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## Research Article

# Development of an Indirect Competitive Enzyme-linked Immunosorbent Assays Method Based on Immunomagnetic-bead for Analyzing *Listeria monocytogenes* in Food

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### Abstract

*Listeria monocytogenes* is an emerging bacterial food borne pathogen, which could survive common stress levels and often cause listeriosis. To control its infection, it is necessary to establish a sensitive, specific, rapid technology for detecting *Listeria monocytogenes*. In this study, invasion-associated-protein (IAP) was used as a target gene. After prokaryotic cloning and expression of the gene, the specific membrane protein P60 of *Listeria monocytogenes* was obtained. The P60 was injected into New Zealand white rabbits to get the antibody. The prepared antibody was conjugated with magnetic-bead to get immunomagnetic-beads. Enzyme-linked immunosorbent assays method based on immunomagnetic-bead (IMB-ELISA) would be developed through immunomagnetic-bead (IMB) collecting *Listeria monocytogenes*. The results showed that the sensitivity of the IMB-ELISA was  $1.2 \times 10^6$  CFU mL<sup>-1</sup>, the limit of detection was 31 CFU mL<sup>-1</sup>. The IMB-ELISA is a quick and accurate method for the detection of *Listeria monocytogenes*.

**Key words:** *Listeria monocytogenes*, IAP, P60, IMB-ELISA

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Listeria monocytogenes* (*L. monocytogenes*) is an emerging bacterial foodborne pathogen responsible for listeriosis, an illness characterized by meningitis, encephalitis and septicaemia. Infection could result in cutaneous lesions and flu-like symptoms (Guo *et al.*, 2011; Churchill *et al.*, 2006). In pregnant women, the pathogen could cause bacteraemia and stillbirth or premature birth of the fetus and the mortality rate for those contracting listeriosis is approximately 20% (Yan *et al.*, 2006). *Listeria monocytogenes* has a complex pathogenicity mechanism with several virulence factors which improve transmission by direct cell-to-cell infection.

As one of four major food borne pathogens by World Health Organization (WHO) (Buzby and Roberts, 1997), *L. monocytogenes* could become one of the important risk factors in food and it is essential to detect the pathogen of low numbers in food samples. Traditional testing methods for *L. monocytogenes* in food include the isolation of the suspected colonies and examination of biochemical reaction, hemolysis and typical motion. For these above methods, selective enrichment culture is the indispensable step, which means the long time, high cost and low sensitivity (Churchill *et al.*, 2006).

In view that the traditional detection is time-consuming in enrichment culture, it is necessary to establish a sensitive, specific and rapid technology for detecting *L. monocytogenes* in food to improve sensitivity and detecting efficiency, several new techniques had been developed based on immunology or molecular biology.

The enzyme-linked immunosorbent assays (ELISA) are widely used as screening method for detecting chemical residue and microorganism in foods and environmental samples under the restricted levels (Wanatabe *et al.*, 2001). The ELISA has several advantages of rapidness with short incubation time and simplicity without complicated clean-up procedures in analysis. The ELISA has been extensively used for screening of bacteria including *Escherichia coli*, *Staphylococcus aureus* and other bacteria in food due to its sensitivity, specificity and rapidity and simplicity (He *et al.*, 2004).

Immunomagnetic separation (immunomagnetic bead-based separation, IMS) is one of the separation technology methods based on the specific antigen-antibody reaction. Immunomagnetic separation makes use of antibody specificity towards a pathogen to concentrate that pathogen before other methods are used to amplify and identify the bacteria. Antibodies are attached to beads and added to a homogenized sample. Any pathogen with affinity for the

antibody should attach to the immunomagnetic beads (IMB) complex. When the sample is mixed with the IMB, the pathogen can be wrapped in the beads because of the specifically reaction between the pathogen and antibody which is conjugated to the Magnetic Beads (MB). The compounds can be rapidly separated by NdFeB magnet (Fitzmaurice *et al.*, 2004; Li and Wu, 2008). Therefore, this technology not only has the advantages of solid phase immunological reactions, but also can reduce the detection time and improve the detecting sensitivity (Wang *et al.*, 2014). With these advantages, IMS is widely used in biomedical and food safety, such as the cell separation and the pathogen detection.

In this study, P60 protein of *L. monocytogenes* was expressed as antigen to prepare the antibody, because P60 protein has hydrolase and amidase activity and can stimulate B-lymphocyte and T-lymphocyte to produce the immune responses, it is a virulence factor of *L. monocytogenes* (Wang *et al.*, 2006a; Bubert *et al.*, 1992; Lu *et al.*, 2010; Zhu and Ye, 2007). Magnetic-bead would be conjugated with polyclonal antibody to get immunomagnetic-beads, enzyme-linked immunosorbent assays method based on immunomagnetic-bead (IMB-ELISA) was established through IMB collecting *L. monocytogenes*.

## MATERIALS AND METHODS

**Animal preparation:** Two New Zealand white rabbits were obtained from the experimental Animal Research Institute of Hangzhou Normal University. Animal welfare and experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals were approved by the animal ethics committee of Hangzhou Normal University, Zhejiang, China.

**Bacterial strains:** Strains of *Listeria monocytogenes*, *Escherichia coli* O157 and *Enterococcus faecalis* were preserved strains in this laboratory; *E. coli* DH5 $\alpha$  and *E. coli* BL21 (DE3) were purchased from CW biotech (Beijing, China).

**Reagents:** Premix polymerase, pMD-18T, DNA Ligation Kit, Quick Cut *Bam*HI and Quick Cut XhoI were purchased from TAKARA (Japan), Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) were purchased from Sigma (USA). Goat anti-rabbit IgG/HRP, nucleic acid stain, protein molecular weight marker, DNA marker, the SanPrep column DNA gel extraction kit, PBS and TBS were purchased from Sangon Biotech (Shanghai, China), the plasmid Mini Kit was purchased from OMEGA (USA), TMB-component color

liquid was purchased from Aladdin (Shanghai, China), immunomagnetic beads were purchased from Huier biotech (Luoyang, China), PVDF membrane, ponceau S staining solution, developing fixing kit and ECL chemiluminescence were purchased from beyotime Biotechnology (Jiangsu, China), other reagents were routine laboratory reagents, analytical grade, they were purchased from Mike Chemical Instrument Corporation Limited (Hangzhou, China).

**Cloning IAP gene:** Genome DNA of *L. monocytogenes* was extracted from overnight cultures and was used as template for PCR amplification of the gene encoding protein P60. The cloning primers were designed as follows: 5'-CGGGATCCATGAATATGAAAAAGCAAC-3' (forward primer, containing *Bam*HI digestion site); 5'-CCCTCGAGTTATACGC GACCGAAGCCAA-3' (reverse primer, containing XhoI digestion site). The PCR amplified products were purified by 1% Agarose Gel Electrophoresis (AGE) and inserted into pMD-18T simple vector. The obtained pMD18T-IAP vector was transformed into competent *E. coli* DH5 $\alpha$  and sequenced to confirm nucleotide identity. The correct pMD18T-IAP plasmid and pET-30a plasmid DNA were digested with *Bam*HI and Xho I overnight at 37°C. The gene was directly ligated into the vector with rapid DNA ligation Kit and the recombinant plasmid pET30a-IAP was transformed into *E. coli* BL21 (DE3).

**Expression and identification of P60 gene in *E. coli*:** Single clone of *E. coli* BL21 (DE3) harboring the pET30a-IAP vector was inoculated into 5 mL LB medium containing 50  $\mu$ g mL<sup>-1</sup> kanamycin and was cultured at 37°C and 150 rpm on a rotary shaker overnight. One milliliter of these cells was inoculated into 50 mL 2 $\times$ YT medium. The cells were incubated at 37°C for 2 h on a rotary shaker until the cell concentration of OD<sub>600</sub> = 0.5-0.7 and IPTG (Isopropylthiogalactoside) was added to induce protein expression by a final concentration of 1 mM. After induction for 8 h at 28°C, cells were harvested by centrifugation at 8500 rpm for 10 min at 4°C. The precipitate was resuspended with PBS (pH 7.4) and the expression protein can be obtained after the sonication. Proteins were detected directly by 12% SDS-PAGE and the protein bands were stained by coomassie brilliant blue.

For Western blot analysis, electroblotting of proteins on PVDF membrane was carried out using wet method in a transfer buffer (25 mM tris, 192 mM glycine and 20% methanol) at 90 mA for 1 h. After blocking the membrane with 5% skim milk in TBS overnight at 4°C, the blot was probed with rabbit antibody *L. monocytogenes* polyclonal antibody as the primary antibody in TBS for 1 h at room temperature by shaking, washed three times (TBS with 0.1% tween20) and incubated with goat anti-rabbit IgG-HRP

as the secondary antibody in TBS for 60 min at room temperature by shaking. The ECL chemiluminescence was used to detect the target band.

**Immunization for rabbits and antiserum preparation:** Two New Zealand white rabbits were immunized with intramuscular injections of the purified protein P60 for three times. After the third booster, each rabbit was bled from the ear vein on the 7th day and the serum was separated through centrifuge. The positive serum of above 1:12800 titers was used in the following experiment. Antibody was purified from separated serum by bitterness-ammonium sulfate precipitation.

**Combination of magnetic bead and the antibody:** Activated beads was been washed with 1 mL MES (pH 6.0) buffer for 2 times. Five hundred microlitter of the antibody proteins were added into the solution of activated beads for overnight at room temperature. The mixed solution was placed on a magnetic separation rack to obtain the magnetic beads-antibody conjugate (IMB). The IMB was washed with 1 mL MES (pH 6.0) buffer for 2 times and resuspended by 1 mL of PBS, which included 0.1% Bovine Serum Albumin (BSA) for storage at 4°C.

**Establishment of the standard curve and the sensitivity determination:** The antibody (antiserum or IMB) was diluted with PBS, antigen-coated was diluted with CBS, *L. monocytogenes* of 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup> and 10<sup>10</sup> CFU mL<sup>-1</sup> were used as the coated antigen to do the phalanx titration. The antibodies and antigen dilutions were selected as the optimal working concentration, when the OD<sub>450</sub> was approximately equal to 1. The optimal working concentration of antigen and antibody (antiserum or IMB) were used to establish the standard curve. The ELISA plates were coated with 100  $\mu$ L 10<sup>5</sup> CFU mL<sup>-1</sup> cultures in each well and the 90  $\mu$ L antibody were mixed with 10  $\mu$ L antigen of final concentration 0, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> CFU mL<sup>-1</sup>. The specificity was determined with the IC-ELISA and the half maximal Inhibitory Concentration (IC<sub>50</sub>, the concentration causing 50% inhibition of binding) and the detectable limitation (IC<sub>10</sub>, the concentration causing 90% inhibition of binding) were calculated.

In the result:

$$\text{Competitive inhibition (\%)} = \frac{B}{B_0} \times 100\%$$

where, B is the absorbance of well with the competitor and B<sub>0</sub> is the absorbance of well without the competitor.

**Specific detection:** The assay specificity was evaluated by testing the cross-reactivity (CR) of the antibody with other pathogenic microorganisms, *Escherichia coli* O157 and *Enterococcus faecalis*. The CR values were calculated according to the following:

$$\text{Cross-reactivity (\%)} = \frac{\text{IC}_{50} \text{ of LM}}{\text{IC}_{50} \text{ of other bacteria}} \times 100\%$$

### Repeatability detection

**Repeatability tests with the same plate:** According to the established ELISA methods, the antiserum and the immunomagnetic beads-antibody conjugate was respectively used as the antibody to determine the *L. monocytogenes* in samples of known concentrations. The colonies concentration was  $10^5$ ,  $10^6$  and  $10^7$  CFU mL<sup>-1</sup>, respectively and each concentration was repeated in 10 different wells. The antibody was replaced by PBS in the blank well. Coefficient of variation of the same plate was calculated under the different operating methods and different colonies.

**Repeatability tests between different plates:** According to the established ELISA methods, the anti-serum and the immunomagnetic beads-antibody conjugate was, respectively used as the antibody to determine the *L. monocytogenes* in samples. The same concentrations of *L. monocytogenes* were determined in three microtiter plates, respectively and each concentration was repeated in 10 different wells in one plate. The antibody was replaced by PBS in the blank well. Coefficient of variation of the same plate was calculated according to the different operating methods and different plates.

**Recovery study in samples:** The different concentrations of *L. monocytogenes* were used as samples in two established methods. In the IC-ELISA, each 0.1 mL sample was coupled with 0.9 mL antiserum; in the IMB-ELISA, each 0.1 mL sample was coupled with 0.9 mL immunomagnetic beads-antibody. The mixture was shocked in the sample mixing device for 45 min at room temperature and separated by NdFeB magnet for 3 min in order to capture the *L. monocytogenes* in samples.

## RESULTS

*Listeria monocytogenes* is a serious threat to the food industry as a psychrotrophic species and could survive the most common stress levels presented in the food processing

environment, such as high salinity, acidity, refrigeration temperatures and low water activity. In this study, the IMB-ELISA method was developed for the detection of the *L. monocytogenes* using the polyclonal antibody raised against P60 protein.

**The cloning of IAP:** The length of amplified product was between 1000 and 1500 bp (Fig. 1) and the sequence of IAP gene was 1437 bp. The BLAST result demonstrated the nucleotide sequence of the cloned DNA shared 99% identity with IAP gene in GenBank and there was no difference of amino acid sequence between them.

**Expression and identification of P60 in *E. coli*:** After double digestion with *Bam*HI and *Xho*I, pET30a and IAP gene were combined into the recombinant vector, which was transformed it into competent *E. coli* BL21 (DE3). The PCR analysis was applied to check the proper insertion of the gene in the recombinant plasmid using T7 promoter-specific primers. The protein expression was monitored by SDS-PAGE

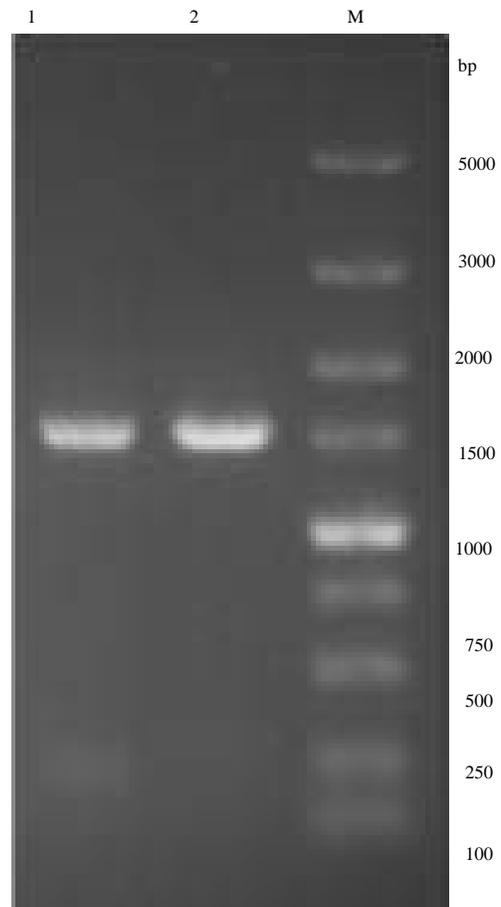


Fig. 1: PCR amplification product of IAP gene, Lanes 1 and 2: PCR identify of IAP gene, Lane M: DNA marker

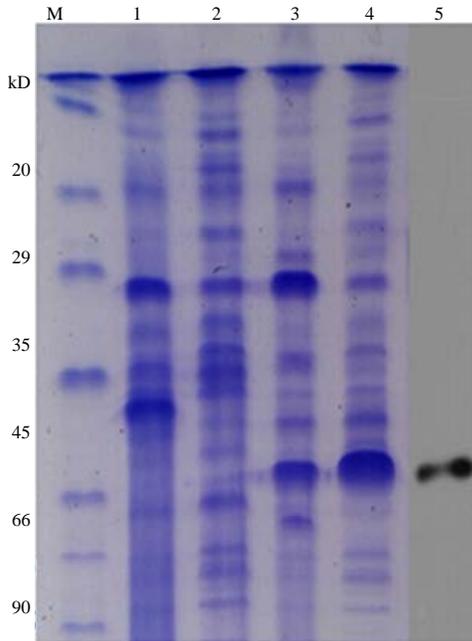


Fig. 2: Analysis of expressed proteins by gradient 12% SDS-PAGE and Western blot: Lane M, protein marker, Lanes 1 and 2: Sonication precipitate and the supernatant fraction of cell lysate with empty vector pET30a, Lane 3: Supernatant fraction of cell lysate, Lane 4: Sonication precipitate of cell lysate, where specific protein band could be found and Lane 5: Western blotting analysis of P60 with polyclonal antibody against *L. monocytogenes* culture as primary antibody and goat anti-rabbit IgG-HRP as secondary antibody

and Western-blot analysis (Fig. 2). There was a clear band with a molecular weight of approximately 60 kD in the lane 4, which was different from lane 2, so we inferred this band maybe the P60, the Western blotting was done with polyclonal antibody against *L. monocytogenes* culture used as primary antibody and goat anti-rabbit IgG-HRP used as secondary antibody, the result was shown in lane 5, it can obtain a specific band, which has a same size with lane 4. It illustrated the protein P60 was successfully expressed.

**Determination of the optimal working concentrations:** The antibodies and antigen dilutions were selected as the optimal working concentration, when the OD450 was approximately equal to 1. In the IC-ELISA, the OD450 value was 0.982, the concentration of antigen was  $10^6$  CFU mL<sup>-1</sup> and the diluted antibody against P60 protein was 1:800. The above step could be repeated for IMB-ELISA examination, in which antibody was replaced by IMB. It was determined that the

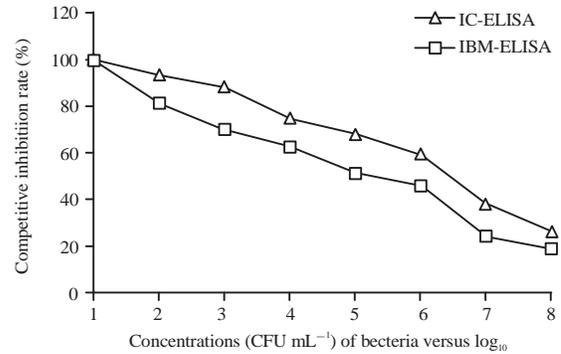


Fig. 3: Standard curves for two different ELISA methods

optimal concentration of antigen was  $10^5$  CFU mL<sup>-1</sup>, when OD450 value was 1.042 and the optimal rate of diluted antibody was 1:800 for IMB-ELISA examination.

**Establishment of the standard curve and the sensitivity determination:**

As is shown in Fig. 3, the calculated curve was  $y = -10.482x + 115.91$ ,  $R^2 = 0.9667$  in the IC-ELISA and  $y = -11.094x + 106.91$ ,  $R^2 = 0.9812$  in the IMB-ELISA. The IC<sub>50</sub> values were  $2 \times 10^7$  and  $1.2 \times 10^6$  CFU mL<sup>-1</sup> and IC<sub>10</sub> values were 316 and 31 CFU mL<sup>-1</sup>, respectively for IC-ELISA and IMB-ELISA. The results indicated the established IMB-ELISA was more sensitive than IC-ELISA for monitoring *L. monocytogenes* in food.

**Specific detection:** The specificity was respectively examined for the IC-ELISA and IMB-ELISA through detecting *Escherichia coli* O157 and *Enterococcus faecalis*. The results showed that CR values were less than 0.1% for the above-mentioned pathogenic microorganisms, which suggested the prepared antibody possessed a high specificity for detection of *L. monocytogenes* in samples.

**Repeatability detection**

**Detection of the repeatability with the same plate:**

According to the determination of *L. monocytogenes* samples of  $10^5$ ,  $10^6$  and  $10^7$  CFU mL<sup>-1</sup>, the coefficient of variation were, respectively 6.87, 4.77 and 2.61 for the IC-ELISA and 4.28, 3.16 and 1.90% for the IMB-ELISA in the same plate. The coefficient of variation of less than 10% indicated two detection methods had a good repeated accuracy and the IMB-ELISA had a better repeatability in the same plate (Table 1).

**Detection of the repeatability between different plates:**

*Listeria monocytogenes* of  $10^5$  CFU mL<sup>-1</sup> was determined in 3  $\mu$ L plates, respectively. For the IC-ELISA, the coefficient of

Table 1: Result of the repeatability test in one plate

Concentrations of bacteria (CFU mL <sup>-1</sup> )	Coefficient of variation <sup>1</sup> (%)	Coefficient of variation <sup>2</sup> (%)
10 <sup>5</sup>	6.87	4.28
10 <sup>6</sup>	4.77	3.16
10 <sup>7</sup>	2.61	1.90

1: IC-ELISA, 2: IMB-ELISA, IC-ELISA: Inhibitory concentration-enzyme linked immuno sorbent assay and IMB-ELISA: Immunomagnetic bead-enzyme linked immunosorbent assay

Table 2: Result of the repeatability test in three plates

	OD <sub>450</sub> of first plate	OD <sub>450</sub> of second plate	OD <sub>450</sub> of third plate	CV (%)
IC-ELISA	0.571	0.632	0.556	6.36
IMB-ELISA	0.525	0.516	0.544	2.69

Table 3: Recovery study of *Listeria monocytogenes* in samples

Bacteria concentration (CFU mL <sup>-1</sup> )	Recovery rate <sup>1</sup> (%)	Recovery rate <sup>2</sup> (%)
10 <sup>7</sup>	89.5	94.5
10 <sup>6</sup>	91.1	108.5
10 <sup>5</sup>	102.1	97.6
10 <sup>4</sup>	83.4	95.3
10 <sup>3</sup>	92.9	99.7
10 <sup>2</sup>	90.7	92.0
10 <sup>1</sup>	82.0	87.4
Average recovery	90.2	96.4

1: IC-ELISA, 2: IMB-ELISA

variation was 6.36 between three different plates and the coefficient of variation was 2.69 for the IMB-ELISA. The coefficient of variation of less than 10% indicated two detection methods have a good repeatability and the IMB-ELISA has better repeatability between different plates (Table 2).

**Recovery study in samples:** According to these established two detection methods (IC-ELISA and the IMB-ELISA), the recovery rates were shown in Table 3. When the concentration of *L. monocytogenes* was more than 10<sup>1</sup> CFU mL<sup>-1</sup>, the average recovery rate was 90.2% in the IC-ELISA and 96.4% in the IMB-ELISA, respectively.

## DISCUSSION

*Listeria monocytogenes* is a serious threat to the food industry as a psychrotrophic species and could survive the most common stress levels presented in the food processing environment, such as high salinity, acidity, refrigeration temperatures and low water activity. In view that the traditional detection is time-consuming in enrichment culture, several new techniques had been developed based on immunology or molecular biology.

After analysis of the virulence genes of *L. monocytogenes*, conventional PCR and the real-time PCR were used in the specific detection (Wang *et al.*, 2006b; Xu *et al.*, 2007) and FRET-PCR (fluorescence resonance energy transfer based PCR), oligonucleotide probe detection and DNA chip technology were developed in the recent years (Moreno *et al.*, 2011; Suo *et al.*, 2010). A combination of PCR and DNA probe was prepared for recognizing a specific region of the internalin gene, which is responsible for the production of one of by most important pathogenic factors of *L. monocytogenes* by Ingianni *et al.* (2001). Five individual oligoprobes were selected for identification of each *Listeria* species by Sergeev *et al.* (2004), in which Cy5-labeled iap DNA from *L. monocytogenes* strain LM82 was hybridized specifically to the oligoprobes corresponding to the *L. monocytogenes* iap gene. It has been shown that fluorescence resonance energy transfer (FRET)-based PCR was used to detect the *L. monocytogenes*, including the TaqMan assay and molecular beacons (Koo and Jaykus, 2003). The limits of these above detection methods were 60-10<sup>3</sup> CFU mL<sup>-1</sup> and the sensitivities were 28-60 CFU mL<sup>-1</sup> based on molecular biology methods for the detection of *L. monocytogenes* (Ingianni *et al.*, 2001; Koo and Jaykus, 2003; Sergeev *et al.*, 2004; Wang *et al.*, 2006b; Xu *et al.*, 2007; Moreno *et al.*, 2011; Suo *et al.*, 2010).

Based on the immune detection, Luo *et al.* (2009) gold immune-chromatography assay coupled with monoclonal antibodies against *L. monocytogenes*. The double-antibody sandwich indirect ELISA method was, respectively developed for detection of *L. monocytogenes* (Chen, 2010; Duan *et al.*, 2010). The bead array and sandwich ELISA was developed for detection of *L. monocytogenes* by Karoonuthaisiri *et al.* (2015). In these ELISA study, the limit of detection for *L. monocytogenes* was 1.7 × 10<sup>5</sup>-3.3 × 10<sup>6</sup> CFU mL<sup>-1</sup>, but the sensitivities were not mentioned in these references (Chen, 2010; Duan *et al.*, 2010; Karoonuthaisiri *et al.*, 2015).

## CONCLUSION

In this study, the IMB-ELISA method was developed for the detection of the *L. monocytogenes* using the polyclonal antibody raised against P60 protein. The sensitivity was 1.2 × 10<sup>6</sup> CFU mL<sup>-1</sup> and the limit of detection was 31 CFU mL<sup>-1</sup>. There was lower detection limit and higher sensitivity than that of PCR methods. However, these PCR detection methods are need to prepare the template from a higher concentration of bacteria culture and more time was needed to improve the

bacterial colony in the sample. Compared with the PCR related methods, IMB-ELISA had a lower detection limit and higher sensitivity. However, these PCR detection methods are need to prepare the template from a higher concentration of bacteria culture and more time was needed to improve the bacterial colony in the sample. To the many of these reported ELISA methods, the detection limit of IMB-ELISA was lower and the sensitivity was higher. In consideration that IMB can quick enrich the target bacteria and the target compounds can be rapidly separated by NdFeB magnet, IMB-ELISA had a better advantage than the reported detecting method of ELISA. On the other hand, the detection method of IMB-ELISA had a better recovery and repeatability, which could basically meet the needs of the detection of the *L. monocytogenes*.

The results of this study demonstrate that the IMB-ELISA using the polyclonal antibody raised against P60 protein is a convenient and sensitive method and could be used for the rapid detection of *L. monocytogenes* in the future.

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