Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Multiplex touchdown PCR assay to enhance specificity and sensitivity for concurrent detection of four foodborne pathogens in raw milk

P. Moezi[1], M. Kargar[1], A. Doosti[2] and M. Khoshneviszadeh[3]

1 Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran
2 Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
3 Department of Medicinal Chemistry, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

Keywords
Escherichia coli O157: H7, *Listeria monocytogenes*, multiplex touchdown PCR, raw milk, *Salmonella enterica*, *Staphylococcus aureus*.

Correspondence
Mohammad Kargar, Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran.
E-mail: mkargar@jia.ac.ir

Parichehr Moezi, Mohammad Kargar, Abbas Doosti, Mehdi Khoshneviszadeh contributed equally to this work.

2019/0070: received 15 January 2019, revised 12 April 2019 and accepted 14 April 2019

doi:10.1111/jam.14285

Abstract

Aims: The aim of this study was to develop a multiplex touchdown PCR (multiplex TD-PCR) for rapid and simultaneous detection of four major foodborne pathogens to avoid mispriming and unwanted production during gene amplification. Touchdown PCR is the modified form of standard PCR, which enhances specificity, sensitivity.

Methods and Results: For this reason, a multiplex TD-PCR assay with a pre-enrichment step was developed to detect four foodborne pathogens namely *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella enterica* serovar Enteritidis in pure culture and raw milk samples. The results showed that this protocol can eliminate the unwanted band or reduce significantly. The detection sensitivity of the single and multiplex TD-PCR was one cell per ml in pure culture. Furthermore, the detection limit of multiplex TD-PCR was one cell per 25 ml for artificially contaminated raw milk. We obtained similar results for detection of aforementioned pathogens in raw milk, after comparing the multiplex TD-PCR method with the traditional culture, except in one or two samples.

Conclusions: Hence, the proposed multiplex TD-PCR method could be confirmed as an effective way for rapid optimization of PCR reactions to increase specificity, sensitivity during gene amplification.

Significance and Impact of the Study: Hence, due to its simplicity, cost-effectiveness and being time-saving, it seems that this method is reasonable and economical for rapid optimization of PCR reactions.

Introduction

According to the United States Center for Disease Control and Prevention, one in six Americans is infected by contaminated food and drink, and annually more than 48 million people are at risk due to foodborne infections (Sulaiman and Hsieh 2017).

Food contamination might occur in various stages. Contamination might be detected in raw products or processed products. Hence, food safety organizations have great concern regarding microbiological contaminants and their consequences in food industry and human health.

Although the use of pasteurized milk and ultra-high-temperature milk has become common, raw milk is still being used by farm workers and people who live in rural areas and has its own advocates. Insufficient pasteurization can lead to the transfer of pathogens in milk; hence, their rapid identification is essential (Oliver *et al.* 2005; Quero *et al.* 2014; Willis *et al.* 2018).

Pathogens such as *Escherichia coli* O157: H7 and *Listeria monocytogenes, Staphylococcus aureus* and *Salmonella enterica* serovar Enteritidis (*S. enterica*) are transmitted through food (Sulaiman and Hsieh 2017). *Escherichia coli* O157: H7 is an important foodborne pathogen, and most

related infections are due to consumption of contaminated milk, ground beef, water, and dairy products (Oberst *et al.* 1998). *Escherichia coli* O157: H7 even in low dosage of 1–100 colony-forming units (CFU) can cause disease (Paton and Paton 1998). *Listeria monocytogenes* is considered as a major species responsible for human infection. Milk, cheese, meat, cream and ready-to-eat foods are the main reason for *L. monocytogenes* infection in humans (McLauchlin 1996; Robinson 2014). *Staphylococcus aureus* produces a wide range of exotoxins including heat-resistant enterotoxins that can lead to food poisoning. Milk and its byproducts as the main source of intoxication by *S. aureus* in humans (Wang *et al.* 2007; Jahan *et al.* 2015; Yoon *et al.* 2018). *Salmonella* lives in the intestine of various animals, and is transmitted to humans via infected animals and their products, such as milk and dairy, meat and eggs, causing salmonellosis. A high percentage of salmonellosis occurs in humans by consuming raw milk or dairy products made from raw milk (Schlosser *et al.* 2000).

Conventional microbiological cultures are valid methods for the identification of foodborne pathogens, and still considered as the "gold standard". However, these methods often require ample time and labour, including enrichment media, selective media, and biochemical and serological tests (Feng 2007; Cheng *et al.* 2012; Garrido *et al.* 2013; Margot *et al.* 2013).

Recently developed molecular techniques are promising alternatives to food microbiology. Molecular methods such as polymerase chain reaction (PCR) and real-time PCR have shown to have high potential in diagnostic laboratories, as well as many advantages over the traditional microbiological techniques, such as specificity, short test time and low detection limits (Burnett and Beuchat 2001; Cocolin *et al.* 2002; Aslam *et al.* 2003; Dwivedi and Jaykus 2011; Zeng *et al.* 2016). Within the past few years, international standards have agreed to use PCR for diagnosing foodborne pathogens (Anonymous 2005) ISO 22174-2005: general requirements and definitions (ISO/TS 20836-2005: performance criteria for thermocyclers; ISO/TS 20837-2006: sample preparation; ISO 20838-2006: amplification and detection for qualitative methods).

In addition, multiplex PCR can detect two or more pathogens in one tube, which is faster, easier, and cheaper to run. Several studies have used multiplex PCR techniques to detect foodborne pathogens with or without pre-enrichment (Germini *et al.* 2009; Silva *et al.* 2011; Kim *et al.* 2014; Park and Ricke 2015; Xu *et al.* 2016; Yu *et al.* 2016).

It is worth stating that the most common problem with classical RCR is the nontarget DNA amplification, the presence of nonspecific products or primer dimers. However, PCR has continuously improved over the years, and the way to overcome the formation of non-specific products is to optimize PCR reaction. This includes testing different concentrations of reaction components, such as Mg^{2+}, Deoxyribonucleotide triphosphate (dNTPs), primers and templates. Nevertheless, one of the most important parameters in the optimization of PCR reaction is the annealing temperature. The annealing temperature for PCR is usually considered to be between 4 and 5°C lower than the T_m of primers. Touchdown PCR is a method for optimizing the conventional PCR that uses a range of annealing temperatures (Don *et al.* 1991; Henegariu *et al.* 1997).

Touchdown PCR is a novel approach to optimize PCR (Don *et al.* 1991; Roux 2009). Since the goal is to avoid low-T_m priming along the initial cycles, it is imperative that TD-PCR is performed with "hot start" modification (D'Aquila *et al.* 1991; Erlich *et al.* 1991; Hecker and Roux 1996). Touchdown PCR should always be performed in combination with a hot start protocol to minimize mispriming during the primary steps of PCR (Green and Sambrook ,2018b, 2018a). Touchdown PCR increases sensitivity, specificity and products, without the need for long optimizations, and redesigning of primers (Korbie and Mattick 2008). Multiplex TD-PCR is a rapid, specific and sensitive molecular approach that uses more than one pair of primer to identify several microbes in a single reaction tube (Luo *et al.* 2012).

Here, we show how TD-PCR expresses an important advantage of added specificity during those cycles above the T_m and enhanced efficiency during the cycles below the T_m to increase product yield. Also, we will show multiplex TD-PCR with a practical modification for the PCR technique, which is the combination of two techniques multiplex and TD-PCR. Several target primers are used to determine multiple DNA targets in multiplex TD-PCR.

Hence, the purpose of this study was to design a rapid protocol for simultaneous detection of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *S. enterica* with prior cultivation. For this aim, we evaluated the efficiency of multiplex TD-PCR method to prevent nonspecific attachment and mispriming to increase the specificity, sensitivity of the replication. In parallel, raw milk samples were also tested using multiplex TD-PCR method to compare with the results of conventional microbiology culture.

Materials and methods

Bacterial strains and culture conditions

All bacterial strains used in this study are shown in (Table 1). The strains used for testing were stored as frozen stock cultures at −80°C. A fresh culture of each pathogen was prepared with inoculating trypticase soy

Table 1 List of target and non-target bacterial species used in this study

Bacterial strains	Source	*rfbE*	*hly A*	*nuc*	*inv A*
***E. coli* O157:H7**	**ATCC 43894**	+	–	–	–
E. coli O157: H7	ATCC 43890	+	–	–	–
E. coli O157:H7	ATCC 43889	+	–	–	–
E. coli O157:H7	ATCC 43888	+	–	–	–
E. coli	ATCC 1330	–	–	–	–
E. coli	PTCC 1399	–	–	–	–
E. coli	LCC	–	–	–	–
E. coli	LCC	–	–	–	–
Pathogenic *E. coli*	LCC	–	–	–	–
Pathogenic *E. coli*	LCC	–	–	–	–
Non-pathogenic *E. coli*	LCC	–	–	–	–
Non-pathogenic *E. coli*	LCC	–	–	–	–
L. monocytogenes	**ATCC 19111**	–	+	–	–
L. monocytogenes	ATCC 13932	–	+	–	–
L. monocytogenes	ATCC 15313	–	+	–	–
L. monocytogenes	ATCC 19115	–	+	–	–
L. monocytogenes	PTCC 1298	–	+	–	–
L. monocytogenes	LCC	–	+	–	–
L. monocytogenes	LCC	–	+	–	–
L. monocytogenes	LCC	–	+	–	–
L. monocytogenes	LCC	–	+	–	–
L. monocytogenes	LCC	–	+	–	–
L. innocua	ATCC 35897	–	–	–	–
L. grayi	ATCC700545	–	–	–	–
Staphylococcus aureus	**ATCC 6538**	–	–	+	–
S. aureus	ATCC 25923	–	–	+	–
S. aureus	ATCC:25923	–	–	+	–
S. aureus	ATCC 33591	–	–	+	–
S. aureus	LCC	–	–	+	–
S. aureus	LCC	–	–	+	–
S. aureus	LCC	–	–	+	–
S. aureus	LCC	–	–	+	–
S. aureus	LCC	–	–	+	–
S. aureus	LCC	–	–	+	–
S. epidermidis	ATCC:3270	–	–	–	–
S. saprophyticus	PTCC 1379	–	–	–	–
***Salmonella enterica* Enteritidis**	**ATCC 13076**	–	–	–	+
S. enterica	ATCC 51741	–	–	–	+
S. enterica Typhimurium	ATCC 14028	–	–	–	+
S. enterica	ATCC 9270	–	–	–	+
S. enterica	ATCC 9150	–	–	–	+
S. enterica Typhimurium	PTCC 1622	–	–	–	+
S. enterica Typhi	PTCC 1609	–	–	–	+
S. enterica	LCC	–	–	–	+
S. enterica	LCC	–	–	–	+
S. enterica	LCC	–	–	–	+
S. enterica	LCC	–	–	–	+
S. enterica	LCC	–	–	–	+
Lactobacillus delbrueckii	ATCC 11842	–	–	–	–
Lactobacillus acidophilus	LCC	–	–	–	–
Lactobacillus plantarum	ATCC 8014	–	–	–	–
Leuconostoc mesenteroides	ATCC 8293	–	–	–	–
Lactobacillus casei	PTCC 1608	–	–	–	–
Streptococcus thermophilus	ATCC 19258	–	–	–	–
Bifidobacterium bifidum	PTCC 9244	–	–	–	–
Clostridium perfringens	LCC	–	–	–	–
Streptococcus faecalis	ATCC 8043	–	–	–	–
Streptococcus faecalis	LCC	–	–	–	–
Yersinia enterocolitica	LCC	–	–	–	–
Bacillus cereus	LCC	–	–	–	–

ATCC, American Type Culture Collection; PTCC, Persian Type Culture Collection; LCC, Laboratory Culture Collection.

broth (TSB) and incubated at 37°C for 24 h in a shaking incubator at 150 rev min^{-1}. The target bacteria were *E. coli* O157: H7 American Type Culture Collection (ATCC) 43894, *L. monocytogenes* ATCC 19111, *S. aureus* ATCC 6538 and *S. enterica serovar* Enteritidis ATCC 13076.

Microbiological experiment

Specific selective enrichment broths and agars

According to ISO (Anonymous, 1998, 1999, 2002a, 2002b), the conventional culture method was used to detect *E. coli* O157: H7, *L. monocytogenes, S. aureus* and *S. enterica* in the raw milk samples.

All media were purchased from (Merck, Germany), which included modified EC broth with 20 mg of novobiocin per litre (mEC + n, Modified EC broth with novobiocin) and Modified Sorbitol MacConkey agar (CT-SMAC) for *E. coli* O157: H7, Fraser Broth (FB) and PALCAM agar for *L. monocytogenes*, Giolliti Cantoni Broth and Baird Parker agar (BP) for *S. aureus*, buffered peptone water (BPW), Muller-Kauffman tetrathionate novobiocin broth, Rappaport Vassiliadis broth, *Salmonella Shigella* Agar and xylose lysine deoxycholate agar for *S. enterica*. The presumptive colonies of the four pathogens were subjected to biochemical testing and serological confirmation.

Enrichment and DNA extraction

The TSB enrichment medium was used for overnight growth of four bacteria. One millilitre from the overnight enriched culture was transferred to microtubes, and bacterial cells were collected via centrifugation at 2000 g for 15 min. Genomic DNA of bacteria was extracted, using the

Genomic DNA Purification Kit (SinaClon BioScience Co., Tehran, Iran). The pellets were suspended in 100 μl prelysis buffer and 20 μl lysozyme, and then incubated at 37°C for 30 min. Followed by adding 10 μl Ributinase and incubated at 55°C for 30 min. After that 400 μl of lysis buffer was added and vortexed. Then, 300 μl of precipitated solution was added and vortexed. The solution was then transferred to a spin column to be centrifuged for 1 min. Then, 400 μl of wash buffer I was added and centrifuged for 1 min. Also, the same washing was performed twice by buffer II. The column was then transferred to a new tube. Thirty microliters of the preheated elution buffer was placed in the column and incubated for 4 min at 65°C. Finally, the solution was centrifuged for 1 min to elute the DNA. The relative centrifugal force was 17 000 ***g*** at all stages. The concentrations of genomic DNA were determined using ND-3800-OD NanoDrop spectrophotometer (Hercuvan, Selangor Darul Ehsan, Malaysia).

Primer design

The primers for detecting *E. coli* O157: H7, *L. monocytogenes, S. aureus* and *S. enterica* were targeted specifically on the *rfbE* (This study), *hlyA* (Wu *et al.* 2004), *nuc* (Brakstad *et al.* 1992) and *invA* genes (Hoorfar *et al.* 2000), respectively.

The *rfbE* gene encoding O157 lipopolysaccharide and for *E. coli* O157: H7 serogroup is unique (Oberst *et al.* 1998), *hlyA* encoding listeriolysin for phagosomal escape into the host cell's cytosol (Wu *et al.* 2004), the *nuc* (thermonuclease) gene in *S. aureus* (Brakstad *et al.* 1992) and *invA* encoding an invasion protein in *S. enterica* (Hoorfar *et al.* 2000). The primers were synthesized by Macrogen Company from South Korea. Primer sequences for the experiments are shown in (Table 2).

Single and multiplex TD-PCR conditions

The TD-PCR reaction was carried out in a final volume of 50 μl with the following components: 25 μl master mix (Taq DNA Polymerase 2x Master Mix RED-AMPLIQON, including all components necessary to perform DNA amplification), 1 μl from 100 to 200 ng μl^{-1} DNA template, and 1 μl from 0·2 μmol l^{-1} forward primer, 1 μl from 0·2 μmol l^{-1} reverse primer, and sterile distilled water up to 50 μl for each strain. In this study, a T-100 thermal cycler system (BioRad, Munich, Germany) was used.

The cycling plan of touchdown PCR was included in two separate stages. Stage 1 started with an annealing temperature above the T_m of the primers and changed to a low annealing temperature during consecutive cycles. The first cycling stage began with an annealing temperature of T_m + 10°C, and then decreases the annealing temperature by 0·5°C per cycle until the T_m of the primers for a total of 20 cycles. Stage 2 consisted of 15 cycles, using the annealing temperature at 55°C. The total number of cycles in stages 1 and 2 should not exceed 35 cycles, as additional cycles risk the creation of nonspecific products and primer dimers (Korbie and Mattick 2008). Positive and negative control reactions were set up using, a genomic DNA template containing the sequence of interest as a positive control and a reaction lacking template as the negative control.

Accordingly, an initial denaturation at 95°C for 5 min, followed by 20 cycles at 95°C for 30 s, 65°C (0·5°C decrease per cycle) for 30 s, 72°C for 1 min, then another 15 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step was carried out at 72°C for 7 min. The PCR products were analyzed on 2% agarose gels in Tris/Borate/EDTA (TBE) Buffer 1X and visualized by Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

At first, the reactions by single TD-PCR were performed, with the purpose of getting the best amplification conditions with the multiplex TD-PCR. After establishing the conditions to perform the single reaction, conditions for multiplex reactions were setup and run.

Specificity assays

DNA template of the 60 strains of bacteria listed in (Table 1) was tested, using a combination of all primers pairs of the four target bacteria and assessed the specificity of the target primers by multiplex TD-PCR protocol. Specificity was experienced by examining the ability of this protocol to distinguish nontarget bacterial strains among these four target pathogens.

Sensitivity assays

Four standard strains of *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* were cultured overnight in TSB at 37°C. To test the sensitivity of single TD-PCR assay in pure culture was carried out using a genomic DNA from 10-fold serial dilution (10^7–10^0 cell per ml) of the target pathogens. One microlitre of each genomic DNA dilution was used for single TD-PCR assay. Each experiment was repeated twice.

To test the sensitivity of multiplex TD-PCR assay in pure culture, four standard strains of bacteria were cultured overnight in TSB at 37°C and then each one diluted 10-fold from (10^7–10^0 cell per ml) with sterile saline. The DNA of each dilution of target bacteria was extracted separately and mixed. One microlitre of genomic DNA from each dilution of the four target bacteria

Table 2 Primers used in the single and multiplex TD-PCR assays

Microorganism	Target gene	Primers	Sequence (5′–3′)	PCR products (bp)
Escherichia coli O157:H7	*rfbE*	FR	AACGGTTGCTCTTCATTTAGCTGATGATTTTATATCACGA	580
Listeria monocytogenes	*hlyA*	FR	ATCATCGACGGCAACCTCGGAGAC CACCATTCCCAAGCTAAACCAGTGC	404
Staphylococcus aureus	*nuc*	FR	GCGATTGATGGTGATACGGTTAGCCAAGCCTTGACGAACTAAAGC	270
Salmonella enterica Enteritidis	*invA*	FR	TCGTCATTCCATTACCTACCAAACGTTGAAAAACTGAGGA	119

was used to perform multiplex TD-PCR. Each experiment was repeated twice.

Multiplex TD-PCR evaluation in inoculated raw milk samples

Raw milk samples were confirmed negative for four target pathogens by culturing in corresponding enrichment media followed by CT-SMAC agar for *E. coli* O157: H7, PALCAM agar for *L. monocytogenes,* BP agar for *S. aureus*, and SS agar for *S. enterica*. The overnight culture of each of the four target bacterial strains containing *E. coli* O157:H7, *L. monocytogenes, S. aureus* and *S. enterica* in TSB was subjected to serial 10-fold dilutions (10^4, 10^3,10^2,10^1,10^0 cell per ml), separately. To obtain the desired bacterial concentration, from an overnight culture, initial turbidity was adjusted to 0·5 McFarland by measuring the optical density at 600 nm (OD 600) using a spectrophotometer (UV-2102 PCS, Unico, China). Viable counts were gained by plating bacterial culture on the corresponding culture medium and were incubated overnight at 37°C. We find out the starting concentration of target bacterial: $0{\cdot}5 \times 10^8$ cell per ml for *E. coli* O157: H7, 2×10^8 cell per ml for *L. monocytogenes,* $1{\cdot}7 \times 10^8$ cell per ml for *L. monocytogenes* and 1×10^8 cell per ml for *S. enterica.* Then 1 ml of all target bacterial culture from each dilution was added to 25 ml of raw milk. These raw milk samples before adding the pre-enrichment medium, was centrifuged at 1100 g for 10 min at 4°C in a refrigerated centrifuge, and then the supernatant containing the fat layer and water was discarded. The pellet was washed twice with phosphate-buffered saline and added it to 225 ml pre-enrichment medium (ELSS) and incubated for 16 h at 37°C in a shaking incubator at 150 rev min^{-1}. (A pre-enrichment broth (ELSS) was formulated to allow concurrent growth of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *S. enterica*. ELSS contained (w/v) 1·4% peptone from casein, 0·4% soya peptone, 0·55% yeast extract, 0·6% beef extract, 0·1% sodium pyruvate, 0·05% esculin hydrate, 0·2% glucose, 0·35% sodium chloride, 1·4% monopotassium phosphate, 0·25% dipotassium phosphate and 0·6% disodium hydrogen phosphate (pH $7{\cdot}1 \pm 1$)). After 16 h, 1 ml of culture broth was subjected to extract DNA using the Genomic DNA Purification Kit (SinaClon). After that, 1 μl of genomic DNA of all target bacteria from each dilution was added to other components which were necessary for the multiplex TD-PCR reaction.

Evaluation of raw milk for natural contamination by multiplex TD-PCR method

According to basic sampling principle, during a 90 days period, 50 raw milk samples were randomly purchased from local stores in four different districts in Shiraz, Iran. Samples were collected in sterile bags and kept at 4°C during sampling and delivery. The microbial test was carried out on the same day. DNA extraction was performed after 16 h pre-enrichment in ELSS and subjected to multiplex TD-PCR. Preparation of raw milk sample for adding to 225 ml pre-enrichment medium (ELSS) was done as previously stated. (See multiplex TD-PCR in inoculated raw milk sample.). After 16 h of incubation in ELSS in a shaking incubator at 150 rev min^{-1} at 37°C, 1 ml of each culture broth was used to extract DNA and subjected to perform multiplex TD-PCR.

A traditional culture technique was applied to detect *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* according to ISO (Anonymous, 1998, 1999, 2002a, 2002b) in the raw milk samples.

Results

Specificity assays

The specificity of the primers is shown in Fig. 1. The specificity of single TD-PCR method by single primer was shown (Fig. 1a). The amplified fragment of *E. coli* O157:H7 was 580 bp, of *L. monocytogenes* was 404 bp, of *S. aureus* was 270 bp and of *S. enterica* was 119 bp. A combination of four primer pairs (multiple primers) and every DNA template from 60 different target and non-target pathogens as listed in (Table 1) was experimented by multiplex TD-PCR method. The results showed that

each primer pair was specific for the corresponding target bacteria, and the specificity of single and multiplex TD-PCR assay were 100%. In addition, the results were the same for the multiplex TD-PCR test to detect individual pathogens and multiple pathogens simultaneously (Fig. 1b).

Sensitivity assays

The sensitivity of a multiplex TD-PCR method was evaluated by preparing a 10-fold serial dilution of the DNA from four target pathogens (10^7–10^0 cell per ml). The detection sensitivity was successfully achieved down to one cell per ml for *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *S. enterica* individually. The results proved the sensitivity of the single TD-PCR assay to be 100% (Fig. 2a–d).

The sensitivity of a multiplex TD-PCR method was evaluated by preparing a 10-fold serial dilution of the DNA from four target pathogens (10^7–10^0 cell per ml) concurrently. The sensitivity for simultaneous detection of the four pathogens was successfully achieved down to one cell per ml. The results verified the sensitivity of the multiplex TD-PCR assay to be 100% (Fig. 3).

Evaluation of the multiplex TD-PCR assay in inoculated raw milk samples

To validate the multiplex TD-PCR assay for its application to raw milk and determine detection limit, the samples of raw milk were inoculated with *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* with five levels of the number of viable cells (10^4, 10^3,10^2,10^1,10^0 cell per ml) were investigated. The results confirmed that the multiplex TD-PCR assay was able to correctly identify the presence of the four food-borne pathogens at all different inoculated levels and detection limits were as low as one bacterial cell per 25 ml of inoculated raw milk after pre-enrichment in ELSS for 16 h by this multiplex TD-PCR. The results are shown in (Fig. 4).

Evaluation of raw milk for natural contamination by multiplex TD-PCR method

To evaluate the practical use of multiplex TD-PCR method for simultaneous recognition of the four pathogenic bacteria in raw milk samples, we compared this method with the conventional culture-based method for detecting four foodborne pathogens in 50 raw milk samples. The detection rate of the *L. monocytogenes* and *S. enterica* was similar in both diagnostic methods, while the rate of *E. coli* O157: H7 and *S. aureus* was different in one and two samples, respectively (Table 3).

Comparisons of classic multiplex PCR and multiplex TD-PCR protocols in raw milk

By comparing classical multiplex PCR with multiplex TD-PCR optimization protocol for detecting the four bacterial pathogens in raw milk sample is shown in Fig. 5. The results showed that multiplex TD-PCR has an advantage over the classic multiplex PCR method in avoiding mispriming and unwanted production during gene amplification. This experiment was repeated twice.

Discussion

Today, the demand for rapid results has increased; hence, the use and development of molecular techniques to detect micro-organisms is of great importance. PCR is one

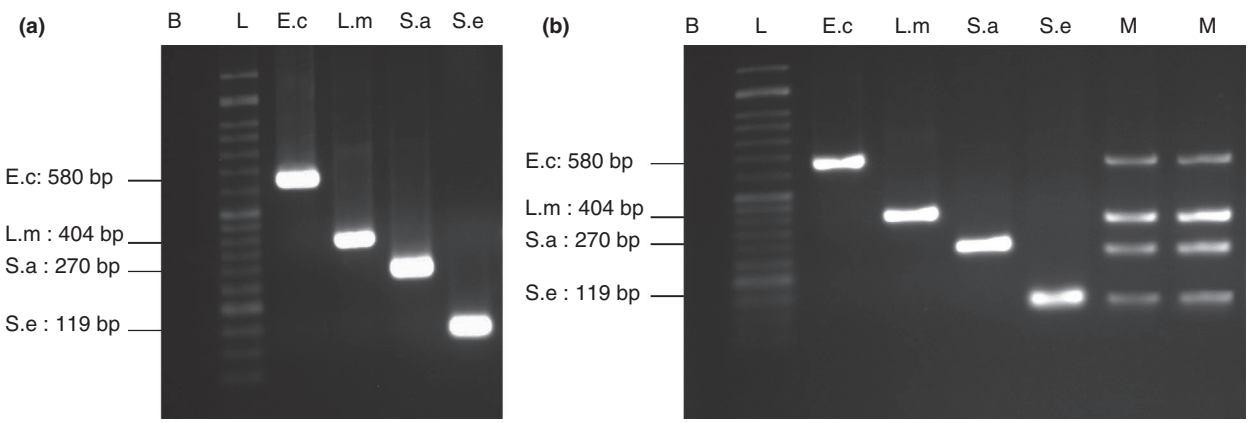

Figure 1 The specificity of the primers by single and multiplex TD-PCR (a) Specificity of the single Touchdown PCR by single primer. (b) Specificity of the multiplex TD-PCR by multiple primers. Target pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica*) were tested individual and multiple using the TD-PCR method. L: 50 bp DNA ladder; M: multiplex TD-PCR; B: blank.

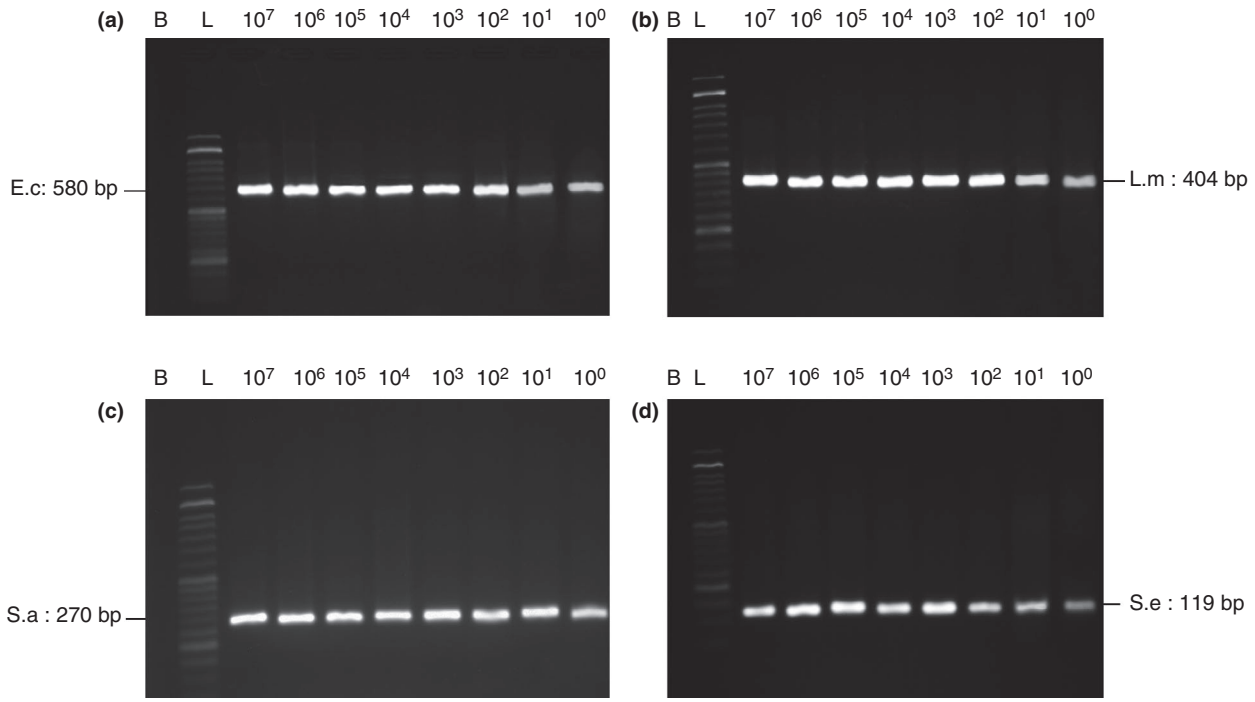

Figure 2 Sensitivity of the single TD-PCR applied to (a) *Escherichia coli* O157:H7 (b) *Listeria monocytogenes* (c) *Staphylococcus aureus* (d) *Staphylococcus enterica*. Serial dilutions (10^7–10^0 cell per ml); L: 50 bp DNA ladder; B: blank.

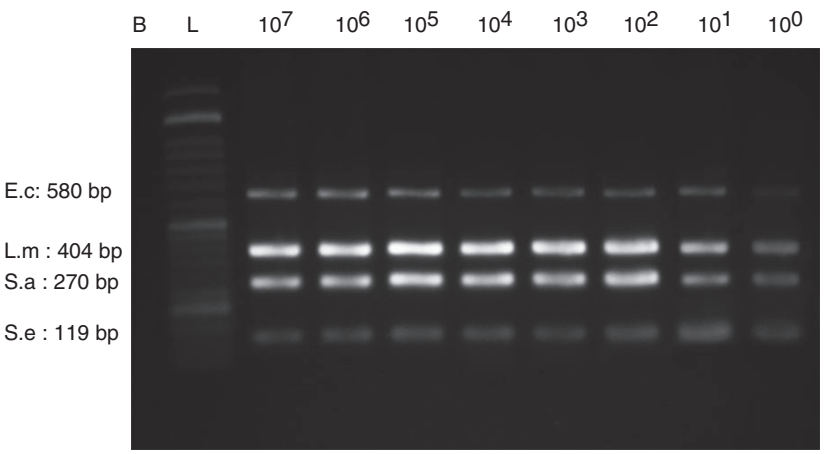

Figure 3 Sensitivity of the multiplex TD-PCR applied to four target bacteria simultaneously. Assessment of multiplex TD-PCR sensitivity obtained from DNA extraction of serial dilutions (10^7–10^0 cell per ml) of the four target pathogens. L: 50 bp DNA ladder; B: blank.

molecular method for replicating DNA; it can produce many copies from a specific part of DNA, swiftly and accurately.

To develop an efficient PCR, several parameters, such as variable concentrations of Mg^{2+}, dNTPs, primers, template, and cycle number should be optimized. Optimizing the number of parameters in the PCR method is laborious and time-consuming. The other method for PCR optimization is touchdown PCR, which uses various annealing temperatures instead of a fixed one. Usage of this method prevents non-specific attachment during the amplification process.

The PCR capacity to detect microorganisms depends on the purity of the template used as the target as well as the presence of an adequate number of target molecules (Estrada *et al.* 2007). Detecting a few foodborne bacteria among the harmless background microflora in a complex matrix of raw milk is extremely challenging. The presence of PCR inhibitors in food samples is a major limitation in PCR-based experiments, which reduces test sensitivity, leading to a false-negative result. Therefore, removing the inhibitory materials and efficient DNA extraction are very essential (Jeníková *et al.* 2000; Fukushima *et al.* 2007).

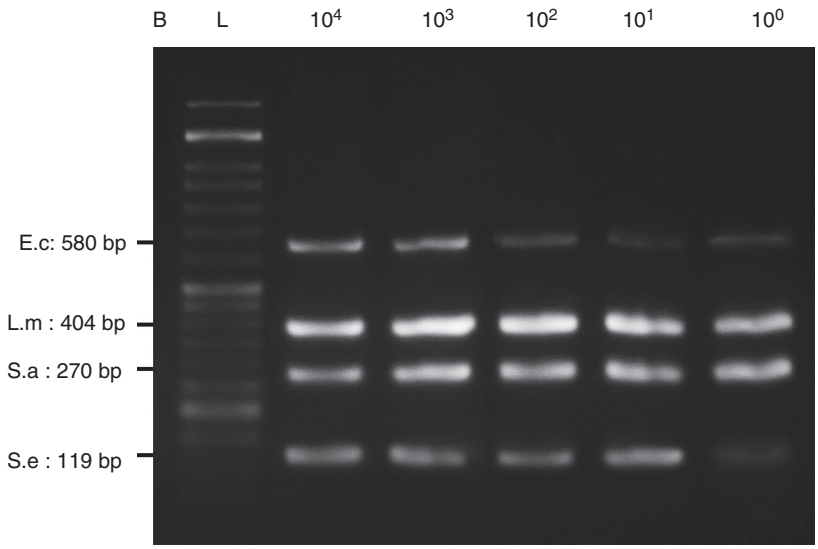

Figure 4 Detection limits of the multiplex TD-PCR for simultaneous detection of four target bacteria from inoculated raw milk. Serial dilutions (10^4–10^0 cell per ml); L: 50 bp DNA ladder; B: blank.

Table 3 Practical application of multiplex TD-PCR and conventional culture in naturally raw milk

	Escherichia coli O157: H7		*Listeria monocytogenes*		*Staphylococcus aureus*		*Salmonella* spp.	
Raw milk number	mTD-PCR	Conventional culture	mTD-PCR	Conventional culture	mTD-PCR	Conventional culture	mTD-PCR	Conventional culture
1	+	–	+	+	+	+	–	–
5	–	–	–	–	+	–	–	–
6	+	+	+	+	+	+	+	+
18	–	–	–	–	+	+	–	–
27	+	+	–	–	+	+	+	+
33	–	–	–	–	+	+	–	–
35	–	–	–	–	–	–	+	+
41	+	+	+	+	+	+	–	–
46	–	–	–	–	–	–	+	+
47	–	–	–	–	+	–	–	–
Total sample	50	50	50	50	50	50	50	50
Positive number (rate)	4 (8%)	3 (6%)	3 (6%)	3 (6%)	8 (16%)	6 (12%)	4 (8%)	4 (8%)

Raw milk is a difficult source to extract DNA with high quality and quantity. Milk protein and fat act as inhibitors and often affect DNA extraction methods. Against all the odds, in this study, we used efficient DNA extraction method to obtain DNA in pure form from raw milk. The centrifugation of raw milk at low speed, prior to DNA extraction might reduce the effect of cream and milk layer inhibitors for PCR.

The following description shows the superior mechanism of the touchdown PCR. In this method, the annealing temperature of the initial cycles is a few degrees higher than melting temperature (T_m) of the primers. Higher temperature minimizes the undesirable amplification and decreases the formation of primer dimers and nonspecific primer–template complexes. Hence, at the start of PCR that promotes specific amplification, it might result in lower PCR yield. To overcome this challenge, the annealing temperature is often decreased by 0·5–1°C at every cycle to reach optimum annealing temperature by producing an acceptable yield of the desired amplicon. Therefore, the products from the initial cycles play the role of target for primers at the subsequent cycles; thus, increasing the amount of yield. In this regard, the desired PCR products will be selectively increased without the enhancement of non-specific targets during PCR cycles (Don *et al.* 1991; Hecker and Roux 1996; Korbie and Mattick 2008; Green and Sambrook, 2018b).

In this study, classical multiplex PCR and multiplex TD-PCR optimization protocol were compared in order

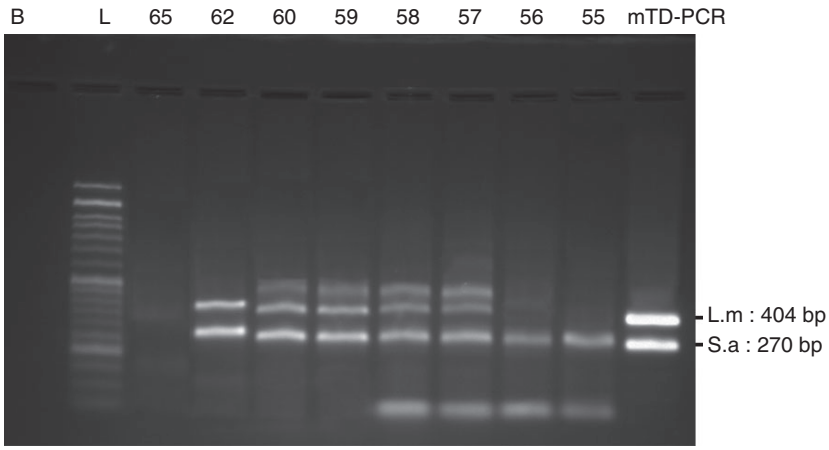

Figure 5 Comparison of multiplex PCR and multiplex TD-PCR protocols in raw milk. Amplicons were generated by multiplex PCR containing 35 cycles at the 8 different annealing temperatures shown above each lane (°C) or by a 35-cycle multiplex TD-PCR. L: 50 bp DNA ladder; mTD-PCR: multiplex TD-PCR; B: blank.

to identify four bacterial pathogens in raw milk samples. An unknown raw milk sample was tested using four specific primers of four target bacteria by two methods of multiplex PCR mentioned above. If this sample of raw milk was infected with all four target bacteria naturally, four amplicons 119, 270, 404 and 580 bp should be created. But we observed that the sample generated just two amplicons 270 and 404 bp which indicates that raw milk was contaminated with two target bacteria naturally. Classic mPCR was performed for eight times at different annealing temperature of 65, 62, 60, 59, 58, 57, 56, and 55°C during 35 cycles. Also, one multiplex TD- PCR was performed from 65 to 55°C temperature for 35 cycles. In classic PCR, low annealing temperature led to mispriming and spurious products, but when 10 out of 35 cycles of the multiplex TD-PCR were performed at annealing temperatures of 55°C, only the target amplicons were observed, clearly demonstrating the advantages of multiplex TD-PCR protocol. The results of this comparison were shown in (Fig. 5) that priming initiated higher than optimum annealing temperature can be an advantage for replicating target amplicons.

The result of the tested raw milk samples showed that the pathogenic contamination of the samples experimented by multiplex TD-PCR was one or two bacteria higher than the conventional culture method. The reason for this difference can be that current molecular methods are not capable of distinguishing DNA from viable and dead cells; consequently, leading to false-positive results (González-Escalona *et al.* 2009; De Medici *et al.* 2015). Also, this might be due to the ability of pre-enrichment media (ELSS) by allowing the recovery of damaged cells. On the other hand, in culture-dependent methods, bacteria that are viable, but noncultured cannot be detected. Another problem of this method is that the interaction of bacteria with competitive bacteria might not lead to the growth of target bacteria (Zoetendal *et al.* 2004; Nocker *et al.* 2007). This explains why in this research the positive detection rate for *E. coli* O157: H7 and *S. aureus* by multiplex TD-PCR was slightly higher than the culture-based method (Table 3).

Conventional multiplex PCR could be applied to detect multiple target organisms in a single tube reaction in order to save time and labour. In a study by Kumar et al., the sensitivity of mPCR was obtained to be 10^3 CFU per ml of *Bacillus cereus*, 10^4 CFU per ml for *S. aureus* and *L. monocytogenes* (Kumar *et al.* 2009). The results by Germini et al. confirmed the detection sensitivity of mPCR to be 10^6 CFU per ml in detecting *E. coli* O157: H7, *Salmonella* spp., and *L. monocytogenes* by multiplex PCR (Germini *et al.* 2009). In a study by Silva et al., the mPCR detection sensitivity was almost 10^3 CFU per ml for *S. enteritidis* and *Salmonella agona* in Peptone Water Phosphate Buffer (Silva *et al.* 2011). Park et al. showed the detection sensitivity of mPCR for simultaneous detection of *Salmonella genus*, *Salmonella* subspecies I, *Salm.* Enteritidis, *Salm.* Heidelberg and *Salm.* Typhimurium was $4{\cdot}6 \times 10^4$ CFU per ml (Park and Ricke 2015). The detection sensitivity of the target-enriched multiplex PCR (Tem-PCR) assay for concurrent detection of *Salmonella*, *S. aureus*, *Shigella, L. monocytogenes and E. coli* O157:H7 from pure cultures and spiked food matrix was 200 CFU per ml (g) for each target (Xu *et al.* 2016). Yu et al. showed that the detection sensitivity of mPCR in pure cultures of *Salmonella*, *S. aureus* and *L. monocytogenes* was 10^3 CFU per ml (Yu *et al.* 2016). In Kim's research, the detection sensitivity of mPCR was 10 cells per ml for simultaneous detection of *L. monocytogenes*, *E. coli* O157: H7, *B. cereus*, *Salmonella* spp. and *S. aureus* in inoculated low-fat milk (Kim *et al.* 2014). Kawasaki et al. showed that the detection sensitivity of mPCR was 5 CFU per ml in 25 g of inoculated food samples for simultaneous detection of *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7 (Kawasaki *et al.* 2009).

Having explained the applied method and the results of this study, it is now possible to make a comparison between the sensitivity of the proposed method and other similar studies as listed above. Conventional multiplex PCR assays have a minimum of 5 CFU per ml sensitivity for simultaneous detection of the four target pathogens. However, in this study greater accuracy was achieved (one cell per ml) by utilizing multiplex TD-PCR optimization method for both single and multiplex TD-PCR. In other words, the proposed protocol is simple, efficient and more sensitive in comparison with the conventional multiplex PCR assays.

In conclusion, since raw milk is often contaminated with *E. coli* O157, *L. monocytogenes*, *S. aureus* and *S. enterica*, raw milk consumption should be considered as a threat to public health. Therefore, finding a rapid and reliable detection method of these foodborne pathogens is imperative. For this reason, we established a multiplex TD-PCR method with prior cultivation. The results confirmed the detection sensitivity of the single and multiplex TD-PCR was one cell per ml in pure culture and detection limit of inoculated raw milk samples, for four target pathogens was as low as one bacterial cell per 25 ml of inoculated raw milk; hence, the proposed multiplex TD-PCR method can be confirmed as an effective way for rapid optimization of PCR reactions to increase specificity, sensitivity during gene amplification. In addition, this protocol can be a reliable method for screening raw milk samples to detect contamination of foodborne pathogens. To the best of our knowledge, this is the first report on the use of multiplex TD-PCR for concurrent detection of foodborne bacteria in food samples. Therefore, we hope for the TD-PCR application to be greater in the near future.

Acknowledgements

The authors are grateful to the Islamic Azad University of Jahrom as well as Food and Drug Control Laboratory of Shiraz for their executive support of this project. The authors thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his cooperation in English editing of this manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

Anonymous, (1998). ISO 11290–2:1998. Microbiology of food and animal feeding stuffs – horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 2: Enumeration method.

Anonymous, (1999). ISO 6888–1: (1999): Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species). Part 1: Technique using Baird-Parker agar medium.

Anonymous, (2002a). ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp.

Anonymous, (2002b). ISO 16654:2002. Microbiology of food and animal feeding stuffs – horizontal method for the detection and enumeration of *Escherichia coli* O157:H7.

Anonymous, (2005) Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. General Requirements and Definitions (EN ISO, 22174).

Aslam, M., Hogan, J. and Smith, K.L. (2003) Development of a PCR-based assay to detect Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in milk. *Food Microbiol* **20**, 345–350.

Brakstad, O.G., Aasbakk, K. and Maeland, J.A. (1992) Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J Clin Microbiol* 1654–1660.

Burnett, S. and Beuchat, L. (2001) Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. *J Ind Microbiol Biotechnol* **27**, 104–110.

Cheng, C.-Y., Huang, M.-J., Chiu, H.-C., Liou, S.-M., Chou, C.-C. and Huang, C.-C. (2012) Simultaneous detection of food pathogens, *Staphylococcus aureus*, *Salmonella enterica*, *Bacillus cereus* and *Vibrio parahaemolyticus* by multiplex real-time polymerase chain reaction. *J Food Drug Anal* **20**, 66–73.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C. and Comi, G. (2002) Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. *Appl Environ Microbiol* **68**, 6273–6282.

D'Aquila, R.T., Bechtel, L.J., Videler, J.A., Eron, J.J., Gorczyca, P. and Kaplan, J.C. (1991) Maximizing sensitivity and specificity of PCR by pre-amplification heating. *Nucleic Acids Res* **19**, 3749.

De Medici, D., Kuchta, T., Knutsson, R., Angelov, A., Auricchio, B., Barbanera, M., Diaz-Amigo, C., Fiore, A., et al. (2015) Rapid methods for quality assurance of foods: the next decade with polymerase chain reaction (PCR)-based food monitoring. *Food Anal Method* **8**, 255–271.

Don, R., Cox, P., Wainwright, B., Baker, K. and Mattick, J. (1991) 'Touchdown'PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* **19**, 4008.

Dwivedi, H.P. and Jaykus, L.-A. (2011) Detection of pathogens in foods: the current state-of-the-art and future directions. *Crit Rev Microbiol* **37**, 40–63.

Erlich, H.A., Gelfand, D. and Sninsky, J.J. (1991) Recent advances in the polymerase chain reaction. *Science* **252**, 1643–1651.

Estrada, C.S.L., del Carmen Velázquez, L., Di Genaro, S. and de Guzmán, A.M.S. (2007) Comparison of DNA extraction methods for pathogenic *Yersinia enterocolitica* detection from meat food by nested PCR. *Food Res Int* **40**, 637–642.

Feng, P. (2007) Rapid methods for the detection of foodborne pathogens: current and next-generation technologies. In *Food Microbiology: Fundamentals and Frontiers* (3rd edn) ed. Doyle, M.P. and Beuchat, L.R. pp. 911–934. Washington, D.C.: ASM Press.

Fukushima, H., Katsube, K., Hata, Y., Kishi, R. and Fujiwara, S. (2007) Rapid separation and concentration of food-borne pathogens in food samples prior to quantification by viable-cell counting and real-time PCR. *Appl Environ Microbiol* **73**, 92–100.

Garrido, A., Chapela, M.-J., Román, B., Fajardo, P., Lago, J., Vieites, J.M. and Cabado, A.G. (2013) A new multiplex real-time PCR developed method for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Control* **30**, 76–85.

Germini, A., Masola, A., Carnevali, P. and Marchelli, R. (2009) Simultaneous detection of *Escherichia coli* O175: H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control* **20**, 733–738.

González-Escalona, N., Hammack, T.S., Russell, M., Jacobson, A.P., De Jesús, A.J., Brown, E.W. and Lampel, K.A. (2009) Detection of live *Salmonella* sp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting invA mRNA. *Appl Environ Microbiol* **75**, 3714–3720.

Green, M.R. and Sambrook, J. (2018a) Hot start polymerase chain reaction (PCR). Cold Spring Harbor Protocols 2018, pdb. prot095125.

Green, M.R. and Sambrook, J. (2018b) Touchdown polymerase chain reaction (PCR). Cold Spring Harbor Protocols 2018, pdb. prot095133.

Hecker, K.H. and Roux, K.H. (1996) High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *Biotechniques* **20**, 478–485.

Henegariu, O., Heerema, N., Dlouhy, S., Vance, G. and Vogt, P. (1997) Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* **23**, 504–511.

Hoorfar, J., Ahrens, P. and Rådström, P. (2000) Automated 5′ nuclease PCR assay for identification of Salmonella enterica. *J Clin Microbiol* **38**, 3429–3435.

Jahan, M., Rahman, M., Parvej, M.S., Chowdhury, S.M.Z.H., Haque, M.E., Talukder, M.A.K. and Ahmed, S. (2015) Isolation and characterization of Staphylococcus aureus from raw cow milk in Bangladesh. *J Adv Vet Anim Res* **2**, 49–55.

Jeníková, G., Pazlarova, J. and Demnerová, K. (2000) Detection of Salmonella in food samples by the combination of immunomagnetic separation and PCR assay. *Int Microbiol* **3**, 225–229.

Kawasaki, S., Fratamico, P.M., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T. and Kawamoto, S. (2009) Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157: H7 in foods and in food subjected to freezing. *Foodborne Pathog Dis* **6**, 81–89.

Kim, J.-H., Rhim, S.-R., Kim, K.-T., Paik, H.-D. and Lee, J.-Y. (2014) Simultaneous detection of *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Bacillus cereus*, *Salmonella* spp., and *Staphylococcus aureus* in low-fatted milk by multiplex PCR. *Korean J Food Sci Anim* **34**, 717.

Korbie, D.J. and Mattick, J.S. (2008) Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat Protoc* **3**, 1452.

Kumar, T.K., Murali, H. and Batra, H. (2009) Simultaneous detection of pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* by multiplex PCR. *Indian J Microbiol* **49**, 283–289.

Luo, Y., Du, P., Zhao, J., Duan, X., Hou, Y., Pan, H. and Shao, S. (2012) A multiplex touchdown PCR for detection of *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and Mycobacterium tuberculosis complex in sputum samples. *Trop Biomed* **29**, 422–428.

Margot, H., Stephan, R., Guarino, S., Jagadeesan, B., Chilton, D., O'Mahony, E. and Iversen, C. (2013) Inclusivity, exclusivity and limit of detection of commercially available real-time PCR assays for the detection of *Salmonella*. *Int J Food Microbl* **165**, 221–226.

McLauchlin, J. (1996) The relationship between Listeria and listeriosis. *Food Control* **7**, 187–193.

Nocker, A., Burr, M. and Camper, A.K. (2007) genotypic microbial community profiling: a critical technical review. *Microb Ecol* **54**, 276–289.

Oberst, R., Hays, M., Bohra, L., Phebus, R., Yamashiro, C., Paszko-Kolva, C., Flood, S., Sargeant, J. et al. (1998) PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157: H7 with an internal fluorogenic probe and the 5′ nuclease (TaqMan) assay. *Appl Environ Microbiol* **64**, 3389–3396.

Oliver, S.P., Jayarao, B.M. and Almeida, R.A. (2005) Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathog Dis* **2**, 115–129.

Park, S. and Ricke, S. (2015) Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm*. Enteritidis, *Salm*. Heidelberg and *Salm. Typhimurium*. *J Appl Microbiol* **118**, 152–160.

Paton, J.C. and Paton, A.W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev* **11**, 450–479.

Quero, G.M., Santovito, E., Visconti, A. and Fusco, V. (2014) Quantitative detection of *Listeria monocytogenes* in raw

milk and soft cheeses: culture-independent versus liquid- and solid-based culture-dependent real time PCR approaches. *LWT – Food Sci Technol* **58**, 11–20.

Robinson, R.K. (2014) *Encyclopedia of food microbiology* (2nd edn). Cambridge: Academic Press.

Roux, K.H. (2009) Optimization and troubleshooting in PCR. Cold Spring Harbor Protocols 2009, pdb. ip66.

Schlosser, W., Hogue, A., Ebel, E., Rose, B., Umholtz, R., Ferris, K. and James, W. (2000) Analysis of Salmonella serotypes from selected carcasses and raw ground products sampled prior to implementation of the pathogen reduction: hazard analysis and critical control point final rule in the US. *Int J Food Microbiol* **58**, 107–111.

Silva, D.S.P., Canato, T., Magnani, M., Alves, J., Hirooka, E.Y. and de Oliveira, T.C.R.M. (2011) Multiplex PCR for the simultaneous detection of *Salmonella* spp. and *Salmonella* Enteritidis in food. *Int J Food Sci Technol* **46**, 1502–1507.

Sulaiman, I.M. and Hsieh, Y.-H. (2017) Foodborne pathogens in milk and dairy products: genetic characterization and rapid diagnostic approach for food safety of public health importance. In *Dairy in Human Health and Disease Across the Lifespan* ed. Watson, R.R., Collier, R.J. and Preedy, V.R. pp. 127–143. Amsterdam, the Netherlands: Elsevier.

Wang, S., Duan, H., Zhang, W. and Li, J.-W. (2007) Analysis of bacterial foodborne disease outbreaks in China between 1994 and 2005. *FEMS Immunol Med Microbiol* **51**, 8–13.

Willis, C., Jørgensen, F., Aird, H., Elviss, N., Fox, A., Jenkins, C. and McLauchlin, J. (2018) An assessment of the microbiological quality and safety of raw drinking milk on retail sale in England. *J Appl Microbiol* **124**, 535–546.

Wu, S.-J., Chan, A. and Kado, C.I. (2004) Detection of PCR amplicons from bacterial pathogens using microsphere agglutination. *J Microbiol Methods* **56**, 395–400.

Xu, Y.-G., Liu, Z.-M., Zhang, B.-Q., Qu, M., Mo, C.-S., Luo, J. and Li, S.-L. (2016) Development of a novel target-enriched multiplex PCR (Tem-PCR) assay for simultaneous detection of five foodborne pathogens. *Food Control* **64**, 54–59.

Yoon, J.-H., Wei, S. and Oh, D.-H. (2018) A highly selective enrichment broth combined with real-time PCR for detection of *Staphylococcus aureus* in food samples. *LWT – Food Sci Technol* **94**, 103–110.

Yu, Q., Zhai, L., Bie, X., Lu, Z., Zhang, C., Tao, T., Li, J., Lv, F. et al (2016) Survey of five food-borne pathogens in commercial cold food dishes and their detection by multiplex PCR. *Food Control* **59**, 862–869.

Zeng, D., Chen, Z., Jiang, Y., Xue, F. and Li, B. (2016) Advances and challenges in viability detection of foodborne pathogens. *Front Microbiol* **7**, 1833.

Zoetendal, E.G., Collier, C.T., Koike, S., Mackie, R.I. and Gaskins, H.R. (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. *J Nutr* **134**, 465–472.