Research Note

Enrichment and DNA Extraction Protocols for the Simultaneous Detection of *Salmonella* and *Listeria monocytogenes* in Raw Sausage Meat with Multiplex Real-Time PCR

XIAOWEN WANG,1 NARAYANAN JOTHIKUMAR,2† AND MANSEL W. GRIFFITHS2,3*

1Department of Food Science, Shanxi Agricultural University, Taigu, Shanxi 030801, People’s Republic of China; and 2Canadian Research Institute for Food Safety and 3Department of Food Science, University of Guelph, 43 McGilvray Street, Guelph, Ontario, Canada N1G 2W1

ABSTRACT

A novel method of DNA extraction and purification was developed and was used in conjunction with a multiplex real-time PCR assay for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in a raw meat sample. The PCR used primers targeting the *invA* gene of *Salmonella* and the *hlyA* gene of *L. monocytogenes*, and PCR products were detected with a LightCycler on the basis of fluorescence from SYBR Green and melting temperature. The assay allowed the detection of 3 *Listeria* cells and 4 *Salmonella* cells per g of the original sausage within 10 h, including an enrichment period of 6 to 8 h.

*Listeria monocytogenes* and *Salmonella* spp. occur widely in the environment and have been isolated from a range of sources, including fruits and vegetables, raw and processed meat, dairy products, and raw and cooked seafood. Foodborne illnesses associated with these two pathogens still occur at unacceptably high frequencies in industrialized and developing countries and can result in severe infections that are sometimes fatal (4, 6, 14, 19).

Many methods, including PCR assays, have been developed for the isolation of *L. monocytogenes* and *Salmonella* spp. directly from food samples, such as ready-to-eat meats in which both organisms may be present, and for the detection of these organisms in such samples (7, 9–11, 20). However, few assays for the simultaneous detection of these two organisms are available. Even the conventional PCR methods of detection are frequently neither rapid nor sensitive enough to detect potential pathogens in food samples. Furthermore, it has been difficult to separate two different targets by gel electrophoresis when the sizes of the PCR products are similar (12). With the advent of real-time PCR with instruments such as the LightCycler, the detection and quantification of suspected foodborne contaminants can be achieved in less than an hour. At the end of the PCR cycles, a melting-curve analysis can be performed to confirm the identity of the product, making it easier to differentiate PCR products of similar sizes.

The reliability of PCR for detection partly depends on the purity of the target template and the presence of sufficient numbers of target molecules. Food samples and a variety of components, such as ground beef, chicken, soft cheese (9, 19–21), heme and its products (1), and acidic polysaccharides (18), have been reported to inhibit PCR, so steps must be taken to limit the effects of any potentially inhibitory compounds present. Many methods for the preparation of inhibitor-free bacterial DNA templates from food samples have been examined, including those involving filtration (14) and immunomagnetic separation (9, 10). However, these methods are often cumbersome and time-consuming.

The purpose of this study was to develop a simple method to purify template DNA and remove PCR inhibitors from raw sausage meat for use in a multiplex real-time PCR for the rapid and sensitive detection of *L. monocytogenes* and *Salmonella* in meat. Raw sausage meat was chosen because it is a complex matrix that has the potential to be contaminated by both bacteria simultaneously.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *Salmonella Enteritidis* SA942451 and *L. monocytogenes* H7764 were obtained from the culture collection of the Canadian Research Institute for Food Safety, University of Guelph. Cells of either *Salmonella Enteritidis* or *L. monocytogenes* from a single colony obtained with tryptone soy agar (BD Diagnostic Systems, Sparks, Md.) were inoculated into 10 ml of tryptone soy broth (TSB; BD Diagnostic Systems) and incubated at 37°C overnight with shaking. One milliliter of the overnight broth culture was transferred to a 250-ml Erlenmeyer flask containing 50 ml of TSB and further incubated at 37°C overnight with shaking. This process resulted in suspensions of both *Salmonella Enteritidis* and *L. monocytogenes* containing ca. 1 × 10⁶ CFU/ml. Equal volumes of the two cultures were mixed, and 10-fold serial dilutions were prepared with TSB.
as the diluent to produce cell suspensions containing 10^6 to 10^8 CFU of both organisms per ml.

**Inoculation of sausage.** Retail packs of raw pork sausage meat were obtained from a local store. The sausage (10 g) was immersed in each of the suspensions described above and held at room temperature for 30 min. The contaminated sausages were left to dry, placed into stomacher bags with filter inserts (Brinkmann Instruments [Canada] Ltd., Mississauga, Ontario, Canada) containing 90 ml of TSB, and homogenized for 2 min in a Stomacher 80 (Brinkmann Instruments). The inoculated samples were incubated at 37°C with shaking, and samples were removed after 4, 6, 8, 10, and 16 h for DNA extraction.

Cell counts were obtained for the diluted cultures used for inoculation by plating 100 μl of each dilution onto CHROMagar Salmonella (DRG International Inc., Mountainside, N.J.) (8) and Oxford Listeria selective agar (Oxoid Inc., Nepean, Ontario, Canada) (5). The plates were incubated at 37°C overnight, and colonies showing the expected morphological characteristics were counted. Cell counts for the original sausages were determined on the basis of the counts for the inocula. Experiments were repeated in triplicate.

**DNA extraction.** Three methods of DNA extraction were compared. For procedure 1, 100 μl of Genereleaser (BioVentures, Inc., Murfreesboro, Tenn.) was added to 100 μl of the preenriched solution, and the mixture was boiled in a water bath for 10 min. After heat treatment, the solution was centrifuged for 5 min in a Microfuge and the supernatant was discarded. Finally, the pellet DNA was dissolved in 10 μl of sterile distilled water, and 1 μl of DNA was used for PCR.

For procedure 2, the NaI treatment described by Makino et al. (16) was used. Two hundred microliters of the preenrichment culture was added to 400 μl of lysis buffer (0.5% N-laurylsarcosine, 50 mM Tris-HCl, 25 mM EDTA [pH 8.0]). After the mixture had been vortexed for 1 min, it was centrifuged at 12,000 × g for 5 min. The pellet was resuspended in 200 μl of lysis buffer containing glycogen at 0.03 μg/μl, heated in a boiling water bath for 10 min, and then chilled on ice for 5 min. Sodium iodide solution (300 μl; prepared as described for procedure 2) and 500 μl of isopropanol were added to the chilled solution. The mixture was added to the Qiagen Spin Miniprep Kit and treated as described for procedure 1.

**PCR.** The primer pair used for the detection of Salmonella Enteritidis and *L. monocytogenes* in the present study has been published previously (13). The primers SF (5'-CTCTT CTCCA TGTC TGTA A-3') and SR (5'-GTTGG TTATC TGCCT GACC) were used to amplify an 85-bp sequence from the gene encoding a fibrin-like protein (fmi) of *Salmonella* Enteritidis (SA 94251). The primer pair comprising LF (5'-CTCGC AAAAA ATGAA GTTC-3'), positions 2539 through 2557 and LR (5'-ACTCC TGGTG TTCTC CGATT-3', positions 2636 through 2617) amplifies a 98-bp sequence from the hemolysin (hly) gene of *L. monocytogenes* (GenBank accession no. M24199). The PCR reaction was carried out in glass capillary tubes (Roche Diagnostics, Laval, Quebec, Canada). The reaction mixture (10 μl) contained 1 μl of FastStart SYBR Green enzyme and buffer (Roche Diagnostics), 3.5 mM magnesium chloride, 0.25 μM of each primer, and 1 μl of DNA template. The PCR protocol consisted of an initial denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 8 s, annealing at 55°C for 5 s, and elongation at 72°C for 10 s. Fluorescence signals obtained were continuously monitored to confirm amplification. Following PCR, melting-curve analysis was carried out. The PCR products were cooled to 65°C and then slowly heated to 95°C at a rate of 0.1°C/s.

The direct amplification of the 98-bp portion of the hly gene of *L. monocytogenes* resulted in a product with a GC content of 49% and a melting temperature (T_m) of 80 ± 0.5°C, and the amplification of the fmiA gene (85 bp) from *Salmonella* strains resulted in a product with a T_m of 86 ± 0.5°C.

**RESULTS AND DISCUSSION**

Effect of preenrichment on detection of *L. monocytogenes* and Salmonella Enteritidis. The length of the preincubation period had a pronounced effect on the detection limit of the PCR assay (Table 1). After overnight (16

<table>
<thead>
<tr>
<th>DNA extraction procedure</th>
<th>Organism</th>
<th>Detection limit (CFU/g) for original sausage sample after enrichment for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>1</td>
<td><em>L. monocytogenes</em></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Enteritidis</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td><em>L. monocytogenes</em></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Enteritidis</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td><em>L. monocytogenes</em></td>
<td>&gt;10^3</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Enteritidis</td>
<td>&gt;10^3</td>
</tr>
</tbody>
</table>

^n^ Sausages were placed into stomacher bags with filter inserts containing 90 ml of TSB and homogenized for 2 min in a Stomacher. The stomached samples were incubated at 37°C with shaking, and samples were removed after 4, 6, 8, 10, and 16 h for DNA extraction. NA, target sequences not amplified or not amplified consistently.

**TABLE 1. Effect of DNA extraction procedure on detection limit of real-time PCR assay**
h) enrichment in TSB at 37°C. *L. monocytogenes* and *Salmonella* Enteritidis could be detected at levels of 3 and 4 CFU/g, respectively, in the original sausage no matter which DNA extraction procedure was used. This finding is in general agreement with those of other studies demonstrating that limits of detection of real-time PCR assays for foods could be increased from about $10^3$ CFU/ml to about 1 CFU/ml following enrichment for 16 h (3). However, the ability to detect small numbers of cells in the raw sausage meat after shorter enrichment times was strongly dependent on the DNA extraction procedure used. After 6 to 8 h of enrichment in TSB, no amplification of the targets by PCR was observed following DNA extraction by procedure 1. The results obtained when DNA was extracted by procedure 2 were inconsistent, perhaps because of losses during the centrifuge and washing steps. When the PCR assay was conducted for DNA extracted by procedure 3, results were consistent, and the minimum detectable levels in the sausage were 3 CFU/g for *Listeria* and 4 CFU/g for the *Salmonella* strain. The results of the melting-curve analysis for sausage samples processed by procedure 3 are presented in Figure 1.

**Comparison of DNA extraction protocols.** With the use of the simple real-time PCR protocol previously described (13), together with the developed template DNA extraction method, low levels of contamination of meat with *L. monocytogenes* and *Salmonella* Enteritidis could be easily detected in 10 h. Burtscher et al. (2) previously described a multiplex PCR assay for the detection of small numbers of *L. monocytogenes* and *Salmonella* Enteritidis in biological waste with the use of a modification of the NaI protocol (16) to extract DNA. These authors claimed that they could detect levels of <10 cells per ml of waste by this method, but the method took 1 to 2 days to carry out. In the present study, the 1-h proteinase K treatment included in the original protocol (2) was replaced by a treatment involving 10 min of heating in boiling water and purification with the use of a commercial DNA spin column. It has previously been reported that boiling is an effective way of lysing cells of foodborne pathogens (12). The revised protocol, from sample preparation to the attainment of results, took about 10 h.

The other DNA extraction procedures tested (i.e., procedures 1 and 2) were not effective in reducing inhibition of the PCR. Makino et al. (16) reported a detection limit of $5 \times 10^2$ CFU/g for *L. monocytogenes* in soft cheese and minced meat without the need for preenrichment. Genereleaser proved ineffective in removing PCR inhibitors from the meat, a finding that is in agreement with previous studies in which Genereleaser was used to purify target DNA for the detection of *Salmonella* Typhimurium in chicken mince or beef mince (15). These authors also reported that the boiling method was ineffective. However, Meng et al. (17) reported that a PCR assay of a DNA template prepared with a boiling method and Genereleaser could detect as few as 25 and 38 CFU of *Escherichia coli* O157:H7, respectively, but their study involved pure cultures and not food samples.

In conclusion, we have described a method to prepare template DNA for inclusion in a real-time multiplex PCR assay for *L. monocytogenes* and *Salmonella* Enteritidis that is capable of detecting as few as 3 to 4 CFU/g of either organism in raw sausage meat within 10 h.

**ACKNOWLEDGMENTS**

The authors thank the Natural Sciences and Engineering Research Council of Canada and Dairy Farmers of Ontario for financial support through a grant to M. W. Griffiths. X. Wang thanks Shanxi Agricultural University for granting leave and the government of the People’s Republic of China for financial support.

**REFERENCES**


