



DNA Extraction Procedures for Real-time PCR Detection of *Listeria monocytogenes* and *Listeria* spp. from Artificially Contaminated Food Samples



K.C. Jinneman¹, K. J. Yoshitomi¹, P.A. Orlandi², S.D. Weagant³, R. Zapata⁴, P.E. Browning⁴, and W.M. Fedio⁴

¹ Food and Drug Administration, Bothell, WA 98021; ² Food and Drug Administration, Rockville, MD 20857; ³ Weagant Consulting, Poulsbo, WA 98370

⁴ New Mexico State University, Food Safety Laboratory, Las Cruces, NM 88003

SUMMARY

Identification of *Listeria* species and specifically *Listeria monocytogenes* by real-time PCR allows rapid detection of the pathogen from contaminated food.

The objective of the current research is to evaluate DNA extraction protocols from food enrichments for detection of *Listeria monocytogenes* by two real-time PCR procedures.

Four DNA extraction protocols from Buffered Listeria Enrichment Broth (BLEB) and DemiFraser/Fraser enrichments of artificially contaminated foods (Asadero cheese, Queso Fresco, Brie cheese, guacamole, coleslaw, and smoked salmon) were evaluated with two real-time PCR procedures for detection of *Listeria monocytogenes* and *Listeria* spp. For DNA extraction, a semi-automated magnetic particle-based extraction instrument (MagNA Pure Compact), with and without enzymatic treatment, PrepSEQTM Rapid Spin columns and wash-spin-boil (WSB) sample preparation techniques were compared. Real-time PCR analysis of the DNA extracts were conducted on the ABI 7500 fast platform using a multiplex *Listeria* spp./*L. monocytogenes* real-time (LIS) PCR assay targeting regions of the *iap* gene and designed to simultaneously detect *L. monocytogenes* as well as all *Listeria* species and the MicroSEQ[®] *Listeria monocytogenes* Pathogen Detection kit.

MagNA Pure Compact DNA extraction with or without enzymatic digestion of the samples was effective for template preparation from BLEB and Fraser Broth enrichments for both PCR procedures for all of the foods tested. The rapid spin columns did not work well with most food samples, causing numerous false positive reactions in uninoculated food samples (16-100%) and inhibition of DNA amplification particularly in Fraser broth enrichments. A 1:10 dilution of the template improved its performance with the LIS assay but not the MicroSEQ[®] *Listeria monocytogenes* assay. The WSB preparations worked well with the LIS PCR assay, but even 1:10 dilutions of the preparations gave poor results with the MicroSEQ[®] *Listeria monocytogenes* assay for most foods.

These results show that template preparation is important for reliable real-time PCR screening of food enrichments for *Listeria monocytogenes*. The MagNA Pure Compact procedure without additional enzyme treatment of the sample resulted in template preparations that were suitable for both PCR assays. The LIS PCR assay was the most reliable test for detection of *Listeria* spp./*L. monocytogenes* from both BLEB and Fraser broth food enrichments.

INTRODUCTION

Identification of *Listeria* species and specifically *Listeria monocytogenes* by real-time PCR allows rapid detection and accurate confirmation of presumptive isolates. A real-time 5'-nuclease assay PCR assay has been developed targeting regions of the *iap* gene and designed to simultaneously detect *L. monocytogenes* as well as all *Listeria* species. The *iap* gene is present in all *Listeria* spp. with conserved and variable regions specific for each species (Bubert et al., 1992).

The purpose of study was to Evaluate DNA extraction protocol from food enrichments using a semi-automated magnetic particle-based extraction instrument (MagNA Pure Compact, Roche, Indianapolis, IN) with and without an enzyme pretreatment, the PrepSEQTM Rapid Spin columns and a wash-spin-boil (WSB) sample preparation technique. Evaluate enrichment screening by a multiplex real-time PCR specific for *Listeria monocytogenes* and *Listeria* spp with internal positive control (LIS) on a high throughput real-time thermocycler, AB7500 Fast (Applied Biosystems/Life Technologies, Foster City, CA) and the ABI MicroSEQ[®] *Listeria monocytogenes* Pathogen Detection System on the same thermocycler platform.

MATERIALS AND METHODS

Sample preparation and enrichment Asadero cheese, brie cheese, coleslaw, queso fresco, guacamole, and smoked salmon were prepared. Food samples were artificially contaminated with the *Listeria* species at low (~0.1 cfu/g), medium (~1.0 cfu/g) and high (~10 cfu/g) levels. Uninoculated control samples were also prepared.

Samples (25g portions) were enriched in BLEB containing pyruvate and, after 4 hour at 30°C, acriflavin, cycloheximide and nalidixic acid added. Samples were further incubated at 30°C for a total of 48h. A parallel set of samples was enriched in Demi-Fraser broth at 30°C for 24h and subcultured into Fraser broth and incubated an additional 24h at 30°C.

For details see poster P3-03.

PCR Procedure

Samples were screened from the 48 h enrichment broths by four template preparation methods. Two PCR procedures (*Listeria* spp. and *L. Monocytogenes* Internal control (LIS) Multiplex PCR assay and the ABI MicroSEQ[®] assay) were used for evaluation of the extraction procedures listed below.

Wash Spin Boil Preparation (WSB)

One mL of each enrichment was transferred to a microcentrifuge tube and centrifuged at 12,000 x g for 3 minutes. The supernatant was removed and the pellet was resuspended in 0.85% saline and centrifuged again at 12,000 x g for 3 minutes. After removing the supernatant, the pellet was resuspended in 1 mL of sterile water, boiled for 10 minutes and centrifuged at 12,000 x g for one minute. The supernatant was used for PCR analysis.

PrepSEQTM Rapid Spin Preparation (RS)

Enrichment broth (750 µl) was loaded onto a PrepSEQTM Rapid Spin column and centrifuged for 3 min at 12,000 x g. The used spin column was discarded and the supernatant removed. Proteinase K lysis buffer (55 µl) was added to the pellet and resuspended. The mixture was incubated at 56°C for 30 min, followed by heating at 95°C for 10 minutes. After heating, samples were centrifuged for one minute at 12,000 x g and the supernatant was used for PCR.

MATERIALS AND METHODS

MagNA Pure Standard Extraction (MagNA Pure)

An aliquot (250 µl) of enrichment broth plus 250 µl of MagNA Pure Bacteria Lysis Buffer (BLB) were combined and vortexed briefly. The sample was boiled for 10 minutes and centrifuged for 3 minutes at 12,000 x g. Boiled sample (400 µl) was transferred to a MagNA Pure Compact Sample Tube for the DNA Bacteria purification protocol with a setting of 50 µL elution volume.

MagNA Pure Standard Extraction plus Enzymatic Digestion (MagNA Pure +)

An aliquot (250 µl) of enrichment broth plus 250 µl of MagNA Pure Bacteria Lysis Buffer (BLB) were combined. An enzyme Cocktail (lysozyme and lysostaphin) were added and vortex briefly. After 20 min incubation at 37°C, 20 µl Proteinase K solution was added and samples were incubated at 65°C for 10 min. Samples were boiled for 10 min. and centrifuged for 3 minutes at 12,000 x g. Boiled sample (400 µl) was transferred to a MagNA Pure Compact Sample Tube for the DNA Bacteria purification protocol with a setting of 50 µL elution volume.

Real-Time PCR detection of Listeria from food enrichments

The ABI 7500 FAST PCR (Applied Biosystems/Life Technologies, Foster City, CA) was used for real-time PCR of the extracted DNA template samples by both the *Listeria* spp./*L. monocytogenes* real-time (LIS) PCR assay and the MicroSEQ[®] *Listeria monocytogenes* assay.

Multiplex Listeria spp./L. monocytogenes real-time (LIS) PCR assay

The composition of the Master mix for real-time PCR is given below.

Table with 3 columns: Master Mix Component, Volume (µl) per Reaction, Volume (µl) for 42 reactions

ABI PCR strip tubes were used for conducting the assays. For each test, 28µl of Listeria Master Mix and 2 µl of template were used.

Computer Set-Up for ABI 7500 FAST:

Assay: Standard Curve (Absolute Quantitation) Run Mode: Fast 7500

The Listeria PRL assay targets the iap gene

Table with 4 columns: Target Name, Reporter, Quencher, Color

The passive reference was ROX.

Thermal cycler Profile

A 2-step PCR protocol was used:

Table with 2 columns: Initial Activation, 45 cycles

ABI MicroSEQ® Listeria monocytogenes Pathogen Detection System

The MicroSEQ[®] *Listeria monocytogenes* Pathogen Detection Kit was used according to the manufacturer's instructions, using 30µL of undiluted or diluted extracts from the *Listeria* enrichment broths.

The MicroSEQ[®] *Listeria monocytogenes* assay beads are contained in 8-tube strips, with one tube for each reaction.

Run Mode: Fast 7500Data was analyzed with ABI 7500 FAST PCR Rapid Finder Express.

The MicroSEQ[®] *Listeria monocytogenes* assay is specific for Listeria monocytogenes only.

Table with 4 columns: Target Name, Reporter, Quencher, Color

The passive reference was ROX.

Thermal cycler Profile

A 2-step PCR protocol was used:

Table with 2 columns: Initial Activation, 40 cycles

Table 1. Listeria spp./L. monocytogenes real-time (LIS) PCR assay- Undiluted

Table with 10 columns: Sample, Inoculum Level, BLEB, DF, WSB

Table 3. MicroSEQ® Listeria monocytogenes assay --Undiluted

Table with 10 columns: Sample, Inoculum Level, BLEB, DF, WSB



Figure 6: Salmon Rapid Spin *Listeria* spp./*L. monocytogenes* real-time (LIS) PCR assay - False Positive

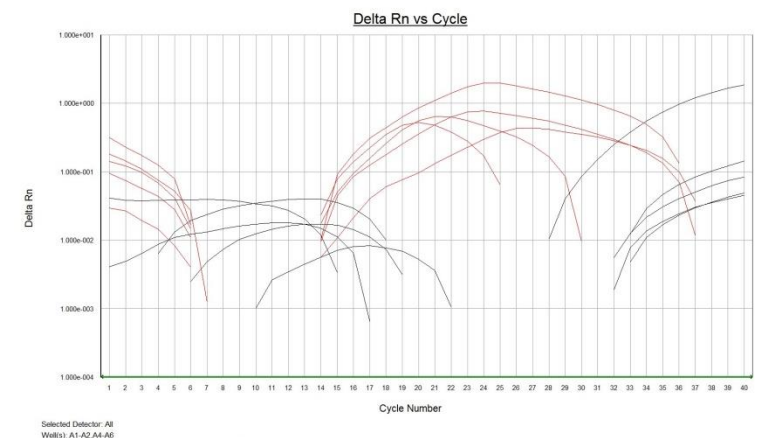


Figure 7: Salmon Rapid Spin MicroSEQ[®] - False Positive

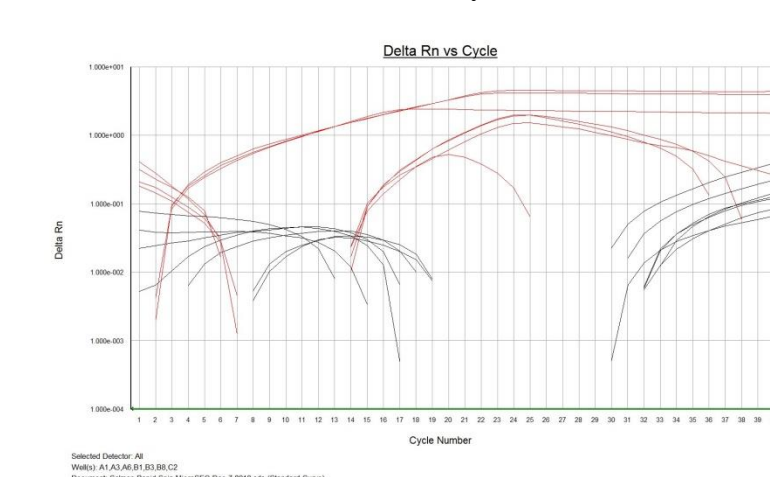


Figure 8: Salmon Rapid Spin MicroSEQ[®] - No Amp

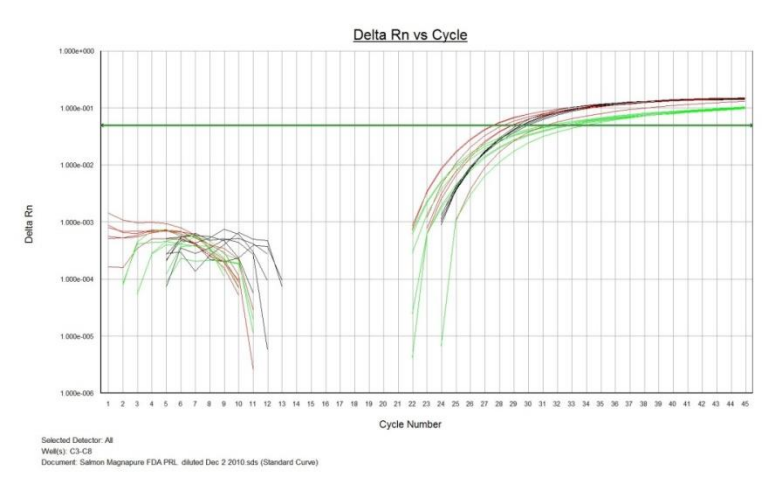


Figure 9: Salmon MagNA Pure *Listeria* spp./*L. monocytogenes* real-time (LIS) PCR assay -- Good Amplification

Table 2. Listeria spp./L. monocytogenes real-time (LIS) PCR assay-- 1:10 Dilution

Table with 10 columns: Sample, Inoculum Level, BLEB, DF, WSB

Table 4. MicroSEQ® Listeria monocytogenes assay-- Diluted 1:10

Table with 10 columns: Sample, Inoculum Level, BLEB, DF, WSB



Figure 1: ABI 7500 Fast Real-Time PCR System



Figure 2: MagNA Pure Compact System

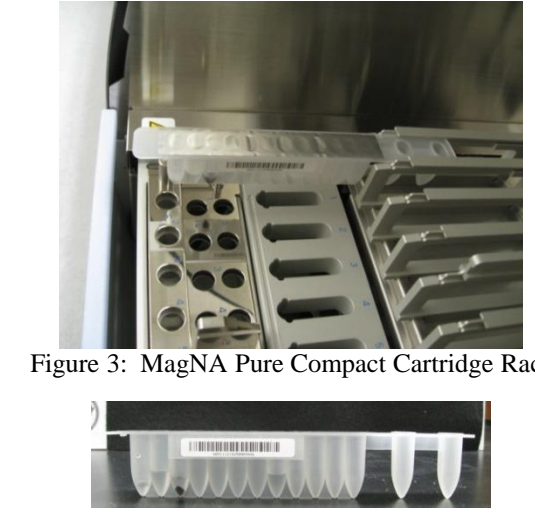


Figure 3: MagNA Pure Compact Cartridge Rack



Figure 5: PrepSEQTM Rapid Spin Column and Collection Tube

RESULTS and DISCUSSION

- Four sample preparation procedures were used for 48h Listeria enrichments (BLEB and DF/Fraser Broth) and examined by two real-time PCR detection procedures - A multiplex Listeria spp./Listeria monocytogenes PCR and MicroSEQ® Listeria monocytogenes assay

CONCLUSIONS

- MagNA Pure extraction was the most effective procedure for template preparation from BLEB and DF/Fraser broth enrichments

REFERENCES

Bubert, A., Kohler, S., and Goebel, W. The homologous and heterologous regions within the iap gene allow genus- and species-specific identification of Listeria spp. by polymerase chain reaction. Appl Environ Micro. 1992. 58:2625-2632.

ACKNOWLEDGEMENTS

We would like to thank Cecilia Garcia, Vivienne Beauchemin and JoAnne Dupre for technical support.