Detection of *Salmonella typhi* Using Multiplex and Monoplex PCR in Tifoid Fever Patients

*Andika aliviameita[1], Meryn Linanda Agustin[1], Anggie Wedha Puspita [1], Miftahul Mushlih[1,2], Puspitasari[1], Yanik Purwanti[1], Arief Wisaksono[1]

[1]Faculty of Health Science, Universitas Muhammadiyah Sidoarjo, Jl. Mojopahit No. 666, Sidoarjo, Jawa Timur. [2]Indonesian Genetic and Biodiversity Community, Jl. Ikan Mujair No. 15, Tunjung Sekar, Lowok Waru, Malang
*Email: aliviameita@umsida.ac.id
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Abstract: Typhoid fever is a disease caused by Salmonella typhi infection. This disease is still a global problem. The purpose of this study was to develop a rapid detection method for Salmonella using molecular methods utilizing Inv A and Fim-C Genes. Two methods compared, which are Multiplex and monoplex PCR. The sample is in the form of the patient's blood, which is stored at 3 ml EDTA vacutainer tube consisting of 10 positive samples and five negative samples. The criteria for samples used previously have tested by widal/ immunoserology (titer more than 1/160). The results showed that multiplex PCR for detection of Salmonella using the Inv A gene and fim c gene not recommended because too many bands produced. The application of monoplex in the Inv A gene gives better results than the Fim C gene. In conclusion, the monoplex application on Inv A gene recommended than Fim C gene used to detect S. typhi in human blood samples.
Keywords: *Salmonella typhi*; Inv A gene; Fim C gene; multiplex PCR.

INTRODUCTION

Typhoid fever, caused by *Salmonella enterica serovar typhi* (*S. typhi*), remains a significant public health problem in many parts of the world (Alba *et al*. 2016), mainly found in southeast Asia (Peter *et al*., 2017). There are no specific clinical symptoms in patients with typhoid fever because people with typhoid fever have symptoms similar to other diseases. During this time, the investigations that are often carried out are serological and bacteriological (Hatta and Smits, 2007).

The standard of S. typhi detection using microbiology methods takes 2-3 days. On the other hand, serological Examination methods still have less specific weaknesses (Hatta and Smits 2007). Now, the development of examinations directed at speed and accuracy. Therefore, molecular life is predicted to be an alternative problem. This study aims to detect S. typhi using molecular methods by utilizing Fim-C and Inv A genes.

Fim-C gene is a gene that can encode flagella. *S. typhi* bacteria have fimbriae or commonly called Fim-C protein, which is a virulence factor that has the potential to be immunogenically preventing typhoid fever infection. Fim-C protein acts as an essential mediator that can be used to interact or attach to an existing host cell or in an external environment (Burrows, 2005; Muktiningsih *et al*., 2009). Fim-C protein acts as an essential mediator that can be used to interact or attach an existing host cell or in an external environment (Burrows, 2005).

Corresponding Author: Andika aliviameita
Faculty of Health Science, Universitas Muhammadiyah Sidoarjo,
Jl. Mojopahit No. 666, Sidoarjo, Jawa Timur
Email: aliviameita@umsida.ac.id

The inv-A gene has a specific sensitivity to identify Salmonella strains (Rahn *et al.*, 1992). The Inv A gene is an invasive regulator of invading epithelial cells in the intestine (Pham, 2015). Several previous studies have reported successfully detecting *S. typhi* in several samples. Shanmugasamy et al. (2011) conducted a survey using the Inv A gene to detect S. typhi in chicken meat. Kumar et al. (2006) identified the presence of S. typhi in food, while this study was designed to detect S. typhi in the blood of typhoid fever patients.

MATERIALS AND METHOD

This design of the research was a descriptive explorative type using the Inv A and Fim C gene in typhoid fever patients. Ethical approval for performing this study obtained from the Research Ethics Committee of Faculty of Dentistry, Universitas Airlangga, Surabaya, Indonesia, with the ethical clearance number: 188/HRECC.FODM/IV/2019 and 196/HRECC.FODM/V/2019. The population in this study were patients affected by *S. typhi*, who had performed laboratory tests. The sample takes from Siti Fatimah Tulangan Hospital in Sidoarjo. Whole blood was collected from10 positive typhoid fever, who had carried out widal serological tests (titers > 1/160) — five samples used as negative controls. A sample stored in EDTA anticoagulants as much as 3 ml and stored at -4oC until used for a maximum of 3 days after collection. DNA isolation method was following gene A id DNA isolation procedure. DNA concentration measured using a UV-Vis Spectrophotometer (Thermo Scientific).

Two set primers were used to detect Inv A and Fim C gene. Inv A primer based on Rahn *et al.*, (1992) which are Inv A-F: 5'-TCGTCATTCCATTACCTACC-3'dan Inv A-R: 5'-AAACGTTGAAAAACTGAGGA-3'with a target of 284 bp. While Fim C gene amplification uses Forward primers (5'-AGCGAGCCCAAAAGTGAAA-3') and reverses primers (5'-ATCTTGAGATGGTTGCCAC-3') (Jawad & Al-Hamadani, 2011) with a target DNA length of 257 bp, multiplex, and monoplex optimization temperature were carried out according to Table 1 and processed by T100 Biorad thermocycler. The results of DNA amplification were analyzed using agarose gel electrophoresis 2%.

Table 1. PCR Treatment

Step	Temperatures and duration (seconds)		
	Multiplex PCR of Inv A gene and Fim C gene	Amplification of Inv A gene	Amplification of Fim C gene
Pra-denaturation	95ºC ; 60	95ºC ; 60	95ºC ; 60
Denaturasi	95ºC ; 30	95ºC ; 30	95ºC ; 30
Annealing	40-60°C ; 30	48.6°C ; 30	51°C ; 30
Extension	72ºC ; 30	72ºC ; 30	72ºC ; 30
Cycles	40	40	40
Final extension	72ºC ; 300	72ºC ; 300	72ºC ; 300

RESULTS AND DISCUSSION

Attempts to obtain alternatives for identification of *S. typhi* have been carried out from the simple methods to the complicated methods (Kumar *et al.*, 2006; Akinade *et al.,* 2015; Radhika *et al.,* 2014; Hatta and Smits, 2007; Ashraf *et al.,* 2013). This study attempts to present the simple identification of *S. typhi* using molecular methods. Previous research rarely used blood samples as the object to detect *S. typhi* (Nader *et al.*, 2015; Shanmugasamy *et al.*, 2011; Salehi *et al.*, 2012).

Phumkhachorn and Rattanachaikunsopon (2017) show that the Inv A gene has a specific sensitivity to detect *S. typhi*. While the Fim C gene can be used to detect salmonella from fecal samples (Jawad and Al-hamadani, 2011). Kumar *et al.* (2016) managed to detect *S. typhi* using pure culture and get an obvious single band. The multiplex PCR examination and directly used to patients with typhoid fever, firstly done by optimization. Optimization is carried out by annealing gradient temperatures, and the results show in Figure 1.A. The specific bands appear at 48,6oC, 46,9oC and 46oC (see arrow). Based on these results, the temperature used is 48,6 and tested on samples (n:10) with positive typhus. Figure 1.B shows much mispriming, and we are not recommended the PCR multiplex method (Fim C and Inv A) to use as a detection method in human samples. Mispriming can cause by the presence of Homology DNA or something similar to the DNA target.
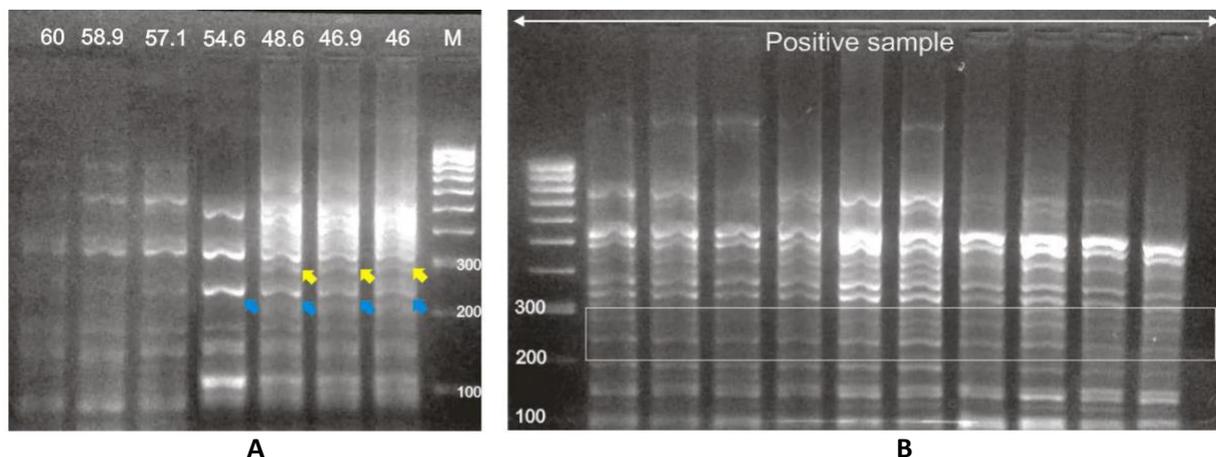

Figure 1. A. PCR multiplex optimization; B. Examination in 10 samples with annealing temperature optimation resulted (48,6^{o}C). The yellow arrow shows the length of the target gene Inv A (284 bp) while the blue arrow shows the length of the target gene Fim C (257 bp). In the boxes are PCR multiplex target

The analysis continued on the monoplex by comparing the results of amplified PCR from the Inv A and Fim C gene. The annealing temperature used to Inv A amplification is the temperature from optimization results in multiplex (48,6 oC). The results of agarose gel electrophoresis using annealing 48,6°C in 10 positive samples of patients with typhoid fever indicates there was a specific band at 284 bp. On the other hand, we do not find it at negative control. This suggests that Inv A gene accurately and can be used for screening *S. typhi* in typhoid fever patients. Besides, it identified several bands that not be targeted, which were at lengths of <100bp, 100bp, and several bands at distances of around 500 bp. The formation of the target gene looks thinner than the group at 100 bp. It probably caused the amount of DNA in *S. typhi* was low. While the band at 100 bp is thicker, it is perhaps mispriming the sample from the sample (human DNA).

S. typhi detection using Fim C genes could not show specific differences in positive and negative samples. The same bands target found around the 257 bp (Figure 3). To ensure these results, were-optimized PCR process on the target genes using positive samples and negative samples. The results show that bands appear in negative samples as well as positive samples (Figure 3.B). Analysis of amplification using the Fim C gene using an annealing temperature of 51°C (according to the results of multiplex optimization) could not show specific differences between positive and negative samples.
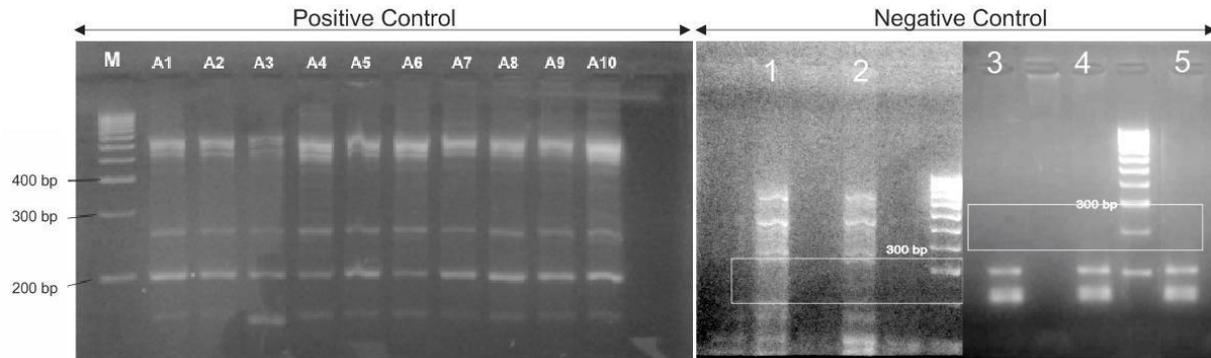

Figure 2. The results of S. typhi identification using Inv A gene (284 bp). Ladder control 100 bp.
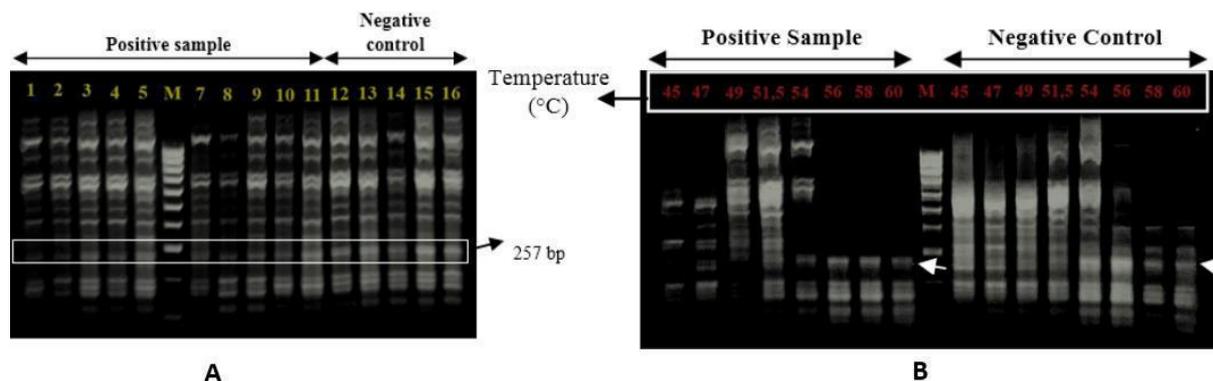

Figure 3. A: visualization of PCR with Anealing 51°C. B: PCR Optimization of positive and negative control.

In a previous study, Jawad and Al-hamadani (2011) succeeded in identifying S. typhi from a fecal sample using the Fim C gene. Nurjayadi, M., et al. (2011) successfully identified S. typhi using the Fim C gene from pure bacterial culture. However, in this study the Fim C gene was used from human blood samples with results 257 bp appears from the negative and positive sample. Mispriming is possible because the primer amplifies homologous genes or primers used are less active, or the initial design used in the study is less specific to the target gene.

CONCLUSION

Multiplex PCR for detection of salmonella using the Inv A gene and Fim C gene not recommended because too many bands produced. The monoplex application on Inv A gene approved than the Fim C gene used to detect S. typhi in human blood samples.

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