Sensitive Detection of Viable _Listeria monocytogenes_ by Reverse Transcription-PCR

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Detection of pathogens in contaminated food products by PCR can result in false-positive data due to the amplification of DNA from nonviable cells. A new method based on reverse transcription-PCR (RT-PCR) amplification of mRNA for the specific detection of viable _Listeria monocytogenes_ was developed. The expression of three _L. monocytogenes_ genes, _iap_, _hly_, and _prfA_, was examined to determine a suitable target for amplification of RT-PCR. Total RNA from _L. monocytogenes_ was isolated, and following DNase treatment, the RNA was amplified by both RT-PCR and PCR with primers specific for the three genes. Amplicon detection was accomplished by Southern hybridization to digoxigenin-labeled gene probes. The levels of expression of these three genes differed markedly, and the results indicated that the _iap_ gene would provide a good target for development of a specific method for detection of viable _L. monocytogenes_ based on RT-PCR amplification. After a 1-h enrichment, the 371-bp _iap-specific_ product was detected with a sensitivity of ca. 10 to 15 CFU/ml from pure culture. Detection of the 713-bp _hly-specific_ amplicon was ca. 4,000 times less sensitive after 1 h, whereas detection of the 508-bp _prfA_ product showed the lowest level of sensitivity, with detection not observed until after a 5-h enrichment period. The amplification of the _iap_ mRNA was specific for _L. monocytogenes_. Overall, the assay could be completed in ca. 54 h. The use of RT-PCR amplification for the detection of viable _L. monocytogenes_ was validated in artificially contaminated cooked ground beef. Following a 2-h enrichment incubation, the _iap-specific_ amplification product could be detected in a cooked meat sample that was originally inoculated with ca. 3 CFU/g. These results support the usefulness of RT-PCR amplification of mRNA as a sensitive method for the specific detection of viable _L. monocytogenes_ and indicate that this method may prove useful in the detection of this pathogen in ready-to-eat, refrigerated meat products.

_Listeria monocytogenes_ is a gram-positive intracellular organism causing severe infections that primarily affect pregnant women, newborns, and immunocompromised individuals (43). While _Listeria_ spp. are ubiquitous in nature, only _L. monocytogenes_ is pathogenic to humans. In recent years, a number of outbreaks of food-borne illness involving a wide range of foods have been linked to _L. monocytogenes_ (16). Elimination of this organism from foods is extremely difficult due to its widespread distribution and ability to grow at refrigeration temperature (4°C). The prevention of further outbreaks of listeriosis will require validation of pathogen interventions around critical control points in food processing. The development of sensitive and specific methods for the detection of _L. monocytogenes_ will play a major role in accomplishing this goal.

Traditional testing methods for the detection of _L. monocytogenes_ have relied almost exclusively on the use of specific microbiological media to isolate and enumerate viable bacterial cells from foods followed by a series of biochemical and serological tests for final confirmation (16, 35). Conventional culture-based methods are labor-intensive and time-consuming, in many instances requiring 5 to 10 days to complete. To overcome these limitations, a number of molecular biology-based techniques for the rapid detection of _L. monocytogenes_ have been developed in recent years, including immunoreassays, nucleic acid-based hybridization assays, and PCR-based methods (for reviews, see references 20, 22, and 44). While the immunogenic approach is rapid, nonspecific detection due to cross-reactivity may occur. Nucleic acid-based hybridization assays are rapid and have high specificity. However, at least 10³ to 10⁴ DNA targets are required to achieve a detectable hybridization signal. Similarly, PCR amplification techniques show a high degree of specificity and have the added advantage of extreme sensitivity (for a review, see reference 44). However, a disadvantage of conventional PCR techniques is that both viable and nonviable cells may be detected (1, 34). This is overcome in many cases by inclusion of an enrichment step to dilute out any nonviable cells that may be present (23, 38, 40). However, the use of enrichment techniques prior to PCR can prolong analysis times, eliminating much of PCR’s high-sensitivity advantage. New types of culture media designed to reduce enrichment times (17, 39) and the use of immunomagnetic particles to concentrate bacteria (12, 18, 26) have been reported. Although these techniques have been somewhat successful at decreasing the time necessary for pathogen detection, they may not be able to unequivocally demonstrate whether the cells are alive or dead. Therefore, an alternative method for detection of bacterial cells that combines sensitivity and specificity with the ability to differentiate between viable and nonviable cells is needed.

The present study was undertaken to develop a sensitive method for the detection of viable _L. monocytogenes_ cells based on amplification of mRNA by reverse transcription-PCR (RT-PCR) technology. The expression of three different _L. monocytogenes_ genes (_iap_, _hly_, and _prfA_) was examined to determine a suitable target for RT-PCR amplification. The _iap_ gene codes for p60, a major extracellular protein of _L. monocytogenes_ that is thought to be associated with invasion of...
nonprofessional phagocytic cells (30). The hly gene encodes the 58-kDa virulence factor listeriolysin O (36), whereas the prfA gene codes for a 27.1-kDa protein that has been shown to positively regulate the expression of several Listeria virulence factors (10, 31, 37). Detection methods based on PCR amplification of the iap (8, 13, 38), hly (3, 4, 11, 17, 21, 23, 25, 38, 40), and prfA (11) gene sequences have been reported. This report details a sensitive RT-PCR-based detection system for viable L. monocytogenes cells. By combining RT-PCR amplification of iap mRNA with a 1-h enrichment incubation and ampiclon detection by Southern hybridization, sensitivity values for the assay were found to be ca. 10 to 15 CFU/ml for pure L. monocytogenes cultures. The ELMIAPF and ELMIAPR primers, used for amplification of an iap-specific sequence, were previously reported by Bubert et al. (8). All other primer sets were designed by using Oligo 5.0 primer analysis software (National Biosciences, Inc., Plymouth, Minn.). External gene primers for RT-PCR amplification of the specific mRNA species (i.e., LMIAP, LMLHY, and LMPRFA) are designated with an E prefix, whereas the internal primer pairs used for making the digoxigenin (DIG)-labeled probes for Southern hybridization are designated with an I prefix. Forward 3’ primers and reverse 3’ primers are indicated with F and R suffixes, respectively. Oligonucleotides were synthesized at the Macromolecular Structure Analysis Facility of the University of Kentucky, Lexington. RT-PCR and PCR amplifications. RT-PCR of Listeria RNA and PCR amplification of Listeria RNA or DNA was conducted with a GenAmp PCR system 9600 thermal cycler and either the GeneAmp EZ r Th Tth DNA polymerase. The temperature cycling routine for denaturation, annealing, and extension was as described above. A 10-μl aliquot of each amplification reaction mixture was electrophoresed through a 1.5% agarose gel in Tris-acetate-EDTA buffer (pH 8.2) and stained with ethidium bromide. For PCR amplification reaction mixtures (total volume, 50 μl) contained the following: either 10 μl of DNase-treated RNA or 5 μl of genomic DNA: 200 μM (each) dATP, dCTP, dGTP, and dTTP; 50 mM MgCl2; 0.01% (vol/vol) gelatin; 0.5 μM each primer; and 2.5 U of AmpliTaq DNA polymerase. The temperature cycling routine for denaturation, annealing, and extension was as described above. A 10-μl aliquot of each amplification reaction mixture was electrophoresed through a 1.5% agarose gel in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate [pH 8.0], 1 mM EDTA), and the gel was then stained with ethidium bromide. DIG-labeled probe preparation. Three internal primer sets (ILMIAPF-ILMIAPR, ILMLHYF-ILMLHYR, and ILMPRFAF-ILMPRFAAR) were employed for the generation of DIG-labeled DNA probes for use in detecting the amplified RT-PCR products. A 119-bp iap-specific probe, a 188-bp hly-specific probe, and a 186-bp prfA-specific probe were generated by incorporation of DIG-DUTP during PCR amplification according to the manufacturer’s protocol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Briefly, PCRs for the production of each gene-specific probe were performed in a final volume of 50 μl that contained the following: 200 μM (each) dATP, dCTP, and dGTP; 10 μM dTTP; 70 μM DIG-11-DUTP (Genius Systems; Boehringer Mannheim); 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris-HCl (pH 8.3); 0.01% (vol/vol) gelatin; 0.5 μM each primer; and 2.5 U of AmpliTaq DNA polymerase. Digestion of DNA with restriction endonucleases and Southern hybridization were performed as described previously (4). DIG-labeled probes were hybridized to agarose gels at 42°C in Church’s hybridization buffer (13). Positive DIG-labeled ampiclon bands were visualized immediately after completion of the hybridization. The supernatant fluid was aspirated, and the cell pellets were immediately frozen in a dry ice-ethanol bath. For all experiments, the cells were collected by centrifugation for 10 min at 4°C. Ten-ml aliquots were removed for both RNA and DNA extraction. The cells were then heat killed by autoclaving for 15 min at 121°C. Following incubation for the extraction of total bacterial RNA, 50 ml of diethyl pyrocarbonate-treated sterile water was added to the sample. RNA was then incubated at 65°C for 10 min to inactivate the DNase. Half of the sample was then used in RT-PCR amplifications, and the other half was DNase-treated RNA; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris-HCl (pH 8.3); 0.01% (wt/vol) gelatin; 0.5 μM each primer; and 2.5 U of AmpliTaq DNA polymerase. The temperature cycling routine of denaturation, annealing, and extension consisted of 1 cycle at 60°C for 35 min for RT of the RNA followed by a temperature cycling routine of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min. After 40 cycles, a final extension at 72°C for 5 min was performed and the tubes were cooled to 4°C. The samples were incubated at 4°C for 15 min to inactivate the DNase, and the sample was then incubated at 65°C for 10 min to inactivate the DNase. The temperature cycling routine for denaturation, annealing, and extension was as described above. A 10-μl aliquot of each amplification reaction mixture was electrophoresed through a 1.5% agarose gel in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate [pH 8.0], 1 mM EDTA), and the gel was then stained with ethidium bromide. DIG-labeled probe preparation. Three internal primer sets (ILMIAPF-ILMIAPR, ILMLHYF-ILMLHYR, and ILMPRFAF-ILMPRFAAR) were employed for the generation of DIG-labeled DNA probes for use in detecting the amplified RT-PCR products. A 119-bp iap-specific probe, a 188-bp hly-specific probe, and a 186-bp prfA-specific probe were generated by incorporation of DIG-DUTP during PCR amplification according to the manufacturer’s protocol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Briefly, PCRs for the production of each gene-specific probe were performed in a final volume of 50 μl that contained the following: 200 μM (each) dATP, dCTP, and dGTP; 10 μM dTTP; 70 μM DIG-11-DUTP (Genius Systems; Boehringer Mannheim); 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris-HCl (pH 8.3); 0.01% (vol/vol) gelatin; 0.5 μM each primer; and 2.5 U of AmpliTaq DNA polymerase. Digestion of DNA with restriction endonucleases and Southern hybridization were performed as described previously (4). DIG-labeled probes were hybridized to agarose gels at 42°C in Church’s hybridization buffer (13). Positive DIG-labeled ampiclon bands were visualized immediately after completion of the hybridization. The supernatant fluid was aspirated, and the cell pellets were immediately frozen in a dry ice-ethanol bath. For all experiments, the cells were collected by centrifugation for 10 min at 4,100 × g. The supernatant fluid was aspirated, and the cell pellets were immediately frozen in a dry ice-ethanol bath.

RNA and DNA isolations. Total RNA from Listeria spp. was isolated from frozen cell pellets by using a commercial FastPrep RNA isolation kit according to the manufacturer’s protocol (Bio101/Savant, La Jolla, Calif.). The precipitated RNA was resuspended in 55 μl of diethyl pyrocarbonate-treated sterile water and stored at −20°C for further use. Total genomic DNA was extracted from the frozen cell pellets by using the G-NOME DNA isolation kit according to the manufacturer’s protocol (Bio101/Savant). The precipitated DNA was resuspended in 50 μl of sterile distilled water and stored at −20°C for further use.

TABLE 1. RT-PCR primers used for the detection of L. monocytogenes mRNA

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
<th>Target gene</th>
<th>Size (position of amplified product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELMIAPF</td>
<td>CAACCTGTCATMCCAGCCTACT</td>
<td>iap</td>
<td>371 (1178–1549)</td>
</tr>
<tr>
<td>ELMIAPR</td>
<td>GCACCTGAATTGCTTATTG</td>
<td>iap</td>
<td>119 (1240–1359)</td>
</tr>
<tr>
<td>ILMIAPF</td>
<td>ACCACGACCTCCAGTATTA</td>
<td>hly</td>
<td>713 (160–2333)</td>
</tr>
<tr>
<td>ILMIAPR</td>
<td>GCCTCTGCAAACCTCTTTTA</td>
<td>hly</td>
<td>188 (1846–2034)</td>
</tr>
</tbody>
</table>

*Sizes are in base pairs; positions are in nucleotides.*
RT-PCR FOR DETECTION OF VIABLE L. MONOCYTOGENES

RESULTS

Optimization of the RT-PCR assay. Experiments were conducted to determine the optimal salt concentration for RT-PCR amplification for each of the three L. monocytogenes primer pairs. Multiplex RT-PCRs were performed with the GeneAmp EZ tRh DNA polymerase kit (Perkin-Elmer) as recommended by the manufacturer, except that the Mn(OAc)₂ concentration was changed from 1.5 to 3.5 mM. The primers for amplification of hly, prfA, and iap were chosen such that the theoretical primer melting points for each primer pair were similar, allowing for a single annealing temperature of 60°C. When RT-PCR amplification mixtures contained 1.5 mM Mn(OAc)₂, three products of 713, 508, and 371 bp were observed, corresponding to the hly, prfA, and iap fragments, respectively (Fig. 1, lane 1). Increasing the Mn(OAc)₂ concentration to 3.5 mM resulted in an increase in amplification of the hly and iap products; however, amplification of the prfA product was inhibited (Fig. 1, compare lanes 1 and 3). Therefore, in subsequent experiments with either the hly or iap primer set, RT-PCRs were performed with 3.5 mM Mn(OAc)₂ whereas amplification of the prfA product was performed in reaction mixtures containing 1.5 mM Mn(OAc)₂.

Sensitivity of the RT-PCR. The suitability of each of the three L. monocytogenes genes to provide a sensitive target for detection of low levels of viable bacteria was examined by performing RT-PCRs on RNA isolated from broth cultures inoculated with serial dilutions of an overnight L. monocytogenes culture after various enrichment times. After 1 h of enrichment, the 371-bp iap-specific product was detected by Southern hybridization at levels corresponding to an initial inoculum of ca. 3 CFU/ml (Fig. 2, 1-h enrichment, lane 1). Enumeration on TSA plates indicated that after 1 h of incubation the culture contained ca. 10 to 15 CFU/ml. The iap product was the result of amplification of mRNA and not DNA, as evidenced by the lack of an amplification signal following PCR with AmpliTaq DNA polymerase (Fig. 2, lanes 6 to 10). When the RT-PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining after 1 h of enrichment, the 371-bp iap fragment was first detected in the sample initially inoculated at a level of ca. 3,000 CFU/ml (data not shown). Enumeration on TSA plates revealed that this sample contained ca. 5,000 CFU/ml indicating that detection of the RT-PCR product by Southern hybridization was approximately 500 times more sensitive than detection by ethidium bromide staining. The hly-specific fragment was also detected by RT-PCR after a 1-h incubation period. The level of sensitivity for this message, however, was approximately 4,000-fold lower than that for the iap message (Fig. 3, 1-h enrichment, lane 5). The hly amplified product was detected only in cultures initially inoculated at the highest inoculum level of 3 × 10⁴ CFU/ml. Enumeration of this culture on TSA after 1 h of enrichment indicated that it had a bacterial count of approximately 4 × 10⁴ CFU/ml. Detection of the amplified hly message at each successive enrichment time was not observed until the culture reached a density of ca. 4 × 10⁵ CFU/ml (Fig. 3, 3-h enrichment, lane 4; 5-h enrichment, lane 3; 7-h enrichment, lane 2). Detection of the prfA message by RT-PCR showed the lowest level of sensitivity among the three genes examined (Fig. 4). The 508-bp prfA product was first...
primers were used for RT-PCR amplification and product detection. L. monocytogenes Scott A mRNA amplified with L. monocytogenes that prfA primer pair (Table 1) has previously been shown to specifically amplify product was observed following Southern hybridization (data not shown). These results indicate that RT-PCR amplification of mRNA with the iap primers can be used for the specific identification of L. monocytogenes.

**Ability of the RT-PCR assay to discriminate between viable and nonviable cells.** The ability of the RT-PCR assay to distinguish between viable and nonviable L. monocytogenes cells was examined by performing RT-PCR as well as PCR assays with RNA and DNA isolated from both live and heat-killed cells. When L. monocytogenes mRNA and DNA were extracted from live cells and amplified by RT-PCR and PCR, respectively, the 371-bp iap product was observed as expected (Fig. 6, lanes L). Furthermore, the iap gene sequence was detected by conventional DNA-based PCR immediately following heat treatment of the cells and for at least 6 h after heating (Fig. 6, PCR panel, lanes 0, 2, 4, and 6 h). Enumeration of the culture on TSA plates revealed that autoclaving had rendered the cell culture nonviable, indicating that the bacterial DNA was quite...
stably and capable of being amplified for hours following the loss of viability. In contrast, iap mRNA from heat-killed cells could not be amplified by RT-PCR at any time following the heat treatment (Fig. 6, RT-PCR panel, lanes 0, 2, 4, and 6 h) due to the rapid degradation of the mRNA following the loss of cell viability. Thus, the detection of mRNA by RT-PCR amplification should provide a more sensitive indicator of cell viability than detection of gene sequences by DNA-based PCR amplification.

Validation of the RT-PCR detection assay with a food product. The ability to detect viable L. monocytogenes cells in a food product by RT-PCR amplification of iap mRNA was examined. Cooked beef samples (autoclaved at 121°C for 15 min) were artificially contaminated with serial dilutions of L. monocytogenes ranging from ca. 0.3 to 30 CFU/g, and following enrichment incubation the RNA was isolated and then amplified by RT-PCR. Following a 2-h enrichment incubation, the iap-specific product was detected in cooked meat samples that had been originally inoculated with ca. 3 CFU/g (Fig. 7, 2-h enrichment, lane 3). Enumeration on TSA plates was unable to give an accurate bacterial count for this sample, as the bacterial population was too low; however, following the 2-h enrichment incubation, an iap-specific RT-PCR product was also detected in the cooked meat sample initially inoculated with 30 CFU/g, and enumeration of this sample on TSA indicated that there had been originally inoculated with ca. 3 CFU/g (Fig. 7, 2-h enrichment, lane 3).

FIG. 7. Detection of iap mRNA in artificially contaminated cooked beef by RT-PCR. Cooked ground meat was inoculated with serial dilutions of L. monocytogenes cells, and the samples were incubated for 2, 6, or 8 h. Following enrichment, RNA was extracted, DNase treated, and amplified by RT-PCR (lanes 1 to 4) or PCR (lanes 5 to 8). The amplification products were colorimetrically detected following transfer to nylon membranes and hybridization with the DIG-labeled internal iap probe. Lanes: 1 and 5, un inoculated control; 2 and 6, 0.3-CFU/g initial inoculum; 3 and 7, 3-CFU/g initial inoculum; 4 and 8, 30-CFU/g initial inoculum; M, DIG-labeled molecular size markers.

To overcome the potential for false-positive results in PCR-based assays, we have developed a method for the specific detection of L. monocytogenes based on RT-PCR amplification of mRNA. Most bacterial mRNAs have a very short half-life, on the order of 0.5 to 2 min, due to rapid degradation by endogenous RNases (27). Processes which render cells nonviable and hence disrupt cellular transcription will result in the rapid loss of cellular mRNA. An assay system based on detection of mRNA should provide a sensitive indicator of cell viability compared with methods that rely on the amplification of DNA or rRNA. Enstrøm et al. (14) used an RT-PCR method for the detection of Helicobacter species; however, their method relied on the amplification of 16S rRNA, not mRNA. Like DNA, rRNA is extremely stable (27) and, therefore, may not be a suitable target for discriminating between viable and nonviable cells. We were unable to detect iap mRNA amplification by the RT-PCR assay following exposure of the cells to extreme heat (autoclaving at 121°C for 15 min), indicating the sensitivity of bacterial mRNA to rapid degradation as well as the tight association between cellular mRNA and cell viability. To our knowledge, this is the first report of a sensitive and specific method for the detection of viable L. monocytogenes based on RT-PCR amplification of mRNA.

A major limitation of RT-PCR-based detection systems is the difficulty of rapidly isolating undegraded mRNA from bacterial cultures due to its very short half-life. To address this problem, we evaluated the newly developed FastPrep RNA isolation system (Bio101/Savant) for the extraction of total RNA from bacterial cells. This system, which includes a FastPrep cell disruption instrument in conjunction with a FastRNA kit, was able to efficiently lyse cells and stabilize the RNA...
before degradation occurred. The system was rapid, with total RNA extraction from cells accomplished within 1 h. We compared RNA isolation with the FastPrep system to isolation with TRIzol reagent (Gibco BRL). Although both systems were rapid and easy to use, the FastPrep system was more efficient at isolation of RNA from small numbers of cells (data not shown). The FastPrep system was found to rapidly yield mRNA suitable for RT-PCR amplification, requiring far less time and labor than conventional RNA isolation systems (41). However, some DNA contamination was present in the RNA samples. To avoid subsequent amplification of the DNA, a short DNase treatment step was incorporated prior to RT-PCR.

Successful detection of L. monocytogenes by RT-PCR of mRNA requires the selection of a suitable target gene for amplification. This target gene should possess several characteristics, including abundant transcript expression, expression throughout the growth cycle, and little or no transcriptional regulation. In the present study, we examined the amplification of three different L. monocytogenes mRNAs, iap, hly, and prfA, for suitability as targets for development of an RT-PCR detection system. DNA-based PCR detection methods using primers from these three genes have been developed (3, 4, 11, 13, 17, 21, 23, 25, 38, 40). In the present system, detection of the three mRNAs required a short enrichment incubation prior to total RNA extraction. This was necessary to increase the sensitivity of the detection assay. Confirmation of the RT-PCR products was accomplished by Southern hybridization with internal DIG-labeled probes from each of the three genes. Southern hybridization appeared to be at least 500 times more sensitive in amplicon detection than ethidium bromide staining of agarose gels. Comparison of the iap, hly, and prfA genes by this method indicated that the iap message was an ideal target for amplification by RT-PCR. Following a 1-h incubation step, we were able to detect the iap-specific product at a sensitivity of ca. 10 to 15 CFU/ml in pure broth cultures. Amplification of the hly message was approximately 4,000 times less sensitive, and amplification of prfA mRNA showed the lowest level of sensitivity of the three genes examined. The iap gene codes for p60, a major extracellular protein of Listeria species (9, 30). Wuenesch et al. (47) have demonstrated that the iap gene from L. monocytogenes is essential for cell viability and suggested that p60 is an essential housekeeping protein that is required during a late step in cell division. Furthermore, Köhler et al. (29) have shown that expression of the iap gene of L. monocytogenes is controlled at the posttranscriptional level. In mutants with reduced levels of p60, transcription of the iap gene appeared normal and wild-type levels of iap mRNA accumulated (29). These results indicate that the iap gene is constitutively expressed and, therefore, that iap mRNA should be present throughout the L. monocytogenes growth cycle. The present RT-PCR results with iap mRNA support this conclusion. In addition, the sensitivity observed when amplifying the iap mRNA indicates that this message is highly abundant within the cell. In contrast to the expression of the iap gene, the hly and prfA genes are not constitutively expressed (37) but are transcriptionally controlled. The prfA gene encodes a 27.1-kDa DNA-binding protein (19) that has been shown to positively regulate the expression of many of the L. monocytogenes virulence genes, including hly and prfA itself (10, 31, 37). Furthermore, the PrfA protein has also been shown to down regulate its own expression (19). Transcription of both prfA and hly is environmentally modulated and is subject to growth phase and thermal regulation (32, 37). Transcription of hly and prfA shows two peaks along the Listeria growth curve, one during early exponential growth and a second in early stationary phase (37). In addition, growth of listeria at temperatures less than 30°C inhibits transcription of both the hly and prfA genes (32). Based on these observations, the hly and prfA mRNAs would not be good candidates for the rapid detection of L. monocytogenes by RT-PCR. This observation is confirmed by the results of the present study. Both mRNAs appeared to be expressed at very low levels. We found that detection of the amplified hly product required ca. 4 \times 10^4 CFU/ml whereas detection of the prfA amplon required ca. 2.5 \times 10^6 to 3 \times 10^6 CFU/ml. In contrast, the iap-specific product was detected after a 1-h incubation from cultures containing ca. 10 to 15 CFU/ml.

The present results indicated that the iap primer set used for RT-PCR amplification was specific for L. monocytogenes and that the sensitivity of the assay was similar for all four strains tested. The iap gene is present in all Listeria species except L. grayii (28). No sequence homologous to an iap gene probe was detected in E. coli, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pyogenes, or Bacillus subtilis (28). The iap primer set used for RT-PCR in the present study and designed by Hubert et al. (8) for the specific detection of L. monocytogenes is derived from a region of the iap gene that is unique to L. monocytogenes. Based on the sensitivity and specificity observed with the iap primer set employed, it is concluded that the iap gene is the ideal target for sensitive and specific detection of L. monocytogenes by RT-PCR amplification of mRNA.

We have validated the use of the RT-PCR assay as a sensitive detection method for viable L. monocytogenes in artificially contaminated cooked meat. This organism is known to occur in both raw and cooked meats (6, 24) and is a pathogen of concern in cook-chill, ready-to-eat foods because of its ability to grow at refrigeration temperatures (45). Furthermore, there is sufficient evidence that L. monocytogenes can survive in ground meat following some thermal processing methods (5, 15, 42). In the present study, we chose to simulate a cooked meat product by autoclaving ground meat for 15 min at 121°C. Autoclaved meat may not be identical to most cooked meat products as far as the microbial population present; however, in our initial experiments we chose not to complicate the system with the presence of background microflora. Initially, these experiments were performed to ensure that the food matrix itself would not be inhibitory for the isolation of bacterial mRNA and its subsequent amplification by RT-PCR. Further studies must be conducted to determine what effect the presence of background flora might have on the RT-PCR assay we have developed. These studies will indicate whether a selective enrichment medium will be required for the isolation of L. monocytogenes in the presence of a natural microbial population. The results from the present study indicate that L. monocytogenes mRNA can be isolated from a food matrix artificially contaminated with very low levels of this organism. When cooked meat was contaminated with L. monocytogenes at an initial inoculum of ca. 3 CFU/g, the iap-specific RT-PCR product could be detected following a 2-h enrichment incubation. Furthermore, following a 6-h enrichment, we were able to detect iap mRNA by RT-PCR in a sample initially inoculated with <1 CFU/g, indicating that the food matrix did not inhibit bacterial RNA extraction or the RT-PCR assay itself. Although a short enrichment incubation (ca. 2 h) was necessary to increase the sensitivity of the RT-PCR assay, our results and those of others (1, 34) indicate that nonviable cells would be detected following this short enrichment period if DNA-based PCR detection systems were used. Thus, the present assay incorporating RT-PCR technology for the detection of bacterial mRNA would yield fewer false-positive results.
In conclusion, we have developed an RT-PCR assay for the sensitive detection of viable \textit{L. monocytogenes}. This method is based on amplification of the \textit{iap} mRNA by RT-PCR and combines a short enrichment incubation with total RNA extraction prior to amplification. Overall, the assay required ca. 54 to 55 h to complete. This is ca. 2 to 7 days shorter than traditional, culture-based testing methods for the identification of \textit{L. monocytogenes}. We are currently examining alternative methods for ampiclon detection in an effort to decrease the assay time further. The use of a chemiluminescence enzyme immunoassay for the detection of the RT-PCR product could reduce the assay time to less than 12 h while still providing a level of sensitivity comparable to that of Southern hybridization (2). Furthermore, experiments are under way to examine the effect of thermal injury on the isolation and detection of viable \textit{L. monocytogenes} by RT-PCR in artificially as well as naturally contaminated food products.

REFERENCES


