Rapid identification of *Salmonella enterica* serovars Typhi and *Salmonella enterica* serovars Paratyphi A from chicken meat

D Raharjo¹,², R Yulistiani³,⁴, W Setyarini², R Y Arizandy³, W Prayoga⁵, and T Shirakawa⁶

¹Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. 60115.
²Gastroenteritis and Salmonellosis Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. 60115
³Department of Food Technology, Faculty of Engineering, Universitas Pembangunan Nasional Veteran Jawa Timur, Jalan Rungkut Madya Gunung Anyar, Surabaya, Indonesia. 60294
⁴Graduate School Program of Sebelas Maret University (UNS), Jalan Ir. Sutami 36A, Keningan, Surakarta, Indonesia, 57126.
⁵Salmonella Research Centre, Animal Biosafety Level 3, Airlangga University, Surabaya, Indonesia. 60115
⁶Graduate School of Science, Technology and Innovation, Kobe University, Kobe Japan

E-mail: Dadik_tdc@yahoo.co.id

Abstract. Rapid diagnosis of pathogenic bacteria in food is closely related to safety. We modified a Culture-based methods for *Salmonella* sp. which rapidly identified *Salmonella enterica* serovars Typhi and *Salmonella enterica* serovars Paratyphi A from chicken meat samples. The World Health Organization method consists of six steps of the test, sequentially pre-enrichment, selective enrichment, selective diagnostic isolation, pick presumptive *Salmonella* colonies, biochemical and serological confirmation. Modifications made at three stages of pick presumptive *Salmonella* colonies, biochemical confirmation and serological confirmation into a single stage of molecular testing to detect genes *rfbE* and, using *tyv* and *prt* primer. In summary, from 120 samples, the modified technique successfully identified 39 (32.5%) *Salmonella enterica* serovars Typhi and one *Salmonella enterica* serovars Paratyphi A (0.83%) compare with 13 (10.8%) and negative result with conventional protocol. The modified were faster and more sensitive than conventional techniques.

1. Introduction

*Salmonella* sp is one the major source to morbidity and mortality in the world. Infections by *Salmonella* manifest as Typhoid fever and Nontyphoidal salmonellae. Typhoid fever, a life-threatening infection caused by *Salmonella enterica* serotype Typhi, every year there are 17 million cases of typhoid fever and 600,000 die. Clinical symptoms include fever, fatigue, headache, nausea, abdominal pain, constipation or diarrhea [1,2]. Nontyphoidal salmonellae estimated to cause 153 million cases of gastroenteritis and 57,000 deaths globally each year. Gastroenteritis is the most common clinical presentation, commonly manifested as acute diarrhea, abdominal pain, fever, and sometimes vomiting. Clinical symptoms of salmonellosis are generally mild and patients will recover without treatment, except in pediatric and elderly patients, severe dehydration and death can occur.
**Salmonella** is a member of the **Enterobacteriaceae**, there are two species, *Salmonella bongori* and *Salmonella enterica* with 2,500 different serotypes [3], some serotypes have limited host such as *Salmonella enterica* serotype Typhi in human, *Salmonella enterica* serotype Gallinarum in poultry, *Salmonella enterica* serotype Dublin in cattle, *Salmonella enterica* serotype Choleraesuis in pigs but all serotypes of *Salmonella* can cause disease in humans [4].

Consumer protection in Indonesia regulated by Indonesian National Standard No 3924-2009 which states that carcass and chicken meat should free from *Salmonella* sp., the total plate count less than 10⁶ cfu/g, the total coliform less than 10³ cfu/g, the total *Staphylococcus aureus* less than 10³ cfu/g [5]. *Salmonella* sp. characterized using the Kauffmann-White scheme basis of surface antigen identification using antiserum to detect the O (somatic) and H (flagellar) antigen [6] unfortunately, there are difficulties in getting antiserum in Indonesia so that genoserotyping can be used as an alternative for *Salmonella* sp. detection [7,8]. Availability of reliable and rapid tests for food-borne pathogen detection play an important role in the food industry and regulators in food security.

2. **Experimental**

One hundred and twenty samples chicken leg collected from the wet market in Surabaya, Indonesia. Samples were packed in sterile plastic bags, transferred directly to the laboratory in the ice box for an immediate bacteriological testing.

Isolation and identification of bacteria. Twenty five grams of each chicken leg incised using a sterile scalpel and forceps, then transferred to sterile Erlenmeyer containing 225 ml of buffered peptone water (Oxoid CM0509, UK), the mixture incubated aerobically at 37 °C for 24 h. From each sample, 1 ml added to 9 ml of Selenite Cystine Broth Base in 0.4% sodium biseleinite (Oxoid LP0121A, UK) and incubated aerobically at 37 °C for 24 h. one hundred micro liter transferred and streaked on xylose lysine deoxycholate (XLD) agar (Oxoid CM 0469, UK). The inoculated plates incubated aerobically at 37 °C for 24 h. Five of presumptive salmonella colonies from XLD agar plates identified biochemically using IMViC test. Biochemically confirmed *Salmonella* isolates by genoserotyping for identification of *Salmonella* serovars [9, 10]. The developed of rapid test for identification of *Salmonella* serovars Typhi and *Salmonella* serovars Paratyphi A from chicken meat done by directly scrub colony on XLD agar for identification of *Salmonella* serovars by genoserotyping. This work carried out at the Gastroenteritis and Salmonellosis Laboratory, Institute of Tropical Disease Airlangga University, Surabaya, Indonesia.

The DNA samples for conventional method obtained from isolate that tested with IMViC by plated on nutrient agar (Merck, Darmstadt, Germany). The DNA samples for rapid method obtained from scraping of the bacterial colonies on XLD agar (Oxoid CM 0469, Basingstoke, UK). The bacterial colonies suspended in 1000 µl of distilled water in 1.5 ml microcentrifuge tube. Tubes boiled for 15 min, and cooled to room temp. The cell debris was pelleted by centrifugation at 10,000 RPM for 10 min. 5 µl of supernatant used as the template for PCR [11].

The specific primer sets and the cycling of the PCR for *Salmonella enterica* serovars Typhi and *Salmonella enterica* serovars Paratyphi A presented in Table 1. PCRs carried out in a Bio-rad PCR system T100™ Thermal Cycler (BioRad). A total of 25 µl PCR mixture contained 12.5 µl PCR mix (MytaqTM HS Redmix 2X, Bioline, USA), 10 µM of each primer, 5 µl PCR template and 5.5 µl ddH2O. The PCR condition was 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and last stage of 72°C for 5 min (6). PCR products loaded on 2% (wt/Vol) agarose gels, DNA marker used sizerTM-100 (Intron Biotechnology Inc.), stained with ethidium bromide and visualized on a UV Transilluminator.
Figure 1. Conventional and Rapid Method for the identification of *Salmonella enterica* serovars Typhi and *Salmonella enterica* serovars Paratyphi [9, 10]

| Primer | Primer sequence (5’ to 3’) | PCR product (bp) | Reference |
|--------|-----------------------------|------------------|-----------|
| tyv-for | GAGGAAGGGAAATGAAGCTTTT       | 614              | 11, 12    |
| tyv-rev | TAGCAAAACTGTCTCCCACCATA      |                  |           |
| prt-for | CTTGCTATGGAAGCACATAACGAACC   | 256              | 11, 12    |
| prt-rev | CGTCTCCATCAAAAGCTCCATAGA     |                  |           |

3. Results and Discussion
The results showed that modified method was more sensitive and rapid, from 120 samples, the modified technique successfully identified 39 (32.5%) *Salmonella enterica* serovars Typhi and one
Salmonella enterica serovars Paratyphi A (0.83%) compare with 13 (10.8%) and negative result with conventional protocol. Prt gene is found in both Salmonella enterica serovars Typhi and Salmonella enterica serovars Paratyphi while the tyv gene is only obtained in Salmonella enterica serovars Typhi. PCR used prt and tyv primers was proven abilities to detect Salmonella enterica serovars Typhi and Salmonella enterica serovars Paratyphi from bacterial mixtures obtained from XLD agar as well as from purified isolates [12]. Result of PCR using prt primer showed in figure 2, and result of PCR using tyv primer showed in figure 3.

![Figure 2. PCR products of prt primers and DNA marker Sizer TM-100 (Intron Biotech, Inc).](image)

![Figure 3. PCR products of tyv primers and DNA marker Sizer TM-100 (Intron Biotech, Inc).](image)

The bacteria that can grow on XLD agar and have the same colony properties are Salmonella, Edwardsiella, Shigella, Providencia, H2S-negative Salmonella and bacteria with different colonies properties are Escherichia, Enterobacter, Klebsiella, Citrobacter, Proteus, Serratia [13]. Conventional method for the isolation and identification of Salmonella sp carried out by picking five presumptive Salmonella colonies from selective agar[12]. Selection of colonies, IMViC test and Serotyping requires special technical expertise and laborious. Rapid diagnostic allowing for direct testing of various types of colonies in the XLD agar and avoided the colony's misrepresentation and worked it out more simply. PCR method has advantages related to sensitivity, specificity, speed, simplicity, as well as in a quantitative [14]. Based on Indonesia National Standard No 3924-2009, chicken meat required free of salmonella sp. and the amount of total plate count did not exceed $10^6$ cfu/g. Staphylococcus aureus did not exceed $10^2$ cfu/g [5]. Identify and count of bacteria on chicken carcasses will be very difficult to carry out conventionally, but make it possible to carry out used molecular method [14]

4. Conclusion

These results showed that the rapid test using the molecular technique which applied directly to the colonies on selective media has a higher success rate than conventional methods, the modified technique has identified 39 from 120 samples (32.5%) Salmonella enterica serovars Typhi comparative conventional method which only identified 13 from 120 samples (10.8%). The modified technique successfully identified one Salmonella enterica serovars Paratyphi A from 120 samples (0.83%) where the conventional technique has failed. Modified method has advantages on rapidity, sensitivity, and simplicity where detection of Salmonella enterica serovars Typhi and Salmonella enterica serovars Paratyphi A from chicken meat only needed four steps compared six steps on conventional method.

References

[1] Typhoid World Health Organization http://www.who.int/news-room/fact-sheets/detail/typhoid
Accessed July 10, 2018

[2] Background document 2003 The diagnosis, treatment and prevention of typhoid fever Communicable Disease Surveillance and Response Vaccines and Biologicals (Switzerland: World Health Organization) chapter 1 pp 1-3
[3] Salmonella (non-typhoidal). World Health Organization. http://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal). Accessed July 17, 2018

[4] Foley SL, TJ Johnson, SC Ricke, R Nayak, J Danzeisen 2013 Salmonella Pathogenicity and Host Adaptation in Chicken-Associated Serovars Microbiology and Molecular Biology Reviews 77 582–607

[5] Badan Standarisasi Nasional. Mutu karkas dan daging ayam. SNI 3924:2009. ICS 67.120.20.

[6] Forbes BA, DF Sahm, AS Weissfeld 2007 Bailey & Scott’s Diagnostic Microbiology 12th Ed. (Missouri : Mosby Elsevier) chapter 28 pp 362-384

[7] Malorny B, J Hoofar, C Bunge, and R Helmuth 2003 Multicenter Validation of the Analytical Accuracy of Salmonella PCR: towards an International Standard Applied and Environmental Microbiology 69 290–296

[8] Berghaus RD, SG Thayer, BF Law, RM Mild, CL Hofacre, RS Singer 2013 Enumeration of Salmonella and Campylobacter spp. in Environmental Farm Samples and Processing Plant Carcass Rinses from Commercial Broiler Chicken Applied and Environmental Microbiology. 79 4106–4114

[9] Perilla PJ 2003 Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. (Switzerland: World Health Organization) chapter 7 pp 103-162

[10] Hendriksen RS 2003 Global Salm-Surv A global Salmonella surveillance and laboratory support project of the World Health Organization Laboratory Protocols Level 1 Training Course Isolation of Salmonella (Switzerland: World Health Organization) chapter 1 pp 1-7

[11] Levy H, S Diallo, SM Tennant, S Livio, SO Sow, M Tapia, P Fields, M Mikoleit, B Tamboura, KL Kotloff, R Lagos, JP Nataro, JE Galen, and MM Levine 2008 PCR Method to Identify Salmonella enterica Serovars Typhi, Paratyphi A, and Paratyphi B among Salmonella Isolates from the Blood of Patients with Clinical Enteric Fever. J. Clin. Microbiol 46 1861–1866

[12] Hirose K, K Itoh, H Nakajima, T Kurazono, M Yamaguchi, K Moriya, T Ezaki, Y Kawamura, KTamura, and H Watanabe 2002 Selective amplification of tyv (rfbE), prt (rfbS), viaB, and fliC genes by multiplex PCR for identification of Salmonella enterica serovars Typhi and Paratyphi A. J. Clin. Microbiol. 40 633–636

[13] XLD Agar. Thermo scientific. Oxoid Microbiology Product http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0469&c=UK&lang=EN. Accessed July 17, 2018.

[14] Dennis Lo YM, W Rossa WK Chiu, KC Allen Chan 2006 Clinical Applications of PCR. 2nd Ed. (New Jersey : Humana) chapter 4 pp 33-45