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Development and Evaluation of an Immunochromatographic Assay for Screening *Listeria* spp. in Pork and Milk

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Abstract Rapid immunochromatographic assay (ICA) kits were developed using flagella-specific monoclonal antibodies (MAbs) and rabbit polyclonal antibodies for screening *Listeria* spp. in food. The establishment of different formats, MAb 2B1 as capture antibody and MAb 7A3 or rabbit polyclonal antibodies as detector antibody, was compared. The 2 formats of the ICA kit were shown to have specific reactions with *Listeria* and no cross-reactivity with any of the non-*Listeria* including *Escherichia coli* O157:H7 and *Salmonella enteritidis*. The detection limits of the ICA kit using the combination of gold-labeled MAb 7A3 and MAb 2B1 showed 1×10^5 and 1×10^6 CFU/ 0.1 mL at 22 and 30°C, respectively. The other format of the ICA kit using the combination of gold-labeled rabbit polyclonal antibodies and MAb 2B1 showed 1×10^6 CFU/ 0.1 mL at 22°C but weak signal at 30 culture. The format utilizing MAb was more sensitive than the one using polyclonal antibodies for capture antibody. Samples contaminated with *L. monocytogenes* 4b culture (9-10, 5-6, and 1-2 CFU/mL) on pork and pasteurized milk were confirmed as positive results. Current data suggests that this ICA kit is a rapid, simple and effective tool to screen for *Listeria* spp. in food.

Keywords: *Listeria*, immunochromatographic assay, food, flagellum, monoclonal antibody (MAb), rabbit polyclonal antibody

Introduction

Listeria monocytogenes was first identified in 1924 as a causative pathogen of disease in laboratory animals (1). Since then, this bacterium has been linked to food-borne outbreaks involving a wide range of foods such as cheese, milk, fish, vegetables, and ready-to-eat meat products (2). The countries such as the United States, Australia, and Korea have established legal limits with regard to the number of *L. monocytogenes* which can be present in meat products. These countries have adopted a zero tolerance policy for *Listeria* in 25 g of raw meat.

L. monocytogenes from inoculated or naturally contaminated foods can be isolated by the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) methods. However, both conventional methods require at least 7-10 days for the isolation and identification of *Listeria* spp.

Polymerase chain reaction (PCR) based techniques are quite sensitive and more rapid than the conventional culture methods (3). Wang *et al.* (4) reported the detection limit to be 4-10 CFU per food sample in direct detection methods, although they could not confirm the reproducibility of results. The key element associated with this technique involves the recovery of *L. monocytogenes* from meat without the PCR reaction inhibitors. In order to circumvent such drawback, Duffy *et al.* (5) used a surface adhesion-based extraction procedure involving limited in their use for detection of *Listeria* spp., due to PCR reaction-inhibitory substances in food, nucleic acids from other bacteria, and sample volumes (6, 7). Furthermore, PCR methods are still relatively expensive, and procedurally less user-friendly to be used as routine screening tools.

Enzyme immunoassays constitute another method for the detection of *Listeria* spp. in foods. Various monoclonal antibodies (MAbs) which react with different *L. monocytogenes* antigens have been developed by many researchers (8-13). These MAbs have not proven specific for *L. monocytogenes*, and have also reacted with other *Listeria* spp.

Until now, rapid methods, including immunoassays and PCR assays (14, 15), generally have required 48 hr of sample enrichment in order to obtain detectable levels of bacteria (10^5-10^6 CFU/mL) (16) as the level of *L. monocytogenes* in meats can be as low as 100 CFU/g (17). Following 48 hr of enrichment, 2 methods mentioned above, although they are sensitive and specific, take at least 2 or 3 additional hr for the completion of testing. Slow turnaround time for results has thus become an issue, and the development of a rapid, inexpensive, and convenient method, which requires no instruments when used in mass-screening surveys is therefore needed.

In this study, we developed a rapid immunochromatographic assay (ICA) for screening of *Listeria* spp. using flagella-specific MAbs and rabbit polyclonal antibodies,

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and compared the reliability of this ICA kit with the sandwich enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Bacteria The 13 strains of *Listeria* and 10 species of non-*Listeria* used in this study are shown in Table 1. *Listeria* spp. were grown in tryptic soy broth supplemented with 0.2% glucose and 0.6% yeast extract (TSB, Difco, Becton Dickinson Co., Sparks, MD, USA) at 22 for 48 hr. Some of non-*Listeria* organisms were isolated from animals in Jeju island. Two *Staphylococcus aureus* and *Salmonella enteritis* were isolated from raw milk and skin of cow, and chicken, respectively. The bacteria isolates were obtained by direct cultivation on blood agar and confirmed at the species level on the basis of Gram stain, catalase, coagulase, urease, and oxidase reactions (18). The selective agar media, MacConkey agar, Baired-Parker agar (BP), and Xylose Lysine Deoxycholate agar (XLD) (Merck, Darmstadt, Germany) were used for selective plating. These bacteria were identified using API Staph strips and VITEK (bioMérieux, Marcy l'Etoile, France). *Rhodococcus equi* has been previously described (19). The cells were inoculated into 10 mL of brain heart infusion (BHI, Difco) or TSB for 24-48 hr at 37°C. The bacteria were determined their concentrations by optical density at 600 nm of 1.0 (approximately 10^9 CFU/mL) and applied to the ICA kits. All bacteria used were inactivated by heating at 100°C for 20 min.

Production of MAbs and rabbit polyclonal antibodies The flagella specific MAbs, 7A3, and 2B1, whose characteristics have already been reported were used for developing the ICA kit (11). Rabbit antiserum against *L. monocytogenes* 4b flagella was raised by immunization of 16-week-old New Zealand White rabbits. One hundred μg/mL of purified flagella from *L. monocytogenes* 4b (ATCC 19115) by Peel' method (20) in complete Freund's adjuvant (Sigma, St. Louis, MO, USA) was injected at different sites on the back subcutaneously for the first dose and incomplete adjuvant for 2 weekly doses. Blood was collected at each immunization and the presence of antibodies checked by ELISA. MAbs and rabbit antibody were purified by immuno-affinity chromatography method (11).

Table 1. Specificity analysis of the ICA kit

Species	Sero-types	Strains	ICA kits	
			The combination of MAb 7A3 and MAb 2B1	The combination of rabbit polyclonal antibodies and MAb 2B1
Listeria monocytogenes	1/2a	HPB 410[1)]	+	+
L. monocytogenes	1/2b	HPB 503	+	+
L. monocytogenes	1/2c	HPB 12	+	+
L. monocytogenes	3a	ATCC 19113[2)]	+	+
L. monocytogenes	4a	ATCC 19114	+	+
L. monocytogenes	4b	ATCC 19115	+	+
L. monocytogenes	4c	ATCC 19118	+	+
L. monocytogenes	4d	ATCC 19117	+	+
L. seeligeri		ATCC 35967	+	+
L. innocua		ATCC 33090	+	+
L. ivanovii		ATCC 19119	+	+
L. welshimeri		ATCC 35897	+	+
Streptococcus agalactiae		ATCC 13813	-	-
S. dysgalactiae		ATCC 27957	-	-
S. pyogenes		ATCC 21059	-	-
S. uberis		ATCC 25025	-	-
Escherichia coli O157:H7		ATCC 43894	-	-
Salmonella Typhimurium		ATCC 14021	-	-
S. enteritidis		Isolated (Chicken)	-	-
Staphylcoccus aureus 1		" (Bovine)	-	-
S. aureus 2		" (Bovine)	-	-
Rhodococcus equi		" (Horse)	-	-

[1)]HPB, Health Protection Branch, Health and Welfare Canada.
[2)]ATCC, American Type Culture Collection.

Preparation of colloidal gold The 20 nm gold colloids were produced according to Frens' method (21). For making 40 nm gold colloids, 100 mL of deionized water was added to 88 mL of 20 nm gold colloids while boiling, after which, 8 mL of 1% sodium citrate was added. The 0.8 mL of 1% tetrachloroauric acid ($HAuCl_4$, Sigma) mixed with 80 mL of deionized water, was dropped slowly at a rate of 1 mL/min onto the above suspension, and boiled for another 20 min. Gold colloids with optical density at 520 nm of 3.0 were used to conjugate antibodies. The size of gold colloids in the preparations were checked by transmission electron microscopy (TEM, 1200EXII; Jem, Amagasaki, Japan).

Antibody-gold conjugate Optimal concentrations of MAb 7A3 and rabbit polyclonal antibodies were added to 10 mL of gold particle suspension, which was then adjusted to pH 7.5, using 10% NaOH. The mixture was then allowed to stand for 30 min for conjugation. The pH of the suspension was adjusted again to 9.0 using 10% NaOH. The reaction was then blocked by the addition of 500 μL of 10% bovine serum albumin (BSA, Sigma), and incubated for 10 min. Conjugates were collected by centrifugation at 11,000×g for 1 hr at 20°C, and the supernatant was discarded. The conjugates were finally suspended in 100 mM phosphate buffer containing 6% sucrose, 2% Tween 20, 2% BSA, and 0.05% NaN_3.

Immunochromatographic test Goat anti-mouse IgG or goat anti-rabbit IgG (1.5 mg/mL in distilled water; Sigma) for the Control line (C) and MAb 2B1 (3 mg/mL) for the Test line (T) were applied 2 and 1.6 cm, respectively, from the bottom of the nitrocellulose membrane (pore size 12 μm, MDI, Ambala Cantt, India), and dried for 1 hr at room temperature (RT). Each MAb 7A3- and rabbit polyclonal antibodies-gold conjugate was soaked in a sheet of glass fiber pad (0.45 mm thick, MDI) for 1 hr at 37°C. This sheet was attached to the other end of the nitrocellulose membrane strip. The ICA kit was assembled with the plastic cassette (MDI), absorbing pad (0.8 mm thick, MDI), nitrocellulose membrane which was coated with goat-anti mouse or rabbit IgG and MAb 2B1 on the surface, conjugate pad which was adsorbed with 40 nm gold-labeled MAb 7A3 or rabbit polyclonal antibodies, and sample pad (0.35 mm thick, MDI).

This assay was carried out with pre-enriched culture initially applied to the sample pad, followed by 100 μL of dilution buffer (3% sucrose, 1% Tween 20, 1% BSA, 0.05% NaN_3). After 15 min, samples with red-colored bands at both the T and goat anti-mouse IgG or goat anti-rabbit IgG at the C were considered as positive for *Listeria* spp.

Detection of *L. monocyotogenes* 4b in foods Twenty five g of pork and 25 mL of milk were artificially contaminated with 9-10, 5-6, and 1-2 CFU/mL of *L. monocytogenes* 4b grown in TSB at 22°C. Colony numbers were determined by plating triplicate 1 mL of each dilution onto tryptic soy agar (TSA, Difco), followed by incubation at 37°C overnight. Samples were pipeted over the surface of 10 cm^2 pork with 1 mL of above concentrations of the cells and allowed to stand for 15 min at RT. The pork was sampled by surface swabbing using sterile moistened cotton bud in TSB. Each swab was placed in 10 mL of UVM broth (Merck) and enriched following the USDA method (22), but the second enrichment temperature was changed from 35 to 30°C for utilization of temperature-dependent flagella-specific MAbs. Pasteurization of milk was also performed following the FDA method (23). Enrichment was carried out on 27 samples, of which 3 and 6 were contaminated at each concentration to pork and milk, respectively. All samples were heated at 100°C for 20 min. Culture fluids were then analyzed using the ICA kit and compared with sandwich ELISA for sensitivity. The latter method has been described in a previous paper (11). Briefly, each well was coated with MAb 2B1 in 0.05 M carbonate buffer (pH 9.5) by incubating at 37°C for 1 hr and at 4°C for overnight. Samples were added into wells and incubated for 30 min at RT. Optimally diluted MAb 7A3 labeled horse radish peroxidase (HRP) was added into each well followed by adding 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; ABTS). Absorbance was checked at 405 nm using ELISA plate reader (SLT, Grödig, Austria).

Results and Discussion

Immunochromatographic assays have been used for the detection of various antigens such as hepatitis B virus (24), *Leishmania infantum* (25), and *Escherichia coli* O157:H7 (26). In this study, the ICA kit using flagella specific MAbs was successfully employed for rapid detection of *Listeria* spp. in food.

Colloidal gold, produced by the reduction of tetrachloric acid, was used for the development of this ICA kit. Gold colloids are commonly used for ICA techniques compared to latex because of their stability and long shelf life, as well as better mobility and sensitivity (27, 28). On TEM, individual particles of gold were round with expected size of nearly 40 nm (data not shown). Chaudhuri and Raychaudhuri (29) have previously reported 40 nm gold particle size to be optimum for the development of diagnostic assays because of visibility and steric hindrance. This was confirmed by the high sensitivity of the ICA kit using 40 nm gold in the present study.

The pilot designs using MAb 7A3 or rabbit polyclonal antibodies as capture antibody were shown to be not reliable; i.e., the nonspecific line appeared at negative sample (data not shown). Asturias *et al.* (30) reported that specific polyclonal antibodies would avoid the loss of detection produced by conformational change affecting epitopes. Therefore, MAb 7A3 and rabbit polyclonal antibodies conjugated with gold colloids were used as detector antibodies in this ICA kit. Moreover, optimal concentration of antibodies for gold conjugation was 160 μg/mL which was determined when the gold colloids were stabilized using salt titration.

The specificity of the ICA kits was proven with 13 strains of *Listeria* and 10 species of non-*Listeria*. Positive lines were observed for all tested *Listeria* on pure culture with both formats of the ICA kit (Table 1). The ICA kit also demonstrates that other bacteria were not detected by detector antibodies as shown in Table 1.

Since 2 MAbs, MAb 7A3, and MAb 2B1, and rabbit

Table 2. Determination of detection limit of the ICA kit for *L. monocytogenes* 4b (ATCC 19115)[1)]

L. monocytogenes (CFU/0.1 mL)	Combination of MAb 7A3 and MAb 2B1		Combination of rabbit polyclonal antibodies and MAb 2B1	
	22°C	30°C	22°C	30°C
10^7	+	+	+	±
10^6	+	+	+	±
10^5	+	±	±	±
10^4	-	-	-	-
10^3	-	-	-	-
Negative	-	-	-	-

[1)]+, positive reaction; ±, not clearly distinguishable; -, negative reaction.

Table 3. Detection of spiked *L. monocytogenes* 4b by the ICA kit and sandwich ELISA using MAb 7A3 and MAb 2B1

Type of product	Contamination levels of *L. monocytogenes* 4b (CFU/mL)							
	0		1-2		5-6		9-10	
	ICA	ELISA	ICA	ELISA	ICA	ELISA	ICA	ELISA
Pork	0/3[1)]	0/3	3/3	3/3	3/3	3/3	3/3	3/3
Milk	0/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6

[1)]No. of positive samples/ No. of tested samples.

polyclonal antibodies demonstrated positive reactivity for flagella whose expression is dependent on temperature, we compared sensitivity of 2 combinations in the ICA kit at culture temperatures of 22 and 30°C. *L. monocytogenes* 4b at the combination of gold-labeled MAb 7A3 and MAb 2B1 showed the detection limit to be 1×10^5 and 1×10^6 CFU/0.1 mL, respectively (Table 2). The combination of gold-labeled rabbit polyclonal antibodies and MAb 2B1 demonstrated the detection limit to be 1×10^6 CFU/0.1 mL in culture at 22°C while the ICA kit was weak signal at 30 culture (Table 2). Consequently, the evaluation of the ICA kit using a combination of gold-labeled rabbit polyclonal antibodies and MAb 2B1 was not pursued any further. In this case, rabbit polyclonal antibodies as detector did not appear to be suitable for detection of *Listeria* in enrichment samples of FDA procedure. This low reactivity with *Listeria* might be due to the competition amongst antibodies specific for different regions in detecting a small portion of flagella.

Therefore, the combination of gold-labeled MAb 7A3 and MAb 2B1 was utilized for developing the ICA kit. As described above, due to the fact that *Listeria* spp. are motile and express abundant flagella at 22-26°C (20), the detection limit of the ICA kit for the sample cultured at 22°C was 10 times higher than that associated with the sample at 30°C. In comparison, the detection limit of the sandwich ELISA was same at both 22 and 30°C (10^4 CFU/0.1 mL) (11). This result suggests that the sandwich ELISA is more sensitive than the ICA on pure culture. Watanabe *et al.* (31) also reported that the sensitivity of the ICA was 10 times lower than that of sandwich ELISA for the detection of dihydrostreptomycin in milk. However, the ICA kit we developed has an advantage that it takes only 15 min to detect Listerial contamination while ELISA requires at least 2 hr.

To make similar condition with natural contamination, 25 g of pork or mL portions of milk were artificially inoculated. Figure 1 shows the results of ICA kit, which detected low numbers of *L. monocytogenes* 4b artificially contaminated in food. All samples were notified to be positive only 5 min of observation. When we compared the ICA kit with the previously reported method of sandwich ELISA, *L. monocytogenes* 4b was detected in all 27 of pork and milk samples (Table 3). This result has same sensitivity as 1-10 CFU/25 g of commercially

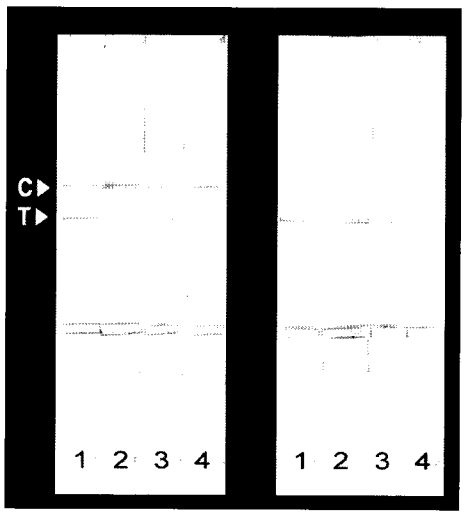

Fig. 1. The ICA kit in the detection of *L. monocytogenes* in pork (left) and milk (right) samples artificially contaminated with 9-10 (1), 5-6 (2), 1-2 (3), and 0 (4) CFU of *L. monocytogenes* 4b (ATCC 19115). The samples were enriched following USDA method, but second enrichment temperature 35°C was changed 30°C, and FDA method. C, control line; T, test line.

available rapid kit, Reveal for *Listeria* (Neogen, Lansing, MI, USA) that has been approved by Association of Official Analytical Chemists (AOAC).

The developed ICA kit shows sufficiently sensitive for the detection of *Listeria* spp. Although all strains may not be detected with equal sensitivity, 48 hr of culturing in enrichment medium was sufficient for the accumulation of detectable titers of *Listeria* strains. Further work is needed to verify practical application of this ICA kit with a set of Korean species from food sources.

In conclusion, the ICA kit presented can be used as more convenient and faster approach for early diagnosis of contamination with *Listeria*. Although only *L. monocytogenes* is restricted by laws concerning food safety, the screening of all *Listeria* spp. can indicate levels of hygiene being observed during meat processing and distribution.

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