Molecular beacon based real-time PCR assay for simultaneous detection of Listeria monocytogenes and Salmonella spp. in dairy products

Singh, Virender Batish, Grover

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NOTE

Molecular beacon based real-time PCR assay for simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in dairy products

Jitender Singh · Virender K. Batish · Sunita Grover

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Abstract In this study, a molecular beacon-based duplex real-time PCR assay was developed for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. by targeting their virulence genes *hly* and *invA*, respectively. The detection sensitivity of the assay in reconstituted non-fat dried milk (11%) was 3 and 4 log cfu mL^{-1} for *L. monocytogenes* and *Salmonella* spp., respectively, without any pre-enrichment. However, after pre-enrichment of the samples in brain heart infusion broth for 6 h, the assay could detect as low as 1 log cfu of both the pathogens. The assay was quantifiable for the respective pathogens over 5 and 4 log cfu mL^{-1} with regression coefficient of 0.9956 and 0.9905. On application of the developed assay on 60 dairy products, one sample of raw milk was positive for *L. monocytogenes*, while *Salmonella* spp. was detected in one sample of ice cream. The performance of the duplex assay was validated using microbiological methods and commercial individual pathogen detection kits, which further affirmed the performance of the assay by positively detecting the same samples. The developed assay can be used to monitor the quality of dairy products in context of *L. monocytogenes* and *Salmonella* spp.

摘要 本研究以李斯特单胞菌毒力基因 *hly* 和沙门氏菌的毒力基因 *invA* 为靶点，建立了同时检测上述两种病原菌的分子信标-双重实时PCR (qPCR) 的分析方法。该方法对未经富集的还原脱脂乳 (11%) 中李斯特单胞菌和沙门氏菌的检测灵敏度分别是1×10^{3}和1×10^{4}cfu mL^{-1}。当样品在脑心浸液肉汤 (BHI) 培养基中富集培养6小时候，该方法对两种病原菌的最低检测线可达1×10^{1} cfu

J. Singh · V. K. Batish · S. Grover (✉)
Molecular Biology Unit, Dairy Microbiology Division, National Dairy Research Institute, Karnal 132001, Haryana, India
e-mail: sungro@gmail.com

J. Singh
e-mail: jitender9@gmail.com

Present Address:
J. Singh
Food Biotech Lab, Product and Process Development Group, National Dairy Development Board, Anand 388001, Gujarat, India

mL^{-1}。该方法对李斯特单胞菌和沙门氏菌两种致病菌的定量范围分别为1×10^5和1×10^4, 线性回归系数分别为0.9956和0.9905。采用该方法检对60个乳制品样品进行了检测，其中一份鲜奶样品中检测出李斯特单胞菌，而在一份冰激凌样品中检测出沙门氏菌。根据微生物学方法和商业生产的试剂盒法的验证结果，证明本研究建立的双重实时 PCR 方法是有效的，可以通过检测李斯特单胞菌和沙门氏菌来监控乳制品的质量。

Keywords *Listeria monocytogenes* · Molecular beacon · *Salmonella* · qPCR

关键词 李斯特单胞菌 · 分子信标 · 沙门氏菌 · 实时PCR

1 Introduction

Listeria monocytogenes and *Salmonella* spp. are the two high-risk food pathogens implicated in a number of outbreaks associated with a variety of foods (Centers for Disease Control and Prevention 2008a,b; Elvis et al. 2009; Peccio et al. 2003). *L. monocytogenes* is an ubiquitous, Gram-positive bacterium, responsible for life-threatening infections in humans and animals. It is capable of growth over a wide range of pH, i.e., 4.39–9.40 as well as at refrigerated temperatures commonly employed to control pathogens in foods. The presence of this organism has been reported in various milk and milk-based products (Brito et al. 2008). *L. monocytogenes* is the etiological agent of listeriosis, which predominantly affects certain risk groups, including pregnant women, newborns, elderly people, and immunocompromised patients.

Similarly, salmonellosis caused by *Salmonella* serovars happens to be the second most common cause of gastrointestinal food poisoning in the developed world (Favrin et al. 2003). *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 (Centers for Disease Control and Prevention 2008a). The conventional methods for detection of these pathogens involve identification and confirmation based on culturing on selective media along with biochemical tests and immunological assays. These methods are extremely laborious, cumbersome, and many times remain inconclusive, and results are invariably delayed to make them virtually impractical for any follow up action. Hence, more advanced, sensitive, and rapid microbial detection methods are needed for many applications to complement or replace the traditional microbial culture procedures. Because of these limitations, conventional methods are now giving way to molecular approaches, such as polymerase chain reaction (PCR), real-time PCR (qPCR) for rapid and reliable detection of the pathogens. qPCR is a powerful technique currently in vogue that may allow rapid detection and quantification of pathogens with extremely high specificity and sensitivity. It provides the user with the option to select the detection chemistry of choice from the array of the chemistries, including universal intercalating dyes and highly specific probes such as molecular beacon (MB).

In this context, several attempts have been made to develop real-time PCR assays exploring different chemistries for detection of *L. monocytogenes* and *Salmonella* spp. over a wide range of food products including beef, sea food, fresh produce, and

dairy products (Bohaychuk et al. 2007; Cady et al. 2005; Calvo et al. 2008; Malorny et al. 2007; Rodriguez-Lazaro et al. 2004). However, barring a few (Bhagwat 2003; Jothikumar et al. 2003), most of these assays were explored in uniplex format (Calvo et al. 2008; Catarame et al. 2006; De Martinis et al. 2007; Krascsenicsova et al. 2008; Neves et al. 2008; Nguyen et al. 2004; O'Grady et al. 2008; Rodriguez-Lazaro et al. 2004). Hence, it would be appropriate to develop multiplex assays for simultaneous detection of more than one pathogen in a food sample by targeting more than one gene specific for each of the target organisms. Such assays could prove to be quite handy, as they would not only add rapidity but also reduce the overall cost of the diagnostic test. Prompted by the leads obtained from our previous work (Singh et al. 2009), wherein a duplex MB-PCR assay developed in our lab was able to detect both *Escherichia coli* O157:H7 and *L. monocytogenes* in milk and milk products, the present study was undertaken to extend MB-based qPCR technology in the development of another duplex assay for detection and quantification of *L. monocytogenes* and *Salmonella* spp. concurrently in dairy foods.

2 Materials and methods

2.1 Bacterial cultures and media

Twenty-two different bacterial strains, which included three strains of *L. monocytogenes* and four of *Salmonella* along with 16 other organisms and their sources as recorded in Table 1, were used for determining the sensitivity and specificity of the assay. When not in use, cultures were stored at –80 °C as glycerol stocks and were revived and maintained in brain heart infusion (BHI) broth. Before use, each culture was activated overnight in BHI broth at 37 °C followed by successive processing. The cell number was adjusted individually to approximately 10^8 cfu mL^{-1}, and serial tenfold dilutions were prepared in phosphate-buffered saline and subsequent counts recorded on BHI Agar. All the media used in the study were procured from HiMedia Lab (Mumbai, India), unless specified.

2.2 Enrichment procedure

In order to assess the sensitivity of the duplex assay, pre-sterilized non-fat dried milk reconstituted at the rate of 11% was spiked individually with each of the target pathogen followed by individual enrichment of 1 log cfu mL^{-1} in BHI broth for 6 h. The natural food samples (n=60) comprising ten each of raw milk, pasteurized milk, ice cream, kulfi, paneer, and infant foods screened for presence of *L. monocytogenes* as reported in our previous study (Singh et al. 2009). The sample processing steps included a pre-enrichment of 25 g of each sample in 225 mL of universal pre-enrichment broth for 6 h, followed by the selective enrichment in *Listeria* enrichment broth 37 °C and Rappaport–Vallisiadis broth for *Salmonella* serovar. The samples were drawn at the periodic intervals of 4, 8, 12, 18, and 24 h for real-time PCR analysis as well as for microbiological analysis by plating on selective agar. The microbiological counts of natural food samples were determined by plating appropriate dilution on PALCAM Agar (Diffco Inc,

Table 1 List of the bacterial cultures used in the study

Organism and source	Detection by MB qPCR
E. coli O157:H7 (ATCC35150)	−
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[b]	−
Lactobacillus plantarum[b]	−
Lactobacillus helveticus[b]	−

Different letters give the source of Bacterial cultures procured

Lawrence, KS, USA) for *L. monocytogenes* and xylose lysine deoxycholate agar for *Salmonella* spp., respectively.

2.3 Sensitivity of the assay in spiked skim milk

The sensitivity of the assay was determined in reconstituted skim milk (11%) spiked with the target pathogens over a range of 1–7 log cfu mL^{-1}. Template DNA was extracted from spiked and enriched dairy products samples with the help of commercial DNA extraction purification Kit (PureExtreme, genomic DNA purification kit, Fermentas, Maryland, USA) after pretreatment of the pellet with 100 μL volume of Triton X-100 and trypsin (1%, *w*/*v*). The DNA was dissolved in the TE buffer and stored at −20 °C until further use.

2.4 Primers and probes

For *L. monocytogenes*, *hly* gene (accession no. EJ030599.1), responsible for hemolysin production was targeted to design specific primers (forward, 635-652; reverse, 792-809) with expected product size of 175 bp, whereas for *Salmonella*

spp., *invA* (invasion associated gene; accession no. CP001113.7; forward, 3015018-3015035; reverse, 3015181-3015198) was targeted with expected amplicon of 181 bp. The respective MB probes were labeled with FAM and Quasar 670 at 5′ end for *L. monocytogenes* (probe, 707-724) and *Salmonella* spp. (probe, 3015102-3015117), respectively, and BHQ-1-3 at 3′ end. The primers and probes were custom synthesized from Fluorescentric (Utah, USA) and Biosearch Technologies (Novato, CA, USA). The sequences of the primers and probes used in the study have been recorded in Table 2 along with expected product size of the respective amplicon.

2.5 Real-time PCR assays

All the PCR reactions using homemade mastermix, which comprised of 10× Taq buffer 2.5 μL, dNTPs mix 2.0 μL (2 mmol L^{-1}), 2 U of DNA Taq polymerase, 5.5 mmol L^{-1} of Mg^{2+}, 4% dimethyl sulfoxide, primer concentration of 500 nm each for forward and reverse primer for both organism, and 200 nm (*L. monocytogenes*) and 300 nm (*Salmonella* spp.) probe concentration in 25 μL of final reaction volume. All the reactions were carried out at 54 °C as the annealing temperature on Smart CyclerII (Cepheid, Sunnyvale, CA, USA) in triplicates, and uninoculated BHI broth served as a negative control. The threshold cycle (C_T) or fluorescence crossing point was selected manually on the basis of second derivative kinetics. The standard curves were generated by plotting mean threshold cycle value against log colony-forming units per milliliter. The optics was kept 'ON' during the annealing step for acquisition of the fluorescence in channel 1 (*L. monocytogenes*) and 4 (*Salmonella* spp.) of SmartCycler II.

2.6 Validation of the assay

In order to validate the efficacy of the duplex assay, the samples detected to be positive were subjected to detection by commercial kits, i.e., HiMotility *Listeria* detection kit for *L. monocytogenes* and *Salmonella* detection (Takara, Bioeurope, Inc., Saint-Germain-en-Laye, France.) for *Salmonella* serovar. HiMotility *Listeria* detection kit deploys a battery for biochemical tests for the detection of the pathogen, while *Salmonella* detection kit involves highly specific chimera probe composed of DNA and RNA. On binding to the amplicon, RNA part of probe is cleaved by the RNase H present in the reaction premix, thus leading to the

Table 2 Sequence of primers

Gene	Organism	Type	Sequence	Amplicon size (bp)
hly	*L. monocytogenes*	MB-F	5′ ggc tta cag tga atc aca 3′	175
		MB-R	5′ tct gga agg tct tgt agg 3′	
		MB-P	5′ FAM- ggt cga act tcg gcg caa tca ggc acc- BHQ-1-3′	
invA	*Salmonella* spp.	MB-F	5′ gac aga cgt aag gag gac 3′	181
		MB-R	5′ acg agc agt aat ggt atc 3′	
		MB-P	5′Quasar670- gct ccg gct tcc ggg tca agg gga gc-BHQ-1-3′	

proportional increase in the fluorescence intensity. Both of the commercial kits were used as per respective manufacturer's recommendations.

3 Results

3.1 Sensitivity and specificity of the assay

The optimized MB qPCR duplex assay was evaluated for its sensitivity in spiked milk samples. The assay could successfully detect 3 and 4 log cfu mL^{-1} of *L. monocytogenes* (Fig. 1a) and *Salmonella* spp. (Fig. 1b), respectively, without any pre-enrichment. However, on prior pre-enrichment of the samples in BHI broth for 6 h, the assay could detect as low as 1 log cfu mL^{-1} of both of the target pathogens. The assay was highly specific since all the *L. monocytogenes* and *Salmonella* spp. were detected positively with fluorescence in the respective channels, and no cross-reactivity with any of other non-targeted cultures was recorded in Table 1.

3.2 Application of the duplex assay on market milk samples

The duplex assay was applied on 60 market samples of dairy products comprising ten each of raw milk, pasteurized milk, kulfi, ice cream, paneer, and infant foods in order to determine the microbiological safety with regard to presence of *L. monocytogenes* and *Salmonella* spp. With the help of the duplex assay, the presence of the *L. monocytogenes* and *Salmonella* serovar was detected unequivocally in one sample each of raw milk and ice cream after selective enrichment for 12 and 8 h,

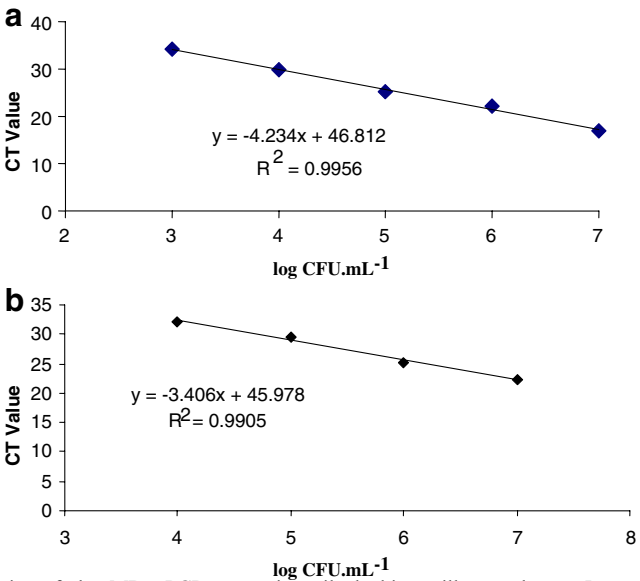

Fig. 1 Sensitivity of the MB qPCR assay in spiked skim milk samples. **a** *L. monocytogenes.* **b** *Salmonella* spp

respectively. However, none of the samples of pasteurized milk, infant food, and kulfi examine during this investigation was found to be positive for any of the two pathogens with qPCR assay. Even by microbiological methods that are considered as gold standard, none of the sample was found positive for the target pathogens, except for two samples that were also detected positive by qPCR assay. The validity of the duplex assay developed in our lab was also reaffirmed by testing the positive samples with commercial kits, namely, HiMotility *Listeria* detection kit for *L. monocytogenes* and *Salmonella* detection kit for *Salmonella* spp., which also indicated the contamination of the same samples with respective pathogens.

4 Discussion

Real-time PCR has now emerged as a reliable and powerful tool for the rapid detection and quantification of high-risk food pathogens including *L. monocytogenes* and *Salmonella* spp. in various foods. However, the performance of real-time PCR assays may be affected by various factors involving reaction components, parameters, processing, and inhibitors as dairy foods represent highly complex and heterogeneous matrix system (Elizaquivel and Aznar 2008). In this investigation, we report a new duplex qPCR assay based on MB for simultaneous detection of *L. monocytogenes* and *Salmonella* spp. in dairy products. Although a considerable number of reports are available regarding the uniplex detection of *L. monocytogenes* and *Salmonella* spp. (Chen et al. 2000; Hein et al. 2006; Liming et al. 2004; Malorny et al. 2007; O'Grady et al. 2009; Omiccioli et al. 2009), there are currently a few limited studies pertaining to the application of multiplex assays based on real-time PCR for simultaneous detection of both of these pathogens (Bhagwat 2003; Jothikumar et al. 2003; Omiccioli et al. 2009). The performance of our MB qPCR assay with regard to sensitivity in dairy food samples spiked with the two target pathogens was comparable to those of previously published reports. In this context, Jothikumar et al. (2003) were the first to report qPCR assay for simultaneous detection of *L. monocytogenes* and *Salmonella* spp. with detection limit of 10 and 10^2 cfu per PCR in pure culture. Using almost the similar approach, Bhagwat (2003) was able to detect simultaneously *L. monocytogenes* and *Salmonella* spp. along with *E. coli* O157:H7 using pathogen-specific commercial BAX system at a sensitivity level of one to ten cells for *Salmonella* spp. and 10^3 cfu mL^{-1} of *L. monocytogenes* in artificially contaminated fresh produce after overall enrichment of more than 20 h. Contrary to this, our assay could detect the targeted pathogens with the same sensitivity with shortened pre-enrichment period. In a related study, a method based on coupling ISO enrichment method to real-time PCR in duplex format for detection of *L. monocytogenes* and *Salmonella* spp. in fresh produce showed detection limit of 1 cfu/25 g with prior enrichment (Badosa et al. 2009), which is comparable to the efficacy of the MB qPCR developed in our lab. Working on similar lines, D'Urso et al. (2009), while using yoghurt as model food, reportedly eliminated the risk of false positive using a novel filtration method in conjunction with real-time PCR for simultaneous detection of both these pathogens. With the technological evolution, the probe-based approach coupled with high resolution melting has also been explored to simultaneous detect *L. monocytogenes* and *Salmonella* spp.

with more or less the same sensitivity level as demonstrated in our duplex assay (Omiccioli et al. 2009).

Dairy products are generally subjected to extreme conditions throughout the processing to storage, which may induce stress, injury, specific growth mode, such as biofilm formation, and viable but not culturable stage in the inhabitants (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.

Currently, advanced technologies such as microarray and microfluidics are being explored for the detection of high-risk food pathogens (Jin et al. 2009; Suo et al. 2010). However, high costs associated with such advanced approaches are the major deterrent, which limit their application in dairy and food industry on routine basis. Our duplex assay, on the other hand, constitutes a relatively simpler and cost-effective proposition for simultaneous detection of target pathogens in dairy foods on account of working efficiently even with a homemade reaction mix developed in our own lab and hence can be applied effectively.

5 Conclusion

From the aforesaid discussion, it is quite evident that, presently, different strategies based on qPCR are being explored across the world for rapid detection of high-risk food pathogens in order to minimize the listeriosis and salmonellosis outbreaks associated with the various food products. However, the presence of high-risk foodborne pathogens in some unexplored indigenous food products, especially those copiously consumed in developing countries (Singh et al. 2009), poses a fresh challenge to the scientific fraternity to come up with the inexpensive technologies to ensure the safety of the consumers. Hence, in the present investigation, we have developed a duplex qPCR assay with considerable specificity, sensitivity, and its application in different milk and milk-based products for assessing their microbiological quality and safety in relation to *L. monocytogenes* and *Salmonell*a spp.

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