Journal of Food Protection, Vol. 71, No. 4, 2008, Pages 781–789

Enhanced Rapidity for Qualitative Detection of *Listeria monocytogenes* Using an Enzyme-Linked Immunosorbent Assay and Immunochromatography Strip Test Combined with Immunomagnetic Bead Separation

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MS 07-222: Received 27 April 2007/Accepted 15 December 2007

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA), immunochromatography (ICG) strip test, and immunomagnetic bead separation (IMBS) system based on a monoclonal antibody were individually developed for the detection and isolation of *Listeria monocytogenes* in meat samples. The three methods showed a strong reaction with *Listeria* species and a weak reaction with *Staphylococcus aureus.* To increase the rapidity of *L. monocytogenes* detection, combinations of the ELISA and ICG strip test with the IMBS system (ELISA-IMBS and ICG-IMBS) were investigated. In comparative analyses of artificially inoculated meat and samples of processed meat, the ELISA and ICG strip test required 24 h of enrichment time to detect the inoculated meat samples with $\geq 1 \times 10^2$ CFU/10 g, whereas the ELISA-IMBS and ICG-IMBS required only 14 h of enrichment. Analyses of naturally contaminated meat samples (30 pork samples, 20 beef samples, 26 chicken samples, 20 fish samples, and 20 processed meat samples) performed by ELISA-IMBS, ICG-IMBS, and API kit produced similar results. The ELISA-IMBS and ICG-IMBS provide a more rapid assay than the individual ELISA and the ICG strip test and are appropriate for rapid and qualitative detection of *L. monocytogenes* (or *Listeria* species) in meat samples. With the ICG-IMBS, *L. monocytogenes* could be detected in meat samples within 15 h and the method has potential as a rapid, cost-effective on-site screening tool for the detection of *L. monocytogenes* in food samples and agricultural products at a minimum detection level of ~100 CFU/10 g.

The *Listeria* genus is a gram-positive aerobic to facultatively anaerobic rod-shaped pathogenic bacterium that can grow between 1 and 45°C, which makes it potentially hazardous for refrigerated products *(8, 24).* In pregnant women, neonates, and elderly or immunocompromised people, *Listeria monocytogenes* causes listeriosis *(11),* which usually is acquired via consumption of contaminated foods *(15).* This microbe is an important foodborne pathogen and is a frequent contaminant of meat products. It is widespread in the environment and can be readily isolated from a number of sources such as soil, water, vegetables, and ready-to-eat foods *(1, 12, 29).*

For more than 50 years, the identification of *L. monocytogenes* has been achieved through traditional culturing methods and immunological and genetic methods. Conventional methods such as culturing are laborious and time-consuming, and results can be difficult to analyze when large numbers of samples are being screened. Initial enrichment and other complicated procedures often are required *(6, 10, 18, 29).* In contrast, immunological and PCR methods provide rapid, specific, reproducible, and reliable detection of target bacteria. However, although these methods provide many advantages compared with conventional techniques, they still require initial enrichment for the detection of low numbers of *L. monocytogenes* in real samples *(1, 9, 21, 30).* During the initial enrichment, various bacteria can be cocultured, which can be an obstacle for *L. monocytogenes* determination. Thus, rapid separation methods for *L. monocytogenes* from food are urgently needed.

In recent years, many studies on the immunomagnetic bead separation (IMBS) method for isolation of pathogenic bacteria from food samples have been reported *(17, 20, 22, 23, 30, 31).* IMBS is a very effective tool for the isolation of target cells from food components or other matrixes. An enzyme-linked immunosorbent assay (ELISA) and PCR as-

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say exploiting IMBS have been described, and these methods are more rapid and specific than the individual ELISAs and PCR assays *(1, 13, 17, 19, 20, 23, 30).* However, even though the IMBS is a useful tool for the separation of *L. monocytogenes* in samples, the performance of ELISA and PCR methods after IMBS separation is still time-consuming and laborious and requires several types of equipment. To overcome these limitations, a convenient and rapid method for detecting *L. monocytogenes* recently has been developed by using a novel concept of immunochromatography (ICG) that depends on the transportation of a reactant to its binding partner that is immobilized on a membrane surface. ICG strips that can detect mycotoxins *(32, 34),* pesticides *(28, 35),* antibiotics *(33),* and pathogenic bacteria *(2, 14)* have been developed and widely used. They combine several benefits of a user-friendly format, short assay time, long-term stability over a wide range of climates, and cost-effectiveness. These characteristics make the ICG method ideally suited for screening of large numbers of samples by personnel who are not skilled analysts *(5, 28).* In preliminary experiments *(27),* we developed an ICG strip test that could detect *L. monocytogenes* within 20 min. However, this assay still involved initial enrichment for 24 h at 37°C.

The ELISA and PCR combined with IMBS for the detection of *L. monocytogenes* has been reported, but an ICG strip test exploiting IMBS has not been described. In this study, we individually developed an ELISA, an ICG strip test, and an IMBS system for the detection and isolation of *L. monocytogenes,* and here we describe improved time to detection for *L. monocytogenes* with the ICG strip test exploiting IMBS.

MATERIALS AND METHODS

Microorganism, culture conditions, and materials. Sixteen *Listeria* and 13 non-*Listeria* species were used in this study (Table 1). The *Listeria* species were *L. monocytogenes* ATCC 19111 (serotype 1), *L. monocytogenes* ATCC 51772 (serotype 1/2a), *L. monocytogenes* ATCC 51780 (serotype 1/2b), *L. monocytogenes* ATCC 51779 (serotype 1/2c), *L. monocytogenes* ATCC 19112 (serotype 2), *L. monocytogenes* ATCC 19113 (serotype 3), *L. monocytogenes* ATCC 19114 (serotype 4a), *L. monocytogenes* ATCC 19115 (serotype 4b), *L. monocytogenes* ATCC 19116 (serotype 4c), *L. monocytogenes* ATCC 19117 (serotype 4d), *L. monocytogenes* ATCC 19118 (serotype 4e), *L. innocua* ATCC 33090 (serotype 6a), *L. ivanovii* ATCC 19119, *L. grayi* ATCC 19120, *L. welshimeri* ATCC 35897 (serotype 6b), and *L. seeligeri* ATCC 35967. These bacteria were grown in a *Listeria* enrichment broth (LEB; Difco, Becton Dickinson, Sparks, Md.) supplemented with nalidixic acid (40 mg/ml) and cycloheximide (50 mg/ml) or on *Listeria* selective agar base (Oxford, Difco, Becton Dickinson) at 37°C for 24 h.

The non-*Listeria* species were *Bacillus cereus* ATCC 21366, *Carnobacterium divergens* KCTC 3675, *Clostridium perfringens* ATCC 3624, *Escherichia coli* O157:H7 ATCC 43888, *Lactobacillus plantarum* ATCC 14917, *Leuconostoc mesenteroides* ATCC 10830, *Pseudomonas fluorescens* ATCC 21514, *Salmonella* Typhimurium ATCC 13311, *Serratia marcescens* ATCC 25419, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175, *Vibrio parahaemolyticus* ATCC 17802, and *Vibrio vulnificus* ATCC 27562. *C. divergens* was grown in tryptic soy broth (TSB; Difco, Becton Dickinson) containing 3% (wt/vol) yeast extract for 24 h at 37°C, and *C. perfringens* was grown in a cooked meat medium (Difco, Becton Dickinson) under anaerobic conditions for 20 h at 37°C or on tryptose sulfite cycloserine agar (Merck KGaA, Darmstadt, Germany) for 24 h at 37°C. *Lactobacillus, Leuconostoc,* and *Pseudomonas* were cultured in deMan Rogosa Sharpe (MRS) broth (Difco, Becton Dickinson) or MRS agar for 24 h at 37°C. *S. aureus* was grown in TSB containing 10% (wt/vol) NaCl or mannitol salt agar (Difco, Becton Dickinson) for 24 h at 37°C, and *S. marcescens* was cultured in brain heart infusion (Difco, Becton Dickinson) containing 0.5% (wt/vol) casein at 37°C for 24 h. *Vibrio* species were grown in TSB containing 3% (wt/vol) NaCl or on thiosulfate citrate bile

TABLE 1. *Specificity of the ELISA, ICG strip test, and IMBS system for* Listeria *species and other pathogenic bacteria*

Strain	Results[a]		
	ELISA	ICG strip test	IMBS system[b]
Listeria monocytogenes, serotype			
1	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
1/2a	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
1/2b	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
1/2c	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
2	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
3	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
4a	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
4b	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
4c	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
4d	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
4e	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
L. innocua	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
L. ivanovii	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^6$)	+
L. grayi	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
L. welshimeri	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^6$)	+
L. seeligeri	+ ($\geq 1 \times 10^5$)	+ ($\geq 1 \times 10^5$)	+
Bacillus cereus	−	−	−
Escherichia coli O157:H7	−	−	−
Carnobacterium divergens	−	−	−
Clostridium perfringens	−	−	−
Lactobacillus plantarum	−	−	−
Leuconostoc mesenteroides	−	−	−
Salmonella Typhimurium	−	−	−
Serratia marcescens	−	−	−
Staphylococcus aureus	+ ($\geq 1 \times 10^7$)	+ ($\geq 1 \times 10^8$)	+
Streptococcus mutans	−	−	−
Pseudomonas fluorescens	−	−	−
Vibrio parahaemolyticus	−	−	−
V. vulnificus	−	−	−

[a] +, the reaction was observed (numbers in parentheses are cell counts, CFU per milliliter); −, no reaction.
[b] IMBS was performed with bacterial levels of 1×10^7 CFU/ml.

salts sucrose agar (Difco, Becton Dickinson) at 37°C for 24 h. The remaining bacteria were grown in TSB or tryptic soy agar (Difco, Becton Dickinson) at 37°C for 24 h.

Complete and incomplete Freund's adjuvant, peroxidase-conjugated anti-mouse immunoglobulin (Ig) G, 2.2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), horseradish peroxidase, tetrachloroauric acid, sodium citrate, and anti-mouse IgG were all purchased from Sigma Chemical Co. (St. Louis, Mo.). The protein G agarose was purchased from Bioprogen (Daejeon, South Korea), and the maxisorp polystyrene 96-microwell plates were obtained from Nunc (Rockilde, Denmark). The nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad were obtained from Millipore (Bedford, Mass.), the semirigid polyethylene sheets were purchased from a local market, and the magnetic beads (M-280) were obtained from Dynal (Oslo, Norway).

Production of monoclonal antibody. A monoclonal antibody (MAb) was produced from a 3B12-17 hybridoma cell that was developed by cell fusion using myeloma cells and spleen cells obtained from mice immunized with formalin-killed *L. monocytogenes* (FKLM) *(27).* The hybridoma cells were grown in cell culture medium and intraperitoneally injected into BALB/c mice that had been pretreated with an intraperitoneal injection of 0.5 ml of pristine. After 1 week, the ascite fluid was taken from the mice and purified by precipitation with saturated ammonium sulfate. A protein G column was used for affinity purification. Protein concentration of the purified MAb was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Development of ELISA. An ELISA for the detection of *L. monocytogenes* was performed with 96-well microtiter plates. *L. monocytogenes* from fresh broth cultures was pelleted by centrifugation (10,000 rpm for 5 min) and suspended in an equal volume of 0.1 M carbonate buffer (CB), pH 9.0. Cells (100 μl) placed in the microtiter plates were incubated for 1 h at 37°C and washed three times with phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween 20 (PBST) with the Immuno Wash 12 microplate washer (Nunc). The residue washing buffer was discarded, and the surface of the wells was blocked with 200 μl of 3% (wt/vol) skim milk for 1 h at room temperature. Blocking reagents were then removed, and the wells were washed four times with PBST. One hundred microliters of MAb diluted 1:2,000 (vol/vol) in PBS was added to 96-well plates, which were incubated for 1 h at room temperature and then washed five times with PBST. A peroxidase-conjugated goat anti-mouse IgG diluted 1:10,000 (vol/vol) in PBS (100 μl per well) was added into each well and incubated for 1 h at 37°C. After a washing step, 100 μl of 0.1 M citrate buffer (pH 4.0) containing 0.025% (wt/vol) ABTS and 0.03% (vol/vol) H_2O_2 was added and incubated for 30 min at 37°C. To determine the reaction spectrum, the enzyme reaction was stopped with 50 μl of 2 M H_2SO_4, and absorbance was measured at 405 nm with a microplate reader (model 550, Bio-Rad).

To determine the specificity of the ELISA, 16 *Listeria* and 13 non-*Listeria* species were individually grown as described above, and the cells were centrifuged, washed, and serially diluted from 10^8 to 10^1 CFU/ml in 0.1 M CB. The adjusted cells were all tested by ELISA.

Development of ICG strip test. Colloidal gold particles (40 nm) used as the marker in the ICG strip test were produced in our laboratory by a reduction method using sodium citrate as previously described *(7, 28),* and these particles were conjugated with the 3B12 MAb using a previously described method *(25, 26).* The gold-MAb conjugates were stored at 4°C before use. The ICG strip test was composed of three pads (sample, conjugate, and absorbent pads) and one nitrocellulose membrane containing test and control zones (Fig. 1). The sample and absorbent pads were treated according to the method described previously *(28).* Five microliters of the gold-MAb conjugate (absorbance at 540 nm was 1.5) was applied to the conjugate pad and allowed to dry at 37°C for 30 min. The test and control lines on the nitrocellulose membrane were treated with 3B12-41 MAb (1.0 mg/ml in PBS) and goat anti-mouse IgG (1.0 mg/ml in PBS) and dried at 37°C for 30 min. The treated pads and membranes were all attached to a semirigid polyethylene sheet. A positive sample produced two red lines, at the test and control zones, whereas a negative sample produced only one red line, at the control zone. The strip test was incorrect if there was no red line on the control zone (Fig. 1). To determine the specificity of this assay, 16 *Listeria* and 13 non-*Listeria* species were individually grown as described above, washed with PBS, and serially diluted from 10^8 to 10^1 CFU/ml in PBS. The adjusted cells were all tested by the ICG strip test.

Development of IMBS system. Magnetic beads (M-280) were directly coupled with the 3B12-17 MAb according to the manufacturer's instructions, but the separation procedures were modified. A 1-ml aliquot of fresh culture or suspended sample was transferred into a 1.5-ml Eppendorf tube, and the cells were harvested by centrifugation and then washed three times. The pellets were suspended in an original volume of PBS, and 30 μl of immunomagnetic beads was added to the suspended solution. After incubation at room temperature for 10 min with gentle agitation, the tubes were placed in a magnet (Dynal) for 5 min. During this time, the magnet was inverted several times to concentrate the beads into a pellet on the side of the tube. The supernatant was completely aspirated, and the beads were washed three times with PBS and recovered with the magnet as described above. The final bead pellet was suspended in 100 μl of PBS, and 50 μl of the suspension was spread on Oxford agar and incubated for 24 h at 37°C for colony counts. The remainder was transferred into LEB and cultured at 37°C for enrichment. The cultures were prepared as described above and tested by both the ELISA and ICG strip test. To determine the specificity of the IMBS system, 16 *Listeria* and 13 non-*Listeria* species were individually grown as described above, washed with PBS, and used in the IMBS system.

To determine the efficiency of the IMBS system, a separation ratio was evaluated by adding different volumes of beads (10, 30, 50, 70, and 100 μl) for *Listeria* species and *S. aureus* (10^7 CFU/ml in PBS). After incubation and isolation by the IMBS system, the final bead pellets were suspended with 100 μl of PBS and spread onto each selective agar. The colonies on the plates were counted after incubation at 37°C for 24 h.

ELISA and ICG strip test exploiting the IMBS system. To enhance the speed of *L. monocytogenes* determination, the ELISA and ICG strip protocols combined with the IMBS system (ELISA-IMBS and ICG-IMBS) were designed and evaluated in this study. To validate the speed and efficiency of the ELISA-IMBS and ICG-IMBS, the artificially inoculated samples were cultured and prepared as described above and analyzed by the ELISA, ICG strip test, ELISA-IMBS, and ICG-IMBS. Six kinds of meat samples (beef, pork, chicken, fish, ham, and sausage) were aseptically cut into several pieces of 10 g each, sterilized for 1 h under UV illumination, and transferred into a stomach bag with a filter lining. The samples were artificially inoculated with *L. monocytogenes* at different levels (10^8, 10^6, 10^4, and 10^2 CFU/10 g), left at room temperature for 1 h, and suspended with 90 ml of PBS (or LEB) in stomacher bags. For individual ELISAs and ICG strip tests, the samples suspended in LEB were incubated at 37°C for 24 h. During incubation, 2 ml of each sample was taken at an

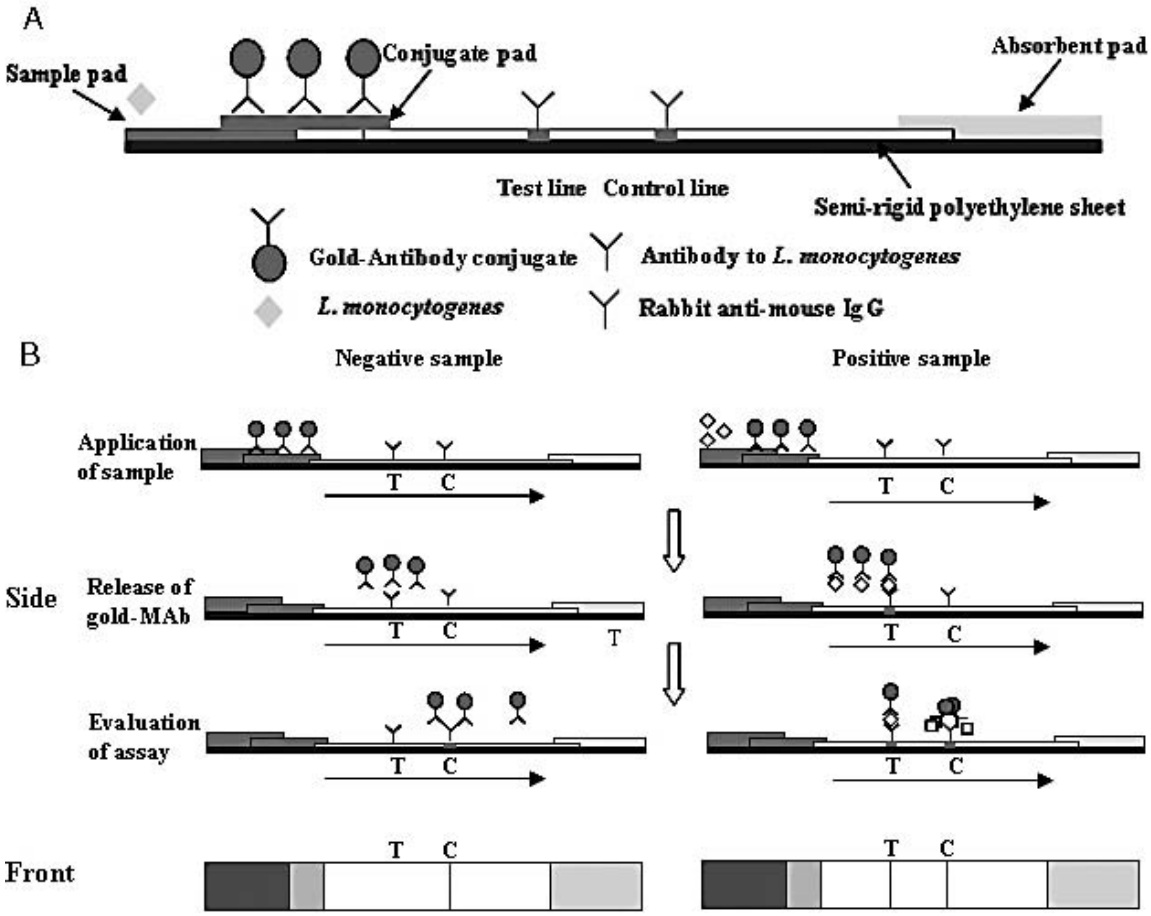

FIGURE 1. *Construction of the ICG strip test (A) and illustration of its results (B). The test is invalid if no line appears in the control zone. C, control line; T, test line.*

interval of 2 h, and cells were harvested by centrifugation, suspended with 1 ml of PBS, and then tested by both the ELISA and ICG strip test. For analysis of ELISA-IMBS and ICG-IMBS, 10-ml aliquots of sample suspensions that were homogenized with PBS were withdrawn into tubes containing 300 μl of beads and incubated at 37°C for 15 min with gentle rotation. The *L. monocytogenes*–bead complex was harvested according to the procedures of IMBS system, inoculated into 5 ml of LEB, and incubated for 24 h. During growth, 2 ml of each sample was collected at an interval of 2 h, and cells in the culture were harvested by centrifugation, suspended with 1 ml of PBS, and tested by both the ELISA and the ICG strip test.

Analysis of meat and processed meat samples by ELISA-IMBS and ICG-IMBS. For the screening of *L. monocytogenes* in meat samples, 116 meat samples (pork, 30 samples; beef, 20 samples; chicken, 26 samples; fish, 20 samples; processed-meat products, 20 samples) were collected from supermarkets and traditional markets. *L. monocytogenes* was isolated with the IMBS and grown in LEB for 14 h at 37C. The cells were then harvested by centrifugation, suspended in PBS, and tested by both the ELISA and ICG strip method. The results were compared with those obtained with the API kit (bioMérieux, Marcy l'Etoile, France). For the API kit analysis, all samples were enriched in LEB at 30°C for 24 h and then transferred to Fraser broth base at 35°C (Difco, Becton Dickinson) for 24 h. The cultures (100 μl) were then spread onto Oxford agar and incubated at 37°C for 24 h. Black colonies on the Oxford plates were picked, inoculated into LEB, and cultured at 37°C for 12 h. The pure cultures were tested with the API kit according to the manufacturer's instructions. To ensure absence of non-*Listeria* species, the black colonies were also streaked onto a selective agar for non-*Listeria* species.

RESULTS AND DISCUSSION

Characterization of MAb. In preliminary work, the immunization of mice was performed with two immunogens: formalin-killed *L. monocytogenes* (FKLM) and heat-killed *L. monocytogenes* (HKLM). The antisera obtained from the mice immunized with the FKLM had a higher titer than did the antisera of the mice immunized with the HKLM. Therefore, we expected that several hybridoma cell lines producing specific MAbs to *L. monocytogenes* would be developed if the mice immunized with FKLM were used in cell fusion. After cell fusion and cloning, five clones (3B12-17, 3B12-19, 3B12-21, 3B12-37, and 3B12-41) were generated. The 3B12-37 hybridoma was selected and expanded for the mass production of MAb because it reacted more strongly to *L. monocytogenes* than to *Listeria* species and showed weak reaction to *S. aureus* in the indirect ELISA *(27)* but no reaction to other bacteria.

In a western blot analysis with the 3B12-37 MAb, *L. monocytogenes* and *S. aureus* adjusted to 1×10^8 CFU/ml in PBS yielded a single reactive band with the same migration distance. The western blot for *Listeria* species pro-

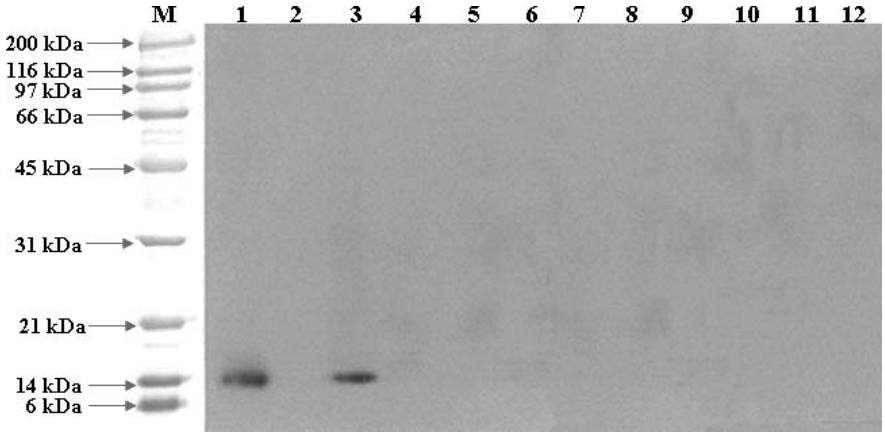

FIGURE 2. *Western blot analysis of various bacteria with FKLM-3B12-37 MAb. Whole cell suspensions were heated at 100°C for 5 min in PBS, mixed 1:1 in Laemmli sample buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% acrylamide gel), transferred to a nitrocellulose membrane, and analyzed with the FKLM-3B12-37 MAb. Lane M, molecular weight standard; lane 1,* Listeria monocytogenes*; lane 2,* Vibrio vulnificus*; lane 3,* Staphylococcus aureus*; lane 4,* Escherichia coli *O157:H7; lane 5,* Bacillus cereus*; lane 6,* Salmonella *Typhimurium; lane 7,* Clostridium perfringens*; lane 8,* Lactobacillus plantarum*; lane 9,* Leuconostoc mesenteroides*; lane 10,* Pseudomonas fluorescens*; lane 11,* Carnobacterium divergens*; lane 12,* Serratia marcescens.

duced a single band at the same position as that for *L. monocytogenes* (data not shown). The molecular mass of the antigen reacted with the 3B12-37 MAb was calculated as 15,000 Da and appeared to be a glycoprotein or lipoprotein moiety. However, no reactive bands for other non-*Listeria* species were observed (Fig. 2). Siragusa and Johnson *(29)* reported on the monoclonal antibody against *L. monocytogenes, L. innocua,* and *L. welshimeri* and the presence of an antigen (molecular mass of 18,500 ± 500 Da) located in the wall of the three *Listeria* species. Carlier et al. *(4)* reported on antigen 2 located in the wall of *L. grayi* and *L. ivanovii.* The molecular mass of the listerial antigen recognized by 3B12-37 MAb is similar to that of the two previously reported antigens. Many other scientists *(3, 29)* have reported the cross-reaction of anti–*L. monocytogenes* antibodies with *S. aureus.* This reaction was explained by the fact that *S. aureus* often expresses protein A in the cell wall, and this protein has a strong affinity to IgG. Unfortunately, the isotype of 3B12-37 MAb developed in the present study is IgG1. We assumed that the 3B12-37 MAb had reacted to the common proteins located in the cell wall fragments of *Listeria* species and to protein A expressed in the cell wall of *S. aureus.*

ELISA. The ELISA based on the 3B12-37 MAb was developed and optimized for the detection of *L. monocytogenes.* A CB without *L. monocytogenes* was used as the negative control and produced an average optical density value of approximately 0.15 at 405 nm. We calculated negative results according to the following formula, negative optical density + 0.1, results of ≤0.25 were considered negative. The detection limit (>0.25 optical density value) of the ELISA was 1×10^5 CFU/ml (optical density of 0.29) (Fig. 2). This assay required 4.5 h (coating step, 1 h; blocking step, 1 h; primary MAb reaction step, 1 h; second antibody reaction step, 1 h; color developing step, 0.5 h) to detect *L. monocytogenes* after enrichment or preparation of cells. The specificity of the ELISA is shown in Table 1. Because the 3B12-37 MAb reacted with *Listeria* species and *S. aureus,* the ELISA had a strong reaction with *Listeria* species and a weak reaction with *S. aureus,* but no cross-reactions to other bacteria were observed. Although the ELISA had a weak reaction with *S. aureus,* this assay was highly specific for *L. monocytogenes* and *Listeria* species. Therefore, this ELISA could be used for screening large numbers of samples for *L. monocytogenes* and *Listeria* species.

ICG strip test. The ICG strip test for the qualitative detection of *L. monocytogenes* contamination was developed and optimized using the 3B12-37 MAb. After sample application to the sample pad of the ICG strip test, the sample solution migrated to the conjugate pad that was treated with the gold-MAb conjugates. When the *L. monocytogenes* count was above the detection limit, the complexes formed between the gold-MAb conjugate and *L. monocytogenes* were sufficient to develop red lines in both the test and control zones. When the *L. monocytogenes* count was below the detection limit, few of these complexes were formed (not enough to develop a red line in the test zone), so free gold-MAb conjugate did not bind to the 3B12-37 MAb immobilized in the test zone but reacted to anti-mouse IgG immobilized in the control zone. The appearance of two red lines on the nitrocellulose membrane is a positive test result for *L. monocytogenes,* and one red line indicates a negative test for *L. monocytogenes.* The ICG strip test developed in this study required 20 min to detect *L. monocytogenes.* The detection limit of the ICG strip test also was 1×10^5 CFU/ml (Fig. 3). The specificity of the ICG strip test is shown in Table 1. The positive results were observed in the test zone when *Listeria* species at approximately $\geq 10^5$ CFU/ml and *S. aureus* at $\geq 10^8$ CFU/ml were applied to the ICG strip test. However, no cross-reaction was observed for other pathogenic bacteria. Although the ICG strip test gave a positive result for *S. aureus* at $\geq 10^8$ CFU/ml, this assay still was highly specific for *L. monocytogenes* and *Listeria* species. Therefore, we thought that the screening of *L. monocytogenes* and *Listeria*

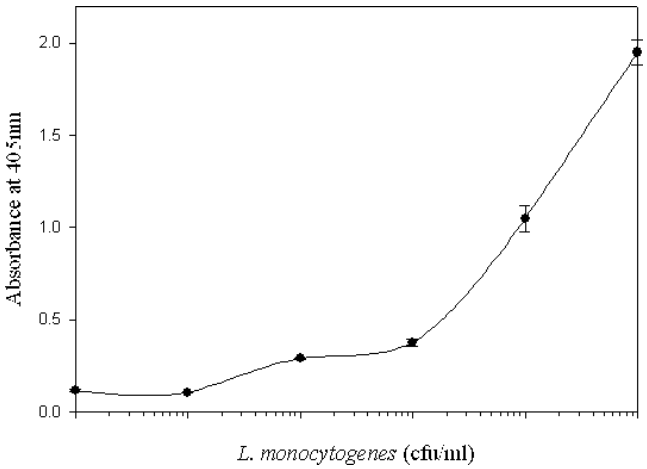

FIGURE 3. *ELISA standard curve for* L. monocytogenes *detection in carbonate buffer (0.1 M, pH 9.0). Each point of the curve represents the mean ± standard deviation of three assays on the same day. NC, negative result for* L. monocytogenes.

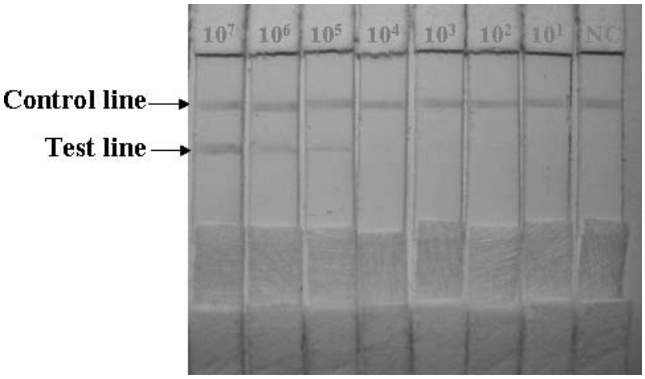

FIGURE 4. *Sensitivity of ICG strip test for the detection of* L. monocytogenes. *The tests were run four times at room temperature using PBS with serial dilutions of* L. monocytogenes *culture. The label (10^7 to 10^1) shows the counts of* L. monocytogenes *in 1 ml of PBS. NC, negative result for* L. monocytogenes.

species with the ICG strip test (Fig. 4) would enhance speed and convenience.

IMBS system. An IMBS system for the rapid isolation and concentration of *L. monocytogenes* in meat samples was developed and optimized. The specificity of the IMBS system to *Listeria* and non-*Listeria* species is shown in Table 1. Many colonies were observed on the Oxford agar and mannitol salt agar when the suspensions of *Listeria* species and *S. aureus* were tested with the IMBS system, spread on each agar plate, and incubated for 24 h at 37°C. However, no colonies were found on agar plates for other bacteria. To determine the efficiency of the IMBS system, the capture efficiency of this system was evaluated. Approximately 1% separation was observed when ≥30 μl of immunomagnetic beads was added to 1 ml of bacterial suspensions (1×10^7 CFU/ml in PBS) (Table 2). For overall economics and efficiency, we selected 30 μl of beads as the optimal volume in the IMBS system. Decreasing the number of *L. monocytogenes* cells increased the capture efficiency. The efficiency of *L. monocytogenes* capture for 10^6, 10^4, 10^2, and 10^1 CFU/ml was 5, 30, 40, and 60%, respectively. The IMBS system developed in this study could be used as a tool for the rapid isolation and concentration of *L. monocytogenes* and *Listeria* species, even though the IMBS system exhibited a weak cross-reaction with *S. aureus.*

Validation of ELISA-IMBS and ICG-IMBS. Before screening of *L. monocytogenes* or *Listeria* species in meat samples by ELISA-IMBS and ICG-IMBS, these methods were compared with the ELISA and the ICG strip test alone with meat samples artificially inoculated with *L. monocytogenes* (10^8, 10^6, 10^4, and 10^2 CFU/10 g of meat) (Table 3). The individual ELISA and the ICG strip test required 24 h of enrichment time to detect 1×10^2 CFU of *L. monocytogenes* inoculated in meat samples, whereas the ELISA-IMBS and ICG-IMBS required 14 h. After enrichment for 14 h, the inoculated samples were all positive with the ELISA-IMBS and ICG-IMBS. Many researchers have described a long enrichment step (≥24 h) as necessary for the detection of *L. monocytogenes* and *Listeria* species in various foods by immunoassays and PCR methods *(11, 12, 16, 20, 29).* In contrast, the ELISA-IMBS and ICG-IMBS require 14 h of enrichment time to detect *L. monocytogenes* and *Listeria* species in meat and processed meat samples. Although the IMBS system exhibited a weak cross-reaction with *S. aureus,* this result is not a significant problem for identification of *L. monocytogenes* and *Listeria* species by both the ELISA-IMBS and ICG-IMBS because the *S. aureus* cannot grow in LEB.

In recent years, immunoassays and commercial kits

TABLE 2. *Efficiency of isolation by adding different volumes of immunomagnetic beads*[a]

Inoculated strain	*n*	Isolation (CFU) in bead volumes of:				
		10 μl	30 μl	50 μl	70 μl	100 μl
Listeria monocytogenes[b]	3	2×10^4	1×10^5	1.1×10^5	1×10^5	1.1×10^5
L. innocua	3	2.1×10^4	1.1×10^5	1.1×10^5	1.1×10^5	1.2×10^5
L. ivanovii	3	1.8×10^4	9.1×10^4	1.0×10^5	1.1×10^5	1.1×10^5
L. grayi	3	1.7×10^4	6×10^4	9.6×10^4	1×10^5	1×10^5
L. welshimeri	3	2×10^4	7.3×10^4	1×10^5	1×10^5	1×10^5
L. seeligeri	3	1.9×10^4	9.8×10^4	1×10^5	1.1×10^5	1.0×10^5
Staphylococcus aureus	3	7.3×10^3	3×10^4	4.5×10^4	6.2×10^4	8.8×10^4

[a] Initial cell count was 1×10^7 CFU/ml in PBS.
[b] *L. monocytogenes* ATCC 19115 was used for this experiment.

TABLE 3. *Determination of enrichment time of inoculated meat and processed meat samples by four immunoassays developed in this study*

Sample	Inoculated *L. monocytogenes* (CFU/10g)[a]	Enrichment time (h) for positive results			
		ELISA	ICG strip test	ELISA-IMBS	ICG-IMBS
Pork ($n = 3$)	10^6	≥22	≥22	≥10	≥10
	10^4	24	24	≥10	≥14
	10^2	24	−	≥14	≥14
	NI[b]	−[c]	−	−	−
Beef ($n = 3$)	10^6	≥22	≥22	≥10	≥10
	10^4	24	24	≥14	≥14
	10^2	24	−	≥14	≥14
	NI	−	−	−	−
Chicken ($n = 3$)	10^6	≥22	≥22	≥10	≥10
	10^4	24	24	≥14	≥14
	10^2	24	−	≥14	≥14
	NI	−	−	−	−
Fish ($n = 3$)	10^6	≥22	≥22	≥10	≥10
	10^4	24	24	≥10	≥14
	10^2	24	24	≥14	≥14
	NI	−	−	−	−
Sausage ($n = 3$)	10^6	≥22	≥22	≥10	≥10
	10^4	24	24	≥10	≥10
	10^2	24	24	≥14	≥14
	NI	−	−	−	−

[a] *L. monocytogenes* ATCC 19115 was used.
[b] NI, no inoculation.
[c] −, negative result.

based on immunoassays have been widely used for screening samples for *L. monocytogenes* or *Listeria* species. Kim et al. *(15)* developed MAbs and chicken Ig against the flagella of *Listeria* species. The detection limit of a sandwich ELISA using their antibodies was 1×10^5 CFU/0.1 ml. The method produced cross-reactions with other *Listeria* species and required 24 h of enrichment time to detect target bacteria. Geng et al. *(8)* developed an immunosensor with polyclonal antibodies and MAbs against *L. monocytogenes.* The immunosensor could detect 4.3×10^3 CFU/ml and required 20 h of enrichment. The immunosensor had a significantly stronger signal in response to *L. monocytogenes* than to other *Listeria* species. The commercial kits used to screen samples for *L. monocytogenes* or *Listeria* species possess high detection limits ($\geq 1 \times 10^5$ CFU/ml), but the kits could be used to detect *Listeria* species at 1 to 10 CFU/25 g of sample after enrichment for 24 to 48 h.

Consequently, the ELISA-IMBS and ICG-IMBS developed in this study are satisfactorily rapid for *L. monocytogenes* and *Listeria* species determination compared with the recently reported immunoassays and commercial kits. Specifically, the ICG-IMBS detected *L. monocytogenes* in meat samples within the same day after samples were obtained (IMBS step, 30 min; enrichment step, 14 h; washing step, 10 min; ICG test strip analysis, 20 min).

Screening for *L. monocytogenes* in naturally contaminated samples. Ninety-six meat samples were analyzed by the ELISA-IMBS, ICG-IMBS, and API kit (Table 4), and 36 samples and 34 samples were positive for *Listeria* species by the ELISA-IMBS and ICG-IMBS, respectively. With the API kit, 30 samples and 27 samples were confirmed to be positive for *Listeria* species and *L. monocytogenes,* respectively. Twenty-seven samples that were positive for *L. monocytogenes* with the API kit were all confirmed to be positive by ELISA-IMBS and ICG-IMBS analyses, and most of them were simultaneously contaminated with *Listeria* species such as *L. innocua* and *L. ivanovii.* Thus, the results of the ELISA-IMBS and ICG-IMBS were in close agreement with those obtained with the API kit. According to these results, we thought that the ELISA-IMBS and ICG-IMBS could be applied as a primary screening method for *L. monocytogenes* or *Listeria* species detection. In addition, the processing time for *L. monocytogenes* detection was faster for the ELISA-IMBS and ICG-IMBS assays. In particular, the ICG-IMBS to detect *Listeria* species could be completed within 1 day.

Various procedures such as culture, biochemical methods, immunoassays, and PCR assays are reliable tools for screening samples for the presence of *L. monocytogenes.* However, these methods are not suitable to screen large numbers of samples because they often require complicated isolation steps and long enrichment periods. To develop a rapid screening method for *L. monocytogenes* in meat and processed meat samples, we individually developed the ELISA, ICG strip test, and IMBS system, and then the ELISA-IMBS and ICG-IMBS were designed and developed to enhance the speed of *L. monocytogenes* detection.

TABLE 4. *Results for* L. monocytogenes *and* Listeria *species screening in meat and processed meat samples by ELISA-IMBS, ICG-IMBS, and API kit*

Sample	No. of samples tested	No. of positive samples[a]			
		ELISA-IMBS	ICG-IMBS	API for *Listeria* spp.	API for *L. monocytogenes*
Pork	30	11 (2, 4, 10, 14, 15, 16, 19, 22, 23, 25, 29)	10 (2, 4, 10, 15, 6, 19, 22, 23, 25, 29)	7 (4, 9, 10, 15, 22, 25, 29)	9 (2, 4, 10, 15, 16, 19, 22, 23, 25)
Beef	20	7 (1, 5, 12, 13, 17, 19, 20)	7 (1, 5, 12, 13, 17, 19, 20)	4 (1, 5, 12, 20)	6 (5, 12, 13, 17, 19, 20)
Chicken	26	14 (1, 2, 4, 5, 6, 9, 11, 14, 15, 16, 17, 21, 22, 25)	14 (1, 2, 4, 5, 6, 9, 11, 14, 15, 16, 17, 21 22, 25)	16 (1, 2, 4, 5, 6, 8, 9, 11, 14, 15, 16, 17, 19, 21, 22, 25)	10 (1, 2, 4, 5, 9, 14, 15, 16, 17, 21)
Fish	20	4 (2, 14, 17, 18)	3 (14, 17, 18)	3 (3, 14, 17)	2 (14, 18)
Processed meat	20	0	0	0	0
Total	116	36	34	30	27

[a] Sample identification numbers are given in parentheses.

The IMBS system provides a rapid and simple method for the detection of *L. monocytogenes* from real samples and does not require long enrichment. In our study, the minimum enrichment time after *L. monocytogenes* and *Listeria* species isolation by IMBS was 14 h to detect low counts of *L. monocytogenes* and *Listeria* species (1×10^2 CFU/10 g) by ELISA and the ICG strip test. ICG-IMBS to detect *L. monocytogenes* and *Listeria* species in meat samples could be completed within 1 day (15 h). We thought that initial enrichment and detection time could be decreased if an IMBS were combined with immunoassays. Actually, the inoculated samples (10^6, 10^4, and 10^2 CFU/g) were verified to be positive within 15 h by the ICG-IMBS. Comparative analyses of natural meat and processed meat samples by the ELISA-IMBS, ICG-IMBS, and API kit resulted in close agreement (Table 4). Many methods involving IMBS have been used, but the ICG strip test exploiting the IMBS system has not been reported previously. The ICG-IMBS developed in this study has potential as a rapid, cost-effective on-site screening tool for pathogenic bacteria contaminating food and agricultural products.

ACKNOWLEDGMENTS

This research was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (03-PJ1-PG1-CH11-0003). W. B. Shim was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2006-352-F00029). J. G. Choi was supported by the Brain Korea 21 program from the Ministry of Education, Republic of Korea.

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