Highly efficient and specific separation of *Staphylococcus aureus* from lettuce and milk using Dynabeads protein G conjugates

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Received May 27, 2016
Revised June 28, 2016
Accepted June 28, 2016
Published online October 31, 2016

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pISSN 1226-7708
eISSN 2092-6456

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Abstract An immunomagnetic separation method using antibody-coated Dynabeads® Protein G was developed for specific and efficient separation of *Staphylococcus aureus* in lettuce and whole milk. The amount of immunomagnetic beads (IMBs) and conjugation conditions were optimized. A high capture efficiency was obtained with 0.4 mg of IMBs, an immunoreaction time of 20 min, and a separation time of 1 min without wash. Under optimal conditions, the capture efficiency (CE) for 10^0 - 10^5 CFU/mL of *S. aureus* was higher than 91.46%. The IMBs showed high specificity even with a high constant number (10^7 CFU/mL) of *Bacillus cereus*, *Micrococcus luteus*, and *Lactobacillus plantarum*. The CE of IMBs against *S. aureus* at concentrations from 10^2 to 10^5 CFU/mL ranged from 78.70 to 94.77% for lettuce and 60.0 to 73.27% for milk samples. This IMS can be an appropriate selection for combining with bacterial detection method or efficient isolation procedure for *S. aureus* from foods.

Keywords: *Staphylococcus aureus*, immunomagnetic separation, Dynabeads® Protein G

Introduction

Staphylococcus aureus is a gram-positive, facultative, anaerobic, non-motile, catalase-positive, and coagulase-positive coccus. It can produce staphylococcal enterotoxins and cause staphylococcal food poisonings (1). Furthermore, *S. aureus* is one of the top five pathogen-food category pairs, resulting in outbreak-associated illness in America according to the Centers for Disease Control and Prevention 2016 (2).

S. aureus has been isolated from several types of food products that were originally contaminated during or after the food processing. Milk (3), dairy products (4), and vegetables (5) have been associated with *S. aureus* contamination. *S. aureus* is of particular concern to the dairy industry because the bacteria have been isolated from contaminated milk in numerous countries including Japan, America, Argentina, Brazil, Ireland, and Turkey (3,6). Lettuce is a very popular vegetable that is eaten raw without heating or cooking. As in Korea, several traditional and popular foods such as barbecue and dakgalbi contain lettuce as an essential supplementary part. Since a lot of people carry *S. aureus*, manually handled foods and minimally processed or cut lettuce could be contaminated (7). In addition, *S. aureus* has been detected in lettuce samples (8) and is one of the predominant bacteria isolated from ready to eat vegetables such as cabbage and cucumber (9). Furthermore, out of 24 gastroenteritis

outbreaks examined, *S. aureus* was recovered from two of the outbreaks linked to lettuce consumption (5). Therefore, effective methods for isolation of *S. aureus* from these foods are of importance to ensure food quality and safety.

Immunomagnetic separation (IMS) method has been applied to reduce the total experiment time and concentrate sample solutions into smaller volumes using a magnetic particle concentrator. It can improve the detection sensitivity and help decrease the interference of food matrices (10-12). IMS could effectively separate the target bacteria from competitive microflora and complex food samples. This selective separation relies on the purity and specificity of the utilized antibody (13). In this study, Dynabeads® Protein G was applied to conjugate anti-*S. aureus* antibodies to form immunomagnetic beads (IMBs) to capture the target bacteria *S. aureus*.

After capturing and separating the target bacteria using IMS, IMBs bacteria can be detected by several methods such as real-time PCR, flow cytometry (14), adenosine triphosphate (15), as well as matrix-assisted laser desorption ionization-time of flight mass spectrometry (16). With these techniques, detection methods become more sensitive and rapid. However, expensive instruments and experienced persons are required, which make them unsuitable for routine laboratory detections. As a result, conventional plating method is a proper selection for bacterial detection and isolation since it still remains the most reliable and accurate technique for routine food-

borne pathogen detection in normal laboratories.

The purposes of this study are (i) to develop an IMS method to capture *S. aureus* by optimizing the amount of IMBs, immunoreaction time, separation time, and wash times; (ii) to evaluate the developed IMS method for separating *S. aureus* in artificially contaminated lettuce and whole milk samples.

Materials and Methods

Bacteria strains and culture media *S. aureus* (ATCC 13150, 13565, and 25923), *Bacillus cereus* (ATCC 14579), *Micrococcus luteus* (ATCC 10240), and *Lactobacillus plantarum* (ATCC 8014) used in this study were maintained at 70°C in tryptic soy broth (TSB; Becton Dickinson and Company, Sparks, MD, USA) containing 20% glycerol and thawed at room temperature. Each strain was cultured in TSB at 35°C for 18–24 h except *L. plantarum* that was cultured in deMan, Rogosa, and Sharpe (MRS; Becton Dickinson and Company) broth. Ten-fold dilutions of cultures in phosphate buffered saline (PBS, Gibco, Life Technologies, Rockville, MD, USA) were prepared and plated onto tryptic soy agar (TSA; Becton Dickinson and Company) and *L. plantarum* was plated onto MRS agar (MRSA). After incubation at 35°C for 24 h, the colonies on the agar plates were enumerated to determine the number of viable cells.

Preparation of IMBs Anti-*S. aureus* rabbit polyclonal antibody (2.8 mg/mL, Prajna Biology, Shanghai, China) was coated to Dynabeads® Protein G (30 mg/mL, Life Technologies) to form IMBs. Briefly, Dynabeads® were vortexed for 40 s and 1 mg (33.3 µL) was transferred to a 1.5 mL tube. The tube was placed on a magnetic particle concentrator (DynaL MPC™-S, Life Technologies) to separate the beads from the solution. The supernatant was removed using a pipette.

An aliquot of 5.4 µL of anti-*S. aureus* antibody was diluted in 200 µL PBS containing 0.02% Tween®-20 (Sigma-Aldrich, St. Louis, MO, USA) and used to coat 1 mg of Dynabeads®. The mixture was incubated at room temperature for 30 min with rotation at 15 rpm on a rotator (HulaMixer; Life Technologies). IMBs were placed on the magnetic particle concentrator and the supernatant was discarded. Finally, the IMBs were stored in 200 µL PBST (PBS containing 0.02% Tween®-20) at 4°C.

Procedure of IMS *S. aureus* (ATCC 25923) was incubated in TSB at 35°C for 18–24 h and serially diluted to 10⁴–10⁵ CFU/mL in PBS. An aliquot of 1 mL *S. aureus* at the concentration of 10⁴–10⁵ CFU/mL was mixed with IMBs. The mixture of IMBs and bacteria was incubated on the rotator (15 rpm) at room temperature for 20 min and separated by the magnetic particle concentrator. An aliquot of 100 µL portion from the supernatant was spread on Baird Parker Agar with egg yolk and tellurite emulsion (BPA, Oxoid, Basingstoke, England) for enumeration after appropriate dilution. All enumeration

experiments were performed in triplicate.

Key parameters of IMS Capture capacity of these IMBs against *S. aureus* was studied for six amounts of IMBs (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg), five immunoreaction times (10, 20, 30, 40, and 60 min), five magnetic separation times (1, 3, 5, 7, and 10 min), and a series of *S. aureus* concentrations (10⁰–10⁵ CFU/mL).

Specificity test assay The specificity of the IMBs was conducted using *S. aureus* (ATCC 13150, 13565, and 25923) and *S. aureus* (ATCC 25923) mixed with other non-*S. aureus* bacteria. All *S. aureus* were cultured in TSB at 35°C for 18–24 h and then serially diluted to 10⁴–10⁵ CFU/mL. One mL of *S. aureus* was mixed with IMBs to test the adequacy of the *S. aureus* antibody. A total of 0.25 mL of *S. aureus* cells at concentrations of 10³ and 10⁵ CFU/mL were mixed with 0.75 mL of 10⁷ CFU/mL of a competitive flora (comprising *B. cereus*, *M. luteus*, and *L. plantarum*). Different wash times were performed for the IMBs-bacteria conjugates with once or twice wash or without wash. An aliquot of 500 µL PBS was used for each wash. Moreover, the same amount of Dynabeads® was mixed with the bacteria as a blank control. An aliquot (0.1 mL) from the washing solution was spread on BPA after appropriate dilution, whereas *B. cereus* and *M. luteus* were plated onto TSA and *L. plantarum* was plated onto MRSA (17).

Detection of *S. aureus* in artificially contaminated food samples

Lettuce and whole milk were purchased from a local retail market and used for the artificial contamination experiment. Unedible, wilted, and damaged portions of lettuce were trimmed. The lettuce and milk samples were then tested as negative samples for *S. aureus* to exclude potential false positive results by plating on BPA after 24 h enrichment in TSB at 35°C.

S. aureus was incubated in TSB at 35°C for 18–24 h and serially diluted in PBS. Lettuce (25 g portions) was inoculated by depositing droplets at 20 locations using 0.5 mL of the appropriate dilutions of the culture to achieve a final inoculation concentration of 10² to 10⁵ CFU/mL. Milk (25 mL) was inoculated with 0.5 mL of the appropriate dilutions of the culture to achieve a final inoculation concentration of 10² to 10⁵ CFU/mL. Following inoculation, the samples were held at 2–5°C overnight prior to analysis to allow the organisms to equilibrate with the sample. After equilibration, each inoculated sample was homogenized with 225 mL PBS in a WhirlPak bag using a Labblender 400 (Seward, London, UK) for 2 min. For each type of food sample, a blank control was prepared by adding 0.5 mL of PBS instead of the *S. aureus* culture. A total of 1 mL of each sample was mixed with 0.4 mg IMBs and incubated at room temperature for 20 min with rotation at 15 rpm. After separation, an aliquot of 100 µL from the supernatant was spread onto BPA plates for enumeration after appropriate dilution. All enumeration experiments were performed in triplicate.

Capture efficiency and statistical analysis The capture efficiency

Fig. 1. Effect of the amount of immunomagnetic beads on capture efficiency (CE) against *S. aureus* at concentration of 6.80×10^4 CFU/mL. Data are presented as means of $CE \pm SD$; Bars labeled with different letters at different amounts of IMBs indicate significant ($p < 0.05$) difference in CE.

Fig. 2. Effect of immunoreaction time on capture efficiency (CE) of 0.4 mg immunomagnetic beads against *S. aureus* at concentration of 8.25×10^4 CFU/mL. Data are presented as means of $CE \pm SD$; Bars labeled with different letters at different immunoreaction time indicate significant ($p < 0.05$) difference in CE.

(CE) was defined as the percentage of the total bacteria retained on the surface of the beads and calculated using the equation published previously (18). The following equation was used for calculating CE: $CE (\%) = (1 - C_u/C_o) \times 100\%$, where C_o is the total number of cells present in the sample (CFU/mL) and C_u is the number of cells unbound to the IMBs (CFU/mL, in supernatant and washed solution). Microbial data and CE were reported as mean results of the triplicate experiments. They were analyzed using SPSS (version 21.0; SPSS Inc., Chicago, IL, USA). Fisher's exact test was used to calculate CEs. Statistical significance was considered when p value was less than 0.05.

Results and Discussion

Optimization for the amount of IMBs with Dynabeads® The amount of IMBs, immunoreaction time, magnetic separation time, and wash times that could affect the capture efficiency against *S. aureus* was optimized. Six amounts of IMBs were used to react with 6.8×10^4 CFU/mL of *S. aureus* and their respective CE was determined. As shown in Fig. 1, the CE significantly ($p < 0.05$) increased from 4.93 to 97.06% when the amount of IMBs increased from 0.05 to 0.4 mg. There was no significant ($p > 0.05$) difference in the CE between the quantity of IMBs from 0.4 to 0.5 mg. Thus, 0.4 mg of IMBs was used in subsequent experiments as the optimized amount.

In this study, 0.4 mg of IMBs was used to capture 6.8×10^4 CFU/mL of *S. aureus*, which had 2.68×10^7 magnetic particles, and the CE was 97.06%. Based on the theoretical calculations, approximately 1.9×10^7 of magnetic particles were required to cover the entire surface of 6.8×10^4 CFU/mL of *S. aureus* (18). However, a higher amount of magnetic particles was used in the test compared with the theoretically estimated number of magnetic particles required to capture 100% of *S. aureus*, but the CE was not 100%. Varshney *et al.* (18) reported a similar decrease in the CE caused by the cluster formation of most of the target bacteria *Escherichia coli* O157:H7 with magnetic particles. Thus, the aggregations are common in the IMS procedure as one magnetic particle could capture more than

one cell or clusters could be formed with several magnetic particles and cells (18,19). Moreover, a cell colony is not necessarily derived from one single bacteria cell and underestimation of CEs could occur by plating count method based on the cells bound to the magnetic particles conjugates (20). Varshney *et al.* (18) found that CE calculations with surface plate counts of cells bound to magnetic particles were less than those based on unbound cells in the supernatant. Therefore, CE calculation based on the unbound cells in the supernatant would give useful quantitative result on the separation of the bacteria by IMS when the initial amount of bacteria is known (21). Shukla *et al.* (19) also applied a similar indirect method to calculate the binding rate based on bacterial cells in a supernatant fluid, which were not bound to the immunomagnetic particles.

Selection of immunoreaction time and separation time Immunoreaction times of 10, 20, 30, 40, and 60 min were used with a concentration of 8.25×10^4 CFU/mL. The CE with 20 min of immunoreaction time was 96.97%. Longer immunoreaction times did not show significant increase in CE ($p > 0.05$) (Fig. 2). Therefore, an immunoreaction time of 20 min was used for the conjugation of IMBs and *S. aureus*. Under this condition, IMBs-bacteria complexes were separated from PBS for 1, 3, 5, 7, or 10 min. No significant difference ($p > 0.05$) was found in CE when different separation times were used (Fig. 3). However, for all separation times, the CEs were higher than 90%. Thus, separation time of 1 min was chosen for subsequent assays. The immunoreaction time from 15 to 45 min has been optimized in several studies, with longer reaction time failing to show significant increase in CE (12,18,22,23). In addition, the separation process could help get rid of non-specific binding. Usually, the separation time is between 2 and 5 min (12,19,23), even for larger-volume (10 mL) sample solutions because magnetic beads are uniform and superparamagnetic and they can be easily separated from sample solutions using a magnetic separator (24).

Specificity test assay and characterization of IMS When 10^4 CFU/mL of *S. aureus* (ATCC 13150, 13565, and 25923) was tested by the

Fig. 3. Effect of separation time on capture efficiency (CE) of 0.4 mg immunomagnetic beads against *S. aureus* at concentration of 1.48×10^5 CFU/mL with an immunoreaction time of 20 min. Data are shown as means of CE $\pm$ SD; Bars labeled with different letters at different separation time indicate significant ($p < 0.05$) difference in CE.

Table 1. Capture efficiency (CE) of immunomagnetic beads against *S. aureus* from a constant competitive flora of *B. cereus*, *M. luteus*, and *L. plantarum* with different wash times

<i>S. aureus</i> / competitive flora (CFU)	Number of washes	CE ¹⁾ (%)
$2.0 \times 10^5 / 5.15 \times 10^7$	0	85.5 $\pm$ 6.5 ^a
	1	70.5 $\pm$ 4.5 ^a
	2	65.0 $\pm$ 5.0 ^a
$2.0 \times 10^3 / 5.15 \times 10^7$	0	87.8 $\pm$ 2.3 ^a
	1	76.3 $\pm$ 4.8 ^{ab}
	2	67.0 $\pm$ 6.0 ^b

¹⁾CE values are means $\pm$ standard deviation ($n=3$); means within the same column of the same bacteria concentration with different letters indicate significant ($p < 0.05$) difference.

IMS, the CEs of three *S. aureus* were 85.0, 91.7, and 93.4%, respectively. *S. aureus* mixed with a competitive flora (comprising *B. cereus*, *M. luteus*, and *L. plantarum*) at a ratio of 1:100 or 1:10,000 was used to evaluate the exclusivity of IMBs. There was no significant difference ($p > 0.05$) between the CEs obtained with different wash times (Table 1). However, the CE slightly decreased when the wash time increased from 0 to 2 times for both the concentrations of 10^3 and 10^5 CFU/mL. Therefore, IMBs-bacteria were not washed after IMS in subsequent experiments. The wash step was used to remove interfering and inhibiting materials. However, target bacteria also could be washed out, thus the CE decreased. Yang *et al.* (22) also reported a similar IMS method without a washing step. Under the optimal conditions, the CEs for different concentrations of *S. aureus* were tested. As shown in Fig. 4, the CEs were higher than 90% when the bacterial concentration was from 4.1×10^0 CFU/mL to 4.1×10^5 CFU/mL.

In recent years, magnetic nanoparticles become popular in the

Fig. 4. The capture efficiency (CE) of 0.4 mg immunomagnetic beads against *S. aureus* at a concentration of 4.10×10^0 CFU/mL to 4.10×10^5 CFU/mL.

IMS because of a large surface-to-volume ratio and being more mobile in complex samples, thus it helps improve the capture efficiency and provide a better detection limit (19,23). However, smaller beads contain less magnetic materials in their cores so longer separation time or higher magnetic intensity is required, which could cause the magnetic beads to form clusters (25,26). In contrast, larger beads can travel through a larger volume of sample solution with more opportunity to interact with the target bacteria than smaller beads of the same density. It has been reported that 2.8 μ m magnetic beads are more effective in capturing bacteria than those of smaller 1 μ m magnetic beads (27). Dynabeads (2.8 μ m diameter) have been used as a magnetic carrier in numerous reported immunoassays studies (28,29).

Recovery of *S. aureus* from food samples To investigate the effect of food matrix interference on CE of IMBs, the application of IMBs for capturing *S. aureus* in whole milk and lettuce was evaluated. As shown in Table 2, the CE of IMBs was higher than 78% when the bacteria concentration in whole milk was between 10^2 and 10^5 CFU/mL. For spiked lettuce samples, the CE was higher than 60% when the bacteria concentration was between 10^2 and 10^5 CFU/mL. The CE of IMS in lettuce samples was lower than that in whole milk samples since various interferences such as tiny vegetable particle and background bacteria in lettuce could decrease the CE.

Several studies have reported that the IMS could help improve the sensitivity of culture-based detection. IMS has been used to improve the isolation of target bacteria from food samples with high levels of natural microflora (10,11,30) or complex matrices (17). A study conducted by Fedio *et al.* (10) showed that the addition of IMS significantly increased the cultural recovery from raw ground beef samples. Prentice *et al.* (30) used IMS for the isolation and detection

Table 2. Capture efficiency (CE) of immunomagnetic beads against *S. aureus* in spiked lettuce and milk samples

Food samples	CE ¹⁾ (%) against different concentration of <i>S. aureus</i> (CFU/mL)			
	10^2	10^3	10^4	10^5
Lettuce	73.27 $\pm$ 3.96 ^a	65.22 $\pm$ 6.09 ^a	69.41 $\pm$ 8.24 ^a	60.00 $\pm$ 4.00 ^a
Whole milk	94.77 $\pm$ 9.80 ^a	83.23 $\pm$ 4.19 ^a	84.42 $\pm$ 7.67 ^a	78.70 $\pm$ 4.63 ^a

¹⁾CE values are means $\pm$ standard deviation ($n=3$); means within the same row with different letters indicate significant ($p < 0.05$) difference.

of *E. coli* O157:H7 from salad samples, which enhanced the target pathogen recovery on selective agar plates with a high microflora of non-target bacteria. Weagant *et al.* (17) reported that the use of either Pathatrix or Invitrogen IMS system could help increase the isolation rate from alfalfa sprouts to 100%. Thus, the developed method of IMS against *S. aureus* with high CE is required and could be a choice for pathogen detection or cultural isolation.

In conclusion, the IMS developed in this study could be used for efficient separation of *S. aureus* from food samples. We optimized the amount of IMBs, immunoreaction time, separation time, and wash times for a better CE. We demonstrated that the IMS of *S. aureus* in a wide range of concentrations showed high specificity and CE (>90%) with 20 min of immunoreaction time and 1 min of separation time. Under the optimal conditions, the IMBs could capture *S. aureus* with high specificity even with a high constant number of competitive flora of *B. cereus*, *M. luteus*, and *L. plantarum*. Thus, this IMS method can be a selection for combining with bacterial detection methods or efficient isolation procedures for *S. aureus*.

Acknowledgments This research was supported by the Export Promotion Technology Development Program (313010-3) funded by the Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea. Shuai Wei thanks the financial support received from the China Scholarship Council.

Disclosure The authors declare no conflict of interest.

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