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Research paper

Detection Method for Salmonella Typhimurium and Salmonella Enteritidis using Real-Time Polymerase Chain Reaction

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Abstract

Salmonella is a pathogenic bacterium that can cause serious harm to humans. Chicken carcasses have been reported contaminated by *Salmonella*, especially *S.* Typhimurium and *S.* Enteritidis. These two serovars are very difficult to be confirmed and distinguished using biochemical analysis, therefore a rapid method for detection and differentiation of both is required. The objective of this study was to evaluate designed primer for detection and differentiation of *S.* Typhimurium and *S.* Enteritidis on chicken carcasses using *real time Polymerase Chain Reaction* (rt-PCR). Detection of *Salmonella* spp. was conducted using primer sequence from invA gene. Differentiation of both *Salmonella* serovars was conducted using specific target gene from *S.* Typhimurium (STM) and specific virulence plasmid of *S.* Enteritidis (Prot6E). The result showed that invA primer effective to detect all species *Salmonella* tested and has good specificity that could not detect *Escherichia coli* and *Shigella dysenteriae* in the similar melting temperature. Two specific primers STM and prot6E have distinguished between *S.* Typhimurium and *S.* Enteritidis. Sensitivity of method showed very good with 0.5 µM primer concentration of invA, STM and prot6E that were 0.2 pg, 22 pg and 28 pg respectively. Initial trial showed that this method can be applied for detection of *Salmonella* spp. and two serovars in chicken carcasses.

Keywords: *chicken carcasses, detection method, real-time PCR,* Salmonella *Enteritidis,* Salmonella *Typhimurium*

1. Introduction

Salmonella was first discovered and isolated from the intestines of classic swine fever infected by pig, by Theobald Smith in 1855. The bacterial strain was named by Dr. Daniel Elmer Salmon, an American pathologist who worked with Smith [1]. Salmonella is characterized as a rod-shaped Gram-negative bacterium that does not form spores. *Salmonella* generally grows on media that has water activity above 0.94 and pH 4.1-9.0 with optimum pH 7.0-7.5. *Salmonella* can grow in a temperature range of 5.2 - 43^{o}C with an optimum temperature of 35-43^{o}C and will die on media with salt content above 9% [2]. Salmonellosis is a disease caused by food contamination by *Salmonella* bacteria [3].

Salmonella is distributed in the environment and commonly colonize the intestinal tract of animals, especially of poultry. During chicken slaughtering process to be carcasses, feather and internal organs are removed. Chicken carcasses can be exposed by manure from intestinal tract during eviscerating and others handling step. *Salmonella* contamination in chicken carcasses can occurred due to contamination in the supply chain starting from the process of production, distribution, seller, and handling by consumers. If this chain is not well controlled, it will result the increasing number of whole pathogenic bacteria, include *Salmonella* [4]. *S. enterica* serovar Typhimurium (then witten as *S.* Typhimurium) and *S. enterica* serovar Typhimurium (then witten as *S.* Typhimurium) are two pathogenic serovars that commonly contaminated in chicken carcasses.

Detection and identification of pathogenic bacteria in food based on molecular method is one of the most widely used recently, such as polymerase chain reaction (PCR) assay. The principle of the PCR method is to amplify specific DNA segments such as virulence factor that is contained in certain bacteria. This technique has been proven to be more sensitive, specific, and faster in detecting the presence of pathogenic bacteria [5]. SYBR green is commonly used for fluorescent dye that could use for label during real-time PCR (rt-PCR) running. SYBR is the simplest and cheapest compared to others dies for rt-PCR. SYBR green rt-PCR provides fluorescence while it binding with the targeted double-stranded DNA that formed during amplification [6]. Fluorescent intensity showed the quantity of PCR products.

The InvA gene target is a gene that functions to improve intracellular survival by regulating the level of toxic nucleotides induced by stress during the infection process [7]. InvA has high specificity and sensitivity to *Salmonella* gene targets [8]. Study conducted by Rahn *et al.* [9] showed a positive invA gene for 630 *Salmonella*, only negative in *S. enterica serovar* Senftenberg and Lichtfield, and internationally validated [10]. The target of the STM gene is the gene responsible for fimbrial biosynthesis [11]. Long polar fimbrial is absent in all *Salmonella* strains, specific to *S.* Typhimurium. Long polar fimbrial from *S* Typhimurium mediates adhesion and is required for full virulence [12]. Fimbrial is generally responsible for the initial adhesion of *Salmonella* bacteria to eukaryotic cells [10]. The Prot6E gene target is located in *S.* Enteritidis) specific to 60-kb virulence plasmid [13]. Tests of 119 strains of 54 non *S.* Enteritidis showed negative results, but four of 79 *S.* Enteritidis were tested, negative for the Prot6E gene due to the absence of 60-kb virulence plasmid in the strain [10]. This shows that plasmid virulence is important in the pathogenicity of *S.* Enteritidis [13].

S. Typhimurium and *S.* Enteritidis are very difficult to be confirmed and distinguished and also to be quantified using biochem-

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ical analysis, therefore a rapid and accurate method for detection, differentiation and quantification of both strains is required. The objective of this study was to evaluate designed primer for detection and differentiation of *S.* Typhimurium and *S.* Enteritidis on chicken carcasses using *real time Polymerase Chain Reaction* (rt-PCR). Parameters for evaluation of rt-PCR method consisted of specificity of primers, sensitivity and efficiency of standard curve.

2. Materials and Method

2.1. Bacterial strains, growth condition and bacterial DNA

Bacteria used in the study were *S. enterica serovar* Hadar (then written as *S.* Hadar) (BCCB2908) from Central Veterinary Research, *S.* Typhimurium (ATCC 14028) and *S.* Enteritidis (ATCC13076) from BPOM RI (Indonesia National Agency for Food and Drug Control). All of bacteria were grown in Brain Heart Infusion Broth (BHIB) at 35°C for 20 h. *S.* Hadar was used as representative of *Salmonella* group outside two serovars tested. Frozen stored DNA of *Shigella dysenteriae* (ATCC 9290) and *Escherichia coli* (NCCP 13717) were used as comparative DNA.

2.2. DNA extraction

DNA was extracted from overnight culture using chelex-100 reagent method [14] with some modification, i.e without using microwave. Each culture was grown in BHIB medium overnight at 35°C, then 2 mL of the bacterial cultures were harvested by centrifugation at 3000 rpm for 5 min at 4°C. Bacterial pellets were washed with 1000 µL TE buffer (10mM TRIS-Base, pH 7.5; 1 mM EDTA, pH 8.0) and centrifuged again under the same conditions. The cell were resuspended in 100 µL TES lysis buffer (10mM TRIS-Base, pH 7.5; 1 mM EDTA, pH 8.0; 0.5% SDS) and incubated on waterbath for 5 min at 65°C. Immediately thereafter, 7.5 µL proteinase K 20 mg/mL (Amresco, catalogue No. 0706-100MG), RNAse A (Thermo, catalogue No. EN0531) were added to the suspension and incubated on waterbath for 5 min at 65°C. After lysis, the suspension was incubated for 2 min at room temperature (25°C), and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant containing the DNA was recovered and quantified. The purified DNA was precipitated 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of 95% ethanol. The supernatant was incubated for 20 h at -20°C. The supernatant was centrifuged 8000 rpm for 5 min at 4°C. Finally, the DNA was dried at room temperature and resuspended in 100 µL deionized water. DNA quantified with Nano Drop 2000 spectrophotometer (Thermo Scientific).

2.3. Primer used and amplification condition

Three primers used were listed as pairing with particular *Salmonella* template (Table 1); and used with two level of concentration (0.5 µM and 0.1 µM). This method used SYBR as fluorescent dye for rt-PCR (SwiftTM Spectrum 48, ESCO, Singapore). Each reaction consisted of Thunderbird® SYBR® qPCR Mix (Code QPS-201, QPS-201T), primer *invA* (F and R), primer STM (F and R), primer *Prot6E* (F and R), and 1 µL of DNA template, with 20 µL total volume of mixture. The reaction were subjected to an initial denaturation (pre-denaturation) at 95°C for 1 min, followed 35 cycles (denaturation, annealing and extension) consisting of denaturation at 95°C for 15 s, annealing at 52°C for 30 s, extension step at 72°C for 35 s, and end-extension at 5 min. Amplification was followed by melting analysis of the PCR products. Melt curve was generated by increasing the temperature from 60 to 90°C at 0.5°C/s.

2.4. Evaluation of specificity

Specificity of primers has checked using Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and MEGA Software (www.megasoftware.net). Specificity of primer also evaluated during rt-PCR running, by cross checking the primer against to non-pairing *Salmonella* template to check between serovars. The genus specificity test was evaluated by checking against another species (*Shigella dysenteriae* and *Escherichia coli*). Selection of *S. dysenteriae* and *E. coli* was based on a high genetic relationship with *Salmonella* [15].

Table 1: Primer used for *Salmonella* spp. detection

Pairing *of Salmonella* & primer	sequence of primers	Size (bp)
Salmonella spp. (InvA) [16]	(F): TCG TCA TTC CAT TAC CTA CC (R) : AAA CGT TGA AAA ACT GAG GA	119
Typhimurium (STM) [17]	(F) :AAC AAC GGC TCC GGT AAT GAG ATT G (R) : ATG ACA AAC TCT TGA TTC TGA AGA TCG	311
Enteritidis (Prot6E) [18]	F: GGC ACC GCA GCA ATG GTT GG R : GGT CGA GCT ACA GAG AGT CAC AC	135

2.5. Evaluation of sensitivity and efficiency of standard curve

This work was carried out by artificially contaminated of *Salmonella* spp. on chicken carcasses. Sensitivity was carried out on samples of chicken contaminated with *S.* Hadar, *S.* Typhimurium and *S.* Enteritidis, respectively. Amount of 25 g of chicken meat was dipped into boiling water for 30 seconds for decontamination process. Then it was putted into sterile plastic, added with 1 mL of *Salmonella* culture (10^5 CFU/mL), homogenized in 50 mL BHIB and allowed to stand for 3 minutes. DNA was extracted from contaminated chicken meat samples using chelex-100 method [14]. DNA was quantified with Nano Drop 2000 spectrophotometer (Thermo Scientific) and diluted to final concentration from 200 – 0.0002 ng/µL in nuclease free water) and stored at -20°C. Then DNA in several concentrations was run at rt-PCR to determine the cycle threshold value (Ct). Standard curve was generated by plotted the log DNA concentration as X-axis and Ct value as Y-axis.
Amplification efficiency (E) was estimated using standard curve slopes and the formula:

$$E = \{(10^{-1/slope}) - 1\}\, x\, 100\%$$

3. Results and Discussion

Real-time PCR method for bacterial detection was evaluated by determination the cycle threshold value (Ct), melting temperature Tm), specificity, sensitivity and efficiency of standard curve. Ct value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e exceeds background level). Ct value can used to determine detection limit, therefore the lowest of Ct value or shortest time for detect showed the good capacity of detection. Ct value also equivalent with the number of bacterial cell, the more number of bacterial cell in the sample the faster detection or the lowest of Ct value.
Melting temperature is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. Tm can used to determine the specificity of the primer or PCR method. Different bacteria should give different Tm result.

3.1. Threshold cycle and melting temperature of gene target

The primer and its pairing *Salmonella* template provided the good threshold detection, indicated by the lower of Ct value (Table 2) than non-pairing bacteria. As an example, the pairing of STM- *S.* Typhimurium showed the Ct value lower than (12.65) the Ct of STM against *S.* Enteritidis (26.76). The highest concentration of primer (0.5 µM) provided the lowest Ct value; therefore this concentration was kept for further study.

Three primers showed different melting temperature (Tm), i.e 79.0, 83.5, and 84.0 for InvA, STM and prot6E respectively. Tm is the temperature at which one half of the DNA duplex will dissociate to become single stranded. The point of this temperature is determined by base sequences and size of DNA amplicon, also by degree of GC content of DNA amplicon. Similar Tm indicates the similar DNA sequences, whereas different Tm indicates the different DNA sequences, that meaning different bacterial strain. In this study, Tm of each primer showed the different sequence of amplicon and can be used to differentiate *S.* Typhimurium and *S.* Enteritidis.

Table 2: Cycle threshold and melting temperature of *Salmonella* spp.

Primer and Strains	Ct value at primer conc. (cycle)		Tm (°C)
	0.1 µM	0.5 µM	
InvA 119			
S. Hadar	12.22 ± 0.10	11.72 ± 0.06	79.0 ± 0.00
S. Typhimurium	12.72 ± 0.12	12.21 ± 0.10	
S. Enteritidis	13.33 ± 0.10	12.78 ± 0.01	
STM311			
S. Hadar	26.96 ± 0.03	25.67 ± 0.06	83.5 ± 0.00
S. Typhimurium	13.29 ± 0.05	12.65 ± 0.01	
S. Enteritidis	26.97 ± 0.01	26.76 ± 0.05	
Prot6E			
S. Hadar	22.29 ± 0.06	22.25 ± 0.02	84.0 ± 0.00
S. Typhimurium	26.92 ± 0.02	26.85 ± 0.02	83.5 ± 0.00
S. Enteritidis	11.72 ± 0.06	9.77 ± 0.01	84.0 ± 0.00

3.2. Specificity

Primer specificity testing was carried out to determine the ability of primer to distinguish the target bacterial DNA from other bacteria. Combination between Primer BLAST and MEGA software for primer checking showed that all primers have high specificity for the serovar target. Amplification output curve showed the results of the test of the specificity between serovars by cross-checking them (Table 2), also showed the results of the genus specificity test (*Shigella dysenteriae* and *Escherichia coli*) (Table 3).

Test of strain specificity showed that the target primer was amplified in cross-test. Product amplification occurred in the 25th cycle on STM primer and the 22nd cycle on Prot6E primer showed the non-specific product or primer dimer products. This product was formed because SYBR Green can bind to non-specific double-chain DNA or the occurrence of primer dimers. Primer dimers are process binding between forward and reverse primer pair that are amplified and quantified to produce false-positive data [19]. Based on these results, there was threshold cycle cut off for detection of *Salmonella* spp. using invA primer was 30 cycles, S. Typhimurium using STM primer was 25 cycles and S. Enteritidis using prot6E primer was 22 cycles.

Primer InvA, STM and Prot6E can be used for detection and differentiate *Salmonella* spp. with other genus, indicated by different Tm value of *S. dysenteriae* and *E. coli* from Tm of *Salmonella* spp. (Table 3). In line with Tm, based on the Ct value primer InvA can use for detection and differentiation of *Salmonella* spp. with other genus, due to *S.dysenteriae* and *E. coli* only can be amplified after cycle of 30 (32.76 and 30.08 respectively). InvA has high specificity and sensitivity for *Salmonella* [8]. *S.dysenteriae* and *E. coli* can be amplified by primer STM and Prot6E after cycle of 21, but showed the really different Tm value from Tm of *Salmonella* spp.

Table 3: Specificity or primer against other bacteria

Primer	*Shigella dysenteriae*		*Escherichia coli*	
	C_t ± SD	Tm ± SD (°C)	C_t ± SD	Tm ± SD (°C)
InvA	32.76 ± 0.04	73.0 ± 0.00	30.08 ± 0.02	72.5 ± 0.00
STM	25.80 ± 0.06	75.6 ± 0.01	25.84 ± 0.03	75.5 ± 0.00
Prot6E	21.36 ± 0.02	85.0 ± 0.00	21.27 ± 0.01	88.5 ± 0.00

3.3. Sensitivity

Sensitivity of primer and method is determined by the highest dilution of DNA or bacterial cell number that can remain be amplified by the PCR. Sensitivity was determined for particular primer and its *Salmonella* template pairing. Sensitivity of rt-PCR with InvA primer for *Salmonella* spp. (representative by *S.* Hadar) detection showed that method could detect bacterial DNA concentration up to lowest limit of 0.0002 ng (0.2 pg) (Fig. 1) for less than 30 cycles. This DNA concentration was equivalent with 3.8 x 10^1 bacterial cells on the sample.

Sensitivity of detection for *S.* Typhimurium using STM primer showed that method could detect bacterial DNA concentration up to lowest limit of 0.022 ng (22 pg) (Fig. 2) for less than 25 cycles. This DNA concentration was equivalent with 4.1 x 10^3 bacterial cells on the sample. Sensitivity of detection for *S.* Enteritidis using Prot6E primer showed that method could detect bacterial DNA concentration up to lowest limit of 0.218 ng (218 pg) (Fig. 3) for less than 22 cycles. This DNA concentration was equivalent with 2.6 x 10^4 bacterial cells on the sample.

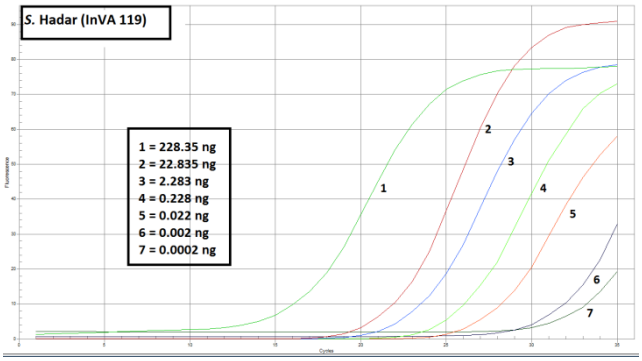

Fig. 1 : Sensitivity curve of *Salmonella* spp. using InvA

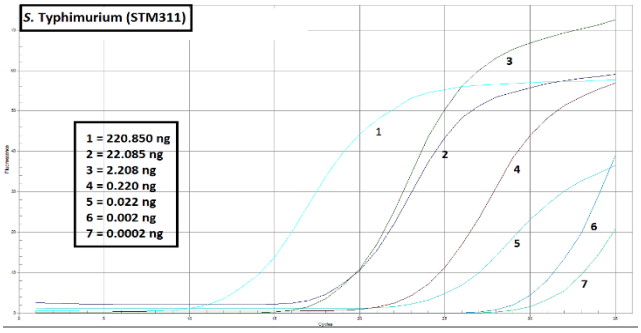

Fig. 2: Sensitivity curve of S. Typhimurium using STM

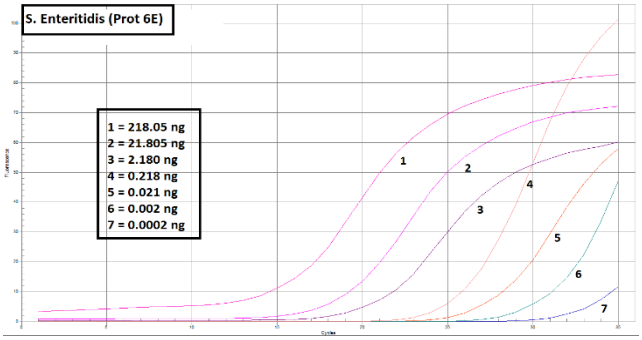

Fig. 3: Sensitivity curve of S. Enteritidis using prot6E

This detection limit for *S.* Hadar and *S.* Typhimurium showed better sensitive than the result of research conducted by Nurjanah *et al*. [6] using SYBR Green for detection pathogenic *Escherichia coli* (0.1 ng DNA concentration). The detection limit for *S.* Hadar showed better sensitive than the result conducted by Singh and Mustapha [20] using SYBR Green for detection of *Salmonella* spp. (0.002 ng DNA concentration) and compare to the research conducted by Oliveira *et al.* [21] using simplex qPCR (0.0023 ng DNA concentration).

However, this result less sensitive when compared with the research conducted by Gunnel *et al*. [22] that use a probe as a label instead of SYBR to detect *Francisella tularensis* (which is 250 fg - 2.5 pg DNA concentration). This less sensitivity is due to usage of SYBR fluorescent dye that need more amount of DNA formed to detect the presence of target.

3.4. Efficiency of standard curve

Standard curve with good linearity is very important to quantify the number of *Salmonella* cell found in the sample. All of primers resulted good linearity (r square close to 1.0) of standard curve. The calculated PCR efficiency ranged between 100 –115% (Fig. 4) with coefficient of determination (r^2) 0.96 – 0.97. The slope values on the curves for the target genes invA, STM and Prot6E were -2.99, -3.20 and -3.30 respectively. Efficiency is indicated of how far the PCR running condition can detect broadest ranges of DNA quantities from the samples [6]. Acceptable efficiency value is range between 90-110% with slope -3.6 to -3.1 [23]. Good PCR efficiency was obtained from targeted gene STM and Prot6E. Efficiency above 110% (corresponds with slope less than -3.1) is indicated of amplification of non-specific products occurred, and presence of primer-dimer [19]. The presence of an inhibitor in rt-PCR reaction can be demonstrated by increasing the efficiency value due to an increase in Ct value and a decrease in the absolute value of the slope [6].

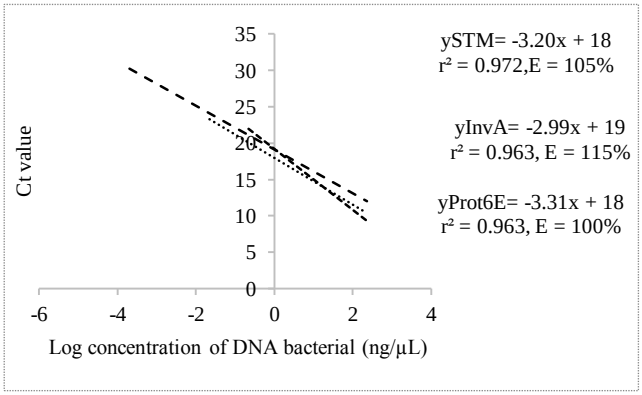

Fig. 4: Standard curves simplex rt-PCR

- - - - - - - InvA ..... STM ---------- Prot6E

3.5. Application on chicken carcasses

Application trials to detect *Salmonella* contaminated on 20 chicken carcass samples from 3 different markets using rt-PCR showed the similar result with standard biochemical assay for *Salmonella* spp. analysis. This rt-PCR method was suitable to use as rapid method detection for detecting and distinguishing *Salmonella* spp., *S.*Typhimurium and *S.* Enteritidis in chicken carcasses.

4. Conclusion

All of primer and its pairing *Salmonella* template provided the good threshold detection, indicated by the lower of Ct value. Three primers showed different melting temperature (Tm), i.e 79.0, 83.5, and 84.0 for InvA, STM and prot6E respectively. Melting temperature of each primer can be used to differentiate both of serovar of *Salmonella* and also with other genus (*Escherichia coli* and *Shigella dysenteriae).* Sensitivity of three primers showed different lowest limit of DNA concentration or bacterial cell number; the InvA showed the highest sensitivity. Good PCR efficiency was obtained from targeted gene STM and Prot6E. Initial trial showed that this method can be applied for detection of *Salmonella* sp. and two serovars in chicken carcasses.

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