Rapid Determination of *Listeria monocytogenes* by Automated Enzyme-Linked Immunoassay and Nonradioactive DNA Probe

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A rapid and reliable analytical method was developed to detect and confirm the presence of *Listeria monocytogenes* in raw and partially processed foods. Forty-nine food samples (25 mixed cut vegetable salad, 12 smoked salmon, and 12 sterile smoked salmon) were individually inoculated with high levels [10–100 colony forming units (cfu)/25 g sample] and low levels (1–10 cfu/25 g sample) of *L. monocytogenes*, and were screened using the Vitek Immuno Diagnostic Assay (VIDAS) *Listeria monocytogenes* (VIDAS LMO). Positive test results were confirmed as *L. monocytogenes* by nonradioactive DNA probe. All samples inoculated with high levels of *L. monocytogenes* were detected by VIDAS and 96% were confirmed as *L. monocytogenes* by DNA probe. VIDAS LMO detected 89% of samples inoculated with low levels of *L. monocytogenes*, and 87% of these were confirmed as positive by DNA probe. In addition, 12 other samples (4 from each of mixed cut vegetable salad, smoked salmon, and sterile smoked salmon) were inoculated with high levels of *L. ivanovii, L. seeligeri, L. welshimeri, L. innocua, L. grayi, and L. murrayi*. Samples were assayed by the same protocol and all gave negative results. Compared with the cultural method, the VIDAS LMO nonradioactive DNA probe combination is highly specific, discriminates between *L. monocytogenes* and all other *Listeria* species, and reduces analytical time.

Microbiological safety of food is an increasingly important concern of consumer, industry, and regulatory agencies. A serious threat to public health is the presence of *Listeria monocytogenes* in food. This pathogen has been reported to survive in food from 9 months to 10 years under various circumstances (1, 2). Because *L. monocytogenes* is the only known human pathogen among the genus *Listeria*, its presence on raw, freshly prepared salad vegetables and on ready-to-eat processed seafood has caused concern about the risk to food safety (3, 4). Listeriosis in humans may cause any of the following problems: spontaneous abortions, meningitis, septicemia, and encephalitis. Groups at risk include pregnant women, neonates, immunocompromised patients, and the elderly (5, 6). Determination of the presence of *L. monocytogenes* in a presumptive positive food sample using methods of AOAC INTERNATIONAL (7) and the U.S. FDA’s *Bacteriological Analytical Manual* (BAM; 8) often takes 2 weeks. However, advances in analytical biotechnology can provide more rapid identification of pathogenic microorganisms that may cause a public health concern (9). In contrast to the current cultural methodology for detecting *L. monocytogenes* in food, combining the VIDAS *L. monocytogenes* (VIDAS LMO) test (bioMérieux Vitek, Inc., Hazelwood, MO) as a screening method with the nonradioactive DNA probe hybridization technique (based on the cloned *L. monocytogenes* hlyA gene and its flanking DNA) as a confirmatory test, is rapid and highly specific.

Experimental

**Media and Reagents**

(a) *Trypticase soy broth* (TSB).—Prepared according to BAM (8).

(b) *Butterfield’s phosphate buffer* (BPB).—Prepared according to BAM (8).

(c) *Tryptic soy agar with yeast extract* (TSAYE).—Prepared according to BAM (8).

(d) *Fraser broth* (FB) without ferric ammonium citrate.—Prepared according to Difco package insert (Difco Laboratories, Detroit, MI); 55 g was dissolved in 1 L distilled water and boiled to dissolve completely. After autoclaving at 121°C for 15 min, the solution was cooled to 45°–50°C, 10 mL Bacto Fraser broth supplement (Difco) was added aseptically, and then it was mixed well.

(e) *Fraser broth with ferric ammonium citrate*.—Prepared according to Difco package insert; 55 g was dissolved in 1 L distilled water and boiled to dissolve completely. After autoclaving at 121°C for 15 min, it was cooled to 45°–50°C, 10 mL Bacto Fraser broth supplement (Difco) and 10 mL 5% solution of ferric ammonium citrate (Sigma Chemical Co., St. Louis, MO) sterilized by passage through 0.20 μm filter (Nalgene Labware Division, Sybron Corporation, Rochester, NY) were aseptically added, and the solution was well mixed.
Denaturation solution.—Prepared according to BAM (8).
Neutralization solution.—Prepared according to BAM (8).
ECL direct nucleic acid labeling and detection system.—Amersham Life Science, Little Chalfont, Buckinghamshire, UK.
Primary wash buffer.—Prepared according to Datta et al. (10).
Tris-EDTA buffer.—Prepared according to BAM (8).
Each liter contains 0.1% sodium dodecyl sulfate (Sigma).
2X Standard saline citrate.—Prepared according to BAM (8).
Hind III restriction enzyme.—New England Biolabs, Beverly, MA.

Bacterial Strains

*L. monocytogenes* ATCC 19118 was used to inoculate samples. To test the method’s ability to detect low-temperature exposed cells as found in refrigerated foods, a cold-treated inoculum was prepared according to the method of Weagent et al. (11) by resuspending washed cells from an overnight culture grown in TSB in BPB. A decimal dilution was then made to extinction (until there is no growth on plating), spreading (0.1 mL) in duplicate on TSAYE plates and incubating at 35ºC for 24 h to determine the number of colony forming units (cfus). This approximate number was used to determine the number of cells needed to artificially contaminate samples. Suspensions were held at 4ºC for 24 h to prevent growth of washed *L. monocytogenes* cells and for inoculum uniformity before appropriate dilutions were selected for use as inocula. The amount of potential cold stress (12) injury in the cold treated population was not determined. High levels of *L. ivanovii* (ATCC 19119), *L. seeligeri* (ATCC 35967), *L. welshimeri* (ATCC 33090), *L. grayi* (ATCC 19120), and *L. murrayi* (ATCC 25402) were used to inoculate 12 other samples (4 each from mixed cut vegetable salad, smoked salmon, and sterile smoked salmon).

**VIDAS L. monocytogenes**

The fully automated stand alone VIDAS system (bioMérieux Vitek, Inc.) was used to screen for the presence of *L. monocytogenes* in the food samples using the VIDAS LMO test. A pipet-like solid-phase receptacle (SPR) is used for immunocapture of the pathogen from the food matrix after a 48 h incubation at 30ºC in enrichment broth. A plastic strip is preloaded with wash solutions; conjugate (antibody conjugated to alkaline phosphatase) and substrate (4-methyl umbelliferyl phosphate) are used in the assay. In a “sandwich” reaction, the bound anti-*L. monocytogenes* antibody in the SPR captures the corresponding bacterial antigen, which is then detected by a second antibody conjugated to alkaline phosphatase. The bound enzyme catalyzes the cleavage of the substrate into a fluorescent product which is then measured and expressed as a relative fluorescence value. Reactions and readings are automatically performed in the VIDAS instrument without user intervention, and results are available in 70 min.

**Sample Preparation**

A 25 g sample was aseptically added to 225 mL Fraser broth with ferric ammonium citrate, blended, and inoculated with a high (10–100 cfu/25 g) or low (1–10 cfu/25 g) level of inoculum in a 0.1 mL volume. High levels of *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. grayi*, and *L. murrayi* were used to inoculate 12 other samples (4 each from mixed cut vegetable salad, smoked salmon, and sterile smoked salmon). The mixed vegetable salad contained shredded carrots, lettuce, and cabbage. A portion of the smoked salmon was sterlized in an Amsco 3021 Eagle series for 25 min at 121ºC (American Sterilizer Company, Erie, PA). After a 24 h incubation at 30ºC, 1 mL of each culture broth was transferred to 10 mL Fraser broth without ferric ammonium citrate and incubated for 24 h at 30ºC. Samples were used to check the specificity of the VIDAS LMO test. Uninoculated samples were used as negative controls.

**Nonradioactive DNA Probe**

For this study, a 5.8 kb DNA probe (pAD319) based on a cloned *L. monocytogenes* hlyA gene and its flanking DNA was generously provided by Atin Datta of the FDA (13). This probe was labeled according to the instructions of the ECL kit (Amersham Life Science) and of Datta et al. (10). Samples that gave positive results by the VIDAS were spotted on TSAYE plates using a colony replica plater (Sigma Chemical Co.). After a 24 h incubation at 35ºC, colonies were lifted onto a Hybond-N+ nylon transfer membrane (Amersham Life Science). To release bacterial DNA, the lifted colonies on the nylon membrane were exposed to microwaves for 30 s in a kitchen microwave at a high setting (Spacesaver, Tappans, Columbus, OH). To denature the double-stranded DNA, filters were soaked in denaturation solution for 3 min at room temperature. The pH of the filters was adjusted to pH 7.0 with a solution of 1.0M Tris-HCl containing 2.0M NaCl. Cross-linking was achieved by exposing the filters to short wave UV light for 45 s. The filters were then washed in a solution of Tris-EDTA (TE) containing 0.1% sodium dodecyl sulfate (SDS), followed by another wash in TE. Cross-linking was performed again by exposing the filters to short UV light for 45 s. The filters were then air-dried. DNA on the nylon membrane filters was hybridized with previously labeled DNA probe pAD319-HRP overnight at 42ºC. To remove or prevent nonspecific hybridization, the hybridized nylon membrane filters were washed twice in primary wash buffer. The filters were then soaked in equal volumes of the 2 detection reagents (provided in the ECL kit). Positive results were detected as spots on X-ray film through photo signal transduction.

**Results and Discussion**

Forty-nine food samples (25 mixed cut vegetable salad, 12 smoked salmon, and 12 sterile smoked salmon) were individually inoculated with high levels (10–100 cfu/25 g sample) and low levels (1–10 cfu/25 g sample) of *L. monocytogenes*,
and were screened by the VIDAS LMO test. Twelve other samples (4 each from mixed cut vegetable salad, smoked salmon, and sterile smoked salmon) were inoculated with a high level (10–100 cfu/25 g sample) of the combination of \textit{L. ivanovii}, \textit{L. seeligeri}, \textit{L. welshimeri}, \textit{L. innocua}, \textit{L. grayi}, and \textit{L. murrayi} and were screened by the VIDAS LMO test. Forty-nine samples inoculated with high levels of \textit{L. monocytogenes} were detected by VIDAS and 47 were confirmed positive by DNA probe. Forty-four of 49 samples inoculated with low levels were detected by VIDAS, whereas 38 samples were confirmed positive by DNA probe (Table 1).

The poorer detection of \textit{L. monocytogenes} at low spiking levels in mixed cut vegetable salad may be due to both inhibition of bacterial growth by natural antimicrobial agents released from the vegetables (14) and to possible microflora competition. The 12 samples that were inoculated with the high level of the combination of \textit{L. ivanovii}, \textit{L. seeligeri}, \textit{L. welshimeri}, \textit{L. innocua}, \textit{L. grayi}, and \textit{L. murrayi} tested negative by VIDAS LMO. One hundred percent of the samples that were tested without inoculation (negative controls) gave negative results. This protocol was highly successful because the monoclonal antibody used in the VIDAS assay is specific for \textit{L. monocytogenes}, making it a sensitive screening tool that can discriminate between \textit{L. monocytogenes} and other strains of \textit{Listeria} spp. (15).

The nonradioactive DNA probe colony hybridization technique is complementary to the antibody test because it uses a highly specific DNA probe consisting of only the \textit{L. monocytogenes} listeriolysin gene (\textit{hlyA}) and its flanking DNA (10) and can be used as a confirmatory test to determine the presence of \textit{L. monocytogenes} in food. A further advantage of the technique is that the nonradioactive labeled DNA probe eliminates radioactive waste and reduces health hazards associated with the use of a radioactive labeled DNA probe. Furthermore, the longer shelf life of the nonradioactive labeled DNA probe is superior to the shorter half-life of the radioactive labeled DNA probe. The combination of the 2 techniques is superior to the present lengthy conventional method which lacks specificity and is time consuming (4 days versus 15 days by the BAM methodology for confirming presumptive positives).

**Acknowledgment**

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**References**


**Table 1. Determination of \textit{L. monocytogenes} by VIDAS and DNA probe**

<table>
<thead>
<tr>
<th>Product</th>
<th>No. of samples</th>
<th>VIDAS</th>
<th>DNA Probe</th>
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<tr>
<td></td>
<td></td>
<td>High level&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Low level&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Smoked salmon</td>
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<td>12</td>
<td>0</td>
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</tbody>
</table>

<sup>a</sup> High level of inoculum (10–100 cfu/25 g).
<sup>b</sup> Low level of inoculum (1–10 cfu/25 g).