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Research Article

Optimizing Real-Time PCR methods for detection of *ssaN* gene *Salmonella enterica subsp. enterica* in the blood specimen

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Abstract

Background: Typhoid fever caused by *Salmonella typhi* is a common acute infection of the reticuloendothelial system, intestinal lymphoid tissue, and gall bladder. The diagnosis of typhoid fever is often enforced based on clinical symptoms and serological tests only. Widal test is one of the serological tests which is still widely used especially in developing countries. This test has several limitations including poor specificity, not easily interpreted especially in endemic areas due to cross-reactions to other infectious diseases.

Objective: The aim of this study was to optimize the real-time PCR method include cross-reaction test and minimum detection limit to develop a *Salmonella enterica* detection system by detection of a new target gene (*ssaN* gene) from blood specimens in typhoid fever patients.

Methods: An experimental laboratory study was performed from March to October 2016. The extraction of *Salmonella typhi* DNA was used as templates for the optimization of a real-time PCR reaction. The blood sample was from 50 patients suspected with typhoid fever obtained from the Menteng Sub-district Health Center according to the inclusion criteria.

Results: Real-time PCR showed that the primers and probes used are not cross-react against other microorganisms. The minimal detection limit is at 10 CFU/ml of the blood specimen. In blood clinical specimens, real-time PCR could detect 19 (38%) positive samples of 50 blood specimens from suspected typhoid fever patients.

Conclusion: Real-time PCR optimization with the new *ssaN* gene target can detect *Salmonella enterica subsp. enterica* in blood specimens from a patient with suspected typhoid fever.

Keywords: *Salmonella enterica subsp. enterica*; typhoid fever; *ssaN* gene; real-time PCR

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INTRODUCTION

Salmonella enterica is an important food-borne pathogen in humans that causes enteric disease. There are 2 main types of diseases caused by *S. enterica*, which are acute gastroenteritis and typhoid fever.¹ Typhoid fever is an acute and often life-threatening febrile illness transmitted via the fecal-oral route caused by *Salmonella enterica* serotype *Typhi*. It spread almost all over the world and still a public health problem in the world.^{2,3}

Humans are the only reservoir for *Salmonella Typhi*. Infection occurs fecally-oral usually through the consumption of food or water contaminated by human feces. Typhoid and paratyphoid infections are relatively common in countries with poor sanitation conditions and have limited access to clean water especially South Asia, Southeast Asia, and sub-Saharan Africa.^{3,4,5,6}

Typhoid fever caused by *Salmonella enterica serovar Typhi* is an acute common infection of the reticuloendothelial system, intestinal lymphoid tissue,

and gallbladder. The clinical manifestations of acute infection vary depending on the host, the pathogen strain, the size of the inoculum, and the carrier of the transmission. Clinical manifestations in older children or adults are characterized by prolonged high fever, abdominal discomfort, malaise, and headache.⁷

Salmonella strains are not easily detected in clinical samples that contain microorganisms in small concentrations. Isolation of organisms remains the most effective diagnostic method in cases of suspected typhoid fever and is a gold standard examination for typhoid diagnosis.⁸ Examination of culture takes a long time, requires at least 2 to 5 days until the organism is identified.⁹

The diagnosis of typhoid fever is often enforced based on clinical symptoms and serological tests only. In many developing countries, the Widal test is one of the serological tests which is still widely used in the diagnosis of typhoid fever.¹⁰ Widal tests have several limitations including poor specificity, not easily interpreted especially in endemic areas due to cross-reactions to other infectious diseases and a cutoff titer that differs according to the endemicity of the disease.¹¹

The PCR assay is the preferred molecular technique for in vitro primer-mediated enzymatic amplification of specific segments of DNA for the detection of Salmonella pathogens. The real-time PCR assay detects accumulated PCR products by monitoring the increased fluorescence signal using the integrated real-time thermocycler and fluorescence detector within a system. It has several advantages compared to conventional PCR in that minimized post-amplification cross-contamination because using closed systems, and test time are more efficient by replacing electrophoresis with real-time monitoring of fluorescence.¹²

The target genes most often used to detect specific Salmonella are related to virulence, including *invA* (Salmonella invasion protein gene) *fimA* (the main gene coding for fimbrial subunits), *spv* (virulence gene), *stn* (enterotoxin gene), *fliC* (flagellin gene), and *hilA* (activator of invasion gene transcription).⁽¹³⁾ The new target sequence used by Jing Chen *et al* to detect *Salmonella enterica* is a nucleotide sequence in the *ssaN* gene (putative type III secretion ATP synthase gene) obtained through comparative genomic analysis.

A study on the *ssaN* gene conducted by Jing Chen *et al* provides test results with 100% sensitivity and specificity. The *ssaN* gene is a gene that is conserved and is always found in all strains of *Salmonella enterica*. Some genes have been reported to be absent in some Salmonella serovars because they are unstable and some give false-positive results when the detection method is tested against non-Salmonella strains.¹⁴

SsaN encodes proteins that are part of type 3 ATP synthase secretion in Salmonella pathogenicity island 2 (SPI-2).¹⁴ The *ssaN* is very important for Salmonella secretion and virulence. The functional characterization of type III secretion ATPase SsaN is encoded by SPI 2. Functional T3SS requires five types of proteins including chaperone, translocator, effector, and transcription protein and regulator apparatus. The *ssaN* is a gene that encodes the structure of the secretory system apparatus.¹⁵

Until now, molecular testing of the target *ssaN* gene has only been used to detect Salmonella from food samples. The application of molecular testing with the target *ssaN* gene in clinical samples has not been done so that the potential for its application to clinical samples is not yet known in diagnosing patients with typhoid fever. Based on the above data, in this study, the putative type III secretion ATP synthase gene was detected in patients with typhoid fever so that it is expected to be an alternative target gene in clinical samples.

MATERIALS AND METHODS

Research design

An experimental laboratory study was performed from March to October 2016

Study subjects

The blood samples were from 50 patients suspected with typhoid fever obtained from the Menteng Sub-district Health Center according to the inclusion criteria; fever complaints ≥ 3 days, temperature ≥ 38 °C and additional complaints according to clinical symptoms of typhoid fever (headache and indigestion such as nausea, vomiting, diarrhea or constipation, abdominal pain). Samples were stored in cold temperatures (temperatures of -2 to -6°C) and are processed no more than 72 hours. Specimens were taken to the Virology and Molecular Biology Laboratory, Faculty of Medicine, the University of Indonesia using a cooler bag. Unused specimens are stored at -80 °C.

Laboratory analysis

The standard bacterial DNA was obtained from the extraction of the bacterial culture of *Salmonella typhi* which is a collection from the Department of Microbiology, Faculty of Medicine, University of Indonesia. The extraction of *Salmonella typhi* DNA in this study used the QIAamp DNA Mini Kit (Qiagen). The DNA was used as templates for the optimization of the real-time PCR reaction and determine minimal detection limit. Extracting DNA from whole blood samples used High Pure PCR Template Preparation Kit Roche. The parameters optimized in this study were annealing temperature, primer concentration, probe concentration, and inhibitor test including elution volume test and DNA template volume test. Optimization was done by using a probe-based real-time PCR method using the IQTM5 iCycler multicolor real-time PCR detection system. The primers and probes used as reported by Jing Chen *et al* were designed based on the *ssaN* gene.

Annealing temperature optimization was carried out by a real-time PCR method with 8 gradients at a temperature range of 54-64 °C. The real-time PCR reaction is carried out in a final volume of 10 μ L, consisting of 5 μ L qPCR Kappa probes fast, primer (forward and reverse) with a volume of 0.2 μ l at a concentration of 10 μ M, probes with a volume of 0.2 μ l at a concentration of 10 μ M, standard bacterial DNA template of 1.5 μ l and add distilled water to a total volume of 10 μ l. Amplification was carried out for 45 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 54-64 °C for 1 minute. The optimum condition was determined based on the Ct value (threshold cycle) and the number of fluorescent signals formed.

Primer optimization was done by comparing it to different concentrations and consists of 3 reactions. Each reaction was done in duplicate. Amplification carried out in 20 µl PCR reaction mix. The reaction consisted of 10 µl Kappa probe fast, a primer at a concentration of 10 µM with a volume of 0.5 µl, 0.7 µl, and 1 µl respectively, probes at a concentration of 10 µM with a volume of 0.4 µl. The DNA template was reacted in a 1 µl PCR master mix. The annealing temperature used at this stage is the optimum temperature obtained in the gradient test.

Optimization of probe concentration was done by comparing it to different concentrations and consists of 3 reactions. Each reaction was done in duplicate. Amplification was carried out in 20 µl PCR reaction mix. The reaction consisted of Kappa probe fast 10 µl, the primer at the concentration of 10 µM with the primer volume obtained from the primer optimization results, probes at concentrations of 10 µM with volumes of 0.5 µl, 0.7 µl and 1 µl respectively, and 1 µl DNA template. Inhibitor tests conducted to find out that DNA extraction methods from blood samples do not include certain substances into the extraction results, which can inhibit the DNA amplification process. This test consists of a final elution volume and a DNA template volume.

In the final elution volume test, the reaction consisted of 1 positive control, 1 negative control, and 1 sample of healthy people's blood which was added with *S.typhi* bacteria (spiked blood) then extracted the DNA. Previously, it was confirmed that blood samples came from healthy people (not infected with Salmonella) with a real-time PCR test. DNA extraction used High Pure PCR Template Preparation Kit Roche with different final elution volumes, 50 µL, 60 µL, 70 µL, and 80 µL. Then see which elution results are optimal.

Test for DNA template volume was conducted to determine the optimal volume used as a template for a real-time PCR reaction on a blood specimen. This test is done in duplicate. Blood samples were tested with different template volumes namely 3 µl, 4 µl, 5 µl, and 6 µl with optimal elution volume obtained in the previous step.

The cross-reaction test of real-time PCR was tested against the DNA of some various pathogens that can be isolated from blood specimens. This test was conducted to determine the primer and probes cross-reaction against other pathogens that have the potential to cause false positives. Real-time PCR was tested by using of non-salmonella bacterial DNA and viral DNA including *Bacillus cereus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Herpes simplex virus*, *Varicella zoster virus*, *Epstein-Barr virus*, and *Cytomegalovirus*.

Minimal detection limit real-time PCR reaction was conducted to determine the real-time PCR assay capability in detecting a minimum amount of CFU/ml of blood specimens. Standard bacteria that have been carried out by Mcfarland 0.5 dilution are serially diluted (serial dilution). Dilution was carried out until 10⁻¹. Centrifuge all standard bacteria tubes that have been serially diluted and discard the supernatant. Add blood to each tube containing pellets. Perform DNA extraction from each dilution before the real-time PCR test was

carried out. The PCR reaction was only tested at 10²-10⁻¹ dilution to determine the minimum detection limit, which was the lowest dilution that still gives positive results in real-time PCR.

Blood clinical specimens test was carried out after obtaining the optimal real-time PCR condition to determine the ability of real-time PCR to detect *Salmonella enterica subsp.enterica* using 50 blood specimens of patients suspected with typhoid fever.

Ethical clearance

This study obtained ethical clearance from the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia, under no.539/UN2.F1/ETIK/2016. This study also obtained approval from the Jakarta One-Door Integrated Service Board (*Badan Pelayanan Terpadu Satu Pintu Jakarta*), recommendation, and research permit from the Jakarta Health Service head office (*Dinas Kesehatan Jakarta*).

RESULTS

Optimizing the annealing temperature

In this study, the annealing temperature optimization was carried out by 8 gradients which ranged from temperatures of 54 °C to 64 °C. Eight (8) gradient of annealing temperature were conducted to find the best result. The results of the gradient of thermal cycler temperature obtained the optimal annealing temperature was 56 °C (Figure 1)

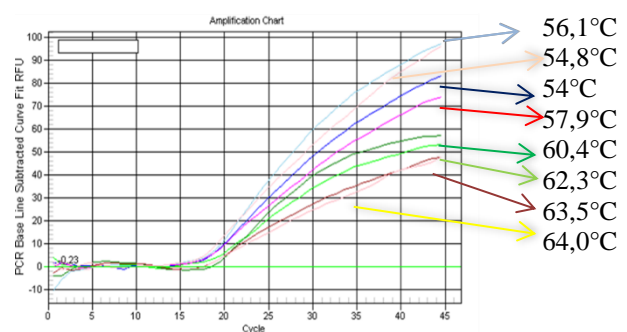


Figure 1. Results of the real-time PCR assay to optimize the annealing temperature of *Salmonella enterica subsp.enterica serovar Typhi* with a temperature gradient of 54-64 °C. Caption: light blue line 56.1 °C, pink line 54.8 °C, dark blue line 54 °C, red line 57.9 °C, dark green line 60.4 °C, light green line 62.3 °C, dark red line 63.5 °C, yellow line 64 °C

Optimizing the primer concentration

The forward and reverse primer concentrations used for the primer optimization test were 0.25 µM, 0.35 µM, and 0.5 µM with a total volume of 20 µl. In this study, the optimal primer concentration obtained was at a volume of 1 µl so that the concentration was 0.5 µM or 500 nM (Figure 2). In the research conducted by Jing Chen *et al.*, the primer concentration used was 400nM.

Optimizing the probe concentration

The concentration of probes used for the probe optimization was 0.25 µM, 0.35 µM, and 0.5 µM with a total volume of 20 µl. The optimum probe concentration obtained in this study was at a volume of 0.7 µl so that the concentration was 0.35 µM or 350 nM (Figure 3). In a study conducted by Jing Chen *et al.*, the concentration of probes used was 200 nM.

Optimizing the elution DNA Volume and DNA template volume of *Salmonella enterica subsp. enterica*

In this test, the results of elution volume which show optimal final elution volume was at 60 μ L (Figure 4). At the volume of 50 μ l elution, the results of amplification showed lower efficiency. This was probably due to the presence of inhibitors contained in the suspension resulting from DNA extraction in blood specimens with a final elution volume of 50 μ l. At a larger final elution volume, the results were not optimal. This was not due to the presence of inhibitors in the suspension but due to the lower concentration of DNA. The results of the DNA template test show that the optimal volume that can be used as a template from a blood specimen is 4 μ l. (Figure 5)

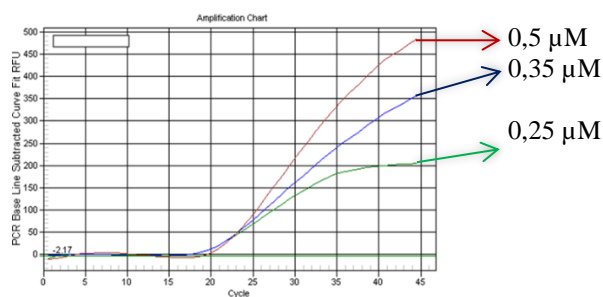


Figure 2. Results of the real-time PCR assay in optimizing the primer (forward and reverse) concentration. Caption: 0.5 μ M (red line), 0.35 μ M (dark blue line), 0.25 μ M (green line).

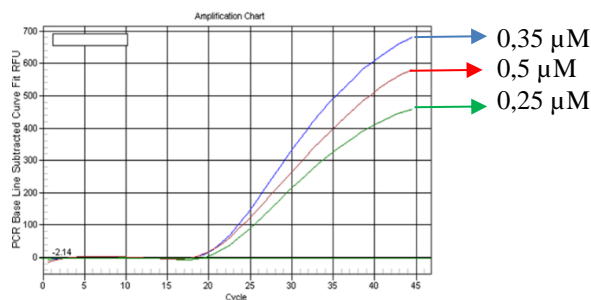


Figure 3. Results of the real-time PCR assay in optimizing the probe concentration. Caption: 0.35 μ M (dark blue line), 0.5 μ M (red line), 0.25 μ M (green line).

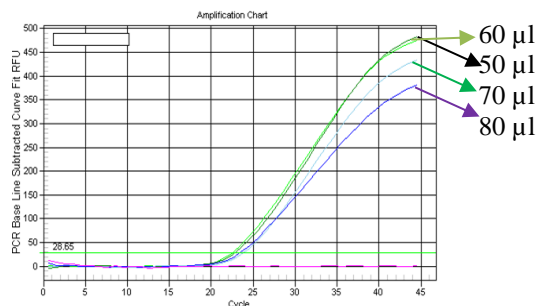


Figure 4. Results of the real-time PCR assay in optimizing the final elution volume of *Salmonella enterica subsp. enterica* DNA in spiked blood specimens. Caption: 60 μ l (dark green line), 50 μ l (light green line), 70 μ l (light blue line), 80 μ l (dark blue line).

Cross-reaction test

A cross-reaction test was tested against several microorganisms that might be found in the blood.

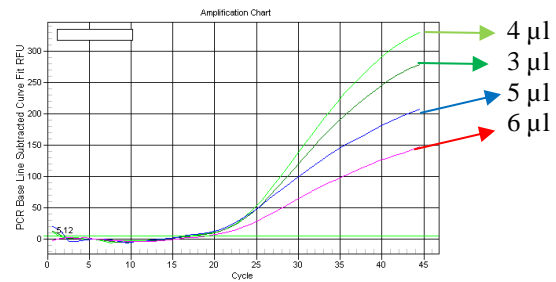


Figure 5. Results of the real-time PCR assay in optimizing the DNA template volume of *Salmonella enterica subsp. enterica* in spiked blood specimens. Caption: 4 μ l (light green line), 3 μ l (dark green line), 5 μ l (dark blue line), 6 μ l (pink line).

Microorganisms used were *Bacillus cereus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Herpes simplex virus (HSV)*, *Cytomegalovirus (CMV)*, *Epstein-Barr virus (EBV)*, and *Varicella zoster virus (VZV)*. The results of this test showed that the primers and probes used in this study did not cross-react with these microorganisms. (Figure 6 and Figure 7).

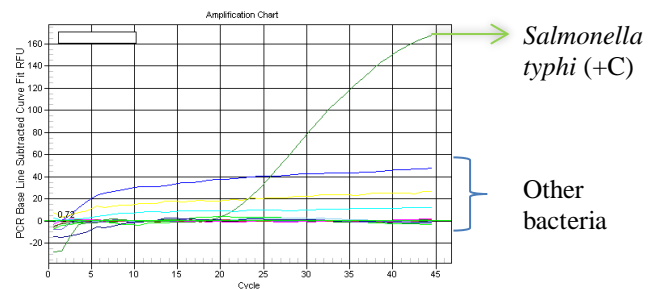


Figure 6. Results of the real-time PCR cross-reaction test of *Salmonella enterica serovar Typhi* DNA with several microorganisms. Caption: DNA of *Salmonella typhi* as a positive control (green line).

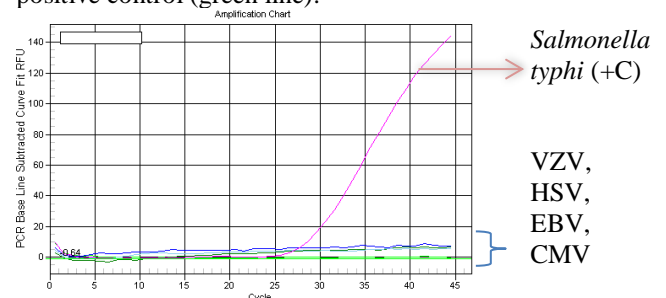


Figure 7. Result of real-time PCR cross-reaction test for DNA viruses. Caption: DNA of *Salmonella typhi* as a positive control (pink line).

Minimum detection limit

This test was carried out to determine the minimum detection limit in CFU/ml of blood specimens using a real-time PCR assay. From the results of this test, the minimum number detected using the real-time PCR assay was 10^1 CFU/ml (Figure 8).

Blood Clinical Samples

The real-time PCR application of clinical samples was carried out on 50 blood specimens of patients suspected with typhoid fever. The results of the comparison between the Widal and qPCR methods are shown in table 1.

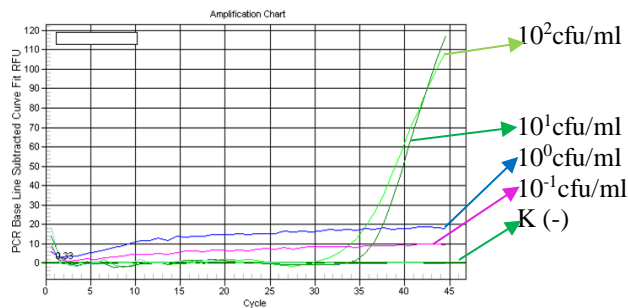


Figure 8. Results of minimum detection limit test. Description of image: light green line 10^2 cfu/ml, dark green line 10^1 cfu/ml, dark blue line 10^0 cfu/ml, red line 10^{-1} cfu/ml.

volume of 1 μ l so that the concentration was 0.5 μ M or 500 nM. In the research conducted by Jing Chen *et al.*, the primer concentration used was 400nM. The different primer concentrations obtained are due to different enzymes, the total volume of reaction, probe concentration, and the molecular weight of primers used in this study.¹³

In the PCR reaction primer is important to initiate the reaction and determine the amount of amplified DNA. Primers play a role in the process of initiating DNA polymerization reactions and limiting the area to be amplified in the PCR reaction. Higher primer concentrations can cause primers to be non-specific and

Table 1. Results of comparison between Widal and qPCR serological methods for 50 samples from patients suspected with typhoid fever.

| Bacteria | rPCR (%) specimen | | | | Total Widal+ | Total rPCR+ |
|------------------------|-------------------|--------------|--------------|--------------|--------------|-------------|
| | rPCR+ Widal+ | rPCR+ Widal- | rPCR- Widal+ | rPCR- Widal- | | |
| <i>S. enterica</i> | 8(16) | 11(22) | 4(10) | 27(52) | 12(24) | 19(38) |
| <i>Subsp. enterica</i> | | | | | | |

+ : positive . - : negative

Blood Clinical Samples

The real-time PCR application of clinical samples was carried out on 50 blood specimens of patients suspected with typhoid fever. The results of the comparison between the Widal and qPCR methods are shown in table 1.

DISCUSSION

Culture is a gold standard examination for the diagnosis of typhoid fever but it requires at least 2 to 5 days until the organism is identified.^{8,9} In many developing countries, the Widal test is one of the serological tests which is still widely used but it has numerous limitations including poor specificity, not easily interpreted especially in endemic areas due to cross-reactions to other infectious diseases.^{(10),(11)}

Molecular testing of the new target *ssaN* gene (putative type III secretion ATP synthase gene) has only been used to detect *Salmonella* from food samples. In this study, real-time PCR optimization is performed so that it can be used to detect salmonella from clinical samples.

In this study, it was observed that the annealing temperature of 56 °C gave the optimal result. The annealing temperature optimization was carried out by 8 gradients which ranged from temperatures of 54 °C to 64 °C. Determining the temperature of annealing is based on the lowest cycle threshold (Ct value) and a high fluorescent signal. The temperature of annealing in a real-time PCR reaction will affect the efficiency of the primer attachment and the probe used. When the temperature used is higher it will cause the failure of primer and probe attached to the complementary DNA template. When it is lower, it can be attached to an appropriate place. This can cause low amplification signal results.⁽¹⁶⁾

The forward and reverse primer concentrations used for the primer optimization test are 0.25 μ M, 0.35 μ M, and 0.5 μ M with a total volume of 20 μ l. In our study, the optimal primer concentration obtained was at a

can form primer-dimers When it is lower, it can also affect amplification efficiency.¹⁶

The probe is a DNA fragment labeled fluorescent and has a complementary nucleotide sequence with PCR amplification products. The probe attachment efficiency is influenced by the annealing temperature and also the concentration of the probe.⁽¹⁶⁾ The concentration of probes used for the probe optimization was 0.25 μ M, 0.35 μ M, and 0.5 μ M with a total volume of 20 μ l. The optimum probe concentration obtained in this study was at a volume of 0.7 μ l so that the concentration was 0.35 μ M or 350 nM. In a study conducted by Jing Chen *et al.*, the concentration of probes used was 200 nM. The difference in concentration is caused by differences in PCR machines, PCR methods, probes, and enzymes used. Jing Chen used an internal amplification control (IAC) probe that was generated by randomly shuffling the sequence of the target probe.⁽¹³⁾

The cross-reaction test of the primer was tested against several microorganisms that might be found in the blood. The results of this test showed that the primers and probes used in this study did not cross-react with these microorganisms. In the study conducted by Jing Chen, the specificity of the same primer was also observed. It was tested against a total of 40 strains of *S. enterica* and 23 bacterial strains from 11 other genera. All PCR-reactions using those bacteria gave also negative results. The primer set was specific for the detection of *S. enterica*.⁽¹³⁾

Minimal detection limit real-time PCR reaction was conducted to determine the real-time PCR assay capability in detecting a minimum amount of CFU/ml of blood specimens. We found that the detection of the minimum number detected using the real-time PCR assay was 10^1 CFU/ml.

In a real-time PCR test that has been optimized for clinical samples of a patient's blood, the potential for contamination is a matter of great concern. To avoid contamination between samples, at the stage of working on real-time PCR always includes negative controls as

quality control to determine the presence of contamination. The laboratory is also designed to avoid contamination by the existence of three separate spaces (preparation, extraction, PCR process) with the one-way flow.⁽¹⁶⁾

Blood clinical specimens test was carried out after obtaining the optimal real-time PCR condition to determine the ability of real-time PCR to detect *Salmonella enterica subsp. enterica* using 50 blood specimens of patients suspected with typhoid fever. From 50 samples, the real-time PCR test can detect 19 (38%) *S. enterica*. A total of 8 samples with positive Widal test results also gave positive qPCR results. Eleven samples with negative Widal test gave positive results on qPCR. Thus, the qPCR used in this study can increase the positive test level by 22% of the total specimens. This can be caused by the formation of antibodies to the *S. typhi* O antigen not yet formed because O antibodies increase on day 6-8 from the beginning of the disease. Widal examination should be done again 1-2 weeks later to see an increase in titers 4 times from the acute phase but the second Widal test was not performed. So from this result, it can be seen that real-time PCR can be used for the initial detection of cases with suspected typhoid fever in blood specimens. There were 4 samples with positive Widal results but the results of the qPCR test were negative. This can be caused by false positives from the Widal test. Among several issues, a higher percentage of false-positive results is a major problem. Several febrile illnesses such as tuberculosis, malaria, and hepatitis B-associated polyarteritis nodosa are associated with a high incidence of false-positive Widal test.^{(17), (18)}

Based on research conducted by Reynold, diagnosing typhoid fever based only on serology is not accurate. There are several false-positive cases in endemic areas and patients with a history of typhoid fever⁽¹⁹⁾. Now are recognized cross-reacting antigenic determinants of typhoidal, nontyphoidal *Salmonella* organisms and other *Enterobacteriaceae* as are several other diseases caused by non-*Salmonella* organisms such as malaria, dengue, and TB.⁽²⁰⁾

One of the limitations of this study was the fact that no culture examination was performed as a gold standard for the diagnosis of *Salmonella* infection. This is because there are limitations in terms of research funding and time. Whereas culture is costly and time-consuming.

CONCLUSION

The optimal condition of the qPCR test to detect *S. enterica* is at an annealing temperature of 56 °C, SEF primary concentration and SER 0.5 µM, SEP probe concentration 0.35 µM. The optimal elution volume is 60 µl. The DNA template for *S. enterica* is 4 µl. The qPCR test does not cross-react with other microorganisms. The minimum detection limit in CFU/ml from a blood sample is 10 CFU/ml. From 50 samples, the qPCR test can detect 19 (38%) *S. enterica*. The qPCR used in this study can increase the level of positive testing by 22% of the total specimens. Real-time PCR is a sensitive tool for the early detection of *S. enterica* in clinical specimens. Real-time PCR optimization with the new *ssaN* gene target can detect

Salmonella enterica subsp. enterica in blood specimens from a patient with suspected typhoid fever.

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