Specific Detection of Five Bacterial Foodborne Pathogens by Oligonucleotide Macroarray

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Abstract

Milk and dairy products can be contaminated with a variety of microorganisms. Thus, a rapid method for simultaneous detection of multiple foodborne pathogens should be considered. In this investigation, a combination of multiplex PCR (m-PCR) and oligonucleotide array hybridization was performed to specifically detect multiple foodborne pathogens. Specific genes, including Enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes, were selected as targets for detection of *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*, respectively. The suitable probes for specific detection of five foodborne pathogens were selected based on the specificity of detection. The hybridization signals of digoxigenin (DIG) incorporated into the PCR target regions were observed by naked eyes. Differences of hybridization patterns were observed among isolated and reference strains of *B. cereus* and *S. aureus*. High accuracies of specific probes, including, probes BC1, BC3 and BC5 (30–100% accuracy); probes EC1–EC4 (83–100% accuracy); probes LM1, LM2, LM4 (87–100% accuracy); probes SA1–SA5 (75–100% accuracy); and probes SM1, SM3 (66–100% accuracy), were selected for detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Salmonella* spp., respectively. For future work, the DNA target of each bacterium will be amplified by PCR followed by hybridization with the suitable probes obtained from this work.

Keywords: Multiplex PCR (m-PCR), Oligonucleotide array, Probes, Foodborne pathogens

1. Introduction

The most widespread of health problems in the world are foodborne diseases. Foodborne pathogens could contaminate various foods including milk and dairy products. Milk and dairy products are basic components of human diet. They provide a dietary source of proteins, vitamins and minerals. However, it also serves as a good medium for the growth of many microorganisms, especially bacterial pathogens including those belonging to the families Enterobacteriaceae, Streptococcaceae, and Bacillaceae (Bartoszewicz *et al*., 2008).

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In Thailand and many countries, the prevalence of foodborne pathogens especially *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* in raw milk and dairy products have been reported (Chye *et al.*, 2004; Van Kessel *et al.*, 2004; Padungtod and Kaneene, 2006; Chitov *et al.*, 2008; Bianchi *et al.*, 2013; Kanungpean *et al.*, 2014; Ombarak *et al.*, 2016). Therefore, detection of these organisms with high accuracy methods is required.

Molecular based methods such as oligonucleotide array and multiplex polymerase chain reaction (m-PCR) have been applied to detect multiple pathogens for time and labor saving (Yoo *et al.*, 2004; Nugen and Baeumner, 2008). M-PCR involves the simultaneous amplification of more than one target gene per reaction by mixing multiple primer pairs with different specificities. The PCR amplicons of different molecular weight is separated by agarose gel electrophoresis (Settanni and Corsetti, 2007). This method has been widely used and adapted for the rapid detection of single and multiple bacterial species, for example, *B. cereus*, *E. coli*, *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *L. monocytogenes* and *S. aureus* (Whyte *et al.*, 2002; Yeh *et al.*, 2002; Li *et al.*, 2005; Nierop *et al.*, 2005; Germini *et al.*, 2009; Fusco *et al.*, 2011; He *et al.*, 2011; Bang *et al.*, 2013; Kupradit *et al.*, 2017). However, m-PCR validation steps are still needed to improve the detection accuracy of each pathogen. Even though m-PCR is able to amplify multiple targets in a single tube, the detection capability is still limited to a few targets per assay because of the low resolution of agarose gels in traditional PCR (Severgnini *et al.*, 2011). Therefore, a simple and high accuracy method, such as Deoxyribonucleic acid (DNA) hybridization are considered to improve the accuracy of m-PCR.

An essential feature of the DNA array technique is the hybridization of labeled DNA fragments with arrays of immobilized probes (Gauthier and Blais, 2003). For oligonucleotide array assay, specific probe selection step is very important. Both 16S Ribosomal Ribonucleic acid (16S rRNA) and species-specific genes can be used as target for probe design. The advantage of the 16S rRNA gene amplification followed by DNA hybridization is that several pathogens can be detected using a single pair of primers for PCR amplification. However, limitation of 16S rRNA gene was its diversity (Kupradit *et al.*, 2014). In this research, bacterial pathogen identifications using species-specific genes as targets were performed to avoid the cross-reactivity of 16S Ribosomal Deoxyribonucleic acid (16S rDNA) probe.

In this study, a pathogen detection methods using combination of m-PCR and oligonucleotide array were developed and evaluated. The oligonucleotide probes were designed from target genes including the enterotoxin *FM*, *uspA*, *prfA*, *fim Y* and *eap* genes for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *S. aureus*,

respectively. M-PCR products were distinguished from each other in single array by post-PCR labeling using digoxigenin (DIG) and hybridizations. Easy systems for hybridization signal detection and result interpretation from oligonucleotide array using immunological chromogenic reaction which could be observed by naked eyes were performed. The suitable probes in this research were selected and would be used to detect the multiple foodborne pathogens in milk samples.

2. Materials and Methods

2.1 Bacterial strains and cultivation

All bacterial reference and isolated strains used to validate oligonucleotide array probes are listed in Table 1. Isolated strains used in this study were identified as described by the United States Food and Drug Administration – Bacteriological Analytical Manual (United States Food and Drug Administration, 1998). All target bacteria 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, at 37 ºC for 24–48 h.

Table 1 Bacterial strains used for the validation of oligonucleotide array

Species	Number of strains	Strain number and sources
Bacillus cereus	10	*B. cereus* TISTR[a] 687, 1474, 1449, 1453, 1527, *B. cereus* isolate BC[b] PTC_3, PTC_6, PTC_9, PCNS_1, PM_1
Escherichia coli	6	*E. coli* TISTR[a] 361, 371, 887 *E. coli* isolate EC[b] 2PCNS_2, 3SK_1, 3PTC_1
Listeria spp.	8	*L. monocytogenes* DMST[a] 1327, 17303, 2871, 20093, 21164, 23136, 23145, 31802
Staphylococcus aureus	8	*S.aureus* TISTR[a] 517, 746 *S. aureus* isolate SA[b] 3PM_1, 3PM_5, 3PC_2, CP_5, PK_1, 2SK_3
Salmonella spp.	3	*S.* Enteritidis JCM[a] 1652 *S.* Typhimurium TISTR[a] 292, 1470

Note: [a] Reference strain: DMST, The Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand; JCM, Japan Collection of Microorganisms; TISTR, Thailand Institute of Scientific and Technology Research

[b] Strains isolated from raw milk of Milk Collection Center in Nakhon Ratchasima, Thailand: BC, *B. cereus*, isolated on Mannitol Yolk Polymyxin agar (MYP; Himedia); EC, *E. coli* bacteria isolated on Eosin-Methylene Blue agar (EMB; Himedia); SA, *S. aureus* isolated on Baird Parker agar (Himedia).

2.2 Oligonucleotide probe design

The species-specific genes, including enterotoxin *FM*, *uspA*, *prfA*, *fimY* and *eap* genes were used as target for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *S. aureus*, respectively. The nucleotide sequences of amplified products from each target gene obtained from PrimerSlect DNASTAR Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA) were used for probe design.

Probes for each target pathogen detection by oligonucleotide array were designed based on the conserve regions of each target genes using the PICKY oligonucleotide design program (Chou *et al*., 2004).

2.3 Target gene amplification by m-PCR technique

The genomic DNA templates for target gene amplification were prepared from 24 h grown pure cultures on TSA using the simple protocol of phenol-chloroform based method (Kupradit *et al*., 2013a, 2013b).

Primer designed and optimum condition for m-PCR amplifications were described previously (Kupradit *et al*., 2017). Briefly, a total volume of 25 µL m-PCR reaction contained 1X GoTaq Flexi buffer (Promega, Madison, USA), 1 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), primers of 0.04 µM enterotoxin *FM*, 0.12 µM *uspA*, 0.16 µM *prfA*, 0.04 µM *fimY*, and 0.2 µM *eap*, and 0.5 U GoTaq Flexi DNA polymerase (Promega), 10 ng DNA templates of each target bacteria (Kupradit *et al*., 2017). Sequences of gene specific primers are shown in Table 2. The PCR reactions were heated at 95 ºC for 3 min and then, 35 cycles at 95 ºC for 30 s, 52 ºC for 45 s, and 72 ºC for 60 s followed by a final step of 5 min incubation at 72 ºC. The products of m-PCR were analyzed by electrophoresis on 1.5% agarose gel (Invitrogen Life Technologies, California, USA) and purified using PureLink PCR Purification Kit (Invitrogen Life Technologies). The concentration of genomic DNA template and PCR products were measured by Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). All purified m-PCR products from each target bacteria were used for probe validation.

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

Target bacteria	Target gene	Primer name	Primer sequences (5'->3')	PCR product size (bp)	References
B. cereus	enterotoxin *FM*	BC_Ent*FM*_F200	TGCTGATGTATTAAATGTTCGTTC	513	Kupradit *et al*., 2017
		BC_Ent*FM*_R713	GCGTTGTATGTAGCTGGGCCT		
E. coli	*uspA*	EC_*uspA*_F	CCGATACGCTGCCAATCAGT	884	Chen and Griffiths, 1998
		EC_*uspA*_R	ACGCAGACCGTAGGCCAGAT		
L. monocytogenes	*prfA*	LM_*prfA*_F	CACAAGAATATTGTATTTTTCTATATGAT	398	Kupradit *et al*., 2017
		LM_*prfA*_R	CAGTGTAATCTTGATGCCATCA		
S. aureus	*eap*	SA_*eap*_F1	TTAAATCGATATCACTAAATACCTC	230	Hussain *et al*., 2008
		SA_*eap*_R1	TACTAACGAAGCATCTGCC		
Salmonella spp.	*fimY*	SM_*fimY*_F	GCCTCAATACAGGAGACAGGTAGCG	315	Kupradit *et al*., 2017
		SM_*fimY*_R	GCAGGGAAAGACACCGCCGTTTAA		

2.4 Oligonucleotide array preparation and detection

Nylon membranes (Roche, Mannheim, Germany) were used as the array matrix. Single strand probes (Table 3) were heated at 95 ºC for 5 min, and 200 pmol were spotted on a specific position on the dry nylon membrane (Figures 1a and 2a). The membranes spotted with probes were exposed to UV for 3 min to allow cross-linking.

For oligonucleotide array probes validation and selection, the hybridization process were performed as described by Kupradit *et al*. (2013a, 2013b). Briefly, 100 ng of purified m-PCR products of each target bacteria were denatured at 99 ºC for 10 min and quickly chilled on ice. The denatured DNA were labeled with 2 µL of DIG High Prime (Roche) and incubated at 37 ºC for 1 h. Membranes with spotted probes were pre-hybridized in a pre-warmed DIG Easy Hybridization solution (Roche) at 35 ºC with gentle shaking for 30 min. Prior to hybridization, 10 µL of labeled PCR products reactions were heated to 99 ºC for 5 min, then immediately cooled on ice and added to 2 mL of newly pre-warmed hybridization solution. The hybridizations were carried out at 35 ºC with gentle rotating for 4 h. After hybridization, the membranes were washed. The membranes were then incubated for 30 min in blocking solution (Roche) and 30 min in antibody solution (Roche). After washing twice in washing solution (Roche) for 15 min each, the membranes were equilibrated in detection buffer (Roche) for 2 min, and in freshly prepared NBT/BCIP (Roche) color substrate solution in the dark for 4–8 h. The results were visualized and photographed.

2.5 Multiple pathogen detection using oligonucleotide array

For multiple target bacteria detection, mixtures of genomic DNA from each target bacteria were used as templates. Genomic DNA of 4 or 5 bacterial species (10 ng each) were mixed. The mixture of DNA templates were amplified by the m-PCR followed by oligonucleotide array. The m-PCR products were purified using PureLink PCR Purification Kit (Invitrogen Life Technologies). Two hundred ng of total purified m-PCR products were labelled and hybridized with specific probes on a single array (Figure 2a). The hybridization process was performed as described in 2. 4. The hybridization results were visualized and photographed.

3. Results and Discussion

3.1 Oligonucleotide probe design

To detect multiple target bacteria using combination of m-PCR and oligonucleotide array, 5 oligonucleotide array probes against each target gene were designed. The gene specific including enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes were selected as target genes for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*, respectively. The nucleotide sequences of amplified products from specific genes were then used for probe design using PICKY oligonucleotide design program (Chou *et al.*, 2004). By this reason, the probes would bind within the amplified PCR fragments. Probes obtained from this step are shown in Table 3. Although some of target genes, including *uspA* of *E. coli* and *prfA* of *L. monocytogenes*, were similar as previous reports (Kupradit *et al.*, 2013a, 2013b), almost all of the probe sequences were different, except for the probe EC2 of *E. coli*. These results indicated that the different probe sequences depend on target bacteria and region of target genes used for probe design. To confirm that the probes obtained from this research can be used for pathogen detection, the probes (Table 3) were tested for hybridization with the target bacteria of interests.

Table 3 Sequences of gene specific probes spotted on the oligonucleotide array

Species	Probe name and sequences (5' to 3')	Target gene	References
B. cereus	BC 1: AAA TCA ACT TCA ATG GTG GAA CT	Ent*FM*	This work
	BC 2: GGC CAA GTG CTA CAA GTA GTT		This work
	BC 3: GTG CTG GTA CAG GAC ATA ACG T		This work
	BC 4: TGG TGG CAA AAC AGG AAC GAC		This work
	BC 5: ACG AAA CAC AAC AAC CAA CT		This work
E. coli	EC 1: CGA AGA AGA ATA ATC TTC CCT CTA CGA CGT	*uspA*	This work
	EC 2: AAG AGA CAC ATC ATG CAC TGA CCG AGC T		(Kupradit *et al.*, 2013 a and b)
	EC 3: ACC TAT ACA CCG GGC TTA TTG ATG TGA AT		This work
	EC 4: ATG CGA AAG TTT CTC TGA TCC ACG T		This work
	EC 5: AGG CGG ATT GAC GGA TCA TC		This work
L. monocytogenes	LM 1: ATT GAT ACA GAA ACA TCG GTT GGC TAT	*prfA*	This work
	LM 2: ACC TAT GTG TGG ATG GTA AAG AAA CTC CT		This work
	LM 3: AAA GAA CTA CTG AGC AAA AAT CTT ACG C		This work
	LM 4: TTT TCT ATG TTT TCC AAA CCC TAC		This work
	LM 5: ACG CAC TTT TTC TAT GTT TTC CA		This work

Table 3 Sequences of gene specific probes spotted on the oligonucleotide array (Continue)

Species	Probe name and sequences (5' to 3')	Target gene	References
Salmonella spp.	SM 1: AGT GAC TCA ATG AAT AGC CGA GGT AGT TGT	*fimY*	This work
	SM 2: ACG GCG GTG TCT TTC CCTGC		This work
	SM 3: ATT ATT GCC TGA GAA ATG ATA CCA GAC C		This work
	SM 4: ACC CCT TTT CAA TCC ACA AAG AAA		This work
	SM 5: AAG AAA TGT CAT CTA ATG AGC		This work
S. aureus	SA 1: ATC GAT ATC ACT AAT ACC TCT AT	*eap*	This work
	SA 2: AGA ATG TCC ATG GTG TAA TGT ACT		This work
	SA 3: AGC TTG ATA AAA TGT TTT GG		This work
	SA 4: AAA ATG TTT TGG CTT GTA CCG		This work
	SA 5: CTT GAT GAT TTA TCT AAT GGC T		This work

3.2 Probe specificity tests

For probe specificity tests, only the m-PCR products from each target bacteria were labeled and tested for the specificity of the probes (Table 3). As report in our previous works, the specific genes of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* were screened and selected. Only the expected sizes of m-PCR products were observed in target bacteria. Cross-amplification from non-target bacteria isolated from raw milk samples were not detected (Kupradit *et al.*, 2017). Thus, only the m-PCR products of target bacterial pure cultures including reference and strains isolated from raw milk samples (Table 1) were labeled using post-PCR labeling technique. Each labeling target PCR product was hybridized individually with the nylon membrane containing specific probes at specific positions as shown in Figure 1a. After hybridization, signals on the array were unambiguously distinguished for each of the bacterial species as shown in Figure 1b. The accuracy of each probe is summarized in Table 4.

(a)

SM1	SM2	SM3	SM4	SM5			
			SA1	SA2	SA3	SA4	SA5
	P						
	EC1	EC2	EC3	EC4	EC5		
			BC1	BC2	BC3	BC4	BC5
LM1	LM2	LM3	LM4	LM5			

(b)

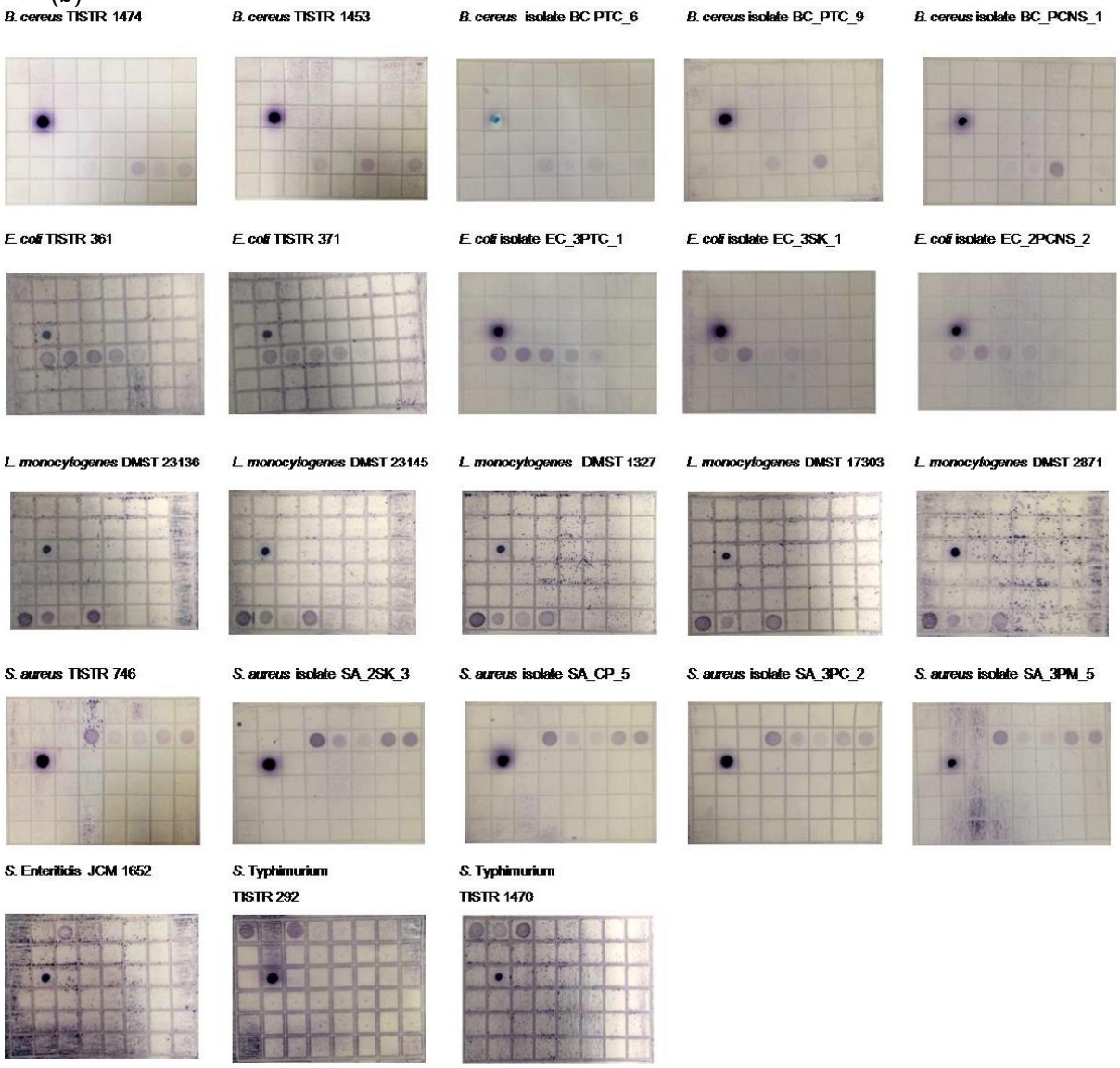

Figure 1 Probe validation and specific hybridization patterns of target bacteria. (a) Position of specific probes on nylon membrane. Positive controls (P) are 100 pmole DIG-label 16S rDNA forward primer (Kupradit *et al*.,2014). (b) Specific hybridization pattern of each target bacteria. The detections of several bacteria were performed by hybridization of labeled 100 ng of m-PCR products with 200 pmol probes. The oligonucleotide probes on nylon membrane were hybridized individually with labelling m-PCR amplification products of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*

Table 4 Probe validation and specificity test using isolated and reference strains of target and non-target bacteria.

Target bacteria	Probe name	Number of target bacterial strains (T)[a]		Number of non-target bacterial strains (NT)[b]		%Accuracy[c]
		Total number of target bacteria tested (No. T)	Positive signal detection (T^+)	Total number of non-target bacteria tested (No. NT)	Positive signal detection (NT^+)	
B. cereus	BC 1	10	7	25	0	70.0
	BC 2	10	2	25	4	4.0
	BC 3	10	10	25	0	100.0
	BC 4	10	1	25	0	10.0
	BC 5	10	3	25	0	30.0
E. coli	EC 1	6	6	29	0	100.0
	EC 2	6	6	29	0	100.0
	EC 3	6	5	29	0	83.3
	EC 4	6	6	29	0	100.0
	EC 5	6	3	29	0	50.0
L. monocytogenes	LM 1	8	8	27	0	100.0
	LM 2	8	7	27	0	87.5
	LM 3	8	5	27	0	62.5
	LM 4	8	8	27	0	100.0
	LM 5	8	0	27	0	0.0
Salmonella spp.	SM 1	3	2	32	0	66.7
	SM 2	3	1	32	0	33.3
	SM 3	3	3	32	0	100.0
	SM 4	3	0	32	0	0.0
	SM 5	3	0	32	0	0.0
S. aureus	SA 1	8	7	27	0	87.5
	SA 2	8	7	27	0	87.5
	SA 3	8	8	27	0	100.0
	SA 4	8	7	27	0	87.5
	SA 5	8	6	27	0	75.0

Note: [a] Total number of target bacteria tested (No. T); Number of target bacteria showing positive signal (T^+)

[b] Total number of non-target bacteria tested (No. NT); Number of non-target bacteria showing positive signal (NT^+)

[c] % Accuracy (Kupradit *et al*., 2013a) = [(T^+ × 100)/ No. T] - [(NT^+ × 100)/ No. NT]

The specific hybridization patterns between the specific probes with their specific targets were found in *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *S. aureus* (Figure 1b). In the case of *L. monocytogenes* and *Salmonella* spp. detections, only reference strains were tested because these two bacteria are low prevalence in raw milk (Padungtod and Kaneene, 2006; Jami *et al*., 2010; Bianchi *et al*., 2013) and could not be isolated in our works (data not shown). Strong hybridization signals were found with probes LM1, LM2, LM4 (87–100% accuracy, Table 4) and probes SM1, SM3 (66–100% accuracy, Table4) for *L. monocytogenes* and *Salmonella* spp. detection, respectively. All *Salmonella* spp. (SM) and *L. monocytogenes* (LM) probes had no cross-activities with non-target m-PCR product (Table 4). Based on specificity and high accuracy results, probes LM1, LM2, LM4 and probes SM1, SM3 were selected for further detection of *L. monocytogenes* and *Salmonella* spp. in milk samples, respectively.

For *E. coli*, hybridization patterns of these bacteria were similar from both reference and isolated strains except isolate EC3_SK1. These results indicated that although the minor variations in the biochemical characteristics were found in the isolated strains of *E. coli*, the hybridization patterns of almost all isolates were similar among these bacteria. The probe EC2 was similar as UA1 or UA2 (Kupradit *et al*., 2013a, 2013b) showed 100% accuracy. These investigations confirmed that the nucleotide sequence of this *uspA* region (probe EC2) was highly conserved for *E. coli* and suitable for using as *E. coli* specific probe. In this research, probes EC1–EC4 (83–100% accuracy) are suitable for detection of *E. coli* in milk sample.

After the hybridization process, the differences in hybridization patterns were observed among isolated and reference strains of *B. cereus* and *S. aureus* (Figure 1b). The results indicated that their target regions might contain the variation of nucleotide sequences. For *B. cereus*, cross-reactions were found from the probe BC2 with m-PCR products of *E. coli* isolates (EC 3PTC_1, 3SK_1, and 2PCNS_2) and *S. aureus* isolate (SA 3PM_1). Accuracy of the *B. cereus* and *S. aureus* probes ranged from 4–100% and 75–100%, respectively. Therefore, to avoid cross-reaction of the probes, probes BC1, BC3 and BC5 of *B. cereus* and probes SA1-SA5 of *S. aureus* which have high accuracy and strong hybridization signal were selected for specific detection of these target bacteria in enrichment cultures.

One of the major advantages of the oligonucleotide array assay over agarose gel analysis of the PCR products was that detection required the fragments to contain sequences that were complementary to the oligonucleotide probes on the microarray (Kim *et al*., 2010). Thus, the hybridization signal of each probe might be different because of the nucleotide

variation of each target gene. Only the suitable probes were selected for further application in multiple foodborne pathogen detection from milk sample.

In the future, all suitable probes of each target bacteria with high accuracy, specificity and strong hybridization signal will be applied to detect multiple target bacteria in enrichment culture from milk samples.

3.3 Multiple pathogen detection using DNA macroarray

For multiple target bacteria detection, results indicated that the hybridization patterns were found to be accurate and specific hybridization patterns of each target bacteria were seen (Figure 2b). In Figure 2b, all 4 target bacteria in mixed DNA samples were detected using the combined method with high accuracy and specificity. As reported in our previous investigation, the separation of all m-PCR products on an agarose gel by electrophoresis was not sufficient and difficult for result interpreting (Kupradit *et al.*, 2017). Thus, these research finding demonstrated that the oligonucleotide array could enhance the accuracy and simplicity of the resultant interpretation of the m-PCR detection.

However, the detection of 5 target bacteria using this combine method showed weak signal and low accuracy (data not shown). Low sensitivity of m-PCR amplification with multiple templates and primers might cause complexity of the reaction. As reported previously, m-PCR and oligonucleotide array assays were developed for the specific detection of *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp., in chicken meat (Kupradit *et al.*, 2013b). The results were found that the amplification of the target gDNA templates from the fresh chicken meat samples using m-PCR was less sensitive than using traditional PCR with a single primer pair (Kupradit *et al.*, 2013b). Therefore, the combined traditional PCR and oligonucleotide array method was developed and successful in detecting *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, and *E. coli* in fresh chicken meat (Kupradit *et al.*, 2013a). Thus, to avoid the problems of false negative and low sensitivity from m-PCR amplification, the traditional PCR will be used to separately amplify each target gene of interest using the same PCR amplification condition followed by oligonucleotide array hybridization. Combining m-PCR with oligonucleotide array could increase detectability and accuracy of the detection systems which can be applied to detect multiple target bacterial species in milk sample.

(a)

SM1	SM2	SM3	SM4	SM5			
			SA1	SA2	SA3	SA4	SA5
	P						
	EC1	EC2	EC3	EC4	EC5		
			BC1	BC2	BC3	BC4	BC5
LM1	LM2	LM3	LM4	LM5			

(b)

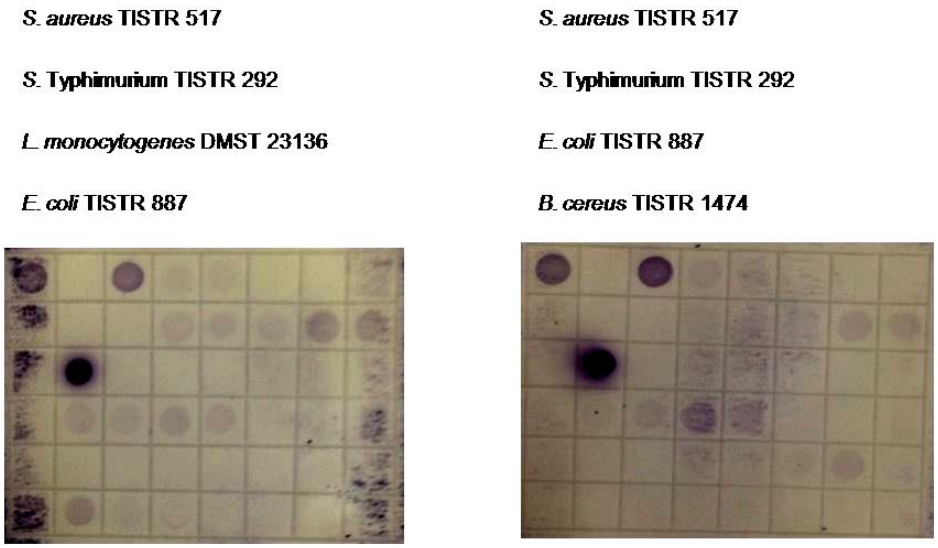

Figure 2 Multiple target bacteria detection by m-PCR-oligonucleotide array hybridization based method. (a) Positions of specific probes on nylon membrane. Positive controls (P) are 100 pmole DIG-label 16S rDNA forward primer (Kupradit *et al*., 2014). (b) Hybridization patterns of each target bacteria used in each test as label on top of each blot are combined in single array

4. Conclusions

The developments of multiple foodborne pathogen detection techniques with high accuracy and high sensitivity are still required. Advantages of both oligonucleotide array and m-PCR for multiple pathogen detection are that multiple target bacteria can be detected simultaneously. By these methods, labor, cost, and identification time can be reduced. In this research, the multiple target bacteria were successfully detected by the probe designed in this work.

As all available data now, this is the first report to combine target genes including enterotoxin *FM*, *uspA*, *prfA*, *fimY*, and *eap* genes, together for specific detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* in a single reaction of m-PCR followed by oligonucleotide array hybridization. For oligonucleotide array assay, probe selection step is very important. In our finding report, high accuracy of specific probes, including, probes BC1, BC3 and BC5 (30–100% accuracy); probes EC1–EC4 (83–100% accuracy); probes LM1, LM2, LM4 (87–100% accuracy); probes SA1–SA5 (75–100% accuracy); and probes SM1, SM3 (66–100% accuracy), were selected for detection of *B. cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Salmonella* spp., respectively (Table 4).

In the future, all target bacteria in milk sample will be enriched in suitable enrichment medium and all target genes will be separately amplified using traditional PCR. The amplicon products will be mixed together and distinguished from each other in a single array. By this method, the accuracy of multiple foodborne pathogen detection in milk products could be improved.

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