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DNA-based bead array technology for simultaneous identification of eleven foodborne pathogens in chicken meat

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ABSTRACT

A DNA-based bead array method was successfully developed to simultaneously discriminate 11 pathogens namely *Listeria grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. welshimeri*, *Escherichia coli*, *E. coli* O157:H7, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and *Salmonella* spp. for multi purposes: food safety, hygiene indication, antibiotic resistance treatment. The bead array technology is based on fluorescent-barcoded paramagnetic beads with unique 24 oligonucleotide (anti-TAG) sequences which can capture biotinylated PCR product with complementary TAG sequence. R-phycoerythrin labeled streptavidin is used to report the presence of the biotinylated PCR products. After optimizing assay conditions, amplification and biotinylation steps can be performed in a single reaction without further purification before hybridization between the biotinylated TAG products and anti-TAG beads. To ensure that the developed method could provide accurate testing with the real food sample, a total of 311 bacterial isolates from 194 chicken meat samples were tested. The results were compared with those from the conventional ISO methods and revealed the relative accuracy, relative specificity, and relative sensitivity of 96%, 100%, and 95%, respectively. Therefore, the developed method was demonstrated to be useful to distinguish 11 bacteria species at the same time with high accuracy, specificity, and sensitivity.

1. Introduction

Foodborne pathogens can be found in many sources such as water, soil, and food. The most common bacterial pathogens contaminated in food causing illness, hospitalization, and death were *Campylobacter* spp., Shiga toxin-producing *Escherichia coli* (*E. coli*) O157, *Listeria monocytogenes* (*L. monocytogenes*), nontyphoidal *Salmonella* spp., and *Staphylococcus aureus* (*S. aureus*) (Centers for Disease Control and Prevention, 2011). Not only is the contamination from these pathogens harmful to human health, but also it indicates inadequate hygiene in food preparation, retail, and consumption (Kotzekidou, 2013). For instance, *Listeria* spp. can be used to indicate an endemic contamination in food processing environments (Nyenje, Odjadjare, Tanih, Green, & Ndip, 2012). Diarrheagenic *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* were commonly found to be contaminants in food product packaging process (Yang et al., 2016). Therefore, it is very important to detect such contamination in timely manner with a cost-effective method.

Although the conventional detection methods for these pathogens followed by the International Organization for Standardization (ISO) are available, there are several pitfalls to be addressed. First, these ISO protocols rely on specific enrichment and morphological, physiological, serological, and biochemical tests for identification for each pathogen, thus they are time-consuming and labor intensive, especially when many pathogens and samples are required to be tested. Second, these protocols sometimes fail to distinguish some closely related species within the same genus due to the similar morphology and biochemical profiles of the bacteria species. For example, some *Listeria* strains such as *L. innocua*, *L. ivanovii,* and *L. seeligeri* could give similar results to *L. monocytogenes*, resulting in difficulty to identify species by biochemical test (Gouin, Mengaud, & Cossart, 1994; Johnson et al., 2004; Volokhov et al., 2007). Moreover, to detect methicillin-resistant *S. aureus* (MRSA), an important pathogen in livestock and food with prevalence in turkey, pork, beef, chicken of 3.5%, 1.9%, 1.7%, and 0.3%, respectively (Ge

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et al., 2017), a complicated ISO protocol to identify the presence of its drug resistance is required (Datta et al., 2011). With these multi-step and time-consuming protocols, skilled microbiologists are often needed to interpret the results (Marlowe & Bankowski, 2011).

Alternatively, a molecular technique based on polymerase chain reaction (PCR) has been employed for single and multiplex detection because of its relatively fast speed, high sensitivity, and high specificity. Previously, several DNA-based multiplex PCR protocols have been used to simultaneously detect six species of *Listeria* (Ryu et al., 2013), to distinguish 1/2a serotype of *L. monocytogenes* from other serotypes (Sheng et al., 2018), and to detect three foodborne pathogens: *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Nguyen, Van Giau, & Vo, 2016). In addition, a quantitative PCR (qPCR) technique had been developed to detect and quantitate multiple genes of foodborne pathogens (Fukushima et al., 2010; Rodriguez-Lazaro, Cook, & Hernandez, 2013), to distinguish *Staphylococcus aureus* and MRSA by detecting two different target genes (*nuc* and *mecA*) and one virulence factor (Panton-Valentine Leukocidin; PVL) (Velasco, Sherwood, Rojas-García, & Logue, 2014). Although these PCR-based techniques allow multiplex detection with high sensitivity and specificity, their maximum capacity for multiplex detection is up to 5 targets per sample depending on instrument.

To address the limitation of number multiplex capacity, a DNA-based bead array has been developed by Luminex company as an efficient and sensitive method for multiplex detection. The principle of the bead array replies on different fluorescently barcoded paramagnetic bead sets; each set was internally filled with a distinct ratio of red and infrared dyes, allowing a maximum capacity of 50-plex for a MAGPIX model (Reslova, Michna, Kasny, Mikel, & Kralik, 2017). The paramagnetic feature helps eliminating unwanted constituent in sample after washing process. The surface of each bead set is covalently precoupled with a unique 24-base oligonucleotide, called anti-TAG. Genomic DNA from test sample can be used as a template for PCR amplification with specific primers containing a complementary TAG sequence and biotin-labeled nucleotide. The obtained biotinylated PCR product containing a complementary TAG sequence can then be hybridized to the anti-TAG bead sets. A fluorescently (R-phycoerythrin, RPE) labeled streptavidin is used to report the presence of the target of interest. Red and green lasers in an instrument are used to identify type of bead set and measure fluorescent signals from the RPE streptavidin, respectively (Angeloni et al., 2014).

With its multiplex capacity and ability to reduce the background from sample metrics, this technology was applied to simultaneously detect multiple pathogens in a single biological sample. For instance, it has been developed to detect twenty pathogens in acute respiratory tract infections (Chen et al., 2016) and to serotype *Salmonella* (Dunbar, Ritchie, Hoffmeyer, Rana, & Zhang, 2015; Fitzgerald et al., 2007; Liang et al., 2016; Zheng, Zheng, Wang, Pan, & Pu, 2017). Up to date, there were several commercial test kit based on this bead array technology for simultaneous detection of gastrointestinal pathogens and toxins, including nine bacteria and bacterial toxins, three viruses, and three parasites (Reslova et al., 2017). However, to the best of our knowledge, there is no kit available for detection of crucial foodborne pathogens such as *L. monocytogenes*, *S. aureus*, *Salmonella,* and MRSA.

Therefore, we developed a DNA-based bead array method with a single step of DNA amplification and biotinylation for multipurpose: (1) to simultaneously analyze four foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonell*a spp.) for food safety, (2) to identify six species (non-pathogenic *E. coli*, *Listeria grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*) for indication of hygiene in food processing, and (3) to detect an antibiotic resistant pathogen (methicillin-resistant *S. aureus*) for treatment in human and livestock. The developed bead array method was also validated with actual chicken meat samples collecting from markets, and the results were compared to those obtained from the ISO methods.

Table 1
Bacteria strains used in this study.

Bacteria strain	Source
Listeria grayi	ATCC 19120
L. innocua	DMST 9011
L. ivanovii	ATCC 700402
L. monocytogenes	ATCC 19115
L. seeligeri	ATCC 35967
L. welshimeri	DMST 20559
Escherichia coli	ATCC 25322
E. coli O157:H7	DMST 12743
Staphylococcus aureus	ATCC 25923
Methicillin-resistant *S. aureus*	ATCC 33591
Salmonella Choleraesuis	DMST 5580
Salmonella Dublin	DMST 30404
Salmonella Enteritidis	ATCC 13076
Salmonella Hadar	DMST 10634
Salmonella Infantis	DMST 26426
Salmonella Mbandaka	DMST 17377
Salmonella Senftenberg	DMST 17013
Salmonella Typhimurium	ATCC 13311
Salmonella Virchow	DMST 32758

ATCC: American Type Culture Collection.
DMST: Department of Medical Sciences Thailand.

2. Materials and Methods

2.1. Bacterial strains and culture

Bacterial reference strains were purchased from American Type Culture Collection (ATCC, USA.), and Department of Medical Sciences (DMST, Thailand) (Table 1). All bacterial strains were streaked on 2xYT agar plate (16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, 15 g/L agar) and incubated at 37 °C for 16–18 h. A single colony of each bacterial strain from agar plate was inoculated in 10 mL 2xYT media broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride) and incubated at 37 °C with shaking at 250 rpm for 16–18 h before being harvested for DNA extraction.

2.2. Genomic DNA extraction

Genomic DNA (gDNA) of all bacterial strains were extracted using a QIAamp® DNA Mini kit (#51304, Qiagen) according to the manufacturer's instruction. Briefly, bacteria cells (1 mL) were harvested by centrifugation at 5,000 × *g* for 5 min. The pellet was suspended in 180 µL extraction buffer (20 mg/mL lysozyme, 20 mM TrisHCl, 2 mM EDTA, 1.2% Triton™ X-100, pH 8.0) before being treated with proteinase K at 56 °C for 2 h, and RNase A at room temperature (RT) for 2 min, respectively. The gDNA was purified by QIAamp mini spin columns and eluted with sterilized water, and kept at −20 °C until used. The concentration and purity of gDNA were measured at absorbance (A) at 260 and 280 nm using a UV-spectrophotometer (NanoDrop 8000 spectrophotometer, USA).

2.3. Primer design and specificity test

A total of twelve primer sets were used to detect eleven pathogens and one internal control. Nine primer sets for each target gene were from previous reports (Brakstad, Aasbakk, & Maeland, 1992; Daum et al., 2002; Gannon, Rashed, King, & Thomas, 1993; Liu, Ainsworth, Austin, & Lawrence, 2004, 2003; Nadkarni, Martin, Jacques, & Hunter, 2002; Ryu et al., 2013). Three target genes, namely *lwe1801, uidA, mecA*, were designed to detect *L. welshimeri*, *E. coli*, and methicillin-resistant *S. aureus*, respectively. Each primer was synthesized with a unique TAG sequence (complementary to the anti-TAG sequence on each bead) at 5' position (Bio Basic Inc, Canada, Table 2).

For TAG-labeled primers specificity, each primer set was evaluated

Table 2
Primer sequences for multiplex detection used in this study.

Bacteria	Gene	Primer[a]	TAG-labeled primer (5′-3′)[b]	PCR product size (bp)	Reference
L. grayi	*oxidoreductase*	tJOgrayi-F	CATAAATCTTCTCATTCTAACAAAGCGGATAAAGGTGTTCGGGTCAA	249	Ryu et al. (2013)
		tJOgrayi-R	CATAAATCTTCTCATTCTAACAAAATTTGCTATCGTCCGAGGCTAGG		
L. innocua	*lin0464*	tlin0464-F	TCTCATCTATCATACTAATTCTTTCGCATTTATCGCCAAAACTC	797	Liu, Ainsworth, Austin, and Lawrence (2003)
		tlin0464-R	TCTCATCTATCATACTAATTCTTTTCGTGACATAGACGCGATTG		
L. ivanovii	*namA*	tliv22-228-F	AATAACAACTCACTATATCATAACCGAATTCCTTATTCACTTGAGC	511	Liu et al. (2004)
		tliv22-228-R	AATAACAACTCACTATATCATAACGGTGCTGCGAACTTAACTCA		
L. monocytogenes	*lmo1030*	tlmo1030-F	CTTTATCAAATTCTAATTCTCAACGCTTGTATTCACTTGGATTTGTCTGG	557	Ryu et al. (2013)
		tlmo1030-R	CTTTATCAAATTCTAATTCTCAACACCATCCGCATATCTCAGCCAACT		
L. seeligeri	*lmo0333*	tlseelin-F	TTTACAAATCTAATCACACTATACGTACCTGCTGGGAGTACATA	721	Ryu et al. (2013)
		tlseelin-R	TTTACAAATCTAATCACACTATACCTGTCTCCATATCCGTACAG		
L. welshimeri	*lwe1801*	tKlwe-F	ATACTTTACAAACAAATAACACAGAACGTGGCACAATAGCAA	466	In this study
		tKlwe-R	ATACTTTACAAACAAATAACACACCGCAAATAATGGAATGGCT		
E. coli	*uidA*	tKEuidA-F	AACTTTCTCTCTCTATTCTTATTTTACCGACGAAAACGGCAAGA	382	In this study
		tKEuidA-R	AACTTTCTCTCTCTATTCTTATTTTGTCTGGCTTTTGGCTGTGA		
E. coli O157:H7	*eaeA*	tAE1-F	TTAACAACTTATACAAACACAAACCAGGTCGTCGTGTCTGCTAAA	1135	Gannon et al. (1993)
		tAE2-R	TTAACAACTTATACAAACACAAACTCAGCGTGGTTGGATCAACCT		
Staphylococcus aureus	*nuc*	tnuc1-F	TTAATACAATTCTCTCTTTCTCTAGCGATTGATGGTGATACGGTT	328	Brakstad et al. (1992)
		tnuc2-R	TTAATACAATTCTCTCTTTCTCTAAGCCAAGCCTTGACGAACTAAAGC		
Methicillin-resistant *S. aureus*	*mecA*	tKMecA-F	CTAAATCACATACTTAACAACAAATGGTATGTGGAAGTTAGATTGGGA	603	In this study
		tKMecA-R	CTAAATCACATACTTAACAACAAATGGTATGTGGAAGTTAGATTGGGA		
Salmonella spp.	*invA*	tinvA-F	ACACTCATTTAACACTATTTCATTGCGTTCTGAACCTTTGGTAATAA	150	Daum et al. (2002)
		tinvA-R	ACACTCATTTAACACTATTTCATTCGTTCGGGCAATTCGTTA		
Internal control	16sRNA	t16s-F	CTATCATTTATCTCTTTCTCAATTTCCTACGGGAGGCAGCAGT	515	Nadkarni et al. (2002)
		t16s-R	CTATCATTTATCTCTTTCTCAATTGGACTACCAGGGTATCTAATCCTGTT		

[a] t represents TAG-labeled primer.
[b] Underlined segment of primer indicates the MagPlex-TAG sequences provided by Luminex, which binds to the complementary anti-TAG sequenced on the bead.

by a single polymerase chain reaction (PCR) method against 19 bacteria strains (6 species of *Listeria*, 2 strains of *E. coli*, 2 strains of *S. aureus*, 9 serovars of *Salmonella*). Briefly, a total of 25 μL of PCR mixture consisted of 25 ng genomic DNA template, 1.25U of *Taq* DNA polymerase (#M0273S, BioLabs), 100 μM each of dNTP (#25152, iNtRON biotechnology), 1.5 mM $MgCl_2$, and TAG-labeled primer (25 nM each for *L. grayi* and *E. coli*, 35 nM each for internal control (16sRNA), 50 nM each for *L. inoccua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. welshimeri*, *E. coli* O157:H7, and *S. aureus*, 100 nM each for MRSA, and 150 nM each for *Salmonella* spp.). PCR was performed with 30 cycles with the following conditions: denaturing at 95 °C for 30 s, primer annealing at 58 °C for 30 s, and DNA extension at 72 °C for 1 min. The PCR products were analyzed by a gel electrophoresis technique using 2% (w/v) agarose (#2125, OmniPur) in 0.5x TBE buffer (44.5 mM Tris, 44.5 mM Boric acid, and 1 mM EDTA), stained with ethidium bromide, and visualized under UV light. The size of PCR product was indicated by a DNA ladder marker (100-bp, 2 kb, 3 kb, #M25, SibEnzyme).

2.4. Multiplex detection using a DNA-based bead array method

To simultaneously detect multiple targets using a bead array method, three steps, DNA amplification and biotinylation, DNA hybridization with anti-TAG beads, and signal detection, were performed (Fig. 1). For DNA amplification and biotinylation, gDNA sample (25 ng) was amplified and biotinylated by a multiplex PCR method using a PCR master mix solution consisting of 1.25U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, a mixture of 15 μM biotin-labeled-14-dCTP (#19518018, Invitrogen) and 12.5 μM each dNTP, and a mixture of twelve TAG-labeled primer pairs (25 nM each for *L. grayi*, *E. coli* and *S. aureus* primers, 50 nM each for *L. inoccua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. welshimeri* and *E. coli* O157:H7 primers, 100 nM each for MRSA primers, 150 nM each for *Salmonella* spp. primers, and 35 nM for internal control (16sRNA) primers). The PCR condition was the same as the single PCR method as described in section 2.3.

For DNA hybridization, a bead mixture was prepared from 12 anti-TAG bead regions (1,250 beads per region per well, 25 μL) in 2xTm hybridization buffer (0.2 M Tris-HCl pH 8.0, 0.4 M NaCl, and 0.16% Triton X-100). To the bead mixture in each well, PCR product (10 μL) and 15 μL distilled water were added and incubated at 96 °C for 60 s, and 37 °C for 30 min, respectively. Subsequently, beads were washed with 1xTm hybridization buffer by an automatic magnetic washer machine (Bio-Plex Pro, USA). R-phycoerythrin-labeled streptavidin (50 μL of 1 μg/mL SAPE, #S866, Life technology™, USA) in 1xTm hybridization buffer was added and incubated in a microplate shaker incubator (Hercuvan Lab systems, USA.) at 37 °C for 15 min followed by a washing step. The washed bead mixture was suspended in 1xTm hybridization buffer (75 μL) for the signal detection. Fluorescent signals were measured and reported in median fluorescent intensity (MFI) values by a Luminex instrument (MAGPIX™, Luminex, USA). Each sample was tested at least three times. The signals were considered positive when they were above three times of the background or negative control (Dunbar, Vander Zee, Oliver, Karem, & Jacobson, 2003). Distilled water was used as a negative control.

2.5. Sample collection and the International Organization for Standardization (ISO) methods

To validate the bead array method, raw chicken samples (n = 194) were collected from markets (194 samples) in the north of Thailand. Each sample was tested for presence or absence of the bacteria of interest by the ISO methods: ISO11290-1, 16649, 6888-3, and 6579 for. *L. monocytogenes*, *E. coli*, *S. aureus*, and *Salmonella* spp. detection, respectively.

2.6. Comparison of results between the ISO methods and bead array method

To evaluate the bead array method, genomic DNA samples from the colonies were extracted and tested by the bead array method (two replicates), the results were compared with those from the conventional methods. The relative accuracy, relative specificity and relative sensitivity were calculated using the following equations (Banoo et al., 2006):

$$\text{Relative accuracy} = (PA + NA)/N \times 100\% \quad (1)$$

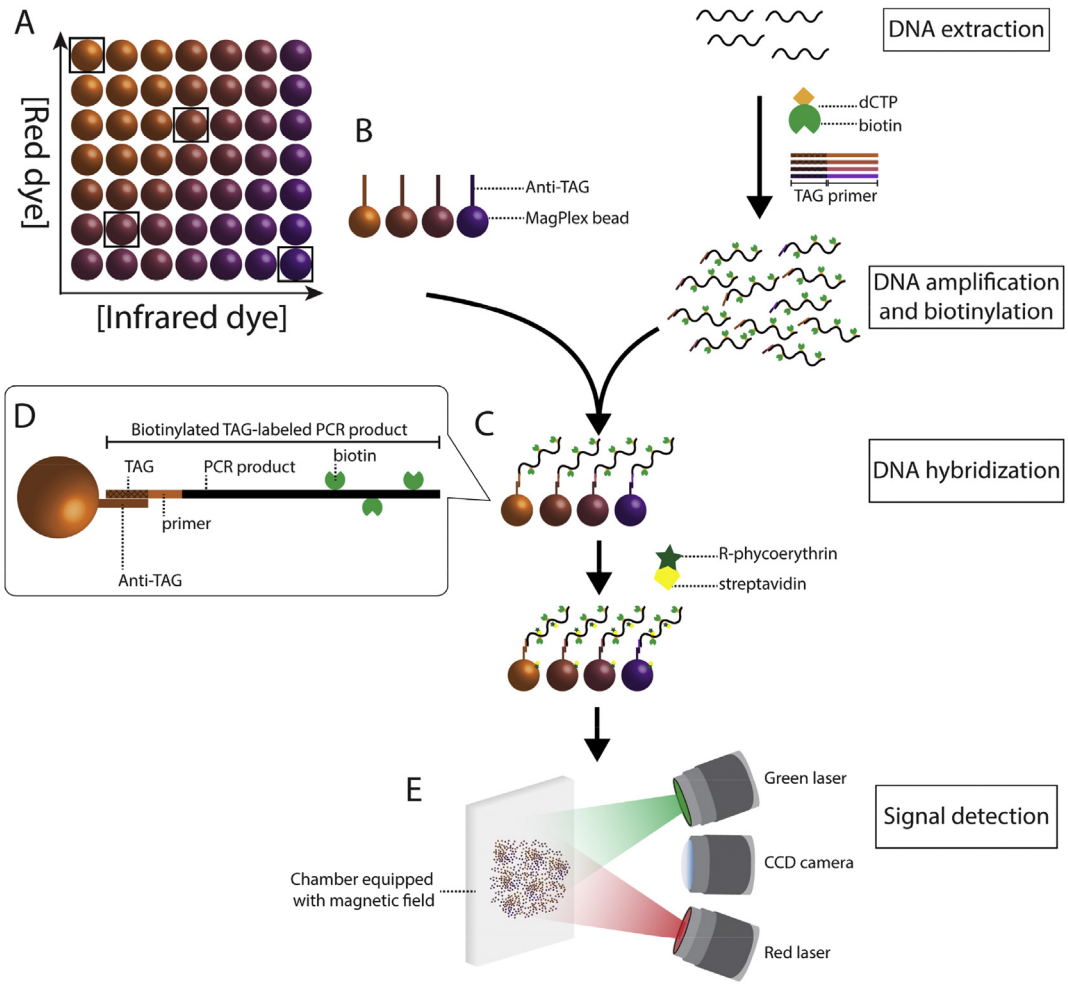

Fig. 1. Scheme of the bead array method. (A) Each paramagnetic bead set contains a unique ratio between infrared and red dyes. (B) Oligonucleotide sequence was pre-coupled on the beads surface providing DNA barcode (anti-TAG bead). (C) Products from polymerase chain reaction using TAG-labeled primers and biotin labeled dNTPs were hybridized to anti-TAG beads. (D) An enlarged scheme of DNA hybridization on each bead. (E) Green laser was used to detect fluorescent signal from R-phycoerythrin streptavidin which was used to report the presence of biotinylated PCR product, and red laser was used to identify anti-TAG beads. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$$\text{Relative specificity} = (NA/(PD + NA)) \times 100\% \quad (2)$$

$$\text{Relative sensitivity} = (PA/(PA + ND)) \times 100\% \quad (3)$$

Where

PA is positive agreement or true positive.
PD is positive deviation or false positive.
NA is negative agreement or true negative.
ND is negative deviation or false negative.
N is total number of samples (PA + PD + ND + NA)

3. Results and discussion

3.1. Specificity of TAG-labeled primers

For a DNA-based bead array method, each bead set was commercially pre-coupled with a unique 24-base DNA sequence (anti-TAG). Thus, each specific primer pair was synthesized with the complimentary sequence with anti-TAG at 5' position (Table 1). In order to ensure that TAG-labeled primer pairs were able to specifically amplify their corresponding targets, the primer concentrations were optimized (data not shown) and each TAG-labeled primer pair was tested against 19 different bacteria strains by a single PCR method and analyzed by gel electrophoresis. The PCR results showed that each TAG-labeled primer pair could specifically amplify its corresponding target when using primer concentration at 25 nM for *L. grayi*, and *E. coli*, 35 nM for internal control, 50 nM for *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. welshimeri*, *E. coli* O157:H7, and *S. aureus*, 100 nM for MRSA, and 150 nM for *Salmonella* spp. detection (Fig. 2). For multiplex PCR, the total of 12 primers sets were mixed in a PCR master mix solution and tested with each bacterial strain. The results demonstrated that the mixture of 12 TAG-labeled primers accurately amplify their corresponding targets (Supplementary Fig. 1). Each genomic DNA sample could give two bands indicating type of bacterial strain and internal control, except in case of *E. coli* O157:H7 and MRSA which exhibit three bands indicating species, strain or drug resistance, and internal control. For instance, in the *E. coli* O157:H7 detection, three bands of 382, 515, and 1135 bp. were observed in gel electrophoresis corresponding to identification of *E. coli* O157:H7 strain, and internal control, respectively.

In addition, while visualization of the multiplex PCR product on gel could not differentiate between *L. ivanovii* or *L. welshimeri,* and internal control because of the similarity of their PCR product sizes, the bead array method could solve this problem because it relies on the specific TAG sequences instead of the size differences of PCR products. Therefore, these 12 primer sets were subsequently used to develop bead array method for multiplex detection.

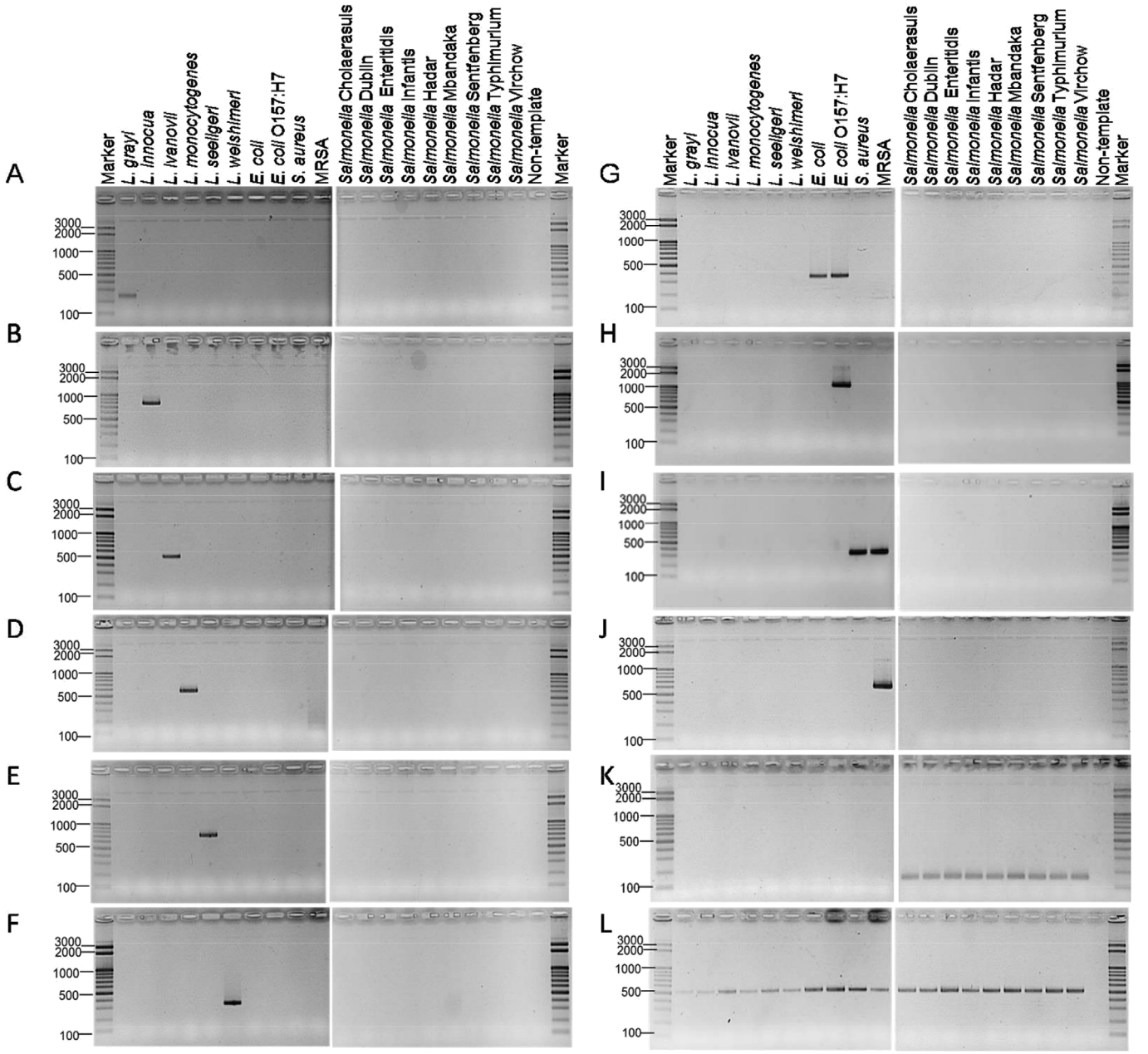

Fig. 2. Specificity of each TAG-labeled primer pair by a PCR method. The genomic DNA samples of 19 bacteria strains were amplified by using each primer pair for (A) *L. grayi* (expected size of 249 bp), (B) *L. innocua* (797 bp), (C) *L. ivanovii* (511 bp), (D) *L. monocytogenes* (557 bp), (E) *L. seeligeri* (721 bp), (F) *L. welshimeri* (466 bp), (G) *E. coli* (382 bp), (H) *E. coli* O157:H7 (1135 bp), (I) *Staphylococcus aureus* (328 bp), (J) methicillin-resistant *S. aureus* (MRSA, 603 bp), (K) *Salmonella* spp. (150 bp), and (L) an internal control (16sRNA, 515 bp). Distilled water was used as a negative control. PCR products were separated by 2% agarose gel. Marker is a 100-bp, 2 kb, 3 kb DNA ladder.

3.2. Multiplex detection using a DNA-based bead array method

In the development of a DNA-based bead array method, several assay formats have been explored. First, a direct DNA hybridization (DDH) method amplifies and labels targets by using specific primer pairs with fluorescent dye at the 5' end position (Reslova et al., 2017), and the labeled multiplex PCR products would be detected by the beads linked with specific sequences. This assay format was successfully applied to determine H antigens for typing of *Salmonella* (McQuiston, Waters, Dinsmore, Mikoleit, & Fields, 2011) and to simultaneously detect multiple enteric pathogens in stool samples (Liu et al., 2012; Onori et al., 2014). Although the system could detect multiple targets at the same time, it is rather complicated and expensive because it requires oligonucleotide probe design before coupling those specific probes on the beads.

Another assay format, called target specific primer extension (TSPE) method, avoids the hassles of specific probe design and reduces the probe-bead coupling step, by utilizing commercially pre-coupled anti-TAG bead sets, containing unique 24 oligomers. The PCR amplification was performed before the purified PCR products were then amplified by using anti-TAG-labeled target specific primers and biotin-dCTP. This method has been applied in a multiplex detection of viroid plant pathogens (van Brunschot et al., 2014). However, this TSPE method needed two steps of amplification and purification, resulting in time-consuming process and expensive detection.

In our bead array format, we developed a multiplex PCR system that was able to amplify and biotinylate in one single step. The biotinylated PCR products were then directly hybridized to a mixture of 12 anti-TAG

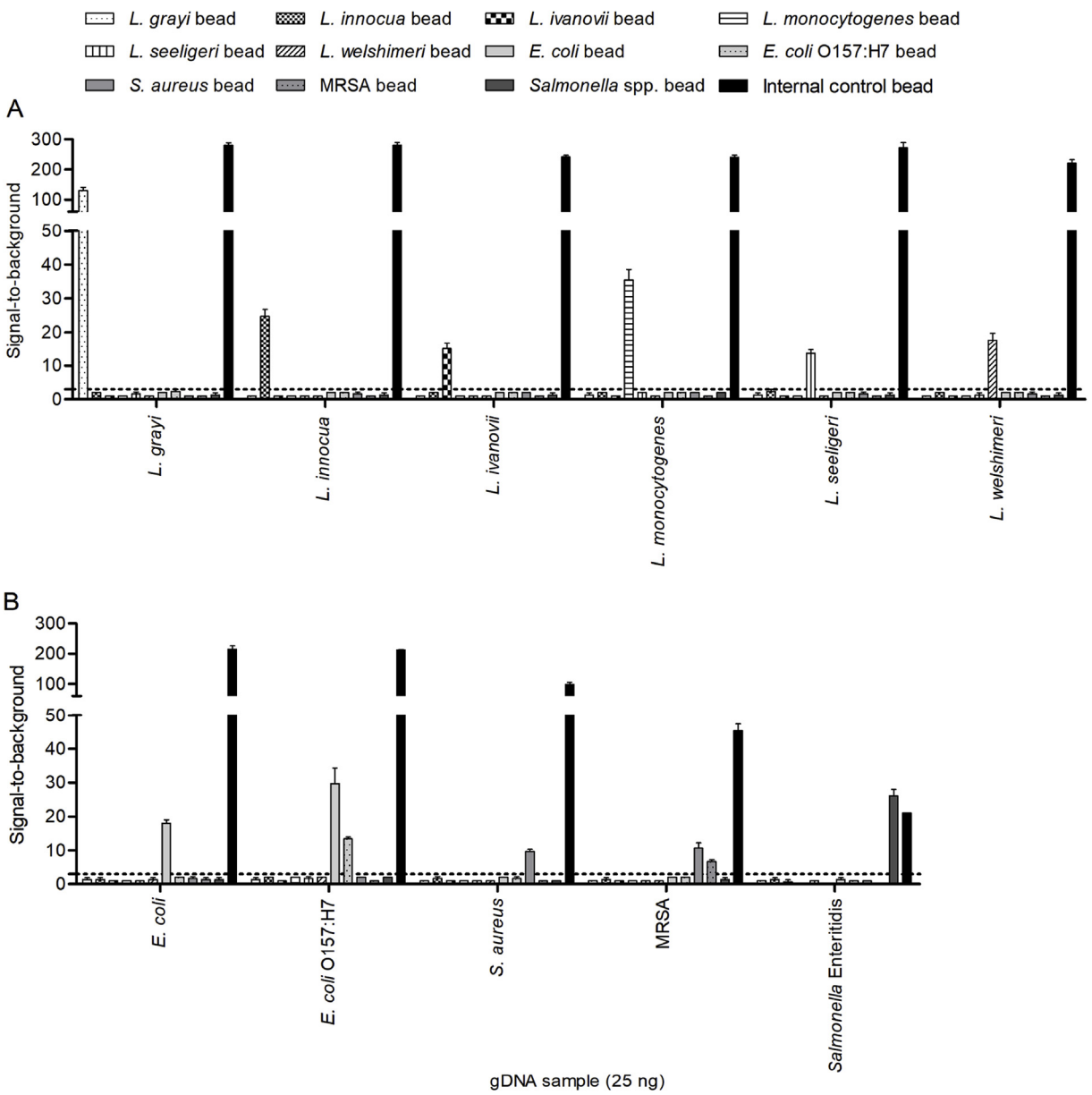

Fig. 3. Each genomic DNA sample (25 ng) of (A) *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri* and (B) *E. coli*, *E. coli* O157:H7, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and *Salmonella* Enteritidis were tested by a bead array method. Each data set was plotted as an average of triplicates with an error bar indicating a standard deviation. Dotted line represents a cut-off value which is three times of the intensity from negative control (distilled water).

bead regions without a requirement for purification. Many parameters such as concentration of each TAG-labeled primer, ratio between biotin-labeled-14-dCTP and dNTP, and hybridization temperature were systematically optimized (data not shown), and the optimal conditions reported in Section 2.4 were used to specifically identify each bacterial strain (Fig. 3). For example, in the detection of all bacterial strains, except *E. coli* O157:H7, and MRSA, bead array showed fluorescent signals from two types of beads, indicating type of pathogen, and internal control, whereas for *E. coli* O157:H7, and MRSA detection, three types of beads showed fluorescent signals, indicating type of pathogen, bacterial strain or resistance, and internal control. All bead array results were in agreement with the results from gel electrophoresis analysis. Furthermore, the bead array method could distinguish *L. ivanovii* or *L. welshimeri* from the internal control, whereas a gel electrophoresis method cloud not because of the similar sizes of the PCR products. The bead array method was illustrated that it could specifically identify type of bacteria by using one-step multiplex PCR combining amplification and biotinylation without a requirement of further purification.

3.3. Validation of developed bead array method

To validate the developed bead array method, a total of 311 isolated colonies from 194 chicken meat samples were tested in the bead array and the ISO protocols. From colonies identification by ISO protocols, we found *L. monocytogenes* (82 isolates), *Listeria* spp. (1 isolate), *E. coli* (116 isolates), *S. aureus* (45 isolates), *Salmonella* spp. (43 isolates), and other bacteria not of our interest (24 colonies) contaminated in the chicken samples. The bead array method could identify *L. monocytogenes* (82/82 isolates), *L. innocua* (1/1 isolate), *E. coli* (113/116 isolates), and *S. aureus* (35/45 isolates), *Salmonella* spp. (43/43 isolates). No other *Listeria* species, *E. coli* O157:H7, and MRSA were found in these samples.

For most of the target pathogens detection, the results from the bead array agree with those from ISO methods. The detection for *S. aureus* by

Table 3
Summary of the validation results from chicken samples.

Detection	Number of comparison results (n = 311) (ISO method[a]/the bead array method)			
	Positive agreement (+/+)	Positive deviation (-/+)	Negative agreement (-/-)	Negative deviation (-/+)
Listeria spp.	1	0	0	0
L. monocytogenes	82	0	0	0
E. coli	113	0	0	3
S. aureus	35	0	0	10
Salmonella spp.	43	0	0	0
Other bacteria not of our interest	0	0	24	0
Total	274	0	24	13
Relative accuracy (%)[b]				96
Relative specificity (%)[c]				100
Relative sensitivity (%)[d]				95

[a] ISO11290-1 for detection of *L. monocytogenes*, ISO16694 for detection of *E. coli*, ISO6579 for detection of *Salmonella* spp., and ISO6888-3 for detection of *S. aureus*.
[b] Equation (1) in Materials and Methods.
[c] Equation (2) in Materials and Methods.
[d] Equation (3) in Materials and Methods.

bead array (35 isolates) was the only test with relatively large discrepancy from the ISO method (45 isolates). Given that the primers for *S. aureus* were found to be specific to its target (Fig. 2I), the possible reason for this discrepancy might be from the fact that the ISO protocol for *S. aureus* identification requires several tests such as gram staining, coagulase testing, and mannitol fermentation testing; some of which have been reported to give false positive. For example, the agglutination assay for the coagulase testing gave false-positive results when detecting *Staphylococcus capitis*, *Staphylococcus saprophyticus*, and *Staphylococcus warneri* (Berke & Tilton, 1986). Therefore, it is not surprising that the ISO protocol result is different from the bead array result.

When compared the result of the bead array and the ISO methods, the relative accuracy, relative specificity, and relative sensitivity of our bead array method were found to be 96, 100, and 95%, respectively (Table 3). These numbers are within the commonly found range as it had been reported that comparison between DNA-based detection and standard culturing method for *Salmonella* spp. detection in animal feed samples were not significantly different with the relative of accuracy, specificity, and sensitivity were 96%, 97%, and 98, respectively (Löfström, Axelsson, & Rådström, 2008). The relative of sensitivity was considered acceptable at 95% or higher according to the guideline of NordVal protocol (Qvist, 2007). Therefore, our bead array was illustrated that it could be an alternative method with high accuracy, specificity, and sensitivity when testing with actual chicken samples.

4. Conclusions

In this study, we successfully developed a DNA-based bead array method to detect foodborne pathogens with one single step of multiplex amplification and biotinylation. The method could specifically identify the 11 bacteria species of interest in chicken meat samples with high accuracy, specificity, and sensitivity when were compared with the conventional ISO methods. This bead array method was validated and demonstrated that it could be an alternative method for food safety, indication of hygiene in food processing, assistance to proper treatment in human and livestock.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.02.014.

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