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ORIGINAL ARTICLE

Simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in dairy products using real time PCR-melt curve analysis

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Abstract The present investigation reports development of post real time PCR (RTi-PCR) - melt curve analysis for simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. The optimal Sybr Green I (SG-I) concentration of 1.6 μM resulted in two specific peaks with melting temperature (Tm) of 79.90±0.39 °C and 86.29± 0.13 °C for *L. monocytogenes* and *Salmonella* spp respectively. The detection sensitivity of the assay in reconstituted non-fat dried milk (NFDM; 11%) spiked with the target pathogens at different levels was 3 log cfu per ml of each pathogen. However, the sensitivity was improved up to 1 log cfu per ml by including pre-enrichment step of 6 h. On application of assay on 60 market samples, one sample each of raw milk and ice cream was detected positive for *L. monocytogenes* and *Salmonella* spp. Assay was quite specific as no cross reactivity with non target cultures could be observed. The developed assay can find valuable application in monitoring dairy products for the presence of *L. monocytogenes* and *Salmonella* spp. to ensure their microbiological quality and safety.

Keywords *Listeria monocytogenes* · *Salmonella* spp · Melt curve analysis · Real time PCR · Dairy products

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Introduction

Listeria monocytogenes and *Salmonella* spp. are the two high risk food pathogens of considerable health concern commonly implicated in a number of food poisoning outbreaks associated with milk and milk products (Makino et al. 2005; CDC 2008a, b; Neves et al. 2008; Dominguez et al. 2009). *L. monocytogenes,* capable of growth over a wide pH range of 4.39 to 9.40, and at refrigeration temperatures, is the causative agent of listeriosis. Similarly, *Salmonella* spp. with more than 2,500 serovars is the causative agent of salmonellosis which is the second most common cause of gastrointestinal food poisoning in the developed world (Elvis et al. 2009). *Salmonella* spp. enter the food chain mainly through agricultural produce and foods of animal origin, including poultry, beef, pork, milk and dairy products, eggs, and seafood (CDC 2008a).

The culture based approaches for diagnosis of *L. monocytogene*s and *Salmonella* spp. are quite laborious and many times remain inconclusive. More advanced, sensitive and rapid microbial detection methods need to be developed to complement or replace the traditional culture based procedures for the prompt detection of these pathogens. RTi-PCR is one such technology that may allow the rapid and quantitative detection of pathogens with high

specificity and sensitivity. Amongst the available chemistries for RTi-PCR, intercalating dyes, such as SG-I, are most commonly used due to universal applicability and associated low cost. After the amplification, the specificity of the product is established by post PCR melt curve analysis that involves heating of the amplified product in a closed system, and determining the Tm. Although, the Tm of a product is specific under a set of reaction conditions but can fluctuate with varying reaction composition and components (Giglio et al. 2003; Monis et al. 2005).

In food science, there are numerous reports on development of SG-1 based assay for detection of *L. monocytogenes* and *Salmonella* spp. in a wide range of products (Cady et al. 2005; Catarame et al. 2006; Bohaychuk et al. 2007). However, most of these assays except a few (Bhagwat 2003; Jothikumar et al. 2003) involved usage of either uniplex format or expensive probe based approaches (Nguyen et al. 2004; De Martinis et al. 2007; Calvo et al. 2008; Krascsenicsova et al. 2008; O'Grady et al. 2008). Multiplex assays by targeting more than one gene would not only add rapidity but also reduce the overall reaction cost of the diagnostic tests. Furthermore, the use of homemade reaction premixes along with the intercalating dyes such as SG-I, may lower the associated cost leading to wide application of the technique. Hence, the present study was undertaken to develop a melt curve analysis based duplex RTi-PCR assay for simultaneous detection of *L. monocytogenes* and *Salmonella* spp. along with homemade premix and its application in pre-selected dairy products.

Materials and methods

Bacterial cultures and media

Twenty two different bacterial strains, which included 3 *L. monocytogenes* and 4 *Salmonella* spp. along with 16 other organisms as recorded in Table 1 were used for determining the efficacy of the assay in terms of sensitivity and specificity. Prior to use, each culture was activated overnight in brain heart infusion (BHI) broth at 37 °C followed by successive processing. The cell number of each culture was adjusted individually to approximately 10^8 cfu per ml and serial 10-fold dilutions were prepared in the phosphate buffer saline and subsequent counts were recorded after plating on BHI agar paltes. The media used in the study were procured from HiMedia Lab, Mumbai, India, unless specified.

Sensitivity of the assay in skim milk and processing of market samples

The sensitivity of the assay was determined in reconstituted NFDM (11%) spiked with the target pathogens over a range

Table 1 List of the bacterial cultures used in the study

Organism and Source	Detection by SG RTi-PCR
E. coli O157:H7 (ATCC35150)[e]	–
E. coli O157:H7 (AIIMS, India)[c]	–
E. coli O157:H7 (CMC)[a]	–
L. monocytogenes (ATCC 53135)	+
L. monocytogenes (ATCC 7644)	+
L. monocytogenes Scott A	+
E. coli (NCDC-134) [b]	–
E. coli (Lab isolate)	–
E. coli (Lab isolate)	–
Shigella flexneri (AIIMS, India) [c]	–
Shigella boydii (AIIMS, India) [c]	–
Shigella dysentriae (AIIMS, India) [c]	–
Salmonella enteritidis (AIIMS, India) [c]	+
Salmonella typhi (AIIMS, India) [c]	+
Salmonella paratyphi (AIIMS, India) [c]	+
Salmonella typhimurium (AIIMS, India) [c]	+
Staphylococcus aureus (MTCC-1144) [d]	–
Campylobacter jejuni (AIIMS, India) [c]	–
Yersinia enterocolitica (AIIMS, India) [c]	–
Lactobacillus fermentum (Lab isolate)	–
Lactobacillus plantarum (Lab isolate)	–
Lactobacillus helveticus (Lab isolate)	–

[a] CMC: Christian Medical College, Vellore, Tamil Nadu, India

[b] NCDC: National Collection of Dairy Cultures, National Dairy Research Institute, Karnal, India

[c] AIIMS: All India Institute of Medical Sciences, New Delhi, India

[d] MTCC: Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India

[e] ATCC: American Type Culture Collection

of 1 to 7 log cfu per ml without and with pre-enrichment for 6 h in BHI broth. DNA was extracted from spiked and enriched dairy products using commercial DNA extraction purification Kit (PureExtreme, Genomic DNA purification Kit, Fermentas, Maryland, USA) after pretreatment of the pellet. Briefly, to 1 ml of spiked and enriched food, 120 μL of Triton X-100 was added followed by vigorous vortexing to allow uniform distribution. To this suspension 1% (*w*/*v*) trypsin powder was added and incubated at 37 °C/30 min. The whole mix was centrifuged at 2,500×g/10 min and the supernatant was discarded. The rest of the process was followed as per manufacturer's instruction for DNA extraction kit. The market samples were collected from Karnal town (Haryana, India) and adjoining areas. A total number of 60 market samples that were analysed for *L. monocytogenes* in one of our previous study based on molecular beacons (Singh et al. 2009), were used in this study as well to comparatively evaluate the performance of SG-I based assay. Market samples, comprising ten each of raw milk, pasteurized milk, ice cream, kulfi, paneer and infant food, were enriched, processed and analyzed by taking 25 g of each of the sample in to 225 ml of universal preenrichment broth (UPB) followed by incubation for 6 h. The enrichment duration of 6 h was selected on the basis of lab trial whereby it was observed that after specified incubation period even one log cfu per ml of each pathogen spiked in NFDM could be detected by RTi-PCR. An aliquot of 1 ml from respective UPB for each sample was added to 9 ml of Listeria enrichment broth and Rappaport Vallisiadis broth respectively and incubated at 37 °C. Samples were drawn at interval of 4, 8, 12, 18 and 24 h for microbiological analysis on PALCAM agar (Difco, Lawrence, KS) and Rappaport Vallisiadis agar and, for DNA extraction.

Real time PCR analysis

For *L. monocytogenes*, *hly* gene responsible for hemolysin production was targeted to design specific primers (F-5′ CAG GAA TGA CTA ATC AAG ACA 3′; R- 5′AGG TTC ATT AAC ATT CAC G 3′) with expected product size of 314 bp whereas for *Salmonella* spp., *invA* (F-5′ CTT TGA TAA ACT TCA TCG CAC 3′; R-5′ TCG TTA TTA CCA AAG GTT CAG 3′) was targeted with specific amplicon of 204 bp. PCR reaction mix components used in the study were procured from BangloreGenei, Banglore, India, unless specified. The primers were custom synthesized by M/S. Biosearch Technologies, Novato, CA USA. All the reactions were carried out with Smart CyclerII (Cepheid, Sunnyvale, CA, USA) using homemade mastermix, which comprised 10X Taq Buffer 2.5 μL, dNTPs mix 2.0 μL, and 4% dimethyl sulfoxide (DMSO) (Sigma, MO, USA). For the optimal performance of the assay, individual PCR reaction conditions and parameters viz. annealing time and temperature, primer, Mg^{2+}, Taq DNA polymerase, and SG concentrations (Molecular Probe, OR, USA) were optimized. All the runs were carried out in triplicates and un-inoculated BHI broth processed along with samples served as negative control. The threshold cycle (C_T) or fluorescence crossing point was selected manually on the basis of second derivative of amplification curve. Each reaction was followed by melt curve analysis involving heating of the PCR product from 60 to 95 °C.

Results

Optimization of duplex assay

For optimal performance, the assay was optimized for reaction conditions and components, first individually and then in duplex format. One of the major optimization was in respect of SG-I concentration and amplification facilitators. The optimal concentration of SG-I was found to be 1.6 μM since at this concentration, two specific peaks could be resolved unambiguously and further increase in the concentration did not result in further improvement of the intensity of the peaks. However, it led to higher C_T values, as depicted in Table 2. Similarly, amplification facilitator concentration of 4% DMSO was selected on the basis of lowest C_T and better fluorescence intensity in comparison to the Betain, Glycerol and Bovine serum albumin (data not shown). DMSO was included in the homemade mix to minimize the inhibitory effect of various food matrices that may interfere with the amplification of the template. It is noteworthy to mention here that with the inclusion of the DMSO in the reaction mix, the Tm of the product decreased. The optimal results were obtained with reaction conditions of 95 °C for 300 s, followed by assigned 35 cycles each of 95 °C/10 s, 59 °C/20 s, 72 °C/20 s. After optimizing the aforesaid parameters and reaction conditions in the duplex assay, a home made cocktail of the reaction mix was developed that comprised 500 nM each of primer set, 1.6 μM of SG-I, 1 unit of Taq polymerase and 5.5 mM of Mg^{2+}.

Table 2 Optimization of SG concentration for duplex assay

SG conc. (μM)	Mean C_T values	Peak resolution
1.0	25.82±0.21	+
1.2	25.67±0.13	+
1.4	25.59±0.17	++
1.6	25.61±0.09	+++
1.8	25.56±0.12	+++
2.0	26.28±0.31	+++

+ one peak; ++ two small peaks; +++ two prominent peaks

Sensitivity and specificity of the assay

The optimized SG RTi-PCR duplex assay was evaluated for its sensitivity in reconstituted (NFDM) non fat dried milk. The assay successfully detected 3 log cfu per ml of each target pathogens with specific Tm for *L. monocytogenes* (79.90±0.39 °C) and *Salmonella* spp (86.29±0.13 °C) without any pre-enrichment. A non specific product, most probably primer dimer, (Tm 71.05 °C) was also observed in the reaction but could be easily distinguished from the target due to lower Tm value (Fig. 1). However, on pre-enrichment of the samples in BHI broth for 6 h, assay could detect as low as 1 log cfu per ml of both pathogens. The assay was highly specific as no cross-reaction with any of other non-targeted tested cultures was recorded while all the *L. monocytogenes* and *Salmonella* serovar tested were detected positive with respective Tm of 79.90±0.39 °C and 86.29±0.13 °C in the melt curve analysis. The assay was tested on 60 market milk and dairy product samples. Same sample of raw milk that was earlier detected positive by molecular beacon based assay, was detected positive this time as well for *L. monocytogenes* after 12 h of selective enrichment by SG-I based assay (Fig. 2a). One ice cream sample was also positive for *Salmonella* spp after 8 h enrichment (Fig. 2b). None of the remaining samples was positive for any of the organisms even after enrichment of 24 h. The percent positive rate for both pathogens in market milk samples was 1.6% each. Both of these samples were also confirmed positive on selective microbiological agar. These results indicate that the developed SG assay can be of considerable use with more or less same sensitivity level as that of specific probe upon appropriate optimization.

Discussion

Post PCR melt curve analysis has emerged as a powerful tool for molecular diagnostic of food pathogens. Generally, the

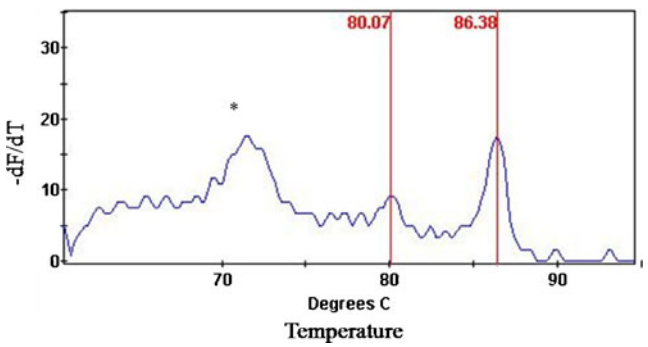

Fig. 1 Melt curve profile showing detection limit of assay (3log cfu per ml) for *L. monocytogenes* (Tm 80.07 °C), *Salmonella* spp. (Tm 86.38 °C) in spiked non fat dried milk, *indicate peak of non specific product

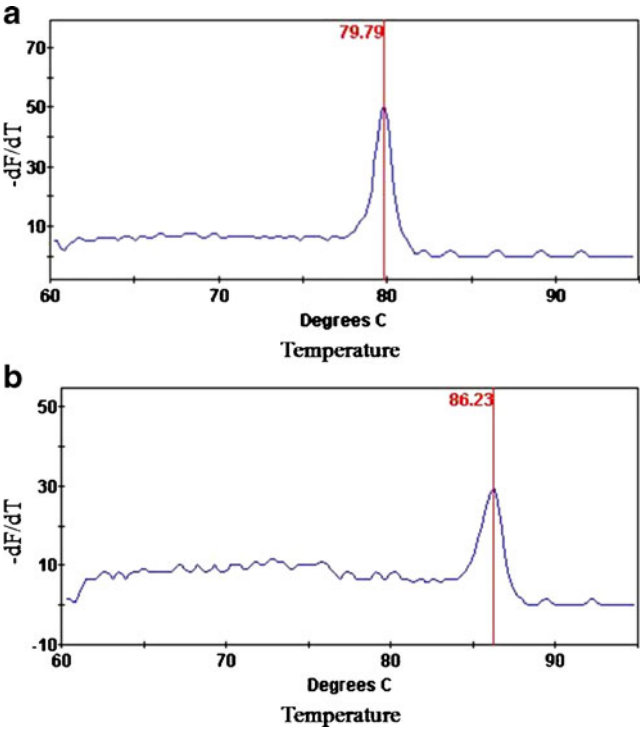

Fig. 2 Melt curve profile showing positive market samples. **a** Raw milk samples positive for *L. monocytogenes*. **b** Ice cream sample positive for *Salmonella* spp

probe based approaches are more expensive than intercalating dyes, although the performance of these chemistries has been reported to be more or less same in some studies (Buh Gasparic et al. 2010). In the present investigation, selection of SG-I concentration was one of the most critical steps in assay optimization and it was observed that at 1.6 μM concentration, both specific peaks could be resolved. Amongst the various chemistries, SG-I is one of the most common intercalating dye used in these assays. Although economical, its non specific binding to any double strand DNA, as observed in this study as well, daunts its application in assays where high specificity is required. It has been also reported to display preferential binding to the longer amplicons and products with higher GC content (Giglio et al. 2003). This inherent tendency may interfere with the development of multiplex assays (Monis et al. 2005). Moreover at higher concentration, SG-I exerts inhibitory effects as evident from the increasing C_T value in current study and as well as earlier reports (Zipper et al. 2004; Monis et al. 2005). Inclusion of DMSO as an amplification facilitator in reaction mix, led to the reduction in Tm of the amplicon. DMSO is known to exert this effect by destabilizing the double helix structure resulting in denaturation temperature (Frackman et al. 1998). Overall, use of SG-I is still more rational than other intercalating dyes and could be the first choice for quantitative assay in uniplex format and multiplex qualitative assay after appropriate optimization.

The performance of the developed SG RTi-PCR assay in the present study is comparable to those of already published reports. Our results with regard to sensitivity of the duplex assay in spiked samples are comparable to those of previously published reports in reference to artificially contaminated fresh produce (Bhagwat 2003) and sausage samples (Wang et al. 2004). O'Grady et al. (2008) had also reported more or less same level of sensitivity for detection of *L. monocytogenes* albeit with a prolonged enrichment of 30 h. In the context of detection of *Salmonella* spp., the sensitivity of the duplex assay developed in this study can be corroborated with those of Hein et al. (2006) and Malorny et al. (2007) who too reported almost similar detection limit of 10 genome/PCR and 2×10^2 cfu of *Salmonella* spp. in 25 g of milk powder in their TaqMan based assay. Recently using a multiplex approach for simultaneous detection of *Escherichia coli* O157:H7, *L. monocytogenes* and *Salmonella* spp., (Omiccioli et al. 2009; Suo et al. 2010; Kawasaki et al. 2010) almost similar level of sensitivity was achieved in other food products, but most of these studies deployed probe based approach which is quite expensive.

Amongst 60 market sample tested, one sample each of raw milk and ice cream was detected positive for *L. monocytogenes* and *Salmonella* spp. after enrichment of 8 and 12 h, respectively. Positive detection of the same sample for *L. monocytogenes* and at same enrichment interval by SG based assay, which was earlier detected by molecular beacon based assay, implies that both chemistries exhibited same sensitivity level. These findings could be of considerable significance towards exploring application of melt curve analysis approach in the diagnostic of food pathogens especially in dairy products. Although, an extensive comparative study with higher number of samples would be more conclusive.

Dairy products are generally subjected to extreme conditions throughout the processing and storage, that may induce stress, injury, specific growth mode such as biofilm formation and viable but not culturable stage in the inhabitants (Foong and Dickson 2004; Oliver 2005; Pagedar et al. 2010). The positive detection of two samples at extended enrichment beyond 6 h could be due to the required resuscitation after the stress imposed by any of these conditions. None of the other samples was positive to any of the target pathogens because probably they were not contaminated as confirmed by extended enrichment up to 24 h. Moreover, the possibility of false negative due to PCR inhibitors and background flora was minimized to a larger extent by using specific pre-enrichment broth for each of the organisms and inclusion of DMSO in reaction premix. Conclusively, the outcome of this study could be of substantial significance to monitor microbiological quality and safety of the dairy products in view of considerable performance at reasonable cost.

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