Evaluation of Reverse Transcriptase 5' Nuclease Polymerase Chain Reaction assay for the Detection of Viable Heat-Injured and Resuscitated *Listeria monocytogenes* in Ground Pork

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An anaerobic resuscitation-enrichment system was combined with a 5' nuclease reverse transcriptase (RT) protocol for detecting *Listeria monocytogenes* Scott A from artificially inoculated ground pork. When irradiation-sterilized ground pork containing *L. monocytogenes* (~6 x 10^5 CFU/g) was heated (60°C, 14 min), 100% of the cells were injured, as indicated by no growth on selective Modified Oxford (MOX) agar plates incubated aerobically. After resuscitation and enrichment (37°C) in anaerobic Penn State University (PSU) broth, *L. monocytogenes* was detected within 24 hours both by plating to MOX agar incubated in air and by a fluorogenic 5' nuclease real-time RT-PCR assay. The RT-5' nuclease polymerase chain reaction (PCR) assay targeting the hemolysin gene (*hlyA*) detected viable *L. monocytogenes* directly from the PSU within 24 hours, although a stronger signal was detected after 48 hours of resucitation. The RT-5' nuclease PCR assay bypassed the need for subsequent plating of ground pork to selective agar and thus may shorten the interval to detect low numbers of viable *L. monocytogenes* following heating of naturally contaminated meat.

Introduction

The 1999 multistate outbreak of delicatessen meats contaminated with *Listeria monocytogenes* as well as mandated zero tolerance for its presence in ready to eat (RTE) products have heightened interest for its rapid detection (2). Improper thermal processing sublethally injures *Listeria,* that retain their virulence upon subsequent recovery (4). Sublethally injured *L. monocytogenes* are sensitive to oxygen and thus enrichment techniques which use aerobic systems may underestimate their presence (6). PSU broth combines anaerobic resuscitation to avert oxygen toxicity with lithium chloride to suppress the growth of competing microflora to detect low levels of injured and uninjured *L. monocytogenes* in ready-to-eat foods, such as pasteurized milk and hot dogs by both conventional bacteriology and PCR-based assays (6,7,8,12).

The fluorogenic 5' nuclease PCR format for real time analysis has improved detection of *L. monocytogenes* (5,9,11). Fluorogenic detection further enhanced sensitivity of the RT-PCR assay to detect mRNA present only in viable cells (10*).* In this study we combined the 5' nuclease RT-PCR with anerobic resuscitation in PSU to detect viable heat-injured *L. monocytogenes* in artificially inoculated ground pork.

Materials and Methods

Bacterial strains and culture conditions. *L. monocytogenes* Scott A (NADC 2045) was maintained (4°C) in tryptic soy agar supplemented with 0.6% yeast extract (TSAYE; Difco Laboratories, Detroit, MI) slants and subcultured monthly. To inoculate pork, *L. monocytogenes* Scott A was grown (37°C, 18 h at 150 rpm) in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; Difco Laboratories). For inoculation into irradiated ground pork, 1 ml of the culture (~10^9 CFU) was centrifuged (5,000 x g for 10 min), the pellet was resuspended in 10 ml of 0.1% peptone water, and the cell suspension was adjusted to ~10^8 CFU/ml.

Heat treatment of *L. monocytogenes* in ground pork. Ground pork was purchased from a local grocery store and irradiated (40 kGy) at Iowa State University Irradiation Facility (Ames, IA). Two replicate experiments were conducted on separate days with the same batch of irradiated ground pork. Ground pork (~10 g) was placed in a sterile whirl-pak bag (Nasco, Fort Atkinson, WI) to which was added 0.1 ml of *L. monocytogenes* cell suspension (~10^7 CFU/ml) to yield ~10^6 CFU/g of ground pork. All packages were mixed to evenly distribute the inoculum and then compressed to ~ 2-3-mm thickness before vacuum packaging (model A300/51; Multivac, Wolfertschwenden/Allgau, Germany). Samples were submerged in a circulating waterbath (60°C, Lindberg/Blue M, model 91, Asheville, NC). After equilibration (~1 min to reach 60°C), the meat packs were withdrawn at selected intervals (0, 2, 4, 6, 8, 10, 12, 13, 14 min), and immediately placed in an ice water bath (0°C). After cooling, each pork sample was combined with 90 ml of 0.1% peptone water and homogenized (1.0 min at medium speed; Seward Lab-blender 80, Seward Ltd., London, England).

Resuscitation/enrichment medium. Survivors were enumerated after heating by spreading the pork slurry (~1

ml) onto selective Modified Oxford (MOX) agar incubated in air as well as to TSAYE agar plates, which were incubated either aerobically or anaerobically (30°C for 48 h). Aliquots (~1 ml) of meat slurry were also transferred to PSU (19 ml) in duplicate and incubated (37°C) as described (8). At 24, 48, and 72 h, 10-fold serial dilutions of the ground pork slurry were plated onto MOX agar and incubated aerobically as well as to TSAYE plates, which were incubated aerobically or anaerobically (30°C for 48 h).

RNA isolations. Heat-injured *L. monocytogenes* Scott A cells (~10^5 CFU/ml) recovered from PSU broth (37°C, 48 h) were harvested from ~5 ml of PSU, pelleted (5,000 x g, 10 min at 4°C) (Sorvall Heraeus RC 5C plus centrifuge; Kendro Laboratory Products, Newtown, CT), and washed twice in 10 ml of 0.1% peptone water. Two procedures were evaluated for extracting *L. monocytogenes* RNA templates. For procedure 1, the cell pellet was resuspended in 100 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) containing of lysozyme (3 mg/ml), incubated (15 min, 37°C) and the mixture was transferred to a sterile 1.5-ml microfuge tube (Fisher Scientific, Pittsburg, PA). Total RNA was prepared using the RNeasy mini-kit (QIAGEN, Valencia, CA) as per the manufacturer's specifications. In procedure 2, RNA was extracted using the three-detergent method in which cell lysis was achieved through a combination of SDS, Tween 20 and Triton X-100 (SST). Genomic DNA was reduced by acid depurination and buffered phenol was used to denature proteins (3). Extracts from both of the procedures were incubated (30 min at 37°C) with RQ1 DNase (final concentration of 10 U/ml; Promega, Madison, WI) before use as a template for the RT-5' nuclease PCR assay.

RT-5' nuclease PCR assay. cDNA was made using a random hexamer primer kit as described by the manufacturer (GeneAmp EZ *rTth* RNA PCR kit, Applied Biosystems, Union City, CA). Each 50-µl reaction mixture contained 1 x EZ buffer, 3.0 mM manganese acetate, 0.3 mM each dATP, dCTP, dGTP, and dTTP, 0.45 µM each primer, 26 mM probe HLYAP15, 10 U of *rTth* DNA polymerase, and 5 µl of DNase-treated template, as described (10). Amplification was performed in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The samples were held initially for the reverse transcription step (60°C, 30 min) followed by 40 amplification cycles each consisting of denaturation (95°C for 15 s), primer annealing (60°C for 30 s), and primer extension (72°C for 90 s). A final extension step at 72°C for 10 min was followed by a hold at 4°C.

The reaction threshold was set at 10 times the standard deviation of the normalized fluorescent emission of three no-template control reactions to ensure a 99% confidence level in detecting a positive sample (Sequence Detector software, version 1.6.3; Applied Biosystems). Unknown samples whose fluorescence exceeded the threshold were scored as positive. Data are reported as the threshold cycle (C_T) or the amplification cycle at which fluorescence exceeds the reaction threshold. The ΔRn value is a measure of the relative fluorescence, with a higher ΔRn value indicating a stronger signal than a lower ΔRn score.

Results

Heating ground pork, which was artificially contaminated with *L. monocytogenes* Scott A (~ 6 x 10^5 CFU/g), at 60°C for 14 min, injured 100% of the cells, as indicated by the absence of colonies on selective MOX agar and markedly reduced growth on nonselective TSAYE incubated either anaerobically (~5 x 10^1) or anaerobically (~2 x 10^0).

As shown in Figure 1, heat-injured cells were resuscitated by 48 h in PSU broth containing 7 g/liter of LiCl, as indicated by subsequent growth on MOX agar as well as on TSAYE incubated either anaerobically (~2 x 10^6 CFU/ml) or aerobically (1.8 x 10^5 CFU/ml). Whereas no colonies were initially observed on MOX (100% injury), the *L. monocytogenes* populations in PSU broth increased after 24 (~1.3 x 10^3), 48 (1 x 10^5), and 72 (5 x 10^8) h.

After resuscitation in PSU broth (37°C, 48 h) *L. monocytogenes* was detected by RT-5' nuclease PCR assay using two different RNA isolation protocols. By procedure 1, the fluorescent signal exceeded the threshold by 14 amplification cycles (C_T = 14) with the ΔRn value of 3.24 (Fig 2A). In contrast, for RNA templates generated by procedure 2, fluorescence occurred later at 24 amplification cycles (C_T = 24) with a lower ΔRn value of 1.62 (Fig 2B). The RNA yield (procedure 1) was further improved by including two additional washing steps to remove residual PSU (data not shown). Because of its sensitivity, based on lower C_T and higher ΔRn values, procedure 1 was adapted for subsequent RNA template preparation.

Mendonca and Knabel (8) demonstrated that 7 g LiCl/liter PSU broth was optimal for both suppressing the growth of the background microflora, such as *Enterococcus faecium*, and resuscitating heat-injured *L. monocytogenes*. In our study, LiCl concentrations of 0, 1.75, 3.5, 5.25, and 7 g/liter were evaluated for potential inhibition of the RT-5' nuclease PCR reaction. As depicted in Fig 3A, increasing concentrations of lithium chloride interfered with the RT-PCR assay. Specifically, in the absence of LiCl (0 g LiCl/liter PSU), the C_T was achieved by amplification cycle 13 (C_T = 13) and was delayed until amplification cycle 20 at the highest concentration evaluated (C_T = 20, 7 g LiCl/liter). That the ΔRn value was higher in the absence of lithium chloride (0 g LiCl) than in the presence of 7 g/liter PSU, also indicates inhibition of the RT-PCR reaction. Consistent detection was achieved in strictly anaerobic PSU broth in the presence of LiCl (1.75 to 7 g/liter of PSU) because both oxygen toxicity and background microflora were inhibited, whereas resuscitation of heat-injured

cells was not altered. Similar results were observed when very low concentrations of NaCl (0.5%), and LiCl (0.7%) were used to resuscitate heat-injured cells in pasteurized milk (8). As seen in Fig 3B, *L. monocytogenes* was detected by RT-5' nuclease PCR assay in PSU broth after 24 hours (C = 26, plot 1), which was the earliest time point evaluated. Stronger signals were observed (C_T = 14) after 48 h (plot 1) and 72 h (plot 3) of resuscitation.

In summary, this is the first report of combining the RT-5' nuclease PCR assay with PSU to shorten the time to detect viable cells in heated pork. Ultimately, these sensitive protocols may improve the ability to test the effectiveness of thermal processing and thus eliminate *L. monocytogenes* from ready-to-eat products.

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Figure 1.The recovery of heat injured *L. monocytogenes* cells (log_{10} CFU/ml) following recovery/enrichment (in hours) in PSU broth. The populations at 0 min represent the survivors immediately after heating (60°C, 14 min). The number of cells recovered from PSU broth is shown after plating to TSAYE and incubating anaerobically (TSAYE/anaerobic) and aerobically (TSAYE/aerobic) as well as plating to MOX agar and incubating aerobically (MOX/aerobic). Each line represents a composite of two replicates.

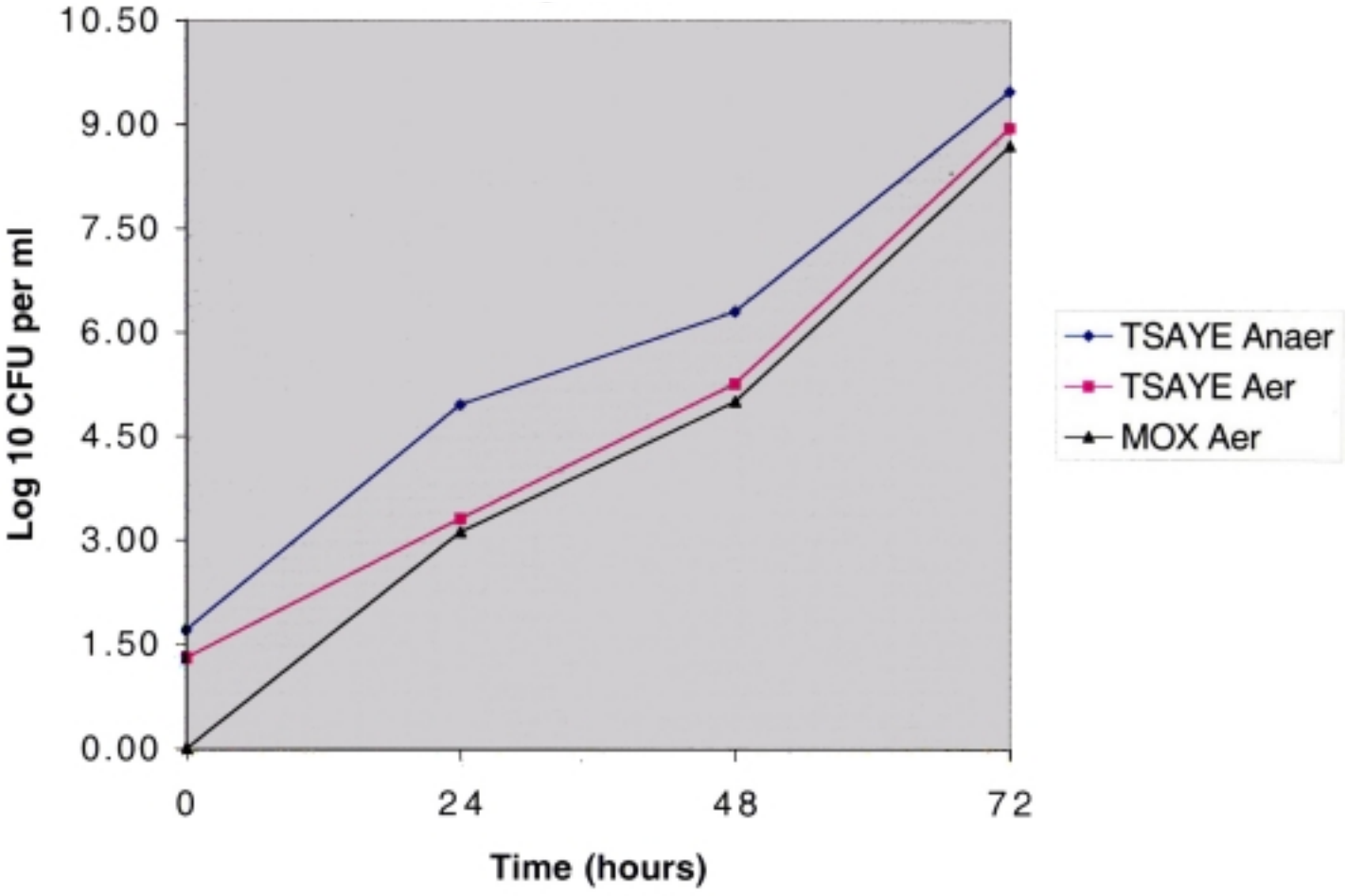

Figure 2. The sensitivity of the 5' nuclease RT- PCR assay was evaluated for two RNA isolation techniques. RNA was extracted after 48-h recovery in PSU broth. (A) Using QIAGEN RNeasy mini-extraction kit, the C_T = 14 (B). Using the three-detergent extraction protocol (2), the fluorescence exceeded the threshold later at 24 amplification cycles (C_T = 24).

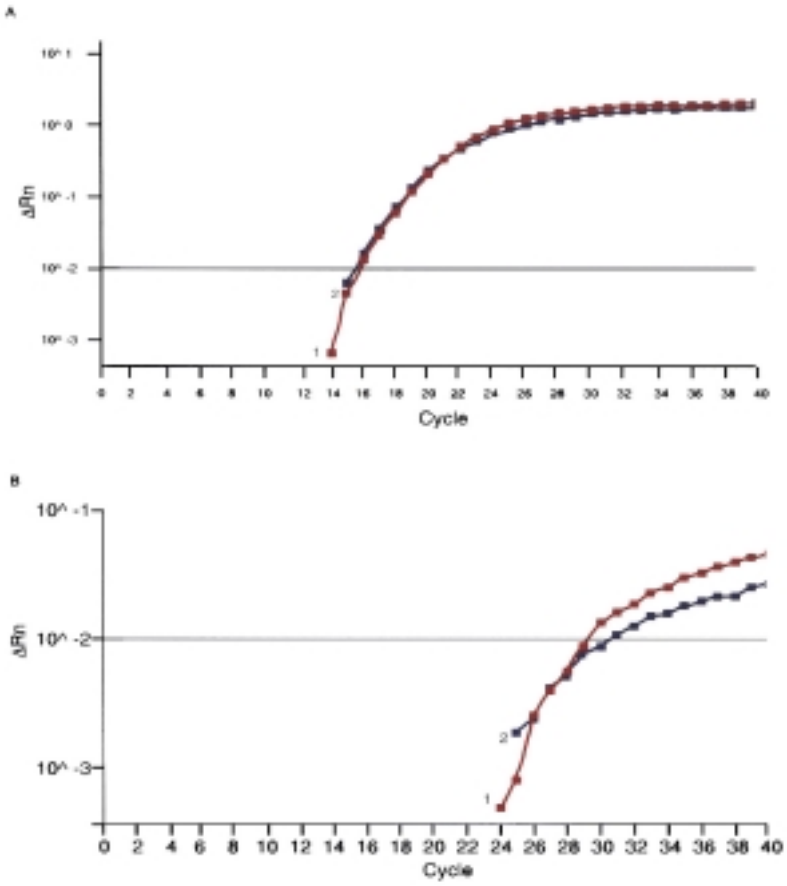

Figure 3. (A) Sensitivity of the 5' nuclease RT-PCR assay at different concentrations of lithium chloride used in the PSU (g/liter) broth: 0 g/liter (plot 1), 1.75 g/liter (plot 2), 3.5 g/liter (plot 3), 5.25 g/liter (plot 4), and 7 g/liter (plot 5). RNA isolated from heat-injured *L. monocytogenes* after 48-h recovery in PSU was used as a template. Each line represents a composite of two replicates. (B). Detection of heat injured *L. monocytogenes* cells after 24, 48, and 72h in PSU broth. RNA was prepared from PSU broth after 24 h (plot 1), 48 h (plot 2), 72 h (plot 3) of resuscitation. Each line represents a composite of two replicates.