Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens

J. Chen, J. Tang, J. Liu, Z. Cai and X. Bai

College of Life Science and Technology, Southwest University for Nationalities, Chengdu, China

Keywords
detection method, enrichment, foodborne pathogens, multiplex PCR.

Correspondence
Junni Tang, College of Life Science and Technology, Southwest University for Nationalities, Chengdu 610041, China. E-mail: junneytang@yahoo.com.cn
Juan Chen and Junni Tang contributed equally to this work.

2011/1683: received 2 October 2011, revised 15 January 2012 and accepted 15 January 2012

doi:10.1111/j.1365-2672.2012.05240.x

Abstract

Aims: To develop a rapid multiplex PCR method for simultaneous detection of five major foodborne pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* Enteritidis and *Shigella flexneri*, respectively).

Methods and Results: Amplification by PCR was optimized to obtain high efficiency. Sensitivity and specificity assays were investigated by testing different strains. With a multipathogen enrichment, multiplex PCR assay was able to simultaneously detect all of the five organisms in artificially contaminated pork samples. The developed method was further applied to retail meat samples, of which 80% were found to be positive for one or more of these five organisms. All the samples were confirmed by traditional culture methods for each individual species.

Conclusions: This study reported a rapid multiplex PCR assay using five primers sets for detection of multiple pathogens. Higher consistency was obtained between the results of multiplex PCR and traditional culture methods.

Significance and Impact of the Study: This work has developed a reliable, useful and cost-effective multiplex PCR method. The assay performed equally as well as the traditional cultural method and facilitated the sensitive detection both in artificially contaminated and naturally contaminated samples.

Introduction

Foodborne pathogens pose a significant threat to human public health, leading to a substantial economic burden both in developed and less developed countries. Among the foodborne bacteria currently observed in a wide range of food products, *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Shigella flexneri* are frequently reported as the causative agents in food poisoning.

Salmonella is responsible for infection of the intestinal epithelium, known as 'salmonellosis'. *Salmonella* Enteritidis strains, the most often isolated *Salmonella* serovar, are one of the important agents of foodborne illness in humans (De Freitas *et al.* 2010). Enterohaemorrhagic *E. coli* (EHEC) strains, a subset of Shiga toxin-producing *E. coli* (STEC) strains, are responsible for haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans (Yoon and Hovde 2008). Among serogroup members of the EHEC, *E. coli* strain of the O157 serogroup is responsible for the most severe cases of these diseases (Kaper *et al.* 2004). *Listeria monocytogenes*, which can be widely distributed in various foodstuffs, is a significant foodborne pathogen. Listeriosis has a high mortality rate (Mook *et al.* 2011) and is caused by the ingestion of *L. monocytogenes*-contaminated foods (Gasanov *et al.* 2004). *Staphylococcus aureus* causes a variety of diseases, ranging from simple skin infections to life-threatening diseases including food poisoning, cutaneous infections, endocarditis, pneumonia, septic arthritis and osteomyelitis (Pragman and Schliever 2004; Tang *et al.* 2006). In addition to these four pathogens, *Shigella* is another concern in raw and ready-to-eat foods, which annually cause an estimated 164·7 million cases of shigellosis worldwide,

resulting in 1·1 million deaths (Vu *et al.* 2004). Thus, rapid and specific methods to detect these five pathogens are necessary.

Traditional detection methods depend upon selective cultivation techniques combined with standard biochemical identifications. These methods are time-consuming and laborious and introduce sampling and enumeration errors, as these pathogenic bacteria occur in low numbers. In fact, the low-throughput of these traditional methods does not allow rapid screening of large numbers of food samples for the presence of one or more pathogens (Abubakar *et al.* 2007). In the last 10 years, rapid nucleic acid amplification and detection technologies have been increasingly applied to pathogen detection in food industry. Recently, the real-time polymerase chain reaction has successfully been performed to identify pathogens in various food products. However, these methods are not only very expensive for routine use in common testing laboratories, but also limited to two or three different types of pathogenic bacteria per detection assay (Wang *et al.* 2007; Elizaquível and Aznar 2008; Suo *et al.* 2010). In fact, the real-time PCR approach is still uncommon. Multiplex PCR simultaneously detecting several pathogens in a single-tube reaction has the potential of saving time and effort, lowering testing-related laboratory costs (Perry *et al.* 2007). The objective of the present study was to develop a reliable and effective multiplex PCR assay to simultaneously detect five different foodborne pathogens (*Staph. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salm. enterica* and *Sh. flexneri*). The application and efficacy of this method for pathogen detection were also evaluated in meat products.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. Some of the reference strains were purchased from the China Center of Industrial Culture Collection (CICC), and some were collected and preserved in our laboratory. Different isolated strains came from food samples were identified and preserved in our laboratory. Bacterial strains were propagated and maintained on trypticase soy agar (TSA) (Scharlau Chemie, Barcelona, Spain) for 18 h at 37°C. Fresh liquid cultures were prepared in Luria–Bertani broth (LB) (Oxoid, Basingstoke, UK) for 18 h at 37°C with agitation at 150 rev min^{-1}. Plate count method was used to measure the level of colony-forming unit (CFU) of each bacterium. Artificially contaminated samples and naturally contaminated meat samples were inoculated by using SSSLE broth (Patent pending). SSSLE represents a multipathogen selective enrichment broth allowing the concurrent growth of *Staph. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salm. enterica* and *Sh. flexneri*.

Oligonucleotide primers

The *Staph. aureus* 16S rDNA genes primer sets were derived from Tang *et al.* (2006). The other DNA sequences of *hlyA*, *eaeA*, *invA* and *ipaH* genes from the targeted bacteria were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and aligned using BLAST program. On the basis of the sequence alignment of specific genes from different strains, a conserved sequence region for each bacterium was selected for

Table 1 Bacterial strains for the evaluation of specificity of PCR primers

Strains	16S rDNA	*hlyA*	*eaeA*	*invA*	*ipaH*
Salmonella Enteritidis CICC 21482	–	–	–	+	–
Salm. Enteritidis ATCC 13076	–	–	–	+	–
Salmonella isolates (6 strains)	–	–	–	+	–
Escherichia coli O157:H7 CICC 21530	–	–	+	–	–
E. coli O157:H7 ATCC 43890	–	–	+	–	–
E. coli O157 isolates (2 strains)	–	–	+	–	–
Listeria monocytogenes ATCC 19111	–	+	–	–	–
L. monocytogenes ATCC 19112	–	+	–	–	–
L. monocytogenes isolates (4 strains)	–	+	–	–	–
Other *Listeria* isolates (4 strains)	–	–	–	–	–
Staphylococcus aureus ATCC 6538	+	–	–	–	–
Staph. aureus ATCC 25923	+	–	–	–	–
Staph. aureus isolates (6 strains)	+	–	–	–	–
Shigella flexneri CICC 1.1868	–	–	–	–	+
Sh. flexneri CICC 21534	–	–	–	–	+
Shigella isolates (3 strains)	–	–	–	–	+

Other *Listeria* is non-*Listeria monocytogenes*.

Table 2 Primer sequences and product sizes used in this study

Bacteria	Target gene	Primers	Sequence (5′ → 3′)	Product sizes (bp)	References
Staphylococcus aureus	16S rDNA	16S-F	GTGCACATCTTGACGGTACC	565	Tang *et al.* (2006)
		16S-B	CGAAGGGGAAGGCTCTATC		
Listeria monocytogenes	Listeriolysin O	*hlyA*-F	CAAGTCCTAAGACGCCAATC	1412	This study
		hlyA-B	ATAAAGTGTAGTGCCCCAGA		
Escherichia coli O157:H7	Intimin	*eaeA*-F	AGGTCGTCGTGTCTGCTA	255	This study
		eaeA-B	CCGTGGTTGCTTGCGTTTG		
Salmonella Enteritidis	Invasion protein	*invA*-F	TCCCTTTGCGAATAACATCC	786	This study
		invA-B	ATTACTTGTGCCGAAGAGCC		
Shigella flexneri	Invasion plasmid antigen H	*ipaH*-F	GGGAGAACCAGTCCGTAAA	1088	This study
		ipaH-B	CGCATCTCTGAAACATCTTGA		

designing the PCR primers using Primer Premier 5.0. Five primers pairs of specific gene targets and sizes of expected amplification products are listed in Table 2.

Genomic DNA extraction

Genomic DNA from pure cultures of Gram-positive bacteria was extracted using the method described by Tang *et al.* (2006); genomic DNA from pure cultures of Gram-negative bacteria was extracted using the method described by Fratamico and Strobaugh (1998). For food samples, to extract enough DNA, the culture samples were divided into two portions: One portion was extracted according to Gram-positive bacteria protocol; the other portion was extracted according to Gram-negative bacteria protocol; then, both portions were mixed together as the total DNA of samples. DNA was suspended in a final volume of 50 μl of sterile distilled water and stored at −20°C for use.

Optimization of multiplex PCR amplification

Optimization of Mg^{2+} concentration, primers concentration and annealing temperature were conducted. The Mg^{2+} concentration was tested using 1·0, 1·25, 1·5, 1·75, 2·0, 2·25 and 2·5 mmol l^{-1} $MgCl_2$. Both equal combinations and different combinations of five primers pairs were investigated. Equal combinations were tested at 0·2, 0·4 and 0·6 μmol l^{-1} for each primer pair; different combinations were tested as follows: (i) 0·25 μmol l^{-1} each of *ipaH*, *invA* and 16S, 0·3 μmol l^{-1} each of *hlyA* and *eaeA*; (ii) 0·25 μmol l^{-1} each of *ipaH*, *invA* and 16S, 0·4 μmol l^{-1} each of *hlyA* and *eaeA*; and (iii) 0·25 μmol l^{-1} each of *ipaH*, *invA* and 16S, 0·4 μmol l^{-1} of *hlyA* and 0·3 μmol l^{-1} of *eaeA*. Annealing temperature was tested at 56·2, 57·2, 58·2, 59·3, 60·2, 61·0 and 61·5°C. Each 20-μl reaction mixture contained 2 μl of 10× PCR buffer (Mg^{2+} free; Takara, Dalian, China), 0·15 mmol l^{-1} of each dNTP (25 mmol l^{-1}; Takara), 1·5 U rTaq DNA polymerase (5 U μl^{-1}; Takara). PCR amplification was performed in triplicate with the following programme: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, annealing temperature for 50 s, 72°C for 50 s, then followed by incubation at 72°C for 10 min and cooling at 4°C. Negative control reaction mixtures contained sterile distilled water instead of template DNA.

Determination of detection sensitivity

Quantification of DNA concentrations was determined by using a SmartSpecTM plus (Bio-Rad, Hercules, CA, USA). Each DNA template was extracted from pure culture of one target bacteria, was decimally diluted, and then subjected to simplex PCR. The DNA mixture template was made by mixing the five DNA templates with equal concentration, decimally diluted, and then subjected to multiplex PCR.

Examination of artificially contaminated samples

Raw 90% lean pork was purchased from a local store. The sample was divided into different parts (10 g for each) and sterilized with radiation. The absence of five target pathogens in the samples was confirmed by GB/T 4789-2008 (GB is the National Standard of the People's Republic of China). Each portion of ten grams of pork samples was transferred into a sterile 250-ml Erlenmeyer flask and inoculated fresh overnight cultures of five target pathogens. The spiked cell was seen in Table 3 with two levels. Then, the samples were held at room temperature for 15 min to allow bacterial adsorption. Subsequently, 90 ml of SSSLE broth were added to each pork sample; the samples were incubated at 37°C for 24 h with shaking at 150 rev min^{-1}. Bacterial DNA was extracted from enriched samples and subjected to the multiplex PCR. In parallel, the same sample was plated for viable cell counts

Table 3 Detection of five target bacterial strains in artificially contaminated pork by the multiplex PCR and the traditional culture method

Sample inoculation level	Five target pathogens	Spiked cells (CFU g^{-1})	The cell numbers after 24-h enrichment (CFU ml^{-1})	Multiplex PCR	Traditional culture methods
Low level	*Salmonella* Enteritidis	12–17	$2{\cdot}2 \times 10^8$–$3{\cdot}7 \times 10^8$	+	+
	Escherichia coli O157:H7	12–17	$2{\cdot}0 \times 10^8$–$3{\cdot}2 \times 10^8$	+	+
	Staphylococcus aureus	12–16	$4{\cdot}2 \times 10^7$–$7{\cdot}1 \times 10^7$	+	+
	Listeria monocytogenes	10–15	$9{\cdot}2 \times 10^5$–$1{\cdot}8 \times 10^6$	+	+
	Shigella flexneri	13–17	$4{\cdot}2 \times 10^7$–$6{\cdot}9 \times 10^7$	+	+
High level	*Salm.* Enteritidis	1245–1623	$2{\cdot}2 \times 10^8$–$1{\cdot}0 \times 10^9$	+	+
	E. coli O157:H7	1283–1735	$1{\cdot}9 \times 10^8$–$3{\cdot}8 \times 10^8$	+	+
	Staph. aureus	1284–1637	$4{\cdot}9 \times 10^7$–$8{\cdot}4 \times 10^7$	+	+
	L. monocytogenes	1203–1532	$8{\cdot}7 \times 10^5$–$1{\cdot}5 \times 10^6$	+	+
	Sh. flexneri	1302–1485	$4{\cdot}2 \times 10^7$–$7{\cdot}4 \times 10^7$	+	+

on the corresponding selective agars (all purchased from Hangzhou Microbial Reagent Co., Ltd, China): Baird–Parker (BP) agar for *Staph. aureus*; PALCAM agar for *L. monocytogenes*; Modified Sorbitol Maconkey (CT-SMAC) agar for *E. coli* O157:H7; desoxycholate hydrogen sulfide lactose (DHL) agar for *Salm. enterica*; and Salmonella–Shigella (SS) agar for *Sh. flexneri*. The experiment was independently repeated three times.

Examination of naturally contaminated meat samples

Twenty-five meat samples including beef, pork and chicken were purchased from different retail, kept at 4°C, and analysed on the same day. Two portions of 25 g of each meat were tested. Each 25 g meat sample was placed into a sterile Whirl-Park sample bag (Nasco, Fort Atkinson, WI, USA) and transferred to a sterile 250-ml Erlenmeyer flask, respectively; then, 225 ml of SSSLE broth was added. The enrichment of the pathogens in these naturally contaminated meat samples was carried out with the same procedure as for the spiked samples. After 24-h enrichment, multiplex PCR analysis and culture-based detection were performed in parallel.

Results

Primer specificity

To evaluate the specificity of primers used in the study, each DNA template prepared from each one of the strains was tested using the mixture of all five pairs of primers. Similarly, each pair of primers was also tested by using both the single DNA and the mixture of five DNA templates. As shown in Table 1, amplification products of the expected bands were obtained by PCR with five pairs of primers (at 0·5 μmol l^{-1} per pair of primers) on each tested bacterial strain; the specificity of primers was validated. After adding each of the five pairs of primers (at 0·5 μmol l^{-1}), the amplification products of the expected sizes were obtained. The PCR products were sufficiently different in size to be distinguishable by agarose gel electrophoresis. In Fig. 1, the 16S rDNA gene amplicon (565 bp) of *Staph. aureus*, the *hlyA* gene amplicon (1412 bp) of *L. monocytogenes*, the *eaeA* gene amplicon (255 bp) of *E. coli* O157: H7, the *invA* gene amplicon (786 bp) of *Salm. enterica* Enteritidis and the *ipaH* gene amplicon (1088 bp) of *Sh. flexneri* were present in lane 1, 2, 3, 4 and 5, respectively. Only one amplicon per lane was observed.

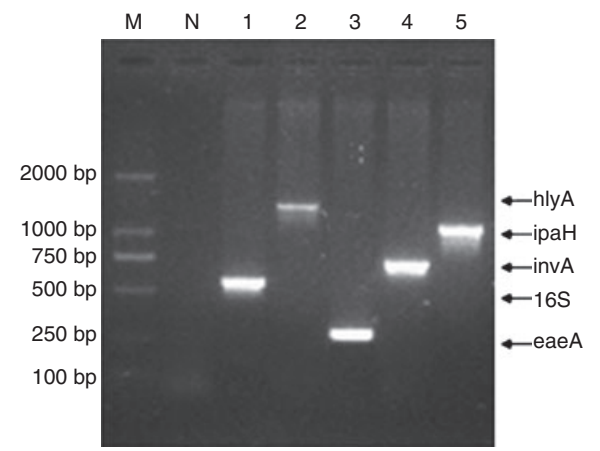

Figure 1 Agarose gel electrophoresis analysis of simplex PCR-amplified target genes from five pathogens. Lane M, 2000-bp DNA ladder; Lane N, negative control; Lane 1, 16S rDNA amplicon of *Staphylococcus aureus* ATCC 6538; Lane 2, *hlyA* amplicon of *Listeria monocytogenes* ATCC 19111; Lane 3, *eaeA* amplicon of *Escherichia coli* O157:H7 CICC 21530; Lane 4, *invA* amplicon of *Salmonella enterica* enteritidis CICC 21482; Lane 5, *ipaH* amplicon of *Shigella flexneri* CICC 1.1868.

Optimization of multiplex PCR

To develop the multiplex PCR assay, five pairs of primers were combined and a mixture of DNA was prepared from five target DNA templates. A systematic study was performed to optimize the multiplex PCR conditions. Based on a set of amplification parameters from the optimization, the Mg^{2+} concentration at 2·25 mmol l^{-1} seemed most effective in producing high yields of all five target genes. When equal combinations and different combinations of five primers were tested, bands of variable intensities were obtained. The primer combination found to be optimal for multiplex PCR was 0·25 μmol l^{-1} each of *ipaH*, *inv*A and 16S rDNA, 0·4 μmol l^{-1} of *hlyA* and 0·3 μmol l^{-1} of *eaeA*. Maximal band intensities of each amplicon were found with 59·3°C of annealing temperature.

Sensitivity of simplex PCR and multiplex PCR assay

The sensitivity evaluation of PCR assay was carried out using a series of target pathogen genomic DNA in 10-fold dilution. There was a qualitative decrease in the intensity of the amplicons with the decrease of the DNA concentration. The detection limit of genomic DNA in the simplex PCR was 5·89 fg for *Staph. aureus*, 17·4 pg for *E. coli* O157:H7, 543 pg for *L. monocytogenes*, 73·35 fg for *Sh. flexneri* and 22 pg for *Salm. enterica* Enteritidis. The detection limit of genomic DNA in the multiplex PCR was 6·4 pg for *Staph. aureus*, 32 pg for *E. coli* O157:H7, 800 pg for *L. monocytogenes*, 160 pg for *Sh. flexneri* and 32 pg for *Salm. enterica* Enteritidis. The results showed that the detection limits of simplex PCR for five pathogens were relatively lower than that of multiplex PCR. Especially for *Staph. aureus* and *Sh. flexneri*, the detection limits of simplex PCR were *c.* 10^3 times lower than that of multiplex PCR. From the above-mentioned, the DNA extraction method described in Materials and methods could successfully isolate DNA from PCR inhibitory components (such as fats, glycogen, organic and phenolic compounds, etc.). And the multiplex assay developed in this study was effective for the detection of target pathogens.

Multiplex PCR and traditional culture method detection of artificially contaminated samples

For artificially contaminated lean pork samples, the detection of five target pathogens grown in SSSLE after 24 h of enrichment was investigated. As shown in Table 3, the growth levels of five bacterial strains could be divided into three patterns by traditional culture method. The cell numbers of *Salm.* Enteritidis and *E. coli* O157:H7 were very similar, reaching 10^8 CFU ml^{-1}; the cell numbers of *Staph. aureus* and *Sh. flexneri* reached 10^7 CFU ml^{-1}; the level of growth of *L. monocytogenes* which reached 10^6 CFU ml^{-1} was slightly lower than those of the other four pathogens. Multiplex PCR assay of the same contaminated pork samples also showed positive PCR-amplified products for the target pathogens. Consequently, the 24-h enrichment allowed both assays to detect samples contaminated with lower level (10–17 CFU g^{-1} sample) and higher level (1203–1735 CFU g^{-1} sample) of target pathogens (Table 3). The multiplex PCR developed in the present study was capable of detecting all five pathogens simultaneously in the spiked pork after 24-h enrichment.

Multiplex PCR and traditional culture method detection of naturally contaminated samples

According to the results shown in Table 4, after 24-h enrichment, a total of 20 samples, or 80%, tested positive for one or more of these organisms by multiplex PCR assay. Among the 25 naturally contaminated samples, 15, 4 and 13 samples, respectively, were positive for *Salm.* Enteritidis, *E. coli* O157:H7 and *Staph. aureus* by both methods. One sample was *L. monocytogenes*-positive by multiplex PCR method, and two samples were positive by the culture method. Two samples were *Sh. flexneri*-positive by the multiplex PCR method, and one sample was positive by the culture method. Comparing the results of two methods, beef sample number four was found to be contaminated with *L. monocytogenes* by the culture method, whereas this sample was found to be negative for *L. monocytogenes* by multiplex PCR method. Chicken sample number two was found to be contaminated with *Sh. flexneri* by multiplex PCR method, whereas this sample was demonstrated to be absent of *Sh. flexneri* by traditional culture method. Despite some minor differences occurred, the results from testing naturally contaminated samples validated the feasibility of the multiplex PCR assay combined with multipathogen enrichment for detecting these target pathogens in food samples.

Discussion

Rapid and accurate methods for simultaneous identification of foodborne pathogens are becoming more and more important (Díaz-López *et al.* 2011). Previously reported methods of multiplex PCR usually assay only for two or three pathogens. In the present study, a multiplex PCR assay that used five pairs of primers was developed to simultaneously detect *Salm.* Enteritidis, *L. monocytogenes*, *Staph. aureus*, *E. coli* O157:H7 and *Sh. flexneri*. The applications of this method in artificially contaminated and naturally contaminated meat samples were also verified. Therefore, the current multiplex PCR assay

Table 4 Detection of five target bacterial strains in naturally contaminated samples by the multiplex PCR and the traditional culture method

	Salmonella Enteritidis		*Escherichia coli* O157:H7		*Staphylococcus aureus*		*Listeria monocytogenes*		*Shigella flexneri*	
Samples	Multiplex PCR	Culture method	Multiplex PCR	Culture method	Multiplex PCR	Culture method	Multiplex PCR	Culture method	Multiplex PCR	Culture method
Beef 1	–	–	–	–	–	–	–	–	–	–
Beef 2	+	+	–	–	+	+	–	–	–	–
Beef 3	+	+	+	+	–	–	–	–	+	+
Beef 4	+	+	–	–	+	+	–	+	–	–
Beef 5	+	+	+	+	–	–	–	–	–	–
Beef 6	–	–	–	–	–	–	–	–	–	–
Beef 7	+	+	–	–	+	+	–	–	–	–
Beef 8	–	–	–	–	+	+	–	–	–	–
Pork 1	+	+	+	+	–	–	–	–	–	–
Pork 2	+	+	–	–	+	+	–	–	–	–
Pork 3	–	–	–	–	+	+	–	–	–	–
Pork 4	+	+	–	–	–	–	–	–	–	–
Pork 5	–	–	–	–	+	+	+	+	–	–
Pork 6	+	+	–	–	+	+	–	–	–	–
Pork 7	–	–	–	–	–	–	–	–	–	–
Pork 8	+	+	–	–	–	–	–	–	–	–
Pork 9	+	+	–	–	+	+	–	–	–	–
Chicken 1	+	+	+	+	–	–	–	–	–	–
Chicken 2	–	–	–	–	+	+	–	–	+	–
Chicken 3	+	+	–	–	+	+	–	–	–	–
Chicken 4	+	+	–	–	–	–	–	–	–	–
Chicken 5	–	–	–	–	–	–	–	–	–	–
Chicken 6	+	+	–	–	+	+	–	–	–	–
Chicken 7	–	–	–	–	+	+	–	–	–	–
Chicken 8	–	–	–	–	–	–	–	–	–	–

combined with a multipathogen enrichment procedure could reliably and effectively detect the five major foodborne pathogens.

The detection method contained an enrichment procedure to enhance detection of pathogens in samples. Potential inhibitory substances in foods, including fats, glycogen, organic and phenolic compounds, can affect the PCR (O'Regan *et al.* 2008). The overnight pre-enrichment step (in SSSLE) was required to increase the number of viable cells and to effectively dilute inhibitory substances present in the sample.

For development of the multiplex PCR assay, designing primers for the five targets is very crucial. It is reported that nearly 2000 *Salmonella* serovars contain the *invA* gene, which is a virulence gene encoding an invasion protein. Generally, the *invA* gene has been proven to be Salmonella-specific (D'Souza *et al.* 2009; Suo *et al.* 2010), which was also verified to exist only in the *Salm.* Enteritidis tested in this study and not in the other isolates tested. It has been proved that the EHEC *eaeA* gene encodes an outer membrane adhesin (intimin), which mediates intimate attachment to host cell and is essential for establishment of attaching and effacing lesion (Louie *et al.* 1993; Dean-Nystrom *et al.* 2003). Molecular-based methods developed for EHEC applied the *eaeA* gene, one of the genes involved in pathogenicity (Critzer *et al.* 2008; Aspán and Eriksson 2010). Hence, in this study, the identification of *E. coli* O157 serotype using *eaeA* as a target was carried out. The *hlyA* encoding the 58-kDa listeriolysin O, which has been classically regarded as one of the three significant virulence markers (Jung *et al.* 2009), is unique to the species *L. monocytogenes* (Deneer and Boychuk 1991). The *hlyA* gene is most commonly used for detecting *L. monocytogenes* (Nogva *et al.* 2000; Suo *et al.* 2010). For *Staph. aureus*, a species-specific region of 16S rDNA has been successfully employed and its specificity has been validated by Tang *et al.* (2006). The invasion plasmid antigen H (*ipaH*) gene is carried by four *Shigella* species. It has been considered that the *ipaH* gene was the main target for detection of *Shigella* in many researches (Vu *et al.* 2004; Shao *et al.* 2011). In this study, a set of primers targeting five major pathogens were designed and adapted to the multiplex assay. BLAST results also show that nontargeted organisms do not contain the primer

sequences of the target organisms. Further, their specificity was thoroughly tested by both simplex PCR and multiplex PCR, demonstrating their suitability for detecting these five major pathogens.

For a multiplex PCR assay to be successful, the relative concentrations of primers, PCR buffer concentration, the balance between magnesium and DNA, cycling temperatures, amounts of template DNA and *Taq* DNA polymerase are very important (Markoulatos *et al.* 2002). In this study, to develop an efficient multiplex PCR, reaction conditions (Mg^{2+} concentration, concentration of primers and annealing temperature) were optimized. The multiplex PCR developed in this study yielded five bands showing the specificity for each pathogen, thus confirming the specificity of the five sets of primers.

A potential advantage of PCR is that it has a high level of sensitivity. In this study, the sensitivity evaluation between simplex PCR and multiplex PCR assay was performed. The detection limit of simplex PCR was relatively lower than that of multiplex PCR. However, the experiments still supported the facts that the DNA extraction method could successfully isolate DNA from PCR inhibitory components, and the multiplex PCR assay developed in this study was effective for the detection of target pathogens. Hereby, our results demonstrated that amplification of the five sets of primers was efficient with five clear bands.

During the naturally contaminated meat sample assays, some of the multiplex PCR bands gave weak signals. We applied simplex PCR to re-amplify the samples and reconfirm the result. Results observed with multiplex PCR and traditional cultures were highly consistent with the exception of two samples. Beef sample number four gave a positive result for *L. monocytogenes* by the traditional culture method but was negative in the PCR method. Analysis of the result may be that other *Listeria* species like *L. innocua*, which is a nonpathogen and lacks the *hlyA* gene, can also grow on the PALCAM agar as *L. monocytogenes*. The suspicious colonies of *Listeria* further detected by using PCR did not contain the *hlyA* gene. Hence, the positive result by traditional culture method should be combined with PCR methods to further identify *L. monocytogenes*. Chicken sample number two gave a positive result for *Sh. flexneri* by the PCR method, but a negative result by the culture method. A 230-kb virulence-associated invasion plasmid, required for entry into host cells, involving a cluster of genes, appears in *Shigella* and enteroinvasive *E. coli* (EIEC) strains (Lan *et al.* 2003; Vu *et al.* 2004; Hien *et al.* 2008). Consequently, further investigation should be carried out with respect to the potential virulence factors co-existing in several species.

Taken together, this study reported a rapid multiplex PCR assay using five primers sets for detection of multiple pathogens. The assay performed equally as well as the traditional cultural method and facilitated the sensitive detection both in artificially contaminated and naturally contaminated samples. Higher consistency was obtained between the results of multiplex PCR and traditional culture methods, indicating that multiplex PCR was a comparatively reliable and useful for rapid screening of these pathogens. To sum up, the multiplex PCR assay has the potential to be used in routine diagnostic laboratories and also might be as a rapid screening tool in food testing laboratories to quickly identify food samples.

Acknowledgements

This work was jointly supported by the Special Foundation for Young Scientists of Sichuan Province, China (2011JQ0043); the National Natural Science Foundation of China (31071515); the Fundamental Research Funds for the Central Universities, Southwest University for Nationalities (11NZYTH08, 11NZYTD08).

References

Abubakar, I., Irvine, L., Aldus, C.F., Wyatt, G.M., Fordham, R., Schelenz, S., Shepstone, L., Howe, A. *et al.* (2007) A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. *Health Technol Assess* **36**, 1–216.

Aspán, A. and Eriksson, E. (2010) Verotoxigenic *Escherichia coli* O157:H7 from Swedish cattle; isolates from prevalence studies versus strains linked to human infections-a retrospective study. *BMC Vet Res* **6**, 7.

Critzer, F.J., Dsouza, D.H. and Golden, D.A. (2008) Transcription analysis of *stx*1, *mar*A, and *eae*A gene in *Escherichia coli* O157:H7 treated with sodium benzoate. *J Food Prot* **71**, 1469–1474.

D'Souza, D.H., Critzer, F.J. and Golden, D.A. (2009) Real-time reverse-transcriptase polymerase chain reaction for the rapid detection of *Salmonella* using *inv*A primers. *Foodborne Pathog Dis* **6**, 1097–1106.

De Freitas, C.G., Santana, A.P., da Silva, P.H., Gonçalves, V.S., Barros Mde, A., Torres, F.A., Murata, L.S. and Perecmanis, S. (2010) PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat. *Int J Food Microbiol* **139**, 15–22.

Dean-Nystrom, E.A., Melton-Celsa, A.R., Pohlenz, J.F., Moon, H.W. and O'Brien, A.D. (2003) Comparative pathogenicity of *Escherichia coli* O157 and intimin-negative non-O157 Shiga toxin-producing *E. coli* strains in neonatal pigs. *Infect Immun* **71**, 6526–6533.

Deneer, H.G. and Boychuk, I. (1991) Species-specific detection of *Listeria monocytogenes* by DNA amplification. *Appl Environ Microbiol* **57**, 606–609.

Díaz-López, A., Cantú-Ramírez, R.C., Garza-González, E., Ruiz-Tolentino, L., Tellez-Luis, S.J., Rivera, G. and Bocanegra-García, V. (2011) Prevalence of foodborne pathogens in grilled chicken from street vendors and retail outlets in reynosa, tamaulipas, Mexico. *J Food Prot* **74**, 1320–1323.

Elizaquível, P. and Aznar, R. (2008) A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. *Food Microbiol* **25**, 705–713.

Fratamico, P.M. and Strobaugh, T.P. (1998) Simultaneous detection of *Salmonella* spp and *Escherichia coli* O157:H7 by multiplex PCR. *J Ind Microbiol Biot* **21**, 92–98.

Gasanov, U., Hughes, D. and Hansbro, P.M. (2004) Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiol Rev* **29**, 851–875.

Hien, B.T., Scheutz, F. and Cam, P.D. (2008) Diarrheagenic *Escherichia coli* and *Shigella* Strains isolated from children in a hospital case–control study in Hanoi, Vietnam. *J Clin Microbiol* **46**, 996–1004.

Jung, H.J., Park, S.H., Ha, S.D., Lee, K.H., Chung, D.H., Kim, C.H., Kim, M.G., Kim, K.Y. *et al.* (2009) Species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the *prf*A virulence gene cluster. *Biosci Biotechnol Biochem* **73**, 1412–1415.

Kaper, J.B., Nataro, J.P. and Mobley, H.L. (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123–140.

Lan, R., Stevenson, G. and Reeves, P.R. (2003) Comparison of two major forms of the *Shigella* virulence plasmid pINV: positive selection is a major force driving the divergence. *Infect Immun* **71**, 6298–6306.

Louie, M., de Azavedo, J.C., Handelsman, M.Y., Clark C.G., Ally., B., Dytoc., M. Sherman, P. and Brunton, J. (1993) Expression and characterization of the *eae*A gene product of *Escherichia coli* serotype O157:H7. *Infect Immun* **61**, 4085–4092.

Markoulatos, P., Siafakas, N. and Moncany, M. (2002) Multiplex polymerase chain reaction: a practical approach. *J Clin Lab Anal* **16**, 47–51.

Mook, P., Patel, B. and Gillespie, I.A. (2011) Risk factors for mortality in non-pregnancy-related listeriosis. *Epidemiol Infect* **21**, 1–10.

Nogva, H.K., Rudi, K., Naterstad, K., Holck, A. and Lillehaug, D. (2000) Application of 5′-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl Environ Microbiol* **66**, 4266–4271.

O'Regan, E., McCabe, E., Burgess, C., McGuinness, S., Barry, T., Duffy, G., Whyte, P. and Fanning, S. (2008) Development of a real-time multiplex PCR assay for the detection of multiple Salmonella serotypes in chicken samples. *BMC Microbiol* **8**, 156.

Perry, L., Heard, P., Kane, M., Kim, H., Savikhin, S., Domínguez, W. and Applegate, B. (2007) Application of multiplex polymerase chain reaction to the detection of pathogens in food. *J Rapid Methods Autom Microbiol* **15**, 176–198.

Pragman, A.A. and Schliever, P.M. (2004) Virulence regulation in *Staphylococcus aureus*: the need for *in vivo* analysis of virulence factor regulation. *FEMS Immunol Med Microbiol* **42**, 147–154.

Shao, Y., Zhu, S., Jin, C. and Chen, F. (2011) Development of multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) to detect *Salmonella* spp. and *Shigella* spp. in milk. *Int J Food Microbiol* **148**, 75–79.

Suo, B., He, Y., Tu, S.I. and Shi, X. (2010) A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. *Foodborne Pathog Dis* **7**, 619–628.

Tang, J.N., Shi, X.M., Shi, C.L. and Chen, H.C. (2006) Characterization of a duplex PCR assay for the detection of enterotoxigenic strains of *Staphylococcus aureus*. *J Rapid Methods Autom Microbiol* **14**, 201–217.

Vu, D.T., Sethabutr, O., Von Seidlein, L., Tran, V.T., Do, G.C., Bui, T.C., Le, H.T., Lee, H. *et al.* (2004) Detection of *Shigella* by a PCR assay targeting the *ipa*H gene suggests increased prevalence of Shigellosis in Nha Trang, Vietnam. *J Clin Microbiol* **42**, 2031–2035.

Wang, L., Li, Y. and Mustaphai, A. (2007) Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, S*almonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. *J Food Prot* **70**, 1366–1372.

Yoon, J.W. and Hovde, C.J. (2008) All blood, no stool: enterohemorrhagic *Escherichia coli* O157:H7 infection. *J Vet Sci* **9**, 219–231.