P3-35

Development of a Rapid Detection Method for *Listeria* with Idaho Technology's R.A.P.I.D.® LT System in Soft Cheese and Deli Meat, and on Environmental Surfaces

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ABSTRACT

Introduction: *Listeria monocytogenes* has been linked to illness from the consumption of many foods. The R.A.P.I.D. LT system is optimal for rapid detection of *Listeria* in foods because of the sensitivity and specificity of PCR and rapid real-time thermocycling (30 min to result). A *Listeria* LT Food Security System (FSS) protocol, similar to the *Salmonella* LT FSS, has been designed to this end for *Listeria* species.

Purpose: To develop a rapid *Listeria* detection method that minimizes the complexity and length of enrichment, based on the principles of the *Salmonella* LT FSS (single abbreviated enrichment, 5 × 1 sample pooling, cell lysis, internal controls, and automatic analysis), for soft cheeses, deli meats, and environmental surfaces, an assay specific for *Listeria* species was developed.

Methods: Several media were tried for each matrix, and minimal enrichment times determined. 25 g portions of food were inoculated with ~1 CFU/25 g of several *Listeria* strains. Samples were enriched at 30° C in 225 ml of media and tested at different time points. Ceramic, stainless steel, and plastic surfaces (4 × 4 in.) were inoculated with low levels (~1 CFU recovered) of *Listeria*, dried, swabbed with a sponge hydrated with neutralizing broth, enriched at 30° C in 100 ml of media, and tested at different time points. All samples were evaluated individually and pooled.

Results: The optimal medium for *Listeria* detection from surfaces is Buffered *Listeria* Enrichment Broth (BLEB), while for soft cheeses and deli meat a more selective media is required. Enrichment times vary from 24 h for environmental and cheese samples to 26 h for deli meat samples. Downstream sample processing and PCR analysis results in a final protocol time of 25 to 29 h. The system specifically detects *Listeria* species. The system is ready for method comparison and for sensitivity, and specificity evaluations.

Significance: With further evaluation, this PCR-based system is expected to provide a rapid, effective *Listeria* detection system that is faster than other methods.

ISSUE

Current methods can take approximately 4 days to identify *Listeria* species in food and environmental samples. The goal of the *Listeria* LT FSS is to provide a sensitive and robust system that is faster than currently available detection systems.

TEST METHODOLOGY

The *Listeria* LT FSS identifies *Listeria* spp. bacteria through a series of sequential steps that include sample collection, enrichment, sample preparation, cell lysis to release DNA, DNA amplification in the Idaho Technology R.A.P.I.D. LT instrument, and automatic result interpretation by the R.A.P.I.D. LT software.

Food matrices are sampled and added to a sterile stomacher bag, while environmental samples are obtained with a sponge and added to a sterile collection bag. Enrichment medium is added and the samples are homogenized. Samples are incubated 24 to 28 hours at 30° C ± 2° C. After incubation, a small aliquot is removed for cell lysis and PCR analysis. These enriched samples may be tested individually or tested as pooled composite samples.

CLAIMS

- **Analytes:** *Listeria* spp.
- **Matrices:** Turkey deli meat, Mexican soft cheese, and environmental surfaces (ceramic tile, plastic and stainless steel which are sampled with a sponge)
- **Limit of Detection:** One colony forming unit (CFU)/25g of food sample, or equivalent recovery from a 100 cm^2 (approximately 4x4 inch) environmental surface.
- **Inclusivity Claims:** Detects naturally occurring *Listeria* species with the exception of *Listeria grayii*.
- **Exclusivity Claims:** Does not detect closely related and other naturally occurring non-*Listeria* organisms.
- **Sensitivity:** Rates of sensitivity, specificity, and false positives equivalent or superior to the FDA Bacteriological Analytical Manual (BAM), or USDA Microbiology Laboratory Guide (MLG) method.

These claims are currently being evaluated for the AOAC by Idaho Technology as well as by sponsor and independent laboratories.

The *Listeria* LT FSS evaluation will include an evaluation of a post enrichment pooling protocol. A number of significant benefits that the pooling provides are:

- Increased sample throughput i.e. up to 5 times
- Significant labor savings i.e. >60%
- Substantial cost reduction i.e. >60%
- Original sample integrity maintained

This pooling method relies on combining up to 5 sub-samples to create a single composite sample. If a positive pooled sample is obtained, then the individual post-enrichment samples can be re-analyzed to achieve rapid confirmation.

LISTERIA LT FSS PROTOCOL

SUMMARY OF DEVELOPMENT AND ONGOING AOAC EVALUATION

SUMMARY OF PROTOCOL DEVELOPMENT

Many liquid media were evaluated for use with food samples with the *Listeria* FSS including: UPB, Fraser, Half-Fraser, BLEB, BLEB with selective additives and Oxoid's ONE Broth with supplements. ONE Broth provided consistently detectable enrichment of fractionally inoculated samples for both Mexican style soft cheeses and turkey deli meat within 26-28 hours. Initial evaluations were performed at 24 hours post enrichment.

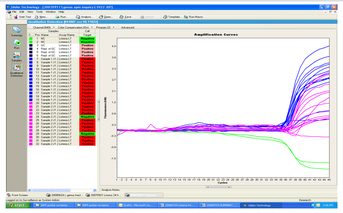


Figure 1. Example of 24 hour enrichment of Mexican style soft cheese inoculated with ~4 CFU *Listeria* in Universal Pre-enrichment Broth.

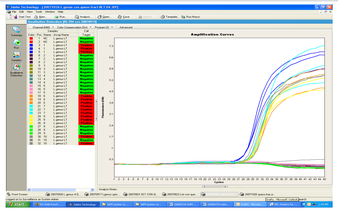


Figure 2. Example of 24 hour enrichment of fractionally inoculated Mexican style soft cheese in Oxoid ONE broth with supplements. Capillary in position 31 is a failed reaction (would be prompted as an invalid call/retest in FSS software). Positive calls verified by culture.

Turkey deli meat, of varying quality, from different producers was evaluated with multiple broths. Oxoid ONE Broth provided optimal enrichment for a range of fractionally inoculated samples after 26 hours of enrichment.

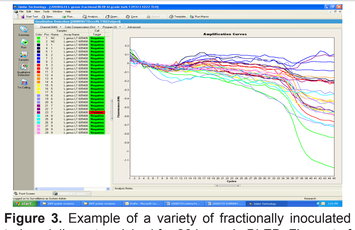


Figure 3. Example of a variety of fractionally inoculated turkey deli meat enriched for 26 hours in BLEB. Five out of these ten samples tested positive both by culture and PCR after 48 hours of enrichment.

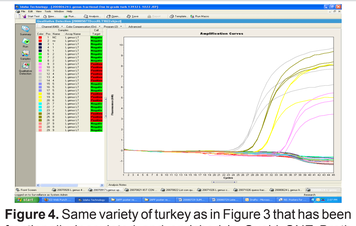


Figure 4. Same variety of turkey as in Figure 3 that has been fractionally inoculated and enriched in Oxoid ONE Broth with supplements for 26 hours. Calls verified by culture.

Figure 5A. Method Comparison

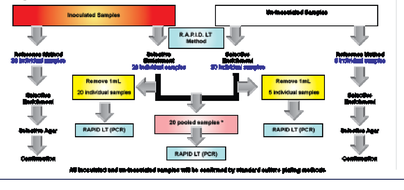
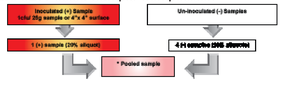


Figure 5B. Pooled Sample Composition



PRELIMINARY RESULTS OF ONGOING AOAC EVALUATION

Method comparison results for the ongoing AOAC evaluation study of the *Listeria* LT FSS have been obtained for Mexican style soft cheese and for plastic environmental samples by the Marshfield Clinic (sponsor laboratory). Exclusivity claims have also been evaluated by the Marshfield Clinic.

Food

A batch of Mexican style soft cheese was fractionally spiked with *Listeria welshimeri* ATCC 35897 (1.85 MPN/25g) and allowed to equilibrate for 70 hours. This batch was split into a reference batch and a batch to be tested with the *Listeria* LT FSS. The inoculated reference batch was divided into of 20 samples of 25 grams each. These samples were combined with media and tested by the reference method (FDA BAM). Meanwhile, the *Listeria* LT FSS batch was split into 20 additional samples that were combined with 225 mLs Oxoid ONE Broth with supplements (Oxoid CM1066 and SR0234), enriched for 24 hours at 30° C and then tested by the *Listeria* LT FSS. Twenty-five uninoculated 25g samples were also prepared. Five of these samples were treated according to the reference method while the other 20 were enriched in 225 mLs Oxoid ONE Broth with supplements for 24 hours at 30° C. Five of these were sub-sampled and tested by the *Listeria* LT FSS. All 20 of the inoculated individual samples were then pooled with the uninoculated samples (50mLs of individual spiked sample + 50mLs of 4 separate uninoculted samples = 250 mL composite sample). (Figures 5A and 5B summarize the workflow).

Of the reference samples 15 out of 20 samples were reported as positive. For the samples that were tested by the *Listeria* LT FSS, 16 of 20 were reported as positive and were verified by culture. There were no false positive or false negatives observed. These individual samples were then pooled with uninoculated samples (as described above), and the same positive calls were observed.

Environmental

Forty 4"x4" plastic environmental samples were inoculated with 100 µL of a liquid dilution that contained approximately 11 CFU of *Listeria innocua* ATCC 33090 per inoculation. Twenty five additional samples were not inoculated with organism but were otherwise treated identically. All of the plastic samples were then allowed to dry at room temperature for 22 hours and then sampled with a sterile sponge that had been rehydrated with neutralizing broth. The sponges were then placed in sterile bags. Twenty of the inoculated and 5 of the uninoculated samples were treated and tested according to the reference method while the other 20 inoculated and 20 uninoculated samples were combined with 100 mLs of pre-warmed Buffered Listeria Enrichment Broth (BLEB, Oxoid CM0897) and allowed to incubate for 24 hours at 30° C (Figure 5A summarizes the workflow). After enrichment these samples were tested with the *Listeria* LT FSS and confirmed by culture. All 20 of the inoculated samples were then pooled with the uninoculated samples (20 mLs of individual spiked sample + 20 mLs of 4 separate uninoculted samples = 120 mL composite sample) and tested with the *Listeria* LT FSS.

Of the reference samples 14 out of 20 samples were reported as positive. For the samples that were tested by the *Listeria* LT FSS, 13 of 20 were reported as positive and were verified by culture. There were no false positive or false negatives observed. These individual samples were then pooled with uninoculated samples (as described above), and the same positive calls were observed.

FOOD (Cheese: Individual and Pooled Samples)

Figure 6A. Individual Mexican style soft cheese samples. Negative control (position 1), 16/20 fractionally spiked positive samples confirmed by culture (positions 2-21) and 5 uninoculated samples (positions 22-26).

Figure 6B. Pooled Mexican style soft cheese samples. Negative control (position 1), 50 mL of individual cheese samples from above (Fig. 6A) combined with 50 mL of 4 separate uninoculated samples (positions 2-21).

Table I. Method Comparison Results, Individual Samples

Matrix	Inoculating organism	Level	MPN CFU/ sample	No. test portions	Reference Method: Positive	Test kit: Presump. Positive	Test kit: Confirmed Positive	Chi Square	Test Kit Performance: Relative Sensitivity	False negative	Specificity rate	False positive
Soft Cheese	*Listeria welshimeri* ATCC 35897	Low	1.85	20	15	16	16	0.14	107%	0	100%	0
		Control	0	5	0	0	0	-	-	-	-	-
Plastic	*Listeria innocua* ATCC 33090	Low	NA	20	14	13	13	0.11	93%	0	100%	0
		Control	0	5	0	0	0	-	-	-	-	-

Table II. Method Comparison Results, Pooled Samples

Matrix	Inoculating organism	Level	MPN CFU/ sample	No. test portions	Reference Method: Positive	Test kit: Presump. Positive	Test kit: Confirmed Positive	Chi Square	Test Kit Performance: Relative Sensitivity	False negative	Specificity rate	False positive
Soft Cheese	*Listeria welshimeri* ATCC 35897	Low	1.85	20	15	16	16	0.14	107%	0	100%	0
		Control	0	5	0	0	0	-	-	-	-	-
Plastic	*Listeria innocua* ATCC 33090	Low	NA	20	14	13	13	0.11	93%	0	100%	0
		Control	0	5	0	0	0	-	-	-	-	-

Environmental Samples (Plastic: Individual and Pooled Samples)

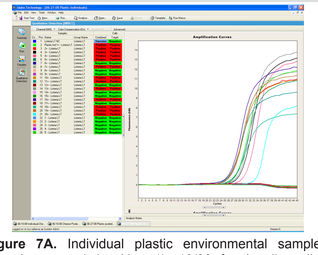


Figure 7A. Individual plastic environmental samples. Negative control (position 1), 13/20 fractionally spiked positive samples confirmed by culture (positions 2-21) and 5 uninoculated samples (positions 22-26).

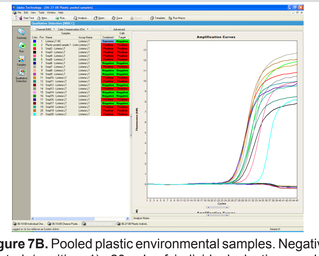


Figure 7B. Pooled plastic environmental samples. Negative control (position 1), 20 mL of individual plastic samples from above (Fig. 7A) combined with 20 mL of 4 separate uninoculated samples (positions 2-21).

EXCLUSIVITY

Thirty-five strains of non-*Listeria* bacteria and *Listeria grayii* were grown overnight (22-24 hours) in non-selective media and tested with the *Listeria* LT FSS. None of the strains were detected by the system.

Exclusivity Panel

Species	Source
Bacillus cereus	ATCC 13061
Bacillus mycoides	ATCC 6462 (soil)
Brochothrix thermosphacta	ATCC 11509 (pork sausage)
Carnobacterium divergens	ATCC 35677 (beef)
Carnobacterium gallinarum	ATCC 49517 (chicken)
Citrobacter braakii	Clinical
Citrobacter freundii	Clinical
Corynebacterium amycolatum	ATCC 49368 (NCFB)
Corynebacterium bovis	ATCC 7715 (milk)
Escherichia coli (Generic)	ATCC 51813
Escherichia coli O55:H7	USDA
Escherichia coli O145:NM	USDA
Escherichia coli O157:H7	Food
Enterobacter sakazakii	ATCC 51329
Enterococcus malodoratus	ATCC 43197 (cheese)
Erysipelothrix rhusiopathiae	ATCC 35428 (sheep dip)
Klebsiella pneumoniae	ATCC 13883
Kurthia gibsonii	ATCC 43195 (meat)

Species	Source
Lactobacillus delbrueckii subp. *lactis*	ATCC 12315 (swiss cheese)
Lactobacillus plantarum	ATCC 14917 (pickled cabbage)
Leuconostoc gelidum	ATCC 49366 (chill-stored meats)
Listeria grayii	ATCC 25401
Micrococcus luteus	ATCC 10240
Morganella morganii	ATCC 25829
Pantoea agglomerans	Clinical
Propionibacterium freundenreichii	ATCC 9617
Proteus hauseri	ATCC 13315
Proteus vulgaris	ATCC 33420
Pseudomonas aeruginosa	ATCC 27853
Rhodococcus equi	ATCC 6939
Salmonella enteriditis	USDA
Shigella flexneri	ATCC 9199
Staphylococcus aureus	ATCC 6538
Staphylococcus xylosus	ATCC 29971 (skin)
Streptococcus pneumoniae	ATCC 6303

REFERENCES

(1) The U.S. Food and Drug Administration Bacteriological Analytical (http://www.cfsan.fda.gov/~ebam/bam-10.html)
(2) United States Department of Agriculture Food Safety Inspection Service Microbiological Laboratory Guidelines (http://www.fsis.usda.gov/PDF/MLG_8_06)