plate readers (Figure 2).

The comparison between spectrophotometer and plate reader measurements indicated that both instruments yielded a similar linear relationship between the observed absorbance increase and the bacterial colony-forming units per milliliter in solution. However, the values obtained by the plate reader were lower. For spectrophotometer measurement, the wavelength was fixed at 400 nm and the cell light path was 1.0 cm. For the plate reader, the detection filter used had the light transmission centered at 405 nm and the light path (equal to the liquid column height in the well) close to 1.1 cm. The results (Figure 2) indicated that the plate reader could be used.

Specificity of magnetic-bead-associated APase detection of the E. coli

The specificity for targeted bacteria was determined by the antibodies used. The antibody coated on the immunomagnetic beads was labeled anti-*E. coli* O157. On the other hand, the antibodies containing biotin or APase label were labeled anti-*E. coli* O157:H7. It has been reported that the polyclonal antiserum raised against *E. coli* O157 exhibits certain cross-reactivity to other bacteria, e.g., *E. hermanii*, *E. coli* O148:NM, *E. coli* O117:H27 and *Salmonella urbana* [13].

To measure cross-reactivity with other bacteria, the same capture and concentration procedure was applied to solutions containing *E. coli* O157:NM and *Shigella dysenteriae*. Comparisons of the immunomagnetic bead capture and observed APase activity are shown in Table 1.

Table 1 Cross-reactions of bacteria with immunomagnetic beads and APase-labeled anti-*E. coli* O157:H7 antibodies

Bacteria	cfu (10^6)/ml[a]		APase[b]
	Applied	Captured	
E. coli O157:H7	3.52	0.42	1.24
E. coli O157:NM	3.79	0.42	1.10
Sh. dysenteriae	12.0	0.00024	0.08

[a]The bacterial suspensions were treated with the same amount of immunomagnetic beads as described in the text and captured bacterial samples were diluted and spiral-plated for enumeration.
[b]Absorbance readings at 405 nm above the background were recorded. The background (contained the same ingredients as the sample, except no bacteria) gave an average absorbance reading of 0.32. Data represent the averages from triplicate experiments with an error range of ±10%.

There was almost identical capture with *E. coli* O157:NM and O157:H7, but only minimal capture of *Sh. dysenteriae*. The APase activities associated with captured cells were in agreement with the extent of cell capture. As discussed by Goodridge *et al.* [8], detection of these false-positive but pathogenic organisms could be advantageous from the point-of-view of enhancing food safety.

The approach exhibited greater than 10^4 to 1 preference for *E. coli* O157:H7 than for non-pathogenic *E. coli* K12 (Figure 3). The signal obtained with 10^5 cfu/ml of *E. coli* K12 is considerably less

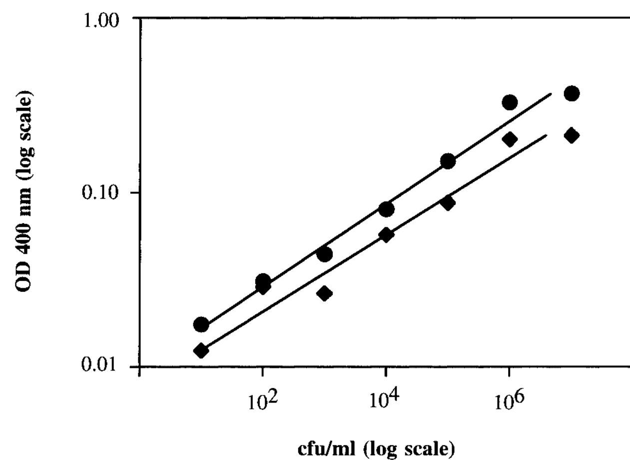

Figure 2 Comparison between spectrophotometer and plate reader measurement. Serially diluted strain B1409 of *E. coli* O157:H7 from 10^8 to 10^1 cfu/ml in 1 ml total volume. Bacterial suspensions in 1 ml were treated with 20 μl of phosphatase-labeled goat anti-*E. coli* O157:H7 antibody and captured with 10 μl of anti-*E. coli* O157 immunomagnetic beads. Captured bacteria were washed with TBS, pH 8, using a Dynal magnetic concentrator. The bead pellet was suspended in 1 ml of TBS, pH 8. After adding 20 μl of 20 mM *p*-NPP, the suspension was incubated at 22°C for 1 h for color development. Absorbance at 400 nm was determined by a Beckman spectrophotometer (open circles) or at 405 nm by a Perkin-Elmer plate reader (filled triangles).

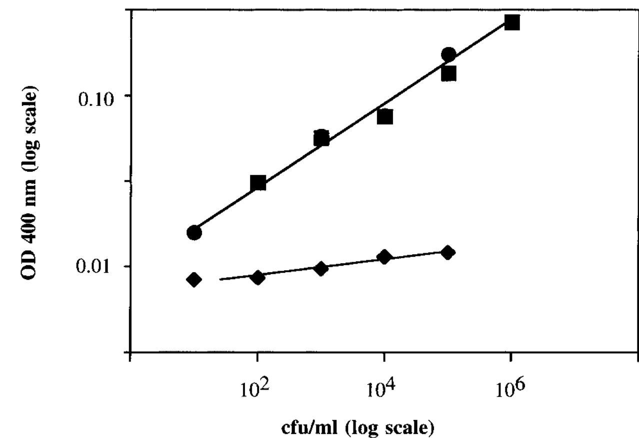

Figure 3 Specificity for *E. coli* O157:H7. Strains of freshly grown *E. coli* were serially diluted from 10^8 to 10^1 cfu/ml. Bacterial suspensions in 1 ml were treated with 20 μl of phosphatase-labeled goat anti-*E. coli* O157:H7 antibody and captured with 10 μl of anti-*E. coli* O157 Dynal beads. Captured bacteria were washed with TBS, pH 8, using a Dynal magnetic concentrator. The bead pellet was suspended in 1 ml of TBS, pH 8. Aliquots of 20 μl of 20 mM *p*-NPP were added to the 1 ml suspensions and incubated for 60 min at room temperature for color development. The absorbance at 405 nm was determined using a plate reader.

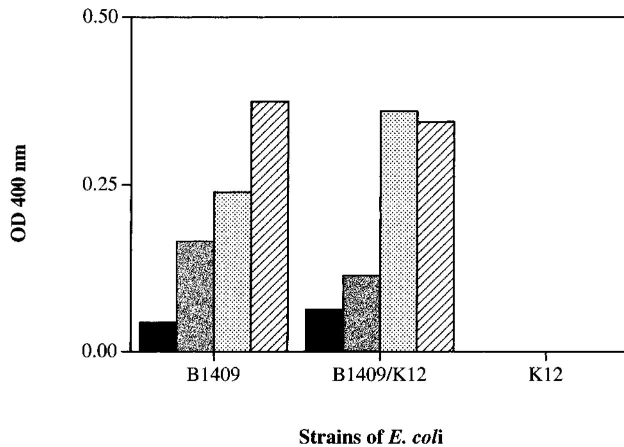

Figure 4 Detection of *E. coli* O157:H7 spiked in hamburger. Portions of 25 g of beef hamburger spiked with 1 cfu/g of indicated that *E. coli* were incubated in 225 ml of novobiocin-containing modified EC broth at 37°C. Aliquots were taken out at the end of 4 and 6 h and filtered through glass wool. The *E. coli* bacteria in filtered samples, after being tagged with APase-labeled anti-*E. coli* O157 antibodies, were captured by either immunomagnetic beads or streptavidin magnetic beads conjugated with biotin-labeled antibodies. Captured bacteria were washed with TBS, pH 8, and concentrated using a magnetic concentrator. The bead pellets were suspended in *p*-NPP-containing TBS as described. Absorbance at 405 nm was obtained using a plate reader. The bars indicate the APase activities detected using immunomagnetic beads (balk bar, 4-h enrichment; dotted bar, 6-h enrichment) and streptavidin-coated beads (gray bar, 4-h enrichment; striped bar, 6-h enrichment).

than that observed with 10 cfu/ml of *E. coli* O157:H7 (strain B1409 or 1558). We also noted that the signals were not significantly affected by the presence of K12 cells (up to the same concentration level) in samples containing O157:H7 cells. Thus, the approach had a reasonable selectivity toward targeted *E. coli* O157:H7.

Application to a hamburger system

The goal of our current study was to determine whether the developed procedure could be applied to actual food systems. To test this, hamburger purchased from local markets was spiked with 1 cfu/g of *E. coli* O157:H7. After a brief incubation in restricted media for 4–6 h at 37°C, the developed procedures were applied to capture, concentrate and detect targeted *E. coli*. As shown in Figure 4, both immunomagnetic beads and labeled streptavidin-coated magnetic beads approaches could detect the presence of less than 1 cfu of *E. coli* O157:H7 in 1 g of beef hamburger after 4 h of enrichment at 37°C. The hamburger spiked with the same level (1 cfu/g) of *E. coli* K12 did not show a detectable signal. Furthermore, co-spiking the hamburger with 1 cfu/g each of *E. coli* O157:H7 and *E. coli* K-12 did not significantly affect the signal intensities associated with targeted pathogen. These results demonstrated that the developed procedures were capable of detecting low levels of *E. coli* O157:H7 with a reasonable specificity.

Conclusions

To promote a wider adoption of pathogen detection as a routine practice by the food industry, relatively inexpensive methodologies with a reasonable accuracy and limit of detection should be developed. In this study, the use of magnetic beads and the application of APase determination were combined to achieve a sensitive detection with reasonable specificity for *E. coli* O157:H7 in hamburger using common microplate readers. Presumably, the approaches developed may be easily adopted to detect *E. coli* O157:H7 in other food matrices such as bottled drinking water, juices and ciders. While the approach is not specific enough for identification of bacteria, it could be used for screening purposes. Since the approach requires only a 4-h enrichment, the detection can be completed well within a standard working shift. The immunomagnetic beads cross-reactivity with certain pathogenic bacteria may be advantageous for applying the method as an informative screening process to enhance the safety of foods. Presumably, by replacing the anti-*E. coli* O157:H7 antibody with proper antiserum against other pathogenic bacteria, the developed approaches could become a general method for food safety applications.

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