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Novel multiplex polymerase chain reaction and an oligonucleotide array for specific detection of the dominant foodborne bacterial pathogens in chicken meat

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Oligonucleotide array hybridisation and multiplex polymerase chain reaction (m-PCR) can be used to screen and detect multiple foodborne pathogens. In our study, m-PCR and oligonucleotide array assays for the specific detection of the dominant foodborne bacterial pathogens, including *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Shigella* spp., in chicken meat were developed. The combination of m-PCR and an oligonucleotide array targeting the 16S rRNA, *uspA*, *prfA*, *fimY*, and *ipaH* genes displayed a high discriminatory power among the aforementioned genera and species with low or no incidence of false negative results. Our combined methods could detect all 4 target bacteria at amounts as low as 1 ng of each from mixed genomic DNA extracted from pure cultures, which is equivalent to 10^4-10^6 CFU/ml. After enrichment steps for the target bacteria, *E. coli*, *L. monocytogenes*, and *Salmonella* sp. could be detected simultaneously from fresh chicken samples. Combining the two methods could enhance accuracy and sensitivity for foodborne pathogen detection and identification. The problems of cross-reactivities from non-target bacteria isolated from an enrichment culture and the difficulties in result interpretation by m-PCR could be solved using our oligonucleotide array hybridisation method.

Key words: Oligonucleotide array, multiplex PCR, foodborne pathogens, target bacteria.

INTRODUCTION

In Thailand and many countries, foodborne pathogens and microbial food safety indicators that are prevalent in poultry, especially *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes*, have been reported (Sackey et al., 2001; Bangtrakulnonth et al., 2004; Angkititrakul et al., 2005; Nierop et al., 2005; Cortez et al., 2006; Padungtod and Kaneene, 2006; Lekroengsin et al., 2007; Vindigni et al., 2007; Minami et al., 2010; Stonsaovapak and Boonyaratanakornkit, 2010). To minimise the prevalence of foodborne diseases and reduce microbial contamination in food supplies, effectively monitoring the occurrence and distribution of

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Abbreviations: m-PCR, Multiplex polymerase chain reaction; **DIG,** digoxigenin; **TSA,** trypticase soy agar; **TSC,** tryptose sulphite cycloserine agar; **EMB,** Eosin-methylene blue agar; **XLD,** xylose lysine deoxycholate; agar; **BS,** bismuth sulphite; agar; **BPW,** buffered peptone water.

bacterial pathogens in food is essential.

The most common tools of standard methods used for pathogen detection are cultural based method, immunelogical based method, and molecular based methods (United States Food and Drug Administration, 1998; Lazcka et al., 2007). Classical cultural methods including step of pre-enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification have been applied to detect foodborne pathogens (United States Food and Drug Administration, 1998; Boera and Beumer, 1999; Lazcka et al. 2007). Conventional methods for detecting enteropathogens are very laborious and time consuming. To overcome these limitations, multiplex polymerase chain reaction (m-PCR), real-time PCR, and oligonucleotide arrays have been applied to detect multiple pathogens simultaneously (Yoo et al., 2004; Huang et al., 2007; Mao et al., 2008; You et al., 2008; Severgnini et al., 2011).

m-PCR is a reaction that amplifies more than one target gene simultaneously by mixing multiple primer pairs. m-PCR-based methods have been widely used and adapted for the rapid detection of single and multiple bacterial species, for example, *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* (Yeh et al., 2002; Li and Mustapha 2004; Thiem et al., 2004; Jofré et al., 2005; Li et al., 2005; Germini et al., 2009). Although m-PCR can amplify multiple targets in a single tube, its detection capability is still restricted to only a few targets per assay due to the complexity of the amplification (Wang et al., 2007; Settanni and Corsetti, 2007). For these reasons, typically only 2 (Jofré et al., 2005) or 3 (Li and Mustapha 2004; Li et al., 2005) bacterial species are simultaneously detected using m-PCR. These different m-PCR amplicons could be differentiated by real-time PCR with a high efficiency (Huang et al., 2007). However, real-time PCR requires special and expensive equipment, specific fluorescent probes, fluorescent detectors to detect several m-PCR products and expensive reagents (Nugen and Baeumner, 2008; Bai et al., 2010; Suo et al., 2010; Hu et al., 2012). Therefore, simple methods are required to improve the sensitivity and accuracy of m-PCR. An essential feature of the DNA array technique is the hybridisation of the labelled target DNA fragments with the array's immobilised probes. It can then be applied for multiple pathogens and microbial community detection in food samples (Gauthier and Blais, 2003; Cremonesi et al., 2009). Nucleic acid hybridisation occurs between the target DNA from the target organisms and DNA probes of approximately 15-30 nucleotides on the array (Boera and Beumer, 1999). The signal generated by the bound and labelled target on the array allows for identifications based on the known locations of the probes (Rasooly and Herold, 2008).

Among many pathogenic bacteria, consensus sequences can be amplified using a single pair of universal primers (Hong et al., 2004; Chiang et al., 2006; Hu et al., 2012). However, the limitation of using consensus sequences is a cross-reactivity with some other closely related bacteria, such as the cross-reactivity between *Salmonella* spp. and *E. coli* when the 23S rRNA gene is used as the target (Hong et al., 2004) or between *E. coli* and *Shigella* spp. (Chiang et al., 2006; Hu et al., 2012) when the 16S rRNA or *groEL* genes are used as the targets. Therefore, combinations of m-PCR amplification of species- and genus-specific genes with a DNA microarray were used in this study. Previously, these combined methods have been applied for multiple pathogen detection in meat product samples (Suo et al., 2010) and clinical samples (Kim et al., 2010) using fluorescent signal detection. Several laboratories have also addressed the development of simple and specific methods with minimal instrumentation requirements (Hong et al., 2011).

In this study, a low-density pathogen detection method using a m-PCR-oligonucleotide array to simultaneously detect 3 foodborne pathogens, including *Shigella*, *Salmonella*, *L. monocytogenes*, and 1 microbial food safety indicator, *E. coli*, which are frequently found in fresh chicken meat were developed and evaluated. Digoxigenin (DIG) was used to label the DNA. No special equipment was required for the material array construction or for signal detection. The m-PCR products for the 16S rRNA, *uspA*, *prfA*, *fimY*, and *ipaH* genes were distinguished from each other by DIG post-PCR labelling and hybridised to the oligonucleotide array. The applicability of this assay to fresh chicken samples was also addressed.

MATERIALS AND METHODS

Bacterial strains

The reference and isolated bacterial strains used to validate the m-PCR and oligonucleotide array probe detection are listed in Table 1. All isolated strains 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 except for *Clostridium 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), at 37°C for 24-48 h. The cultivation of *C. perfringens* was performed on tryptose sulphite cycloserine agar (TSC; Biomark, Pune, India) under anaerobic conditions at 37°C for 24 h.

Primer and probe design

To obtain the consensus sequence of each pathogen, the sequences were downloaded from the National Center for Biotechnology Information (NCBI) database and aligned using MegAlign DNAStar Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA). Specific genes and 16S rDNA primers (Table 2) were designed using PrimerSelect DNAStar Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA) based on the conserved regions of each specific gene and the conserved regions of all the target bacteria, which contained variable regions in the amplicons. All the primers in Table 2 were tested for their specificity with the reference and isolated bacterial strains (Table 1). For the oligonucleotide

Table 1. Bacterial strains used for the validation of m-PCR and the oligonucleotide array.

Specie	Number of strains	Strain name and sources
Escherichia coli	7	*E. coli* TISTR[a] 887, *E. coli* E[b] 1, 2, 3, 4, 6, 7
Clostridium perfringens	1	*C. perfringens* CP[b]5
Listeria monocytogenes	9	*L. monocytogenes* DSM[a] 12464, DMST[a] 1327, 2871, 17303, 20093, 21164, 23136, 23145, 31802
Salmonella spp.	9	*Salmonella* serotype *enteritidis* (*S. enteritidis*) JCM[a] 1652, TISTR 2394, *Salmonella* serotype *typhimurium* (*S. typhimurium*) TISTR 292, *Salmonella* sp. S[b]2-7
Shigella spp.	12	*Shigella boydii* DMST 3395, 28180, 30245, *S. dysenteriae* DMST 2137, 5875, 15111, *S. flexneri* DMST 17559, 17560, 30581, *S. sonnei* DMST 17561, 23595, *Shigella* sp. Sh[c]1
Staphylococcus aureus	1	*S. aureus* TISTR 517
Non-target bacteria found in enrichment culture	10	C[b]2, 3, 4, 6, RV[b]2, 3, TT[b]1, L[b]2, 4, 5

[a]Reference strains: 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 Nakhon Ratchasima, Thailand: C, non-*E. coli* bacteria isolated on EMB agar; 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.; TT, non-*Salmonella* bacteria enriched using TT broth and isolated on XLD agar. [c]Strains isolated from food in Khon Kaen, Thailand: Sh, *Shigella* sp.

array, probes specific for each pathogen (Table 3) were designed based on the variable regions of the 16S rDNA and the conserved regions of each target gene using the PICKY oligonucleotide design program (Chou et al., 2004).

Target gene amplification by m-PCR

Genomic DNA (gDNA) from cultures grown on TSA or TSC (Biomark) for 16-24 h was extracted using a phenol-chloroform-based method (Liu et al., 2011). The concentrations and purity of the gDNA and m-PCR products were detected by measuring the absorbance at 260 and 280 nm using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Genomic DNA was used as a template for the target gene amplification by m-PCR. The reactions were performed in a total volume of 25 µl and contained 1× GoTaq Flexi buffer (Promega, Madison, WI, USA), 1 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.5 U GoTaq Flexi DNA polymerase (Promega), 100 ng DNA templates and primers. In all the m-PCR reactions, the amplified 16S rRNA gene was used as control. The concentrations of each primer pair and the annealing temperature were optimised. The PCR reactions were maintained at 95°C for 3 min and then 35 cycles of 95°C for 30 s, 50-59°C for 45 s, and 72°C for 60 s were performed followed by a final extension step at 72°C for 5 min. The m-PCR products were analysed by electrophoresis on a 4% (w/v) agarose gel and purified using a QIAquick PCR Purification kit (Qiagen, GmbH, Hilden Germany).

Oligonucleotide array preparation and detection

Nylon membrane (Roche, Mannheim, Germany) was used as the array matrix. Single stranded probes were heated at 95°C for 5 min, and 200 pmol was spotted at a specific position on a dry nylon membrane (Figure 2A). The membranes spotted with the probes were exposed to UV for 3 min to allow for cross-linking. Two hundred nanograms of purified m-PCR product was denatured at 99°C for 10 min and quickly chilled on ice. The denatured DNA was labelled with 2 µl of DIG High Prime (Roche) followed the manufacture protocol for 1 h at 37°C. Membranes with spotted probes were pre-hybridised in a pre-warmed DIG Easy hybridisation solution (Roche) at 35°C with gentle shaking for 30 min. Ten microlitres of the labelled PCR product reaction was heated to 99°C for 5 min, then immediately cooled on ice and added to 2 ml of the newly pre-warmed hybridisation solution. The hybridisations were performed with gentle rotation at 35°C for 4 h. After hybridisation, the membranes were washed twice for 5 min each in 2× SSC (Roche) and 0.1% sodium dodecyl sulfate (SDS) (25°C), twice for 10 min each in 0.5× SSC (Roche) and 0.1% SDS (45°C) and briefly washed in washing solution (Roche) at room temperature. Then, the membranes were incubated for 30 min in blocking solution (Roche) and 30 min in antibody solution (Roche). After 2 washes in a washing solution (Roche) for 15 min each, the membranes were equilibrated in detection buffer (Roche) for 2 min and in a freshly prepared NBT/BCIP (Roche) colour substrate solution in the dark for 4 h. The results were visualised and photographed.

Application of the oligonucleotide arrays

Four fresh chicken meat samples including 2 breasts (Cb1 and Cb2), 1 wing (Cw3), and 1 thigh (Ct4), were divided into 2 portions and used as natural samples (non-bacteria spiked sample) and target bacteria spiked samples. For spiked samples, a 10-fold dilution series of each bacterial culture including *Salmonella* serotype *enteritidis* (*S. enteritidis*) JCM 1652, *L. monocytogenes* DSM 12464, and *Shigella boydii* DMST 28180 were prepared using 0.85% sodium chloride solution. One hundred microliters of each cell dilution solution was spread onto TSA plates for viable cell count. At the same time, 25 g of each divided portion of each meat sample was placed in a stomacher bag and spiked with 100 µl cell dilution solution (ranging from 1-200 CFU) of each target bacteria. Sample Cb1_1 and Cb2_1 were chicken breast sample 1 (Cb1) and sample 2 (Cb2) (25 g each) spiked with *L. monocytogenes* 1 CFU, *S. boydii* 1 CFU and *S. enteritidis* 20 CFU, respectively. Samples

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

Specie	Target gene	Accession number in GenBank	Primer sequences (5' to 3')	Primer position on gene sequences	Amplicon size (bp)	References
Campylobacter jejuni	16S rRNA	Y19244	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	316-332 925-941	626	This work
Clostridium perfringens	16S rRNA	AB075767	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	302-318 909-925	624	This work
Escherichia coli	16S rRNA	EU337124	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	338-354 970-986	649	This work
Listeria spp.	16S rRNA	EU090894	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	337-353 971-987	651	This work
Salmonella spp.	16S rRNA	EU014687	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	294-310 928-944	651	This work
Staphylococcus aureus	16S rRNA	FJ895583	16S rRNA _F: AGACTCCTACGGGAGGC 16S rRNA _R: GGTAAGGTTCTTCGCGT	283-299 917-933	651	This work
Escherichia coli	*uspA*	X67639	*uspA*_F: CCGATACGCTGCCAATCAGT *uspA*_R: ACGCAGACCGTAGGCCAGAT	4-23 868-887	884	Chen and Griffiths (1998)
Listeria monocytogenes	*prfA*	EU294567	*prfA*_F: CACAAGAATATTGTATTTTTCTATATGAT *prfA*_R: CAGTGTAATCTTGATGCCATCA	101-129 477-498	398	This work
Salmonella spp.	*fimY*	L19338	*fimY*_F: CGGCTAAAGCTTTCCGATAAGCG *fimY*_R: AAATGCTAAAGACTGCGCCTGCCG	194-216 659 - 682	489	This work
Salmonella spp.	*invA*	EU348365	*invA*_F: GAAATTATCGCCACGTTCGGGCAA *invA*_R: TCATCGCACCGTCAAAGGAACC	289-312 550-571	283	Mao et al. (2008)
Shigella spp.	*ipaH*	M32063	*ipaH*_F: GAGGACATTGCCCGGGATAAAG *ipaH*_R: TAAATCTGCTGTTCAGTCTCACGC	1358 -1379 1756 -1779	422	This work
Shigella spp.	*virA*	AF047364	*virA*_F: CTGCATTCTGGCAATCTCTTCACATC *virA*_R: TGATGAGCTAACTTCGTAAGCCCTCC	1358-1379 1756-1779	215	Mao et al. (2008)

Cb1_2 and Cb2_2 were chicken breast sample 1 (Cb1) and sample 2 (Cb2) (25 g each) spiked with *L. monocytogenes* 10 CFU, *S. boydii* 3 CFU and *S. enteritidis* 200 CFU, respectively. Sample Ct4_1 was chicken thigh sample Ct4 (25 g) spiked with *L. monocytogenes* 20 CFU, *S. boydii* 80 CFU and *S. enteritidis* 8 CFU. Sample Cw3_1 was chicken wing sample Cw3 (25 g) spiked with *L. monocytogenes* 20 CFU, *S. boydii* 80 CFU and *S. enteritidis* 8 CFU. All natural (Cb1, Cb2, Cw3, and Ct4) and spiked (Cb1_1, Cb1_2, Cb2_1, Cb2_2, Cw3_1, and Ct4_1) samples were added to 225 ml of pre-enriched buffered peptone water (BPW) (United States Food and Drug Administration, 1998) for *Salmonella* and *E. coli* enrichment, to Half Fraser broth (HF; OXIOD, Basingstoke, United Kingdom) for *L. monocytogenes* enrichment or to Shigella broth (United States Food and Drug Administration, 1998) for *S. boydii* enrichment. Samples were homogenised at normal speed for 1 min using a laboratory blender stomacher 400 (Seward Laboratory System Inc., New York, USA). All homogenised mixtures were incubated for 24 h at 37°C under aerobic conditions for *Salmonella* and *E. coli* enrichment, under anaerobic conditions at 42°C for *Shigella* enrichment, and at room temperature (25°C) for *L. monocytogenes* pre-enrichment. One hundred microlitres of BPW culture was transferred to 10 ml Rappaport-Vassiliadis broth (RV; Himedia, Mumbai, India) and 10 ml tetrathionate (TT) broth (Himedia), followed

Table 3. Sequences of the probes spotted on the oligonucleotide array.

Specie	Target gene	Accession number in GenBank	Probe name and sequences (5' to 3')	Probe position on gene sequences	Reference
Campylobacter spp.	16S rRNA	Y19244	CJ 1: AGGCAGATGGAATTGGTGGTGTAGG	621-645	This work
			CJ 2: AGCGTAAACTCCTTTTCTTAGGGA	405-428	This work
Clostridium perfringens	16S rRNA	AB075767	CP 1: AAGCTCTGTCTTTGGGGAAGATAATGACGG	397-426	This work
			CP 3: TCCAAACTGGTTATCTAGAGTGCA	578-601	This work
			CP 4: GGCGGATGATTAAGTGGGATGT	525-546	This work (modified from Mao et al., 2008)
Escherichia coli	16S rRNA	EU337124	EC 1: AGGAAGGGAGTAAAGTTAATACCTTTGCT	450-478	This work (modified from Chiang et al., 2006; Mao et al., 2008)
			EC 2: CTGCATCTGATACTGGCAAG	633-652	This work
Listeria spp.	16S rRNA	EU090894	LM 1: GCTTGTCCCTTGACGGTATCTAACC	471-495	This work
			LM 2: GTTTTCGGATCGTAAAGTACTGTTGTTAGAGA	418-449	Mao et al. (2008)
Salmonella spp.	16S rRNA	EU014687	SM1: AGGAAGGTGTTGTGGTTAATAAC	406-428	This work
			SM2: TCTGTCAAGTCGGATGTGAA	548-567	This work (modified from Chiang et al., 2006)
Staphylococcus aureus	16S rRNA	FJ895583	SA 1: AGAACATATGTGTAAGTAACTGTGC	396-420	This work (modified from Mao et al., 2008)
			SA 2: CGCAGAGATATGGAGGAACA	646-665	This work
Escherichia coli	*uspA*	X67639	UA 1: AAGAGACACATCATGCGCTGACCGAGCT	533-560	This work
			UA 2: GGTAGAGAAAGCAGTCTCTATGGCTCGCCC	399-428	This work
			UA 3: ACCGTTCACGTTGATATGCTGATTGTTCCG	727-756	This work
			UA 5: AAGGTAAGGATGGTCTTAACACTGAAT	205-231	This work
			UA 6: GGTGACGTAACGGCACAAGAAACGCTAGCT	276-305	This work
Listeria monocytogenes	*prfA*	EU294567	PA 2: ACGGGAAGCTTGGCTCTATTTTGCGG	410-435	This work
			PA 3: AGCTTACAAGTATTAGCGAGAACGGGACCA	140-169	This work
			PA 4: ACAAAGGTGCTTTCGTTATAATGTCTGGCT	188-217	This work
			PA 5: AATTTAGAAGTCATTAGCGAACAGGCT	250-276	This work
			PA 7: AAACATCGGTTGGCTATTATAAGTTTAG	230-257	This work
Salmonella spp.	*fimY*	L19338	FY 1: GCCTCAATACAGGAGACAGGTAGCGCC	395-421	This work
			FY 2: ATATCGCTTTGTTGCCAACTGAGCGC	353-378	This work
			FY 3: AAATAAGTAGTGACTCAATGAATAGCCGAG	514-543	This work
			FY 4: AGTTGTAATTATTGCCTGAGAAATGATAC	553-581	This work
Shigella spp.	*ipaH*	M32063	IH 1: GGGAGTGACAGCAAATGACCTCCGC	1495-1519	This work
			IH 2: CGGCACTGGTTCTCCCTCTGGGGACCA	1563-1588	This work
			IH 3: TGTGGATGAGATAGAAGTCTACCTGG	1396-1421	This work
			IH 4: AGAATGAGTACTCTCAGAGGGTGGCTGAC	1662-1690	This work
			IH 5: AGAAACTTCAGCTCTCCACTGCCGTGA	1443-1469	This work

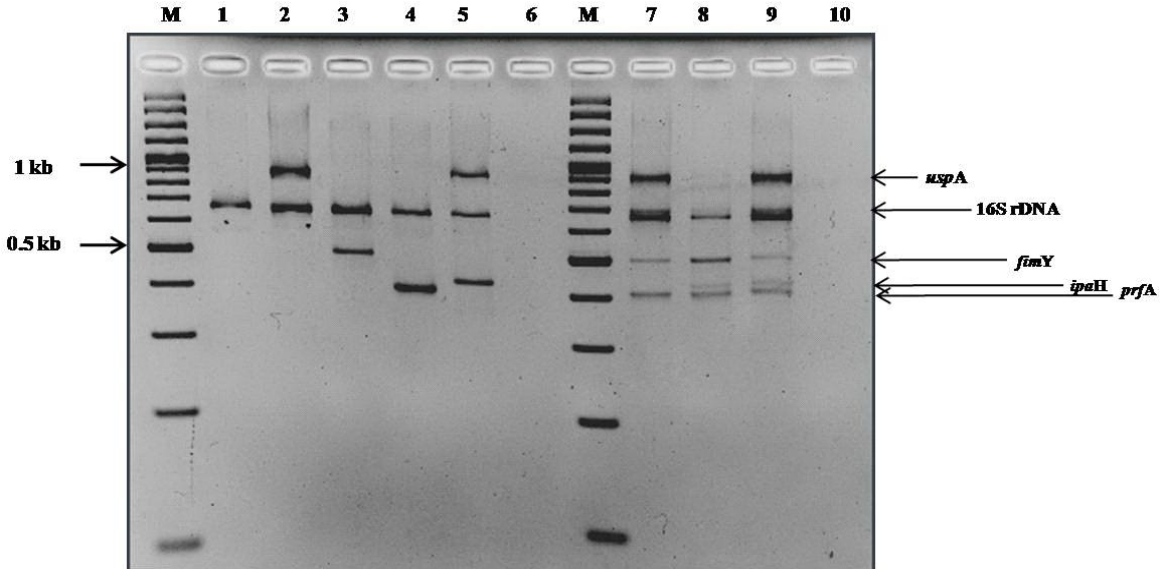

Figure 1. Multiple target pathogen detection using the m-PCR technique. Lanes: 1, *S. aureus* TISTR 517; 2, *E. coli* TISTR 887; 3, *S. enteritidis* JCM 1652; 4, *L. monocytogenes* DSM 12464; 5, *Shigella* sp. isolate Sh1; 6, negative control; 7, mixed templates of *E. coli*, *L. monocytogenes*, *S. enteritidis*; 8, mixed templates of *L. monocytogenes*, *S. enteritidis*, *Shigella* sp.; 9, mixed templates of *E. coli*, *L. monocytogenes*, *S. enteritidis*, *Shigella* sp.; 10, negative control; M, 100 bp DNA marker (Fermentas).

by incubation at 42°C for 24 h for *Salmonella* detection. For *L. monocytogenes* detection, 100 µl of HF culture was transferred to 10 ml Fraser broth (OXIOD) and incubated at 37°C for 24 h. After 24 or 48 h incubation, an aliquot of each enrichment culture from each sample was subjected to the conventional analyses and oligonucleotide array assay.

The accuracy of m-PCR-oligonucleotide array assay was evaluated and compared with the conventional analysis. The cultures of *Shigella* broths were streaked on MacConkey agar (Himedia) for the conventional analysis of *S. boydii* detection. For *E. coli* detection, the BPW cultures were streaked on Eosin-Methylene Blue agar (EMB; Himedia). For *Salmonella* detection, the cultures of RV and TT broth were streaked on xylose lysine deoxycholate (XLD) agar (OXIOD) and bismuth sulphite (BS) agar (OXIOD). *L. monocytogenes* was detected by streaking the Fraser culture on PALCAM agar (OXIOD). The inoculations of the target bacteria on selective agar were incubated at 37°C for 24 h for *E. coli*, *Shigella*, and *Salmonella* detection and 48 h for *L. monocytogenes* detection. The suspected colonies of each target bacterium on the selective agar were re-streaked. Single colonies were picked and mixed in 20 µl water, heated at 100°C for 10 min and 1 µl of supernatant was used directly as templates in the m-PCR reactions for bacterial colony confirmation.

For the oligonucleotide array assay, 1 ml of BPW, RV, TT, Shigella, and Fraser culture were separately collected. Cell pellets were harvested by centrifugation and washed once in 0.85% sodium chloride solution, and gDNA was extracted using a phenol-chloroform-based method (Liu et al., 2011). The gDNA pellet was dissolved in 50 µl TE, pH 8. An equal volume of the gDNA solution obtained from each enrichment culture was mixed, and 1 µl of the gDNA mixture was used as the template for the m-PCR amplification. For *L. monocytogenes* detection, 1 µl of the gDNA extracted from the Fraser culture was used separately as a template. Ten microlitres of the m-PCR products from the mixed enrichment culture and Fraser culture were individually labelled and applied to separate oligonucleotide arrays. The hybridisation patterns of both arrays were combined for the 4 target bacteria detected for each sample.

RESULTS AND DISCUSSION

Optimisation of m-PCR

The specificities of the *fimY*, *invA, ipaH*, *prfA, uspA*, and *virA* genes (Table 2) were tested using the gDNA templates extracted from the pure cultures of *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., and the non-target bacteria (Table 1). The *fimY*, *ipaH*, *prfA*, and *uspA* were suitable target genes for detection of *Salmonella* spp., *Shigella* spp., *L. monocytogenes,* and *E. coli* because of the specificity and ability of amplification in the m-PCR reaction. The optimum annealing temperature was 52°C and the optimum concentrations of the primers in the m-PCR reaction were 0.02 µM *ipaH*, 0.036 µM *fimY*, 0.06 µM *uspA*, 0.12 µM *prfA*, and 0.4 µM 16S rRNA. The 16S rRNA gene amplified from all the target bacteria was used as a control for the presence of amplifiable bacterial DNA in the m-PCR amplification. Using m-PCR amplification, only the 16S rRNA gene product was detected from the non-target bacteria (data not shown). The expected PCR products of 884, 489, 422, and 398 bp were detected from the specific amplification of the reference and isolated strains of *E. coli, Salmonella* spp*., Shigella* spp., and *L. monocytogenes*, respectively (Figure 1, lanes 2-5).

A

SM 1	SM 2	FY 1		CJ 1	CJ 2		SA 1	SA 2
FY 2	FY 3	FY 4		CP 1	CP 3	CP 4		
	P		16S				EC 1	EC 2
				UA 1	UA 2	UA 3	UA 5	UA 6
LM 1	LM 2			IH 1	IH 2	IH 3	IH 4	IH 5
PA 2	PA 3	PA 4	PA 5	PA 7				

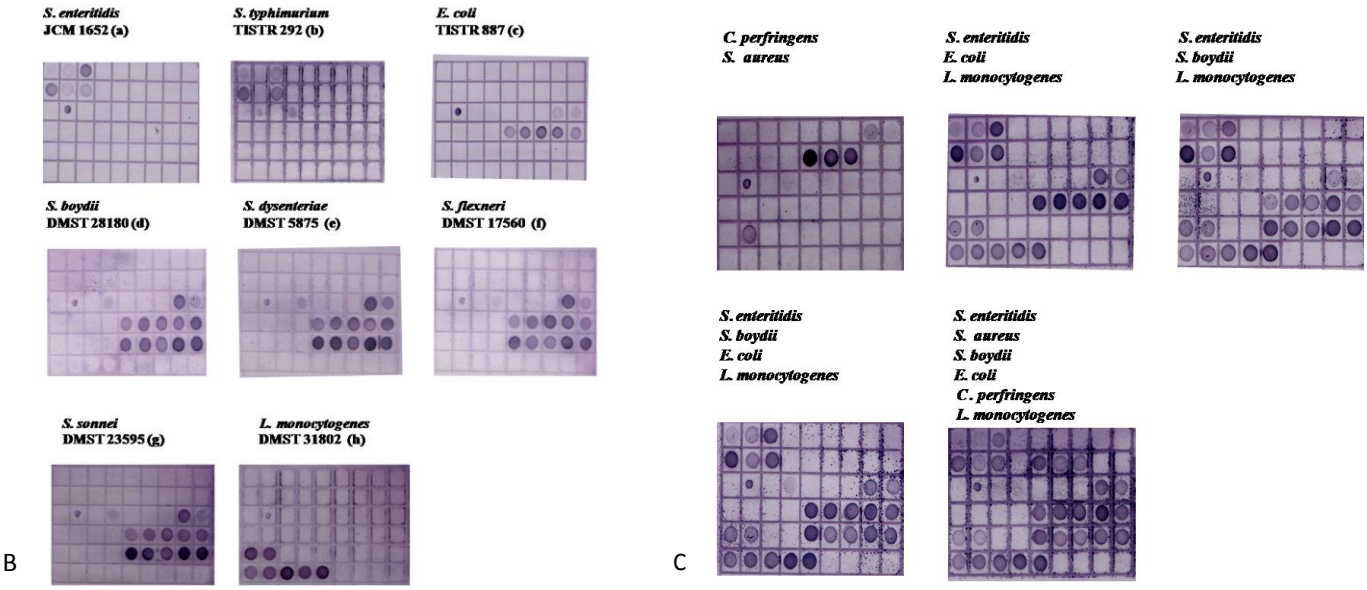

Figure 2. Specific hybridisation patterns of the target bacteria. (A) Position of specific probes on the nylon membrane. Positive controls are 0.1 ng of DIG-labelled control DNA (pBR328 DNA, linearised with *Bam*HI) (P) and 200 pmol 16S rDNA forward primer (16S). The abbreviated letters in the grids are the probe names shown in Table 3. (B) Specific hybridisation of individual m-PCR amplification products from each target bacteria with specific probes on the array. (C) Detection of multiple target bacteria using the m-PCR-oligonucleotide array hybridisation-based method.

The amplification of the *uspA* gene fragment, which encodes for a highly conserved universal stress protein present in all *E. coli* (Chen and Griffiths, 1998), was also detected from the *Shigella* spp. This gene could be amplified not only from *E. coli* but also from all 4 *Shigella* species due to the high identity of the genes between *E. coli* and *Shigella* (Chen, 2007). However, *Shigella* can be differentiated from *E. coli* by the presence of the *ipaH* gene product. These results demonstrated that the specific detection of *E. coli*, *Salmonella* spp., *L. monocytogenes*, and *Shigella* spp. could be performed using the m-PCR developed during this investigation. However, our results indicated that the separation of all 5 amplicons on an agarose gel by electrophoresis was less sensitive and not sufficient (Figure 1, lanes 7-9). Therefore, oligonucleotide array was used to solve the problem of m-PCR result interpretation.

Probe validation and specificity testing

The target genes used for probe design were the 16S rRNA genes and genus- or species-specific genes included *fimY*, *ipaH*, *prfA*, and *uspA* genes. Our preliminary results indicated that the detection of *E. coli* and *Salmonella* using the probes targeted to the 16S rRNA genes resulted in some cross-reactivity with the non-*Salmonella* and non-*E. coli* bacteria from the enrichment culture (data not shown). Moreover, *E. coli* and the *Shigella* spp. could not be differentiated using the 16S rDNA probes (Figure 2B). A reliable genus- or species-

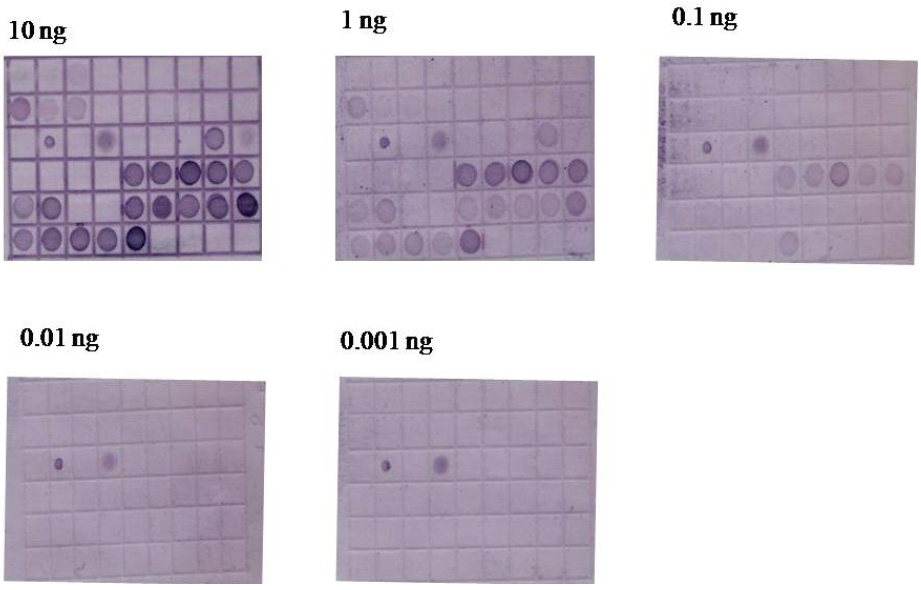

Figure 3. Sensitivity of the oligonucleotide array for the detection of multiple target bacteria. Genomic DNA extracted from each target bacteria were mixed at the same final concentration. A series of 10-fold dilutions of gDNA mixtures, ranging from 10-0.001 ng, from the 4 target bacteria were used as templates for m-PCR amplification followed by the oligonucleotide array hybridisation.

specific gene was required for differentiation between *Shigella* and *E. coli*. To detect multiple target bacteria using a combination of m-PCR and an oligonucleotide array, oligonucleotide array probes specific for each gene and that would bind within the amplicon were designed (Table 3). DNA amplified from the bacterial strains listed in Table 1 was employed to evaluate the performance of the assay. After hybridisation, the signals on the array were unambiguously distinguished (Figure 2B). Cross-reactivities of the m-PCR products from *Shigella* with the *E. coli* probes (UA and EC probes) were found for all 4 species of *Shigella* (Figure 2B). But *Shigella* can be differentiated from *E. coli* through a positive signal from the IH probes (Figure 2B). A mixture of gDNA from each target bacteria was also used as a mixed template for the detection of multiple target bacteria. The hybridisation patterns were determined to be accurate (Figure 2C). These results indicated that the developed oligonucleotide array could enhance the accuracy and simplicity of the resultant interpretation of the m-PCR detection. Using these techniques, the detection of the PCR products did not solely rely on the length of the PCR products but also required the fragments to contain sequences that were complementary to the oligonucleotide probes on the microarray (Kim et al., 2010).

In previous reports using DIG or biotin for the oligonucleotide array assay, only conserved genes, including the 16S rRNA (Chiang et al., 2006), 23S rRNA (Hong et al., 2004) and *groEL* genes (Hu et al., 2012), were used as targets. The detection of multiple pathogens was performed in pure culture, food samples, and foodborne infectious samples (Hong et al., 2004; Chiang et al., 2006; Hu et al., 2012). However, the problem of a low discriminatory ability among target and non-target bacteria was reported. Considering this problem, in our work primers and probes identifying the 4 target bacteria were also designed against genes specifically found in their respective pathogens to prevent false-positive and false-negative results.

Sensitivity of the m-PCR-oligonucleotide array detection

The detection sensitivity of the assay was determined using a gDNA mixture extracted from *S. enteritidis* JCM 1652, *E. coli* TISTR 887, *S. boydii* DMST 28180 and *L. monocytogenes* DSM 12464. A 10-fold dilution series of gDNA mixtures ranging from 10-0.001 ng were used as templates for m-PCR amplifications. Ten microlitres of the m-PCR products was labelled with 2 µl of DIG High Prime (Roche) followed by hybridisation with the specific probes. The detectability of the 4 target bacteria from pure cultures by our assay was 1 ng of each gDNA (Figure 3), which corresponds to approximately 2×10^5 copies of the bacterial genome and was equivalent to 10^4 CFU/ml *S. boydii*, 10^5 CFU/ml *S. enteritidis* and *E. coli*, and 10^6 CFU/ml *L. monocytogenes*. The m-PCR products amplified from the mixture of templates (1 ng of each gDNA) were not sufficiently separated, and all the target gene products could not be observed on an agarose gel (data not shown). Thus, the m-PCR method followed by a hybridisation of the labelled products to the oligonucleo-

Table 4. Application of the oligonucleotide array for foodborne pathogen detection from fresh chicken samples.

Sample	Target bacteria inoculation and final cell concentration in 25 g chicken meat[a]	m-PCR-oligonucleotide array test	Colony confirmation from the isolation agar [b]
Cb1	Unspiked sample	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Cb1_1	*L. monocytogenes* 1 CFU *S. boydii* 1 CFU *S. enteritidis* 20 CFU	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Cb1_2	*L. monocytogenes* 10 CFU *S. boydii* 3 CFU *S. enteritidis* 200 CFU	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Cb2	Unspiked sample	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *L. monocytogenes* *Salmonella* sp.
Cb2_1	*L. monocytogenes* 1 CFU *S. boydii* 1 CFU *S. enteritidis* 20 CFU	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Cb2_2	*L. monocytogenes* 10 CFU *S. boydii* 3 CFU *S. enteritidis* 200 CFU	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *L. monocytogenes* *Salmonella* sp.
Cw3	Unspiked sample	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Cw3_1	*L. monocytogenes* 20 CFU *S. boydii* 80 CFU *S. enteritidis* 8 CFU	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *L. monocytogenes* *Salmonella* sp.
Ct4	Unspiked sample	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
Ct4_1	*L. monocytogenes* 20 CFU *S. boydii* 80 CFU *S. enteritidis* 8 CFU	*E. coli* *L. monocytogenes* *Salmonella* sp.	*E. coli* *Salmonella* sp.

[a] Final cell concentration in 25 g chicken samples; initial cell concentration inoculated into sample were calculated from result of viable cell count on TSA. [b]colony confirmation: presumptive colonies on selective agar from each sample were confirmed by m-PCR.

tide array could improve the detectability. Although our detection limit level was less sensitive than that of the microarray using fluorescence detection, as reported by others (Kim et al., 2010; Suo et al., 2010), our system is still simpler and does not require any expensive or special equipment for microarray construction and fluorescent signal detection.

Application of the oligonucleotide array

The application of oligonucleotide array was tested with a total of 4 unspiked and 6 spiked fresh chicken samples (Table 4). In raw meat, pathogens are often present at low concentration (1-2 cells/25 g food) in a relatively high background of microbiota (Suo et al., 2010). Therefore, enrichment steps are very important to increase the target bacterial cells in samples. Detection of *L. monocytogenes* in BPW is poor due to the significant growth of *Salmonella* (Jofré et al., 2005). Therefore, pre-enrichment and enrichment steps specific for each target bacteria were performed in our study. Performing an enrichment step on a suspect food sample adds time to the overall detection regime and precludes the ability to enumerate the original density of the target pathogen. However, enrichment is necessary and, of course, extremely common for target bacteria detection.

In food sample applications, the total gDNA extracted from enrichment cultures contains both the target and non-target bacteria of a high microbiota background. The presence of these non-target DNAs may interfere with the amplification and/or hybridisation of the target DNAs and, hence, affect the detection sensitivity (Kim et al., 2010). Therefore, optimisation of each primer for the amplification of several target bacteria from food samples was necessary. The optimum primer concentrations for amplification of the multiple target bacteria in the fresh chicken samples using m-PCR were 0.032 µM *ipaH* and *uspA*, 0.036 µM *fimY*, 0.28 µM *prfA*, and 0.14 µM 16S rRNA. We also found that the efficiency of our assay for *L. monocytogenes* detection in samples with very low conta-

mination levels decreased when all the gDNAs extracted from each enrichment culture were mixed and used as templates (data not shown). Therefore, only the gDNA extracted from the Fraser culture was used as a template for m-PCR amplification prior to the application of the oligonucleotide array. The results for the detection of multiple target bacteria using our protocol are summarised in Table 4.

Our protocol could simultaneously detect 3 target bacteria from the fresh chicken samples. All unspiked and spiked samples were found to be indigenously contaminated with *Salmonella* and *E. coli*, which could be detected using our methods and the conventional culture assay. An indigenous contamination of *L. monocytogenes* was found in only 1 of the unspiked samples (sample Cb2; Table 4). After the enrichment step using our combined methods, the sensitivity of *L. monocytogenes* detection in the fresh chicken samples was at least 10 CFU of initial contamination in 25 g samples. At this contamination level, positive hybridisation signals from the PA probes were detected while the PCR product for the *prfA* gene was not visible on agarose gels (data not shown). This result indicated that our oligonucleotide array could increase detectability compared to the PCR method. However, *Shigella* could not be detected from all the spiked samples using either our assay or conventional culturing. These problems might be due to the lower sensitivity of m-PCR amplification or the choice of the target genes (Ojha et al., 2013). In our preliminary investigation, the selected gene, *ipaH*, was specific for all 12 strains included reference and isolated strains of *Shigella* species (data not shown). This result indicated that the *ipaH* gene was suitable for specific detection of *Shigella*. Therefore, equal volumes of gDNA extracted from each enrichment culture were mixed and used as a template to individually amplify with each specific primer. An *ipaH* gene amplicon of the expected size and positive hybridisation signals from the IH probes were observed in the 5 spiked samples (Cb1_2, Cb2_1, Cb2_2, Cw3_1, Ct4_1), which contained an initial cell concentration of at least 1 CFU of *S. boydii* in a 25 g sample (data not shown). This result indicated that problems was due to the amplification of the target gDNA templates from the fresh chicken samples using m-PCR was less sensitive than using conventional PCR with a single primer pair. In m-PCR, a mixture of several primer sets might lead to a poor amplification efficiency (Chiang et al., 2006). Thus, to increase the specificity and sensitivity of the m-PCR–oligonucleotide array for multiple pathogen detection, a determination of how many genes (that is, pathogens) can be used for the m-PCR in a single reaction without sacrificing the sensitivity of the hybridisation to the array is required (Kim et al., 2010). To avoid this problem in future studies, all target genes could be amplified from mixed gDNA templates using a separate pair of primers by conventional PCR. Each target amplicon could be labelled, mixed together and distinguished from each other on a single array. When comparing the conventional culture method to the array, 3 target bacteria could be detected from only 2 of the 6 spiked samples while the oligonucleotide array could detect 3 target bacteria simultaneously from 5 of the 6 spiked samples (Table 4). Thus, the detection of multiple foodborne pathogens using our assay was easier and had a higher accuracy compared to the conventional culture and PCR methods. However, sensitivity of our technique was not sufficient to detect 1 cell of *L. monocytogenes* and *Shigella* in 25 g sample. In sample contaminated with very low initial cell concentration, all the factors, including stressed environment in food, antibiotic selection, homogenisation, among others, could make the lag phase of cell growth longer. Therefore, detecting pathogens in food without enrichment or with inappropriate enrichment time and media might result in an underestimation or even a false-negative assessment of the pathogen contaminations in food (Suo et al., 2010). In our further works, optimisation of the enrichment steps of all target bacteria follow by PCR amplification and hybridisation will be tested to improve the sensitivity of simultaneous multiple pathogen detection in food.

In conclusion, oligonucleotide arrays and m-PCR can be successfully applied to detect multiple foodborne pathogens. To avoid cross amplification by m-PCR in food samples with a high bacterial background, a combination of m-PCR and oligonucleotide array hybridisation can be performed to specifically detect multiple target bacteria after enrichment steps. Although multiple pathogen detection using this protocol requires an additional 10-15 h for labelling, hybridisation and signal detection, compared with a conventional PCR method, the analysis time is still shorter and the protocol is simpler compared to traditional cultivation approaches. Our protocol is simple and has minimal instrumentation requirements, and, thus, a general molecular laboratory, especially in a developing country, is sufficient for performing this protocol.

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