Comparative Evaluation of Culture- and BAX Polymerase Chain Reaction–Based Detection Methods for Listeria spp. and Listeria monocytogenes in Environmental and Raw Fish Samples

ADAM D. HOFFMAN AND MARTIN WIEDMANN*

Department of Food Science, 412 Stocking Hall, Cornell University, Ithaca, New York 14853, USA

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ABSTRACT

Two commercial polymerase chain reaction (PCR)-based Listeria detection systems, the BAX for Screening/Listeria monocytogenes and the BAX for Screening/Genus Listeria, and a culture-based detection system, the Biosynth L. monocytogenes Detection System (LMDS), were evaluated for their ability to detect L. monocytogenes and Listeria spp. in raw ingredients and the processing environment. For detection of L. monocytogenes from raw fish, enrichment was performed in Listeria enrichment broth (LEB), followed by plating on both Oxford agar and LMDS L. monocytogenes plating medium (LMPM). Detection of Listeria and L. monocytogenes from environmental samples was performed using LMDS enrichment medium, followed by plating on both Oxford agar and LMPM. A total of 512 environmental samples and 315 raw fish were taken from two smoked fish processing facilities and screened using these molecular and cultural Listeria detection methods. The BAX for Screening/L. monocytogenes was used to screen raw fish and was 84.8% sensitive and 100% specific. The BAX for Screening/Genus Listeria was evaluated on environmental samples and had 94.7% sensitivity and 97.4% specificity. In conjunction with enrichment in LEB, LMPM had a sensitivity and specificity for detection of L. monocytogenes from raw fish of 97.8 and 100%, respectively. Use of LMDS enrichment medium followed by plating on LMPM allowed for sensitivity and specificity rates of 94.8 and 100%, respectively, for detection of L. monocytogenes from environmental samples. We conclude that both the BAX systems and the use of LMPM allow for reliable and rapid detection of Listeria spp. and L. monocytogenes. While the BAX systems provide screening results in about 3 days, the use of LMPM allows for L. monocytogenes isolation in 4 to 5 days.

Listeria monocytogenes is a foodborne pathogen with a zero-tolerance limit in ready-to-eat foods. L. monocytogenes has forced the recall of a wide variety of contaminated food products and is responsible for an estimated 500 foodborne deaths each year in the United States (22). Current conventional detection techniques take approximately 1 week to complete and require additional species-differentiating tests to distinguish L. monocytogenes from other members of the Listeria genus (8, 23).

L. monocytogenes is able to grow at refrigerated temperatures commonly used to preserve ready-to-eat foods (20). Techniques to detect L. monocytogenes before product distribution need to be rapid and have low detection limits. Selective enrichment is generally used to allow detection of low numbers of Listeria in a mixed microflora. Early techniques relied on cold enrichment to select for Listeria spp. (8). Since some cold enrichment protocols required several months of incubation, antibiotics have become the selective agents of choice today (5, 8, 26). Detection methods have evolved from Listeria-selective agars to genetic tests and plating media that are highly specific for Listeria spp. and L. monocytogenes. Methods currently entering the market to screen for and identify L. monocytogenes have cut detection times from 1 week to 1 to 4 days (6, 13, 23, 25, 27, 30).

Significant efforts have been dedicated to the development of enrichment media and protocols for L. monocytogenes and Listeria spp. (2, 4, 5, 7, 8, 15, 16, 19, 26, 31). Ideal enrichment media would facilitate recovery of injured Listeria cells and enrichment of Listeria spp. and L. monocytogenes over competing microflora. Detection of L. monocytogenes after enrichment is complicated by the fact that other Listeria spp. may overgrow L. monocytogenes during enrichment, thus masking the presence of this pathogenic species (5). In traditional culture-based assays, it becomes very difficult to detect L. monocytogenes at any level when it is greatly outnumbered by other Listeria spp. (5, 9). This is of particular concern since other Listeria spp. and specifically L. innocua often appear to be present together with L. monocytogenes (7, 10, 29). Consequently, detection of Listeria spp. is often used as an indicator for the presence of L. monocytogenes (17).

Recently, a new plating medium has been described that can be used to differentiate L. monocytogenes and Listeria ivanovii from other Listeria spp. based on phosphatidylinositol-specific phospholipase C activity (21, 27). L. ivanovii is rarely found in foods and can subsequently be distinguished from L. monocytogenes using a confirmatory medium and screening for rhamnose utilization (27). This
differential plating medium thus should allow specific detection and isolation of \textit{L. monocytogenes}, even in the presence of relative high numbers of other \textit{Listeria} spp. (5,27).

Polymerase chain reaction (PCR) assays provide another approach for sensitive and specific detection of \textit{L. monocytogenes}, even in a background of other \textit{Listeria} spp. Several PCR-based detection methods for \textit{L. monocytogenes} and \textit{Listeria} spp. overall are currently commercially available, including the Probelia and BAX systems, manufactured by BioControl and Qualicon, Inc., respectively. The BAX PCR detection system is offered in two kits that allow the detection of either \textit{L. monocytogenes} or \textit{Listeria} genus. The BAX PCR detection system has been modified since evaluated by Norton et al. (23) with the addition of a built-in positive control to each reaction tube. The positive control is comprised of an internal competitor DNA present in each reaction mix. This competitor will yield a smaller PCR amplification product than that obtained with either \textit{Listeria} or \textit{L. monocytogenes} in the respective kits. The positive control for each sample is important to confirm that substances present in the food sample or enrichment did not inhibit the PCR. The absence of the smaller control band in a PCR-negative sample indicates a PCR failure—e.g., the presence of inhibitors in the samples tested. In the previous version of the BAX system, an additional control PCR had to be run in parallel to every sample, requiring additional time and materials.

In our study, we use the cold-smoked fish industry as a model industry to evaluate commercial PCR and culture-based detection methods for \textit{L. monocytogenes} and \textit{Listeria} spp. Potential sources of \textit{L. monocytogenes} in cold-smoked fish include the food processing plant as well as raw materials (24). This study was thus specifically designed to evaluate (i) the BAX for Screening/\textit{L. monocytogenes} for monitoring raw fish for \textit{L. monocytogenes} contamination, (ii) the BAX for Screening/Genus \textit{Listeria} for monitoring the processing environment, and (iii) the culture-based \textit{L. monocytogenes} detection system (LMDS), which includes a differential plating medium for \textit{L. monocytogenes}, for detection and isolation of \textit{L. monocytogenes} from environmental and raw fish samples.

**MATERIALS AND METHODS**

**Fish samples.** A total of 315 raw fish samples were analyzed in two sampling periods: February through April (133 fish) and July through September (182 fish). Raw fish were shipped overnight on ice from two smoked fish processing facilities and processed immediately upon arrival. Fish analyzed were lake whitefish (n = 68), sablefish (n = 55), and salmon (n = 192). Samples arrived as fillets, as whole fish, or as collar, tail, and belly trimmings. A combined 60-g sample was collected from the collar, belly flap, tail, and anal regions. The sample was combined with 60 ml of 0.8% NaCl solution in a sterile Stomacher 400 closure bag (Seward Ltd., London, UK) and stomached for 60 s on normal speed using a Stomacher 400. The resulting fish homogenate was split and tested by the two methods described below.

**BAX for Screening/\textit{L. monocytogenes} in fish samples.** The BAX system protocol (Qualicon, Inc., Wilmington, Del.) was followed (1). Briefly, 50 g of the fish homogenate (equivalent to 25 g of fish) was enriched with 200 ml of Demi-Fraser (Difco Laboratories, Detroit, Mich.) broth. After 24 h of enrichment at 30°C, a 0.1-ml aliquot of enrichment broth was inoculated into 10 ml MOPS-buffered \textit{Listeria} enrichment broth (LEB; Difco) and incubated for 24 h at 35°C. Lysate preparation and PCR were performed according to manufacturer’s directions. A positive control is built into each BAX system reaction mixture, and a single negative control from unincubated broth was run with each batch. PCR was performed using a GeneAmp PCR System 9600 (Perkin Elmer Applied Biosystems, Foster City, Calif.) (1).

**Bacteriological analysis of fish samples.** A modification of the Food and Drug Administration method was used for isolation of \textit{L. monocytogenes} from raw fish (18). Samples were enriched in LEB, with no recovery time before addition of antibiotics, before being plated on both (i) the Food and Drug Administration method’s Oxford agar with antibiotic supplement, and (ii) a chromogenic \textit{L. monocytogenes} plating medium (LMPP; Biosyn Biochemica & Synthetica [BCM], Naperville, Ill.) (Fig. 1A). Specifically, a 50-g sample of fish homogenate (equivalent to 25 g of fish) was added to 200 ml of LEB in a sterile Stomacher 400 self-closing bag and incubated at 30°C. After 24 and 48 h of incubation, 0.05 ml of enrichment was streaked onto Oxford plates containing antibiotic supplement (Difco) and onto LMPM. LMPM and Oxford plates were incubated for 48 h at 35 and 30°C, respectively. Up to five colonies typical for \textit{L. monocytogenes} were selected from LMPM and streaked onto \textit{L. monocytogenes} confirmatory medium (LMCM; BCM) and onto purple broth base agar (Difco) containing 1.0% rhamnose and scored according to manufacturer’s directions (27). \textit{L. monocytogenes} isolates identified by LMDS were confirmed by \textit{hlyA} PCR as described below. Oxford plates were scored for \textit{Listeria}-like colonies based on esculin hydrolysis and morphology. For all samples that were negative for \textit{L. monocytogenes} using LMPM and LMCM, but that showed \textit{Listeria}-like colonies on Oxford, one \textit{Listeria}-like colony from the Oxford plate was screened using a previously described PCR assay, which detects the presence of the \textit{L. monocytogenes}-specific listeriolyisin \textit{O} gene, \textit{hlyA} (23). The gold standard for \textit{L. monocytogenes}.
*L. monocytogenes* detection was defined as all samples positive by *L. monocytogenes* detection on LMPM followed by LMCM confirmation as well as all LMPM/LMCM-negative samples, which were positive hly PCR screening of a colony from Oxford plates.

**Environmental samples.** A total of 512 environmental samples were collected by sponge from two smoked fish processing plants. Samples were taken at 16 sites from each plant for 8 weeks in March through April and July through August. The 16 sample sites were constant during the study and included drains, floors, doorknobs, condensate lines, plastic crates, and equipment. Sponges, neutralizing buffer (containing sodium thiosulfate and aryl sulfonate), and sterile sampling gloves (Bacti-Sponge Sampling Kit; Hardy Diagnostics, Santa Maria, Calif.) were provided to plant personnel. The sampling technique was demonstrated for each sample location, and samples were taken by a single individual to normalize sampling. Samples were shipped overnight on ice and processed immediately upon arrival.

**BAX for Screening/Genus *Listeria* in environmental samples.** The BAX for Screening/Genus *Listeria* protocol (Qualicon) was used (1). Half of the sampling sponge was added to 50 ml of Demi-Fraser broth (Difco) and incubated for 24 h at 30°C.

A 0.1-ml aliquot of enrichment medium was inoculated into MOPS-buffered LEB and incubated for 24 h at 30°C. The manufacturer's protocol was followed to prepare lysates and perform DNA amplification. A positive control is built into each BAX system reaction mixture, and a single negative control from unincoculated broth was run with each batch. PCR was performed using a GeneAmp PCR System 9600.

**Bacteriological analysis of environmental samples.** A modification of the BCM LMDS was used to enrich and detect *L. monocytogenes* from environmental samples using half of the sampling sponge (Fig. 1B) (27). For samples from weeks 1 to 8 (256 samples), half of the sampling sponge was added to 50 ml of *L. monocytogenes* preenrichment broth (BCM) and incubated at 30°C. After 24 and 48 h, 0.05 ml of *L. monocytogenes* preenrichment broth was streaked onto each LMPM and Oxford plate. For samples from weeks 9 to 16 (256 samples), half of the sampling sponges collected were added to 50 ml of *L. monocytogenes* preenrichment broth. After incubation at 30°C, 0.5 ml of this preenrichment media was added to 10 ml *Listeria*-selective enrichment broth (BCM). After 24 h of incubation at 35°C, 0.05 ml of the *Listeria*-selective enrichment broth was streaked onto both LMPM and Oxford plates. LMPM and Oxford plates were incubated for 48 h at 35 and 30°C, respectively. Up to five colonies characteristic of *L. monocytogenes* were picked from LMPM and streaked onto biplates LMCM and purple broth base agar with 1% rhamnose and incubated at 35°C for 24 h for confirmation according to manufacturer's directions. *L. monocytogenes* isolates identified on LMPM were confirmed by PCR as described below. Oxford plates were scored for *Listeria*-like colonies based on esculin hydrolysis and colony morphology. *Listeria*-like colonies were confirmed to be *Listeria* spp. using API test strips (bio-Merieux Vitek, Inc., Hazelwood, Mo.). *Listeria* spp. detection on Oxford plates was used as a gold standard to evaluate the performance of the BAX for Screening/Genus *Listeria* kit. The gold standard for *L. monocytogenes* detection was defined as all samples positive by *L. monocytogenes* detection on LMPM followed by LMCM confirmation, as well as all LMPM/LMCM-negative samples, which were positive hly PCR screening of a colony from Oxford plates.

**Confirmation of *L. monocytogenes* isolates by PCR.** At least one putative *L. monocytogenes* colony from LMPM was confirmed using a PCR assay targeting the *L. monocytogenes* listeriolysin O gene, *hlyA*, as previously described (23).

**RESULTS AND DISCUSSION**

To control contamination of ready-to-eat food products, the food industry needs rapid methods to screen for the presence of *L. monocytogenes* and *Listeria* spp. *Listeria* spp. are often used as indicator organisms for *L. monocytogenes*, particularly when testing environmental samples (11). Raw materials and the food processing environment are two potential primary contamination sources for *L. monocytogenes* in cold-smoked fish (3, 10, 23). We thus evaluated a PCR-based method to screen for *L. monocytogenes* and *Listeria* spp. in raw fish and environmental samples, respectively. We furthermore evaluated a cultural method to specifically isolate *L. monocytogenes* from raw fish and environmental samples. Our sampling protocol of two plants yielded about 40% *Listeria* spp.-positive and 23% *L. monocytogenes*-positive environmental samples as well as 14% *L. monocytogenes*-positive raw fish samples. Similar prevalence data were previously described for samples from the smoked fish industry (3, 10, 23, 28).
followed by MOPS-buffered LEB suggested by Qualicon. Our sensitivity rate of 94.7% possibly reflects a higher sensitivity of the BAX system assay when used with the manufacturer’s recommended enrichment protocol. The specificity rate of 96.2% found by Norton et al. (23) is similar to the specificity found in the current study (Table 1).

The PCR-based BAX system results in an easy-to-read positive–negative result, and unlike plating methods, it requires minimal interpretation. The high sensitivity and specificity values make this system a reliable and rapid method to determine the presence of Listeria spp. Thus, this assay may overcome some of the problems with false-positive results other groups have described when analyzing seaweed for Listeria spp. (12).

BAX for Screening/L. monocytogenes. We used the BAX for Screening/L. monocytogenes to screen 315 raw fish to evaluate its use to monitor raw materials entering a processing facility, an important control point for minimally processed seaweed. This BAX system assay had a sensitivity of 84.8% and a specificity of 100% (Table 1). The false-negative rate for the L. monocytogenes system was thus slightly higher than that for the Listeria spp. system.

To determine if the false negatives were due to a limitation of the BAX system’s PCR primers, a cultural isolate from each BAX-system-negative sample was tested in pure culture using the BAX system. Each of these isolates yielded a positive result, indicating that the PCR primers were capable of detecting these isolates and suggesting that false-negative results were due to low L. monocytogenes numbers in the enrichment. The BAX for Screening/L. monocytogenes is more susceptible to false negatives than the BAX for Screening/Genus Listeria, because during enrichment, L. monocytogenes may be outcompeted by other strains of Listeria including L. innocua (4, 7, 8, 26). The issue of competition becomes very important when using traditional culture methods, ELISA, or genetic techniques to detect L. monocytogenes. Genetic and ELISA techniques require the presence of L. monocytogenes. Therefore, if only four randomly chosen colonies after enrichment are speciated, a significant background of Listeria spp. will often result in false-negative results for L. monocytogenes. In interpreting our results for the BAX for Screening/L. monocytogenes, it is important to consider that the gold standard for this comparison was the detection of L. monocytogenes on LMPM plates or on Oxford after LEB enrichment, which provides more sensitive detection compared to plating only on Oxford or Listeria plating media.

Another possible cause for the occurrence of false negatives from food samples is the influence of sample partitioning. Because of oils contained in the fish, the enrichment contains a substantial amount of oil phase in which Listeria cells and listerial DNA will not be soluble. Another PCR-based detection method overcame this obstacle by allowing the phases to separate in glass cylinders for 4 h and sampling the aqueous phase (14). When testing an oily food

### Table 1. Performance of BAX systems and BCM LMDS system for detection of Listeria monocytogenes and Listeria spp.

<table>
<thead>
<tr>
<th>Detection method</th>
<th>No. positive</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value of positive (%)</th>
<th>Predictive value of negative (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental samples (n = 512)</td>
<td></td>
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<td></td>
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<tr>
<td>BAX for Listeria spp. Gold standard for Listeria spp. detection</td>
<td>196</td>
<td>94.7</td>
<td>97.4</td>
<td>96.1</td>
<td>96.4</td>
<td>96.3</td>
</tr>
<tr>
<td>BCM LMDS Gold standard for L. monocytogenes detection</td>
<td>207</td>
<td>94.8</td>
<td>100.0</td>
<td>100.0</td>
<td>98.5</td>
<td>98.8</td>
</tr>
<tr>
<td>Raw fish samples (n = 315)</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>BAX for L. monocytogenes Mod. BCM LMDS Gold standard for L. monocytogenes detection</td>
<td>39</td>
<td>84.8</td>
<td>100.0</td>
<td>100.0</td>
<td>97.5</td>
<td>97.8</td>
</tr>
<tr>
<td>BAX for L. monocytogenes Gold standard for L. monocytogenes detection</td>
<td>44</td>
<td>97.8</td>
<td>100.0</td>
<td>100.0</td>
<td>99.6</td>
<td>99.7</td>
</tr>
</tbody>
</table>

* Sensitivity (%) = [(true positive/(true positive + false negative)] × 100.
  
* Specificity (%) = [(true negative/(true negative + false positive)] × 100.
  
* Predictive value of a positive test (%) = [(true positive/(true positive + false positive)] × 100.
  
* Predictive value of a negative test (%) = [(true negative/(true negative + false negative)] × 100.
  
* Accuracy (%) = [(true positive + true negative)/(true positive + true negative + false positive + false negative)] × 100.

Two hundred fifty-six samples were tested with and without a secondary selective enrichment broth (see “Materials and Methods”).

Samples positive for Listeria spp. after LMDS enrichment and plating on Oxford.

Samples positive by LMDS detection system as well as LMDS-negative samples, which were positive hly PCR screening of a colony from Oxford plates, were used as a gold standard for L. monocytogenes detection.

Samples positive by L. monocytogenes detection on LMPM followed by LMCM confirmation as well as LMPM/LMCM-negative samples, which were positive hly PCR screening of a colony from Oxford plates.
such as fish, we thus recommend allowing the enrichment to settle and taking the sample from the middle aqueous phase. This precaution excludes food sediments, which clog pipettes and contain PCR-interfering substances, and it avoids the oil phase that is unlikely to contain *Listeria* cells.

Like the BAX for Screening/Genus *Listeria* test, the BAX for Screening/*L. monocytogenes* has a rapid turn-around time and requires minimal interpretation of results, and its reliability is good when considering the added complexity of testing a food directly. Despite several false-negative samples, the BAX system PCR primers were shown to react with all *L. monocytogenes* isolates from our study. Our results using both BAX systems are comparable to other studies using PCR-based detection systems. Norton et al. (23) found the BAX for Screening/*L. monocytogenes* to have a sensitivity of 82.2% and the BAX for Screening/Genus *Listeria* to have an 89.9% sensitivity. Both our study and the previous study show the BAX for Screening/*L. monocytogenes* to be less sensitive than the BAX for Screening/Genus *Listeria*. Importantly, the new version of the BAX system used in this study utilizes an internal PCR control, unlike the BAX systems evaluated by Norton et al. (23), which required a separate control reaction for each sample.

**BCM *L. monocytogenes* detection system.** The BCM LMPM, which allows differentiation of *L. monocytogenes* from other *Listeria* spp., was used in conjunction with a one-step enrichment in LEB to detect *L. monocytogenes* in 315 fish samples. The calculated sensitivity (true positive) rate for detection in raw fish was 97.8% (Table 1). The complete LMDS system (LMDS enrichment medium and LMPM plates) was used to detect *L. monocytogenes* in 512 environmental samples. The calculated sensitivity (true positive) rate was 94.8% for environmental samples (Table 1). Our reported sensitivity is higher than a previously published sensitivity of 85.7% (27), which was obtained on environmental samples using the recommended LMDS enrichment protocol. We tested the LMDS environmental testing protocol with and without a secondary selective enrichment broth on 256 samples. The preenrichment broth is a resuscitation medium designed to inhibit gram-negative organisms, while the selective enrichment broth also inhibits gram-positive organisms (27). The dual-enrichment system yielded five false negatives (256 samples, 91.2% sensitivity) compared to one false negative (256 samples, 98.3% sensitivity) without a secondary selective enrichment. Without the secondary selective enrichment, plates for isolation had to be restreaked frequently due to high microbial background growth. Recently, Hitchins et al. (19) showed that one-step enrichments can be as efficacious as a two-step method and that they can reduce detection time (9). However, the LMDS plating medium may require the addition of gram-positive inhibitors to reduce background microflora when the result is needed, the laboratory resources available, and the necessity of an isolate for further analysis.

The BAX system delivers a result in 3 days, but it requires specialized laboratory equipment for PCR and electrophoresis. While PCR-based methods such as the BAX are screening tests that do not lead to isolation of a pure *L. monocytogenes* colony, use of the LMPM plating medium requires only basic microbiology equipment and yields a culture isolate, which may be used in molecular subtyping methods for further analyses such as strain tracking within the plant or supply chain. Thus, the advantages and disadvantages of each approach need to be considered when selecting the best monitoring tool for a given application—e.g., the background microflora, when the result is needed, the laboratory resources available, and the necessity of an isolate for further analysis.

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