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Rapid detection of *Salmonella* in food and beverage samples by polymerase chain reaction

Radji, M.,* Malik, A. and Widyasmara, A.

Laboratory of Microbiology and Biotechnology, Department of Pharmacy,
Faculty of Mathematics and Sciences, University of Indonesia, Kampus UI, Depok, 16424, Indonesia.
E-mail: maksum@farmasi.ui.ac.id

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ABSTRACT

Polymerase chain reaction (PCR) assay had been used to detect *Salmonella* in food and beverage samples using suitable primers which are based on specific *invA* gene of *Salmonella*. Twenty nine samples were collected from street food counters and some canteens in Margonda Street, Depok, West Java, Indonesia. It was found that five of twenty nine samples were detected to contain *Salmonella* and showed the presence of the amplified product of the size 244 bp. The method of PCR demonstrated the specificity of *invA* primers for detection of *Salmonella* as confirmed by biochemical and serological assay. The results of this study revealed that PCR was a rapid and useful tool for detection of *Salmonella* in food and beverage samples.

Keywords: Salmonella, PCR, primer, *invA*

INTRODUCTION

Typhoid fever, also known as enteric fever occurs worldwide, primarily in developing countries, including Indonesia. Typhoid fever is a systemic infection caused primarily by *Salmonella* serotype Typhi. The disease remains an important public health problem in developing countries. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216,000 deaths and that more than 90% of this morbidity and mortality occurred in Asia (Crump *et al*., 2004). In Indonesia, the incidence of typhoid was 148.7 per 100,000 person-years (Ochiai *et al*., 2008).

The transmission of typhoid fever occurs by oral transmission via food or beverages handled by an individual who chronically sheds the bacteria through stool and via sewage-contaminated water sources which could possibly be due to fecal contamination from human and animal. The unsanitary practices of food and beverages processes lead to contamination of foods by *Salmonella*. The previous study showed that 25%-50% of beverage samples which are sold on the street food counters in Bogor, Indonesia, were contaminated predominantly by *Salmonella paratyphi* A. The contamination of bacteria possibly comes from the uncooked water (Anita, 2002).

The increased frequency of food-borne *Salmonella* has been causing recurring outbreaks, sometime with fatal infections. The exceedingly variable manifestations of typhoid fever have lead to the development of numerous diagnostic techniques. The routine detection of *Salmonella* in the environment including in foods and beverages is a necessary component of public health programs.

Standard cultural methods for detection of *Salmonella* are sensitive enough to detect *Salmonella* in food samples. However, the cultural methods also require multiple sub-culturing stages followed by biochemical and serological confirmatory tests with can take up to seven days to get a confirmed positive result. Therefore, these methods may be too time-consuming in cases where rapid pathogen identification is critical. In addition, sensitivity of cultures can be affected by antibiotic treatment, inadequate sampling, variations of bacteremia and a small number of viable organisms in samples (Miller and Pegues, 2000).

The development of molecular methods for diagnosis of infectious diseases has improved the sensitivity, specificity, quality and availability of diagnosis and treatment. Several polymerase chain reaction (PCR) assays for detection of *Salmonella* have been developed, and different targets DNAs for amplification have been applied. PCR enables the detection of *Salmonella* in different sources, such as human or animal feces (Stone *et al*., 1994; Juck *et al*., 1996), soil (Way *et al.,* 1993), environmental water samples and other sources (Way *et al.,* 1993; Pathmanathan *et al*., 2003; Aurélie *et al*., 2005).

PCR studies have also been carried out to evaluate the specificity of *invA* primers to detect *Salmonella* by PCR technique (Chiu and Ou, 1996; Jenikova *et al*., 2000; Carli *et al*., 2001**;** Gentry-Weeks *et al*., 2002; Ziemer and Steadham, 2003; Moganedi *et al.,* 2007). The oligonucleotide primer pairs were developed according to the sequences of the chromosomal *invA* gene (Chiu and Ou, 1996), which is essential in the invasion of *Salmonella* to enter the epithelial cells (Galan *et al.,* 1992). Rahn *et al*. (1992) reported that the *invA* primers were able to

***Corresponding author**

discriminate between *Salmonella* and non-*Salmonella* species. The detection limit was 300 cfu/mL of pure culture, however they did not evaluate the methods on environmental samples. Moganedi *et al.* (2007) demonstrated that the *inv A* primers were specific for the detection of *Salmonella* in drinking and surface waters and the limit of detection of PCR was 2.6 x 10^4 cfu/mL. PCR analysis offers several advantages including the specificity and rapidity. In order to develop the simple and rapid method for detection of *Salmonella*, in this study we used the *invA1* and *invA2* primers to detect *Salmonella* in food and beverage samples which were collected from street food counters and some canteens in Margonda Street, Depok, West Java, Indonesia.

MATERIALS AND METHODS

Food and beverage samples

Twenty nine food and beverage samples were collected from street food counters and some canteens in Margonda Street, Depok, West Java, Indonesia. The samples were selected for the possibility of contamination of *Salmonella* during the handling, processing and storage of raw material of the foods and beverages. All samples that were labeled and recorded have to be analyzed as soon as possible. Samples can be refrigerated on 0-4 °C for not more than 24 h after collection. The pre-enrichment of samples was performed according to the method described by Medici *et al*. (2003) with some modification. Briefly, 25 g or 25 mL samples were homogenized with 225 mL of Buffered Peptone Water medium and incubated at 37 °C for 24 h. The pre-enrichment culture was then divided into two aliquots. The first aliquot was subjected to DNA extraction by boiling method and the second aliquot was used to confirm the presence of *Salmonella* by standard cultural method, and followed by biochemical and serological confirmatory tests.

Preparation of template DNA samples for PCR by boiling method

Preparation of template DNA samples was performed by boiling method according to the method described by Medici *et al*. (2003). Briefly, 1 mL of the pre-enriched sample was transferred to a 1.5 mL micro-centrifuge tube and centrifuged for 10 min at 14,000 × *g*. The supernatant was discarded carefully. The pellet was resuspended in 300 µL of DNase-RNase-free distilled water (Sigma) by vortexing. The tube was centrifuged at 14,000 × *g* for 5 min, and the supernatant was discarded carefully. The pellet was re-suspended in 200 µL of DNase-RNase-free distilled water (Sigma) by vortexing. The micro-centrifuge tube was incubated for 15 min at 100 °C and immediately chilled on ice. The tube was centrifuged for 5 min at 14,000 × *g* at 4 °C. The supernatant was carefully transferred to a new micro-centrifuge tube and an aliquot of 10 µL of the supernatant was used as the template DNA in the PCR.

Preparation of template DNA *Salmonella typhi* as a positive control

Salmonella typhi that was used as a positive control provided by Laboratory of Microbiology and Biotechnology, Department Pharmacy, Faculty of Mathematics and Sciences, University of Indonesia. DNA template of *Salmonella typhi* was prepared by a modified method of Murray and Thompson (1980), using cetyltrimethylammonium bromide (CTAB). Briefly, 5 mL of an overnight culture grown in LB broth culture was harvested by centrifugation. The pellet was resuspensed in STET buffer (sucrose, Tris base, ethylene diamine tetra acetic acid/EDTA, Triton X-100), 10 µL lysozyme (concentration 10 mg/mL; Sigma), 30 µL 10 % sodium dodesil sulphate (SDS; Sigma), and 4 µL proteinase-K. The mixture was incubated at 37 °C for 60 min. After incubation, 65 µL 4 M NaCl and 80 µL CTAB were added and incubated at 65 °C for 30-60 min. DNA was purified by extraction with 624 µL chloroform and 26 µL isoamyl alcohol (24:1, v/v), and the nucleic acid was then pelleted by centrifugation, washed with 70 % ethanol and dried under vacuum. The DNA pellet was resuspended in TE buffer (10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA) and quantified by UV spectrophotometry at 260 and 280 nm. Tenfold serial dilution with sterile distilled water were performed and 5 µΛ (0.25 µg) of DNA suspension was used as DNA template and then subjected to PCR amplification.

Primers

The sequences of the primer pairs used for DNA amplification of *invA* gene region of *Salmonella* were prepared according to Chiu and Ou (1996): *invA1* (*forward*): ACA GTG CTC GTT TAC GAC CTG AAT (AIT Biotech) and *invA2* (*reverse*): AGA CGA CTG GTA CTG ATC GAT AAT (AIT Biotech).

PCR Protocol

PCR was carried out according to Chiu and Ou (1996) in a programmable thermal controller (MJ Mini Biorad). PCR reaction mixture (25 µL) contained 10 µL template DNA, 12.5 µL PCR Master Mix (PCR Buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP, 1.25 U *Taq polymerase*; Fermentas), 1 µM of each primer and ddH_2O to the total volume of 25 µL. The PCR was performed using the following condition: denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, and extension at 72 °C for 2 min for a total 30 cycles and a final extension step at 72 °C for 10 min.

PCR amplicons were electrophoresed in a 2 % agarose gel. After staining with ethidium bromide, the amplified fragments in the gel were visualized. The molecular mass marker used was 1 kb plus DNA ladder marker (Invitrogen). PCR was carried out in duplicate for each sample. One positive control (DNA from *Salmonella typhi*) and negative control were included. A product of 244 bp was considered the *invA* gene (Chiu and Ou, 1996).

Limit of PCR detection

An overnight culture of *Salmonella typhi* was serially diluted 10-fold with nutrient broth. A 1 mL aliquot of each dilution was centrifuged (10,000 x *g*) for 5 min. The supernatant was discarded carefully. The pellets of bacteria were washed with ddH_2O and finally re-suspended in 300 µL ddH_2O and boiled for 15 min, snap-cooled on ice and then centrifuged at 14,000 x *g* for 5 min at 4 °C. After centrifugation, 10 µL of supernatant were used as template in the PCR. Viable counts were obtained by plating 1 mL of each dilution of bacterial culture on nutrient agar plates and incubating overnight at 37 °C.

Spiked food sample

Spiking of food sample was performed according to Pathmanathan *et al*., (2003) with some modification. Briefly, 25 mg of food sample free from *Salmonella* was homogenized with 225 mL Buffered Peptone Water medium and incubated for 24 h at 37 °C. The pre-enrichment broth was then divided into two aliquots: (a) the first aliquot was used as a negative control; (b) the second aliquot was spiked with an appropriate quantity of *Salmonella typhi* suspension to obtain a final concentration from 10 cfu/mL to 10^9 cfu/mL. The cell suspension were then centrifuged (10,000 x *g*) for 5 min. The pellets of bacteria were washed with ddH_2O and finally re-suspended in 300 µL ddH_2O and boiled for 15 min, chilled on ice and then centrifuged at 14,000 x *g* for 5 min at 4 °C. After centrifugation, 10 µL of supernatant were used as template in the PCR.

Standard cultural method

Twenty five mg or 25 mL samples were homogenized with 225 mL buffered peptone water medium and incubated for 18-24 h at 37 °C. After incubation, 1 mL of pre-enrichment broth culture were transferred to 9 mL of selenite cystein enrichment broth culture and incubated at 37 °C for 24 h. A loop from each of cultures was streaked onto a plate salmonella-shigella agar (SSA) and incubated at 37 °C for 24 h. Presumptive *Salmonella* colonies were characterized by biochemical assay using lactose, glucose, and sucrose culture, triple sugar iron agar and urea agar media.

Serological test

Serological testing of samples was performed using the *Salmonella* test kit (Oxoid). Briefly, 25 mg or 25 mL samples were homogenized with 225 mL buffered peptone water medium and incubated for 18-24 h at 37 °C. After incubation, 1 mL of pre-enrichment broth culture were transferred to 9 mL of selenite cystein enrichment broth culture and incubated at 37 °C for 24 h. One drop of the selenite cystein enrichment broth culture using a Pasteur pipette were dropped within one circle on the reaction card and mixed with 1 drop of the Oxoid *Salmonella* Latex Reagent to the broth on the card. The suspension was mixed thoroughly using a clean mixing stick or inoculating loop, and then rocked the reaction card gently for 2 or 3 times. The agglutination was examined within a maximum of 2 min.

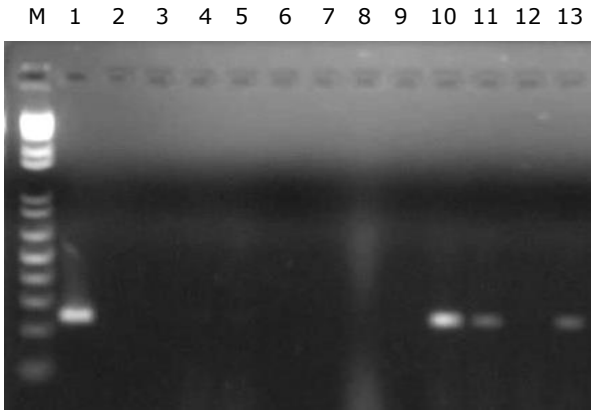

Figure 1: Representative samples determined by PCR and detected by 2 % agarose gel electrophoresis. Lane M: 1kb molecular size marker ladder; lane 1: positive control (*Salmonella typhi*); lanes 2 – 9 and 12: negative samples; lanes 10, 11 and 13: positive samples

RESULTS AND DISCUSSION

The *invA* gene primer pair specific for *Salmonella* was used in PCR reaction on both the genomic DNA isolated from samples and on the genomic DNA isolated from pure cultures of *Salmonella typhi* produced an intense band of the expected 244 bp. In this study we found that the limit of detection of PCR using genomic DNA obtained by boiling method from pure cultures of *Salmonella typhi* and in the *Salmonella*-spiked food samples was 2.85 x 104 cfu/mL and 2.85 x 105 cfu/mL respectively (data not shown). Therefore the pre-enrichment samples in non-selective medium is a necessary step to increase the number of *Salmonella* in food and beverage samples. Similar results have reported previously that at least 103 - 105 cells/mL must be present to give positive results by PCR without a pre-enrichment step (Pathmanathan *et al.*, 2003; Oliviera *et al.*, 2003; Myint *et al.*, 2006; Moganedi *et al.*, 2007). Pre-enrichment culture has been done in order to increase the viable number of *Salmonella* in the samples prior to the detection of *Salmonella* by PCR technique. Boiling method was selected for preparation of the DNA template, because this method was simple and rapid to extract DNA. Centrifugation and washing steps were sufficient to remove the PCR inhibitors from culture medium. Boiling of the bacterial cells and then immediately cooling them on ice was efficient for releasing and denaturing the DNA template, resulting the suspension was directly subjected to the PCR without DNA isolation and purification steps (Herich *et al.,* 2004).

Table 1: Food and beverage samples tested for *Salmonella* by PCR, biochemical and serological assay

Samples	PCR assay	Biochemical and Serological assay
Fruits salad	N	N
Fruits salad	N	N
Fruits	N	N
Fruits	N	N
Cooked vegetables salad	N	N
Vegetables salad	N	N
Mixed vegetables salad	N	N
Vegetables salad	N	N
Coconut juice	positive	positive
Coconut juice	N	N
Coconut juice	positive	positive
Ice Fruits cocktail	positive	positive
Mixed cold drink	N	N
Traditional cold drink	N	N
Traditional cold drink	N	N
Coconut juice	N	N
Traditional cold drink	positive	positive
Ice tea	N	N
Mixed herbal cocktail	N	N
Mixed herbal cocktail	N	N
Coconut juice	N	N
Vegetables salad	positive	positive
Mixed herbal cocktail	N	N
Traditional cold drink	N	N
Cooked vegetables salad	N	N
Ice Fruits cocktail	N	N
Coconut juice	N	N
Mixed herbal cocktail	N	N
Fruits salad	N	N

N: negative

Five out of twenty nine samples were detected to contain *Salmonella* and revealed the presence of the amplified product of the size 244 bp (Figure 1). No false positives or negatives for *Salmonella* were observed with PCR as confirmed by standard cultural and serological methods (Table 1). This result proved the specificity of PCR compared to the conventional culturing and serological method. All *Salmonella* carry the *invA* gene, which is not carried by any other bacterial species. Therefore if 244 bp amplified product appeared in the PCR with the *invA1* and *invA2* primers it would indicate that the sample contains an *invA* gene of *Salmonella* (Chiu and Ou, 1996).

Traditional approaches for analysis of *Salmonella* has relied on cultural techniques and several selective-differential media have used for differentiation. However, biochemical analysis for an enzyme associated with the particular pathogenic trait could be cross reactive with other enteric bacteria. In addition, the cultural methods also require multiple sub-culturing stages followed by biochemical and serological confirmatory tests with can take up to 7 days to get a confirmed positive result. In contrast to the 7 days culture method, in this study, 24 h pre-enrichment-PCR assay using *invA1* and *invA2* primers, offer a rapid and good diagnostic tool for the routine monitoring for detection of *Salmonella* in food and beverage samples. The presence of Salmonella in foods and beverages could be due to several reasons such as contamination of raw material, poor hygienic conditions, contamination of water sources and unsanitary processes of foods and beverages.

The presence of *Salmonella* in foods beverages which are sold on the street food counters is an important environmental issue because of the health problems can be involved.

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