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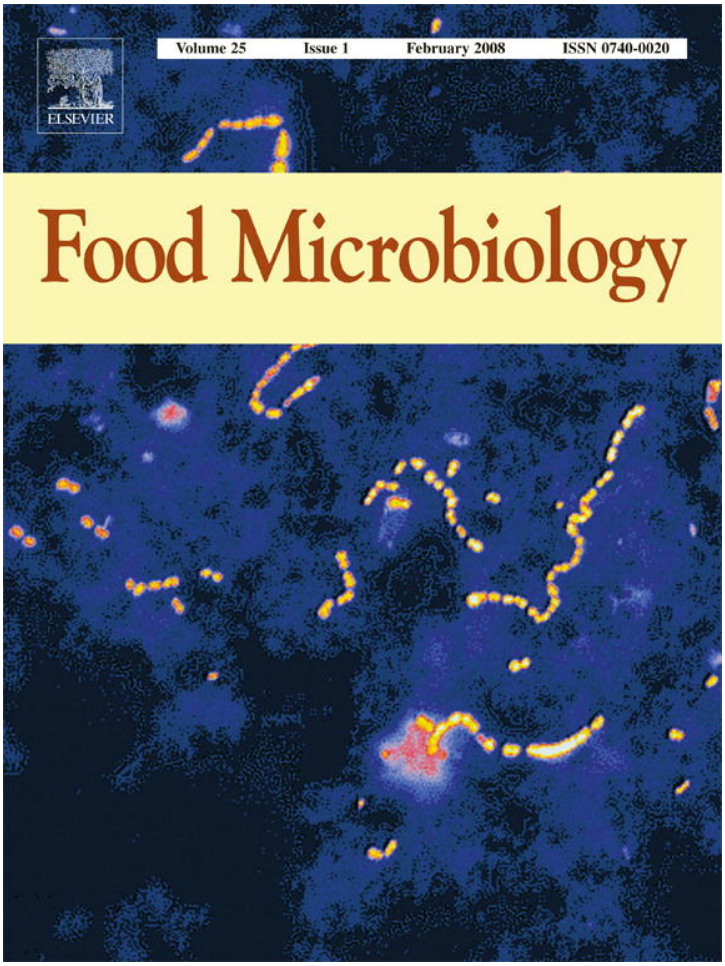

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Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the *ssrA* gene, a novel diagnostic target

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Abstract

A real-time PCR assay was designed to detect a 162-bp fragment of the *ssrA* gene in *Listeria monocytogenes*. The specificity of the assay for *L. monocytogenes* was confirmed against a panel of 6 *Listeria* species and 26 other bacterial species. A detection limit of 1–10 genome equivalents was determined for the assay. Application of the assay in natural and artificially contaminated culture enriched foods, including soft cheese, meat, milk, vegetables and fish, enabled detection of 1–5 CFU *L. monocytogenes* per 25 g/ml of food sample in 30 h. The performance of the assay was compared with the Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit'. Both methods detected *L. monocytogenes* in all artificially contaminated retail samples ($n = 27$) and *L. monocytogenes* was not detected by either system in 27 natural retail food samples. The method developed in this study has the potential to enable the specific detection of *L. monocytogenes* in a variety of food types in a time-frame considerably faster than current standard methods. The potential of the *ssrA* gene as a nucleic acid diagnostic (NAD) target has been demonstrated in *L. monocytogenes*. We are currently developing NAD tests based on the *ssrA* gene for a range of common foodborne and clinically relevant bacterial pathogens.
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Keywords: *Listeria monocytogenes*; Real-time PCR; Internal amplification control (IAC); Food; *ssrA* Gene/tmRNA

1. Introduction

Listeria monocytogenes is the primary human pathogen of the genus *Listeria*, although there have been reports of illness caused by *L. seeligeri*, *L. ivanovii* and *L. innocua* (Perrin et al., 2003; Gasanov et al., 2005). Those most at risk of infection are pregnant women, neonates, newborns, immunocompromized persons and the elderly (Schuchat et al., 1991). Infection has been associated with a variety of foods, including cheese, meat, milk, vegetables and fish (Davies et al., 1984; Tham et al., 2000; de Valk et al., 2001; Lunden et al., 2004; Makino et al., 2005). Symptoms range from flu-like illness to severe complications including meningitis, septicaemia, spontaneous abortion or listeriosis of the newborn. Although the number of *L. monocytogenes* infections reported each year is low, a mortality rate as high as 30% indicates that it is one of the most important foodborne pathogens (Berche, 2005).

Conventional methods for the detection of *L. monocytogenes* in food involve selective culture enrichment with subsequent culturing on selective media, followed by serological and/or biochemical tests for species identification (Cox et al., 1998). One of the current recommended standard methods for the isolation of *L. monocytogenes* from foods, ISO 11290-1, takes five days to confirm a negative result and up to 10 days to confirm a positive result (Anon, 1997). An alternative method for detection and identification of *L. monocytogenes* with similar or better performance, which reduces cost and turnaround time to results, would be of great value to the food industry. PCR-based methods, in particular real-time PCR-based technologies, have the potential to enable the rapid and specific identification of foodborne pathogens.

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Although direct PCR-based detection methods have been described for *L. monocytogenes*, pre-enrichment procedures are still necessary to ensure the detection of low numbers of viable *L. monocytogenes* in foods (Norton, 2002).

Previous studies have described PCR and real-time PCR assays combined with culture enrichment for the specific identification of *L. monocytogenes* in foods in a shorter time than can be achieved by standard culture methods alone (Jothikumar et al., 2003; Somer and Kashi, 2003; D'Agostino et al., 2004; Nguyen et al., 2004; Kawasaki et al., 2005; Rudi et al., 2005). In this study, the development of a qualitative real-time PCR assay for the LightCycler employing fluorescence resonance energy transfer (FRET) hybridization probe technology (Wittwer et al., 1997) targeting the *ssrA* gene of *L. monocytogenes* and an internal amplification control (IAC) is described.

The *ssrA* gene codes for tmRNA and has been identified in all bacterial phyla (Keiler et al., 2000). The function of tmRNA in bacteria is to rescue stalled ribosomes and to clear the cell of incomplete polypeptides. tmRNA functions both as tRNA and mRNA, with the mRNA portion encoding a peptide tag that is incorporated at the end of the aberrant polypeptide which targets it for proteolysis (Keiler et al., 2000).

FRET hybridization probes were used, in the real-time PCR assay developed in this study, for the specific detection of the *ssrA* gene target in *L. monocytogenes*. The two probes, which comprise the hybridization probe pair, hybridize adjacently on the single-stranded nucleic acid target, typically 1–5 bases apart, during the primer annealing phase of the PCR. The donor probes are 3′ terminally labeled with a donor fluorophore and the acceptor probes are 5′ terminally labeled with an acceptor fluorophore. The donor fluorophore is excited by light and the excitation energy is transferred by FRET from the donor to the acceptor fluorophore and read by the real-time PCR instrument (Anon, 2000). The probe with the lowest T_m is known as the reporter probe and the other probe is referred to as the anchor probe. Melt peak analysis, which is performed after PCR amplification, utilizes the specificity of the T_m of the reporter probe to differentiate binding of the probe to the target sequence from binding to non-target sequences. The reporter probe produces the melt peak, as when it melts from the target the fluorophores are separated and FRET ceases to occur (Wilhelm and Pingoud, 2003).

A strategy combining a modification of ISO 11290-1 with the real-time PCR assay developed in this study was optimized and applied to detect *L. monocytogenes* in a range of natural and artificially contaminated foods, including soft cheese, meat, milk, vegetables and fish. A comparison of the developed assay with the Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit' for the detection of *L. monocytogenes* was also performed.

2. Methods

2.1. Bacterial strains, culture media and growth conditions

Forty eight *Listeria* strains (28 *L. monocytogenes*, 15 *L. innocua*, 2 *L. grayi*, 1 *L. ivanovii*, 1 *L. seeligeri* and 1 *L. welshimeri* strain) and 26 non-*Listeria* strains were used in this study (Tables 1 and 2). All *L. monocytogenes* and *L. innocua* food isolates and *Bacillus cereus*, *Brochotrix thermosphacta*, *Kurthia gibsonii*, *Kurthia zopfii*, *Lactobacillus casei*, *Lactobacillus lactis* and *Streptococcus thermophilus* were kindly donated by Dr. Ingeborg Hein, Institute of Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna, Austria. All other bacterial species and strains were laboratory stocks previously obtained from culture collections. *Listeria* strains were grown on TSB-Y broth/agar plates [3% tryptone soy broth, 0.6% yeast extract (2% agar for plates)] at 30 °C overnight. All non-*Listeria* were grown in TSB-Y or LB (Luria–Bertani) broth/agar plates at either 30 °C or 37 °C overnight. All media were purchased from Oxoid (Hampshire, United Kingdom).

2.2. Culture enrichment of food samples

Twenty seven retail samples, comprising nine foods purchased on three occasions including soft cheese (brie and cottage cheese), meat (hotdog, sliced ham, pâté and sliced turkey), milk, vegetables (coleslaw) and fish (smoked salmon) were investigated in this study. Three independent experiments were performed in which nine foods were culture-enriched and tested by real-time PCR. For culture enrichment, two samples of each food were prepared as follows: 25 g/ml of food was added to 225 ml of half Fraser broth (half content of selective components as recommended by the manufacturer) (Oxoid, Hampshire, United Kingdom), in sterile plastic Seward Stomacher filter bags (Norfolk, United Kingdom) and homogenized in a stomacher (Seward Stomacher 400 Lab System, Norfolk, United Kingdom) for 2 min. After homogenization, the mixture was poured into sterile 250 ml Pyrex screw cap flasks. One sample served as a 'naturally contaminated' control. The second sample was inoculated with 100 µl of a 10^{-8} dilution of an overnight culture of *L. monocytogenes* strain NCTC 7973 in TSB-Y which was determined to contain 10–50 CFU ml^{-1} by plate counts on TSB-Y. The flasks were incubated at 30 °C for 22 h on a rotary shaker at 200 rpm. Following incubation, a secondary enrichment step was performed. One hundred microlitres of naturally contaminated and inoculated enriched food culture was used to inoculate 10 ml Fraser broths, respectively, in sterile plastic universals and incubated at 37 °C for 4 h on a rotary shaker at 200 rpm. DNA was extracted from 1.5 ml aliquots of the secondary enrichment cultures for the *L. monocytogenes* real-time PCR assay. In parallel, a loopful of secondary enrichment culture was streaked onto PALCAM *Listeria* selective agar (Oxoid, Hampshire,

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Table 1
Listeria species and strains used in this study

Bacteria	Strain designation	Serovar	Origin
Listeria monocytogenes	NCTC 7973	1/2a	N/A[a]
	1	1/2b	Cheese
	2	1/2a	Cheese
	3	1/2a	Cheese
	4	1/2c	Cheese
	5	1/2b	Cheese
	6	4b	Cheese
	7	1/2b	Cheese
	8	4b	Cheese
	9	1/2b	Abortus (sheep)
	10	1/2b	Shrimp
	11	4b	Cheese
	12	1/2b	Cheese
	13	1/2b	Cheese
	14	1/2b	Cheese
	15	1/2a	Cheese
	16	4b	Cheese
	17	1/2b	Salad
	18	1/2a	Cheese
	19	3b	Cheese
	20	1/2a	Cheese
	21	1/2a	Lax Gravad (salmon dish)
	22	4d	Dairy plant (smear)
	23	1/2a	Turkey
	24	4d	Salad with ham
	25	1/2a	Frying sausage
	26	1/2b	Fermented sausage
	27	3c	Fresh meat
Listeria innocua	NCTC 11288	6a	N/A[a]
	1	6b	Cheese
	2	6a	Cheese
	3	6a	Brine
	4	6b	Cheese
	5	6b	Brine
	6	6a	Brine
	7	6b	Brine
	8	6b	Cheese
	9	6a	Brine
	10	6a	Brine
	11	6a	Brine
	12	N/A[a]	Salami
	13	N/A[a]	Salad with ham
	14	N/A[a]	Paella
Listeria grayi subsp. *grayi*	NCTC 10815	N/A[a]	N/A[a]
Listeria grayi subsp. *murrayi*	NCTC 10812	N/A[a]	N/A[a]
Listeria ivanovii subsp. *ivanovii*	NCTC 11846	5	N/A[a]
Listeria seeligeri	NCTC 11856	1/2b	N/A[a]
Listeria welshimeri	NCTC 11857	6a	N/A[a]

[a]N/A = not available.

United Kingdom), incubated under microaerophilic conditions for 24–48 h at 37 °C and examined for typical *Listeria* colonies to confirm the presence or absence of *L. monocytogenes* in the enriched food sample.

Table 2
Non-*Listeria* species used in this study

Bacteria	Strain designation
Aeromonas hydrophila	NCTC 8049
Bacillus cereus	NCTC 7464
Brochotrix thermosphacta	DSM 20171
Campylobacter jejuni	ATCC 33560
Citrobacter diversus	ATCC 27156
Citrobacter freundii	ATCC 8090
Enterobacter cloacae	ATCC 23355
Enterobacter intermedius	ATCC 33110
Enterococcus faecalis	NCTC 775
Escherichia coli	NCTC 10418
Klebsiella aerogenes	NCTC 9528
Klebsiella pneumoniae	ATCC 13883
Kurthia gibsonii	CCM 3321
Kurthia zopfii	ATCC 33403
Lactobacillus casei	ATCC 393
Lactobacillus delbrueckii	ATCC 12315
Salmonella Enteritidis	NCTC 12694
Salmonella Poona	NCTC 4840
Salmonella Senftenberg	NCTC 9959
Salmonella Thompson	ATCC 8391
Salmonella Typhimurium	ATCC 13311
Serratia liquefaciens	ATCC 27592
Staphylococcus aureus	NCTC 6571
Staphylococcus epidermidis	NCTC 11047
Streptococcus pyogenes	ATCC 12344
Streptococcus thermophilus	ATCC 19258

2.3. DNA isolation and quantification

Genomic DNA was isolated from 1.5 ml of cultures (TSB-Y, LB or enriched food culture) using the 'Bacterial Genomic DNA Purification Kit' (Edge BioSystems, Gaithersburg, Maryland, USA) according to the manufacturer's instructions and resuspended in 50 µl nuclease free dH_2O (Ambion, Austin, Texas, USA). DNA concentrations were determined for specificity and sensitivity studies using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon, USA) and the TBS-380 mini-fluorometer (Turner BioSystems, Sunnyvale, California, USA). DNA samples were stored at −20 °C.

2.4. PCR primer pairs and FRET hybridization probes for real-time PCR

Oligonucleotide primers and a FRET probe pair for the *L. monocytogenes ssrA* gene were designed according to recommended general guidelines (Anon, 2000; Landt, 2001) following alignment of *ssrA* gene sequences of species and strains (Tables 1 and 2) generated for this study or available on the tmRNA website (Williams, 2000) and the tmRNA database (Knudsen et al., 2001). PCR primers were supplied by MWG-BIOTECH AG (Ebensburg, Germany) (Table 3). Hybridization probes labeled with 5′ LC (LightCycler) Red 640 and 3′ Fluorescein were manufactured by TIB MOLBIOL (Berlin, Germany) (Table 3).

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Table 3
Oligonucleotide primers and probes used in this study

Name	Type	Sequence (5′-3′)
L*ssrA* F	Forward assay primer	GCATCGCCCATGTGCTAC
L*ssrA* R	Reverse assay primer	TCTACGAGCGTAGTCACCG
IAC F	Forward composite primer for IAC generation	GCATCGCCCATGTGCTACATACCCAAC TTGGAATG
IAC R	Reverse composite primer for IAC generation	TCTACGAGCGTAGTCACCGTCTTCACC AGAATAAAATTG
Hybprobe 1	*L. monocytogenes* hybridization probe	CCATTCAGCTAGTCTGATTAAGCTCT-fluorescein
Hybprobe 2	*L. monocytogenes* hybridization probe	LC Red 640-CTATTTAACCCCAGACGGAGA-phosphate
ALS1-FLU	IAC hybridization probe	TGAATGTATCCCCTGGA-fluorescein
ALS1-LC	IAC hybridization probe	LC Red 705-TGGCACTGGTACCATCTAA-phosphate

2.5. Development of an IAC for real-time PCR

An IAC was developed for the assay using the composite primer approach for competitive PCR described by Hoorfar et al. (2000, 2004). The IAC was based on the *ALS1* gene of *Candida albicans* and a real-time PCR assay for this gene previously developed in this laboratory (O'Connor et al., 2005). Composite primers IAC F/R were used to amplify a chimeric PCR product by conventional PCR which was cloned and sequenced (TA Cloning kit, Invitrogen, De Schelp, Netherlands). Plasmid DNA was extracted from a clone (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) confirmed to contain the correct insert and included as the IAC in the real-time PCR assay. IAC primers and hybridization probes labeled with 5′ LC Red 705 and 3′ Fluorescein were manufactured by TIB MOLBIOL (Table 3).

2.6. L. monocytogenes real-time PCR assay

Real-time PCR amplification was performed on the LightCycler using the 'LightCycler FastStart DNA master hybridization probes' kit (Roche Diagnostics, Mannheim, Germany). PCR was performed in a final volume of 20 μl including 2 μl of template DNA and 2 μl IAC plasmid in 10 × LightCycler hybridization buffer with $MgCl_2$ adjusted to 5 mM concentration. PCR primers (0.5 μM concentration) and FRET hybridization probes for *L. monocytogenes* and IAC targets (0.2 μM concentration) were added to the reaction mixture and the volume was increased to 20 μl by addition of nuclease free dH_2O. The cycling parameters consisted of a 95 °C incubation for 10 min for enzyme activation and DNA denaturation, followed by 45 PCR amplification cycles consisting of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 10 s. The temperature transition rate for all cycling steps was 20 °C/s. Fluorescence acquisition was at the end of the annealing stage of each cycle. The thermocycling program was followed by a melting program of 95 °C for 1 min (denaturation), 45 °C for 30 s (annealing), and then 45–80 °C at a transition rate of 0.1 °C/s with continual monitoring of fluorescence. All subsequent analysis was carried out in the F2/BackF1 (*ssrA* gene target) and F3/BackF1 (IAC) channels with color compensation using the second derivative maximum option of the LightCycler software (version 3.5). A no-template negative control was included in each run.

2.7. Roche Diagnostics 'LightCycler foodproof Listeria monocytogenes Detection Kit'

A 24–48 h culture enrichment, depending on the food matrix, and DNA extraction using the Roche Diagnostics '*Listeria* ShortPrep Kit' or 'High Pure *Listeria* Sample Preparation Kit' is recommended by the manufacturer prior to application of the Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit' (Schneider et al., 2002). In this study all food types (brie, coleslaw, cottage cheese, ham, hotdog, milk, pâté, smoked salmon and turkey) were culture enriched for 26 h (as described for the in-house *L. monocytogenes* real-time PCR assay) and DNA was extracted as described previously using the 'Bacterial Genomic DNA Purification Kit' (Edge BioSystems, Gaithersburg, Maryland, USA). The Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit' assay was performed according to the manufacturer's instructions with the modification of using 2 μl template DNA instead of 5 μl in the real-time PCR.

3. Results

3.1. Design and optimization of L. monocytogenes ssrA gene specific real-time PCR assay

PCR primers, L*ssrA* F and L*ssrA* R, were designed to amplify a 162-bp fragment from the *L. monocytogenes ssrA* gene. FRET hybridization probes, Hybprobe 1 and Hybprobe 2, were designed to enable the specific detection of the *L. monocytogenes* 162-bp PCR fragment by real-time PCR on the LightCycler using melt peak analysis. To distinguish *L. monocytogenes* from *L. innocua*, the reporter probe (Hybprobe 2) was designed with the single base pair difference in the *ssrA* genes between the two species,

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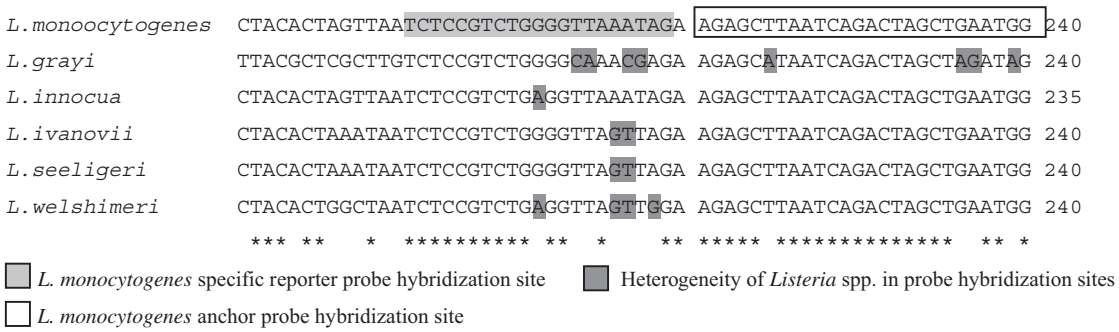

Fig. 1. Clustal W multiple sequence alignment of a 60 nucleotide region of the *ssrA* gene from the *Listeria* species. The alignment shows the FRET hybridization probes designed for *L. monocytogenes* and highlights the sequence differences in the probe regions found in the other *Listeria* species.

located in the center of the probe. *L. ivanovii* and *L. seeligeri* had a common two base pair sequence difference to the *L. monocytogenes ssrA* gene at the 3′ end of the reporter probe binding site. *L. grayi*, *L. welshimeri* and all non-*Listeria* species had three or more base pair differences to the *L. monocytogenes ssrA* target gene in the reporter probe binding site (Fig. 1). Each mismatch between the reporter probe and a non-target PCR product will cause the probe to melt from this PCR product approximately 4 °C lower than the primary target, *L. monocytogenes*, resulting in species-specific melt peaks.

The BLAST-N program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) was used to confirm that the PCR primers and FRET hybridization probes did not recognize any other microbial DNA sequence in GenBank. Initial optimizations of the assay were performed to establish $MgCl_2$, PCR primer, FRET hybridization probe concentrations and thermocycling conditions to enable the PCR amplification and specific detection of the *L. monocytogenes ssrA* gene fragment on the LightCycler.

An IAC was developed for the assay, amplified by the same primers as *L. monocytogenes* (L*ssrA* F/R) but producing a 213-bp *C. albicans ALS1* gene product. A second pair of FRET hybridization probes (ALS1-FLU and ALS1-LC) for detection of the IAC was included in the assay and analyzed in the F3 channel on the LightCycler. The optimum number of IAC plasmids for inclusion in the PCR reaction was determined by performing titrations of serial dilutions of the IAC plasmid (10^4–10 copies) with known concentrations of *L. monocytogenes* DNA (10^4–1 cell equivalents). An IAC concentration of 1000 copies per reaction enabled amplification of the IAC without affecting the detection limit of the *L. monocytogenes ssrA* gene target in the real-time PCR assay (Fig. 2).

3.2. Specificity of the assay

Evaluation of the specificity of the real-time PCR assay was performed by including DNA from the range of bacteria listed in Tables 1 and 2 in the real-time PCR. The 28 *L. monocytogenes* strains (including serovars: 1/2a, 1/2b, 1/2c, 3b, 3c, 4b and 4d), 15 *L. innocua* and two of the four other *Listeria* species produced amplification curves in the assay. Two *Listeria* species, *L. grayi* and *L. welshimeri* and all the non-*Listeria* ($n = 26$) did not yield amplification curves. When melt peak analysis was applied, the *L. monocytogenes* strains ($n = 28$) had melting temperatures (T_m's) of 61.5 °C. The *L. innocua* strains ($n = 15$) had T_m's of 54 °C. *L. ivanovii* ($n = 1$) and *L. seeligeri* ($n = 1$) had T_m's of 56 °C. *L. grayi* strains ($n = 2$) had T_m's of 52 °C. *L. welshimeri* species and all non-*Listeria* had T_m's of <52 °C and produced no melt peaks. The assay was performed at an annealing temperature of 55 °C, too high for the reporter probe to anneal to *L. grayi* and *L. welshimeri* and non-*Listeria* PCR products, therefore no amplification curves were obtained for these species. A melt peak was obtained for *L. grayi* as melt peak analysis was performed from 45–80 °C on denatured PCR product after PCR. Melt peak analysis detected and discriminated *L. monocytogenes* from all other *Listeria* species (Fig. 3).

3.3. Sensitivity of the assay

The detection limit of the real-time PCR assay was determined using genomic DNA isolated from an overnight culture of *L. monocytogenes* NCTC 7973. Duplicate serial dilutions of DNA corresponding to 100,000–1 cell equivalents, based on plate count analysis and a genome size of 2.94 fg DNA (Glaser et al., 2001; Rodriguez-Lazaro et al., 2004a), were included in the real-time PCR assay. Three independent experimental assessments of the detection limit of the real-time PCR assay determined a sensitivity of 1–10 *L. monocytogenes* cells or genome equivalents.

3.4. Detection of L. monocytogenes in food

Twenty seven natural retail food samples and 27 inoculated retail food samples were culture enriched and tested with the in-house *L. monocytogenes* real-time PCR assay and with the Roche Diagnostics 'LightCycler food-proof *Listeria monocytogenes* Detection Kit' as described. Table 4 summarizes the C_T values obtained with the two

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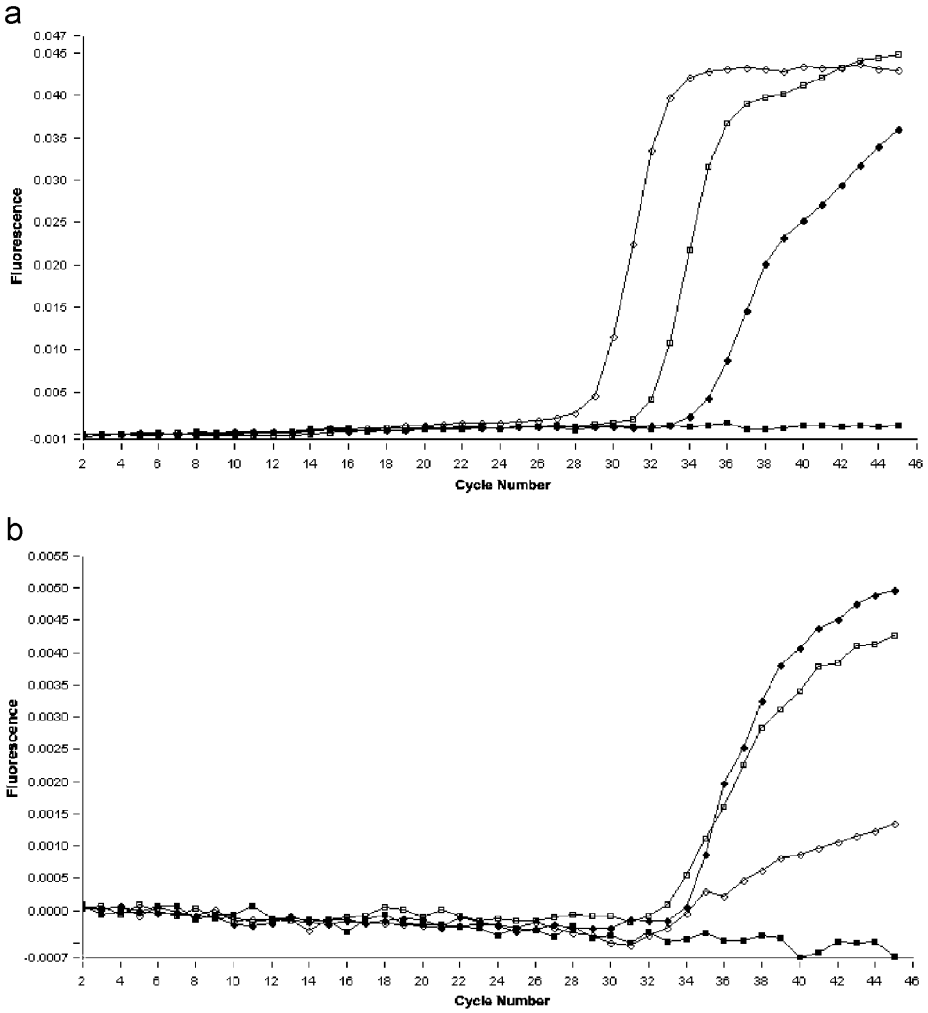

Fig. 2. Real-time PCR amplification of serial dilutions of *L. monocytogenes* DNA and 1000 copies of the IAC in the *L. monocytogenes ssrA* gene FRET hybridization probe real-time PCR assay. (a) Detection of serial dilutions of *L. monocytogenes* DNA ranging from 100–1 cell equivalents. (b) Detection of IAC (1000 copies) with varying concentrations of *L. monocytogenes* DNA. *L. monocytogenes* cell equivalents: 100 cell equivalents (○), 10 cell equivalents (□), 1 cell equivalents (●), dH_2O negative control (■).

assays for nine foods on one of three occasions when testing was performed. *L. monocytogenes* was not detected in the natural retail culture enriched food samples by either assay. The IAC included in both assays was positive for all natural samples validating the negative results. Both assays detected *L. monocytogenes* in all inoculated culture enriched samples. The presence of *L. monocytogenes* in inoculated samples was confirmed by isolation of typical *L. monocytogenes* colonies from the enriched food samples on PALCAM selective agar plates.

4. Discussion

Current conventional methods for *L. monocytogenes* detection and identification take between 5 and 10 days (Rodriguez-Lazaro et al., 2004b). Recently conventional and real-time PCR assays have been developed for the detection of *L. monocytogenes* in foods (Nogva et al., 2000; Hein et al., 2001; Bhagwat, 2003; Koo and Jaykus, 2003; Somer and Kashi, 2003; D'Agostino et al., 2004; Rodriguez-Lazaro et al., 2004b; Wang et al., 2004; Rudi et al., 2005). Many are based on the detection of virulence genes in *L. monocytogenes* and have been tested in a variety of foods. Some of the assays were quantitative and did not require enrichment but they were generally less sensitive than those including an enrichment step (Koo and Jaykus, 2003; Rodriguez-Lazaro et al., 2004b). Reported sensitivities range from 1000 CFU/ml (Bhagwat, 2003) to 1–5 CFU per 25 ml/g (Somer and Kashi, 2003). The reported real-time PCR assays employed a range of fluorescent detection chemistries including SYBR Green and TaqMan or 5′ exonuclease technologies. In contrast, the assay developed in this study employed FRET hybridization probe technology for the specific detection of *L. monocytogenes*.

Commercial kits based on real-time PCR and other molecular techniques are available for the identification of *L. monocytogenes* in foods including the BAX system, VIT technology and the LightCycler foodproof *Listeria monocytogenes* detection kit (Hoffman and Wiedmann, 2001;

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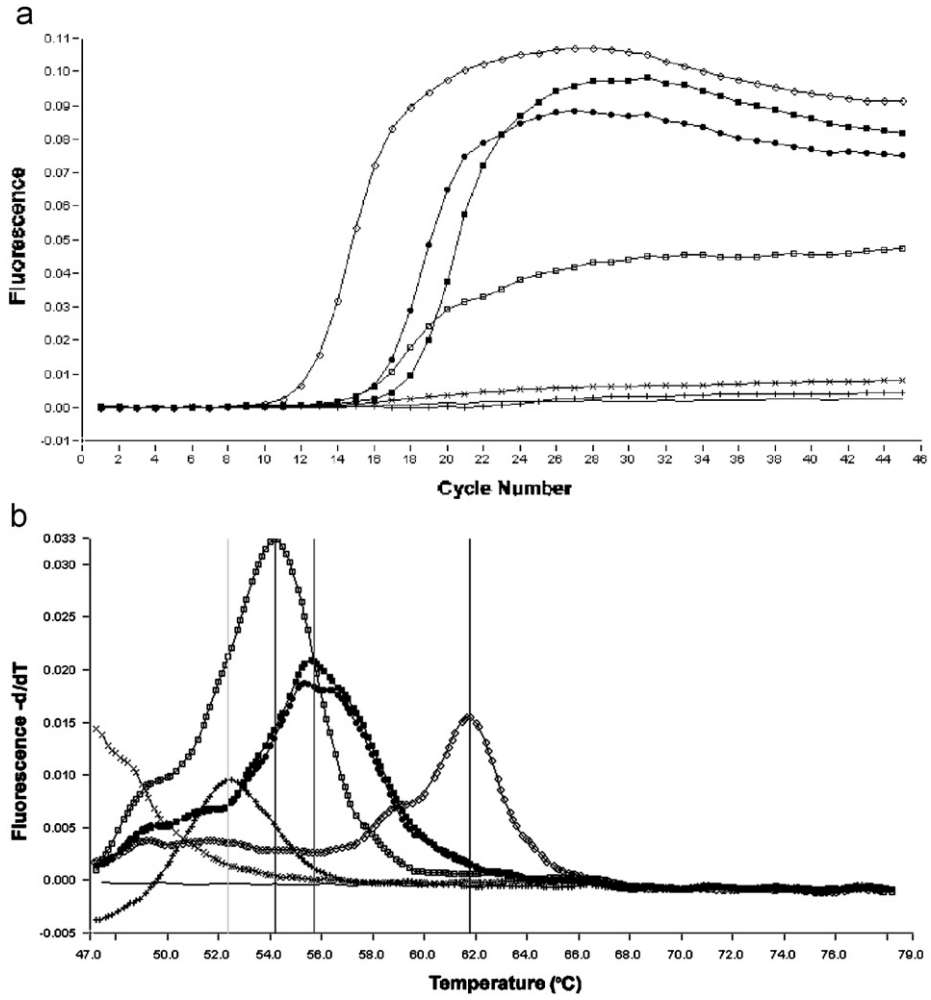

Fig. 3. (a) Amplification curves for the detection of *Listeria* species using the *L. monocytogenes ssrA* gene FRET hybridization probe real-time PCR assay on the LightCycler. (b) Melt peaks for the *Listeria* species demonstrating *L. monocytogenes* specific peak at 61.5 °C, *L. innocua* melt peaks at 54 °C, *L. ivanovii* and *L. seeligeri* melt peak at 56 °C and *L. grayi* melt peak at 52 °C. *L. monocytogenes* (○), *L. grayi* (+), *L. innocua* (□), *L. ivanovii* (●), *L. seeligeri* (■), *L. welshimeri* (×), dH_2O negative control (–).

Schneider et al., 2002; Stephan et al., 2003). Countries including the United States, Australia and New Zealand apply a zero-tolerance policy (absent in 25 g/ml of food) for *L. monocytogenes* in foods (Anon, 2005b). All AOAC international approved *L. monocytogenes* detection tests, including culture, immunological and molecular-based tests, are required to detect 1 CFU per 25 g food sample, therefore, all approved tests require culture enrichment (Gasanov et al., 2005). In 2006, new EU legislation (EC No 2073/2005) was introduced requiring absence of *L. monocytogenes* in 'ready to eat' (RTE) food for certain consumer groups and allowing limits of 100 CFU/g in other categories of RTE foods. Food producers must also perform environmental monitoring where certain types of RTE foods are produced. The legislation provides for the use of alternative test methods provided they are validated according to internationally accepted protocols (Anon, 2005a).

Table 4
C_T values for *L. monocytogenes* detection in the real-time PCR assay developed in this study and the Roche Diagnostics 'LightCycler foodproof *Listeria monocytogenes* Detection Kit' in foods artificially contaminated with 1–5 CFU per 25 g/ml in one of three independent experiments

Food	C_T	
	Real-time PCR assay	Roche Diagnostics kit
Brie	26.21	28.257
Coleslaw	24.78	24.5
Cottage cheese	22.89	24.213
Sliced ham	22.97	26.18
Hotdog	22.80	24.45
Milk	25.99	25.593
Pork liver pâté	22.05	22.463
Smoked salmon	32.23	31.723
Sliced turkey	24.38	27.383

Natural retail food samples were negative for *L. monocytogenes* contamination.

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In this study, a real-time PCR assay for the detection of *L. monocytogenes* based on the *ssrA* gene [a single copy gene in *E. coli* (Oh et al., 1990)] was developed. The detection limit of 1–10 cell equivalents per PCR reaction was comparable with published real-time PCR assays for *L. monocytogenes* (Hein et al., 2001; Rodriguez-Lazaro et al., 2004a; Berrada et al., 2006). Also, based on inoculation of a variety of different foods, the assay was capable of detecting 1–5 CFU per 25 g/ml with no interference from the microflora present in these food samples. Melt peak analysis enabled *L. monocytogenes* to be specifically identified. The assay also detected *L. innocua*, *L. ivanovii*, *L. seeligeri and L. grayi* based on melt peak analysis. Therefore, the assay has the potential to detect four *Listeria* species in addition to *L. monocytogenes* although further analysis with more *L. ivanovii*, *L. seeligeri* and *L. grayi* strains would have to be performed to confirm this. One of the advantages of FRET hybridization probe technology is the ability to perform melt peak analysis to differentiate target from non-target sequences enabling identification of closely related bacterial species based on species-specific melt peaks. Furthermore, a positive result from the amplification curve analysis can be confirmed using melt peak data. The ability to rapidly discriminate *L. monocytogenes* from other *Listeria* species could be of value in the food industry in preventing the unnecessary recall of valuable food products (Rodriguez-Lazaro et al., 2004a). Moreover, as hygiene monitoring is required as an integral part of food safety assurance, molecular tests which can rapidly detect the presence of *L. monocytogenes* and other *Listeria* species in the food processing environment have the potential to provide an 'early warning' for producers.

An IAC was included in the assay to prevent false negative results caused by malfunction of the thermal cycler, incorrect PCR mixture, poor DNA polymerase activity or the presence of PCR inhibitors in the reaction. If the target is amplified but the IAC is not, the result is valid because it is assumed that there is more target DNA than IAC present in the reaction. If neither target nor IAC DNA is amplified the result is invalid and the sample must be repeated (Hoorfar et al., 2004). The European standard for PCR methods for the detection of foodborne pathogens, ISO 22174:2005, states that the presence of PCR inhibition shall be demonstrated using appropriate controls and that an internal or external amplification control shall be performed with every PCR reaction (Anon, 2005c). The IAC included in this assay fulfills these requirements.

The *L. monocytogenes ssrA* gene real-time PCR assay was tested on nine foods with a culture enrichment step included ensuring that only viable *L. monocytogenes* were detected. The culture enrichment step was based on the European standard method ISO 11290-1. It was possible to reduce culture enrichment time from the recommended 72 to 26 h when combined with the real-time PCR assay and detect *L. monocytogenes* in foods at low levels (1–5 CFU per 25 g/ml) in 30 h.

The performance of the *L. monocytogenes ssrA* gene real-time PCR assay was compared to the 'LightCycler foodproof *Listeria monocytogenes* detection kit' from Roche Diagnostics. This kit in combination with the 'ShortPrep foodproof II kit' was granted Performance Tested Methods status by the AOAC Research Institute in 2004. In a recent study by Berrada et al. (2006), the 'LightCycler foodproof *Listeria monocytogenes* detection kit' was used for the detection and quantification of *L. monocytogenes* in salads and results were comparable to the classical microbiological method used. The Roche Diagnostics method recommends a similar enrichment protocol to ISO 11290-1 including a 24 h primary enrichment and an optional 24 h secondary enrichment, followed by a FRET hybridization probe real-time PCR assay. The total test time is 26–50 h depending on the food sample type being tested. In this study, DNA was extracted from culture enriched (26 h) natural retail samples and inoculated food samples and tested for *L. monocytogenes* using the Roche Diagnostics kit and the developed real-time assay. Comparable C_T values were obtained which suggests similar sensitivities of detection with both assays.

The food samples analyzed in this study were not naturally contaminated with *L. monocytogenes* when tested with the developed assay or the AOAC approved Roche Diagnostics kit. Future work may include inoculation of food samples with stressed *L. monocytogenes* cells to mimic naturally contaminated food samples. We are planning to test a large number of food samples with the real-time PCR test and the ISO 11290-1 method and to compare results.

This is the first description of a real-time PCR diagnostic assay using the *ssrA* gene as the diagnostic target. This gene is present in all bacteria (Keiler et al., 2000) and is an excellent candidate for nucleic acid tests with the presence of conserved regions at extremities that flank divergent sequences. We have demonstrated the versatility of the target for the specific identification of *L. monocytogenes* and the detection of other *Listeria* species in a single test. This platform target is currently being exploited for the development of diagnostic assays for a range of important food and clinical pathogens (Glynn et al., 2006). tmRNA is coded for by the *ssrA* gene and it is present in high copy numbers in the cell (~1000 copies per cell) (Schonhuber et al., 2001). We have established that several hundred copies of tmRNA are present per cell in *L. monocytogenes* during exponential and stationary growth phases (unpublished data). We are currently investigating the potential of the natural high copy number of the target to increase the sensitivity of detection of *L. monocytogenes* by reverse transcription real-time PCR. RNA targets can also be used as indicators of cell viability, therefore, RNA amplification-based assays may increase in importance; particularly if testing for the presence of pathogens in foods can be performed directly, without the need for an enrichment step (Norton, 2002).

In conclusion, a rapid and sensitive qualitative method for the detection of *L. monocytogenes* was developed,

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combining culture enrichment and a FRET hybridization probe real-time PCR assay including an IAC. The assay has potential for the detection of other *Listeria* species including *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. grayi* using melt-peak analysis. The assay may be suitable for use in the food industry for hygiene monitoring and early identification of contaminated food products.

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