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RAPID DETECTION OF *Salmonella* IN SHRIMP BY POLYMERASE CHAIN REACTION

[Deteksi Cepat Salmonella pada Udang dengan Polymerase Chain Reaction]

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ABSTRACT

Shrimp is an important non-oil commodity for foreign trade in Indonesia. However, rejection of shrimp exports by the importing countries is still commonly encountered. In 2011, the USFDA recorded two cases of Salmonella spp. contamination in shrimp products from two shrimp processing companies in Indonesia. Analysis of Salmonella spp. in seafood is generally performed using a conventional method which takes at least 5 days. The objective of the study is to get a Salmonellae rapid detection method in shrimp by PCR. In this study, optimization of PCR protocol method to detect Salmonella invA gene was conducted using six different annealing temperatures (59, 59.5, 60.8, 62, 64 and 64.5°C). The results showed that 64°C was the optimum annealing temperature to detect the 284 bp fragment of Salmonella invA gene. The PCR based detection method has a DNA detection limit of 27.81μg/mL and 10°CFU/mL of viable salmonellae with 100% specificity. The PCR protocol is capable of detecting six different Salmonella serovars (S. Enteritidis, S. Hadar, S. Heidelberg, S. Kentucky, S. Paratyphi and S. Typhimurium) but none of the non salmonellae isolates. Application of the PCR assay on Salmonella in shrimp after the selective enrichment step suggested that all 16 samples were positive for Salmonella. At the same time, the conventional method could only detected 3 samples for Salmonella positive.

Keywords: invA gene, optimization, PCR , Salmonella spp., shrimp

ABSTRAK

Udang merupakan sumber non migas yang penting dalam perdagangan internasional di Indonesia. Namun, kasus penolakan ekspor udang oleh negara pengimpor masih sering ditemui. Pada tahun 2011, USFDA mencatat dua kasus kontaminasi Salmonella spp. pada produk udang dari dua industri pengolahan udang di Indonesia. Pengujian Salmonella spp. pada seafood umumnya menggunakan metode konvensional yang membutuhkan waktu minimal 5 hari. Penelitian ini bertujuan untuk mendapatkan metode deteksi cepat Salmonellae dalam udang dengan metode PCR menggunakan sekuens gen invA. Pada penelitian ini, dilakukan optimasi protokol PCR untuk mendeteksi Salmonella spp. dengan 6 suhu annealing yang berbeda (59; 59.5; 60.8; 62; 64 dan 64.5°C). Hasil penelitian menunjukkan bahwa suhu annealing 64°C optimum untuk mendeteksi fragmen gen invA Salmonella spp. pada 284 bp. Protokol PCR ini memiliki limit deteksi terhadap DNA sebesar 27.81 μg/mL dan kultur Salmonella spp. sebesar 10°CFU/mL dengan spesifisitas 100%. Metode PCR tersebut mampu mendeteksi 6 serovar Salmonella yang berbeda (S. Enteritidis, S. Hadar, S. Heidelberg, S. Kentucky, S. Paratyphi dan S. Typhimurium), dan tidak mengenali non Salmonella. Aplikasi protokol PCR terpilih pada Salmonella spp. dalam udang segar setelah tahap pengayaan selektif menunjukkan bahwa ke-16 sampel positif mengandung Salmonella spp. Metode konvensional hanya mampu mendeteksi 3 sampel sebagai positif mengandung Salmonella spp.

***Kata kunci**: gen invA, optimasi, PCR, Salmonella spp., udang*

INTRODUCTION

For Indonesia, shrimp is an important food commodity for export (Ministry of Marine Affairs and Fisheries, 2012). However, export and rejection is still frequently encountered due to Salmonellae, *Vibrio parahaemolyticus* (Ministry of Marine Affairs and Fisheries, 2010) as well as chemical residues (Ministry of Marine Affairs and Fisheries, 2012). Between January–December 2010, the import rejection of fisheries products from Indonesia due to *Salmonella* spp. was 65%. Presence of *Salmonella* spp. in fish and fisheries products from Indonesia have been reported. Dewanti-Hariyadi *et al*. (2005) reported that 2 of 32 ocean shrimp samples contaminated with *Salmonella* spp., whereas in aquaculture shrimp samples 4 of 32 samples were contaminated by *Salmonella* spp. *Salmonella* spp. was found in aquaculture shrimp located Tangerang (2.4%) and Karawang (24-29%), West Java (Rusyanto, 2005). Contamination of S*almonella* was also found in frozen cooked shrimp (Norhana *et al.* 2010).

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Salmonella spp. can contaminate shrimp from the aquatic environments during harvest and handling. Absence of *Salmonella* spp. in shrimp is generally tested using conventional methods which take more than 5 days. With advances in technology, a variety of detection methods have been developed. Detection method of *Salmonella* spp. based on PCR has been reported by Hyeon *et al.* (2010) and Upadhyay *et al*. (2010).

Devananda (2011) reported that testing *Salmonella* spp. using conventional method is effective and has been used to isolate and identify *Salmonella* spp. in food, but the procedure is very complex and resources consuming. *Polymerase Chain Reaction* (PCR) based method is more sensitive, specific and faster than conventional method. It also improves the limit of quantitative detection for *Salmonella* (Ellingson *et al*. 2004).

Various detection limits and accuracies have been observed with the use of different primers to detect *Salmonella* (Malorny *et al.* 2003). Several primers were reported to amplify and detect *Salmonella* spp. with the following gene targets: replication *oriC Salmonella* spp. (Devananda, 2011), *invA* and E (virulence genes) (Trafny *et al.* 2006; Shanmugasamy *et al.* 2011; Zou *et al.* 2011). The objective of this study was to develop a PCR based method to detect *Salmonella* spp. in shrimp employing an *invA* gene primer. The use of these genes as a potential target for Salmonella identification has been suggested since these genes were shown to be present in a number of Salmonella strains. Primers amplify and detect a fragment of *invA*, a highly conserved gene present in almost all salmonella serotypes (Upadhyay *et al.* 2010; Devananda, 2011).

MATERIALS AND METHODS

Bacterial isolates

Bacteria used for this study were 6 different *Salmonella* serovars, i.e. *S.* Enteritidis*, S.* Hadar*, S.* Heidelberg*, S.* Kentucky*, S.* Paratyphi obtained from SEAFAST CENTER (Dewanti-Hariyadi *et al.* 2005) and *S.* Typhimurium obtained from Microbiology Lab, CRDPPMBF, Jakarta, Indonesia. Five non-Salmonellae which frequently contaminate food were used as negative controls, i.e. *E. coli, L. monocytogenes, P. mirabilis, S. aureus, V. parahaemolyticus.* These bacteria were provided by Microbiology Lab, CRDPPMBF, Jakarta, Indonesia.

DNA extraction for PCR (invitrogen™)

Purification of genomic DNA was conducted using The PureLink™ Genomic DNA Mini Kits (invitrogen™) by a spin column based centrifugation procedure. DNA was extracted from 1 mL aliquots of overnight bacterial culture in Tryptone Soya Broth (TSB) (Oxoid, England). In brief, one mL aliquots of bacterial culture was centrifuged at 4.000 g for 10 minutes and the cell pellets were suspended in 190 µL TE Buffer (invitrogen™) and 10 µL Lysozyme (invitrogen™) (for Gram negative) or 100 µL TE Buffer dan 100 µL Lysozyme (for Gram positive) and incubated at 30°C for 30 minutes. The cell suspensions were centrifuged at 4.000 g for 5 minutes and the cell pellets were resuspended in 400 µL of DNA *Lysis Buffer T* (invitrogen™)*,* added with 20 µL Proteinase K (invitrogen™), 15 µL RNaseA (invitrogen™) and mixed well by brief vortexing. The mixture was incubated at 70°C for 30 minutes and 200 µL of DNA *Binding Buffer* (invitrogen™) was added. Purification was conducted by adding the mixture into spin column and centrifugation at 10.000 g for 1 minutes. The spin column containing solid phase was moved to new tubes, added with 650 µL DNA *Wash Buffer* (invitrogen™) containing 100% ethanol, and centrifuged at 10.000 g for 10 minutes. The spin column was placed in a new sterile 1.5 mL microcentrifuge tube and added with 50 µL DNA *Elution Buffer* (invitrogen™), incubated at room temperature for 3 minutes. DNA precipitation was carried out by centrifugation at 6.000 g for 1 minute. To recover more DNA, a second elution step was performed using the same volume of the same elution buffer. The clear supernatant (DNA) was collected and stored at -20°C for PCR. The concentration of DNA was determined by optical density (OD) at the wavelength of 260 nm (Bio-Spectrometer, Eppendorf) and its purity was evaluated by the ratio of OD at the wavelength of 260/280. The DNA were kept at -20°C until further use.

Optimization of PCR method to detect *Salmonella invA* gene (modified Rahn *et al.* 1992)

The specific primers for *invA* gene amplification consisted of a forward primer F139 and a reverse primer R141 with sequences 5'GTGAAATTATCGCCACGTTCGGGCAA-3' and 5'TCATCGCACC GTCAAAGGAACC-3', respectively (Rahn *et al.* 1992). These primers amplify gene encoding for *invA* located within 287 to 312 and 571 to 550 bp regions (AIT-Biotech Singapore). PCR amplification was performed with PCR Applied Biosystem 2720 Cycler (Foster City, California) using PCR mixtures containing 1.5 µL of primers, 7.5 µL PCR Master Mix (0.05 U/µL Taq DNA polymerase, PCR buffer, $MgCl_2$ and dNTP) (Thermo Scientific). The thermocycling conditions were 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59; 59.5; 60.8; 62; 64; or 64.5°C for 30 seconds and elongation at 72°C for 30 seconds, with a final extension at 72°C for 4 minutes. The PCR products were visualized by electrophoresis on 1.5% agarose gel (Fermentas, USA) at 100 V for 30 minutes, stained with SYBR SAFE (0.6 µg/100 mL) (invitrogen™), visualized under UV transilluminator (Bio-Rad) and photographed. DNA from reference strain *S.* Typhimurium and *S. aureus* was used as positive and negative control, respectively.

Sensitivity and specificity of PCR (Upadhyay *et al.* 2010)

The sensitivity of PCR assay was evaluated in various concentration of DNA and various concentration of viable *S.* Typhimurium. For sensitivity test based on DNA concentration, DNA extract was sequentially diluted ten folds. For sensitivity test based on viable bacterial count, *S.* Typhimurium was sequentially diluted from 10^9 to 1 CFU/mL and DNA was extracted from each dilution. DNA extracted from *S.* Typhimurium and *S. aureus* was used as positive and negative control, respectively in each PCR run. The PCR products were visualized by electrophoresis and observed as described above. The minimum concentration of DNA giving a positive signal was recorded. The specificity of PCR was evaluated by comparing

with 6 different *Salmonella* serovars (*S.* Enteritidis, *S.* Hadar, *S.* Heidelberg, *S.* Kentucky, *S.* Paratyphi and *S.* Typhimurium) and cross-tested with 5 non-*Salmonella* isolates, i.e. of *E. coli, L. monocytogenes, P. mirabilis, S. aureus, V. parahaemolyticus*.

Application of the PCR assay on *Salmonella* isolated from shrimp in fresh markets

The PCR method developed was applied to detect *Salmonella* from shrimp samples. Briefly, 16 shrimp samples were collected from 5 fresh markets in Jakarta. Samples were pre-enriched in 225 mL of Buffered Peptone Water (BPW) (Oxoid, England) and incubated at 37°C for 24 hour, and then selectively enriched in Muller Kauffmann Tetrathionate Broth (MKTTn) (Oxoid, England) for 24 hours at 37°C. A conventional method was carried out according to ISO 6579:2002. Using this method, selective enrichment was conducted in Rappaport-Vassiliadis Broth (RV) (Oxoid, England) and MKTTn and incubated for 24 hours at 42°C and 37°C, respectively, followed by selective isolation onto Xylose Lysine Deoxycholate Agar (XLDA) (Oxoid, England) and Brilliance Salmonella Agar (BSA) (Oxoid, England) plates and incubated for 24 hours at 37°C. Typical colonies were streaked onto Triple Sugar Iron Agar (TSIA) (Oxoid, England) and Lysine Iron Agar (LIA) (Oxoid, England), and incubated for 24 hours at 37°C. Single colony was confirmed with Microgen GN A-ID System (Microgen Bioproducts). Growth in MKTTn was centrifuged at 20.000 g for 5 minutes and the DNA was extracted for the cell pellets as above for PCR assay.

RESULTS AND DISCUSSION

Annealing temperature maximum

Isolation of DNA from bacteria is the initial step in determining the genotypic characteristics of bacteria. In this study, the chromosomal DNA was extracted by spin-column technology. The bacterial cell wall was removed by lysozyme and proteinase K. Addition of ethanol was applied to wash the DNA or oligonucleotide-DNA extraction from small oligo-nucleotides, while detergent and organic solvent were used to remove proteins. The genomic DNA concentration of *S.* Typhimurium extracted by this method was 2.486 μg/mL. According to Wilkerson *et al.* (1993), the concentration of DNA for PCR ranged from 0.5 to 6.5 μg/mL. The purity of DNA extracted obtained in this study was 1.88 which was complied with the standard value of 1.8 to 2.0 (Sambrook *et al.* 2001).

Amplified primer of gene encoding for *invA* of *S.* Typhimurium at different annealing temperatures 59°C up to 64.5°C resulted clear and thick band of 284 bp. The results indicate that annealing temperatures used in this study were suitable for the amplification of the *Salmonella invA* gene. One way to optimize detection method by PCR is directed at the factors that affect annealing or extension (Devananda, 2011). Yuwono (2006) states that annealing temperatures at 55 to 65°C increase the specificity of the amplification reaction, although decrease the overall efficiency. In this study, annealing temperature of 64°C provides the thickest band, accordingly selected for further use (Figure 1).

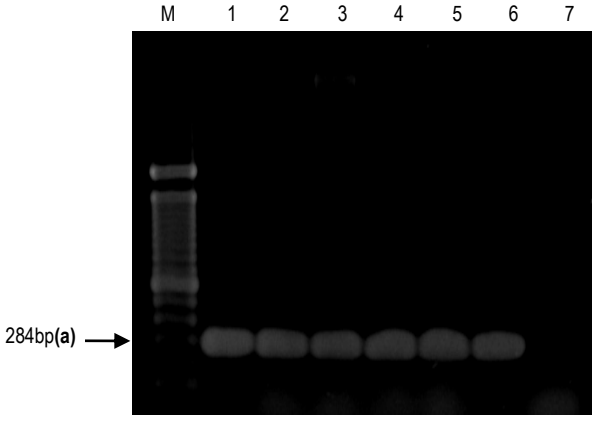

Figure 1. Visualization of DNA amplification products *invA* gene *S.* Typhimurium at different annealing temperatures, lanes 1-6: 59; 59.5; 60.8; 62; 64; 64.5°C respectively, lane 7: *S. aureus* as a negative control, lane M is a marker 100 bp DNA ladder

Sensitivity and specificity PCR methods

The specific bands of *invA* gene at 284 bp can be detected at DNA dilution up to 10^{-5} (Figure 2 (a)), i.e. a DNA concentration of 27.81 μg/mL as measured by Bio-Spectrometer. The sensitivity was evaluated in various concentration of viable cell showed that *invA* gene can be detected at the highest dilution concentration (1 CFU/mL) (Figure 2 (b)). The negative control, *S. aureus* did not give any amplicons. The results showed better sensitivity than Upadhyay *et al.* (2010) who reported a sensitivity of 10^4 CFU/mL.

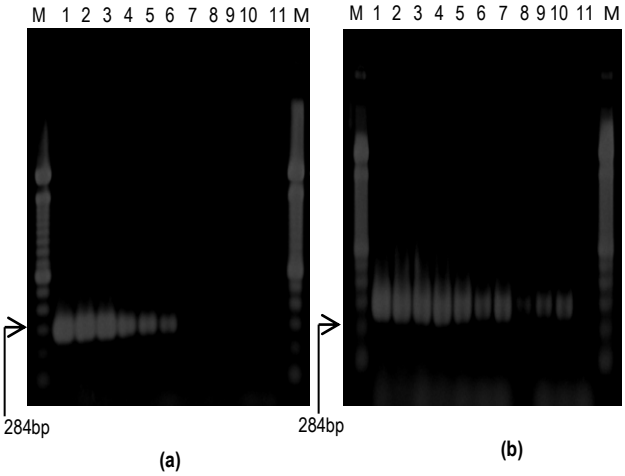

Figure 2. *invA* gene *Salmonella* amplification products on a 1.5% agarose gel (TBE). Detection limit of PCR (a). Lanes 1–10: Dilution of *S.* Typhimurium DNA extracted from10^0-10^{-9}; lane 11: *S.aureus* DNA as a negative control; lane M: DNA marker 100 bp ladder. (b). Lanes 1-10: Dilution of *S.* Typhimurium culturesfrom 10^9-1 CFU/mL; lane 11: *S. aureus* DNA as a negative control; lane M: DNA marker 100 bp ladder

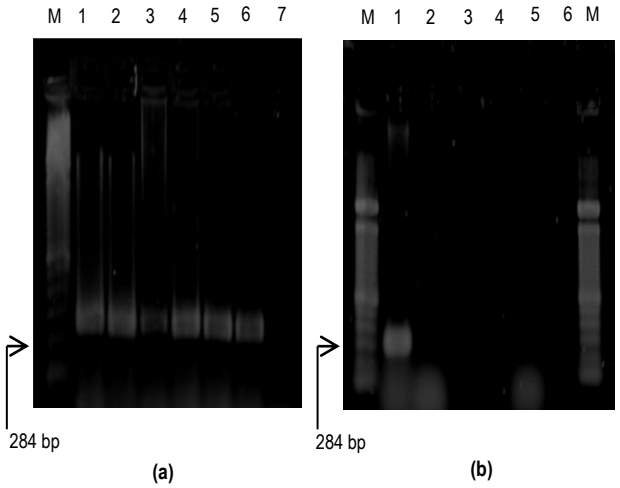

Figure 3. PCR specificity was evaluated by 5 different isolates of *Salmonella* serovar cross-tested with 5 non-*Salmonella* isolates a) lanes 1-6: *S.* Enteritidis*, S.* Hadar*, S.* Heidelberg*, S.* Kentucky*, S.* Paratyphi*,* and *S.* Typhimurium; lane 7: *S. aureus* as a negative control; lane M: 100 bp ladder DNA marker, b) lane 1: *S.* Typhimurium as a positive control; lanes 2-6 respectively: *E. coli, L. monocytogenes, P. mirabilis, S. aureus* and *V. parahaemolyticus* and lane M: DNA marker 100 bp ladder

Results of the specificity test showed that the PCR protocol could be used for detecting of 284 bp single band at overall 6 *Salmonella* isolates (Figure 3 (a)). The protocol did not detect any signal for 5 non-*Salmonella* isolates (Figure 3 (b)). *InvA* gene is a virulence gene in *Salmonella* that is important for invasion into the epithel cell of host. Invasion of intestinal epithelial cells is an important step in the pathogenicity of *Salmonella* infection. Karunasagar *et al.* (2012) suggested 17 areas of Salmonella Pathogenicity Island (PAI) and *invA* gene belong to the SPI 1, which is responsible for the invasion and infection by *Salmonella* (Knodler and Steele-Mortimer, 2005). Those studies indicated that the *inv*A gene was specific for *Salmonella* as reported by Shanmugasamy *et al.* (2011).

Application of the PCR based on *invA* gene for detection *Salmonella* from fresh markets shrimp

Detection of *Salmonella* by conventional method consist of pre and selective enrichment which takes 48 hours shows that 3 out of 16 shrimp samples were positive (18.75%) for *Salmonella*, with satisfactory final identification (80.9%). However, the PCR which takes 2 hours showed that all samples were positive for *Salmonella* (Figure 4). The PCR method showed can more sensitive and faster than the conventional one. These results were similar to previous studies in shrimp, poultry, animal and clinical samples suggesting that PCR after enrichment detected more *Salmonella* as compared to the conventional method (Eyigor and Carli, 2003; Maciorowski *et al.* 2005; Upadhyay *et al.* 2010; Germini *et al.* 2011). The use of growth in selective medium for DNA extraction was expected to increase the number of presumptive *Salmonella*. Soumet *et al.* (1999) reported that using poultry directly for DNA extractionwas not sufficient due to the high amounts of inhibitor, interference and growth inhibition by other competing bacteria. The inability of the conventional method to detect *Salmonella* in shrimp samples may be due to the viable but non culturable state of the pathogen (Guo *et al.* 2000).

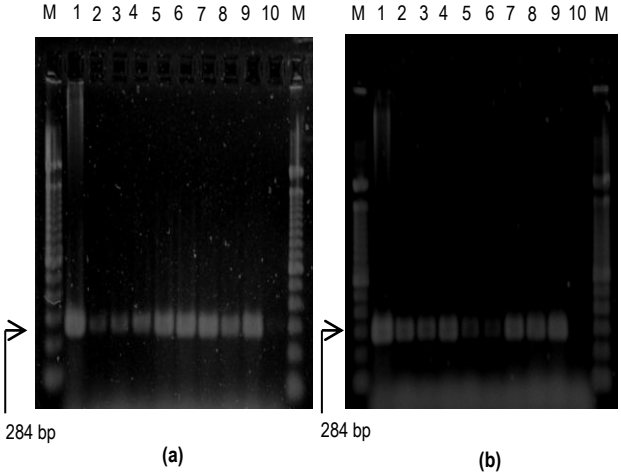

Figure 4. DNA visualization of *invA* gene *Salmonella* amplification products on a 1.5% agarose gel (TBE). PCR to detect *Salmonella invA* gene (a). Lane 1: *S.* Typhimurium DNA as positive control, lane 2-9: shrimp samples 1-8, respectively, lane 10: *S. aureus* DNA as a negative control (b) Lane 1: *S.* Typhimurium DNA as positive control, lane 2-9: shrimp samples 9-16, respectively, lane 10: *S. aureus* DNA as a negative control, lane M: DNA marker 100 bp ladder

CONCLUSIONS

Detection of *Salmonella* spp. based on *invA* gene could be performed with the following PCR protocol: pre denaturation (95°C for 1 minutes), denaturation (95°C for 30 seconds), annealing (64°C for 30 seconds), elongation (72°C for 30 seconds), and final extension (72°C for 4 minutes), with 35 cycles. Primer pairs F-139 and R-141 could amplified *Salmonella invA* gene specifically and had a high sensitivity with detection limit of 27.81 μg/mL. Application of PCR assay in this research gave better detection of *Salmonella* spp. in shrimp than that of conventional method. Time required for PCR assay is faster than the conventional one, i.e. 2 hours for the PCR and 48 hours for enrichment.

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