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ORIGINAL ARTICLE

Simultaneous detection of *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* by multiplex PCR in milk

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Abstract

Escherichia coli O157:H7, *Staphylococcus aureus*, and *Salmonella* are food-borne pathogens that cause serious gastrointestinal illness and frequent food safety accidents. This study aimed to develop a practical multiplex polymerase chain reaction (mPCR) technique for the simultaneous detection of these food-borne pathogens in culture broth and artificial food matrix. Pathogen-specific DNA sequences in the *rfb*E, *nuc*, and *inv*A genes were used as targets to design primers for the identification of *E. coli* O157:H7, *S. aureus*, and *Salmonella,* respectively. As expected, the method produced species-specific bands of amplified products without any contaminating non-specific bands. The highest species specificity was established with primer concentrations of 0.1, 0.2, and 0.4 µM for *E. coli* O157:H7, *S. aureus*, and *Salmonella*, correspondingly. The detection sensitivity of this assay was 10^3 CFU/mL in culture broth, and the limit of detection was consistent with singleplex PCR in the food sample. The mPCR assay proposed here is an easy and convenient detection method, which will be valuable for microbial epidemiology and food safety investigations.

Keywords Multiplex PCR · *Escherichia coli* O157:H7 · *Staphylococcus aureus* · *Salmonella* · Detection method

Introduction

In recent years, the situation of food safety has become critical and with the frequent emergence of various types of food safety incidents, it is important to find a solution to resolve those global problems. Among food safety incidents, food-borne pathogens are an important component of food safety, as they increase security risks and are of tremendous threat to people's health and well-being (Zhao et al. 2017a). Therefore, the identification and control of food-borne pathogens are essential for food safety. *Escherichia coli* O157:H7 (Cowley et al. 2016; Poolman and Wacker 2016), *Staphylococcus aureus* (Zhao et al. 2016; Miao et al. 2016), and *Salmonella* (Wallace et al. 2014) are regarded as the three most important food-borne pathogens that are found in many foods and are also the cause of frequent food poisoning (Zhao et al. 2017b). The effective prevention and control of food-borne diseases depends on the accuracy of the detection technology. The current routine detection of food-borne pathogens at an international or national level still relies on the traditional microbiological techniques of separation, cultivation, and biochemical identification of the pathogens. However, the conventional methods have difficulties in meeting the challenges and demands of the new enterprises and the requirements of the related regulatory authorities for rapid and accurate detection of these pathogens (Zhao et al. 2013a; Li et al. 2017; Zhong and Zhao 2017). Therefore, there is some urgency in establishing a rapid, accurate, and efficient method for the detection of these food-borne pathogens.

Compared with the classical microbiological methods, polymerase chain reaction (PCR) is the most widely applied technology to date due to its advantages of short analysis time, low detection limit, and high specificity and automation potential (Forghani et al. 2016; Jiyeon et al. 2010; Löfström et al. 2010; Cantekin et al. 2015). To further increase the advantages of PCR, multiplex polymerase chain reaction (mPCR) has been developed to detect several pathogens together. Multiplex PCR

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technology is based on the PCR technology, which can amplify multiple fragments in a unique reaction system (Elizaquível and Aznar 2008; Kawasaki et al. 2010; Singh et al. 2012). Although the development of PCR is relatively mature, research on mPCR is still being conducted (Ertas et al. 2010; Pelisser et al. 2009). The objective of this study was to develop mPCR method for the detection of *E. coli* O157:H7, *S. aureus*, and *Salmonella* that can be applied to food samples and reduce the potential hazards of these harmful pathogens.

Materials and methods

Bacterial strain

Reference strains of *Salmonella* (ATCC 13076), *S. aureus* (ATCC 27664), and enterohemorrhagic *E. coli* O157:H7 (ATCC 47895) were used as positive controls in this study. *E. coli* (non-O157:H7, ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Listeria monocytogenes* (ATCC 15313, 19115, 19119, 19111, 19113), *Bacillus cereus* (ATCC 13061, 11778, 12480, 14579, 25621, 53522), and *Vibrio parahaemolyticus* (ATCC 17802) were utilized as negative controls. All the strains (Table 2), obtained from the American Type Culture Collection (ATCC, USA), were preserved at − 20 °C in our laboratory until use.

Primers

The gene targets chosen were the *inv*A gene for *Salmonella.*, the *nuc* gene for *S. aureus*, and the *rfb*E gene for *E. coli* O157:H7, since they were described in the recent literature as being among the most specific and reliable genetic targets for the considered microorganisms. The primer sequences employed for the detection of the three test organisms are presented in Table 1. All primers were synthesized by Tianyi Biotech (Wuhan, China).

DNA template extraction

The strains were inoculated on Luria–Bertani (LB) broth solid medium at 37 °C for 24 h and then cultured in LB liquid medium at 37 °C with 190 rpm for 12 h. Genomic DNA was extracted by thermal lysis according to the procedures described in previous research (Zhao et al. 2013b; Sun et al. 2014). Briefly, 1 mL of the cell suspension was centrifuged at 10,000×*g* for 5 min. Then, the supernatant was removed, the cell pellets were washed three times with physiological saline, followed by centrifugation at 10,000×*g* for 5 min. Further, the pelleted cells were used for DNA extraction. The cells suspended with Tris–EDTA Triton buffer solution were boiled in a water bath for 10 min, followed by immediate immersion in ice, where they remained for 10 min, and centrifugation at 10,000×*g* for 10 min. The supernatant containing DNA was treated as a template.

Reaction system and conditions

The PCR was performed in a final volume of the reaction mixture of 25 µL. The following master mix composition was used: 0.5 µL of TaKaRa Taq™ DNA polymerase (5 U/µL), 2.5 µL of TaKaRa Taq™ 10× PCR buffer (Mg^{2+} plus), 2.0 µL of dNTP mixture (2.5 mmoL/L), 1 µL of DNA templates, 1 µL of upstream primers, and 1 µL of downstream primers (TaKaRa Biotech, Dalian, China). Then, the volume was filled up to 25 µL with ultra-pure water. The PCR amplification was performed with an initial denaturation at 94 °C for 5 min, and then 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s, followed by 10 min of final extension at 72 °C in a Life Pro Thermal Cycler (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China). Upon completion of the reaction, all PCR amplification products were detected by 2% agarose gel electrophoresis followed by imaging using the BD-3000 Gel Image Analysis system (Beijing QHBODA Technology Co., Ltd., Beijing, China). According to the protocol detailed in previous investigations (Zhang et al. 2014), the optimal PCR conditions were determined by optimization of

Table 1 Sequences and parameters of primers

Microorganism	Target gene	Primer sequence (5′–3′)	G+C (%)	Tm (°C)	Amplicon length (bp)	References
E. coli O157:H7 (ATCC 47895)	*rfb*E	F:GCCACCCCCATTTTCGTTG R:TCCTCTCTTTCCTCTGCGGT	57.9 47.4	63.2 51.7	601	In this work
S. aureus (ATCC 27664)	*nuc*	F:TACAGGTGACTGCGGGCTTATC R:CTTACCGGGCAATACACTCACTA	50 45.4	60.2 58.3	484	Xu et al. (2006)
Salmonella (ATCC 13076)	*inv*A	F:CTTTAGCCAAGCCTTGACGAAC R:AAAGGCAATACGCAAAGAGGT	54.5 47.8	62.1 60.6	284	Xu et al. (2006)

the ratio of the three bacterial primer concentrations and the annealing temperature of PCR. To optimize the conditions, the reactions were designed with different temperatures and primer concentration ratios.

Specificity of mPCR

To verify the specificity of target primers, three pairs of primers with random combinations of DNA templates (*E. coli* O157:H7, *S. aureus*, and *Salmonella*) were added into the reaction mixture and were amplified by the optimized mPCR conditions. Meanwhile, the negative controls in mPCR reaction were also subjected to evaluation.

Sensitivity of mPCR

The sensitivity of the mPCR assay was tested using serial dilutions of each overnight-grown bacterial liquid culture. *E. coli* O157:H7 (ATCC 47895), *S. aureus* (ATCC 27664) and *Salmonella* (ATCC 13076) were incubated in fresh sterile nutrient broth medium at 37 °C for 12 h with shaking. The three bacterial numbers of colonies were, respectively, confirmed to be 4.36×10^9, 2.14×10^9, and 3.6×10^9 CFU/mL by the plate counting method. All target bacterial cell suspensions were diluted with normal saline ten times, ranging from 10^9 to 10^1 CFU/mL, and DNA was extracted using the above-mentioned method, subsequently tested by the optimized mPCR conditions.

Analysis of food artificially contaminated with *E. coli* O157:H7, *S. aureus*, and *Salmonella*

Each overnight-grown bacterial liquid culture was subjected to counting by the method of enumeration of microorganisms. The quantity of *E. coli* O157:H7 was approximately 5.1×10^8 CFU/mL, of *S. aureus* was about 3.0×10^8 CFU/mL, and of *Salmonella* was around 2.6×10^8 CFU/mL. Then, dilution was performed with physiological saline gradient. Contamination of aseptic deluxe pure milk (Inner Mongolia Mengniu Dairy Co., Ltd. Inner Mongolia, China) was performed with bacterial concentrations ranging from 10^1 CFU/mL to 10^8 CFU/mL. All samples were subjected to DNA extraction and mPCR sensitivity tests.

Results and discussion

Specificity of singleplex PCR

Details on the specificity of single PCR are presented in Fig. 1 and Table 2. The amplified fragment of *E. coli* O157:H7 between 500 and 750 bp was 601 bp, of *S. aureus* near 500 bp was 484 bp, and of *Salmonella* within 250–500 bp was 284 bp (Fig. 1). As can be seen from Table 2, *P. aeruginosa*, *L. monocytogenes*, and other non-target strains had no non-specific amplified bands of the target gene of *rfb*E, *nuc*, and *inv*A. Each PCR amplification reaction generated a unique DNA fragment with the expected size, without producing PCR products from

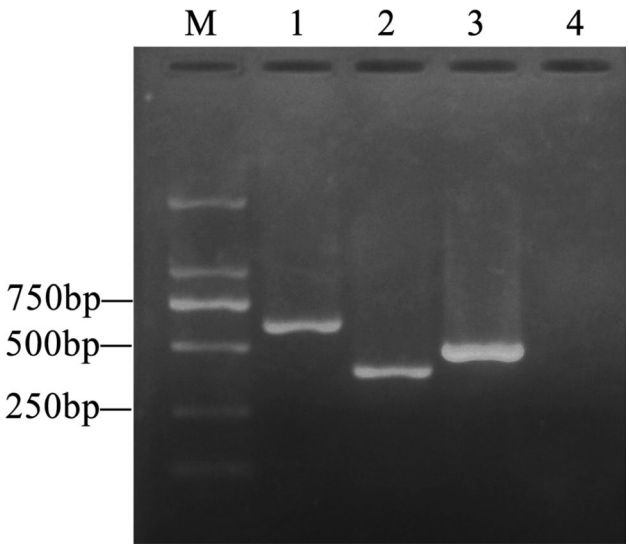

Fig. 1 Agarose gel electrophoresis of simplex PCR products. M: DL 2000 DNA marker; lane 1–4 are *E. coli* O157:H7, *S. aureus*, *Salmonella* spp., and negative control, respectively

Table 2 Experimental bacteria and primer specificity verification

Bacteria	Origin ID (strain ID)	Target gene		
		*rfb*E	*nuc*	*inv*A
E. coli O157:H7	ATCC 47895	+	−	−
S. aureus	ATCC 27664	−	+	−
Salmonella enteritidis	ATCC 13076	−	−	+
P. aeruginosa	ATCC 27853	−	−	−
L. monocytogenes	ATCC 15313			
L. monocytogenes	ATCC 19115	−	−	−
L. monocytogenes	ATCC 19119	−	−	−
L. monocytogenes	ATCC 19111	−	−	−
L. monocytogenes	ATCC 19113	−	−	−
E. coli (non O157:H7)	ATCC 25922	−	−	−
B. cereus	ATCC 13061	−	−	−
B. cereus	ATCC 11778	−	−	−
B. cereus	ATCC 12480	−	−	−
B. cereus	ATCC 14579	−	−	−
B. cereus	ATCC 25621	−	−	−
B. cereus	ATCC 53522	−	−	−
V. parahaemolyticus	ATCC 17802	−	−	−

Result (+/−) indicates positive and negative signals

non-target species, which indicated that each primer set was highly species specific.

Reaction conditions of the mPCR assay

Through the optimization of multiple PCR annealing temperatures, we obtained finally the optimal annealing temperature of 57 °C. The optimized reaction conditions were as follows: pre-denaturation at 94 °C for 5 min, denaturalization at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, 30 cycles of amplification, and a final extension at 72 °C for 10 min. Meanwhile, three types of bacterial target primers of concentration ratio were explored and repeatedly optimized by the optimization of multiple PCR (Fig. 2). As can be seen from the figure, optimal results of the mPCR assay were obtained at primer concentrations of *E. coli* O157:H7, *S. aureus*, and *Salmonella* of 0.1, 0.2, and 0.4, correspondingly. In contrast, under the conditions of the other primer concentrations, not all respective fragments were completely amplified, because of the competition between the three pairs of primers, which affected the PCR amplification results.

Specificity and sensitivity of mPCR

To evaluate the specificity, each reaction mixture, including three pairs of primers with random combinations of DNA templates, was amplified using the optimized mPCR conditions. In Fig. 3a, it can be observed that each primer could amplify the respective fragment only with the target template. The results of sensitivity of mPCR are illustrated in Fig. 3b. As determined by the agarose gel electrophoresis, the sensitivity of the detection limit achieved was 10^3 CFU/mL for the three bacteria. These results are in agreement with the marginal capacity of the PCR detection method.

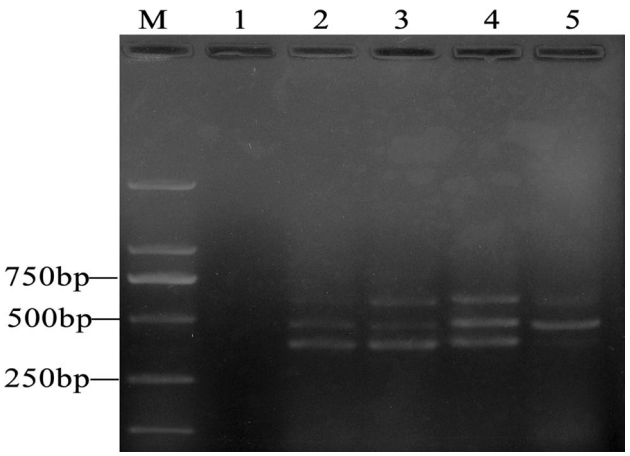

Fig. 2 The optimization of primer concentration ratio. M: DL 2000 DNA marker; the optimization of the primer concentration of *E. coli* O157:H7, *S. aureus*, and *Salmonella* (μM/L) were as follows: 1: 0.08, 0.16, 0.08; 2: 0.1, 0.2, 0.1; 3: 0.4, 0.8, 0.4; 4: 0.1, 0.2, 0.4; 5: 0.1, 0.2, 0.3

Testing of artificially contaminated food produce

The PCR in our examination was so sensitive that the presence of other macromolecules (such as fat, protein, and carbohydrates) in the reaction system might have obstructed the amplification reaction of the target sequences. We found that the complex matrix macromolecules available in the reaction system acted as inhibitors that reduced the sensitivity of PCR. Since more interference factors exist in the complex food matrix composition that influences the detection of the target bacteria, in this study, we used mPCR and singleplex PCR for identification of the artificially contaminated samples. Food samples consisting of aseptic milk, contaminated with *E. coli* O157:H7, *S. aureus*, and *Salmonella*, were utilized in this investigation. The results presented in Fig. 4 indicate the limits for detection of *E. coli* O157:H7, *S. aureus*, and *Salmonella* in the aseptic milk by singleplex PCR: 10^4, 10^5 and 10^4 CFU/mL, respectively. Our findings also indicate that the detection of *S. aureus* was not characterized by higher sensitivity than that of *E. coli* O157:H7 and *Salmonella* in the artificially contaminated food product. The incomplete release of plasmid DNA during the DNA extraction process could be due to the fact that *S. aureus* is a Gram-positive bacterium with thick and tough cell walls. As outlined earlier, the sensitivity of the mPCR assay was evaluated using aseptic milk artificially contaminated with three pathogenic bacteria, which is schematically depicted in Fig. 4d. Although the composition of the food matrix was more complex than that of the pure culture system, the detection sensitivity for the artificially simulated food contamination was slightly lower than that for the pure medium utilized in this experiment. In addition, the mPCR sensitivity was consistent with that of the singleplex PCR performed in aseptic milk. The results obtained clearly suggest that mPCR has good practicability, reliability, and excellent potential for the detection of contamination of food samples.

On the whole, *E. coli* O157:H7, *S. aureus*, and *Salmonella* are the most common causative agents of bacterial food poisoning outbreaks that cause millions of cases of human illness every year, resulting in major public health issues and substantial economic burden (Vugia et al. 2010). Therefore, rapid and accurate pathogen detection methods are required for the reduction of the effects of microbial risk factors. Conventional detection methods are based on selective plating combined with immunological or biochemical identification, which necessitates long time and high labor intensity (Abubakar et al. 2007). PCR assays using specific primers have been indicated to be efficient for the detection of pathogenic bacteria in food products (Lee et al. 2008). They are simpler and quicker than conventional methods.

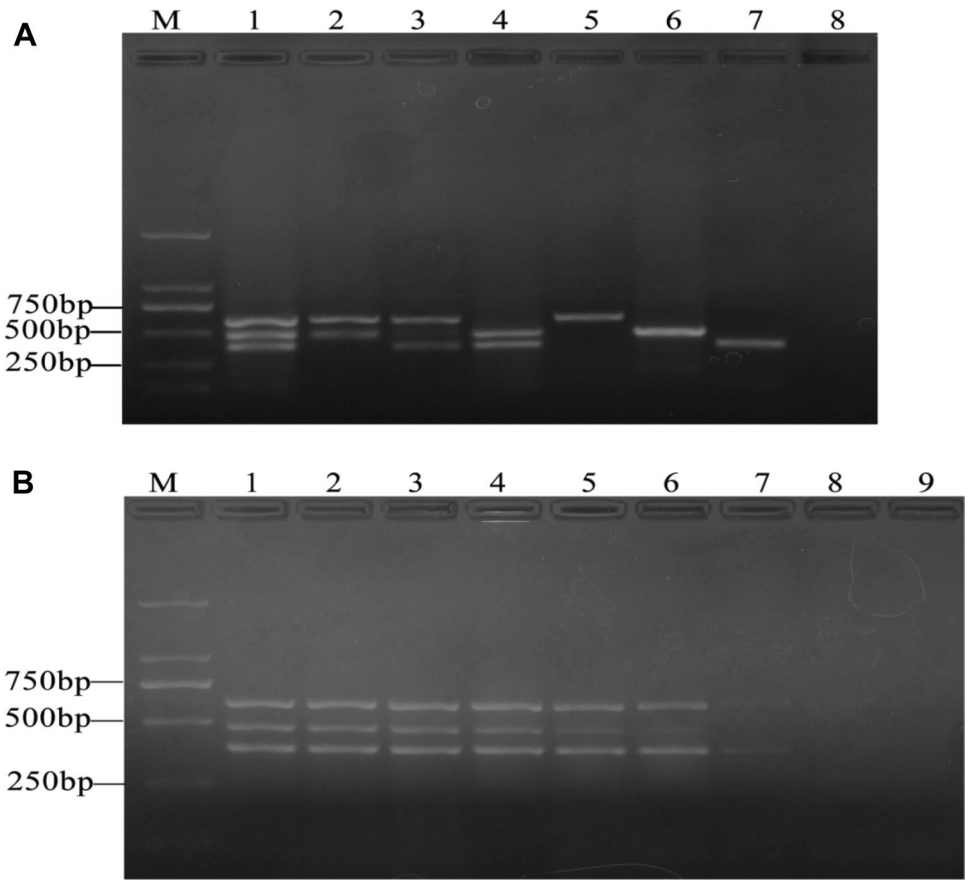

Fig. 3 The specificity and sensitivity of mPCR. **a** The specificity test of the mPCR. M: DL 2000 DNA marker; lane 1: *E. coli* O157:H7, *S. aureus*, *Salmonella*; lane 2: *E. coli* O157:H7, *S. aureus*; lane 3: *E. coli* O157:H7, *Salmonella*; lane 4: *S. aureus*, *Salmonella*; lane 5: *E. coli* O157:H7; lane 6: *S. aureus*; lane 7: *Salmonella*; lane 8: negative control. **b** The sensitivity test of the mPCR. M: DL 2000 DNA marker; lane 1–9, varying concentrations of bacterial suspension (10^9,10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL)

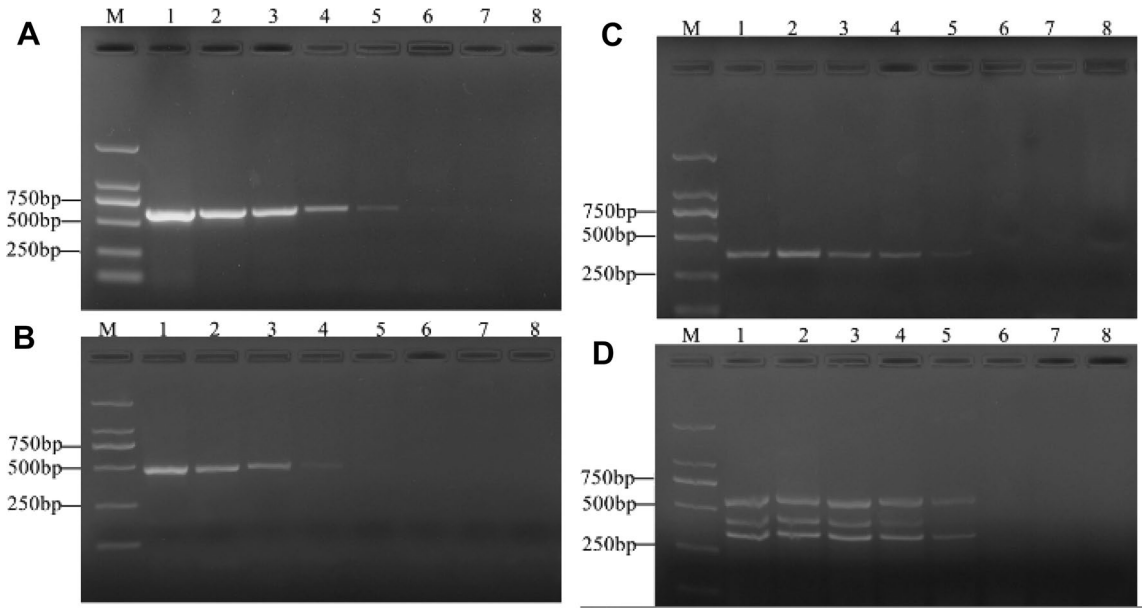

Fig. 4 Sensitivity of PCR in milk. **a** Sensitivity of PCR procedures for detecting *E. coli* O157:H7 in milk. M: DL 2000 DNA marker; lane 1–8, varying concentrations of bacterial suspension (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL); **b** sensitivity of PCR procedures for detecting *S. aureus* in milk. M: DL 2000 DNA marker; lane 1–8, varying concentrations of bacterial suspension (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL); **c** sensitivity of PCR procedures for detecting *Salmonella* in milk. M: DL 2000 DNA marker; lane 1–8, varying concentrations of bacterial suspension (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL); **d** sensitivity of mPCR in food sample. M: DL 2000 DNA marker; lane 1–8, varying concentrations of bacterial suspension (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL)

However, single-species PCR systems are laborious and logistically complex. At the same time, some researchers found that mPCR, used for the identification of pathogens isolated by conventional methods, had the potential to be used in routine diagnostic laboratories and ensured the obtaining of presumptive positive results (Saeki et al. 2013). Owing to the simultaneous amplification of more than one locus in a single reaction tube, mPCR has been identified as a rapid and convenient screening assay, with both clinical and research applications (Park et al. 2006). In this paper, the setting of mPCR method for the simultaneous detection of *E. coli* O157:H7, *S. aureus*, and *Salmonella* has been described and tested. The selection of pathogen-specific target genes and the design of compatible PCR primers are critical steps to achieving high detection specificity and avoidance of the competition in the multiplex target amplifications (Suo et al. 2010). Thus, on the basis of literature search and sequence homology analyses, in this study, we selected the *rfb*E, *nuc*, and *inv*A genes as the target sequences for the detection of *E. coli* O157:H7, *S. aureus*, and *Salmonella,* respectively. In an earlier examination, Park et al. used *Stx2*A, the specific primer for *E. coli* O157:H7; *Its*, the specific primer for *Salmonella*; *Cap8*A-B, the specific primer for *S. aureus*; and *Hly* as the specific primer for *L. monocytogenes* mPCR to detect the pathogen DNA, the concentrations of which ranged from approximately 0.45 to 0.05 pM/μL (Park et al. 2006). Germini et al. (2009) reported that the mPCR assay was employed for the specificity and sensitivity detection of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in liquid whole egg, and the limit of detection could be decreased to 10 cells/25 g. Our results showed that the mPCR assay was able to successfully detect *E. coli* O157:H7, *S. aureus*, *Salmonella* in both culture broth and a model food system. The limit of detection of those three pathogenic bacteria achieved was 10^3 CFU/mL in the culture broth, whereas it was 10^4 CFU/mL in the model food system. The sensitivity difference may be due to the different composition of the complex food matrix from that of the culture broth. In contrast, these detection sensitivity levels are consistent with the ones obtained by PCR for *E. coli* O157:H7, *S. aureus*, and *Salmonella* in the complex food matrix. The sensitivity for detection of *S. aureus* was lower than that for detection of *E. coli* O157:H7 and *Salmonella*. This result can be explained by the fact that *S. aureus* is a Gram-positive bacterium that could not completely release its plasmid DNA during the DNA extraction process. In this study, we chose to use the thermal lysis method to extract DNA because the boiling method saved time and eliminated the need for intensive labor (Zhang et al. 2014), although the microbial DNA isolation kit can be useful in reducing PCR inhibitors in pure bacterial cultures or food matrices (Yang et al. 2012). In addition, the newly developed mPCR system effectively eliminated the inhibitors by the re-suspension of the DNA samples from the boiled bacteria after the template dilution (Ceuppens et al. 2010). Nevertheless, the present method has some shortcomings, and its sensitivity could be further improved using various approaches. However, it is encouraging that the typical amounts of DNA used in any PCR appear to be sufficient for this type of detection. In addition, the sensitivity levels of mPCR were consistent with those for *E. coli* O157:H7, *S. aureus*, and *Salmonella* established in complex food matrix by PCR. In other words, the mPCR detection system has good stability and sensitivity.

In conclusion, a rapid and sensitive mPCR assay was developed to simultaneously detect *E. coli* O157:H7, *S. aureus*, and *Salmonella*, which was performed on a complex food matrix. The use of this method for detection of the here-studied three food-borne pathogens in food matrices is rapid and reliable. It is also an easy and convenient test that can be used for improvements in food sanitation and the maintenance of adequate food safety. Furthermore, it is likely to become a potential and valuable tool for the rapid identification of food-borne pathogens utilized by the personnel of food-monitoring agencies and commercial enterprises, and would potentially facilitate the further development of international trade.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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