Magnetic Nanoparticle-Antibody Conjugates for the Separation of *Escherichia coli* O157:H7 in Ground Beef

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MS 04-547: Received 30 November 2004/Accepted 30 April 2005

**ABSTRACT**

The immunomagnetic separation with magnetic nanoparticle-antibody conjugates (MNCs) was investigated and evaluated for the detection of *Escherichia coli* O157:H7 in ground beef samples. MNCs were prepared by immobilizing biotin-labeled polyclonal goat anti- *E. coli* antibodies onto streptavidin-coated magnetic nanospheres. For bacterial separation, MNCs were mixed with inoculated ground beef samples, then nanoparticle-antibody- *E. coli* O157:H7 complexes were separated from food matrix with a magnet, washed, and surface plated for microbial enumeration. The capture efficiency was determined by plating cells bound to nanoparticles and unbound cells in the supernatant onto sorbitol MacConkey agar. Key parameters, including the amount of nanoparticles and immunoreaction time, were optimized with different concentrations of *E. coli* O157:H7 in phosphate-buffered saline. MNCs presented a minimum capture efficiency of 94% for *E. coli* O157:H7 ranging from 1.6 × 10^1 to 7.2 × 10^7 CFU/ml with an immunoreaction time of 15 min without any enrichment. Capture of *E. coli* O157:H7 by MNCs did not interfere with other bacteria, including *Salmonella enteritidis*, *Citrobacter freundii*, and *Listeria monocytogenes*. The capture efficiency values of MNCs increased from 69 to 94.5% as *E. coli* O157:H7 decreased from 3.4 × 10^7 to 8.0 × 10^0 CFU/ml in the ground beef samples prepared with minimal steps (without filtration and centrifugation). An enrichment of 6 h was done for 8.0 × 10^0 and 8.0 × 10^3 CFU/ml of *E. coli* O157:H7 in ground beef to increase the number of cells in the sample to a detectable level. The results also indicated that capture efficiencies of MNCs for *E. coli* O157:H7 with and without mechanical mixing during immunoreaction were not significantly different (*P > 0.05*). Compared with microbeads based immunomagnetic separation, the magnetic nanoparticles showed their advantages in terms of higher capture efficiency, no need for mechanical mixing, and minimal sample preparation.

*Escherichia coli* O157:H7 is one of the most harmful foodborne pathogenic bacteria and alone is responsible for an estimated 73,000 cases of infection and 61 deaths that occur in the United States each year (4). The infections of *E. coli* O157:H7 may cause life threatening complications—hemolytic uremic syndrome and hemorrhagic colitis in humans (2, 5, 33). Some sources of infection are consumption of beef, sprouts, lettuce, salmon, unpasteurized milk, and juice contaminated with pathogens (11). Since the loss caused by *E. coli* O157:H7 is enormous in terms of medical cost and product recall, it is extremely important to rapidly, sensitively, and specifically detect *E. coli* O157:H7 in food products.

In immunomagnetic separation (IMS) methods, the capture of target bacteria by immunomagnetic particles (or beads) has been a critical factor for the success of the methodology (9, 32). Binding efficiency or capture efficiency (CE), the percentage fraction of the total bacteria retained on the surface of the beads, is used to define the binding capacity of the beads with bacteria (21, 35). Pérez et al. (21) and Yu et al. (35) used conventional plating methods to determine binding kinetics of the microbeads. Although IMS techniques provide simple, rapid, sensitive, and low-cost methods for isolation of target organisms from the mixture of bacteria (3, 9, 17, 19, 20, 22, 33), the food matrix always interferes with the capture of bacteria in a food sample by immunomagnetic particles, resulting in low CE (14). Sample preparation always incorporates tedious and multiple steps of stomaching, filtration, and centrifugation in order to reduce the effect of food matrix on capture of bacteria. In many of the reported immunoassays, Dynabeads (2.8 μm diameter, Dynal Inc., Lake Success, N.Y.) are used as a magnetic carrier (5, 15, 32). For example, Liu et al. (14) developed 2.8-μm diameter Dynabeads-based chemiluminescence fiberoptic biosensor for the detection of *E. coli* O157:H7 in food samples with an overall detection time of 90 min and a detection limit of 3.2 × 10^2 CFU/ml. Some researchers combined Dynabeads with other detection techniques like multiplex PCR and showed that the detection limit of the IMS was 1 CFU of *E. coli* O157:H7 per 25 g of raw ground meat sample (11) with a total detection time of 24 h. The most important aspect of IMS is to capture and concentrate target bacteria and to eliminate the background noise caused by the food matrix.

In recent years, nanoparticles have opened new dimensions in IMS because of their unique advantages over microbeads. Magnetic nanoparticles from 50 to 150 nm exhibit properties of ferrofluids and remain stable colloids, whereas microbeads show particle behavior. The advantages of employing nanoparticles as compared with microbeads are in the kinetics of reaction (8, 12). Liberti et al.
(12) showed that magnetic nanoparticles are 5- to 20-fold faster than 1- to 100-μm magnetic microbeads in reaction kinetics, and the mass of nanoparticles used for separation was much less as compared with microbeads (0.5 μg versus 100 to 300 μg). Nanoparticles are important in numerous applications because of their unique optical properties, high surface-to-volume ratio, and other size-dependent qualities (29). From a functionality point of view nanoparticles find their use as a magnetic carrier or as a label (7, 12, 26, 27) in a variety of applications. Additionally, magnetic nanoparticles do not interfere with chemiluminescence, fluorescence, and PCR reactions in numerous immunoassays (18, 25, 30, 31).

Amagliani et al. (1) developed a method based on the use of DNA adsorbed paramagnetic silica nanoparticles for the detection of Listeria monocytogenes from milk samples with an overall detection time of 7 h and a detection limit of 10 CFU/ml. However, to the best of our knowledge there is no report on the use of magnetic nanoparticle-antibody conjugates (MNCs) for the direct separation of bacteria from food samples. Therefore, in this study nanoparticles were applied for the IMS of E. coli O157:H7 directly from ground beef with minimum sample preparation. Streptavidin-coated magnetic nanoparticles and biotin conjugated anti-E. coli antibodies were selected as immunocapture agents. Nanoparticles were also compared with microbeads in terms of their CE values. The effect of sample preparation (stomaching, filtration, and centrifuge) and mixing (magnetic particles and bacteria) on capture of bacteria by nanoparticles was investigated with microbial surface plating methods and scanning electron microscopy.

MATERIALS AND METHODS

Culture and plating of bacteria. Frozen stocks of E. coli O157:H7 (ATCC 43888), rifampin-resistant E. coli O157:H7 (ATCC 43888), Salmonella enteritidis (ATCC 13076), L. monocytogenes (FDA 101M 4b), and Citrobacter freundii (ATCC 3624) were maintained in brain heart infusion (with 12% glycerol) broth (Remel Inc., Lenexa, Kans.) at ~70°C. All cultures were harvested in brain heart infusion broth maintained at 37°C for 18 to 22 h. Pure cultures were diluted with 0.01 M, pH 7.4 phosphate-buffered saline (PBS). E. coli O157:H7 was surface plated on sorbitol MacConkey (SMAC) agar (Remel), which was incubated at 37°C for 20 to 22 h. Rifampin-resistant E. coli O157:H7 was surface plated on the SMAC agar with 0.3% (wt/vol) rifampin (Sigma Chemical Co., St. Louis, Mo.).

Chemicals and reagents. PBS (0.01 M, pH 7.4) was obtained from Sigma. Bovine serum albumin (BSA; EM Science, Gibbstown, N.J.), 0.5% (wt/vol) was prepared in PBS as a blocking buffer (PBS BSA). PBS (0.05 M, pH 7.0) was used for serial dilution of bacteria before surface plating. All solutions were prepared with deionized water from Millipore (Milli-Q, 18.2 MΩ·cm, Bedford, Mass.). PBS Tween 20 0.05% (wt/vol) (Sigma) was used for washing immunomagnetic particles.

Nanoparticles and microbeads. Magnetic nanoparticles (average diameter of 145 nm, Captivate ferrofluid streptavidin, 0.5 mg Fe/ml) conjugated with streptavidin were obtained from Molecular Probes Inc. (Eugene, Oreg.). Magnetic nanoparticles have more than 85% of oxide as Fe3O4, approximately 80% wt/wt of magnetite, and approximately 4 × 1011 particles per mg Fe. Based on these values, the number of magnetic nanoparticles per micro-liter was estimated as 1.6 × 108. Affinity purified polyclonal goat antibodies against E. coli (specific for O and K antigens) conjugated with biotin were obtained from Biodesignt International (Saco, Maine). The concentration of stock solution of biotin-labeled antibodies was 4 to 5 mg/ml. A 1:10 dilution of the antibodies was prepared in PBS (0.01 M, pH 7.4) before use. This dilution of the biotin conjugated anti-E. coli was used for all tests. Magnetic microbeads (MMBs, 2.8 μm diameter) coated with affinity purified polyclonal antibodies against E. coli O157:H7 were obtained from Dynal, Inc.

Preparation of MNCs. MNCs were prepared in 1.7 ml of sterile polypropylene centrifuge tubes. Biotin-labeled polyclonal goat anti-E. coli antibodies (10 μl) were continuously mixed with streptavidin-coated magnetic nanoparticles (20 μl) in 250 μl PBS BSA at 7 rpm on a variable speed rotator (ATR, Laurel, Md.) for 35 min at room temperature. Then they were ready for use in tests.

CE calculations. CE, defined as the percentage fraction of the total bacteria retained on the surface of the beads, could be calculated using two methods: one is based on the cells bound to immunomagnetic particles, and the other is based on the cells unbound to immunomagnetic particles or left in the supernatant. In both cases, conventional plating method was used to determine the binding kinetics of the immunomagnetic particles (21, 25). The following two equations were used for the two methods:

For CE based on cells bound to immunomagnetic particles

\[
CE(\%) = \frac{C_b}{C_o} \times 100
\]

For CE based on cells unbound to immunomagnetic particles

\[
CE(\%) = \left(1 - \frac{C_u}{C_o}\right) \times 100
\]

where, \(C_o\) is the total number of cells present in the sample (CFU per milliliter), \(C_b\) is the number of cells bound to immunomagnetic particles (CFU per milliliter), and \(C_u\) is the number of cells unbound to immunomagnetic particles (CFU per milliliter).

Assay procedure. Serial dilutions of the pure culture of E. coli O157:H7 were prepared in PBS (0.01 M, pH 7.4) buffer. The whole assay procedure was completed in two steps. The first step was the immunoreaction between immunomagnetic particles and bacteria. Immunoreaction was followed by surface plating as the second step in order to enumerate bacteria. For all tests a sample size of 0.5 ml was used and therefore CFU/0.5 ml was used as the unit in presenting bacterial concentration. The binding kinetics and capacity of the MNCs against E. coli O157:H7 were studied for four immunoreaction times (15, 30, 45, and 60 min) and five bacterial concentrations (10⁶, 10⁷, 10⁸, 10⁹, and 10¹⁰ CFU/ml). Immunoreaction was carried out by mixing 20 μl of immunomagnetic particles with 0.5 ml of a sample at a given cell concentration in a 1.7-ml centrifuge tube at 10 rpm with a variable speed rotator. After respective immunoreaction time, tubes were removed from the rotator and the immunomagnetic particles were separated from the supernatant with a magnetic particle concentrator (Dynal Inc.). An aliquot of 0.1 ml from supernatant was surface plated for bacterial enumeration after appropriate dilution. A total of 0.5 ml of PBS Tween 20 0.05% was used twice to wash immunomagnetic particles with intermittent separation with a magnetic particle concentrator, vortexing in a mini-vortexer (VWR, West Chester, Pa.), and resuspension in 0.5 ml of PBS.
 Tween 20 0.05%. An aliquot of 0.1 ml from supernatants from each washing solution was surface plated after appropriate dilution. Finally, immunomagnetic particles were resuspended in 0.5 ml of PBS BSA, and 0.1 ml of them was used for surface plating after appropriate dilution. Triplets of each sample were plated.

The possible growth of *E. coli* O157:H7 during the test was checked to confirm the microbial data. *E. coli* O157:H7 in PBS BSA was left in a tube for 60 min at room temperature (22°C), surface plated onto SMAC agar plates, and then incubated at 37°C for 18 to 20 h. The plating result was compared with the initial cell number of *E. coli* O157:H7 in the same sample.

For cell concentrations less than or equal to 10 CFU/0.5 ml of *E. coli* O157:H7, after immunoreaction and separation, all supernatant (0.5 ml) was aspirated and 100 μl of it was surface plated on each of five SMAC agar plates. The washing step was done twice, and the washing buffer was also surface plated on five SMAC agar plates. Similar steps were applied for the resuspended immunomagnetic particle–antibody–bacteria complex. Finally, all five plates were counted for the total number of CFU in a particular test. The values of CE were calculated based on equations 1 and 2. Initial cell number per ml in these samples was determined by surface plating 0.1 ml of the sample on each of the 10 agar plates and adding cell numbers on all 10 plates to get a bacterial count per milliliter.

**Comparison of MNCs with immunomagnetic microbeads.** For comparison, MMBs were used in all tests in parallel with MNCs, following the same procedure. For MMBs, the immunoreaction was carried out by mixing 10 μl of MMBs with 0.5 ml of a sample containing *E. coli* O157:H7 at a given cell concentration in a 1.7-ml centrifuge tube rotating at 10 rpm with a variable speed rotator. They were compared in terms of CE for *E. coli* O157:H7 at three concentrations and four immunoreaction times. In this study, immunomagnetic particles were referred to both MMBs and magnetic nanoparticles.

**Food samples preparation and enrichment.** Commercial ground beef was purchased from a local supermarket. Two types of samples were prepared for ground beef: one with enrichment for low cell concentrations (10^3 and 10^4 CFU/ml) in tryptic soy broth (EM Science, Gibbstown, N.J.), and the other without enrichment for all other higher concentrations (10^5 to 10^7 CFU/ml) in buffered peptone water (Remel). For both cases, 25 g of ground beef was homogenized with 225 ml of 0.1% buffered peptone water or tryptic soy broth in a Whirl-pak plastic bag using a laboratory stomacher 400 (Seward, Norfolk, UK) for 2 min. After stomaching, food samples were inoculated with decimally diluted cultures of *E. coli* O157:H7. For enrichment, 1 ml of 8 × 10^3 CFU/ml and 4 × 10^4 CFU/ml of *E. coli* O157:H7 was added to 9 ml of tryptic soy broth homogenized ground beef samples and incubated at 37°C for 6 h. For samples without enrichment, 1 ml of *E. coli* O157:H7 ranging from 6.0 × 10^5 CFU/ml to 3.4 × 10^8 CFU/ml was inoculated to 9 ml of buffered peptone water homogenized ground beef samples. For 1, 10, and 10^3 CFU/ml of inoculum concentrations, rifampin-resistant *E. coli* O157:H7 was used in order to avoid the growth of naturally present bacteria from ground beef samples onto agar plates and was surface plated on rifampin-resistant SMAC (R-SMAC) agar. For both types of food sample preparation, samples without purposely added *E. coli* O157:H7 and rifampin-resistant *E. coli* O157:H7 were used as a negative control and were surface plated on SMAC and R-SMAC agar, respectively, and incubated at 37°C for 18 to 22 h. The total viable count of organisms present in the ground beef samples was enumerated by surface plating onto tryptic soy agar (TSA) plates incubated at 37°C for 18 to 22 h.

To observe the effect of food components on CE of MNCs for *E. coli* O157:H7 in ground beef samples, two sets of tests were compared: one on the sample collected after stomaching only; and the other on the sample after stomaching, filtration with cheese cloth, and centrifugation (two times at 250 × g for 15 min) in order to separate large size particles present in the ground beef stomaching water. *E. coli* O157:H7 was added after sample preparation. Steps for immunoreaction and separation were the same as described before. Only 10^3 to 10^7 CFU/0.5 ml of *E. coli* O157:H7 were compared in order to avoid the enrichment step.

**Specificity test.** Nonspecific binding of MNCs was tested for three nontarget bacteria—*S. enteritidis, L. monocytogenes,* and *C. freundii.* All three nontarget bacteria were added with rifampin-resistant *E. coli* O157:H7 in food samples. The known number of nontarget bacteria added with 2.5 × 10^4 CFU/0.5 ml of *E. coli* O157:H7 was approximately 10^6 CFU/0.5 ml. Binding of MNCs against target and nontarget bacteria was tested by the surface plating method as described earlier. Inoculum level of nontarget bacteria was estimated by surface planting onto TSA agar plates, and rifampin-resistant *E. coli* O157:H7 was grown on R-SMAC agar plates. Uninoculated ground beef samples were surface plated onto SMAC and R-SMAC agar plates to check the natural presence of *E. coli* O157:H7 and rifampin-resistant *E. coli* O157:H7 in the beef samples. The immunoreaction time for MNCs and bacteria was 15 min.

**Mixing test.** To determine the effect of mixing on CE of MNCs and MMBs, two tests were performed—one with mixing in a variable speed rotator at 10 rpm and the other without mixing. PBS BSA was used with 3.9 × 10^4 CFU/0.5 ml of *E. coli* O157:H7 for 15 min of immunoreaction time. All other steps for the calculation of CE were the same as described in the assay procedure.

**Statistical analysis.** The microbial data and calculated CE were reported as mean, standard deviation, and coefficient of variance of three replicates. The mean values were subjected to analysis of variance for significant difference (α = 0.05). The standard deviation of all triplets was plotted as error bars at data points (JMP software, SAS Institute Inc., Cary, N.C.).

**RESULTS AND DISCUSSION**

**CE of magnetic nanoparticle conjugates against *E. coli* O157:H7.** To obtain an accurate bacterial count, 1-ml aliquots from each cell concentration were first prepared. Each aliquot was then divided equally into control and test samples. The controls were appropriately diluted and surface plated to obtain an accurate number of bacteria for each inoculum level before capture by immunomagnetic particles (C_o). Test samples were mixed with MNCs for the determination of fraction of bacteria captured by immunomagnetic particles (C_b). After magnetic separation, supernatant was surface plated to determine fraction of bacteria not bound with immunomagnetic particles (C_m). CE was calculated based on the bacteria count of control and sample using equations 1 and 2 (21, 35). Table 1 shows the relationship between cell concentrations and the fraction of bacteria captured by MNCs in 15 min of immunoreaction time. For 8.0 × 10^3, 2.7 × 10^4, 3.8 × 10^3, 4.2 × 10^3, and 3.6 × 10^7 CFU/0.5 ml of *E. coli* O157:H7, this method was able to obtain CE values of 100, 100, 94, 96, and 95%, respectively. As shown in Figure 1, for 15, 30, 45, and 60
TABLE 1. Capture efficiency of magnetic nanoparticle conjugates against $8.0 \times 10^0$, $2.7 \times 10^1$, $3.8 \times 10^3$, $4.2 \times 10^5$, and $3.6 \times 10^7$ CFU/0.5 ml of E. coli O157:H7 for 15 min of immunoreaction time

<table>
<thead>
<tr>
<th>E. coli O157:H7 concentration (CFU/0.5 ml)</th>
<th>Mean and SD of bacteria captured by 20 μl of nanoparticles (CFU)$^a$</th>
<th>Fraction of total bacteria captured by the nanoparticles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>$8.0 \times 10^0$</td>
<td>0 ± 0</td>
<td>7.0 ± 2.4</td>
</tr>
<tr>
<td>$2.7 \times 10^1$</td>
<td>0 ± 0</td>
<td>$2.5 \times 10^1 \pm 3.0$</td>
</tr>
<tr>
<td>$3.8 \times 10^3$</td>
<td>$3.6 \times 10^3 \pm 4.9 \times 10^1$</td>
<td>$3.5 \times 10^3 \pm 2.9 \times 10^1$</td>
</tr>
<tr>
<td>$4.2 \times 10^5$</td>
<td>$4.1 \times 10^5 \pm 1.0 \times 10^3$</td>
<td>$3.1 \times 10^5 \pm 1.2 \times 10^5$</td>
</tr>
<tr>
<td>$3.6 \times 10^7$</td>
<td>$3.4 \times 10^6 \pm 5.6 \times 10^8$</td>
<td>$2.6 \times 10^7 \pm 6.1 \times 10^6$</td>
</tr>
</tbody>
</table>

$^a$ Three replicates were taken for all readings.
$^b$ Calculation was based on the unbound bacteria present in the supernatant.
$^c$ Calculation was based on the bacteria bound to magnetic nanoparticles.

min of immunoreaction time, we have demonstrated that CE was greater than 94% for all cell concentrations of E. coli O157:H7.

Washing was performed in order to remove bacteria loosely bound to MNCs. After washing and magnetic separation of nanoparticle-antibody–E. coli O157:H7 complexes, the supernatant was surface plated to determine fraction of bacteria lost during washing. Fraction of bacteria washed out was less than 1.5% (data not shown) of the initial concentration, hence neglected for all other tests. As shown in Table 1, CE was greater than 94% for the separation of E. coli O157:H7 from $10^0$ to $10^7$ CFU/0.5 ml even for an immunoreaction time of 15 min. Thus 15 min of immunoreaction time was taken for all further tests. CE calculations based on surface plate counts of cells bound to nanoparticles (equation 1) were generally less than those based on unbound cells in the supernatant (equation 2). Similar observations were presented by other researchers on microbeads (6, 21). Scanning electron micrographs show cluster formation of MNCs and MMBs in the presence of E. coli O157:H7 when a magnetic field was applied (Fig. 2a and 2b). This was also reported by Liberti et al. (12) for MMBs. When CE was calculated based on cells bound to magnetic particle conjugates, which was determined by surface plating method, the CE values could be underestimated.

FIGURE 1. CE of magnetic nanoparticle conjugates against $2.7 \times 10^1$, $3.8 \times 10^3$, $4.2 \times 10^5$, and $3.6 \times 10^7$ CFU/0.5 ml of E. coli O157:H7 with 15, 30, 45, and 60 min of immunoreaction times. For $8.0 \times 10^0$ and $2.7 \times 10^1$ CFU/0.5 ml of E. coli O157:H7, no bacterial growth was observed in the supernatant of all replicates, hence mean CE was taken as 100% with no standard error bars (based on equation 2).

FIGURE 2. (a) Scanning electron microscopy micrographs (×15,000) of the MNCs bound with E. coli O157:H7 after the application of a magnetic field. The clusters around bacteria are formed by nanoparticles. (b) Scanning electron microscopy micrographs (×10,000) of the MMBs bound with E. coli O157:H7 after the application of a magnetic field.
Comparison of nanoparticle-antibody conjugates with microbeads for their CEs. MNCs resulted in significantly higher capacity (CE values) to separate E. coli O157:H7 as compared with MMBs for all combinations of three levels of cell numbers with four immunoreaction times (Fig. 3 for two cases; the rest of the data are not shown). Figure 3a shows CEs of both MNCs and MMBs for detection of E. coli O157:H7 at three levels of cell numbers (10^3, 10^5, 10^7 CFU/0.5 ml) with an immunoreaction time of 30 min. MMBs showed a decrease in CE with an increase in concentration of bacteria. CEs of MMBs were 92, 90, and 83% for 3.6 \times 10^3, 4.2 \times 10^5, and 3.5 \times 10^7 CFU/0.5 ml of E. coli O157:H7, respectively. MNCs showed CEs of 98, 98, and 97% for 3.6 \times 10^3, 4.2 \times 10^5, and 3.5 \times 10^7 CFU/0.5 ml, respectively, and showed no dependence of capture of bacteria on the initial concentrations of E. coli O157:H7.

Figure 3b shows CEs of both MNCs and MMBs at different immunoreaction times (15, 30, 45, and 60 min) in the detection of 3.6 \times 10^5 CFU/0.5 ml of E. coli O157:H7. The CE values of MNCs were greater than 96% for all of the four immunoreaction times, while the CE values of MMBs were 85.8, 89.5, 93.0, and 94.8% for 15, 30, 45, and 60 min of immunoreaction time, respectively. When MMBs are used in the IMS methods, the minimum immunoreaction time of 10 min is recommended by Dynal, but most researchers have used immunoreaction times ranging from 30 to 120 min (14, 21, 35). MMBs showed an increase in CE values with an increase in immunoreaction time. On the other hand, in the case of MNCs, the CE values increased from 96.3 to 98.4% when the immunoreaction time increased from 15 to 30 min. After 30 min, CE remained almost 98% for both 45 and 60 min of immunoreaction time. Hence, MNCs did not show time-dependent characteristics for the separation of E. coli O157:H7 after 30 min immunoreaction time. No significant growth (P > 0.05) was observed when 3.5 \times 10^5 of E. coli O157:H7 present in PBS BSA was incubated at room temperature for 60 min.

In this study, the high binding capacity of MNCs resulted in a CE of 96.3% for 3.6 \times 10^5 CFU/0.5 ml of E. coli O157:H7 within 15 min of immunoreaction time. MNCs were found to separate approximately 10% more E. coli O157:H7 as compared with MMBs in 15 min of immunoreaction time. Fratamico et al. (6) also showed low

because one magnetic particle could have captured more than one cell or clusters could be formed with several magnetic particles and cells. In both cases, the result would be the formation of only one colony on the agar plate, causing an underestimation of separation of bacteria by magnetic particles (28). Thus CE calculated based on surface plating of unbound cells in the supernatant will present useful quantitative information on separation of bacteria by magnetic particles when the initial amount of bacteria is known (35). Therefore, we present the results using CEs based on unbound cells in the supernatant. The CEs based on bound cells to the nanoparticles are also presented in two tables for comparison.

CE of 39 to 60% for the separation of E. coli O157 with MMBs (diameter 4.5 μm) within 30 to 60 min of immunoreaction time. The difference in CE could be attributed to the differences in sizes of the beads, bead coating procedures, and microbial counting methods, as discussed elaborately by Pérez et al. (21). In this study, the higher CE of MNCs may also be attributed to the larger surface area of nanoparticles available for immunoreaction. The surface area of the nanoparticles available for capture of bacteria was 78 times more than that of the MMBs used in the tests. Differences were also found in the movement of particles in the solution. Nanoparticles, because of their smaller size, move by diffusion as compared with gravitational settling of microbeads (hence inertial mixing is required for microbeads) (12, 15). The difference is that biotin–streptavidin conjugation was employed in the present study for the immobilization of antibodies with nanoparticles as compared with the covalent coupling of anti–E. coli antibodies on the surface of the microbeads.
Effect of the amount of MNCs on CE. The amount of MNCs used in this study was in accordance with suggestions made in the company’s literature—40 μl/ml for Captivate Ferrofluid Streptavidin (15). We used 20 μl of MNCs, since our sample volume was 0.5 ml. Twenty microliters of MNCs resulted in a CE of 96% for 4.2 × 10^5 CFU/0.5 ml of E. coli O157:H7 within 15 min of immunoreaction time. To investigate the effect of a lower amount of magnetic nanoparticles, we compared CE values of three different volumes, 10, 15, and 20 μl. All three volumes of MNCs were reacted with 4.2 × 10^5 CFU/0.5 ml of E. coli O157:H7, and their respective CEs were determined. Fifteen microliters of MNCs gave an increase of 8% in CE compared with 10 μl volume, while 20 μl volume gave an increase of 15% in CE compared with 15 μl of MNCs (data not shown). Therefore, we demonstrated that 20 μl of MNCs could be used to achieve higher binding of bacteria.

Theoretical calculations were also made to calculate the optimum number of nanoparticles required for the capture of E. coli O157:H7. Calculations were based on the surface area of bacteria and nanoparticles. Zhao et al. (36) showed that antibody conjugated nanoparticles covered the entire surface of E. coli O157:H7 because of their small size. Based on this assumption, theoretically it was estimated that 2.3 × 10^3, 7.8 × 10^3, 1.1 × 10^6, 1.2 × 10^8, and 1.0 × 10^10 of MNCs were required to cover the entire surface of 8.0 × 10^9, 2.7 × 10^9, 3.8 × 10^3, 4.2 × 10^5, and 3.6 × 10^7 CFU/0.5 ml of E. coli O157:H7. In this study we used 20 μl of MNCs, which had 3.2 × 10^9 particles, for all tests. CE values for 10^8 and 10^5 CFU/0.5 ml of E. coli O157:H7 were 100%, since the number of MNCs used for testing was much higher than the theoretical values. However, for high concentrations of E. coli O157:H7 from 10^3 to 10^5 CFU/0.5 ml, CE values were 94 to 99%, though the theoretically estimated number of MNCs required to capture 100% of E. coli O157:H7 were less than the number of MNCs actually used in the tests. Decrease in CE could be due to the fact that most of E. coli O157:H7 formed a cluster with MNCs as shown by scanning electron microscopy micrographs (Fig. 2a). For 10^7 CFU/0.5 ml of E. coli O157:H7, the theoretical number of nanoparticles estimated was 300% more than the actual number of MNCs used; however, the CE value of MNCs was still 95%. Thus this study clearly showed that for cell concentrations less than 4.2 × 10^5 CFU/0.5 ml of E. coli O157:H7, the majority of the MNCs used for immunoreaction participated in cluster formation; and for 3.6 × 10^7 CFU/0.5 ml of E. coli O157:H7, complete covering of bacterium surface with nanoparticles was not required for an efficient separation. Moreover, only a small fraction of the total MNCs added to the sample participated in the separation of bacteria from the liquid matrix.

CE of magnetic nanoparticle conjugates for E. coli O157:H7 in ground beef samples. E. coli O157:H7 and rifampin-resistant E. coli O157:H7 were not found in uninoculated ground beef samples used in this study, based on surface plating of the samples on SMAC and R-SMAC agars. The sample of stomached ground beef had a total viable count of approximately 10^5 CFU/g. Figure 4 shows CE values for the range of concentration from 4 × 10^0 to 1.7 × 10^7 CFU/0.5 ml of E. coli O157:H7 in the ground beef sample prepared with stomaching only. For 4.0 × 10^0 and 4.0 × 10^1 CFU/0.5 ml of E. coli O157:H7, the mean values of CE were 94.5 and 88.5%, respectively. In our experiments, during enrichment steps E. coli O157:H7 inoculated at 8.0 × 10^0 and 8.0 × 10^1 CFU/ml reached 1.4 × 10^3 and 4.7 × 10^3 CFU/ml in ground beef samples, respectively. Without any enrichment, higher concentrations of E. coli O157:H7 gave the mean values of CE as 74, 73.9, and 68.5% for 1.8 × 10^3, 1.6 × 10^5, and 1.7 × 10^7 CFU/0.5 ml of E. coli O157:H7, respectively.

For 10^3 to 10^7 CFU/0.5 ml of E. coli O157:H7 inoculated in the ground beef samples, presence of larger sized food particles in the stomached samples reduced the CE values by 6 to 12% when compared with the CE values obtained in the samples prepared with stomaching, filtration, and centrifugation (data not shown). The most probable reasons for such observations are the binding of E. coli O157:H7 with the food matrix or the entrapment of nanoparticles in larger sized organic particles present in the ground beef sample that was not filtered or centrifuged. Liu et al. (14) reported a decrease in chemiluminescence signal for the capture of E. coli O157:H7 in beef samples and attributed it to the presence of fat, tissue, and other organic materials in the samples.

Selection analysis. CE of MNCs for 2.4 × 10^4 CFU/0.5 ml of E. coli O157:H7 in ground beef was 94%. When 2.4 × 10^4 CFU/0.5 ml of E. coli O157:H7 was mixed with 10^6 CFU/0.5 ml of each S. enteritidis, L. monocytogenes, and C. freundii in ground beef for the specific separation of target E. coli O157:H7 from food samples, CE value was found to decrease by 12%. MNCs showed some affinity for these nontarget bacteria and other competing microflora in the ground beef samples. However, a substantial
TABLE 2. Effect of mixing on the binding capacity of magnetic nanoparticle conjugates and magnetic microbeads against $3.9 \times 10^3$ CFU/0.5 ml of E. coli O157:H7 for 15 min of immunoreaction time

<table>
<thead>
<tr>
<th>Particles</th>
<th>Supernatant</th>
<th>Nanoparticles</th>
<th>Supematant</th>
<th>Nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixing during immunoreaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>$3.8 \times 10^3 \pm 4.3 \times 10^1$</td>
<td>$2.2 \times 10^3 \pm 1.3 \times 10^2$</td>
<td>95</td>
<td>56</td>
</tr>
<tr>
<td>Microbeads</td>
<td>$2.9 \times 10^3 \pm 5.2 \times 10^1$</td>
<td>$1.4 \times 10^3 \pm 1.3 \times 10^2$</td>
<td>75</td>
<td>49</td>
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<tr>
<td>No mixing during immunoreaction</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>$3.7 \times 10^3 \pm 4.4 \times 10^1$</td>
<td>$2.7 \times 10^3 \pm 5.9 \times 10^2$</td>
<td>94</td>
<td>70</td>
</tr>
<tr>
<td>Microbeads</td>
<td>$2.1 \times 10^3 \pm 5.3 \times 10^1$</td>
<td>$1.4 \times 10^3 \pm 7.4 \times 10^1$</td>
<td>52</td>
<td>34</td>
</tr>
</tbody>
</table>

* $a$ 20-μl nanoparticles and 10-μl microbeads were used in the tests.
* $b$ Three replicates were taken for all readings.
* $c$ Calculation was based on the unbound bacteria present in the supernatant.
* $d$ Calculation was based on the bacteria bound to magnetic nanoparticles.

amount (82%) of E. coli O157:H7 was still separated by MNCs in the presence of nontarget bacteria within 15 min. Unincubated ground beef samples did not show presence of E. coli O157:H7 or rifampin-resistant E. coli O157:H7 on SMAC or R-SMAC agar plates, respectively.

Effect of mixing on CE. Table 2 shows the effect of mixing on CE of MNCs and MMBs for $3.9 \times 10^3$ CFU/0.5 ml of E. coli O157:H7 in PBS BSA buffer for the two samples prepared by mixing and nonmixing. MNCs showed CE values of 95 and 94% for the samples obtained by mixing and nonmixing. There was no significant difference between these two CE values ($P > 0.05$). Thus, results indicated that there was no effect of mechanical mixing on the capture of bacteria by MNCs. There was a significant effect ($P < 0.05$) of mixing on the capture of bacteria by MMBs, as shown by the CE values of 75% and 52% for the samples prepared by mixing and nonmixing, respectively. This could be attributed to difference in size of nanoparticles and microbeads. Small and relatively uniform particle size of the nanoparticles made them ideal for efficient diffusion and rapid kinetics of binding (10, 15, 25), and hence no settling of nanoparticles was observed during immunoreaction, while owing to the larger size of microbeads, gravitational settling was observed and hence inertial mixing was required for efficient immunoreaction of microbeads with bacteria (24). For this reason nanoparticles will be highly useful for microfluidic applications, where mass transfer is dominated by laminar flow. Microfluidic devices, often referred to as “laboratory-on-a-chip” or micro–total analysis systems (μTAS), consist of fluidic channels etched into a suitable solid substrate for processing and analyzing samples in a fast, serial manner. In a laminar flow, which is common for the microfluidic channels, mass transfer is principally governed by diffusion, and where nanoparticles can offer a more efficient mixing and immunoreaction. Research related to beads and microfluidic channels has always suffered from the problems of mixing (13, 16, 23, 24). Therefore, magnetic nanoparticles will offer distinct advantages in terms of no mixing required for the immunoreaction between magnetic particles and bacteria and thus could be a good choice to be used in microfluidic applications.

In this article we showed that MNCs have great potential to be used as a magnetic carrier in IMS of E. coli O157:H7 from food samples. We demonstrated that nanoparticles can be used for the rapid IMS of E. coli O157:H7 in a wide range of concentrations with high specificity and CE (94%) within 15 min of immunoreaction time. The result indicated that even with minimal sample preparation (stomaching only), MNCs could be used with a CE of 69 to 94% for the separation of wide range of concentrations of E. coli O157:H7 directly from ground beef samples. In addition to this, we also demonstrated that other pathogens such as S. enteritidis, C. freundii, and L. monocytogenes did not interfere with the separation of E. coli O157:H7 in food samples, hence MNCs are very specific in capturing E. coli O157:H7. Compared with the microbeads IMS method, nanoparticles showed higher capture of bacteria and no mechanical mixing was required. Finally, we can conclude that nanoparticle-antibody conjugates with high CE, minimal sample preparation, and no need of mixing for immunoreaction have the potential to be combined with bacterial detection techniques for the design of new immunoassay systems.

ACKNOWLEDGMENTS

This research was supported by the Food Safety Consortium. The authors thank Betty Swen for her help in microbiological tests.

REFERENCES

MAGNETIC NANOPARTICLE-ANTIBODY CONJUGATES SEPARATE _E. COLI_ IN BEEF


