Note

Detection of *Listeria monocytogenes* from Food Samples by PCR after IMS-Plating

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For rapid, accurate and sensitive detection of *Listeria monocytogenes* in food samples, colonies developed on the selective agar (Oxford agar) after immunomagnetic separation (IMS) were subjected to polymerase chain reaction (PCR) assay with the prf A1-2 primer pair. The proposed assay system was shown experimentally to be capable of specifically detecting the bacteria from food samples contaminated at more than 10^2 cfu/g. However, the enrichment culture after a short period of 16 h with the appropriate selective broth was needed before IMS-plating, because the bacterial contents in most actual food were as low as less than 10^2 cfu/g. However, even if the enrichment cultivation was employed before IMS, *L. monocytogenes* was detected within 3 days.

Key words : *Listeria monocytogenes* / IMS / PCR / Food

Listeria monocytogenes has been recognized as one of the important foodborne pathogens since the early 1980's when it was demonstrated that outbreaks of foodborne listeriosis were caused by the ingestion of contaminated food such as cheese, meat, ice cream and fish (Schlech, et al., 1983; Linnan, et al., 1988; McLauchlin, 1997). In Japan, listeriosis has occurred only sporadically, and a definite foodborne outbreak has been not reported so far (Okutani, et al., 2004). All 13 serovars of *L. monocytogenes* may cause listeriosis in people, but 95 % of isolates from humans have been found to be 1/2a, 1/2b, or 4b. *L. monocytogenes* is widely distributed in the environment. It is found in vegetation, in soils, animal and human feces, sewage, and water. Approximately 2–6% of healthy people are estimated to be asymptomatic fecal carriers of the organism (Rocourt, 1996), and wild and domestic animals may also be carriers. *L. monocytogenes* can be detected from a wide variety of food, including processed meats, fresh meat and

fish, raw vegetables, prepared retail foods and dairy products (Bell and Kyriakides, 2002). Although numbers are often very low in these foods, it is necessary to monitor the levels of *L. monocytogenes* in food because multiplication of the bacterium can potentially occur during refrigerated storage. Conventional methods require several days to detect *L. monocytogenes* from various food materials by enrichment culturing in selective broth, isolating the bacteria on selective agar and then differentiating them. At the same time, enzyme-linked immunosorbent assays and assays based on DNA probes or polymerase chain reaction (PCR) tests are readily available. Fluit et al. (1993) detected *L. monocytogenes* in cheese with an immunomagnetic separation (IMS) -polymerase chain reaction (PCR) assay. In this study, we attempted to rapidly, specifically and sensitively detect *L. monocytogenes* from naturally or artificially contaminated food samples by combining the IMS-plating technique and PCR assay.

The immunomagnetic beads (IMB) used in this study were Dynabeads® anti-*Listeria* (Dynal A.S., Oslo, Norway) which was coated with polyclonal

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antibodies against *Listeria* species and suspended at the level of 10^8 particles/ml of phosphate buffered saline (PBS) with 0.1% bovine serum albumin. IMS was performed according to the manufacturer's instructions. After 20 μ l of the IMB suspension was added to 10 ml of sample suspension and incubated for 20 min with slight agitation at room temperature, the bead-bacteria complexes were separated using a particle concentrator and washed twice with PBS, pH7.5, containing 0.005% (W/V) Tween 20 (PBS-T), and finally resuspended in 100 μ l of PBS-T. For isolation of typical *L. monocytogenes*, a 50 μ l aliquot of the suspension was streaked onto the selective agar plates, and incubated overnight at 37°C. One typical colony which developed on the selective plate was suspended in 100 μ l of 10 mM Tris-HCl containing 1mM EDTA, pH7.5 (TE buffer), and the suspension was heated for 5 min at 95 °C. After centrifugation at 14,000 $\times$ g for 60s, the straight supernatant was subjected to PCR assay.

Four primer sets for detection of *L. monocytogenes* were synthesized on the basis of the DNA sequences of the *L. monocytogenes* *hly*, *iap* or *prf* genes, as shown in Table 1. PCR was carried out as reported previously (Ueda, et al., 2005). Briefly, a 10 μ l aliquot of the bacterial DNA extract was added to 90 μ l of a reaction solution consisting of 10 μ l of a 10 fold reaction buffer, 8 μ l of 2.5 mM dNTP mixture, 100 p mol of each primer, 2.5 U of *Taq* DNA polymerase (Takara) and double-distilled water. Then, the sample was overlaid with 70 μ l of sterile liquid paraffin. The PCR was run for 35 cycles with denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. For visual detection, a 10 μ l aliquot of the PCR product was subjected to electrophoresis in a 2 % (W/V) agarose gel (Sigma) with Tris-acetate EDTA buffer. The gel was stained with ethidium bromide and the DNA bands were visualized with UV light.

For determining the effectiveness of several selective agar plates, the bead-bacteria complexes after IMS from the *L. monocytogenes* 4b suspension at

10^2 cfu/ml were plated onto several selective agar plates including Oxford agar (Oxoid, Basingstoke, England), PALCAM agar (Oxoid), BCM agar (Brunschwig Chemie, Amsterdam, Netherlands), UVM agar (Oxoid) and/or FDA formulation agar (Oxoid). Almost the same numbers of 4b colonies were recovered on all agar media used. Thus, as the plating agar medium after IMS, Oxford agar, usually utilized for isolation of *L. monocytogenes* (Iida et al., 1998; Bell and Kyriakides, 2002), was used in the following experiments.

The specificity of the IMB to *Listeria* species was examined with 10^2 – 10^3 cfu/ml of different bacteria suspended in TE-buffer. The *Listeria* strains used included 11 strains of *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c and 1/4b, and *L. innocua*. Also, other gram negative and positive bacteria were used as references. Although all *Listeria* spp. including *L. monocytogenes* and *L. innocua* developed typical colonies on the Oxford agar plates after the IMS, other bacterial species did not (Table 2). Thus, the IMB was shown to capture only *Listeria*. For the identification of *L. monocytogenes*, the *Listeria* colonies on the plates were subjected to the PCR assay. Four primer pairs were first selected for the detection of *L. monocytogenes* by means of the PCR assay. In this trial, the assays were performed with the colonies of various bacterial species including *L. monocytogenes* developed on Brain Heart Infusion agar plates (Difco). The *prfA* 1-2 pair reacted exclusively with *L. monocytogenes*, but 3 other pairs cross-reacted minutely or definitely with several species (Table 3). The *prf A* pair was confirmed to react only with *L. monocytogenes* and not with other *Listeria* spp. including *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. grayi* and *L. seeligeri*. Even if the pair did not always react with all *L. monocytogenes* strains, the PCR with the pair was thought to be very effective for detection of the bacteria. Thus, the following experiments were performed using Oxford agar plate as the selective medium for IMS-plating and the *prfA* primer pair for the PCR assay.

TABLE 1. Oligonucleotide primer pairs used for the detection of *L. monocytogenes*.

No.	Primer set	Target	Sequence (5'-3')	bp	Reference
1	LM C LM D	<i>hly A</i>	TTA CGA ATT AAA AAG GAG CG TTA AAT CAG CAG GGG TCT TT	161	Mengaud et al. (1989)
2	Mono A Mono B	<i>iap</i>	CAA ACT GCT AAC ACA GCT ACT GCA CTT GAA TTG CTG TTA TTG	371	Bubert et al. (1992)
3	<i>hly</i> 1 <i>hly</i> -2	<i>hly</i>	CGG AGG TTC CGC AAA AGA TG CCT CCA GAG TGA TCG ATG TT	234	Bubert et al. (1997)
4	<i>prfA</i> 1 <i>prfA</i> 2	<i>prf A</i>	ACC AAT GGG ATC CAC AAG A CAG CTG AGC TAT GTG CGA T	467	Bubert et al. (1997)

TABLE 2. *Listeria* strains and other bacterial species used for confirming the specificity of the immunomagnetic beads for the capture of *L. monocytogenes*.

Species	Serovar/Toxicogenicity/Origin	Capture ^a by the beads
<i>Listeria</i> spp.		
<i>L. monocytogenes</i>	1/2a 880278	+
<i>L. monocytogenes</i>	1/2a 880575	+
<i>L. monocytogenes</i>	1/2b 900638	+
<i>L. monocytogenes</i>	1/2b 900418	+
<i>L. monocytogenes</i>	1/2c 880282	+
<i>L. monocytogenes</i>	1/2c 900616	+
<i>L. monocytogenes</i>	4b 900208	+
<i>L. monocytogenes</i>	4b 900226	+
<i>L. monocytogenes</i>	4b 110571	+
<i>L. monocytogenes</i>	4b CH190	+
<i>L. innocua</i>	SC11	+
Gram negative bacteria		
<i>Escherichia coli</i>	10 strains containing type cultures	—
<i>Klebsiella aerogenes</i>	isolated from food	—
<i>Citrobacter freundii</i>	isolated from food	—
<i>Salmonella Enteritidis</i>	isolated from egg	—
<i>Salmonella Typhimurium</i>	isolated from human faeces	—
<i>Vibrio parahaemolyticus</i>	isolated from fish	—
<i>Aeromonas hydrophila</i>	isolated from water	—
Gram positive bacteria		
<i>Staphylococcus aureus</i>	7 strains containing enterotoxigenic producers	—
<i>Bacillus cereus</i>	3 strains containing toxigenic strains	—
<i>Bacillus subtilis</i>	PCI219	—
<i>Bacillus licheniformis</i>	ATCC14580	—
<i>Bacillus pumilus</i>	isolate from air	—
<i>Clostridium botulinum</i>	E type toxin producer	—
<i>Clostridium perfringens</i>	Hobbs type 13	—

a +, Positive result with the immunomagnetic beads.

—, Negative result with immunomagnetic beads.

The sensitivity of the assay system was evaluated on food samples and human materials contaminated artificially with *L. monocytogenes*. Food samples used were vegetables and vegetable salads, fruits and fruit juices, milk and dairy products, eggs and egg dishes, meat (pork and beef) and dishes prepared from such meat, and chicken purchased from retail shops. Human materials were feces and blood obtained from a healthy person. A 10% suspension of each sample was prepared with sterile saline solution, except in the case of milk and blood, and these fluid samples were inoculated with *L. monocytogenes* 4b at the level of 10^1 or 10^2 cfu/ml. The proposed method was shown to be capable of isolating and identifying *L. monocytogenes* from all samples, if the bacteria were present at more than 10^1 cfu/ml of the suspension (Table 4). That is to say, *L.*

monocytogenes could to be detected by the assay system experimentally if this bacteria were present at more than 10^2 cfu/g. However, it is normal to find low levels of *L. monocytogenes* at less than 10^2 cfu/g in many foods including milk, fish, meat, fruits, and vegetables (Blackburn and McClure, 2002). Therefore, it is predicted that *L. monocytogenes* cannot be detected from the samples contaminated at very low levels of less than 10^2 cfu/g by the proposed method.

Some retail meat samples from which *L. monocytogenes* was isolated by the conventional microbiological method were used for the detection of the bacteria. The bacterial contents of all meat samples were estimated to be less than 10^2 cfu/g, because the bacterial colonies did not appear on the selective agar streaked directly with 0.1ml of the

TABLE 3. Specificity of some primer sets for *Listeria monocytogenes* and other bacterial species.

Species serovar/strain No.	Detection ^a with primer pair:			
	LM C,D	Mono A-B	hly 1-2	prfA 1-2
<i>L. monocytogenes</i>				
1/2a strain 880278	+	+	+	+
1/2a strain 880575	+	+	+	+
1/2b strain 900638	+	+	+	+
1/2b strain 900418	+	+	+	+
1/2c strain 880282	+	+	+	+
1/2c strain 900616	+	+	+	+
4b strain 900208	+	+	+	+
4b strain 900226	+	+	+	+
4b strain 110571	+	+	+	+
4b strain CH190	+	+	+	+
<i>L. innocua</i> SC11	—	—	—	—
<i>Escherichia coli</i> (10 strains)	—	— (2), ± (7), + (1)	— (8), ± (1), + (1)	—
<i>Klebsiella aerogenes</i>	—	±	—	—
<i>Citrobacter freundii</i>	—	—	—	—
<i>Salmonella</i> Enteritidis	—	—	—	—
<i>Salmonella</i> Typhimurium	±	±	—	—
<i>Vibrio parahaemolyticus</i>	—	—	—	—
<i>Yersinia enterocolitica</i>	±	—	—	—
<i>Aeromonas hydrophila</i>	—	—	+	—
<i>Staphylococcus aureus</i> (7 strains)	— (6), ± (1)	— (2), + (5)	—	—
<i>Bacillus cereus</i> (3 strains)	—	— (2), ± (1)	—	—
<i>Bacillus subtilis</i>	±	—	—	—
<i>Bacillus licheniformis</i>	±	±	—	—
<i>Bacillus pumilus</i>	±	±	—	—
<i>Clostridium botulinum</i>	—	+	—	—
<i>Clostridium perfringens</i>	±	±	±	—

a+, positive; —, negative; ±, indefinite.

TABLE 4. Recovery of *L. monocytogenes* inoculated into the suspension of various food samples and human materials by the PCR assay after IMS-plating.

Test sample	Inoculation level ^a at:			Test sample	Inoculation level ^a at:		
	0	10 ¹	10 ²		0	10 ¹	10 ²
Lettuce	— ^b	+	+	Spinach saute	—	+	+
Parsley	—	+	+	Spinach soup	—	+	+
Cabbage	—	+	+	Vegetable salad	—	+	+
Sprouts	—	+	+	Pumpkin salad	—	+	+
Leek	—	+	+	Okara salad	—	+	+
Welsh onion	—	+	+	Potato salad	—	+	+
Tufed stone leek	—	+	+	Seafood salad	—	+	+
Carrot	—	+	+	Mashed potatoes	—	+	+
Tomato	—	+	+	Raw beef	—	+	+
Watermelon	—	+	+	Raw pork	—	+	+
Melon	—	+	+	Raw chicken	—	+	+
Apple juice	—	+	+	Raw swine liver	—	+	+
Orange juice	—	+	+	Hamburger	—	+	+
Liquid whole egg	—	+	+	Roasted beef	—	+	+
Thick omelet	—	+	+	Swab from surface	—	+	+
Milk	—	+	+				
Yoghurt	—	+	+	Human blood	—	+	+
Cheese	—	+	+	Human faeces	—	+	+

a *L. monocytogenes* was inoculated into 10% suspension of materials other than juice and milk at the levels of 10¹ and 10² cfu/ml.

b +, detectable by the proposed method; —, not detectable by the method.

sample suspension. *L. monocytogenes* was isolated only by the enrichment culture (Table 5). Although these samples were directly subjected to the PCR assay after IMS-plating, *L. monocytogenes* was detected from only 2 among 28 samples (Table 5). Therefore, after 1 ml of the 10% sample suspension was incubated at 30°C for 16 h in 9 ml of UVM broth (Oxoid) for the enrichment of *Listeria*, the cultures were subjected to IMS-plating. Consequently, *L. monocytogenes* was captured and identified from the meat samples by PCR with the *prfA* primer pair after

IMS plating (Table 5). Thus, when the bacteria are present in the food sample at a very low level of less than 10^2 cfu/g, it is necessary to carry out the enrichment cultivation with an appropriate selective medium before IMS to detect the bacteria more certainly. The *L. monocytogenes* contamination ratio of most food is estimated to be between 0.5 and 30%, and the bacterial content is reported to be as low as less than 10^2 cfu/g (Iida et al., 1998; Blachburn and McClure, 2002). Therefore, the enrichment culture before the IMS may be important to the detection of bacteria

TABLE 5. Detection of *L. monocytogenes* by the conventional method and PCR after IMS from *L. monocytogenes*-positive meat samples.

Sample No.	Material	<i>L. monocytogenes</i> counts (cfu/g) on Oxford and/or BCM agar ¹⁾	Detection of <i>L. monocytogenes</i> from enrichment and non-enrichment samples by PCR after IMS-plating.			
			Pre-enrichment ²⁾		Post-enrichment ³⁾	
			Oxford agar	BCM agar	Oxford agar	BCM agar
A-135	ground beef	100>	ND	ND	+	+
A-144	ground pork	100>	ND	ND	+	+
A-145	ground pork	100>	ND	ND	+	+
A-146	ground pork	100>	ND	ND	+	+
A-149	ground pork	100>	ND	ND	+	+
A-150	ground pork	100>	ND	ND	+	+
B-115	ground pork	100>	ND	ND	+	+
B-116	ground pork	100>	ND	ND	+	+
B-117	ground pork	100>	ND	ND	+	+
B-119	chicken	100>	ND	ND	+	+
B-125	ground pork	100>	ND	ND	+	+
B-128	ground pork	100>	ND	ND	+	+
B-132	chicken	100>	ND	ND	+	+
B-133	chicken	100>	ND	ND	+	+
B-200	ground pork	100>	ND	ND	+	+
B-208	ground pork	100>	ND	ND	+	+
B-215	chicken	100>	ND	ND	+	+
B-216	chicken	100>	+	ND	+	+
B-217	chicken	100>	+	ND	+	+
B-219	chicken	100>	ND	ND	+	+
B-222	chicken	100>	ND	ND	+	+
E-112	lean pork	100>	ND	ND	+	+
E-172	lean beef	100>	ND	ND	+	+
E-176	lean pork	100>	ND	ND	+	+
E-183	lean pork	100>	ND	ND	+	+
E-187	chicken	100>	ND	ND	+	+
E-188	chicken	100>	ND	ND	+	+
E-191	chicken	100>	ND	ND	+	+
Detection of <i>L. monocytogenes</i> by PCR after IMS-plating (positives/total)			2/28	0/28	28/28	28/28

1) A 0.1 ml aliquot of 10% material suspension was streaked onto Oxford or BCM agar. After incubation for 24 h at 37°C, the colonies on the plate were enumerated.

2) After 1 ml of 10% material suspension was mixed with 20 μ l of immunomagnetic beads for 30 min at room temperature, the beads which captured *Listeria* were suspended in 100 μ l of PBS. A 50 μ l aliquot of the suspension was streaked onto Oxford or BCM agar.

3) After 9 ml of FDA LM broth inoculated with 10% material suspension was incubated for 18 h at 37°C, the culture was mixed with 20 μ l of immunomagnetic beads for 30 min at room temperature. The beads which captured *Listeria* were suspended in 100 μ l of PBS. A 50 μ l aliquot of the suspension was streaked onto Oxford or BCM agar.

from samples contaminated at very low levels.

Even if the enrichment procedure is introduced prior to IMS, the proposed assay system may detect *L. monocytogenes* precisely and rapidly as compared with the conventional method and simultaneously identify the serovar of colonies on the selective agar plate within 3 days.

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