Two-step large-volume magnetic separation combined with PCR assay for sensitive detection of *Listeria monocytogenes* in pasteurized milk

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ABSTRACT

Immunomagnetic separation (IMS) is an effective tool for the preconcentration and purification of food-borne pathogens from complex food samples because of its high capture efficiency (CE). In conventional IMS, antibodies are usually conjugated on the surface of magnetic beads (MB); the random orientation and conformation changes of antibodies on the MB surface can decrease their bioactivity. Moreover, the Brownian motion of immobilized antibodies is weakened, thereby rendering their binding efficiency with bacteria lower than that of free antibodies. Thus, abundant antibodies are commonly required to ensure high CE for IMS, particularly for large volumes. In this study, a 2-step large-volume magnetic separation (10 mL) was proposed to preconcentrate *Listeria monocytogenes* from pasteurized milk. First, the biotinylated anti-*L. monocytogenes* monoclonal antibodies (mAb) were bound with *L. monocytogenes* in 10 mL of diluted milk through an antigen-antibody interaction, and then streptavidin-labeled MB were used to capture biotin-mAb coated with *L. monocytogenes* by biotin and streptavidin interaction. Under optimal conditions, the CE of 2-step magnetic separation was >90% with *L. monocytogenes* concentrations ranging from 8×10^0 to 8×10^4 cfu/mL, whereas the amount of biotin-mAb was 14 fold lower than that of the conventional IMS method. Coupled with a PCR assay, the detection limit of the proposed method was 8×10^0 cfu/mL in pure culture and 8×10^1 cfu/mL in pasteurized milk without any pre-enrichment process. Moreover, the overall detection time, including sample preparation, large-volume magnetic separation, and PCR, took less than 7 h. In summary, the developed 2-step large-volume IMS combined with PCR was highly sensitive and low cost and, thus, has

considerable potential for the rapid screening of food-borne pathogenic bacteria.

Key words: two-step large-volume magnetic separation, *Listeria monocytogenes*, polymerase chain reaction, pasteurized milk

INTRODUCTION

Listeria monocytogenes is a common food-borne pathogen that ubiquitously exists in environments, including plant, soil, water, and human or animal feces. It can grow at refrigeration temperatures, and even in some fairly extreme conditions (Jooste et al., 2016). Newborns, pregnant women, senior citizens, and immunodeficient people are susceptible to *L. monocytogenes* (Camargo et al., 2016). Listeriosis caused by *L. monocytogenes*, mainly through the consumption of contaminated food, can lead to serious infection in humans, with a global mortality rate of 25% (Mead et al., 1999; Allerberger and Wagner, 2010; de Noordhout et al., 2014). Milk and milk products are identified as among the main food vectors for *L. monocytogenes* transmission (Jordan et al., 2016). Approximately 2 to 5% of raw milk products may be contaminated with *L. monocytogenes* from cow feces, soil, straw, and mastitis (Bickley et al., 1996; Laximan et al., 2016). Moreover, *L. monocytogenes* has also been found in pasteurized milk and milk products; for example, Dalton et al. (1997) reported an outbreak of gastroenteritis and fever among persons who drank chocolate milk contaminated with *L. monocytogenes* at a picnic and Weissfeld et al. (2017) reported 2 ice cream companies that had a listeriosis outbreak at about the same time in 2015 and 2016. Traditional methods for *L. monocytogenes* detection are time-consuming because they commonly require 12 to 24 h pre-enrichment for improving the target bacteria concentration, and thereby fail to meet the requirements of rapid detection in less than 8 h of assay time. Therefore, developing a sensitive and low-cost method for rapid detection of *L. monocytogenes* contaminated foods is needed.

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Immunomagnetic separation (IMS) is among the most effective technologies to separate and concentrate target pathogens from complex food matrix; IMS can also eliminate the inhibitory substances in PCR reaction or immunoassay, thereby improving the limit of detection and shortening the assay time (Yang et al., 2013). However, traditional IMS always suffers from its small enrichment factor because it generally applies 1.0 mL of aqueous solution from 250 mL of enrichment culture for separation (Chen et al., 2014). In our previous study, we established a large-volume IMS (10 mL) for *L. monocytogenes* and *Listeria ivanovii* separation and concentration. Owing to a higher enrichment factor, the limit of detection of large-volume IMS combined with PCR reached as low as 10 cfu/g in lettuce samples in less than 7 h of assay time; however, the antibody consumption in a 10-mL volume IMS reaches up to 70 µg/reaction (Mao et al., 2016). In the conventional IMS method, antibodies are usually immobilized on the surface of magnetic beads (MB) to form immunomagnetic beads through covalent coupling or biotin and a streptavidin (SA) system (Cao et al., 2012). Compared with free antibodies, the Brownian movement of antibodies immobilized on MB is very weak, thereby resulting in poor immunoreaction efficiency between immobilized antibodies and epitopes on the surface of bacteria, particularly in a large separation volume. To ensure high capture efficiency (CE) of immunomagnetic beads to target bacteria, a large amount of antibodies and MB are required in a large-volume IMS. Thus, practical application of large-volume IMS is limited because of its high cost.

In the present study, a novel IMS based on 2-step recognition reaction (2-step IMS) was proposed for the first time to decrease antibody consumption in a large-volume IMS, in which biotin-labeled anti-*L. monocytogenes* monoclonal antibodies (mAb) were first premixed with *L. monocytogenes* in a 10 mL volume and then the SA-coated MB (SA-MB) and used to capture mAb-coated *L. monocytogenes* by the biotin and SA interaction. The CE of 2-step IMS is influenced by various parameters, including the labeled ratio of biotin and mAb, biotin-mAb, and SA-MB contents, immunoreaction time between biotin-mAb and *L. monocytogenes*, capture time of SA-MB- and biotin-mAb-coated bacteria, and magnetic separation time, which were systematically investigated and optimized. Owing to the high immunoreaction efficiency of free biotin-mAb and bacteria, the antibody consumption of 2-step IMS in 10 mL of separation volume reduced by approximately 14 fold compared with that of conventional IMS method. Combined with PCR, the developed 2-step IMS was successfully applied in *L. monocytogenes* detection with a limit of detection at 8×10^0 cfu/mL in pure culture

and 8×10^1 cfu/mL in pasteurized milk with a total assay time of less than 7 h.

MATERIALS AND METHODS

Materials and Reagents

Carboxylated MB with a 180 nm diameter (10 mg/mL) were obtained from Allrunnano Technology Co. Ltd. (Shanghai, China). Anti-*L. monocytogenes* mAb were provided by Wuxi Zodoboe Biotech. Co. Ltd. (Wuxi, China). Streptavidin and *N*-hydroxysuccinimide sodium salt (NHSS) activated long-chain biotins were purchased from Hualan Technology Co. Ltd. (Shanghai, China). Bovine serum albumin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl), NHSS, Tris, glycogen, Triton-X100, ammonium acetate, and Na₂EDTA were from Sigma-Aldrich Chemical Co. (St. Louis, MO). BacteriaGen DNA Kit and lysozyme were bought from ComWin Biotech Co. Ltd. (Beijing, China). Both SA and horseradish peroxidase conjugate were from Beijing ZhongShan-Golden Bridge Biological Technology Co. Ltd. (Beijing, China). PALCAM was obtained from Land Bridge Technology Co. Ltd. (Beijing, China). Pasteurized milk was purchased from the local supermarket.

Bacteria and Culture Conditions

The strains used in this study as shown in Table 1. *Listeria monocytogenes* (ATCC13932) was obtained from the American Type Culture Collection (ATCC) and grown in Luria-Bertani broth on a shaking incubator at 180 rpm overnight at 37°C. The bacteria solutions were continuously diluted with sterile PBS to different concentrations for IMS experiments. After magnetic separation, 100 µL of diluents, including supernatants and mAb-MB bacteria pellets, were spread onto selective PALCAM plates and incubated at 37°C for 18 to 24 h. The cell number of *L. monocytogenes* was determined by counting the colonies grown on the plates.

Preparation of SA-MB and Biotin-mAb

The SA-MB were prepared according to our previously described method with some modifications (Mao et al., 2016). Briefly, 5.75 mg of EDC·HCl and 6.52 mg of NHSS were added to 20 mg of carboxylated MB in 10 mL of sterilized PBS (0.01 M, pH 7.4) on an HS-3 Vertical Mixer (NingBoScientz Biotechnology Co. Ltd., Zhejiang, China) at room temperature for 1 h. After washing thrice with PBS, 1.6 mg of SA was added into the activated MB. The mixture was reacted at room

Figure 1. Schematic illustration of 2-step magnetic separation combined with PCR for detection of *Listeria monocytogenes* in a 10-mL volume. SA-MB = streptavidin-coated magnetic beads. Color version available online.

temperature for 2 h and then blocked with 1% (wt/vol) BSA solution for 45 min. The as-prepared SA-MB were purified using an external magnetic field and resuspended with 10 mL of 0.01 M PBS (pH 7.4).

The biotin-mAb were prepared by mixing NHSS activated long-chain biotin and anti-*L. monocytogenes* mAb in 0.01 M PBS (pH 7.4) with molar ratios of 20:1, 10:1, 5:1, and 1:1. After reaction at room temperature for 4 h, the mixture was transferred into a dialysis bag at 4°C for 3 d to remove free long-chain biotin. Finally, glycerin was added to the as-prepared biotin-mAb solution to a final concentration of 50% and then stored at −20°C for further use.

2-Step IMS Procedure

As shown in Figure 1, 5 µg of biotin-mAb (the molar ratio of long-chain biotin and mAb is 20:1, named biotin-mAb_{20:1}) was first added into 10 mL of PBS buffer (0.01 M, pH 7.4) containing a certain concentration of *L. monocytogenes* with gentle shaking at 37°C for 1 h. Then, 700 µg of SA-MB was added into the above mixture to form an MB-SA-biotin-mAb-bacteria complex. After reaction at 37°C for 30 min, the mixture was separated for 3 min using a 0.4-T external magnetic field caused by 2 fabricated magnets (12.0 × 2.0 × 1.0 cm; Mao et al., 2016). The MB-SA-biotin-mAb-bacteria pellets were resuspended with 1 mL of sterilized PBS. Among them, 400-µL solutions were used to extract DNA for PCR amplification and the rest were plated on PALCAM to count the bacteria number after appropriate dilution. The CE was calculated using the equation

$$\text{CE (\%)} = \left[\frac{N_b}{(N_u + N_b)} \right] \times 100 \quad (\text{Mao et al., 2016}),$$

where N_u is the number of unbound bacteria in the supernatant (cfu/mL) and N_b is the number of cells

bound to the SA-MB (cfu/mL). All experiments were performed in triplicate. The means of groups were compared via 1-way ANOVA, standard deviation was calculated from the resultant means of the triplicate experiments, and the statistical significance threshold was determined as $P < 0.05$ (Shan et al., 2014).

PCR Assay

Table 2 shows the primer pairs used in this study. *Listeria monocytogenes* primer for *actA* amplification was designed using Oligo 6.0 software (<http://www.oligo.net/>), and the sequence specificity was verified by using Primer BLAST in the NCBI webpage (<https://www.ncbi.nlm.nih.gov/pmc/>). One pair of primer for internal amplification control (IAC), obtained from

Table 1. Bacteria strains used in this study

Number	Bacteria strains	Source ¹
1	<i>Micrococcus luteus</i>	CMCC28003
2	<i>Shigella flexneri</i>	2a 2457T
3	<i>Cronobacter sakazakii</i>	ATCC29544
4	<i>Staphylococcus aureus</i>	CMCC26003
5	<i>Bacillus cereus</i>	JDZ0043Y
6	<i>Salmonella</i> Enteritidis	CMCC50041
7	<i>Salmonella</i> Typhimurium	ATCC13311
8	<i>Pseudomonas aeruginosa</i>	CICC21562
9	<i>Escherichia coli</i> O157:H7	CMCC44102
10	<i>Escherichia coli</i>	ATCC25922
11	<i>Listeria welshimeri</i>	ATCC35897
12	<i>Listeria seeligeri</i>	ATCC35967
13	<i>Listeria monocytogenes</i>	ATCC13932
14	<i>Listeria monocytogenes</i>	CMCC54007
15	<i>Listeria ivanovii</i>	ATCC19119
16	<i>Listeria innocua</i>	NCTC11288
17	<i>Listeria grayi</i>	ATCC25401

¹ATCC = American Type Culture Collection; CMCC = China Medical Culture Collection; CICC = China Center of Industrial Culture Collection; NCTC = National Collection of Type Cultures; JDZ0043Y was obtained from JX-CDC (Jiang Xi Province Center for Disease Control and Prevention).

Table 2. Primers used in the PCR

Target bacteria	Primer sequence (5'-3') ¹	Product length (bp)	Primer concentration (μM)	Reference
<i>Listeria monocytogenes</i>	<i>actA</i> -F-5'-GCGACAGATAGCGAAGATTCCAG-3' <i>actA</i> -R-5'-GCCACATTATCGCTTTGTTCACTG-3'	257	1.0	This study
16S rRNA	16S rRNA-F-5'-CCTACGGGAGGCGAGCAGT-3' 16S rRNA-R-5'-CGTTTACGGCGTGGACTAC-3'	475	0.1	(Chiang et al., 2006)

¹F = forward; R = reverse.

a previous report (Chiang et al., 2006), was used to indicate false-negative results. The total reaction volume for PCR amplification was 20 μL, including 8 μL of 2.5× reaction mix, 5 U of hot start Taq DNA polymerase (AGCU ScienTech Inc., Wuxi, China), 2 μL of each DNA template containing *L. monocytogenes* and *Escherichia coli* (for IAC), 1.0 μL of *actA* primer, 0.1 μL of 16S rRNA primer, and sterile water. The PCR amplification conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, and a final stretch at 72°C for 10 min. Afterward, the PCR products were run with 2.5% agarose gel electrophoresis stained with ethidium bromide, and imaged under a UV transilluminator (Bio Rad, Hercules, CA).

Detection of *L. monocytogenes* in Spiked Milk Samples

Pasteurized milk was spiked with *L. monocytogenes* at different concentrations and assayed by the proposed method. In brief, 10 mL of milk was added into 90 mL of 0.01 M sterilized PBS to form a 10% homogenate. The *L. monocytogenes* solutions were spiked to the above homogenates with *L. monocytogenes* final concentrations ranging from 8×10^0 to 8×10^3 cfu/mL. A total of 5 μg of biotin-mAb_{20:1} was added into the *L. monocytogenes* spiked milk samples. After incubation at 37°C for 1 h with gentle shaking, 700 μg of SA-MB was added and the solution was incubated for another 30 min at 37°C. Finally, the MB-SA-biotin-mAb-bacteria complex was separated magnetically for 3 min and resuspended in 1 mL of 0.01 M PBS. Among the complex, 400 μL of resuspension liquid was used to extract the target DNA for PCR amplification. The DNA extraction was performed with (Mao et al., 2016) 400 μL of resuspension liquid heated at 90°C for 10 min in a thermostatically controlled water bath to separate the *L. monocytogenes* from MB-mAb-bacteria and a magnet was used to separate the MB. The supernatant was removed by centrifugation ($13,400 \times g$, room temperature, 3 min), and the precipitate was then resuspended in the enzymatic lysis buffer (180 μL) containing Na₂EDTA (2 mM), Tris (20 mM), Triton-

X100 (1.2%), and lysozyme (20 mg/mL) at 37°C for 30 min. Proteinase K (20 μL) and Buffer GL (200 μL; ComWin Biotech Co. Ltd.) were added and vortexed for blending adequately. After incubation for 30 min at 56°C, the mixtures were treated with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1), and then centrifuged at $13,400 \times g$ at room temperature for 3 min. A total of 66.5 μL of ammonium acetate (7.5 mol/L), 1.5 μL of glycogen (20 mg/mL), and 388.5 μL of ethanol were added into approximately 400 μL of supernatant. After standing for 30 min at 0°C, the mixture was centrifuged at $13,400 \times g$ for 30 min (4°C) to discard the supernatant. The precipitate was washed twice with 150 μL of ethanol (70%) and dissolved in 10 μL of Buffer GE for PCR.

RESULTS AND DISCUSSION

Characterization of Biotin-mAb

As shown in Figure 1, the MB bound on the surface of *L. monocytogenes* were based on biotin and SA interaction because the SA is a tetrameric protein that can combined with 4 biotin moleculars (Shan et al., 2014). The labeled amounts of biotin to mAb can influence the amounts and binding efficiency of SA-MB on the surface of the bacteria, and further effect the CE of SA-MB to *L. monocytogenes*. In the present study, we synthesized 4 kinds of biotin-mAb with the labeled biotin-to-mAb ratio at 1:1, 5:1, 10:1, and 20:1. To characterize the amounts of biotin coupled on mAb, various biotin-mAb with concentrations ranging from 0.156 to 5 μg/mL were coated on the ELISA plates and then reacted with SA-horseradish peroxidase conjugate. OD₄₅₀ (optical density at 450 nm) value was used to evaluate the combining capacity of biotin-mAb to SA. As shown in Figure 2, OD₄₅₀ values increased with the increased biotin-to-mAb ratio; when the labeled ratio of biotin to mAb was 20:1, the OD₄₅₀ values was still up to 2.0 even if the concentration of mAb was as low as 0.625 μg/mL, indicating that sufficient amounts of biotin coupled on the mAb. However, excessive biotin coupled on the mAb may block the binding site of mAb to antigen

Table 3. The influence of biotin-monoclonal antibodies (mAb) on capture efficiency (CE)

Item	Molar ratio of biotin to mAb			
	20:1	10:1	5:1	1:1
CE (%)	92.54 ± 3.21	91.67 ± 5.33	76.66 ± 4.54	47.86 ± 0.67

and result in a low CE of *L. monocytogenes*. Therefore, the effects of biotin-to-mAb ratio on CE were further evaluated through a 2-step IMS method. Compared with the labeled ratio of biotin-to-mAb at 5:1 (CE = 76.66 ± 4.54%) and 1:1 (CE = 47.86 ± 0.67%), the CE of biotin-mAb with a 20:1 and 10:1 biotin-to-mAb ratio can reach up to 92.54 ± 3.21 and 91.67 ± 5.33%, respectively (shown in Table 3). Considering these results, biotin-mAb with 20:1 biotin-to-mAb molar ratio were suggested for subsequent experiments because more biotins on the mAb is beneficial to improve the binding affinity of SA-MB to mAb.

Optimization of Experimental Parameters of IMS in 10 mL

In the 2-step IMS system, the contents of biotin-mAb 20:1 and immunoreaction time between mAb and *L. monocytogenes*, the amounts of SA-MB and capture time of SA-MB to biotin-mAb 20:1-coated bacteria, and the magnetic separation time are considered the most important factors that affected the CE of MB to *L. monocytogenes*. The dosage of biotin-mAb 20:1 was optimized by adding 1, 2, 3, 5, 7, 9, and 11 µg of biotin-mAb 20:1 to 10 mL of PBS buffer containing 8×10^4 cfu/mL of *L. monocytogenes*, and 700 µg of SA-MB was

used to capture the biotin-mAb 20:1-coated bacteria complex. The results shown in Figure 3A indicated that the CE of SA-MB to *L. monocytogenes* increased from 46.30 ± 4.83 to 80.37 ± 7.98% with the amounts of biotin-mAb 20:1 increasing from 1 to 5 µg. When the amounts of biotin-mAb 20:1 further increase from 5 to 11 µg, the CE inversely decreased to 28.93 ± 9.79%, indicating that the excessive free biotin-mAb 20:1 occupied too many binding sites of SA-MB, resulting in a low CE. When the amounts of biotin-mAb 20:1 was between 3 and 5 µg, CE were not significantly different ($P > 0.05$, $n = 3$). To ensure sufficient mAb combined with *L. monocytogenes*, 5 µg of biotin-mAb 20:1 was suggested for subsequent experiments. The immunoreaction time was optimized by incubating the mixture of biotin-mAb 20:1 and *L. monocytogenes* at 37°C for 30, 45, 60, 90, and 120 min. The results shown in Figure 3B indicated that the CE increased from 45.62 ± 6.43 to 91 ± 8.66% when immunoreaction time was between 30 and 60 min, and then reached a plateau when the immunoreaction time was further extended to 120 min. Therefore, 60 min of immunoreaction time was enough to ensure a high CE of *L. monocytogenes*. The effects of SA-MB contents on the CE were assayed by adding 100, 300, 500, 700, and 900 µg of SA-MB to capture biotin-mAb 20:1-coated bacteria complex. As shown in Figure 3C, CE gradually increased from 61.38 ± 9.66 to 91.24 ± 3.96% as the amounts of SA-MB increased from 100 to 500 µg, and then stabilized when SA-MB contents further increased to 900 µg ($P > 0.05$, $n = 3$). However, when SA-MB content was 500 µg, CE decreased significantly at low bacteria concentrations of 8×10^0 to 8×10^1 cfu/mL because the increased free biotin-mAb in the solution can consume large amounts of SA-MB, whereas 700 µg of SA-MB was sufficient to ensure a high CE (more than 90%) for the *L. monocytogenes* concentration from 8×10^0 to 8×10^4 cfu/mL (Figure 3D). The results of effects of capture time between SA-MB and biotin-mAb 20:1-coated bacteria complex on the CE are shown in Figure 3E; CE gradually increased and reached a high constant value of 84.94 ± 2.75% with the capture time increasing from 10 to 30 min. Finally, the separation time of SA-MB on the magnetic field was also optimized. The results in Figure 3F show that CE increased from 82.40 ± 3.56 to 91.76% ± 3.39% as the separation time increased from

Figure 2. The characterization of biotin-monoclonal antibodies (mAb). The labeled ratio of biotin to mAb was 20:1, 10:1, 5:1, and 1:1. The absorbance of ELISA were obtained at 405 nm, in which the concentrations of biotin-mAb and mAb ranged from 5 to 0.156 µg/mL. Error bars represent the SD of 3 replicates.

Figure 3. Optimization of experimental parameters via (A) the amounts of biotin-monoclonal antibodies (mAb, ratio of 20:1; 60 min immunoreaction time and 5 min magnetic separation time), (B) immunoreaction time, (C) the amounts of streptavidin-labeled magnetic beads (SA-MB), (D) 500 and 700 μg of SA-MB used in pure culture (8×10^0 – 8×10^5 cfu/mL), (E) capture time, and (F) magnetic separation time. Error bars represent the SD of 3 replicates.

1 to 3 min. Thus, the optimized parameters for 2-step IMS were 5 μg of biotin-mAb 20:1 added to 10 mL of sample volume (the bacteria concentration ranges from 8×10^0 to 8×10^5 cfu/mL). After antibody and bacteria interaction at 37°C for 60 min, 700 μg of SA-MB was added and continually reacted with biotin-mAb and bacteria complex for 30 min. Then, the MB-SA-biotin-mAb-bacteria pellets were separated for 3 min using a 0.4-T external magnetic field.

The specificity of the 2-step IMS in 10 mL volume was investigated by testing the CE for 17 different pathogens, including 7 *Listeria* strains and 10 nontarget strains (Table 1). As seen in Figure 4, CE was more than 80% for *Listeria* strains (10^4 cfu/mL) except for *Listeria grayi* and *Listeria seeligeri* (approximately 60%), whereas CE of nontarget bacteria was less than 4% (10^5 cfu/mL). The results showed that the proposed 2-step IMS method has high specificity for detection of *Listeria* species.

Optimization of PCR Conditions

In the present study, we designed 2 pairs of primers to ensure specificity of PCR assay, including *actA*-F/R for the specific amplification of a 257-bp fragment from the *actA* of *L. monocytogenes*, and 16S rRNA-F/R for the amplification of a 475-bp fragment from the 16S rRNA

Figure 4. Specificity of 2-step magnetic separation. The concentrations of *Listeria* strains were all 10^4 cfu/mL; other nontarget strains were all 10^5 cfu/mL. All experiments were performed under the optimal conditions. Error bars represent the SD of 3 replicates. See Table 1 for a full description of strains used.

Figure 5. Results of 2-step large-volume magnetic separation combined with PCR for detection of *Listeria monocytogenes* in pure culture. Lane M = DL 2000 DNA Marker (TaKaRa, Biotech Co. Ltd., Dalian, China); lane C = negative control; lanes 1–4 = 8×10^3 to 8×10^0 cfu/mL of culture, respectively.

gene that acted as an IAC in PCR. The 16S rRNA gene exists in all bacteria genome and has high conservatism and specificity; it can completely consume deoxynucleotide triphosphates substrates with the *actA* primer during PCR, and thereby decrease the sensitivity for amplification of low concentrations of *L. monocytogenes*. Therefore, we optimized the concentrations of 2 pairs of primers and the annealing temperature at different concentrations of *L. monocytogenes* using an orthogonal experimental design (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13140>). The optimum primer concentrations were 1.0 μ M for *actA* primer and 0.1 μ M for 16S rRNA. The optimal annealing temperature was set at 62°C. Under the optimized PCR conditions, 2 specific PCR fragments can be produced even if the concentration of *L. monocytogenes* was as low as 8×10^0 cfu/mL in pure culture (Figure 5).

Detection of *L. monocytogenes* in Milk Using 2-Step IMS and PCR

To verify the feasibility of 2-step IMS combined with PCR for sensitive detection of *L. monocytogenes* in milk samples, the pasteurized milk samples were diluted with PBS buffer for 10 times, and then the

Figure 6. Results of 2-step large-volume magnetic separation combined with PCR for detection *Listeria monocytogenes* in pasteurized milk. Lane M = DL 2000 DNA Marker (TaKaRa, Biotech Co. Ltd., Dalian, China); lane C = negative control; lanes 1–4 = 8×10^3 to 8×10^0 cfu/mL of culture, respectively.

diluent was spiked with *L. monocytogenes* at different concentrations from 8×10^0 to 8×10^3 cfu/mL. All artificially contaminated milk samples were analyzed by using 2-step IMS combined with PCR. The results in Figure 6 show that the PCR bands of *actA* and 16S rRNA genes were clearly observed in agarose gel even when the *L. monocytogenes* concentration was as low as 8×10^1 cfu/mL, indicating that proposed method possesses an high sensitivity to meet the requirement of *L. monocytogenes* rapid detection in real milk samples without any enrichment process.

CONCLUSIONS

We developed a 2-step magnetic separation combined with PCR for sensitive detection of *L. monocytogenes* in a 10-mL volume without any pre-enrichment. Under optimal conditions, the amount of biotin-mAb 20:1 was reduced by 14 fold compared with a previous study using the same amount of SA-MB. The CE was higher than 90% when the concentration of *L. monocytogenes* was less than 8×10^4 cfu/mL in pure culture, and the limit of detection reached as low as 8×10^0 cfu/mL in pure culture and 8×10^1 cfu/mL in pasteurized milk. Moreover, the total assay time took less than 7 h, including sample preparation, 2-step magnetic separation, and PCR. In summary, the proposed method provided a novel strategy to reduce the antibody consumption in a large separation volume based on a 2-step magnetic separation, and this method exhibits considerable potential for rapid and sensitive detection of *L. monocytogenes* or other common pathogens in complex food samples without further pre-enrichment.

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