

NOVEL MULTIPLEX PCR TO SPECIFICALLY DETECT BACTERIAL FOODBORNE PATHOGENS

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Abstract

Bacterial foodborne pathogens prevalent in poultry, especially *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes*, have been reported in many countries including Thailand. Rapid methods for identification and detection of these dominant foodborne pathogens are still required. In our study, multiplex polymerase chain reaction (m-PCR) was developed for detecting multiple bacterial foodborne pathogens. Specific genes for the m-PCR primers were screened and selected. m-PCR targeting the *uspA*, *fimY*, *ipaH*, and *prfA* gene was used to detect *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, respectively. The optimum conditions for the m-PCR reaction were found to be primer concentrations of 0.02 μ M *ipaH*, 0.036 μ M *fimY*, 0.06 μ M *uspA*, 0.12 μ M *prfA*, and 0.4 μ M 16S rRNA gene (used as internal control) for at least 10 ng of each bacterial total genomic DNA; and 52°C was the annealing temperature. The expected PCR products of 884, 489, 422, and 398 bp were obtained from specific amplification of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, respectively, of the reference strains and strains isolated from fresh chicken intestine. Cross amplification from non-target bacteria which have been frequently found in enrichment culture were not detected. These results indicated that the developed m-PCR could be used to detect multiple foodborne pathogens with no cross-reactivity with the non-target bacteria found in the enrichment culture. Alternatively, this method could also be used to identify the presumptive colonies of interest on selective agar with considerable timesaving when compared with the biochemical characterization of the conventional method.

Keywords: Multiplex PCR, foodborne pathogens, optimization, target bacteria

Introduction

Foodborne diseases are some of the most widespread health problems in the world. Regulations for foodborne pathogens including *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, and *Campylobacter jejuni* in poultry meat are required (Mulder and Hupkes, 2007). The prevalence of the foodborne

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pathogens and microbial food safety indicators in poultry especially *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* have been reported in Thailand and many countries (Sackey *et al.*, 2001; Bangtrakulnonth *et al.*, 2004; Angkititrakul *et al.*, 2005; Padungtod and Kaneene, 2006; Lekroengsin *et al.*, 2007; Vindigni *et al.*, 2007; Minami *et al.*, 2010; Stonsaovapak and Boonyaratanakornkit, 2010). Therefore, the detection of foodborne pathogens in chicken meat is needed. The conventional methods for detecting enteropathogens involve isolation followed by biochemical identification of each pathogen. They are very laborious and time-consuming (de Boer and Beumer, 1999; You *et al.*, 2008). To overcome these limitations, a molecular-based method has been developed as a more rapid tool for pathogenic detection.

Multiplex polymerase chain reaction (m-PCR) involves simultaneous amplification of more than 1 amplicon per reaction by mixing multiple primer pairs with different specificities. It is based on the separation of PCR amplicons of different molecular weights by an agarose gel electrophoresis (Settanni and Corsetti, 2007). The m-PCR based method has been widely used and adapted for rapid detection of single and multiple bacterial species (Chen and Griffiths, 1998; Yeh *et al.*, 2002; Li and Mustapha, 2004; Thiem *et al.*, 2004; Jofré *et al.*, 2005; Li *et al.*, 2005; Germini *et al.*, 2009). However, most research has not combined the detection of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* together. The gene specificity for all the bacteria of interest can be used in the m-PCR to detect each bacterial species. In this investigation, the *invA* gene, the encodes for the inner membrane protein of bacteria (Salehi *et al.*, 2005), and the *fimY* gene, the regulatory gene of the major fimbrial subunit protein (Yeh *et al.*, 2002), were evaluated for specific detection of *Salmonella* spp. For *Shigella* detection, the specificity and accuracy of the *virA*, the virulence gene on virulence plasmid (Villalobo and Torres, 1998; Mao *et al.*, 2008), and the *ipaH* gene, the encodes for

the invasion plasmid antigen H (Thiem *et al.*, 2004), were evaluated. The *uspA* gene, the encodes for universal stress protein (Chen and Griffiths, 1998; Osek, 2001), and the *prfA* gene, the encodes for the transcriptional activator of the virulence factor (Wernars *et al.*, 1992; Germini *et al.*, 2009), were used for specific detection of *E. coli* and *L. monocytogenes*, respectively.

The aim of this investigation was to develop an m-PCR method to specifically detect multiple foodborne pathogens prevalent in chicken meat. Primers were designed and m-PCR conditions were optimized. The dominant target bacteria prevalent in chicken meat, including *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, were chosen as models for method development. The isolated strains used in the investigation were isolated from fresh chicken intestine due to its high diversity of intestinal microflora and poultry foodborne pathogens (Amit-Romach *et al.*, 2004). The target and non-target bacterial isolates with diverse physiological characteristics were used as the tested organisms. Finally, m-PCR primer sets were validated and tested for specificity using reference and isolated strains of the target and non-target bacteria.

Materials and Methods

Bacterial Strains and Cultivation

All bacterial reference and isolated strains used to validate the m-PCR detection system are listed in Table 1. Isolated strains of the target and non-target bacteria were obtained from food sample and chicken intestine isolation (Table 1) and then identified using biochemical characteristic profiles as described by the United States Food and Drug Administration – Bacteriological Analytical Manual (United States Food and Drug Administration, 1998). All target bacteria except for *C. perfringens* were grown on trypticase soy agar (TSA), composed of tryptone 15 g/l, proteose peptone 5 g/l, sodium chloride 15 g/l, and agar 15 g/l,

Table 1. Bacterial strains used for m-PCR validation

Species	Number of strains	Strain number and sources
<i>Escherichia coli</i>	7	<i>E. coli</i> TISTR ^a 887, <i>E. coli</i> E ^b 1, 2, 3, 4, 6, 7
<i>Clostridium perfringens</i>	1	<i>C. perfringens</i> CP ^b 5
<i>Listeria monocytogenes</i>	11	<i>Listeria</i> sp. JCM ^a 7679, <i>L. innocua</i> DSM ^a 20649, <i>L. monocytogenes</i> DSM 12464, DMST ^a 1327, 2871, 17303, 20093, 21164, 23136, 23145, 31802
<i>Salmonella</i> spp.	8	<i>Salmonella</i> serotype Enteritidis (<i>S. Enteritidis</i>) JCM 1652, TISTR 2394, <i>Salmonella</i> serotype Typhimurium (<i>S. Typhimurium</i>) TISTR 292, <i>Salmonella</i> sp. S ^b 2, 3, BC ^b 1, L ^b 6, CM ^b 7
<i>Shigella</i> spp.	12	<i>Sh. boydii</i> DMST 3395, 28180, 30245, <i>Sh. dysenteriae</i> DMST 2137, 5875, 15111, <i>Sh. flexneri</i> DMST 17559, 17560, 30581, <i>Sh. sonnei</i> DMST 17561, 23595, <i>Shigella</i> sp. Sh ^c 1
<i>Staphylococcus aureus</i>	1	<i>Staph. aureus</i> TISTR 517
Non-target bacteria found in an enrichment culture	10	C ^b 2, 3, 4, 6, RV ^b 2, 3, TT ^b 1, L2, 4, 5

^a Reference strain: **DMST**, The Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH German Collection of Microorganisms and Cell Cultures; **JCM**, Japan Collection of Microorganisms; **TISTR**, Thailand Institute of Scientific and Technology Research.

^b Strains isolated from chicken intestine in NakhonRatchasima, Thailand: **BC**, *Salmonella* sp. enriched using RV broth and isolated on XLD agar; **C**, non-*E. coli* bacteria isolated on EMB agar; **CM**, *Salmonella* sp. isolated on mCCDA; **CP**, *C. perfringens*; **E**, *E. coli*; **L**, non-*Listeria* bacteria isolated on PALCAM agar; **RV**, non-*Salmonella* bacteria enriched using RV broth and isolated on XLD agar; **S**, *Salmonella* sp. enriched using TT broth and isolated on XLD agar; **TT**, non-*Salmonella* bacteria enriched using TT broth and isolated on XLD agar.

^c Strains isolated from food in KhonKaen, Thailand: **Sh**, *Shigella* sp.

at 37°C for 24-48 h. For the cultivation of *C. perfringens*, the bacterium was cultured on tryptose sulfite cycloserine agar (TSC) (Biomark, Pune, India) containing egg yolk emulsion (Biomark) and incubated under anaerobic condition at 37°C for 24 h.

Primer Design

Universal 16S rDNA primers were designed based on the consensus sequences which were conserved for all the target bacteria. To obtain the consensus sequence of each pathogen, the sequences were downloaded from the NCBI database and aligned using Meg Align DNA Star Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA). Primers for the specific gene amplification were designed based on the conserved regions of each target gene in each target bacterium of interest using Primer Select DNA Star Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA). For the primer validation and selection, all primers in Table 2 were tested for specificity using reference and isolated strains of the target and non-target bacteria (Table 1).

Target Gene Amplification by m-PCR

Genomic DNA (gDNA) from 16-24 h grown pure cultures on TSA and TSC were extracted using the simple protocol of the phenol-chloroform-based method (Liu *et al.*, 2011). The gDNA pellets were then resuspended in 100 µl of 10 mM Tris-Cl, 1 mM EDTA (TE), pH8 and 10 µg/ml RNaseA. The concentrations and purity of the gDNA were detected by measuring the absorbance at 260 and 280 nm using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The gDNAs were used as template for amplification of the target genes. In all m-PCR reactions, the amplification of the 16S rRNA gene was used as an internal control. The concentration of each primer pair and the annealing temperature were optimized.

The m-PCR reactions were performed in a total volume of 25 µl containing 1× GoTaq Flexi buffer (Promega, Madison, USA),

1 mM MgCl₂ (Promega), 0.2 mM dNTPs (Promega), 0.4 µM 16S rDNA primers (Table 2), 0.5 U GoTaq Flexi DNA polymerase (Promega), 100 ng DNA templates, and gene specific primers. The PCR reactions were heated at 95°C for 3 min and then, 35 cycles at 95°C for 30 s, 50-59°C for 45 s (optimization of annealing temperature), and 72°C for 60 s followed by a final step of 5 min incubation at 72°C. The m-PCR products were analyzed by electrophoresis on 4% agarose gel.

Results and Discussion

Optimization and Validation of the m-PCR

The specificities of the *uspA*, *fimY*, *invA*, *ipaH*, *virA*, and *prfA* genes (Table 2) were tested with gDNA templates extracted from pure cultures of *E. coli*, *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, and the non-target bacteria (Table 1). Primers for amplification of *virA* gene previously published by Mao *et al.* (2008) were evaluated and gave negative results to the *Shigella* sp. isolated strain (Sh1) (data not shown). The *virA* gene is known to locate on virulence plasmids of *Shigellae* (Gall *et al.*, 2005), thus this gene might be lost in some isolated strains. In contrast, detection of the *Shigella* spp. using the *ipaH* gene as a target showed that this gene was conserved among all *Shigella* isolates including isolate Sh1. The *ipaH* gene is located on a 220 kb plasmid and also the bacterial chromosome (Ashida *et al.*, 2007). For these reasons, the *ipaH* gene was the suitable target for *Shigella* spp. detection. Negative results observed from the *virA* gene amplification in isolate Sh1 indicated that some published primers targeted to specific genes might not be able to be applied for detection of some local isolated strains, since false negative foodborne pathogen detection might occur.

In this investigation, the *uspA* gene can be amplified not only from *E. coli* but also from all 4 *Shigella* species found in Thailand (Figure 1(a), Lanes 1-7; (b),

Table 2. Primers used for the target gene amplifications by m-PCR

Species	Target gene	Primer sequences (5' to 3')	PCR product size (bp)	References
All species	16S	F: AGACTCTACGGGAGGC	625-655	This work
	rRNA	R: GGTAAGGTTCTTCGCGT		
<i>E. coli</i>	<i>uspA</i>	F: CCGATACGCTGCCAATCAGT	884	Chen and Griffiths (1998)
		R: ACGCAGACCGTAGGCCAGAT		
<i>L. monocytogenes</i>	<i>prfA</i>	F: CACAAGAATATGTATTCTATATGAT	398	This work
		R: CAGTGTAATCTTGATGCCATCA		
<i>Salmonella</i> spp.	<i>fimY</i>	F: CGGCTAAAGCTTCCGATAAGCG	489	This work
		R: AAATGCTAAAGACTGCGCTGCCG		
<i>Salmonella</i> spp.	<i>invA</i>	F: GAAATTATCGCCACGTTCCGGGCAA	283	Mao <i>et al.</i> (2008)
		R: TCATCGCACCGTCAAGGAACC		
<i>Shigella</i> spp.	<i>ipaH</i>	F: GAGGACATTGCCCGGGATAAAG	422	This work
		R: TAAATCTGCTGTTCAGTCTCACGC		
<i>Shigella</i> spp.	<i>virA</i>	F: CTGCATCTCTGGCAATCTCTTCACATC	215	Mao <i>et al.</i> (2008)
		R: TGATGAGCTAACTTCGTAAAGCCCTCC		

Lanes 1-11) due to the high identity of the gene between *E. coli* and *Shigella* (Chen, 2007). However, *Shigella* can be differentiated from *E. coli* by the presence of the *ipaH* gene product (Figure 1(b), Lanes 1-11). The *uspA* gene was shown to be conserved among all *E. coli* isolates (Figure 1(a), Lanes 1-7) and can be used for differentiation of *E. coli* and non-*E. coli* bacteria from the enrichment culture (Figure 2). For these reasons, the *uspA* gene was used here.

The amplification efficiency of the gene specific primers by the mixed primer set in the m-PCR reaction is an important point that influences the accuracy of the technique. The amplification ability of the *invA* (Mao *et al.*, 2008) and *fimY* primers in the m-PCR reactions for specific detection of *Salmonella* spp. was investigated. Compared with the *fimY* primers, the lower yields of PCR products were observed when *invA* primers were used for the amplification of the same

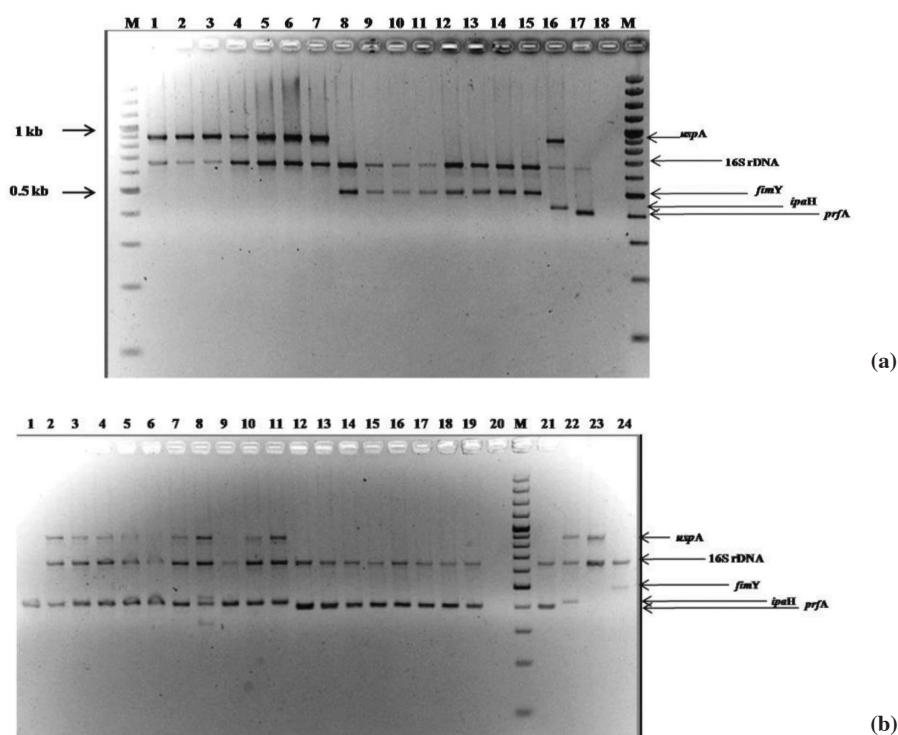


Figure 1. Primer validations for specific detection of the target bacteria using m-PCR technique. (A) Specific detection of *E. coli* and *Salmonella* spp. using m-PCR technique. Lanes: 1-7, *E. coli* isolates E1, 2, 3, 4, 6, 7, TISTR 887, respectively; 8-15, *Salmonella* sp. isolates S2, 3, BC1, L6, CM7, *S. Enteritidis* JCM 1652, TISTR 2394, *S. Typhimurium* TISTR 292, respectively; 16, *Shigella* sp. isolate Sh1; 17, *L. monocytogenes* DSM 12464; 18, negative control (H_2O). (B) Specific detection of *Shigella* spp. and *L. monocytogenes* using m-PCR technique. Lanes: 1-3, *Shigella boydii* DMST 3395, 28180, 30245, respectively; 4-6, *Sh. dysenteriae* DMST 2137, 5875, 15111, respectively; 7-9, *Sh. flexneri* DMST 17559, 17560, 30581, respectively; 10-11, *Sh. sonnei* DMST 17561, 23595, respectively; 12-19, *L. monocytogenes* DMST 1327, 2871, 17303, 20093, 21164, 23136, 23145, 31802, respectively; 20, negative control (H_2O); 21, *L. monocytogenes* DSM 12464; 22, *Shigella* sp. isolate Sh1; 23, *E. coli* TISTR 887; 24, *S. Enteritidis* JCM 1652; M, 100 bp DNA marker (Fermentas)

concentration of gDNA template (data not shown). This might be due to the compatibility of the *invA* primers in the m-PCR reaction being lower than that of the *fimY* primers. In the case of *prfA* gene amplification, the *prfA* gene was amplified from only *L. monocytogenes* but not from *Listeria* sp. JCM 7679, *L. innocua* DSM 20649 (data not shown), nor non-*Listeria* bacteria (Figure 2). The *prfA* gene product regulates the expression of listeriolysin which is a major virulence factor expressed by pathogenic *Listeria* spp. (Wernars *et al.*, 1992). The amplification of the *prfA* gene with primers designed in this work was specific for only *L. monocytogenes*. No-cross reactivity with other bacteria was observed. This result indicated that the *prfA* gene was suitable for specific detection of *L. monocytogenes*.

Based on the specificity and ability to amplify in the m-PCR reaction, the suitable target genes were *uspA*, *fimY*, *ipaH*, and *prfA* for specific detection of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, respectively. The concentration of gene specific primers for *uspA*, *fimY*, *ipaH*, *prfA*, and 16S rRNA gene

amplifications were varied from 0.02-0.4 μ M. The annealing temperatures of the m-PCR reactions were varied from 50-59°C. The optimum concentrations of the primer set for amplification of target bacteria by m-PCR were found to be 0.02 μ M *ipaH*, 0.036 μ M *fimY*, 0.06 μ M *uspA*, 0.12 μ M *prfA*, and 0.4 μ M 16S rRNA (internal control). The optimum annealing temperature for the m-PCR was 52°C. The expected PCR products of 884, 489, 422, and 398 bp were found from specific amplification of both the reference and isolated strains of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, respectively (Figure 1).

Although minor variations of the biochemical characteristic profiles were found from the isolated strains used in this study, the amplification of specific target genes by m-PCR can be used to specifically identify the target bacteria with high accuracy. These results demonstrated that the specific detection of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* can be done using the m-PCR developed from this investigation. As all available data show, this is the first report to combine these target genes together

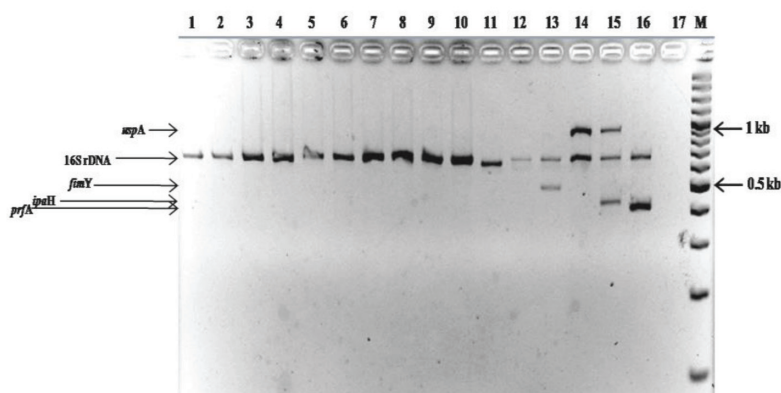


Figure 2. Specificity of the m-PCR amplification using gDNA of target and non-target bacteria as templates. Lanes: 1-4, non-*E. coli* isolates C2, 3, 4, 6, respectively; 5-7, non-*Salmonella* isolates RV2, RV3, TT1, respectively; 8-10, non-*Listeria* isolates L2, 4, 5, respectively; 11, *C. perfringens* isolate CP5; 12-17: *Staph. aureus* TISTR 517, *S. Enteritidis* JCM 1652, *E. coli* TISTR 887, *Shigella* sp. isolate Sh1, *L. monocytogenes* DSM 12464, negative control (H₂O), respectively; M, 100 bp DNA marker (Fermentas)

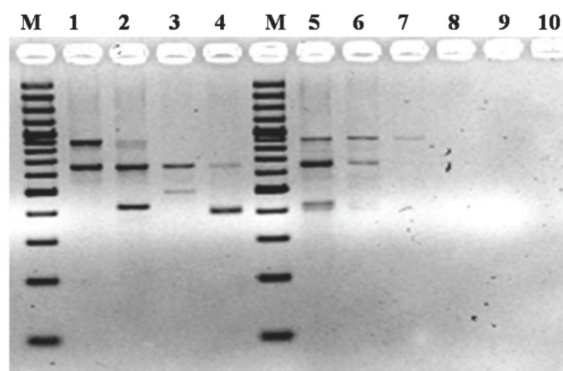


Figure 3. Sensitivity of the m-PCR amplification. A series of 10-fold diluted gDNA mixture of 4 target bacteria were used as templates for m-PCR amplification. Lanes: 1-4, 100 ng of gDNA template extracted from *E. coli* TISTR 887, *Sh. boydii* DMST 28180, *S. Enteritidis* JCM 1652, and *L. monocytogenes* DSM 12464, respectively; 5-9, a 10-fold series dilutions of the gDNA mixture templates ranging from 10-0.001 ng of each gDNA, respectively; 10, negative control (H_2O); M, 100 bp DNA marker (Fermentas)

for specific detection of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*.

Specificity and Sensitivity of the m-PCR Detection

The m-PCR specificity was also tested using non-target bacteria isolated from each enrichment culture (Table 1). The identification of non-target bacteria (Table 1) using several biochemical reactions indicated that these bacteria were Gram-negative, and identified as belonging to either non-*Salmonella*, non-*Listeria*, or non-*E. coli* bacteria (data not shown). Only the 16S rDNA gene product was detected from the non-target bacteria (Figure 2). These results demonstrated that the target genes reported here can be used for specific detection of the target bacteria. Thus these foodborne pathogens could be detected with high accuracy and no cross-reactivity with other non-target bacteria found in an enrichment culture.

The detection sensitivity of the assay was also determined using the gDNA mixture that was extracted from the pure culture of *Salmonella* serotype Enteritidis (*S. Enteritidis*) JCM 1652, *E. coli* TISTR 887, *Sh. boydii* DMST 28180, and *L. monocytogenes* DSM 12464. Ten-fold dilution series of gDNA mixtures ranging from 10-0.001 ng/ μ l were

used as templates for m-PCR amplifications. The sensitivity of the multiple target bacteria detection using m-PCR methods are shown in Figure 3. The results demonstrated that the detection limit of the m-PCR for 4 target bacteria detection was 10 ng of each gDNA which correspondstoapproximately 2×10^6 copies of the bacterial genome and was equivalent to 10^5 - 10^7 cells/ml of each target bacteria (10^5 cells/ml *Sh. boydii*, 10^6 cells/ml *S. Enteritidis* and *E. coli*, and 10^7 cells/ml *L. monocytogenes*). Results indicated that simultaneous detection of the 4 target pathogens was less sensitive than that of the 3 target pathogens (10^6 cells/ml of *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes*) as reported previously (Germini *et al.*, 2009). This might be due to the mixture of several primer sets leading to poor amplification efficiency in the m-PCR reaction. For these reasons, all target bacterial cells in food samples should be enriched by the enrichment steps prior to the application of the m-PCR methods.

Our results indicated that the developed m-PCR in our study could be used to detect multiple foodborne pathogens simultaneously. Alternatively, this method could be applied to identify the presumptive colonies of interest on selective agar with considerable time

saving in comparison to the biochemical characterization of the conventional culture method.

Conclusions

In conclusion, m-PCR can be successfully applied to detect multiple foodborne pathogens in this research. The target genes reported in this study can be used for *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* detection with no cross-reactivity with other non-target bacteria found in the enrichment culture. However, some weak points were also observed. The detection capability is still limited due to the low resolution of agarose gels for traditional PCR. The separation of all 5 amplicons on an agarose gel by electrophoresis was less sensitive and not sufficient. To avoid these problems in further study, the combination of m-PCR and a simple PCR validation step such as oligonucleotide array hybridization can be performed to specifically detect multiple target bacteria after the enrichment steps. Therefore, hybridization of the labelled m-PCR products with the array's immobilised probes will be used to enhance the accuracy and simplicity of the resultant interpretation of the m-PCR detection.

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