Direct, quantitative detection of *Listeria monocytogenes* in fresh raw whole milk by qPCR

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Abstract

A method previously developed for direct (non-enrichment) detection of *Escherichia coli* O157:H7 was adapted for *Listeria monocytogenes*. The sample treatment protocol was successful in concentrating bacteria from 10 mL raw milk samples and reducing PCR inhibition, but qPCR detection sensitivity and reproducibility was poor. Two DNA extraction reagents and multiple extraction conditions were tested to identify an efficient and reproducible DNA extraction method. Two primer/probe sets were evaluated at two concentrations and three annealing temperatures to minimize false-positive results and optimize sensitivity and reproducibility of qPCR detection. Under the selected conditions, DNA was extracted efficiently from the entire milk sample in a volume of 10 μL, and subsequently quantitated by a 5′ nuclease qPCR assay lasting 50 min. The method provided detection of 1 cfu mL^{-1} *L. monocytogenes* in 10 mL raw milk and quantitation from 10 to 1000 cfu mL^{-1} with a total time to result of <3 h.

1. Introduction

Outbreaks of foodborne illness associated with raw milk continue to occur at a significant rate in the US and Europe (De Buyser, Dufour, Maire, & Lafarge, 2001; Langer et al., 2012). Rapid, quantitative detection methods with good sensitivity are important tools for preventing such outbreaks. Although enrichment methods have very low detection limits (~0.04 cfu g^{-1}), they provide only presence/absence information and exhibit long delays (1–4 days) between sampling and results. We previously described a method for the direct detection and quantitation of *Escherichia coli* O157 in raw whole milk samples that provided low detection limits (~1 cfu mL^{-1}) without the use of toxic or flammable reagents in a total assay time of ~3 h (Paul, Van Hekken, & Brewster, 2013). Extension of this approach to other pathogens would provide a general method for rapid, sensitive quantitation of bacteria in raw milk.

Listeria monocytogenes was selected as the target for extension of the method due to its importance as a milk-borne pathogen. *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 all occur with similar frequency (2.8–7.0%) in bulk raw milk (Oliver, Boor, Murphy, & Murinda, 2009), but *L. monocytogenes* exhibits a far higher mortality rate in infected individuals and is the only organism able to grow at refrigerator temperatures (Jay, Loessner, & Golden, 2005). Cells of *L. monocytogenes* are significantly smaller than those of *E. coli* O157:H7 and potentially more difficult to recover by centrifugation. In addition, the rigid cell wall of *L. monocytogenes* and other Gram-positive organisms results in far less efficient DNA extraction than from the Gram-negative organisms such as *E. coli* (Brewster & Paoli, 2013). We report here optimization of the DNA extraction and qPCR conditions and data demonstrating rapid detection and quantitation of *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial cultures and enumeration

L. monocytogenes ATCC 19115 (American Type Culture Collection, Manassas, VA, USA) was grown on Brain Heart Infusion Agar (BHIA) and stored at 4 °C. Overnight (16–20 h) cultures were prepared by mixing a single colony with 3 mL of Brain Heart Infusion (BHI) broth and incubation at 37 °C with shaking at 250 rpm. Nominal bacteria concentrations were estimated from optical absorbance at 600 nm and actual concentrations determined by plating appropriate dilutions in 0.85% (w/v) NaCl saline solution on BHIA plates.

2.2. Milk sample treatment protocol

Samples of raw bovine milk were purchased from local markets, kept at 4 °C and used before the sell-by date. The protocol used for preparation and treatment of spiked milk samples has been described previously (Paul et al., 2013). The same protocol was used here, apart from omission of diatomaceous earth as a pellet marker. Briefly, 10 mL milk was treated with EDTA and warmed to 35–40 °C. *L. monocytogenes* dilutions (20 µL) were added to the warmed milk, mixed, and centrifuged immediately (15 min, 4696× *g*). The fat and supernatant liquid were removed by aspiration, and the pellet containing bacteria was resuspended in lysis buffer containing SDS and treated with DNase I. After recovering the bacteria by centrifugation, the pellet was treated with trypsin, washed (saline solution + 0.1%, w/v, Tween-20), and centrifuged to recover the bacteria.

2.3. DNA extraction

Extraction with 5 µL 5× HotShot reagent followed the protocol described previously (Paul et al., 2013) with heating for 5–20 min at 65–95 °C (5 min at 95 °C in the final protocol) followed by addition of 5 µL neutralizer. Alternatively, 10 µL of *QuickExtract*™ Bacterial DNA Extraction Solution + 0.1 µL Ready-Lyse Lysozyme Solution (Epicentre Biotechnologies, Madison, WI, USA) was added to 0.6 mL tubes containing 2 µL samples. Samples were vortexed, incubated at room temperature for 15 min, and heated to 80 °C for 1–10 min as necessary. The full extract was added directly to PCR tubes for detection. Purified DNA was prepared with a commercial spin-column kit (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA, USA) and quantitated by UV spectrophotometry.

2.4. Quantitative PCR assay

Two 5′ nuclease primer/probe sets were employed for detection of *L. monocytogenes*: one (hlyA-N) targeting an 113 bp amplicon in the listerolysin O (*hlyA*) gene (Nogva, Rudi, Naterstad, Holck, & Lillehaug, 2000) and the other (hlyA-R) targeting a second 63 bp amplicon in the *hlyA* gene (Rodríguez-Lazaro et al., 2004). Probe and primers for both sets were supplied as PrimeTime® assays (Integrated DNA Technologies, Coralville, IA, USA) containing 5 nmoles forward primer, 5 nmoles reverse primer, and 2.5 nmoles probe. Sequences of the hlyA-N set were forward primer: 5′-TGCAAGTCCTAAGACGCCA-3′; reverse primer: 5′-CACTGCATCTCCGTGGTATACTAA-3′; and probe: FAM-5′ CGATTTCATCCGCGTGTTTCTTTTCG-BkFQ. Sequences of the hlyA-R set were forward primer: 5′-CATGGCACCACCAGCATCT-3′; reverse primer: 5′-ATCCGCGTGTTTCTTTTCGA-3′; and probe: FAM-5′ CGCCTGCAAGTCCTAAGACGCCA-BkFQ. Reactions contained 10 µL TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL primer/probe and 9 µL sample. A StepOne real time thermocycler (Applied Biosystems) was used with activation for 20 s at 95 °C, and 45 cycles of 1 s at 95 °C and 20 s at 62 °C.

For optimization of PCR conditions, duplicate wells for each combination of primer/probe concentration (500/250 nM or 250/125 nM) and annealing temperature contained purified DNA at 1, 10, 100, and 1000 pg. Reaction (amplification) efficiency was calculated with the thermocycler software package. For assays of raw milk, samples were inoculated in triplicate with 0, 1, 10, and 100 cfu mL^{-1} *L. monocytogenes* (nominal concentration) and subjected to the sample treatment and DNA extraction protocols above. DNA extracts were assayed by qPCR using the hlyA-N primer/probe set at 250/125 nM with 62 °C annealing temperature. The assay included triplicate positive (purified DNA) and negative (water) controls.

Table 1
Effects of hlyA-N and hlyA-R primer concentration and annealing temperature on C_T values with 1 pg DNA of *L. monocytogenes*.

Primer set	Primer (nM)	Annealing (°C)	C_T mean	C_T σ	Efficiency (%)
hlyA-N	500	60	29.4	0.015	99
	250	60	31.7	0.044	91
	500	62	29.2	0.004	101
	250	62	30.7	0.086	94
	500	64	28.7	0.178	97
	250	64	30.8	0.136	89
hlyA-R	500	63	28.7	0.006	85
	250	63	29.7	0.022	95
	500	65	29.1	0.127	82
	250	65	31.2	0.019	90
	500	67	33.0	0.002	58
	250	67	39.3	0.366	69

3. Results and discussion

3.1. PCR assay optimization

There are many primer–probe combinations available for qPCR assays of *L. monocytogenes* (Dadkhah et al., 2012; Klein & Juneja, 1997; Rantsiou, Alessandria, Urso, Dolci, & Cocolin, 2008). We selected two qPCR primer/probe sets for investigation based on reported high selectivity, sensitivity and inclusivity for *L. monocytogenes* strains: hlyA-N (Barbau-Piednoir, Botteldoorn, Yde, Mahillon, & Roosens, 2013; Nogva et al., 2000) and hlyA-R (Rodríguez-Lazaro et al., 2004). Preliminary experiments (data not shown) using PrimeTime® primer/probe sets at the manufacturer's recommended concentrations (500/250 nM) and literature annealing temperatures exhibited issues with false-positives, reproducibility, and sensitivity in blank milk samples. Experiments were conducted to identify primer/probe concentrations and annealing temperatures that would provide improved performance. Table 1 details C_T values for 1 pg DNA and efficiency calculated from the entire range (1–1000 pg) of DNA tested. For both primer/probe sets, C_T values increased as annealing temperature was raised and as primer concentration was lowered. However, the hlyA-N primer/probe exhibited less variation in C_T and efficiency than hlyA-R, and this more robust primer set was chosen for subsequent experiments. The combination of 250 nM hlyA-N primer and 62 °C annealing temperature was selected for further work as it was expected to produce fewer false-positives than 500 nM primer while providing good sensitivity and high efficiency.

3.2. Comparison of DNA extraction techniques

Previous research (Brewster & Paoli, 2013) indicated that QuickExtract™ (QEX) and HotShot (Truett et al., 2000) were the most effective reagents for direct DNA extraction from *L. monocytogenes*, providing relatively high recovery and minimal PCR inhibition. The QuickExtract™ protocol calls for a 15 min incubation of the cells with the reagent at room temperature. The optional 2 min heating step at 80 °C to kill viable bacteria was not tested in the previous study. Table 2 shows qPCR assay results (500/250 nM hlyA-N primer/probe concentration) after treatment of 500 cfu samples of *L. monocytogenes* with QuickExtract™. Heating at 80 °C significantly improved recovery (evidenced by reduced C_T value), with optimal results obtained with heating for 2 min. Preliminary experiments with the 2× HotShot reagent used previously in detection of *E. coli* (Paul et al., 2013) indicated poor recovery of *L. monocytogenes* DNA (data not shown). Results of experiments with 5× HotShot under a variety of conditions are shown in Table 3. Heating for 5 min at 95 °C gave low, reproducible C_T values (28.6 ± 0.3). Under these qPCR conditions (250/125 nM hlyA-N primer/probe concentration) samples extracted with QuickExtract™ with 2-min heating at 80 °C gave C_T values of 35.1 ± 1.5.

Table 2
DNA extraction of 500 cfu *L. monocytogenes* with QuickExtract™: effect of heating on C_T.[a]

Heating time (min)	C_T mean	C_T σ
0	33.5	0.4
1	30.5	1.0
2	29.9	0.4
4	30.7	0.7

[a] Samples were mixed with reagent, incubated 15 min at room temperature, then heated at 80 °C; hlyA-N primer/probe: 500/250 nM.

Table 3
DNA extraction of 500 cfu *L. monocytogenes* with 5× HotShot: effect of heating on C_T.[a]

Temperature (°C)	Time (min)	C_T mean	C_T σ
65	10	30.7	0.5
65	20	29.2	1.0
75	10	28.4	0.4
85	10	28.2	0.3
95	5	28.6	0.3

[a] Samples were heated with 5 μL reagent, then mixed with 5 μL neutralizer; hlyA-N primer/probe: 250/125 nM.

3.3. qPCR assay performance

DNA was extracted by the method protocol from samples of bacterial cells suspended in saline solution or samples of raw milk that were inoculated just prior to DNA extraction. Results for triplicate samples of both types with various levels of bacteria are shown in Fig. 1. The C_T values were unaffected by the presence of milk, indicating efficient removal of inhibitors during sample processing and extraction. Fewer than 10 cfu per reaction were detected (corresponding to ~1 cfu mL^{-1} for a 10 mL sample) and the response was linear and reproducible from 6 to 6000 cfu.

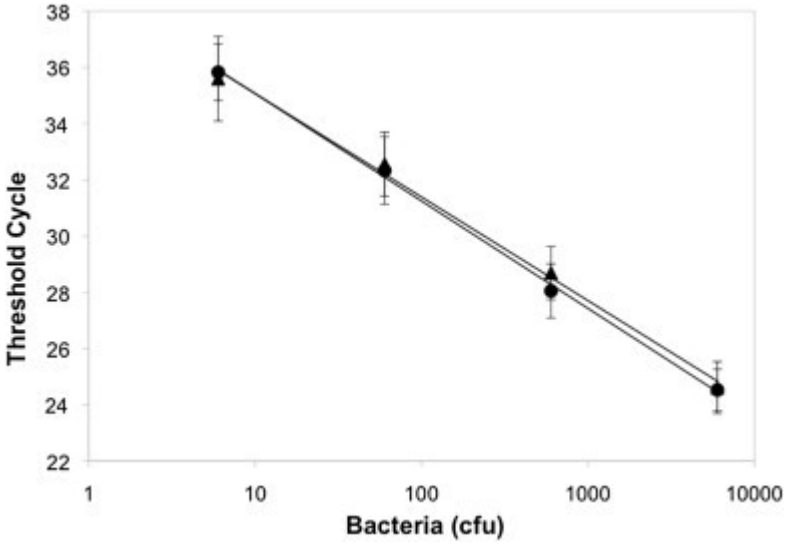

Fig. 1. qPCR results for *L. monocytogenes* in saline and in treated milk. Each qPCR reaction contained the indicated amount of bacteria. Symbols (●, cells in the presence of milk; ▲, cells in saline solution) represent the mean and error bars represent the standard deviation for triplicate samples; the solid line represents a logarithmic fit to the data points.

3.4. Application

Sensitivity and reproducibility were studied using 10 mL raw milk samples inoculated at 0, 1, 10, and 100 cfu mL^{-1} (nominal concentration). The results from five experiments over three days in Table 4 showed detection of 1 cfu mL^{-1}, although one replicate failed to amplify at this level in three trials. No amplification of the negative controls was observed, and one false-positive result (amplification before 40 cycles) was observed in one replicate of the blank milk (0 cfu) sample. Quantitation, within-day and day-to-day reproducibility were considered to be acceptable.

Table 4
Reproducibility of threshold cycle (C_T) values for raw bovine milk spiked with low levels of *L. monocytogenes*.[a]

Spike level (cfu mL^{-1})	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ	Mean	σ
0	U	–	U	–	U	–	38.9[b]	–	U	–
1	39.5[c]	0.01	38.1[c]	2.2	38.4	0.6	37.3	0.8	38.1[c]	1.3
10	34.9	0.3	35.4	0.7	35.6	2.1	35.2	0.7	33.5	0.5
100	32.6[c]	0.2	32.8	1.9	32.5	2.5	32.1	0.5	31.2	0.4
PC	27.6	0.1	27.1	0.1	27.1	0.1	27.3	0.2	28.1	0.4

[a] U, no amplification within 40 cycles; PC, positive control (10 pg purified DNA).
[b] One of three replicate samples exhibited PCR amplification.
[c] Two of three replicate samples exhibited PCR amplification.

4. Conclusions

The sample treatment steps previously reported for detection of *E. coli* O157:H7 were combined with modified DNA extraction and qPCR steps to provide an assay for *L. monocytogenes* with comparable sensitivity and 15 min shorter time to results. The method accommodates relatively large (10 mL) samples and produces data from a set of 12 samples in less than 3 h, including qPCR. *L. monocytogenes* could be detected at 1 cfu mL^{-1} and quantified from 10 to 1000 cfu mL^{-1}, although the precision of the *L. monocytogenes* assay was somewhat lower than that for *E. coli*

O157:H7. The successful detection of both Gram-negative and Gram-positive bacteria with this approach indicates its potential for application to other bacteria in raw milk.

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References

Barbau-Piednoir, E., Botteldoorn, N., Yde, M., Mahillon, J., & Roosens, N.H. (2013). Development and validation of qualitative SYBR®Green Real-Time PCR for detection and discrimination of *Listeria* spp. and *Listeria monocytogenes*. *Applied Microbiology and Biotechnology,* 97, 4021-4037.

Brewster, J.D., & Paoli, G.C. (2013). DNA extraction protocol for rapid PCR detection of pathogenic bacteria. *Analytical Biochemistry, 442,* 107-109.

Dadkhah, H., Bassami, M.R., Hashemi, S., Shahraz, F., Hosseini, H., Karatzas, K.A.G., et al. (2012). Evaluation and comparison of SYBR Green I Real-Time PCR and TaqMan Real-Time PCR methods for quantitative assay of *Listeria monocytogenes* in nutrient broth and milk. *African Journal of Microbiology Research, 6*, 1908-1917.

De Buyser, M.L., Dufour, B., Maire, M., & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology, 67,* 1-17.

Jay, J.M., Loessner, M.M., & Golden, D.A. (2005). *Modern food microbiology* (7th ed.). New York, NY, USA: Springer Science and Business Media, Inc. Chapt. 25.

Klein, P.G., & Juneja, V.K. (1997). Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Applied and Environmental Microbiology, 63,* 4441-4448.

Langer, A.J., Ayers, T., Grass, J., Lynch, M., Angulo, F.J., & Mahon, B.E. (2012). Nonpasteurized dairy products, disease outbreaks, and state laws–United States, 1993–2006. *Emerging Infectious Diseases, 18,* 385-391.

Nogva,, H.K., Rudi, K., Naterstad, K., Holck, A., & Lillehaug, D. (2000). Application of 5′-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Applied and Environmental Microbiology, 66,* 4266-4271.

Oliver, S.P., Boor, K.J., Murphy, S.C., & Murinda, S.E. (2009). Food safety hazards associated with consumption of raw milk. *Foodborne Pathogens and Disease, 6,* 793-806.

Paul, M., Van Hekken, D.L., & Brewster, J.D. (2013). Detection and quantitation of *Escherichia coli* O157 in raw milk by direct qPCR. *International Dairy Journal, 32,* 53-60.

Rantsiou, K., Alessandria, V., Urso, R., Dolci, P., & Cocolin, L. (2008). Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *International Journal of Food Microbiology, 121,* 99-105.

Rodríguez-Lazaro, D., Hernandez, M., Scortti, M., Esteve, T., Vazquez-Boland, J.A., & Pla, M. (2004).

Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of hly, iap, and lin02483 targets and AmpliFluor technology. *Applied and Environmental Microbiology, 70,* 1366-1377.

Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., & Warma, M.L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). *BioTechniques, 29,* 52-54.