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Detection of *Listeria monocytogenes* in Cheese with the Magnetic Immuno-Polymerase Chain Reaction Assay

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A new detection system, the magnetic immuno-polymerase chain reaction (PCR) assay (MIPA) has been developed to detect *Listeria monocytogenes* in food. This method separates *Listeria* cells from PCR-inhibitory factors present in enrichment broths containing food samples by using magnetic beads coated with specific monoclonal antibodies (MAbs). The separated bacteria were lysed, and the supernatant containing the bacterial DNA was subjected to the PCR. Detection of *L. monocytogenes* in three naturally contaminated cheese samples with two different MAbs and PCR primers specific for the gene encoding the delayed-hypersensitivity factor showed that with MAb 55 all three samples were positive whereas with MAb A two samples were positive. A further improvement of the method was obtained by using a PCR step based on the listeriolysin O gene. A MIPA employing MAb 55 and the listeriolysin O gene primer set detected *L. monocytogenes* after 24 h of culture in Listeria Enrichment Broth samples from Port Salut artificially contaminated with 40 CFU/25 g. We could detect 1 CFU of *L. monocytogenes* per g of cheese after a second enrichment for 24 h in Fraser broth. The analysis time including both enrichments is approximately 55 h.

Because outbreaks of listeriosis are known to be caused by food contaminated with *Listeria monocytogenes* (10), rapid and reliable detection methods for *Listeria* spp. and especially *L. monocytogenes* are important. *L. monocytogenes* may be present in small numbers in food along with large numbers of bacteria that are members of the competitive flora (for a review, see reference 7). The classical method to detect *L. monocytogenes* in food involves selective enrichments and subsequent culturing on a selective medium to obtain colonies, followed by biochemical and/or serological confirmation and differentiation of *Listeria* species (7, 13, 16). These methods are laborious and usually take several days to produce a result.

Immunologic methods, such as those based on the enzyme-linked immunosorbent assay (ELISA) (11, 15), offer faster and specific alternatives. Although the assays themselves last only 2.5 h, time-consuming enrichment and plating steps are still required because of the detection limit of 10^5 to 10^7 CFU/ml in these assays. Another disadvantage of these immunologic assays which detect bacterial cells is the absence of discrimination between pathogenic *L. monocytogenes* and nonpathogenic *Listeria innocua*. The major differences between these species are the excretion of some proteins, including listeriolysin O and the delayed-hypersensitivity factor (DTH), by *L. monocytogenes*. Therefore, additional tests to differentiate these species must still be performed to confirm the presence of *L. monocytogenes*.

Recently, the use of specific DNA probes (3, 6, 18) and the polymerase chain reaction (PCR) (1, 2, 24) to detect *L. monocytogenes* has been described. However, inhibition of the *Taq* polymerase by components of food, enrichment media, or large amounts of DNA may lead to false-negative results. An elegant method in which *L. monocytogenes* was separated from enrichment broths by magnetic beads coated with specific antibodies was described (22). *L. monocytogenes* was detected by culture and classical identification techniques. Recently, we described a magnetic immuno-PCR Assay (MIPA), in which we combined immunomagnetic separation with the PCR, for the detection of *Salmonella* species (25). In the present study, the MIPA was used to detect *L. monocytogenes* in food. The MIPA combines magnetic separation of *Listeria* cells by magnetic particles coated with *Listeria*-specific monoclonal antibodies (MAbs) followed by amplification of an *L. monocytogenes*-specific DNA sequence by PCR.

MATERIALS AND METHODS

Bacterial strains. Seventy-nine *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, 6a, and 7; 19 *L. innocua* strains belonging to serotypes 4c, 6a, 6b, and 7; 4 *Listeria ivanovii* strains belonging to serotype 5; 4 *Listeria seeligeri* strains belonging to serotypes 1/2b, and 4ab; 5 strains of *Listeria welshimeri* belonging to serotypes 6a, and 6b; and 3 strains of *Listeria grayi* were obtained from the National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Two *Streptococcus pyogenes* and two *Streptococcus pneumoniae* isolates were obtained from the University Hospital Utrecht. One strain each of *L. welshimeri*, *L. seeligeri*, *L. innocua* serotype 6a, and *L. monocytogenes* serotypes 1/2a and 4b, as well as two strains of *L. grayi* and seven *Streptococcus* spp. isolated from food products, were obtained from Unilever Research Laboratory, Vlaardingen, The Netherlands. One strain each of *L. ivanovii* serotype 5 and *L. innocua* serotype 6b was obtained from the American Type Culture Collection (ATCC 19119) and the National Collection of Type Cultures (NCTC 11289), respectively.

Bacteria were cultured on blood-agar plates and after collection, suspended in phosphate-buffered saline (PBS).

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Bacterial concentration was determined by comparing the optical density at 543 nm with calibrated readings.

Generation of MAb 55. Female BALB/c mice, 6 to 8 weeks old, were immunized for several weeks with antibiotic-treated bacteria. Spleen cells obtained from immune mice were collected and fused with the SP2/0 myeloma cell line. Supernatants from wells containing growing hybridomas were screened by using the magnetic immunoluminescence assay as the detection system. The magnetic immunoluminescence assay was performed as described previously (23). Bioluminescence reagents were from LUMAC BV, Landgraaf, The Netherlands.

Generation of MAb A. *L. monocytogenes* ATCC 19115 was grown in buffered peptone medium plus 10% sucrose for 3 days at 23°C without aeration to promote good production of flagella. The cells thus obtained were homogenized to remove flagella, and the suspension was centrifuged at 4,000 × *g* for 50 min to remove debris. The supernatant was then centrifuged at 20,000 × *g* for 1 h to sediment flagella. The pellet was resuspended in saline for use. BALB/c mice, 8 to 12 weeks of age, were immunized intraperitoneally with 20 to 50 μg of flagella emulsified with an equal volume of complete Freund's adjuvant. Booster injections with incomplete Freund's adjuvant were given four times over several weeks. Mouse sera were tested for anti-*L. monocytogenes* antibodies by ELISA. The mouse with the highest titers was given three further daily injections of immunogen in saline intraperitoneally and intravenously prior to sacrifice. Fusions were performed as described by Gani et al. (9). Antibodies were selected by using ELISA to screen for reactivity with *Listeria* species for nonreactivity with *L. grayi*. MAb A, belonging to the immunoglobulin G class and directed against the flagellin proteins of *L. monocytogenes* and *L. innocua*, was obtained.

Isolation of *L. monocytogenes* from cheese. Cheese (25 g) was homogenized in 225 ml of Listeria Enrichment Broth (LEB; Oxoid, Basingstoke, United Kingdom) (14) and incubated for 24 h at 30°C (enrichment I). From enrichment I, 0.1 ml was transferred into 10 ml of Fraser broth (Oxoid) (8) and incubated for 24 h at 30°C (enrichment II).

After 24 h of incubation in LEB and in Fraser broth, the immunologic capture of *Listeria* cells was carried out with 100 μl of each sample and used for PCR amplification. The *Listeria* viable-cell counts in the enrichment broths were determined by plating on Oxford agar (Oxoid) (4). Two colonies from each plate (six colonies per sample) were Gram stained and assayed for hemolysis and for catalase production. Biochemical characterization was performed by using the Micro-ID test strip (Organon Teknika, Turnhout, Belgium).

Immunomagnetic separation. Magnisort M magnetic chromium dioxide particles coated with goat immunoglobulins specific for murine immunoglobulins G and M were purchased from E. I. DuPont & Nemours, Wilmington, Del.

A 75-μl volume of diluted ascitic fluid in PBS plus 1% gelatin (gPBS) or 75 μl of hybridoma culture supernatant was incubated with 50 μl of Magnisort M particles diluted 1:25 in gPBS in a 96-well microtiter plate for 30 min at room temperature with moderate shaking. The magnetic particles were recovered by magnetic force, and the supernatant was discarded. The particles were washed once with 200 μl of gPBS and resuspended in 100 μl of gPBS. Then 100 μl of a sample to be tested was added to the particles and incubated for 30 min at room temperature. Samples to be tested consisted of either suspensions of pure cultures in 0.85% saline plus 0.1% (wt/vol) peptone or enrichment broths from cheese samples. After incubation, the particles were recovered by magnetic force and washed three times with 200 μl of 0.2 M Tris-citric acid–0.1 M NaCl–0.05% (wt/vol) Tween 20 (pH 7.4).

For PCR with DTH-18 gene-specific primers, the beads were resuspended in 30 μl of distilled water. This suspension was incubated for 5 min at 95°C. For PCR with listeriolysin O-specific primers, the beads were resuspended in 20 μl of 0.05% sodium docecyl sulfate and heated for 5 min at 100°C, and 2.5 μl of 20% Nonidet P-40 (Sigma, St. Louis, Mo.) was added. After a brief centrifugation, 10 μl of the supernatant was subjected to the PCR.

PCR. Primer set A, specific for the *L. monocytogenes* DTH-18 gene described by Wernars et al. (24), was used in the PCR. The PCR mixture (100 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 100 μM (each) deoxynucleoside triphosphate, 0.5 μM (each) primer, and 1.25 U of *Taq* polymerase. Amplification was carried out for 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C in a thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Additional primers for PCR were selected in areas of the listeriolysin O gene which show little homology with streptolysin and pneumolysin sequences. Primer 1 (5′-CTAATCAAGACAATAAAATC) and primer 2 (5′-GTTAGTTCTACATCACCTGA) were located between bp 536 and 555 and between bp 1037 and 1056, respectively, of the published sequence of the listeriolysin O gene (17). Thirty-five amplification cycles were performed, each consisting of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min. The total reaction volume was 50 μl.

Products formed in the PCR were analyzed on a 1% agarose gel with ethidium bromide staining. In addition, PCR products were spotted on Z-probe filters (Bio-Rad) and hybridized with a digoxigenin (Boehringer, Mannheim, Germany)-labeled DNA probe encoding the DTH-18 gene of *L. monocytogenes* described by Notermans et al. (18). The listeriolysin O probe was labeled by using PCR (20). The conditions were similar to those described above, except for a reaction volume of 100 μl which contained 90 μM dTTP and 10 μM digoxigenin-dUTP.

RESULTS

Generation of MAbs. One MAb (MAb 55; immunoglobulin G2a) that showed reactivity toward 103 of 112 *Listeria* spp. tested was obtained. The nine nonreactive *Listeria* spp. consisted of one strain each of *L. monocytogenes* serotypes 4d and 4e, two strains of *L. innocua* serotype 6b, three strains of *L. seeligeri* serotype 4ab, one strain of *L. welshimeri* serotype 6b, and one strain of *L. ivanovii* serotype 5. The MAb does not react with *Escherichia coli* or other members of the family *Enterobacteriaceae*. No difference in reactivity was observed when *Listeria* cells were grown at either 20 or 37°C, indicating that this MAb is not reactive with flagellin.

In addition, a MAb directed against *Listeria* flagellin proteins (MAb A) was obtained. Reaction of MAb A with *Listeria* cells is temperature dependent. MAb A does not react with *L. grayi* and a range of gram-positive and gram-negative organisms.

PCR amplification. The PCR with primer set A directed against the DTH-18 gene of *L. monocytogenes* was evaluated with all strains from Unilever, the *L. ivanovii* strain from the American Type Culture Collection, the *L. innocua* serotype 6b from the National Collection of Type Cultures,

TABLE 1. Detection of *L. monocytogenes* by the MIPA after enrichment II of cheese samples

Sample	Detection by:			
	Electrophoresis		Hybridization	
	MAb A	MAb 55	MAb A	MAb 55
Curd cheese 1	−	−	−	−
Curd cheese 2[a]	+	+	+	+
Port Salut 1	−	−	+	+
Port Salut 2	−	−	−	+
Port Salut 3	−	−	+	+
Brie	−	−	−	−

[a] Curd cheese 2 was an artificially contaminated sample of curd cheese 1.

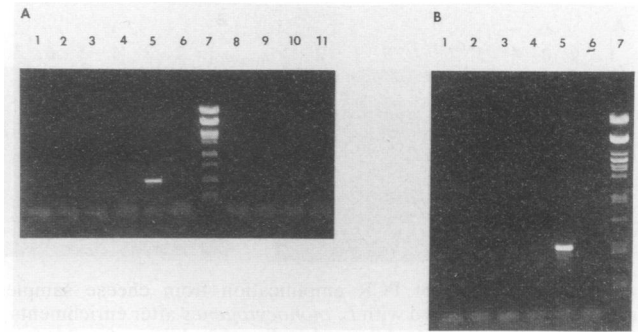

FIG. 1. Products of PCR amplification from cheese samples artificially contaminated with *L. monocytogenes* after enrichment I. (A) Samples of 1 μl (lanes 1 to 4) and 2 μl (lanes 8 to 11) from enrichment I. (B) Results of the PCR after immunomagnetic separation of 100-μl *Listeria* samples (lanes 1 to 4). Before enrichment, 25-g cheese samples were artificially contaminated with either 0 (panel A, lanes 1 and 8; panel B, lane 1), 0.4 (panel A, lanes 2 and 9; panel B, lane 2), 4 (panel A, lanes 3 and 10; panel B, lane 3), or 40 (panel A, lanes 4 and 11; panel B, lane 4) CFU of *L. monocytogenes* per g of cheese. Lanes 6 contain negative controls without *L. monocytogenes*. The positive controls (lanes 5) consisted of 0.05 μg of purified *L. monocytogenes* DNA. A phage lambda DNA *Pst*I digest was used as the marker (lanes 7).

and one each of the *L. monocytogenes* serotypes 1/2b, 3a, 3b, 3c, and 4a from the National Institute of Public Health and Environmental Hygiene. All *L. monocytogenes* serotypes tested except *L. monocytogenes* serotype 4a showed the expected product of 300 bp. In addition, the PCR product hybridized with the DNA probe. *L. seeligeri*, *L. ivanovii*, and *L. grayi* strains showed some products larger than 300 bp, which did not hybridize with the probe. Also, one of the *L. innocua* strains showed a product larger than 300 bp, which did not hybridize with the digoxigenin-labeled listeriolysin O-specific probe.

The sensitivity of the PCR amplification was tested with serial dilutions of an *L. monocytogenes* serotype 1/2b strain. The detection limit with hybridization was approximately 50 CFU. The detection limit with agarose gel electrophoresis was approximately 100-fold less sensitive.

Evaluation of the MIPA by using enrichment broths from cheese. The performance of the MIPA for the detection of *L. monocytogenes* was tested with artificially and naturally contaminated cheese samples. The negative control was a sample of curd cheese which was shown not to be contaminated with *Listeria* spp. The positive control was another part of this curd cheese, which was inoculated with 6 CFU of *L. monocytogenes* serotype 1/2b per 25 g. The three samples of Port Salut were shown to be contaminated with *L. monocytogenes* by conventional detection techniques. The Brie sample was not contaminated with *L. monocytogenes*. Results of the MIPA with primers specific for the DTH-18 gene show that *L. monocytogenes* was detected in the positive control only when agarose gel electrophoresis was used as the detection system. Besides the positive control, Port Salut samples 1, 2, and 3 were positive when MAb 55 and hybridization were used as the detection system (Table 1). However, the MIPA with MAb A failed to detect *L. monocytogenes* in sample 2. Less than 10% of the *Listeria* spp. present in the samples were *L. monocytogenes* as determined by conventional culture.

PCR based on the listeriolysin O gene. Because the primer set described by Wernars et al. (24) failed to detect serotype 4a, a PCR based on primers for the *L. monocytogenes*-specific listeriolysin O gene was developed. Primers for this improved PCR were selected in areas of the listeriolysin O gene which show little homology with the genes encoding the related streptolysin and pneumolysin, which are produced by *S. pyogenes* and *S. pneumoniae*, respectively (17). The primers were tested in the PCR against 79 *L. monocytogenes* strains, 19 *L. innocua* strains, 4 *L. ivanovii* strains, 4 *L. seeligeri* strains, 4 *L. welshimeri* strains, 3 *L. grayi* strains, 2 *S. pyogenes* strains, and 2 *S. pneumoniae* strains. These strains included all known *Listeria* serotypes. We found that 78 of the *L. monocytogenes* strains showed the 521-bp PCR product after agarose gel electrophoresis. No detectable product was formed with any of the other *Listeria* strains, *S. pneumoniae*, or *S. pyogenes* clinical isolates. The single *L. monocytogenes* strain which did not produce a product in the PCR was also negative in a β-hemolysin assay. The identity of the PCR product was confirmed by restriction enzyme analysis with *Hin*dIII, *Dra*I, and *Sca*I.

The listeriolysin-based PCR followed by agarose gel electrophoresis with ethidium bromide staining resulted in the detection of amplified product from 500 cells as determined by both cell counting and culturing on blood agar. When a nonradioactive digoxigenin-labeled DNA probe identical to the amplified sequence was used to detect amplified product on a spot blot, five cells could be reliably detected. No signal was detected in the absence of *L. monocytogenes* cells.

Detection of *L. monocytogenes* in cheese by the listeriolysin O-based MIPA. The performance of a MIPA involving a listeriolysin O-based PCR was evaluated with MAb 55 on artificially contaminated cheese samples. Samples (25 g) of Port Salut were contaminated with 0, 0.4, 4, or 40 CFU of *L. monocytogenes* serotype 1/2a per g of cheese. After enrichment I, a MIPA was performed with the PCR based on listeriolysin O-specific primers. The results in Fig. 1 show that the expected PCR product could be detected in the sample contaminated with 40 CFU/g of cheese. No PCR product could be detected in either 1-, 2-, 5-, or 10-μl samples of enrichment I of any of the cheese samples. No product was detected when no *L. monocytogenes* was added to the cheese samples.

When 25 g of Port Salut was contaminated with 0, 1, 10, or 100 CFU of *L. monocytogenes* serotype 1/2a per g of cheese and a MIPA with listeriolysin O-specific primers was performed after both enrichment I and enrichment II, samples contaminated with 1, 10, or 100 CFU/g of cheese showed the expected PCR product. No product was detected when no *L. monocytogenes* was added to the cheese samples (Fig. 2). Amplifications performed directly on 1-μl samples from enrichment II showed a product of the expected size when

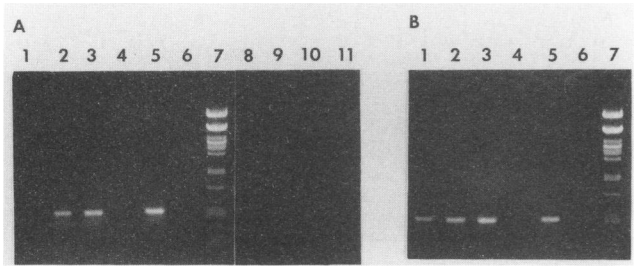

FIG. 2. Products of PCR amplification from cheese samples artificially contaminated with *L. monocytogenes* after enrichments I and II. (A) Samples of 1 μl (lanes 1 to 4) and 2 μl (lanes 8 to 11) from enrichment II. (B) Results of the PCR after immunomagnetic separation of 100-μl *Listeria* samples (lanes 1 to 4). Before enrichment, 25-g cheese samples were artificially contaminated with either 0 (panel A, lanes 4 and 11; panel B, lane 4), 1 (panel A, lanes 1 and 10; panel B, lane 1), 10 (panel A, lanes 2 and 9; panel B, lane 2), or 100 (panel A, lanes 3 and 8; panel B, lane 3) CFU of *L. monocytogenes* per g of cheese. Lanes 6 contain negative controls without *L. monocytogenes*. The positive controls (lanes 5) consisted of 0.05 μg of purified *L. monocytogenes* DNA. A phage lambda DNA *Pst*I digest was used as the marker (lanes 7).

the cheese was contaminated with 10 or 100 CFU of *L. monocytogenes* per g (Fig. 2). No product was observed when the cheese was contaminated with 1 CFU/g. A barely detectable product of the expected size was obtained for the 2-μl sample obtained from cheese contaminated with 10 CFU/g. The other samples did not show the expected amplification product. Because hybridization is both cumbersome and time-consuming, detection was limited to analysis on agarose gels with ethidium bromide staining.

The effect of immunomagnetic separation on template levels and PCR inhibitors. The use of immunomagnetic separation improved the detection of *L. monocytogenes* in cheese either by removing inhibitory substances or by increasing the number of template molecules available for PCR. Depending on the inoculum and batch of cheese, 10^5 to 10^8 CFU of *L. monocytogenes* per ml was obtained in enrichment II. The recovery of *L. monocytogenes* from enrichment II by immunomagnetic separation varied between 5 and 15%. The effect of inhibitors was studied by adding serial 10-fold dilutions of *L. monocytogenes* in either physiologic salt solution, 1 μl of enrichment II, or 2 μl of enrichment II to the PCR. Results showed that the presence of a 10^5-fold excess of template in enrichment II compared with physiologic salt solution did not give a detectable signal in the PCR.

DISCUSSION

The PCR described by Wernars et al. (24), which amplifies a 300-bp sequence of the DTH-18 gene of *L. monocytogenes*, does not detect *L. monocytogenes* serotype 4a, a serotype not often implicated in listeriosis outbreaks (7). A PCR based on this gene was used to demonstrate the feasibility of the MIPA concept for the detection of *L. monocytogenes*. The MIPA detected *L. monocytogenes* in both artificially contaminated curd cheese and naturally contaminated Port Salut samples. The results (Table 1) indicate that MAb 55 is preferred over MAb A. In *Listeria* cells, the production of flagella is dependent on the culture temperature (19). The binding of MAb 55 is independent of the culture temperature of the bacterium, and it may therefore be that MAb A is less useful because it is directed against flagellin proteins. Moreover, flagella may break off easily during immunomagnetic separation.

The experiments show that immunomagnetic separation of specific bacteria can effectively be used as an isolation step before PCR amplification. With MAb 55, approximately 5 to 15% of *L. monocytogenes* cells present in enrichment II were recovered. This value is approximately 10^3 to 10^6 CFU of *L. monocytogenes* in a PCR incubation, 10-fold more than for the direct addition of 1-μl samples. However, the main effect of immunomagnetic separation is on inhibitors of the PCR, since a 10^5-fold increase in template concentration does not yield a PCR product in the presence of 1 or 2 μl of enrichment II. A similar inhibition was described by Wernars et al. (24), who found a strong inhibition when *Listeria* DNA was directly extracted from soft cheese. Furthermore, bacteria which bound nonspecifically to the beads did not interfere with the MIPA because of the specificity of the PCR amplification used for detection of the captured *L. monocytogenes* cells.

MAb A and MAb 55 do not discriminate between *L. monocytogenes* and other, less-pathogenic *Listeria* spp. This drawback is remedied in the PCR step after magnetic separation. To obtain a PCR amplification which included *L. monocytogenes* serotype 4a, we developed a new amplification. Primers were chosen to contain the DNA sequence of the gene encoding listeriolysin O, a hemolysin, since this gene seemed to be specific for *L. monocytogenes* (21). However, there was some ambiguity concerning the specificity, because listeriolysin O shows strong regional homologies with both streptolysin and pneumolysin (5). In addition, cross-hybridization of a 1-kb listeriolysin O probe with *L. ivanovii* and *L. seeligeri* DNAs, which both encode a hemolysin, was reported (12). In contrast, Datta et al. (5) reported no cross-hybridization of a 650-bp listeriolysin O probe and two synthetic probes with either *L. ivanovii* or *L. seeligeri* DNA. In this study we showed that the two primers chosen from the listeriolysin O gene DNA sequence resulted in the expected product with *L. monocytogenes* strains, whereas no product was formed when other *Listeria* species, *S. pneumoniae*, or *S. pyogenes* DNA was used as the target in the PCR.

In this paper, the MIPA for the detection of *L. monocytogenes* is presented. The combination of the immunologic capturing of bacteria and the subsequent detection of the *L. monocytogenes* separated by the PCR overcomes the major disadvantages of both methods when used alone. Non-*L. monocytogenes* bacteria which either bound nonspecifically to the beads or also react specifically with the MAbs are not detected in the PCR when *L. monocytogenes*-specific primers are used. On the other hand, material which inhibits the *Taq* polymerase is removed during the immunomagnetic separation. Although the MIPA is not optimized, it has reached a fair sensitivity, which is amenable to further improvement.

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