Polymerase Chain Reaction-Based Methods for Detection of Listeria monocytogenes: Toward Real-Time Screening for Food and Environmental Samples

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A review is presented of nucleic acid amplification-based methodology, specifically polymerase chain reaction (PCR)-based assays, for the detection of Listeria monocytogenes in food and environmental samples. Until recently, developmental challenges including poor sensitivity, due in part to reaction inhibition by components of the sample matrix, and the potential for false-positive reactions have limited routine application of PCR-based screening assays. Commercial assays address these challenges while offering convenient, standardized protocols, a high level of automation, and results within 2 days after the sampling date. Although sample enrichment is necessary to achieve desired detection limits, continued efforts toward template purification will facilitate the development of assays offering real-time, quantitative results. The development of ribonucleic acid (RNA) amplification-based assays may increase in importance, particularly if end-product testing is prioritized by regulatory agencies, as messenger RNA appears to serve as an accurate indicator of cell viability. Further, the increase in target copy number may improve assay sensitivity. PCR-based screening methods offer efficient, reliable results and are ideal for monitoring the presence of L. monocytogenes in foods and in the food processing environment.

Although Listeria monocytogenes causes only 2500 of an estimated 76 000 000 cases of foodborne illness each year in the United States (1), it is responsible for approximately one-quarter of the foodborne disease-related deaths linked to known pathogens. Thus, L. monocytogenes is a significant public health concern. This organism is capable of causing serious invasive disease in animals and humans with clinical manifestations including spontaneous abortion, perinatal septicemia, meningitis, and encephalitis (2, 3). Although the majority of human listeriosis cases occur in neonates, immunocompromised individuals, and the elderly, L. monocytogenes also causes an influenza-like illness in healthy adults. First recognized as a foodborne pathogen in 1981 (4), L. monocytogenes has been implicated in at least 11 human foodborne epidemics worldwide characterized by case fatality rates of 20–40% (2, 3, 5, 6). In response to this and to the association of this organism with a variety of foods often consumed without cooking (2, 7), the U.S. Food and Drug Administration (FDA) established a zero tolerance for L. monocytogenes in ready-to-eat (RTE) foods in 1989.

Consistent compliance with the zero tolerance ruling for L. monocytogenes in RTE foods presents a daunting challenge to the food industry. L. monocytogenes is ubiquitous in nature, can grow over a wide pH range of 4.6–9.2, grows well at refrigeration temperatures, and is associated with a variety of foods, including milk, cheese, ice cream, raw vegetables, raw and cooked poultry, raw and cooked meat products, fermented sausages, and raw and cooked seafood (2, 3, 8). In 1998 alone, this organism caused 53.6% of Class I–III microbiological recalls in the United States (8). Farber and Peterkin (2) summarized surveys of a variety of foods, most citing a notable 2–10% prevalence of L. monocytogenes. Recent U.S. Department of Agriculture (USDA) data indicated a 2.5% prevalence of this organism in a 1998 survey of RTE foods, and a 4.6 and 2.7% prevalence in sliced ham and luncheon meats, and roast beef and corned beef samples, respectively, tested in 1999 (http://www.fsis.usda.gov/oa/topics/lm_action.htm). Further, studies using molecular typing methods have shown that L. monocytogenes may have the ability to colonize the food processing environment, thus establishing a reservoir for finished product contamination (9–13). Development of improved control strategies for L. monocytogenes requires a better understanding of the ecology of this organism in foods and in the food processing environment. Comprehensive surveillance programs featuring efficient, accurate detection systems and molecular subtyping will greatly facilitate the development of targeted strategies for control of L. monocytogenes.

The 1998–1999 multistate listeriosis outbreak, linked to consumption of contaminated hot dogs and deli meats, resulted in over 100 cases, at least 21 deaths (5), and a dramatic resurgence of regulatory activity. Notably, regulatory agen-
cies plan to expand the range of RTE products to be monitored and to increase the regularity of end product testing (http://www.fsis.usda.gov:80/OA/topics/lm_action.htm). Routine screening of final products by members of the food industry has been recommended as a means of Hazard Analysis Critical Control Point (HACCP) program verification. The FDA, in conjunction with the USDA Food Safety and Inspection Service (USDA-FSIS), recently completed a risk assessment for *Listeria monocytogenes* in RTE foods to develop a thorough, science-based review of current regulatory programs (Fed. Regist. 66, 5515–5517, draft available from http://www.cfsan.fda.gov).

The above discussion highlights the importance of efficient, reliable methods for detecting *Listeria monocytogenes* in foods and in the food processing environment. The focus of this review is nucleic acid amplification-based methodology, specifically the polymerase chain reaction (PCR). Challenges in the development and application of PCR-based assays, commercially available assays, and the utility of this technology as a component of a comprehensive monitoring program for *Listeria monocytogenes* are emphasized.

**Traditional Methodology for Detection of *Listeria monocytogenes***

Due primarily to historical precedence, along with considerations of cost and the perceived technical complexity associated with rapid screening methodology, traditional culture-based methods are by far the most widely used for detection of *Listeria monocytogenes* in food and environmental samples. A brief discussion of conventional methods will highlight inherent disadvantages, which served as the impetus for development of molecular-based detection methods.

An enrichment step is generally used to facilitate recovery of injured or stressed organisms, which might be encountered in samples that have undergone acidification or a thermal processing step, and to select for growth of *Listeria monocytogenes* over other microorganisms present in the sample. Gray et al. (14) described one of the earliest isolation protocols, in which samples are cold-enriched at 4°C in a nonselective medium for up to several months, thus limiting the growth of competing organisms. Although the cold-enrichment method provides high sensitivity, the time to results limits the potential for practical application as a screening method. Efforts to reduce recovery time while optimizing isolation of *Listeria* spp. from the complex matrices presented by food and environmental samples resulted in development of numerous enrichment broths and selective and differential agars (2, 15, 16). Two methods, the FDA method (17) and the USDA-FSIS method (18) have emerged as the most commonly used protocols in the United States. Most reports find these methods comparable to one another and to other methods, each with positive and negative attributes when applied for isolation of *Listeria monocytogenes* from a given sample type (15, 16). For example, the FDA method is most effective for isolation of low numbers of *Listeria* spp. and stressed or injured cells from foods with a low level of background flora. The USDA-FSIS method, which uses a 2-step enrichment method, offers high sensitivity for isolation of *Listeria* spp. from samples with high levels of background flora. The use of Fraser broth, a selective and differential medium, for the secondary enrichment broth allows interpretation of negative results within 3 rather than the 4 days required with the FDA method.

These and other culture-based methods are effective for recovery of *Listeria monocytogenes* from a variety of food and environmental samples. Several disadvantages, however, limit their routine application by testing laboratories and members of the food industry. The time to negative results is at least 3 days from the time of sampling. Further, most methods do not allow for differentiation of *Listeria monocytogenes* and other commonly co-isolated *Listeria* spp. Because *Listeria monocytogenes* is the only *Listeria* species of human public health concern, additional biochemical tests must be performed on at least 5 isolates to confirm the presence of this organism in the sample (17). The time to positive results, a major disadvantage of culture-based methods, is 5–7 days after sample collection. It is often not feasible, both with respect to cost and shelf-life, to withhold a food product from distribution for that length of time. Stressed or injured organisms may fail to recover or *Listeria monocytogenes* may be overgrown by other *Listeria* spp. or competing microorganisms, resulting in false-negative screening results (16, 19, 20). Traditional methods are labor-intensive and not amenable to high throughput, thus increasing the potential for user error and limiting their

![Schematic representation of one PCR cycle](http://www.fsis.usda.gov:80/OA/topics/lm_action.htm)
application in routine monitoring systems for *L. monocytogenes*. Enrichment and isolation procedures result in an increase in the number of viable organisms. Although it is imperative that food processors with on-site laboratories adequately separate them from the processing area and have appropriate biological containment programs in place regardless of the screening methodology in use, culture-based methods present an additional food safety concern. Sample enrichment also precludes quantitation of initial levels of *L. monocytogenes* in samples. As discussed below, molecular methodology has the potential to overcome some of these limitations.

**Molecular Methodology for Detection of *L. monocytogenes***

**Key Attributes of Improved Methods for Detection of *L. monocytogenes* in Food and Environmental Samples**

The limitations inherent in culture-based detection methods for *L. monocytogenes* have helped to define key attributes of improved methods. The ultimate sample screening assay should be rapid. As a function of both sample throughput and time to results, the ideal assay would provide definitive results within minutes to hours after sampling. Because there is a zero tolerance for *L. monocytogenes* in RTE foods and this organism is often present in low numbers (2), the assay should be highly sensitive (i.e., able to detect 1 CFU/25 g sample). Assay specificity is key to the prevention of false-positive results arising from cross-reactivity with other genera or *Listeria* spp. The assay should have a simple protocol to minimize or eliminate the necessity for highly trained or specialized operators. Automation would reduce labor, minimize user error, and remove subjectivity from interpretation of results. A quantitative assay would be particularly useful for risk assessment. The ability to quantitate initial levels of this organism in food samples would strengthen models used in exposure assessment and, therefore, risk characterization (http://www.cfsan.fda.gov). In addition, assay standardization, superior technical support, cost effectiveness, and verification and approval by independent methods validation agencies such as AOAC INTERNATIONAL are also critical to the practical application of detection methods by testing laboratories and members of the food industry.

**Development of Rapid, Molecular Methods for Detection of *L. monocytogenes***

Advances in biotechnology have led to the development of a diverse array of assays for detecting *L. monocytogenes*, which have been comprehensively reviewed by other authors (2, 21–23). Rapid assays that use immunochemical, nucleic acid hybridization, and nucleic acid amplification techniques offer more sensitivity and specificity than culture-based methods as well as dramatic reductions in the time to results. Many methods have also achieved a high level of automation, facilitating their application as routine sample screening assays. However, as with conventional methods, practical application of rapid methods for detection of *L. monocytogenes* in food and environmental samples is a challenge. Antibody-based assays often suffer from poor sensitivity [10^5–10^6 CFU/mL detection limit for enzyme-linked immunosorbent assays]
Thus requiring sample enrichment (21, 22). Cross-reactivity with components of the food matrix and other Listeria spp. necessitates culture-based confirmation of positive results (22, 24). Commercially available nucleic acid probe-based assays also require enrichment to achieve desired detection levels (25). Molecular methods have improved upon some aspects of traditional means for detection, however, and their development continues in earnest. Nucleic acid amplification technologies have been developed in recent years and show tremendous potential for detection and identification of L. monocytogenes.

**Polymerase Chain Reaction**

Few scientific developments have revolutionized as many fields as the polymerase chain reaction (PCR). Conceptualized by K. Mullis of Cetus Corp. and brought to practical reality in 1985, PCR is an in vitro enzymatic process for the amplification of a specific DNA sequence (26, 27). Simple in theory, the assay is based upon oligonucleotide primer-directed DNA synthesis by a polymerase. Briefly, 2 oligonucleotide primers, designed to flank the DNA sequence to be amplified, are provided in a buffered reaction mixture along with DNA polymerase, enzyme cofactors, deoxyribonucleic acids (dATP, dCTP, dGTP, and dTTP), and the DNA template. As shown in Figure 1, the reaction proceeds according to a general protocol involving 3 temperature-based steps: (1) denaturation of template DNA into single strands; (2) annealing, or hybridization, of the primers to complementary regions of the DNA template; and (3) extension, or synthesis of DNA, from the site dictated by the primers, designed so that polymerization proceeds across the region between them. With each 3-step cycle, the number of templates doubles. As each newly synthesized fragment can serve as a template in subsequent cycles, successive rounds of temperature cycling result in an exponential increase in the number of copies of the target DNA region. A single copy of target DNA can theoretically be amplified to $10^6$ copies in only 30–40 cycles, generally completed within 1–3 h. Initially, a heat-labile DNA polymerase was used for PCR. Because each cycle begins with a high-temperature denaturation step, fresh enzyme was required for completion of each cycle. The discovery and application of thermostable DNA polymerase from Thermus aquaticus (Taq) eliminated this requirement, dramatically reducing assay cost, labor, and the potential for error while lending the assay to automation and improving specificity, product yield, and sensitivity (27). PCR is one of several methods for nucleic acid amplification with application potential in the area

![Figure 3](image_url)

Figure 3. (A) Relative location of primers and fluorogenic probe designed for use with an RT-5' nuclease assay targeting the hlyA gene of L. monocytogenes (63). Bold arrow represents the hlyA gene and direction of transcription. Small arrows represent primers. Black bar represents fluorogenic probe. The position of the two transcriptional start sites and forward primer PF, based upon GenBank accession No. M24199 and M29030, are indicated. PF; forward primer; PR-a, PR-b, PR-c; reverse primers. Regions denoted by the primer pairs are indicated. Figure not drawn to scale. Figure adapted from Norton and Batt (63). Primer and probe names were simplified for this review. (B) Effect of reverse primer position on the outcome of an RT-5' nuclease assay performed 0, 3 and 6 h after cell inactivation by heat. ΔRQ reflects an increase of reporter emission intensity as a result of release from probe (proximity of the quencher fluorophore; 63, 65). Solid symbols represent assay results using RNA extract aliquots not treated with DNase (DNA and RNA templates). Open symbols represent assay results using DNase treated RNA extracts (RNA template only). Results shown represent assays performed using primer pairs PF and PR-a (■, □); PF and PR-b (●, ○); and PF and PR-c (▲, △). Figure was adapted from Norton and Batt (63).
of molecular diagnostics. Other methods including Qβ replicase amplification, self-sustained sequence replication (3SR, NASBA™), and the ligase chain reaction (LCR) are reviewed in detail by other authors (21, 23, 28).

Among a myriad of other applications, PCR is a powerful method for detection of foodborne pathogens, including *L. monocytogenes*. Shortly after the advent of this technology, researchers reported the specific detection of *L. monocytogenes* using PCR-based methods (29, 30). Over the next decade, numerous assays for detection of this organism with a variety of detection formats, including agarose gel electrophoresis, Southern hybridization, dot blot, reverse dot blot, ELISA-based formats and, more recently, homogenous (closed tube) detection formats were developed (21, 28, 31). Although many detection formats require extensive post-amplification handling, the adaptation of a microplate format and the advent of methods allowing homogenous detection have greatly reduced labor and facilitated the standardization and commercialization of PCR-based detection methods.

**Challenges in Development of PCR-Based Detection Assays**

PCR is a powerful molecular technique that exponentially amplifies specific target DNA sequences. The utility of this technology, however, can be limited by a number of factors. Challenges in the development of PCR assays often limit their practical application by testing laboratories and members of the food industry.

As with other molecular-based detection methods, selection of the appropriate target sequence is crucial for assay specificity. The sequence should be unique to the organism of interest, but present in all subtypes targeted by the assay. PCR primers are often designed based upon a *priori* knowledge of specific gene sequences and functions that are unique to the organism (e.g., genes encoding virulence factors). The gene encoding Listeriolysin O, *hlyA*, is the most common target of PCR-based assays for the detection of *L. monocytogenes*. **Other targets include iap** (invasion associated protein p60) and *lmaA* (listerial antigen associated with delayed type hypersensitivity in *Listeria*-immune mice; 28). Primers targeting *L. monocytogenes*-specific ribosomal RNA sequences may help to improve the detection limit of the assay because multiple copies of these genes are present on the genome (30, 32). Molecular methods including genomic subtraction (33) and random amplified polymorphic DNA analysis (RAPD; 34) are useful for identification of unique sequences which may not have been previously defined. RAPD analysis was used to identify sequences unique to *L. monocytogenes* and *Listeria* spp. for the development of the BAX® for Screening system (35). Primer validation with a number of nontarget organisms along with multiple subtypes of the target organism is of critical importance for successful application of screening methods. For example, DNA from some *L. monocytogenes* serotype 4c strains was not amplified by primers based upon the *hlyA* gene in a study by Johnson et al. (36). Results from the same study revealed the presence of the *lmaA* gene (previously thought to be exclusive to *L. monocytogenes*) in *L. ivanovii* and *L. innocua*, which are not of concern to human public health.

Although simple in theory, the optimized PCR reaction depends upon the appropriate concentration of reagents and optimized reaction conditions (28, 37). For example, the assay is highly sensitive to the concentration of the polymerase co-factor, MgCl₂. An inappropriately low concentration will limit or prevent amplification, whereas nonspecific priming and a loss of assay specificity will occur if the concentration is too high. The selection of appropriate primer annealing temperature is also critical; hybridization will not occur if the temperature is too high and nonspecific priming will occur if the temperature is too low. Thus, careful reagent and protocol standardization, along with simplification of reaction setup, are essential for reproducible results and the minimization of user error.

Unequivocally, the most challenging aspect of PCR-based detection method development, for *L. monocytogenes* as well as other foodborne pathogens, is the achievement of a low detection limit. Theoretically, PCR-based technology should reliably provide the detection level of ≤1 CFU/25 g food sample mandated by the zero tolerance ruling. Assay sensitivity, however, is complicated by a number of factors, including low contamination levels, uneven distribution of organisms in sample matrices (thus the possibility of a nonrepresentative sample), large sample volumes relative to small ultimate reaction volumes, and inhibition of the PCR reaction by components of the food matrix (38, 39).

Although PCR is a powerful technology, the reactions can be dramatically affected by the presence of inhibitory compounds common to foods and selective microbiological media. As described in a comprehensive review by Wilson, inhibitors may hinder assay performance by interfering with cell lysis and DNA purification, by capture or degradation of the DNA template, and/or by denaturation or inhibition of the DNA polymerase (39). Food components including phenolic compounds, proteins, glycogen, fats, and calcium ions all have inhibitory effects on PCR (38, 39). As a result, sample enrichment is generally required to achieve detection limits below 10⁵–10⁶ CFU/g (28). Components of media commonly used to isolate *L. monocytogenes*, including esculin, bile salts, and acriflavin, also inhibit PCR (38), thus necessitating careful selection of enrichment media or template purification before amplification.

The development of strategies to overcome reaction inhibition, thereby improving assay sensitivity, has received much attention. Significant effort has been dedicated to improved cellular and DNA purification techniques, with methods including centrifugation, sample filtration, immunomagnetic separation (21, 28), and cell concentration via immobilization with metal hydroxides (40, 41). Because it is difficult to standardize such methods for routine application with a variety of samples, the method most commonly used in commercially available assays is dilution of inhibitory compounds by sample enrichment. Although dilution reduces assay sensitivity, the increase in target DNA caused by cell growth helps to overcome this limitation. Sample enrichment, however, precludes quantitation of initial inoculum levels. Interestingly,
some studies have shown that use of the appropriate polymerase or a cocktail of different polymerases could help to overcome reaction inhibition (39, 42). A study evaluating 9 thermostable polymerases, for example, showed that the sensitivity differed among various inhibitors common to food and biological samples (42).

False-positive PCR results, another important issue, may be caused by the introduction of target sequences from the environment, carryover of DNA from one sample to the next during preparation or, most commonly, contamination by amplicons from previous reactions. A number of recommendations, including physical separation of DNA extraction, PCR setup and post-amplification areas, strict adherence to aseptic technique, the use of aerosol-resistant pipet tips, and laboratory surface treatment with sodium hypochlorite or UV irradiation, help to minimize the potential for cross-contamination (43). Photochemical modification of PCR products prevents their amplification if carried over to subsequent reactions (44). The use of the enzyme uracil-n-glycosylase (UNG; or uracil DNA glycosylase, UDG) also greatly reduces the risk of false-positive reactions caused by carryover of amplicons from previous assays (45). Briefly, the assay is designed to incorporate uracil into amplicons during synthesis. Treatment of subsequent reactions with UNG before amplification results in specific degradation of fragments containing uracil. Thus, products carried over from previous assays can no longer serve as templates.

**Commercial Assays for Detection of L. monocytogenes**

Commercially available PCR-based systems for detection of *L. monocytogenes* address many of the challenges previously limiting the routine application of this technology by testing laboratories and members of the food industry. Currently, several systems, including DNA-Detect™ (Vita-Tech, Markham, Ontario, Canada), Foodproof® (BIOTECON Diagnostics, Hamilton Square, NJ), Probelia™ (Bio-Rad Laboratories, Hercules, CA), and the BAX for Screening (Qualicon, Inc., Wilmington, DE), all allow specific detection of *L. monocytogenes*. The DNA-Detect system, developed for research and development applications, has a detection limit of 10–50 CFU/g and has been evaluated for a variety of environmental and food samples (A.F. Choo, Vita-Tech, personal communication). Following a 3–6 h enrichment step in transport media, DNA is prepared with spin-column technology. Following amplification, reaction products are visualized via agarose gel electrophoresis. Included in the system are DNA extraction kits, *L. monocytogenes*-specific primers, and a DNA amplification control to be run with each batch of samples. All other reagents and equipment are supplied by the user. The system is highly sensitive. An internal control for reaction failure or inhibition by components of the food matrix has not yet been developed, however, and most components of the system are not standardized for multiple users. Thus, while the currently available assay is ideal for research and development applications, the manufacturer does not recommend its use for diagnostic purposes.

The Foodproof system, developed for food testing applications, offers specific detection of *L. monocytogenes* in a variety of samples in 1 to 2 days after the sampling date (http://www.bc-diagnostics.com). Preparation of DNA template from a 24–48 h enrichment culture (incubation time dependent upon food matrix) via cell lysis allows a detection limit of approximately 10^2 CFU/mL. Amplification and PCR-product detection can be achieved by 2 different formats. One version of this system uses LightCycler™ technology (Roche Diagnostics Corp., Indianapolis, IN), which allows DNA amplification and real-time automated detection in a single tube and a single instrument. Briefly, amplicons are detected by a fluorescence resonance energy transfer (FRET) assay (46, 47). DNA probes, labeled with donor and acceptor fluorophores, are designed to hybridize to the target DNA region. Upon excitation of the donor fluorophore, energy is transferred between donor and acceptor if they are proximally located, i.e., if the probes are bound to the reaction products. An increase in emission intensity of the acceptor fluorophore indicates the presence of target DNA, thus a positive result. Data analysis and interpretation are performed by accompanying software. False-positive reactions due to product carryover are minimized by lack of post-amplification handling and by the use of UNG. An internal amplification control indicates negative results arising from reaction inhibition or failure. Although initial investment for this system is great, the assay offers a dramatic reduction in time to positive results and high throughput capability. The probes used by the product detection system impart an additional level of assay specificity and the level of automation minimizes labor and potential for user-introduced errors. Further, the detection format provides a quantitative read-out, allowing DNA template quantitation and, potentially, estimation of the levels of *L. monocytogenes* in the sample.

The second version of the Foodproof system uses an ELISA-based sandwich hybridization detection format. Following amplification, the hybridization of PCR products to nonradioactively labeled, target-specific DNA probes is detected photometrically by a color reaction. The internal amplification control is detected in a separate hybridization with a control-specific probe. Although more labor-intensive, the ELISA-based format also provides results in 1–2 days after sampling and may be less cost-prohibitive because much of the required equipment may already be in place.

The Probelia PCR system also provides standardized, specific detection of *L. monocytogenes* from a variety of food samples within 1–2 days after the sampling date. All reagents for DNA preparation, amplification, and product detection are included in 2 (amplification and detection) kits. Following a 24–48 h enrichment (2-step, 48 h enrichment for meat products), DNA is prepared using a cell lysis procedure (Technical Assistance, former distributor BioControl, Inc., Bellevue, WA, personal communication). A centrifugation step concentrates the culture by a factor of 5, thus improving assay sensitivity. This system incorporates UDG to minimize carryover contamination and includes an internal control as a monitor for successful DNA amplification in each reaction. Similar to the Foodproof system, the detection format is an
ELISA-based sandwich hybridization assay using target-specific DNA probes and a colorimetric assay. Results are determined objectively by the user based upon optical density readings. Advantages offered by this system include a high level of standardization, reduced time to results as compared to culture-based methodology, and high throughput capabilities. The Probelia system has been certified by the Association Française de Normalisation (AFNOR), the bureau responsible for validation of rapid methods in France.

The BAX for Screening, available in formats providing specific detection of *L. monocytogenes* or *Listeria* spp., within 2 days after the sampling date, is a highly standardized PCR-based system developed for food and environmental sample testing applications. The system follows a basic 3-step protocol, including sample enrichment and DNA preparation by cell lysis, DNA amplification, and reaction product detection. This system is unique in that PCR-reaction setup is simplified by the provision of reaction tubes prepackaged with tablets containing lyophilized amplification reagents. A reagent supplement designed to minimize reaction inhibition caused by components of the food matrix (Technical Assistance, Qualicon, Inc., personal communication), and internal controls for DNA amplification are also included in the lyophilized tablet (48). The assays have been validated with several enrichment protocols, allowing the user to customize the protocol according to the sample matrix. As few as 1 CFU/25 g sample can be detected when the appropriate enrichment is used (Technical Assistance, Qualicon, Inc., personal communication). Following amplification, reaction products are detected by 1 of 2 formats. One format entails detection by agarose gel electrophoresis of reaction products in a format adapted for use with multichannel pipets. Strict adherence to aseptic techniques, along with physical separation of sample preparation, DNA amplification, and PCR-product detection areas, minimizes the potential for carryover contamination.

The second detection format, recently released by Qualicon, Inc., uses a fully automated system enabling amplification and homogenous amplicon detection in a single cycler/detector instrument. Briefly, detection is achieved by melting curve analysis (31, 49). Reactions are performed in the presence of the DNA intercalating fluorescent dye SYBR Green I (Molecular Probes, Eugene, OR), which fluoresces only when bound to double-stranded DNA. Following amplification, melting curves are generated. The fluorescence is monitored while the sample temperature is increased. The target amplicon denatures, or melts apart, to form single-stranded DNA at a temperature specific for that amplicon. When the amplicon denatures, the SYBR Green I dissociates from the DNA and the fluorescence decreases significantly. Mathematical integration results in a graphical curve with a peak at the temperature at which the amplicon denatured. Because the melting temperature of a DNA fragment is dependent upon its length and nucleotide content, this technology enables detection and identification of specific reaction products.

Data collection and analysis are performed by system software, which provides a positive/negative sample readout for the user. The potential for carryover contamination is minimized (if not eliminated) because amplification and detection are performed in a single closed tube. The system reduces the time to definitive results to 2 days after sampling; it is highly standardized and the detection format is fully automated. Further, the temperature cycling conditions for the BAX for Screening/**L. monocytogenes, Listeria* spp., *Escherichia coli* O157:H7, and *Salmonella* are identical, allowing simultaneous testing for each organism. The system using the electrophoresis-based detection format, while slightly more labor-intensive, may be less cost-prohibitive for smaller laboratories if the equipment is already in place. AOAC INTERNATIONAL Performance-Tested Certification has been granted to the gel-based *L. monocytogenes* system. The certification process for the automated system has been initiated.

**Practical Application of PCR-Based Methods for Detection of *L. monocytogenes***

The development of improved strategies for control of *L. monocytogenes* will be greatly facilitated by a better understanding of its ecology in foods and in the food processing environment. Using the smoked fish industry as a model, Norton et al. (12, 50) applied commercially available PCR-based detection strategies and molecular typing methods to explore the ecology of *L. monocytogenes* in foods and the food processing environment, and to develop a system for routine monitoring of this organism. The results of these studies conclusively demonstrated the utility of PCR-based methodology and highlighted some of the challenges of assay development. Over 500 raw materials, fish in process, cold-smoked fish, and environmental samples collected from 3 smoked fish processors were screened for the presence of *L. monocytogenes* by the BAX for Screening (gel-based detection system). The PCR-based screening assay enabled reliable, efficient detection of *L. monocytogenes* in a variety of sample types. Results indicated a 28 and 8% prevalence of this organism in environmental and raw materials samples, respectively, establishing potential sources of finished product contamination within 2 days after sampling. In comparison, the presence of *L. monocytogenes* in samples using culture-based methodology was confirmed in a minimum of 4 days after sampling.

Using a modification of the FDA method for isolation of *L. monocytogenes* as the standard of comparison, results indicated a sensitivity (true positive rate) of 91.8% and a specificity (true negative rate) of 96.2% for the BAX system after re-examination of samples initially yielding discrepant results from PCR- and culture-based screening (12). False-negative results (PCR system-negative/culture-positive) were likely caused by low levels of *L. monocytogenes* in the enrichment culture. Less than 1 × 10^5 CFU/mL *Listeria*-like colonies, below the PCR system detection limit of 1 × 10^5 CFU/mL, were isolated from corresponding sample enrichment cultures. Pure-culture isolates from these samples were correctly identified by the BAX system, indicating that false-negative results were not due to the presence of nonreacting isolates. The use of an alternative enrichment protocol may improve assay sensitivity. The BAX system false-positive reactions were
likely due to failure of the culture-based method to detect *L. monocytogenes* among a high background of other *Listeria* spp. as opposed to cross-reaction with other species by the BAX system. Several studies have shown that other *Listeria* spp., specifically *L. innocua*, can outcompete *L. monocytogenes* during enrichment (19, 20). Standard culture confirmatory screening requires that only 5 *Listeria*-like colonies from isolation media be characterized (17) and may indicate a negative culture-result if *L. monocytogenes* is present as a small percentage of the entire *Listeria* population. This highlights one of the advantages of DNA amplification-based screening assays, because all the organisms in the sample aliquot are tested. Further, the BAX system demonstrated 100% exclusivity for 60 *Listeria* spp. strains and 44 non-Listeria strains in validation studies (51).

The BAX for Screening/Genus *Listeria* was applied for detection of *Listeria* spp. in a subset of samples (50). *Listeria* spp. were detected in 40% of the samples. Comparison with culture-based methodology indicated 89.9% sensitivity and 96.2% specificity for this assay. Similar to findings with the *L. monocytogenes* assay, false-negative reactions were likely caused by the presence of low levels of *Listeria* spp. in the enrichment culture. Results from a subsequent study screening environmental samples, in which a 2-step enrichment protocol was used, indicated an improved sensitivity of 94.7% and similar specificity for this system (52). Interestingly, results from the same study indicated a sensitivity of 84.4% for the *L. monocytogenes* system when applied for screening raw fish samples enriched by the 2-step procedure. These results highlight the importance of an optimal enrichment protocol for a given sample type and the potential impact of such protocols on sample screening. Thus, manufacturers of the Foodproof and Probelia detection systems provide sample-specific enrichment protocols. As discussed above, the BAX system has been optimized for use with several enrichment protocols, allowing the user to select the protocol appropriate for a given sample type.

Inherent differences in food matrices and enrichment procedures make it difficult to directly compare performance studies for commercially available screening assays. Sensitivity and specificity values reported in the studies discussed above are, nonetheless, cognate with those reported for other assays. Results of a collaborative study evaluating the DNA hybridization-based GENE-TRAK® assay for *Listeria* spp. indicated sensitivity and specificity values of 60–100 and 92–100%, respectively, for a variety of inoculated and naturally contaminated food samples (25). A similar study evaluating the performance of the immunochemical (ELISA-based) assay *Listeria*-Tek™ for detection of *L. monocytogenes* in inoculated food samples reported sensitivity and specificity values of 64–100 and 90–98%, respectively (24). Both BAX systems provided definitive, reliable results within 2 days after sampling, and are thus ideal for industry applications, including routine monitoring of *L. monocytogenes* in food and environmental samples.

### PCR-Based Methodology

PCR-based methodology provides an efficient means for monitoring the presence of *L. monocytogenes* in a variety of food and environmental samples. One potential caveat of PCR, however, is the inability to distinguish between viable and nonviable organisms in the direct testing of food samples. As only viable *L. monocytogenes* can cause disease in humans, the detection of dead cells in a finished product constitutes a false-positive reaction. Several studies have demonstrated the persistence of target DNA following cell inactivation by heat, starvation, desiccation, and acid (53–55). Organisms that contaminated a food prior to a microbial inactivation step would, therefore, still be detectable in the finished product by a PCR-based assay. The use of an enrichment culture helps to overcome this issue, but eliminates the potential for quantitation and relies upon recovery and growth of stressed or injured organisms. Recently, conclusive evidence was presented for the viable but nonculturable (VBNC) state in *L. monocytogenes* (56). Organisms in this state, which is often induced by stress, are nonculturable by standard methods but maintain metabolic activity (57). These organisms may retain their pathogenic potential under favorable conditions. A nonculture-based assay allowing discrimination between viable and nonviable organisms would, therefore, be an attractive sample screening tool.

Messenger RNA (mRNA) is a promising target for detection assays. mRNA, produced only by viable organisms, is rapidly degraded and thus should serve as an accurate indicator of cell viability. Further, gene expression results in the production of multiple copies of mRNA, thereby increasing the number of potential target sequences and assay sensitivity. Reverse transcriptase-PCR (RT-PCR) assays have been successfully developed for detection of pathogens, including *Vibrio cholerae* (58), *Cryptosporidium parvum* (61), *Legionella* (60), and *Giardia* (61).

Several groups have reported the development of nucleic acid amplification-based assays for *L. monocytogenes* targeting RNA (53, 54, 62, 63). A nucleic acid sequence-based amplification (NASBA) method targeting *hlyA* allowed for sensitive (<10 CFU/mL), specific detection of *L. monocytogenes* from dairy products after 48 h enrichment (62). Reaction products were detected by hybridization with a capture probe; a sensitive, albeit labor-intensive format. NASBA technology is advantageous in that the reaction is isothermal, which may reduce equipment costs, and extremely efficient (up to 10³ amplification in 1.5 h). The isothermal reaction conditions result in high specificity for single-stranded nucleic acids and, therefore, the ability to distinguish RNA and DNA targets. NASBA-based assays are, however, subject to the same potential as PCR for contamination by extraneous nucleic acids (including amplimers carried over from previous reactions). Assay optimization is also critical as the use of multiple enzymes and low reactant stringency may lower the inherent specificity (23).

Klein and Juneja (54) reported the development of a one-tube RT-PCR assay targeting the *iap* gene for detection of...
L. monocytogenes. The use of polymerase \textit{rTth} (Applied Biosystems, Foster City, CA), which can function as both a thermostable RNA polymerase and thermostable DNA polymerase (64), simplified the reaction protocol, thus minimizing the potential for user error. A potential caveat of this method is that \textit{rTth} amplifies RNA and DNA (thus a positive reaction could result from the presence of nonviable cells), but RNA specificity was achieved by DNase treatment of the template. The assay enabled detection of 3 CFU/g from inoculated ground beef samples after a 2 h enrichment. Reaction products were detected by Southern hybridization. Although this detection format offered greater sensitivity than gel electrophoresis, it extended the assay time to >50 h and is not amenable to screening large sample numbers.

To reduce the time to results and lend the assay to automation, a fluorogenic 5' nuclease assay targeting the \textit{hlyA} gene was successfully adapted for detection of mRNA and, therefore, viable \textit{L. monocytogenes} using \textit{rTth} (63, 65). Briefly, the 5' nuclease assay capitalizes on the endogenous 5'\textrightarrow;3' exonuclease activity of \textit{Taq} DNA polymerase (Figure 2; 66–68). A fluorescently labeled probe, which is incorporated directly into the master mix, is designed to hybridize to the target DNA region between the oligonucleotide primers. As \textit{Taq} proceeds along the template, the hybridized probe is digested, resulting in release of a reporter fluorophore and a corresponding increase in emission intensity. The assay is highly specific, amenable to automation, and provides a quantitative readout within 10 min of assay completion. Commercial 5' nuclease assays (targeting DNA) have been released for detection of \textit{Salmonella} and \textit{E. coli} O157:H7 from food and environmental samples (Applied Biosystems), highlighting the assay's potential as a routine screening method.

A critical step in the development of an amplification assay targeting RNA is the selection of the appropriate target. As previously discussed with regard to PCR, the sequence should be unique to the organism to be detected while allowing detection of all strains and subtypes. The selection of a target for RNA-based assays is more complex, however, as regulation of gene expression and transcript stability must also be considered. Ribosomal RNA, for example, is quite stable and present in high copy numbers (23, 69). It would not, therefore, serve as a suitable indicator of cell viability. mRNA is less stable, and is generally degraded within minutes of expression (70, 71). Ideally, the target gene would be constitutively expressed, with little or no regulation in response to environmental conditions. Targets ideal for PCR assays for detection of \textit{L. monocytogenes}, including unique but highly regulated virulence genes, may not be suitable for RNA amplification-based assays. Klein and Juneja (54), for example, evaluated the suitability of the genes \textit{hlyA}, \textit{prfA} (which encodes the virulence gene positive regulatory factor PrfA), and \textit{iap}. Markedly different expression levels were observed for each under similar incubation conditions. The \textit{iap} gene, shown previously to be constitutively expressed (72), provided the highest sensitivity level and an accurate indication of cell viability. Conversely, an assay targeting a stringently regulated heat shock gene allowed sensitive detection of viable \textit{Giardia} cysts (61). Gene expression was induced before RNA extraction by exposure to high temperature.

There is also evidence for a pronounced affect of primer location within a gene target on the outcome of an RNA amplification-based assay. Several reverse primers, which altered the position of the 3' end of the amplicon, were evaluated during development of the RT-5'-nuclease assay targeting the \textit{hlyA} gene of \textit{L. monocytogenes} (Figure 3a; 63). Distal primers provided an accurate indication of cell viability following heat activation of cells (Figure 3b). The most internal primer, however, overestimated cell viability. The authors concluded that apparent differences in longevity of the regions defined by the primer sets may have been due to structural features offering protection from cellular RNases and 3' endonucleases (63, 70). Specifically, analysis indicated significant secondary structure in the region of the transcript between primers PR-a and PR-b (Figure 3a).

Inherent challenges in the development of RNA amplification-based assays have limited their widespread application in food microbiology. Such assays have the potential, however, to provide specific detection of viable organisms with improved sensitivity. These features may realize great importance in the future, particularly if regular end-product testing is prioritized by regulatory agencies.

Conclusions

The future of rapid detection methods calls for continued development of assays allowing for real-time (ideally, on-line) detection of low levels of \textit{L. monocytogenes} from a variety of samples. If tolerance levels are established for certain \textit{L. monocytogenes} subtypes or all subtypes in specific foods, as have been adopted in Denmark and Canada (73), the development of a rapid, quantitative assay is critical. Thus, continued efforts toward improved methods for sample preparation and template purification should remain a priority. Further, as increasing evidence is gathered in support of virulence differences among subtypes of \textit{L. monocytogenes} common to foods and the food processing environment (74–78), assays that allow subtype differentiation may become part of routine monitoring programs. Assays allowing rapid differentiation of viable and nonviable cells may be of great importance in the future, particularly if routine end-product testing is prioritized.

However, the overall goal of a specific program must be carefully considered in deciding upon a detection method for \textit{L. monocytogenes}. Rapid methods, for example, are ideal for routine monitoring of this organism in foods and food processing environments. However, the use of traditional methods for detection of \textit{L. monocytogenes} remains an important component of sample analysis for testing laboratories and members of the food industry. In fact, while agencies, such as AOAC INTERNATIONAL, accept negative rapid screening results as definitive, positive sample screening results are regarded as presumptive and must be confirmed using culture techniques (Wallace Andrews, FDA, Center for Food Safety and Applied Nutrition, personal communication). Further, subtyping methods such as ribotyping, MEE, and PFGE (79),
critical to the establishment of in-plant contamination patterns and surveillance of L. monocytogenes, rely on culture-based isolation of this organism.

Current demographic and technological trends in the United States strongly support continued regulatory and research emphasis on L. monocytogenes. Our population is aging, new medical technologies allow individuals to live with conditions that compromise the immune system, and consumers are demanding lightly processed foods with increased shelf lives. As each of these trends is associated with either increased susceptibility to disease or to the increased presence of L. monocytogenes in foods, reduction of the levels of this organism in foods is imperative. As discussed here, comprehensive monitoring programs featuring efficient, reliable molecular detection methods including PCR-based assays, will provide information critical to the development of improved strategies for control of this organism in foods and in the food processing environment.

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