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Development of multiplex PCR assay for detection of *Salmonella* serotypes Enteritidis and Typhimurium

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ABSTRACT

Salmonella enterica is an important zoonotic bacterial pathogen. Consuming *Salmonella* contaminated foods or water is the main route of transmission. Although most of *Salmonella* serotypes can cause moderate sickness such as diarrhea, gastroenteritis, or salmonellosis, the range of illness due to *Salmonella* infection is wide, covering from no symptoms to invasive sickness or death. Aside from the typhoidal serotypes, serotypes Enteritidis and Typhimurium are the most common *Salmonella* serotypes causing 75% salmonellosis cases worldwide. Both Enteritidis and Typhimurium can transmit from animal to human and are persistent in the environments meaning they both can be problematic throughout the food chain. Therefore, developing rapid and reliable method for *Salmonella* Enteritidis and Typhimurium detection is important in order to prevent any consequence losses. Because PCR-based method used for bacterial identification is less time-consuming and less labor intensive in comparison to the conventional method, this study focuses on development of multiplex PCR (mPCR) for *S. enterica* serotype Enteritidis and Typhimurium. This mPCR composes of four primer sets which are En primers targeting Enteritidis, Tp primers targeting Typhimurium, invA primers specific for *Salmonella* genus, and the other primer set for internal positive control (IPC). The sensitivity of this mPCR is 10 pg for Entertidis genomic DNA (approximately 10^3 bacteria) and 100 pg for Typhimurium genomic DNA (approximately 10^4 bacteria). The result also showed that this mPCR did not cross-react with eight other bacteria genera.

KEYWORDS: Enteritidis; Multiplex PCR; *Salmonella*; Serotyping; Typhimurium

INTRODUCTION

Salmonella spp. is important foodborne pathogen. *Salmonella* are normally contaminated in water, vegetables, foods from animal origins (e.g., eggs, dairy products, raw meat, and animals used for food production) (El-Gazzar and Marth, 1992). *Salmonella* can also persist in the environments (e.g., animal farms, slaughter houses, and food processing plants) (Waldner et al., 2012). This bacterium leads to many diseases such as salmonellosis, typhoid fever, or septicemia (Ryu et al., 1995). Although most serotypes cause mild illness, some often cause severe sickness or death depending on the immune system of individuals (Acheson and Hohmann, 2001). Consumption of *Salmonella* contaminated in food or water supply is the main route of transmission from origins to human. Both natural and intentional outbreaks of this bacterium are relatively common. *Salmonella* becomes the major concern in food industry, public health including economic loss especially in developing countries (World Health Organization, 2016).

Salmonella shows high diversity in its genetic components and surface antigens. Currently, there are more than 2,600 serotypes of *Salmonella* (Gal-Mor et al., 2014). Although there are two species in the *Salmonella* genus which are *S. enterica* and *S. bongori*, most of *Salmonella* serotypes belong to *Salmonella enterica* subspecies I enterica (Actmann et al., 2012). Serotypes Enteritidis and Typhimurium, also belonging to *S. enterica* subspecies I enterica, cause 75% of global salmonellosis cases (Gwida and AL-Ashmawy, 2014) i.e., approximately 70.4 cases per year (Majowicz et al., 2010). Serotypes Enteritidis and Typhimurium are reported as the most and second most common infectious serotypes worldwide (Hendriksen et al., 2011).

Serotyping, the subtyping method used for serotype identification in *Salmonella*, is the agglutination reaction between *Salmonella* surface antigens and specific antibodies (Salazar et al., 2015). However, this traditional method is labor intensive, time-consuming, high costs for preservation and storage of typing sera and antigens, and unable to characterize rough and mucoid strain (Kim et al., 2006). Moreover, its sensitivity is often not high enough for foodborne outbreak differentiation (Ranieri et al., 2013). Therefore, polymerase chain reaction (PCR) becomes more widely used because it shows higher sensitivity and specificity, easier to operate with reasonable cost, and easier for result interpretation based on the presence or absence of a PCR product band (Garafutdinov et al., 2017). Multiplex PCR (mPCR) is then developed to amplify more than one target in a single reaction which results in providing more rapid and productive response time.

The objective of this study was to develop mPCR assay to detect and identify *S.* Enteritidis and *S.* Typhimurium. The mPCR included an internal positive control (IPC) in each PCR reaction in order to eliminate false-negative results from PCR inhibition.

MATERIALS AND METHODS

Bacterial strains and culture condition

Salmonella Enteritidis S5-371 and *S.* Typhimurium LT2 were chosen as references strains for mPCR optimization. Eight non-*Salmonella* strains were tested for primer specificity. Bacteria were cultured on tryptic soy agar (TSA) and incubated overnight at 37 °C. A single colony was picked and inoculated into 5 mL of tryptic soy broth (TSB). The culture was incubated overnight at 37 °C with shaking 200 rpm.

DNA extraction

Genomic DNA (gDNA) was obtained by extracting 1 mL of an overnight culture using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taipei, Taiwan) following the manufacturer's instruction. After purification, concentration and quality of extracted DNA was measured by a DS-11 spectrophotometer (DeNovix, Delaware, USA).

Primer design

Primers used in this study were obtained either from literature reviews or manually designed to the specific region. DNA sequences retrieved from NCBI GenBank database were multiple aligned to identify the serotype-specific region using ClustalW2 (www.ebi.ac.uk/tools/clustalw2). Primer designs were performed *in silico* specificity test using Primer Blast on the NCBI website (www.ncbi.nlm.nih.gov). En and Tp primers were respectively specific to Enteritidis and Typhimurium. invA primer was selected as genus *Salmonella* specific and IPC was selected as an internal positive control in order to eliminate the false-negative result.

Single PCR

Size and position of each PCR product were determined by single PCR amplification. The 25 µL reaction mixture was composed of 12.5 µL of 2 × GoTaq Green Mastermix (Promega, Wisconsin, USA), and 10 ng of reference *Salmonella* gDNA template. The primer concentrations were 0.06 µM of En primers, while 0.34 µM of Tp primers, and 0.12 µM of invA primers. In case of IPC amplification, IPC oligo 9×10^7 copies was spiked in each reaction as DNA template with IPC primers at 0.20 µM final concentration. Nuclease-free water was used to adjust the final volume to 25 µL. The single PCR amplification started with an initial denaturation at 95 ℃ for 3 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products

were separated by using 3% agarose gel electrophoresis stained with 0.5 × ViSafe Green Gel Stain (Vivantis, California, USA), and visualized under UV transilluminator by ChemiDoc™ XRS+ System (Bio-Rad, California, USA). Quick-Load® Purple Low Molecular Weight DNA Ladder (NEB, Massachusetts, USA) was used as DNA marker. Equal amount of amplicon was loaded in each lane.

Multiplex PCR

Multiplex PCR reaction was consisted of four primer sets mentioned in previous section which are two serotype-specific, one genus-specific, and internal positive control primer sets. The amplification was performed in a 25 µL reaction volume containing 12.5 µL of 2 × GoTaq Green Mastermix, 0.12 µM invA primers, 0.06 µM En primers, 0.34 µM Tp primers, 0.20 µM IPC primers, 9×10^7 copies of IPC oligonucleotides, and 1 ng of gDNA template. Nuclease-free water was used to adjust the final volume to 25 µL. The PCR reaction and electrophoresis were carried out in the same conditions as described earlier.

Sensitivity and specificity

The limit of detection was determined by sensitivity testing. Ten-fold serially dilution of gDNA template was applied to the mPCR reaction ranging from 10 ng to 1 pg. Non-*Salmonella* gDNA templates replaced *Salmonella* gDNA to define the specificity of this mPCR method. One ng of eight non-*Salmonella* bacteria namely *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Vibrio cholerae* were used as DNA template in each mPCR reaction.

RESULTS AND DISCUSSION

Primer design

invA primers was retrieved from Chiu and Ou (1996) while IPC primers and IPC oligonucleotides was retrieved from Hudlow et al. (2008). En primers target a gene that encodes phage-holin family protein while Tp primers target a gene encoding putative type II restriction enzyme, methylase subunit.

Single PCR

A single PCR with each primer set was performed in order to confirm that each amplification product was at the expected size. Gel electrophoresis result is shown in Figure 1. The amplicon sizes of invA, En, Tp, and IPC primers are at 244, 164, 98, and 77 bp, respectively.

Multiplex PCR

All of 4 primer pairs used in this study were combined in a single reaction. Figure 2 shows four different patterns are observed due to different kinds of template. invA amplicon was present in every reaction with *Salmonella* gDNA and IPC amplicon was present in all samples confirming positive amplification within this mPCR mixture.

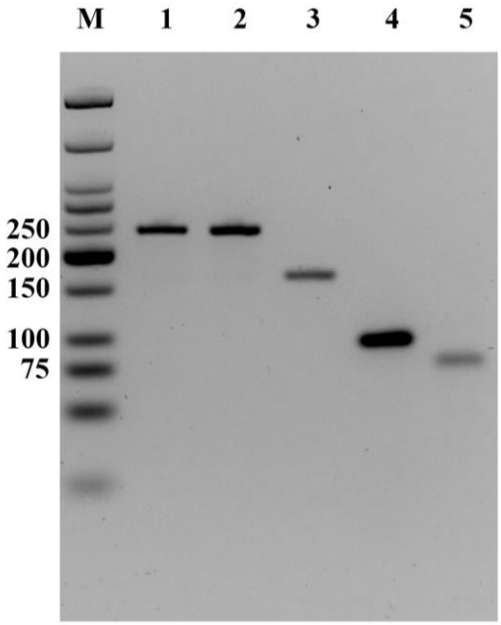

Figure 1. Single amplification result of each primer pair for multiplex PCR assay. (M) DNA marker; (1) invA amplicon from invA primers with 10 ng *S*. Enteritidis gDNA template, 244 bp; (2) invA amplicon from invA primers with 10 ng *S*. Typhimrium gDNA template, 244 bp; (3) Enteritidis-specific amplicon, 164 bp; (4) Typhimurium-specific amplicon, 98 bp; (5) IPC amplicon, 77 bp.

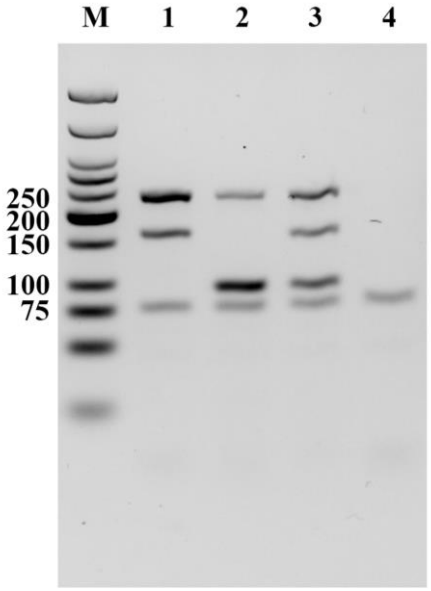

Figure 2. Multiplex amplification results with four different DNA templates. (M) DNA marker; (1) *S*. 1 ng Enteritidis gDNA template; (2) 1 ng *S*. Typhimrium gDNA template; (3) Mixed gDNA templates between *S*. Enteritidis and *S*. Typhimurium, 1 ng each; (4) No *Salmonella* DNA template.

Sensitivity

This mPCR assay was tested with 10-fold serially diluted gDNA templates in order to determine the sensitivity of the developed system. The results showed that the sensitivity with *S*. Enteritidis was 10 pg while *S*. Typhimurium was 100 pg (Figure 3).

Specificity

After DNA templates were changed to eight different non-*Salmonella* gDNA, the results confirmed that this system did not cross-react with these other bacteria genera. Only IPC bands were present as expected (Figure 4).

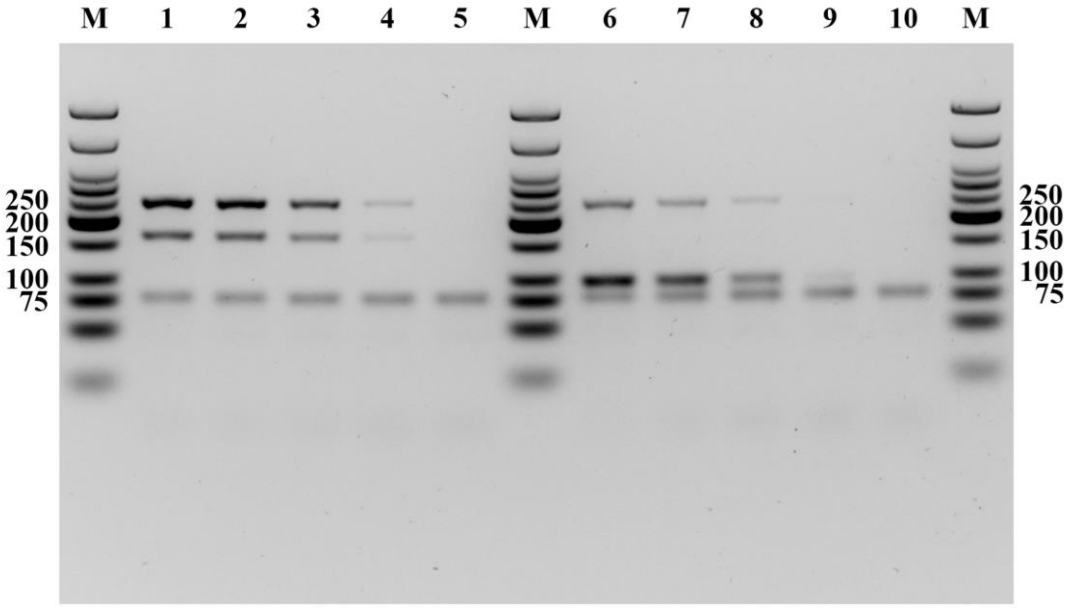

Figure 3. Sensitivity of multiplex PCR assay (M) DNA Marker (1)-(5) *S*. Enteritidis DNA (6)-(10) *S*. Typhimurium DNA at 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg, respectively.

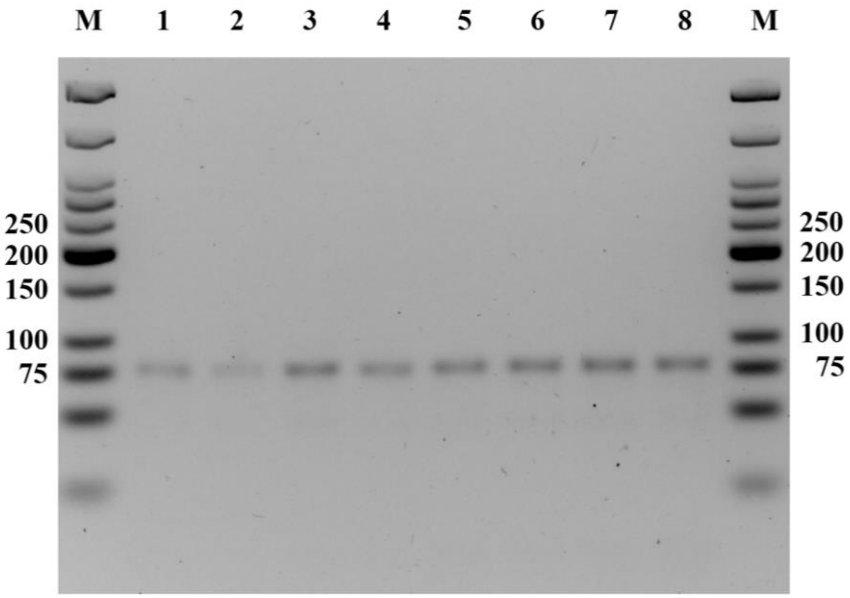

Figure 4. Specificity of multiplex PCR assay (M) DNA Marker and gDNA templates from (1) *Enterobacter cloacae*, (2) *Enterococcus faecalis*, (3) *Escherichia coli*, (4) *Citrobacter freundii*, (5) *Listeria monocytogenes*, (6) *Pseudomonas aeruginosa*, (7) *Vibrio cholera*, and (8) *Staphylococcus aureus*.

CONCLUSION

The multiplex PCR assay for detection of *Salmonella* serotypes Enteritidis and Typhimurium was developed. This assay included genus *Salmonella* specific and internal positive control to avoid the false-negative result. After optimization, the sensitivity of this assay was 10 pg gDNA for serotype Enteritidis and 100 pg gDNA for serotype Typhimurium. The result also suggested that this assay do not cross-amplify with eight other bacteria genera commonly found in foods. The developed system can be used to rapidly identify presence of these problematic *Salmonella* serotypes in enriched food or environmental samples, and subsequently initiate a prompt strategic control before contaminated foods reach the consumers.

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