

# Detection of Heat Injury in *Listeria monocytogenes* Scott A<sup>†</sup>

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## ABSTRACT

Methods of detecting live pathogens in foods that may be growth inhibited following heat treatment are essential to food safety. Among the techniques available, reverse transcription polymerase chain reaction (RT-PCR) amplification of messenger RNA from heat-injured *Listeria monocytogenes* Scott A is preferable to direct PCR in an attempt to avoid false positives from dead cells. The RT-PCR has a detection limit of  $3 \times 10^6$  CFU/g, compared to 3 CFU/g for untreated controls, but may not be suitable for the identification of all viable cells. Physically apparent changes in cellular structures from heat injury in *L. monocytogenes* are expected to result. Ultrastructural analyses did depict notable heat damage as cytoplasmic clearing after 5 min at 60°C. The heat-injured survivors can be readily distinguished from total viable cells using selective media. As a result, combinations of molecular and visual methods including selective media improve detectability of heat-injured, viable *L. monocytogenes* Scott A.

*Listeria monocytogenes* is a gram-positive facultatively anaerobic rod with a reported higher heat resistance than many other foodborne pathogenic nonspore formers (10, 15). It has been isolated from a variety of foods and by-products (3, 27). Most susceptible are the very young with developing immune systems, pregnant women, the elderly, and the immunocompromised. In 1999, 92.2% of the 2,518 illnesses attributed to *L. monocytogenes* resulted in hospitalizations while accounting for 27.6% of the total foodborne deaths due to foodborne illness in the United States (17). The ability of this foodborne pathogen to spread intercellularly is disconcerting (21). Growth at refrigeration temperatures and survivability in adverse environments, including those that occur in minimally processed foods, have made *L. monocytogenes* a challenge to control (10).

Food processing conditions, encountered in minimally processed foods, may heat injure, but not effectively kill all *L. monocytogenes* cells, resulting in eventual repair and growth of damaged survivors (24). Detection of live pathogens is absolutely crucial in food safety. Failure to detect injured pathogens can have adverse health effects as well as substantial economic losses and fatalities. Heat-injured *L. monocytogenes* strains were found to be capable of growth on a rich medium such as tryptic soy agar (TSA) but were inhibited in the presence of 4% NaCl (3). Inadequate heating and possible pathogen protection by meat fat or drying during heating result in underestimations of *L. monocytogenes* cellular heat lethality. This has led to the development of modified media formulations and methods designed specifically to resuscitate heat-injured cells (11, 15). To date, various media formulations have been pro-

posed but then have not been universally accepted to ensure sensitive detection and enumeration of heat-injured *L. monocytogenes* (2, 6, 12).

Likewise, various enrichment, biochemical, immunological, and polymerase chain reaction (PCR)-based DNA probe technologies have been applied with varying sensitivities, specificities, and shortcomings (8, 20, 25, 26). A reverse transcription (RT)-PCR amplification of mRNA specific for the *iap* gene product, a 60-kDa major extracellular house-keeping protein essential to viable cells (22), was shown to be capable of detecting as low as 3 CFU of live, uninjured *L. monocytogenes* per g of inoculated ground beef (13). This study examines the efficacy of this technology in detecting *L. monocytogenes* heat injury while providing visual evidence of injury using electron microscopy.

## MATERIALS AND METHODS

**Detection of heat injury in *L. monocytogenes* using RT-PCR.** Appropriate dilutions of stationary-phase *L. monocytogenes* Scott A cultures, maintained on TSA slants and obtained from the U.S. Department of Agriculture-Agricultural Research Service Eastern Regional Research Center culture collection, were used to inoculate meat to obtain 3 CFU/g, as previously described (13). For measures of heat injury, 2-g samples of meat were inoculated with  $10^7$  CFU of *L. monocytogenes*/g followed by heat treatment at 60°C for 0, 1, 2, and 5 min. Appropriate 10-fold serial dilutions in filter-sterilized phosphate-buffered saline containing 0.1% (vol/vol) Tween 20 were made and homogenized for 2 min in a Stomacher Lab-Blender 400 (Tekmar Co., Cincinnati, Ohio). Samples were then plated in duplicate on TSA for injured and TSA with 4% NaCl for noninjured cell counts. The percentage injury was calculated as  $[1 - (\text{CFU on TSA with 4\% NaCl}/\text{CFU on TSA}) \times 100]$ . After the heat treatments, a 1,000-fold dilution of this initial recorded pathogen concentration resulted at the start of enrichment at 37°C for 5 h. At the end of the enrichment incubation period, only 10% of the total cultures was used for total RNA and genomic DNA extractions (13). Following RT-PCR of isolated

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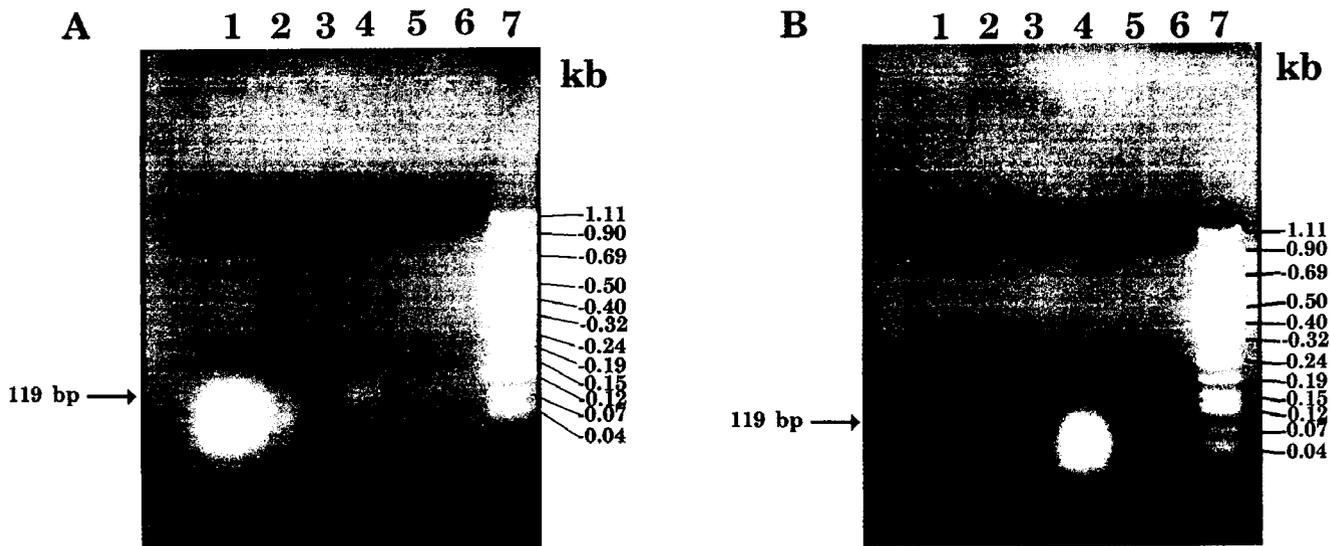


FIGURE 1. Detection of *iap* mRNA and *iap* gene sequences in (A) live versus heat-injured *L. monocytogenes* cells and in (B) heat-killed versus heat-injured *L. monocytogenes* at the lower limits of detection using RT-PCR and PCR. 1A lanes: 1, RT-PCR of mRNA (control:  $1 \times 10^7$  CFU/g viable cells); 2, RT-PCR of mRNA following 60°C for 1 min ( $3 \times 10^6$  CFU/g viable and 60% injury); 3, RT-PCR of mRNA following 60°C for 2 min ( $2 \times 10^6$  CFU/g viable and 98% injury); 4, PCR of total genomic DNA (control:  $1 \times 10^7$  CFU/g viable cells); 5, PCR of total genomic DNA following 60°C for 1 min ( $3 \times 10^6$  CFU/g viable and 60% injury); 6, PCR of total genomic DNA following 60°C for 2 min ( $2 \times 10^6$  CFU/g viable and 98% injury); and 7, DNA molecular size markers. 1B lanes: 1, RT-PCR of mRNA (control: 3 CFU/g viable cells); 2, RT-PCR of mRNA following 60°C for 5 min (3 CFU/g viable cells); 3, RT-PCR of mRNA from heat-killed (autoclaved) cells; 4, same as 1A lane 1; 5, same as 1A lane 3; 6, same as 1A lane 4; and 7, DNA molecular size markers.

mRNA (13) from 5-h enrichments in tryptic soy broth (TSB) or PCR of DNA from 5-h TSB enrichments, amplified products were visualized on 2% agarose gels as previously described (13). The internal primers used for RT-PCR and PCR amplification of the *iap* gene were obtained from Operon Technologies, Inc. (Alameda, Calif.) and were expected to result in a 119-bp amplified DNA fragment (13).

**Ultrastructural analysis of heat injury in *L. monocytogenes*.** Overnight cultures of *L. monocytogenes* Scott A in TSB medium at 37°C were dispensed into 1.5-ml microfuge tubes and either heat treated directly or pelleted and resuspended in 0.1% (wt/vol) peptone water (PW). Following heat treatments at 60°C for 0, 2.5, 5, 10, and 20 min, a portion of the culture was fixed overnight in 2.5% glutaraldehyde-0.1 M imidazole HCl buffer (pH 6.8), and the rest was used for dilution plating on TSA and TSA with 4% NaCl. This was followed by 2% osmium tetroxide-0.1 M imidazole HCl buffer (pH 6.8) for 2 h and dehydration with a serial concentration of ethanol. Dehydrated cells were then embedded in Epon resin for transmission electron microscopy, sectioned, and stained with lead citrate. Micrographs were taken under bright field imaging using a Philips model CM12 transmission electron microscope (FEI Co., Hillsboro, Oreg.).

**Measurements of survival and heat injury.** Using a combination of nonselective (TSA) and selective (TSA + 4% NaCl) plating medium, it was possible to determine the severity of heat injury to surviving cells. As with confirmation of heat injury for RT-PCR, the percentage injury was calculated as  $[1 - (\text{CFU on TSA with 4\% NaCl} / \text{CFU on TSA}) \times 100]$ .

## RESULTS AND DISCUSSION

**RT-PCR of heat-injured cells.** The detection limit of the RT-PCR technique for heat-injured *L. monocytogenes* was  $3 \times 10^6$  CFU/g following 60°C exposure for 1 min.

This represented 60% injury as determined by aerobic plate count calculations (Fig. 1A, lane 2). A heat treatment of 60°C for 2 min resulted in 98% injury without any amplified products detected for the mRNA from  $2 \times 10^6$  CFU/g survivors (Fig. 1A, lane 3). Clearly, the valid use of RT-PCR for heat injury detection of *L. monocytogenes* is limited by the stability of the mRNA in the heat-injured cells. PCR amplification of DNA from the untreated control cells at a concentration of  $10^7$  CFU/g resulted in a noticeable 119-bp amplified product (Fig. 1A, lane 4). At a pathogen level of 3 CFU/g, the RT-PCR method was capable of detecting uninjured *L. monocytogenes* as previously described following 5 h of enrichment (Fig. 1B, lane 1) (13). No RT-PCR products were visible on agarose gels following 5 min of 60°C heat exposure (Fig. 1B, lane 2).

Negative RT-PCR results may be attributed to RNA degradation, which is believed to be one of the first events to occur in heat-treated cells (23). We have shown that under these conditions, the 5-h enrichment in TSB at 37°C was not sufficient to elicit cellular repair and synthesis of mRNA to enable detection of *L. monocytogenes* (Fig. 1B). Therefore, the RT-PCR method was determined unreliable for detection of heat-injured survivors that did not recover to detectable limits during the 5-h enrichment.

**Ultrastructural analysis of heat-injured cells.** The internal structure of *L. monocytogenes* cells can be characterized by a cytoplasm with uniform granular appearance and uniform density distribution of ribosomes and chromosomal structure (Fig. 2A). After 5 min at 60°C, clear evidence of heat damage to cells can be seen as a central clearing and dispersion of the cytoplasmic contents to the

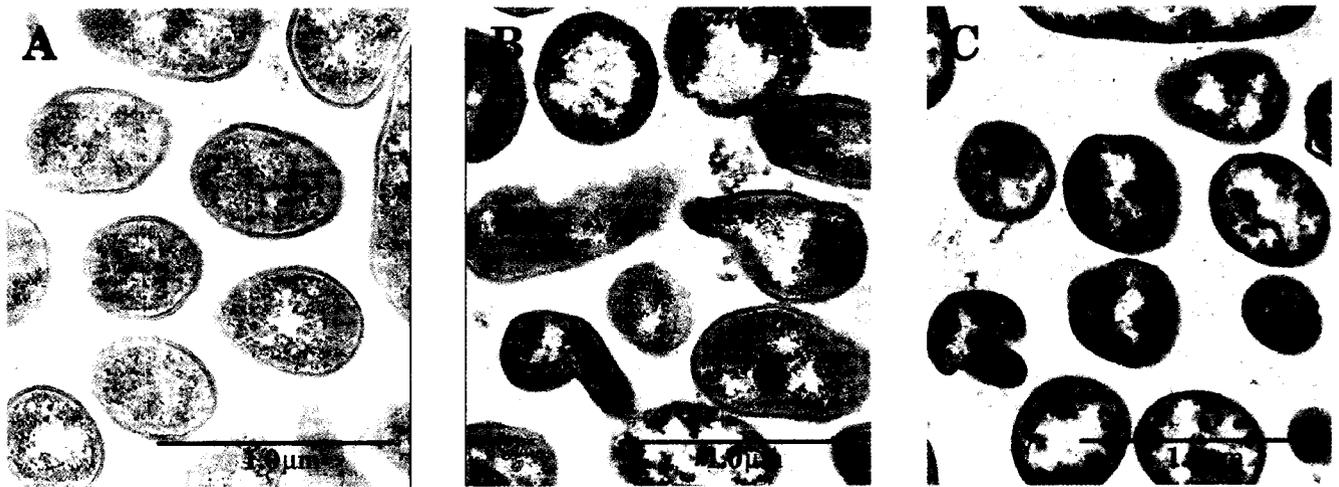


FIGURE 2. Transmission electron micrographs of *L. monocytogenes* Scott A cells (A) prior to heat injury; (B) exposed to 60°C for 5 min; and (C) exposed to 60°C for 10 min. The bar represents 1  $\mu\text{m}$ .

internal periphery of the cells (Fig. 2B). After 10 min at 60°C, the cytoplasmic clearing with particulate density concentrated near the cell edges becomes even more prominent and characterizes nearly every cell (Fig. 2C). A previous study comparing microwave heating damage on *Escherichia coli* and *Bacillus subtilis* cells showed dark spots within

the cytoplasm of the cells thought to be aggregated proteins due to denaturations (28). This was not seen for heat damage in *L. monocytogenes*, but the results are similar to those seen for cells of *L. monocytogenes* sublethally injured following chilled storage at 4°C for up to 4 weeks in duration (9). The long-term chilled storage resulted in a similar cytoplasmic clearing as seen here, except that in the earlier study, there were additional effects, such as damage to the cell walls, retraction of the cytoplasmic cell membranes away from the cell walls, and leakage of nucleic acids and proteins (9).

In this study, there was no clear evidence of cytoplasmic membrane retraction or damage to the cell walls (Fig. 2B and 2C). In addition, the cell-free supernatants from each of the temperature treatments were scanned spectrophotometrically from 200 to 800 nm. There was no discernible cell leakage of proteins (280 nm) or nucleic acids (260 nm) from broken or porous membranes after 60°C treatment for up to 30 min (data not shown). In further support of the results presented herein, a study examining the effects of high pH on *L. monocytogenes* cytoplasmic membranes concluded there was no significant leakage of cytoplasmic materials in gram-positive bacteria due to the presence of a thick peptidoglycan layer for stabilization (18). In accordance, another study scanning supernatant fluids from heat-injured *L. monocytogenes* indicated that any stress to cell membranes was insufficient to allow leakage of nucleotides and proteins (5). However, other cytoplasmic molecules, such as proteins, were expected to precipitate at high pH and become electron dense, compared to DNA that was expected to separate as a liquid phase from the rest of the cytoplasm (18).

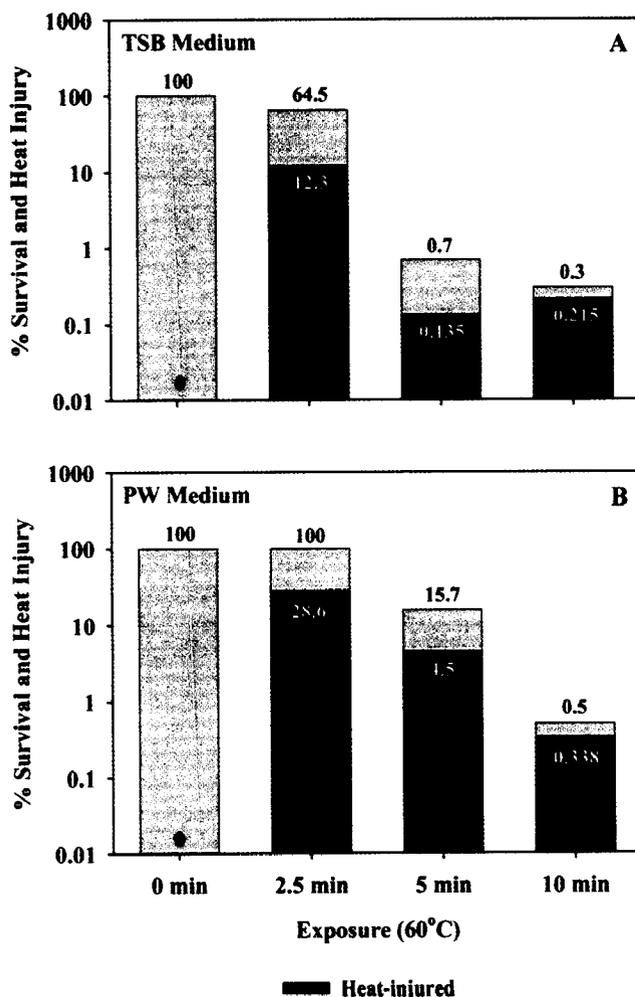


FIGURE 3. The decrease in *L. monocytogenes* Scott A survival (top numbers) in (A) TSB medium over time at 60°C along with heat injury (lower numbers) or (B) in PW medium.

**Using selective medium for heat injury detection.** In establishing the conditions for transmission electron microscopy, it was visually noted that heating of cells in TSB medium resulted in flocculation of particulates. To diminish this occurrence, cells were pelleted and heated in 0.1% PW. Figure 3 depicts the results from heating *L. monocytogenes* at 60°C in TSB medium compared with heating in PW medium. As exposure time to 60°C increased, *L. monocyto-*

## REFERENCES

*genes* appeared to be more resistant to heat in the minimal medium. The explanation for this involves a pH effect rather than any perceived benefit from peptone compared to a tryptic soy digest. The initial pH of the TSB was 7.2 compared to 6.9 for PW. Following culture growth, the *L. monocytogenes* culture in TSB reached a pH of 5.1, after which it was exposed to a 60°C heat treatment. A portion of the culture was centrifuged and resuspended in PW, resulting in a final pH of 6.0 prior to heat treatment. Heating at a pH of 5 for the TSB medium compared to pH 6 for PW most probably resulted in fewer survivors in TSB compared to PW by including an additional stress hurdle for the pathogen.

For heat injury determinations, all cultures of *L. monocytogenes* Scott A were at  $10^9$  cell/ml concentrations prior to heat treatment at 60°C. After 2.5 min at 60°C, *L. monocytogenes* in TSB produced 64.5% total survivors, with 12.3% heat-injured survivors (Fig. 3A). For the same heating exposure, PW medium enabled 100% survival, with 28.6% heat injured (Fig. 3B). Heating in TSB for 5 min at 60°C resulted in 0.7% survivors, with 0.135% heat-injured survivors (Fig. 3A), while 5 min in PW at 60°C produced 15.7% survivors, of which 4.5% were heat-injured survivors (Fig. 3B). Regardless of heating menstroom, as the number of survivors decreased, the percentage of heat injury within those survivors increased (Fig. 3A and 3B). After 10 min at 60°C, total viable cell counts reached  $2.22 \times 10^7$  CFU/ml in the TSB culture and  $9.75 \times 10^6$  CFU/ml in the PW culture following three replicate experiments.

Parameters describing the thermal inactivation of microorganisms have traditionally involved *D*-value calculations based on a linear relationship between  $\log_{10}$  number of surviving cells versus heating time. Unfortunately, survivor curves typically exhibit sigmoidal curves due to heterogeneities within cellular populations resulting from growing conditions, nonuniform heat transfer, stress adaptations, or underestimations of heat-injured cells capable of repair and growth. Various mathematical models have been proposed to avoid underestimation of the heating parameters necessary to ensure zero tolerance for *L. monocytogenes* in the food industry (1, 4, 7, 14, 16, 19).

In conclusion, this study examined a number of methods in order to ensure detection of *L. monocytogenes* heat-injured cells that might be capable of repair and survival. RT-PCR has been shown to be ineffective in detecting low levels of heat-injured cells until mRNA synthesis is reestablished. Therefore, it is proposed that heat injury can be detected using a combination of methods that could include transmission electron microscopy and direct plating on appropriate rich and selective media. Currently, we are assessing the effectiveness of differential fluorescently labeled molecular probes for use in confocal microscopy for the detection of heat injury.

## ACKNOWLEDGMENTS

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