Application of a 'Universal' Real-Time Primer for PCR Detection of *Listeria monocytogenes* from Meats

S. Mitra and P.M. Muriana.

Story in Brief

Listeria monocytogenes is an important foodborne pathogen which is a frequent contaminant in the postprocessing areas where ready-to-eat (RTE) meats are manufactured. *L. monocytogenes* causes numerous foodborne outbreaks and deaths and rapid detection of *L. monocytogenes* may enable processors to reduce the time required for testing. Our objective was to examine the Ampliflour Uniprimer™ real-time PCR technique for rapid and specific detection of *L. monocytogenes* from meat products following primary and secondary enrichment. For real-time PCR detection, primers were selected using the Vector NTI suite primer analysis program that targets the hemolysin gene (*hly*A) specific for *L. monocytogenes*. *L. monocytogenes* ScottA-2 was inoculated by serial dilution into raw and processed meats from 10^7 to 10^0 cfu/25gm. Real-time PCR was performed following primary and secondary enrichment of the inoculated products. In both raw and RTE meats we were able to detect *L. monocytogenes* (after enrichment) when inoculated as low as 1 cfu/25gm of meat with a maximum detection time of 2 days including primary and secondary enrichments. No amplification was obtained with the negative control or samples having non-pathogenic species of *Listeria*. Uniprimer™ PCR targeting the 16S rRNA gene yielded a greater fluorescence at lower threshold cycles due to six copies of the 16S rRNA gene per *Listeria* genome.

Key words: *Listeria monocytogenes*, Detection, Ready-to-Eat Meat, Uniprimer™, Real-time PCR

Introduction

Listeria monocytogenes is a Gram-positive, facultative, psychrotropic, intracellular bacterium which is pathogenic to humans and animals. These bacteria are capable of causing severe infections such as septicemia and meningitis, especially in immunocompromised individuals, newborns and pregnant woman (Hein et al. 2001). Several large outbreaks of listeriosis have been associated with contaminated vegetables, milk, raw and ready-to-eat (RTE) meat products on which the bacteria can multiply even at low temperatures. The threatening characteristics of *L. monocytogenes* is its ability to survive and grow in raw and RTE foods held under refrigeration temperatures and its ability to adhere and form biofilms on food contact surfaces that are resistant to sanitizers (Daeschel et al.1999). In the U.S., there are about 2500 cases of listeriosis per year with 20-40% mortality (Mead et al.1999). The costs of acute listeriosis, the potential for illness, the high fatality rate, have influenced U.S. regulatory agencies to enforce a "zero-tolerance" for *L. monocytogenes* in RTE foods and routine screening of final product in the food industries as a means of HACCP (Norton 2002). Despite the zero-tolerance regulation, multimillion dollar food recalls occur due to contamination (and outbreaks) of RTE foods. Due to the significance of potential product contamination, many processors ascribe to a 'test-and-hold' testing regimen whereby large productions lots of perishable product is held under refrigerated storage until test results come back negative. The seriousness of outbreaks and costs

of refrigerated storage emphasize the need of a rapid, reliable detection systems for a quick detection of *L. monocytogenes* in RTE foods.

The traditional microbiological method for detection and identification of *L. monocytogenes* takes approximately 7-8 days to confirm and identify isolates to species level which is unacceptable for a test-and-hold food testing strategy adopted by the U.S. RTE meat industry. The recently developed Ampliflour system proposes a one-step closed-tube procedure with the use of energy transfer hairpin primers (Uniprimer™) for fluorescence-based PCR detection and has shown potential for use in detection and identification of *L. monocytogenes*. The Uniprimer™ has a specific 3' oligonucleotide sequence called the "Z"-sequence and a 5' hairpin tagged with fluorescein and quencher moieties. The proximity of the fluorophore and the quencher at the base of the hairpin allows quenching of the fluorescence released by the fluorophore (Fig. 1). Theoretically, the opening of the hairpin when incorporated during PCR enables a significantly higher level of fluorescence.

The purpose of our study was to examine and apply the Ampliflour Uniprimer™ real time PCR technique for the detection of *L. monocytogenes* from meats following enrichment. If successful, then subsequent steps may be to examine what can be done to shorten the total detection period.

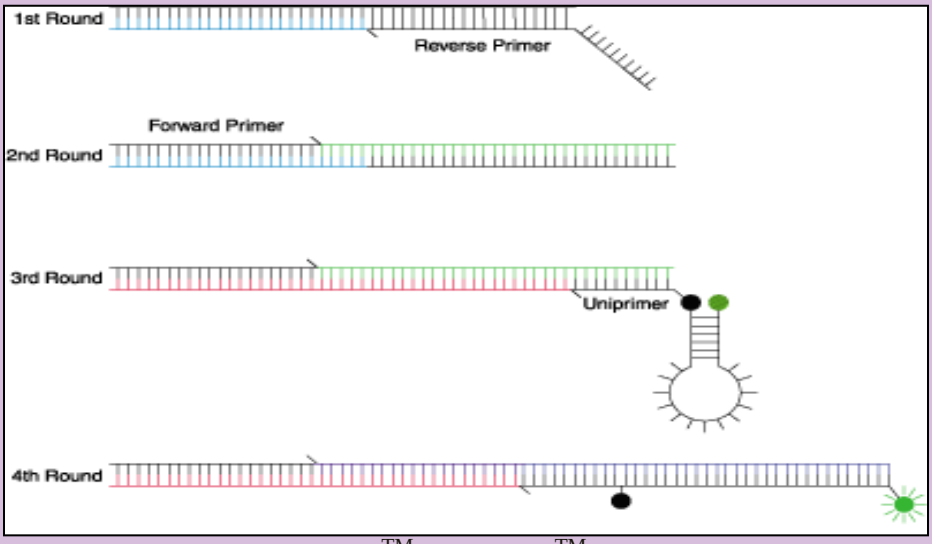

Figure 1. Schematic of the Amplifluor™ Uniprimer™ during real-time PCR. The Uniprimer™ dual label probe uses fluorescein as the fluorophore with excitation at 495 nm and emission at 516 nm that is quenched by a quencher moiety (Dabsyl) when it is present in a hairpin configuration, but not when it is included as part of the amplicon

Materials and Methods

Detection by Real-Time PCR. For testing of pure cultures, *L. monocytogenes* Scott-A2, V7-2, 39-2, 383-2, *L. ivanovii* and *L. innocua* ATCC 19119 were grown overnight in Brain Heart Infusion (BHI) broth at 30°C. Strain Scott-A2 (an outbreak strain) was used as the main target organism for optimization of PCR conditions and for further studies with detection from foods. Prior to the PCR, the DNA of Scott-A2 was extracted by the BAX™ procedure (Qualicon, Wilmington, DE), whereby 5 µl of overnight culture was mixed with 200 µl of BAX lysis

reagent containing protease. Cell lysis was performed by holding the mixture at 55°C for 60 min and then 95°C for 10 min; 5 µl of the BAX lysate (used as the DNA source) was then mixed with 20 µl of PCR reaction mixture. The Uniprimer™ real-time PCR (Chemicon Intl, Temecula, CA), was then performed with different primer sets (Table 1) using the Opticon 2 DNA engine (MJ Research Inc, Waltham, MA) with the following thermal cycling conditions: initial denaturation at 95°C for 4 min, followed by 39 cycles of 95°C for 15 sec, 51°C for 18 sec (annealing), 72°C for 40 sec (extension), a final extension at 72°C for 4 min followed by a final hold period at 4°C. The conditions for Uniprimer™ PCR were optimized by employing the best results obtained by individually assaying different ranges of annealing times, annealing temperatures, $MgCl_2$ levels, extension temperature, and dNTP levels. To determine the minimum number of target cells needed to be detected by real-time PCR within 40 cycles, an overnight culture was serially diluted to the 10^{-9} dilution and all dilutions were checked by real-time PCR.

Table 1. List of primers used in this study.

Primer	Target Gene	Sequence (5' → 3')	Product Size (bp)
Primer I	Hemolysin A (*hly*A)		
	Forward	CAA AAG CTT ATA CAG ATG GAA	110
	Reverse-Z-tail(5')	ACT GAA CCT GAC CGT ACA AAT TTC GTT ACC TTC AGG A	
Primer II	16S rRNA		90
	Forward	TAC ACA CGT GCT ACA ATG GAT A	
	Reverse-Z-tail(5')	ACT GAA CCT GAC CGT ACA CCT ACA ATC CGA ACT GAG AAT A	

Testing of Inoculated Foods. Both raw and RTE meats were used in this portion of the study. *L. monocytogenes* Scott-A2 was serially-diluted (10-fold increments) in 0.1% sterile buffered peptone water until 10^0cfu/ml. A 25-g sample of ground meat (i.e., ground beef) or RTE meat (i.e., hotdogs) was added to 225 mls of primary enrichment broth according to USDA acceptable testing media (Demi-Fraser broth for raw meat; UVM broth for processed meat) and mixed with a stomacher for 1min. Each identically stomached sample bag was inoculated with 1 ml of *L. monocytogenes* dilution from 10^6 cfu/ml to 10^0cfu/ml, stomached again, and incubated at 30°C for 24 hr. After incubation, 0.1 ml of primary enrichment from each sample was inoculated into 10 mls of secondary enrichment broth (MOPS-BLEB), incubated at 37°C for 22-24 hrs, followed by Uniprimer™ real-time PCR detection. This would help identify whether the enrichment protocols are capable of providing sufficient enrichment for detection regardless of the initial level of contaminating *Listeria* in the raw or processed meat.

Testing of Retail Foods for L. monocytogenes. Samples of raw ground products (likely to have *Listeria*) were tested in comparison with the traditional USDA culture test method. Twenty-five grams of raw ground meat (ground beef, pork, or chicken) from different retailers was incubated in enrichment broths and tested by both Uniprimer™ real-time PCR and traditional culture methods. The testing of retail samples and comparison of methods is still currently ongoing.

Statistical Analysis. Data are expressed as the means of triplicate replications. Statistical comparison of maximum fluorescence levels and Ct values, before and after, optimization were performed by one way analysis of variance (Sigma Stat 3.0, SPSS, Chicago, IL). Data were considered signicant when their computed probalities were less than 0.05 (P<0.05).

Results and Discussion

The Ampliflour Uniprimer™ real-time PCR assay using primer set-I that targets the hemolysin gene, amplified only *L. monocytogenes* strains and was negative for *L. innocua* (non-pathogenic), *L. ivanovii* and the non-template control reaction (Fig. 2A). Using a primer set that targets the 16S rRNA gene (primer set-II), we were able to obtain more sensitive detection whereby the fluorescent signal was increased by 3-fold resulting in decrease in the threshold cycle for fluorescence detection.This may be attributed to the presence of six copies of the 16S rRNA gene in *Listeria* in comparison to one copy of the hemolysin gene (Fig. 2B).

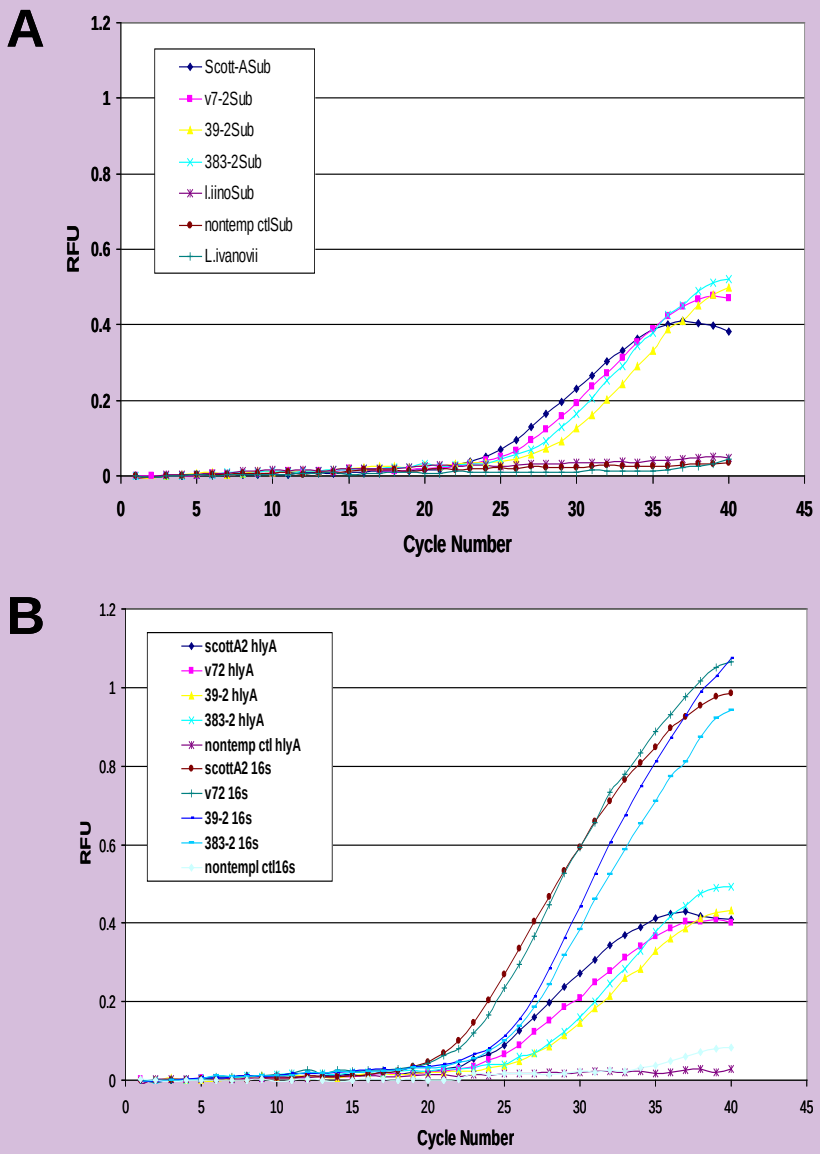

Figure 2. Uniprimer™ PCR amplification using the *hly*A primer set 1 (panel A) or 16S rRNA primer set 2 (panel B). Panel A, PCR using primer set 1 against four strains of *L. monocytogenes* (ScottA, V7, 39, 383), *L. innocua*, *L. ivanovii*, and a non-template control. Panel B, PCR comparing primer set 1 and 2 against four strains of *L.*

monocytogenes and non-template controls.

After optimization, the Ct levels for all the four strains of *L. monocytogenes* improved and were significantly different ($P<0.05$) from their respective Ct levels before optimization (Table 2). Although, the maximum RFU levels for all the strains were higher after optimization, when statistically compared, for some of the strains they were not significantly different due to high standard deviation (S.D.) within the replicates of those strains. However, after optimization the S.D. within the replicates of each strain were smaller and more consistent.

Table 2. Statistical analysis of Uniprimer™ PCR before and after optimization for 4 strains of *L. monocytogenes*

L. monocytogenes strains	Maximum RFU*± S.D. [1]		Ct value*± S.D. [2]	
	Before	After	Before	After
ScottA-2	0.65 ± 0.15 [A]	0.76 ± 0.02 [A]	27.77 ± 0.64 [c]	22.93 ± 0.26 [d]
V7-2	0.63 ± 0.17 [A]	0.73 ± 0.02 [A]	27.97 ± 1.32 [c]	23.43 ± 0.40 [d]
39-2	0.47 ± 0.04 [A]	0.71 ± 0.02 [B]	31.80 ± 0.20 [c]	26.48 ± 0.19 [d]
383-2	0.49 ± 0.15 [A]	0.72 ± 0.00 [B]	31.00 ± 1.82 [c]	26.14 ± 0.17 [d]
Non-template control	0.02 ± 0.01 [A]	0.05 ± 0.01 [A]	0.00 ± 0.00 [c]	0.00 ± 0.00 [d]

*Note: values are the means of triplicate replications. Ct values are calculated by the Opticon Monitor-2 (MJ Research Inc., Alameda, CA) at 0.1 RFU

[1] RFU values in the same row with different upper case letters are significantly different from each other ($P<0.05$)

[2] Ct values in the same row with different lower case letters are significantly different from each other ($P<0.05$)

In our study, we determined the cell concentration required for confident detection in enrichment media to be 10^5 cfu/ml (Fig. 3). This would only be a problem if the method would be used to detect *L. monocytogenes* directly, as most contaminated samples have levels of *Listeria* less than 10^2 cfu/gm. This was not a constraint during detection from food samples as enrichment procedures with either raw or ready-to-eat meat products inoculated with as few as 1 cfu/25 g were able to increase the number of cells after secondary enrichment to at least 10^8 cfu/ml which exceeds our minimum detection level for Uniprimer™ detection (Fig. 4).

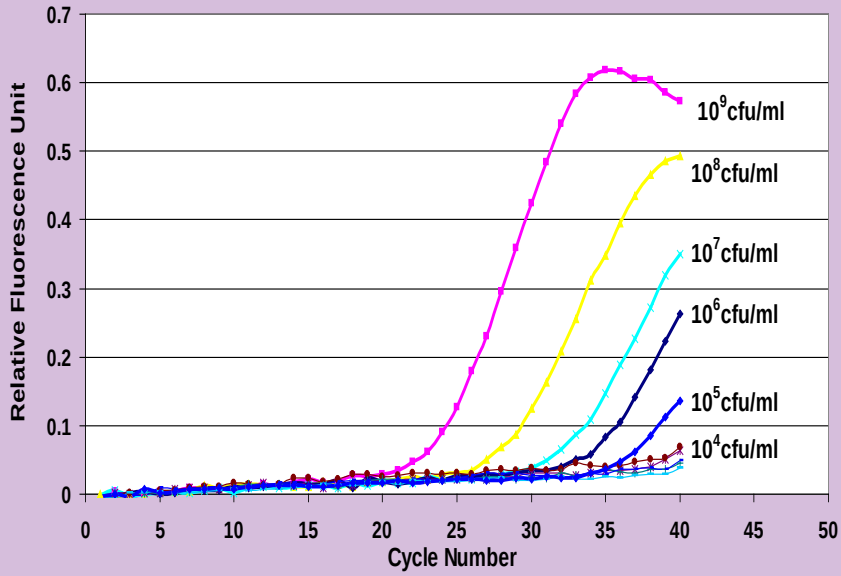

Figure 3. Determination of the minimum cfu/ml required for detection of *L. monocytogenes* by the Uniprimer™ fluorescein-labeled PCR assay.

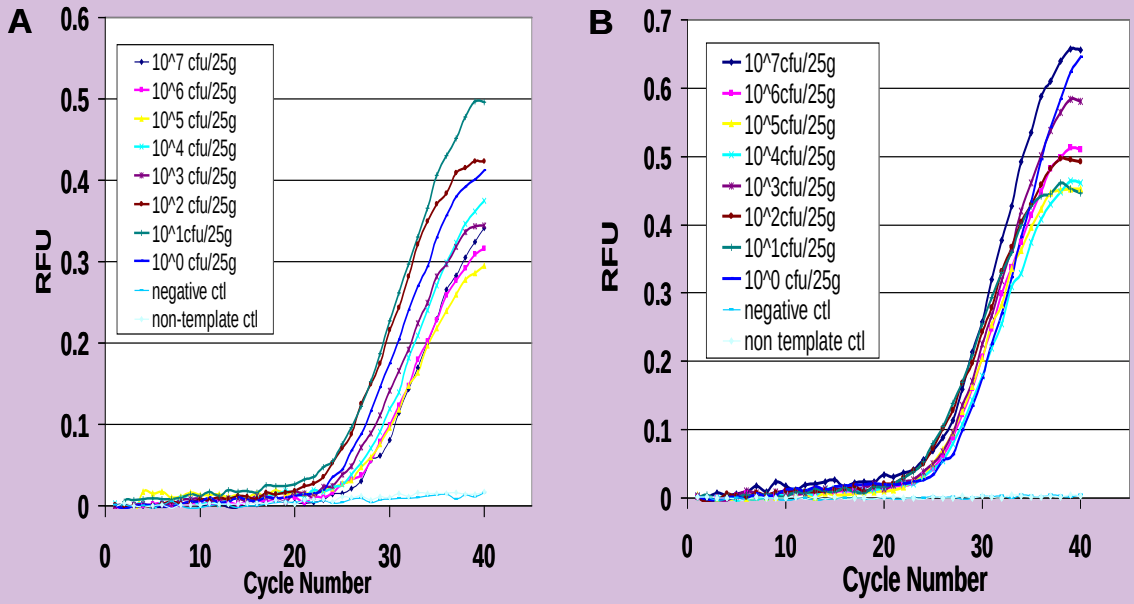

Figure 4. Detection of *L. monocytogenes* Scott-A2 in raw and processed meats inoculated at various levels followed by enrichment and Uniprimer™ real-time PCR assay. Panel A, detection in raw sausage emulsion. Panel B, detection in RTE processed meat (hotdogs).

In addition to identifying how we could apply the Ampliflour™ real-time PCR assay to successfully detect *L. monocytogenes* from meat products, we also examined the procedure for detection of *L. monocytogenes* from a limited number of retail food samples in comparison with the traditional method. We obtained the same results whether using the USDA-FSIS culture method or using the Amplifuor™ PCR assay (Table 3).

Table 3. Detection of *L. monocytogenes* in food samples using the Amplifluor Uniprimer™ in comparison with traditional microbiological detection.				
Food product		**Samples tested**	**Real-time PCR**	**Traditional method**
1. Raw meats		34	5	5
a.	Ground beef	9	3	3
b.	Ground turkey	9	1	1
c.	Pork sausages	9	1	1
d.	Ground pork	4	0	0
e.	Ground chicken	3	0	0
2. Ready-to-eat meats (hotdogs)		10	0	0
3. Cheese products		10	0	0
Total =		88	10	10

There are several advantages of the AmpliflourTM system that are not obtained with other fluorescent detection systems, such as the hybridization probes (i.e., Molecular Beacons, Light Cycler). The AmpliflourTM system makes use of a universal primer (UniprimerTM) that emits fluorescent signal only upon the incorporation of the primers into the amplification product and yields low fluorescent background with unincorporated primers (Nazarenko et al., 1997). This method can be inexpensively adapted to different molecular targets simply by placing the "Z-tail" sequence on PCR primers for the new target and using the same "universal primer" whereas the hybridization probes require the costly synthesis of new labelled primers.

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Author List

Mitra, Suparna. M.S. student, Department of Animal Science, Oklahoma State University

Muriana, Peter. Associate Professor, Department of Animal Science & Food and Ag Products Center, Oklahoma State University