

NOVEL MULTIPLEX PCR ASSAY FOR RAPID DETECTION OF FIVE BACTERIAL FOODBORNE PATHOGENS

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Abstract

Milk and dairy products can harbor varieties of foodborne pathogens especially *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*. In this work, a rapid multiplex polymerase chain reaction (m-PCR) method for simultaneous detection of 5 major foodborne pathogens in milk was developed. Specific primers targetting the enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes were selected for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*, respectively. The optimum concentrations of the primers in the m-PCR reaction were 0.04 μ M enterotoxin *FM*, 0.12 μ M *uspA*, 0.16 μ M *prfA*, 0.04 μ M *fimY*, and 0.2 μ M *eap*. The expected polymerase chain reaction (PCR) products of 513, 884, 398, 315, and 230 bp were detected from the specific amplification of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*, respectively. Cross-amplifications from non-target bacteria isolated from raw milk samples were not detected. The developed m-PCR methods could detect all 5 target bacteria at the level of at least 100 ng of each from mixed genomic DNA extracted from pure cultures. These results indicated that the developed m-PCR using 5 primer sets can be used for *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* detection with no cross-reactivity with other non-target bacteria found in the enrichment culture. For future work, the m-PCR technique will be applied to detect multiple foodborne pathogens in enrichment cultures from milk samples with considerable timesaving and cost-effectiveness compared with the biochemical characterization of the conventional method.

Keywords: Foodborne pathogens, milk, multiplex PCR, target genes

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Introduction

Milk and dairy products are healthy foods for most people because they serve as good sources of calcium, vitamins, protein, and other essential nutrients. However, milk and dairy products can harbor varieties of microorganisms and can be important sources of foodborne pathogens (Oliver *et al.*, 2005). Foodborne diseases are some of the most widespread health problems in the world. Regulations for foodborne pathogens, including *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* in milks, are required (United States Food and Drug Administration, 1998). Therefore, detection of these organisms with rapid, sensitive, and easy methods is considered.

For simultaneous detection of multiple target bacteria, a multiplex polymerase chain reaction (m-PCR) has been applied. An m-PCR involves the simultaneous amplification of more than 1 target gene in a reaction by mixing multiple primer pairs with different specificities. Therefore, several primer sets are combined into a single PCR assay. Then, the PCR amplicons of different molecular weights can be separated by agarose gel electrophoresis (Settanni and Corsetti, 2007; Zhao *et al.*, 2014). Primer design is very important for the development of the m-PCR. The primer sets should have a similar annealing temperature in order to produce a successful m-PCR assay (Law *et al.*, 2015). Methods based on a m-PCR have been widely used and adapted for the rapid detection of single or multiple bacterial species. Li and Mustapha (2004) used a m-PCR to detect *E. coli* O157:H7, *Salmonella*, and *Shigella* in apple cider. After 24 h enrichment, *E. coli* O157:H7, *Salmonella*, and *Shigella* could be detected in low background bacterial samples including apple cider, cantaloupe, watermelon, and tomato and 80 CFU/g of these bacteria could be detected in alfalfa. Jofré *et al.* (2005) applied the m-PCR methods to detect 2 target bacteria, *L. monocytogenes* and *Salmonella* spp., in cooked ham targeted at the *prfA* and *invA* genes, respectively. After 48 h enrichment in buffer peptone water, 100 CFU/g of *L. monocytogenes* and *S. London* could be detected. Chen *et al.* (2012) developed a rapid

m-PCR method for simultaneous detection of 5 foodborne pathogens, *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* Enteritidis, and *Shigella flexneri*. The developed method was applied to retail meat samples. A higher consistency was obtained between the m-PCR results and traditional culture methods (Chen *et al.*, 2012). In 2013, the m-PCR assay was developed in our previous work for specific detection of *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. using the *uspA*, *prfA*, *fimY*, and *ipaH* genes as targets. All 4 target bacteria could be specifically detected in single detection with no cross-reactivity with the non-target bacteria found in the enrichment culture (Kupradit *et al.*, 2013). Tekiner and Özpınar (2015) evaluated the performance of a real-time PCR fourplex assay in simultaneous detection of 4 foodborne pathogenic bacteria: *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*. The artificial milk sample inoculated with 4 target bacteria were tested for the efficiency and sensitivity of the detection method. The best performance of the detection method was using a real-time PCR triplex for detection of *S. aureus*, *L. monocytogenes*, and *Salmonella* in a concentration of 10-100 CFU/ml. However, a m-PCR for 5 bacterial detections, those of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* which are the regulated pathogens in milk, has not been reported.

The purpose of this research was to develop the m-PCR assay to specifically detect *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*. The condition of the m-PCR was optimized and the specificity of the developed method was evaluated. The genomic DNA (gDNA) extracted from target and non-target bacteria isolated from raw milk was used as the DNA templates for the validation of the m-PCR assay.

Materials and Methods

Bacterial Strains and Cultivation

All bacterial reference strains and isolates used to evaluate the m-PCR assay are shown in Table 1. All isolates of target and non-target bacteria were identified using biochemical

characteristic profiles, as described by the United States Food and Drug Administration (1998) (no data shown). All bacteria were grown on trypticase soy agar (tryptose 15 g/L, proteose peptone 5 g/L, sodium chloride 15 g/L, and agar 15 g/L) under aerobic conditions at 37°C for 24 h.

Primer Design

For genus or species specific genes, primers were designed based on the conserved regions of each gene in each target bacterium. Primers were designed using PrimerSelect DNASTar Lasergene 7 (DNASTAR, Inc., Madison, WI, USA) based on the conserved regions of each specific gene. The genus

specific gene was *fimY* for specific detection of *Salmonella* spp. Species specific genes were *uspA* (Chen and Griffiths, 1998) and *prfA* (Kupradit *et al.*, 2013) for specific detection of *E. coli* and *L. monocytogenes*, respectively. For detection of *B. cereus*, primers for specific amplification of the enterotoxin *FM*, *hblA*, and *hblD* genes were designed and validated. The gene-specific primers for detection of *S. aureus* were designed to detect the *eap* (Hussain *et al.*, 2008), *seG*, *seGV*, *seM*, *seI*, and *seIV* genes. Sequences of all forward and reverse primers are shown in Table 2. All primers were also tested for specificity with gDNA extracted from the reference and isolated bacterial strains in Table 1.

Table 1. Bacterial isolates used for the validation of the m-PCR

Bacterial species	Number of isolates	Isolates' numbers and sources
<i>Bacillus</i> spp.	16	<i>B. subtilis</i> TISTR ^a 1248, 1528 <i>B. amyloliquefaciens</i> TISTR ^a 1045 <i>B. cereus</i> TISTR ^a 687, 1474, 1449, 1453, 1527 <i>B. cereus</i> isolate BC ^b PTC_3, PTC_4, PTC_6, PTC_8, PTC_9, PTC_10, PCNS_1, PM_1
<i>Escherichia coli</i>	19	<i>E. coli</i> TISTR ^a 361, 371, 887 <i>E. coli</i> isolate EC ^b PM_1, PCF_6, 4PC_1, CP_8, 4CP_1, 4CP_3, 2PCNS_2, 4PCNS_2, PK_5, 2PK_1, 3SK_1, 4SK_2, PTC_4, 3PTC_1, 4PTC_1, 4SS_1
<i>Listeria</i> spp.	11	<i>Listeria</i> sp. JCM ^a 7679 <i>L. innocua</i> DSM ^a 20649 <i>L. monocytogenes</i> DSM ^a 12464, DMST ^a 1327, 17303, 2871, 20093, 21164, 23136, 23145, 31802
<i>Staphylococcus aureus</i>	31	<i>S. aureus</i> TISTR ^a 517, 746 <i>S. aureus</i> isolate SA ^b 3PM_1, 3PM_5, 2PC_1, 3PC_2, 4PC_1, 4PC_2, 4PC_3, CP_5, 2CP_2, 2CP_8, 3CP_1, 3CP_5, 4CP_1, 4CP_2, 4CP_4, 4CP_5, PK_1, 4PK_1, 4PK_2, 4PK_3, 2SK_3, 3SK_1, 4SK_1, 2KS_4, 3KS_8, 4KS_6, 2PTC_4, 4KS_2, 3KS_7
<i>Salmonella</i> spp.	3	<i>S. Enteritidis</i> JCM ^a 1652 <i>S. Typhimurium</i> TISTR ^a 292, 1470
Non-target bacteria	21	NT ^c CP_R1, 3KS_B1, 4KS_P2, PCT2M_C4, 2PC_BC2, 3PC_BC4, 2PCNS_B2, 3PCNS_C1, 4PCNS_BC1, 4PCNS_W1, PK_B2, 2PK_C1, 2PK_E1, 3PTC_B4, PM_BK1, 3PM_C1, 4PM_Y3, 2SK_BC1, 4SS_B1, 4SS_C1, 4SS_C2

^a Sources of bacteria references: 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 Bacteria were strains isolated from raw milk of the Milk Collection Center in Nakhon Ratchasima, Thailand: BC, *B. cereus*, isolated on MYP agar (Himedia); EC, *E. coli* bacteria isolated on EMB agar (Himedia); SA, *S. aureus* isolated on Baird Parker agar (Himedia)

^c Non-target bacteria were isolated from the Milk Collection Center in Nakhon Ratchasima, Thailand

Target Gene Amplification by m-PCR

Genomic DNA from 16–24 h grown pure cultures on TSA was extracted using the simple protocol of the phenol-chloroform-based method (Kupradit *et al.*, 2013). The gDNA pellets were then resuspended in 100 µl of 10 mM Tris-Cl, 1 mM EDTA (TE), pH 8.0 and 10 µg/ml RNaseA. The gDNAs were used as templates for amplification of the target genes using specific primers. The reference strains of all target bacteria were tested for the specificity of each gene. The suitable specific genes of each target bacterium were used for the m-PCR optimization. In the m-PCR reactions, the concentrations of all gene-specific primers were optimized.

The m-PCR reactions were performed as described by Kupradit *et al.* (2013). For the gene-specific primer test, only 16S rRNA and all gene-specific primers were combined in the m-PCR reaction. A total volume of 25 µl m-PCR reaction contained 1× GoTaq Flexi buffer (Promega Corp., Madison, WI, 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), 10 ng DNA templates, and 0.4 µM of each of the gene-specific primers. The PCR reactions were heated at 95°C for 3 min; then, there were 35 cycles at 95°C for 30 s, at 52°C for 45 s, and at 72°C for 60 s followed by a final step of 5 min incubation at 72°C. For the detection of the 5 target bacteria in the mixed samples, the concentrations of 5 specific primer pairs were optimized. The m-PCR products were analyzed by agarose gel electrophoresis on 1.5% agarose gel.

Results and Discussion

Specific Gene Screening and Selection

Gene-specific primers for detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* were designed. The specific gene selection for the m-PCR reactions was performed using each specific primer pairs and 16S rRNA primers (Table 2). The specificities of the enterotoxin *FM*, *hblA*, *hblD*, *uspA*, *prfA*, *fimY*, *eap*, *seG*, *seGV*, *seM*, *seI*, and *seIV* genes (Table 2) were tested using gDNA extracted from reference strains of each target bacterium (Table 1). Results of the specificity of each

of the gene-specific primers are shown in Table 3. For detection of *L. monocytogenes* and *Salmonella* spp., the *prfA* and *fimY* genes were suitable because of their specificity with no cross-reaction with other non-target bacteria (Table 3). These results were similar to those reported by Kupradit *et al.* (2013). For *E. coli* detection, the *uspA* gene was shown to be conserved among all the reference strains of *E. coli* (Table 1) and strains that were isolated from raw milk samples. For these reasons, the *uspA* gene was used for *E. coli* detection in this work.

For *B. cereus* detection, the enterotoxin *FM*, *hblA*, and *hblD* primers were validated. Results revealed that among 5 reference strains of *B. cereus*, only 1 strain, *B. cereus* TISTR 1474, showed positive results from the enterotoxin *FM*, *hblA*, and *hblD* genes. Similar results were reported by Wiwat and Thiramanas (2014). Only 48% of *B. cereus* isolates showed positive results with the hemolysin BL (HBL), *hblA*, *hblC*, and *hblD* genes, while 47 % showed positive results with all *hbl* genes including the *hblB* gene (Wiwat and Thiramanas, 2014). However, the enterotoxin *FM* gene was also used as a target gene for specific detection of *B. cereus* in this work. PCR products of the enterotoxin *FM* gene were detected from all 5 reference strains of *B. cereus* (Table 3). These results indicated that the enterotoxin *FM* gene was conserved among all 5 reference strains of *B. cereus*. Moreover, this enterotoxin *FM* gene was conserved among *B. cereus* isolated from all 8 raw milk samples with no cross-amplification from other *Bacillus* species (no data shown).

For specific detection of *S. aureus*, the *eap*, *seG*, *seGV*, *seM*, *seI*, and *seIV* genes were tested. Only 1 of 2 reference strains, *S. aureus* TISTR 517, showed positive results for all the enterotoxin genes (*seG*, *seGV*, *seM*, *seI*, and *seIV*) and *eap* gene detection. Staphylococcal enterotoxins (SEs) are a family of structurally related pyrogenic exotoxins consisting of the 5 prototypic SEs (types A to E) and 3 newly characterized SEs (types G to I) produced by *S. aureus*. They also work as superantigens and cause food poisoning and shock symptoms in humans (Abe *et al.*, 2000). The variations of enterotoxin genes in *S. aureus* have been previously reported. Abe *et al.* (2000) characterized and investigated the distribution

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

Target bacteria	Target gene	Primer name	Primer sequences (5'→3')	PCR product size (bp)	References
<i>S. aureus</i>	<i>eap</i>	SA_eap_F1	TTAAATCGATATCACTAATACCTC	230	Hussain <i>et al.</i> , 2008
		SA_eap_R1	TACTAACGAAGCATCTGCC		
	<i>sel</i>	SA_Ent_I_F191	TGATTATAGATTTAAAGGGCGTCACA	515	This work
		SA_Ent_I_R705	GCAGTCCATCTCTGTATAAAACAA		
	<i>seG</i>	SA_Ent_GV_F340	AGGTTAAAACTGAATTAGAAAATAC	312	This work
		SA_Ent_GV_R651	CTTTAGTGAGCCAGTCTCTGC		
	<i>seM</i>	SA_Ent_M_F34	CAATCATAACTTAGTAAAGGAAATGC	430	This work
		SA_Ent_M_R463	CAGTAGAAAATGTTTATGTTGCC		
	<i>seIV</i>	SA_Ent_IV_F269	TGGATATTTTGGCATTTGATTA	265	This work
		SA_Ent_IV_R533	TCTTACCTTTAGCATTGTATTA		
<i>B. cereus</i>	<i>seG</i>	SA_Ent_G_F_35	AGACTGAATAAGTTAGAGGAGGTTTA	700	This work
		SA_Ent_G_R_752	GGAACAAAAGGTACTAGTCTTTTTTA		
	<i>hblD</i>	BC_hblD_F227	GGTTAGATACAGGAAGCCACAG	409	This work
		BC_hblD_R638	GCTCCCAATCCACCACCAAT		
	<i>hblA</i>	BC_hblA_F181	ATTGCAAAATCTATGAATGCC	672	This work
		BC_hblA_R852	GCAACTCCAACTACACGATTAA		
	enterotoxin <i>FM</i>	BC_EntFM_F200	TGCTGATGTATTAAATGTTCTGTTTC	513	This work
		BC_EntFM_R713	GCGTTGTATGTAGCTGGGCCT		
	<i>uspA</i>	EC_uspA_F	CCGATACGCTGCCAATCAGT	884	Chen and Griffiths, 1998
		EC_uspA_R	ACGCAGACCGTAGGCCAGAT		
<i>L. monocytogenes</i>	<i>prfA</i>	LM_prfA_F	CACAAGAATATTGTATTTTCTATATGAT	398	Kupradit <i>et al.</i> , 2013
		LM_prfA_R	CAGTGTAACTTGTATGCCATCA		
<i>Salmonella</i> spp.	<i>fimY</i>	SM_fimY_F	GCCTCAATACAGGAGACAGGTAGCG	315	This work
		SM_fimY_R	GCAGGGAAGACACGCCGTTTAA		
All bacteria	16S rRNA	16S_F	AGACTCCTACGGGAGGC	625-655	Kupradit <i>et al.</i> , 2013
		16S_R	GGTAAGGTTCTTCGCGT		

of a new enterotoxin-related superantigen produced by *S. aureus*. They suggest that *seG*, or *seGV*, is one of the most frequently produced superantigen exotoxins by *S. aureus*. Blaiotta *et al.* (2004) found that only 11 *S. aureus* of 109 wild *Staphylococcus* spp. strains analyzed were SE (enterotoxin gene) and/or TSST1 (toxic shock syndrome toxin 1) PCR-positive. Therefore, these genes were not suitable to be used as targets for *S. aureus* detection using the m-PCR technique because of the variation of these genes among *S. aureus*. In this work, only the amplified product of the *eap* gene was detected from both *S. aureus* TISTR 517 and TISTR 746. Moreover, 93% of coagulase-positive *S. aureus* isolated from the raw milk samples showed the product of *eap* gene amplification. The anchorless extracellular adherence protein (Eap) of

S. aureus designated major histocompatibility complex class II analogous protein (Map), selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. In a previous report, the presence of the Eap-encoding gene (*eap*) was determined to occur in only selected human clinical *S. aureus* isolates (Hussain *et al.*, 2008). In this work, only 7% of coagulase-positive *S. aureus* isolated from raw milk showed PCR-negative results from *eap* gene amplification. These 7% of *Staphylococcus* isolates might be the other coagulase-positive staphylococcal species. As reported by Hussain *et al.* (2008), the *eap* gene was suitable for molecular diagnostics of *S. aureus* because of its sensitivity and specificity. The coagulase-negative strain and other coagulase-positive or -variable

Table 3. Detection of target bacteria using m-PCR amplification with gene-specific primers

Bacterial species	Target gene ^a											
	BC ^b		EC ^b		LM ^b		SM ^b		SA ^b			
	<i>hblD</i>	<i>ent FM</i>	<i>hblA</i>	<i>uspA</i>	<i>prfA</i>	<i>fimY</i>	<i>eap</i>	<i>seIV</i>	<i>seGV</i>	<i>seM</i>	<i>seI</i>	<i>seG</i>
<i>Bacillus subtilis</i> TISTR 1248	-	-	-	-	-	-	ND	-	-	-	-	-
<i>B. subtilis</i> TISTR 1528	-	-	-	-	-	-	ND	-	-	-	-	-
<i>B. amyloliquefacies</i> TISTR 1045	-	-	-	-	-	-	ND	-	-	-	-	-
<i>B. cereus</i> TISTR 687	-	+	-	-	-	-	-	-	-	-	-	-
<i>B. cereus</i> TISTR 1474	+	+	+	-	-	-	-	-	-	-	-	-
<i>B. cereus</i> TISTR 1449	-	+	-	-	-	-	-	ND	ND	ND	ND	ND
<i>B. cereus</i> TISTR 1453	-	+	-	-	-	-	-	ND	ND	ND	ND	ND
<i>B. cereus</i> TISTR 1527	-	+	-	-	-	-	-	ND	ND	ND	ND	ND
<i>Escherichia coli</i> TISTR 887	-	-	-	+	-	-	-	-	-	-	-	-
<i>E. coli</i> TISTR 361	ND	-	ND	+	-	-	-	ND	ND	ND	ND	ND
<i>E. coli</i> TISTR 371	ND	-	ND	+	-	-	-	ND	ND	ND	ND	ND
<i>Listeria</i> sp. JCM 7679	-	-	-	-	-	ND	ND	-	-	-	-	-
<i>L. innocua</i> DSM 20649	-	-	-	-	-	ND	ND	-	-	-	-	-
<i>L. monocytogenes</i> DMST 23136	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 23145	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 21164	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 20093	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 17303	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 1327	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 31802	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DMST 2871	-	-	-	-	+	-	-	-	-	-	-	-
<i>L. monocytogenes</i> DSM 12464	-	-	-	-	+	-	-	-	-	-	-	-
<i>S. aureus</i> TISTR 517	-	-	-	-	-	-	+	+	+	+	+	+
<i>S. aureus</i> TISTR 746	ND	-	ND	-	-	-	+	-	-	-	-	-
<i>Salmonella</i> Enteritidis JCM 1652	-	-	-	-	-	+	-	-	-	-	-	-
<i>S. Typhimurium</i> TISTR 292	-	-	-	-	-	+	-	-	-	-	-	-
<i>S. Typhimurium</i> TISTR 1470	ND	-	ND	-	-	+	-	ND	ND	ND	ND	ND

^a+: The expected sizes of PCR products were detected;

-: The expected sizes of PCR products were not found

^b Target bacteria: BC: *B. cereus*; EC: *E. coli*; LM: *L. monocytogenes*;

SM: *Salmonella* spp.; SA: *S. aureus*

ND: Not determined

staphylococcal species were shown to miss the encoding gene at the DNA, transcriptional, and protein levels (Hussain *et al.*, 2008). For these reasons, the enterotoxin *FM* and *eap* genes were used as specific genes for *B. cereus* and *S. aureus* detection, respectively.

Based on the specificity and ability to amplify in the m-PCR reaction, the suitable target genes were enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*, respectively.

Optimization of the m-PCR

The concentration of gene-specific primers for amplification of the enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes varied from 0.04-0.28 μ M. The optimum concentrations of the primers in the m-PCR reaction were 0.22 μ M 16S rRNA, 0.04 μ M enterotoxin *FM*, 0.12 μ M *uspA*, 0.16 μ M *prfA*, 0.04 μ M *fimY*, and 0.2 μ M *eap* primers. The expected PCR products of 513, 884, 398, 315, and 230 bp were detected from the specific amplification of the reference strains,

B. cereus, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*, respectively (Figure 1(a)). The optimum conditions of the m-PCR were also applied to amplify the target genes from isolates of *E. coli* (16 isolates), *B. cereus* (8 isolates), and *S. aureus* (29 isolates). The results found that all of the *B. cereus* and *E. coli* isolates showed only the expected PCR product of 530 and 884 bp from the enterotoxin *FM* and *uspA* gene amplifications, respectively. For *S. aureus*, 27 of the 29 isolates (93%) showed the expected PCR product of 230 bp from the *eap* gene amplification (no data shown). These results indicated that almost all the reference and isolated target bacteria could be specifically detected by the m-PCR at the optimum condition.

Specificity and Sensitivity of m-PCR

The m-PCR specificity was also tested using gDNA extracted from non-target bacteria isolated from raw milk samples as a template (Table 1). The identification of non-target bacteria using several biochemical

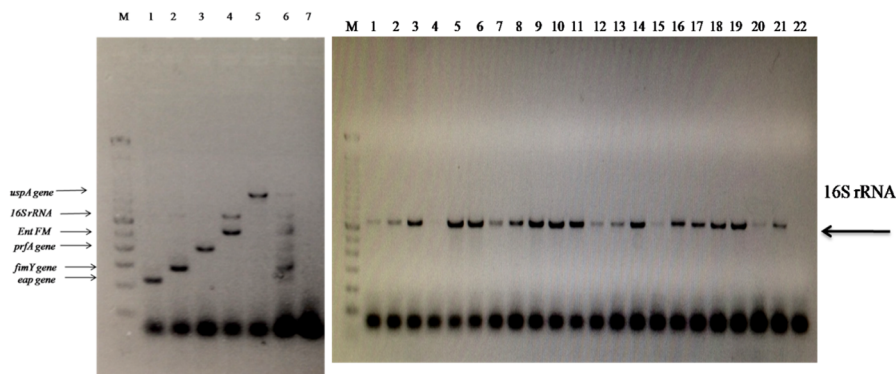


Figure 1. Specific gene amplification from gDNA extracted from pure culture of target bacteria using m-PCR assay containing the combination of 16S rRNA, enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* gene-specific primers. (a) Specific detection of target bacteria by the m-PCR technique using 10 ng of gDNA as a template. Lane: M, Molecular weight marker (100 bp ladder, Invitrogen); Lane: 1-5, *S. aureus* TISTR 517; *S. Typhimurium* TISTR 292; *L. monocytogenes* DMST 23136; *B. cereus* TISTR 1474; *E. coli* TISTR 887; Lane 6: mix gDNA from 5 target bacteria; Lane: 7, negative control (H_2O). (b) Detection of non-target bacteria by m-PCR technique using 10 ng of gDNA as a template. Lane: M, Molecular weight marker (100 bp ladder, Invitrogen); Lane 1-22 (non-target bacterial isolates), non-target bacterial isolate NT_CP_R1; NT_3KS_B1; NT_4KS_P2; NT_PCT2M_C4; NT_2PC_BC2; NT_3PC_BC4; NT_2PCNS_B2; NT_3PCNS_C1; NT_4PCNS_BC1; NT_4PCNS_W1; NT_PK_B2; NT_2PK_C1; NT_2PK_E1; NT_3PTC_B4; NT_PM_BK1; NT_3PM_C1; NT_4PM_Y3; NT_2SK_BC1; NT_4SS_B1; NT_4SS_C1; NT_4SS_C2; H_2O , respectively

reactions indicated that these bacteria were non-target bacteria. Only the 16S rRNA gene product was detected from the non-target bacteria (Figure 1(b)). These results demonstrated that the target genes reported here can be used for specific detection of the target bacteria. Thus, the developed m-PCR in this work can be used to detect multiple target bacteria in an enrichment culture from milk samples with high accuracy and specificity.

The multiple target bacterial detection using 6 primer sets including the 16S rRNA gene by the m-PCR showed that only faint bands of PCR products were detected from each target bacterium and mixed gDNA from all target bacteria (Figure 1(a)). Thus, only 5 gene-specific primers, the enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes were combined at the optimum concentration in the m-PCR reaction to increase the sensitivity of detection. Large amounts of expected PCR products from each target gene were observed on agarose gel when the 10 ng of each gDNA was amplified using 5 primer pairs in the

m-PCR (Figure 2). The developed m-PCR assay was also applied to detect multiple target bacteria using mixtures of gDNA from each target bacterium (10 ng of each) as templates. Results found that the specific products of each target gene detected showed the pattern of the PCR product specific for each of the target bacterium that was contained in the mixture (Figure 3). Thus, sensitivity of detection can be improved by using only 5 primer sets in the m-PCR (Figure 2 and 3). This might be due to the mixture of several primer sets leading to poor amplification efficiency in the m-PCR.

The detection sensitivity of the assay was determined using the gDNA mixture that was extracted from the pure culture of *B. cereus* TISTR 1474, *E. coli* TISTR 887, *L. monocytogenes* DMST 23136, *S. Typhimurium* TISTR 292, and *S. aureus* TISTR 517. The concentrations of gDNA mixtures ranging from 100-1 ng were used as templates for m-PCR amplifications at the optimum conditions. The sensitivity of the detection of the 5 target bacteria using the m-PCR techniques is shown

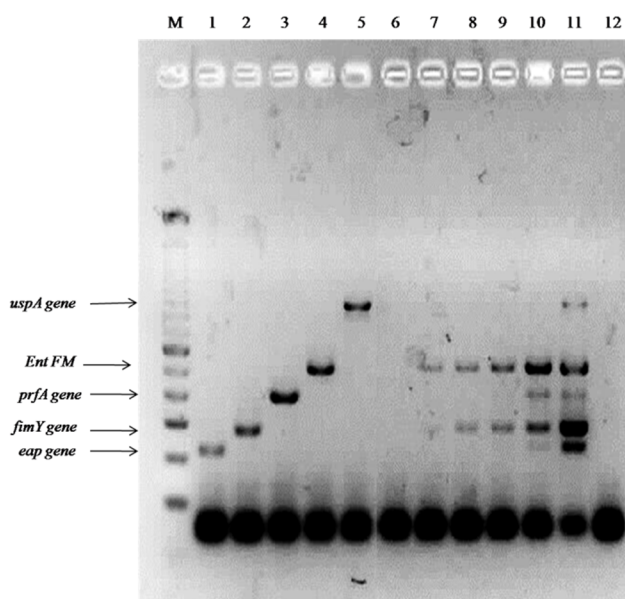


Figure 2. Sensitivity of the m-PCR amplification. The mixtures of gDNA at concentrations of 1-100 ng from 5 target bacteria were used as templates for m-PCR amplification. Lanes: 1-5, 10 ng of gDNA template extracted from *S. aureus* TISTR 517; *S. Typhimurium* TISTR 292; *L. monocytogenes* DMST 23136; *B. cereus* TISTR 1474; and *E. coli* TISTR 887, respectively. Lane: 6-11, the gDNA mixture templates ranging from 1 ng; 3 ng; 5 ng; 7 ng; 10 ng; and 100 ng of each target bacterium, respectively. Lane:12, negative control (H₂O)

in Figure 2. The detection limit of the m-PCR for the detection of the 5 target bacteria was 100 ng of each gDNA. However, the sensitivity of detection using the developed m-PCR in this work was 10 ng of each target bacterium when applied to detect 2 or 3 mixed target DNA templates with high accuracy and specificity (Figure 3). For 4 target bacteria detection in mixed gDNA templates (10 ng of each) containing *L. monocytogenes*, only small amounts of *prfA* product were observed (Figure 3). These results indicated that simultaneous detection of the 4 and 5 target pathogens was less sensitive than that of the 3 target pathogens. Thus, all target bacterial cells in food samples should be enriched with the enrichment steps prior to the application of the m-PCR methods. To obtain highly accurate results of the developed m-PCR assay, gDNA templates of each target bacterium should be maintained in the reaction with at least 100 ng of each.

Our results indicated that the developed m-PCR in our study could be used to detect

multiple foodborne pathogens simultaneously in milk samples with high accuracy and specificity.

Conclusions

The conventional methods for detecting pathogens in food involve isolation followed by biochemical identification for each pathogen. They are very laborious and time consuming (De Boer and Beumer, 1999; You *et al.*, 2008). Therefore, rapid, specific, and sensitive methods such as the m-PCR have been developed in this research for detecting and identifying pathogens.

In conclusion, the m-PCR can be successfully applied to detect multiple foodborne pathogens in this research. To develop an efficient m-PCR, specific genes were screened and the concentrations of primers were optimized. The m-PCR developed in this study showed a specific band pattern for each target bacterium, thus confirming the

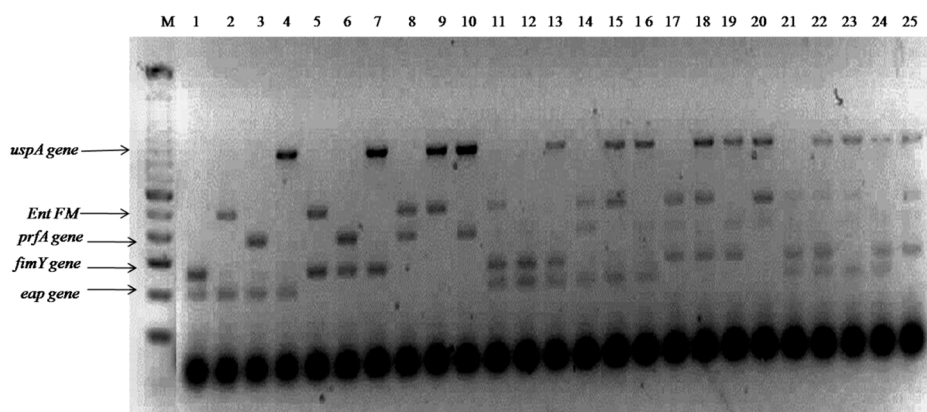


Figure 3. Multiple target bacteria detection using m-PCR containing the combination of enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* gene-specific primers. A mixture of gDNA extracted from *S. aureus* TISTR 517 (SA), *S. Typhimurium* TISTR 292 (ST), *B. cereus* TISTR 1474 (BC), *L. monocytogenes* DMST 23136 (LM), and *E. coli* TISTR 887 (EC) was used as a template at 10 ng. Lane: M, Molecular weight marker (100 bp ladder, Invitrogen); Lane: 1-4, mixture of SA+ST; SA+BC; SA+LM; SA+EC, respectively. Lane: 5-7, mixture of ST+BC; ST+LM; ST+EC, respectively. Lane: 8-10, mixture of BC+LM; BC+EC; LM+EC, respectively. Lane: 11-13, mixture of SA+ST+BC; SA+ST+LM; SA+ST+EC, respectively. Lane: 14-16, mixture of SA+BC+LM; SA+BC+EC; SA+LM+EC, respectively. Lane: 17-18, mixture of ST+BC+LM; ST+BC+EC, respectively. Lane: 19-20, mixture of LM+EC+ST; LM+EC+BC, respectively. Lane: 21-22, mixture of SA+ST+BC+LM; SA+ST+BC+EC, respectively. Lane: 23-25, mixture of SA+LM+EC+BC; SA+LM+EC+ST; ST+BC+LM+EC, respectively.

specificity of the 5 sets of primers. The target genes, the enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes, can be used for *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* detection, respectively, with no cross-reactivity with other non-target bacteria found in the enrichment culture. The detection limit of the m-PCR for detection of the 5 target bacteria was 100 ng of each gDNA. However, 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 the m-PCR validation steps such as oligonucleotide array hybridization can be performed to simultaneously detect multiple target bacteria after the enrichment steps. 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.

In our future work, optimization of the enrichment steps of all target bacteria followed by m-PCR amplification and hybridization will be performed to improve the sensitivity of simultaneous multiple pathogen detection in milk.

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