

## DETECTION OF *SALMONELLA TYPHI* BY NESTED POLYMERASE CHAIN REACTION IN BLOOD, URINE, AND STOOL SAMPLES

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**Abstract.** A nested polymerase chain reaction (PCR) specific for *Salmonella enterica* serovar Typhi was used for the detection of the pathogen in blood, urine, and stool samples from 131 patients with clinical suspicion of typhoid fever. The sensitivity of blood culture, the PCRs with blood, urine, and feces, and the Widal test were 61.8%, 84.5%, 69.3%, 46.9%, and 39.0%, respectively. The sensitivity of the PCRs with blood ( $P < 0.001$ ) and urine ( $P = 0.01$ ) were significantly higher, and the sensitivity of the PCR with feces ( $P > 0.05$ ) was similar to that of blood culture. Combined, the PCRs on urine and feces showed positive results for 16 (70%) of 23 typhoid patients with negative results with blood culture and PCR with blood. These results show that the PCR with blood is a sensitive method for the diagnosis of typhoid fever, and that the PCRs with urine and feces could be useful complementary tests.

### INTRODUCTION

Typhoid fever is an important health problem in many developing countries. Worldwide, an estimated 17 million persons develop this disease annually. Most of this burden occurs among citizens of low-income countries, particular those in Southeast Asia, Africa, and Latin America. In South Sulawesi, Indonesia, typhoid fever is one of the most important infectious diseases.<sup>1</sup> The disease is endemic throughout the region and is the fourth most frequently reported infectious disease in most of its 24 districts. In South-Sulawesi, typhoid is the most important cause of community-acquired septicemia, with a reported incidence rate exceeding 2,500/100,000 in many districts.

Typhoid fever is caused by *Salmonella enterica* serovar Typhi and is transmitted through the fecal-oral route by the consumption of contaminated water and food. The presence of a convalescent patient or a carrier actively shedding the pathogen poses an increased risk for infection. In non-endemic areas, disease outbreaks may occur from a unique source of food or carrier.<sup>2,3</sup> In disease-endemic areas a recent contact with a patient or carrier has been identified as a major risk factor,<sup>4,5</sup> but other risk factors include poverty, low education level, poor hygienic conditions and water supplies, and eating outdoors at food stalls.<sup>1,6–10</sup>

Almost half of the treated patients continue to excrete the pathogen one month after the symptoms have disappeared, and approximately 5% still do so five months later.<sup>11,12</sup> Approximately 3% become carriers and continue to excrete the organism, often lifelong.<sup>13</sup> The carrier stage may also develop after an asymptomatic infection. Molecular detection methods are most suitable to identify pathogens in human excretions because these methods are highly specific and sensitive. In particular the polymerase chain reaction (PCR) is capable of detecting minute quantities of DNA of specific pathogens through amplification of a defined DNA segment, and discriminating in one reaction between different organisms even if they are closely related. In combination with the appropri-

ate sample preparation method, PCR can be applied on almost any specimen including whole blood, stool, and urine.<sup>14,15</sup> Thus, PCR seems to be suitable to identify those patients actively excreting the organism and to investigate the carrier stage through the specific detection the DNA of *S. typhi* in urine and feces samples.

Song and coworkers described a nested PCR for the detection of *S. typhi* DNA in blood specimens.<sup>16</sup> In this study, we assessed the sensitivity of the nested PCR on blood samples and investigated whether the method can be used for the detection of *S. typhi* DNA in urine and stool samples. To this end blood, urine and stools samples from patients with suspected typhoid fever were tested and the results were compared with blood culture and the Widal test.

### MATERIALS AND METHODS

**Patient group and clinical specimens.** A total of 131 (74 males and 57 females) patients (median age = 20 years, range = 6–54 years) with clinical suspicion of typhoid fever who came to two different hospitals and one primary health care center in or located near Makassar, the capital city of South Sulawesi, were included in the study on the basis of continuous high-grade fever (median temperature = 38°C, range = 37.2–40°C), toxic appearance, splenomegaly, and constitutional symptoms. The median duration of illness at consultation was 7 days (range = 4–15 days). Blood samples for culture and PCR, and serum samples for serologic analysis were collected from all patients. A urine sample was collected from 105 patients (60 males and 45 females), and a stool sample was collected from 50 patients (38 males and 12 females). Clinical specimens were collected on the same day or within 1–2 days after the first consultation.

**Ethical considerations.** The study was reviewed and approved by the review boards of the participating institutes and informed consent was obtained from all participants or their parents or guardians.

**Blood culture.** Five milliliters of freshly collected blood was placed in 15 mL of Ox bile broth (Merck, Darmstadt, Germany) and incubated for 24 hours at 37°C. One milliliter of this culture was then plated on *Salmonella Shigella* (SS) agar (Oxoid, Basingstoke, United Kingdom), incubated for 24 hours at 37°C, and examined for growth. If growth was present, individual colonies were examined by Gram staining

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and identification of the bacteria was performed after subculturing on SS agar by biochemical testing with the triple sugar iron test, sulfide indole motility, methyl red Voges' Proskauer reactivity, citrate consumption, urease and decarboxylase activity, and carbohydrate fermentation of glucose, lactose, mannitol, sucrose, and arabinose.<sup>17</sup>

**Serologic analysis.** The Widal test with O antigen (Murex Biotech Ltd., Dartford, United Kingdom) was performed and interpreted according to routine laboratory procedures. A titer  $\geq 1:320$  was considered positive.

**Preparation of DNA from blood, feces, and urine.** DNA was extracted from freshly collected whole blood, urine, and stool samples according to the diatom-guanidinium isothiocyanate (GuSCN) method described by Boom and others.<sup>14</sup> For the extraction of DNA from blood, a 100- $\mu$ L freshly collected blood sample was mixed with 900  $\mu$ L of lysis buffer (50 mM Tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X-100) and centrifuged at  $12,000 \times g$  for 10 minutes. For the preparation of DNA from urine, a 1-mL urine sample was centrifuged at 1,000 rpm for 5 minutes. Supernatants were collected and centrifuged again at 5,000 rpm for 30 minutes, and lysis buffer (900  $\mu$ L) was added to the sediment. For the preparation of DNA from feces, a stool sample with a volume of approximately 100  $\mu$ L was attached to a cotton swab, suspended in 1 mL of sterile water, vortexed vigorously, and centrifuged at 1,000 rpm for 5 min. To obtain the DNA, samples were lysed by incubation for 15 minutes at 18°C and 20  $\mu$ L of diatom suspension was added.<sup>14</sup> The diatom containing the bound DNA was sedimented by centrifugation at  $12,000 \times g$  for 15 seconds. The diatom pellet was washed with washing buffer (5.25 M GuSCN in 0.1 M Tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 minutes. The pellet was mixed with 60  $\mu$ L of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer and the DNA was eluted by incubation at 56°C for 10 minutes. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until PCR was performed.

**Molecular detection.** The nested PCR described by Song and others<sup>16</sup> with a modified forward primer for the first PCR as suggested by Frankel and others<sup>18</sup> to increase specificity is based on the amplification of unique sequences in the VI region of the flagellin gene. The modified PCR amplifies a 458-basepair (bp) fragment corresponding to nucleotides 1063–1530 of the gene in the first reaction and a nested 343-bp fragment corresponding to nucleotides 1072–1435 in the second reaction.<sup>18</sup> The first PCR was performed by the addition of 2  $\mu$ L of extracted DNA to 25  $\mu$ L of PCR mixture and amplification for 40 cycles (1 minute at 94°C, 75 seconds at 57°C, and 3 minutes at 72°C) in a thermocycler (Hybaid, Ashford, United Kingdom). The PCR mixture consisted of standard PCR buffer (100 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% gelatin, 200- $\mu$ M each of all four dNTPs, and 0.625 units of *Taq* DNA polymerase) supplemented with 25 pmol of primer ST1 (5'-ACT GCT AAA ACC ACT ACT-3') and 25 pmol of primer ST2 (5'-ACT GCT AAA ACC ACT ACT-3'). The nested PCR was performed by the addition of 2  $\mu$ L of the PCR product to 20  $\mu$ L of standard PCR buffer supplemented with 25 pmol of primer ST3 (5'-AGA TGG TAC TGG CGT TGC TC-3') and 25 pmol of primer ST4 (5'-TGG AGA CTT CGG TCG CGT AG-3') and amplification for 40 cycles with the same temperature cycle program as for the first reaction. The PCR was

performed without prior knowledge of the classification of the samples.

## RESULTS

Based on the results of blood culture, the evolution of the illness, and response to treatment, a diagnosis of typhoid fever was made in 119 patients of a series of 131 clinical suspected cases of typhoid fever. Culture was positive for 68 (57.1%) patients. The nested PCR on blood showed a positive result in 93 (78.2%) patients, and 45 (37.8%) were positive by the Widal test (titer  $\geq 1:320$ ) (Table 1). Negative test results in blood culture and PCR on blood were obtained for 25 patients with a diagnosis of typhoid fever. A clinical diagnosis other than typhoid fever was made in 12 other culture- and PCR-negative patients. Of these 12 patients, four (33.3%) were positive in the Widal test (titer  $\geq 1:320$ ). At the one-step lower cut-off value (titer 1:160), 57 (47.9%) patients diagnosed with typhoid fever and 11 (91.7%) patients with a diagnosis other than typhoid fever showed positive results in the Widal test.

The PCRs on urine and feces showed positive results in 65.6% and 39.5% of the typhoid fever patients from whom these samples were collected (Table 2). The PCRs with urine and feces showed positive results in 72.9% of the patients diagnosed with typhoid fever, and all three PCRs showed positive results in 95.7% of the patients. Stratification according to the culture results showed that the PCR on urine had a positive result in 61.1% of the blood culture-positive patients and in 71.8% of the blood culture-negative typhoid patients, and that the PCR on feces had a positive result in 23.8% and 58.8% of these two groups of patients, respectively.

Blood samples from 26 typhoid patients tested negative in the PCR. A urine sample was collected from 18 of these patients and a stool sample from 16 of them. Of these, 13 (72.2%) tested positive in the PCR for urine and 10 (62.5%) tested positive in the PCR for feces. The PCRs with urine and feces were also performed for all 12 non-typhoid patients and all showed negative results.

Based on the combined results of blood culture and the three PCRs, the sensitivities of blood culture, PCR on blood,

TABLE 1  
Comparative study of blood culture, Widal test, and polymerase chain reaction (PCR) on blood in suspected typhoid patients

| Patient group/<br>No. of cases<br>with typhoid | Test result and total no. (%) with<br>a positive result in the respective tests |              |            | Median duration<br>of illness (days) |
|--|---|--------------|------------|--------------------------------------|
|  | Blood culture   | PCR on blood | Widal test |                                      |
| 33   | +   | +            | +          | 9                                    |
| 34   | +   | +            | -          | 4                                    |
| 1  | +   | -            | +          | 7                                    |
| 6  | -   | +            | +          | 8.5                                  |
| 20   | -   | +            | -          | 6                                    |
| 5  | -   | -            | +          | 7                                    |
| 20   | -   | -            | -          | 7                                    |
| Total<br>119                                   | 68 (57.1)   | 93 (78.2)    | 45 (37.8)  | 7                                    |
| Non-typhoid                                    |   |              |            |                                      |
| 4  | -   | -            | +          | 6.5                                  |
| 8  | -   | -            | -          | 7                                    |
| Total<br>12                                    | 0 (0)   | 0 (0)        | 4 (33.3)   | 7                                    |

TABLE 2

Results of nested polymerase chain reaction (PCR) on different clinical specimens according to the results of blood culture for typhoid and non-typhoid patients

| Patient group and assay                       | No. positive (%; 95% confidence interval)/no. tested |
|---|--|
| Patients with typhoid fever ( <i>n</i> = 119) |  |
| PCR on blood                                  | 93 (78.2, 71–86)/119                                 |
| PCR on urine                                  | 61 (65.6, 51–71)/93                                  |
| PCR on feces                                  | 15 (39.5, 24–55)/38                                  |
| PCR on feces and/or urine                     | 70 (72.9%, 64–82)/96                                 |
| Any PCR                                       | 110 (95.7%, 91–99)/115                               |
| Culture confirmed ( <i>n</i> = 68)            |  |
| PCR on blood                                  | 67 (98.5, 97–100)/68                                 |
| PCR on urine                                  | 33 (61.1, 48–81)/54                                  |
| PCR on feces                                  | 5 (23.8, 7–42)/21                                    |
| PCR on feces and/or urine                     | 38 (70.4, 58–83)/54                                  |
| Any PCR                                       | 68 (100, 93–100)/68                                  |
| Culture negative ( <i>n</i> = 51)             |  |
| PCR on blood                                  | 26 (51.0, 37–65)/51                                  |
| PCR on urine                                  | 28 (71.8, 58–86)/39                                  |
| PCR on feces                                  | 10 (58.8, 35–82)/17                                  |
| PCR on feces and/or urine                     | 32 (71.1, 56–84)/45                                  |
| Any PCR                                       | 42 (82.3, 72–93)/51                                  |
| Non-typhoid patients ( <i>n</i> = 12)         |  |
| PCR on blood                                  | 0 (0, 0–30)/12                                       |
| PCR on urine                                  | 0 (0, 0–30)/12                                       |
| PCR on feces                                  | 0 (0, 0–30)/12                                       |

PCR on urine, PCR on feces, and the Widal test were 61.8%, 84.5%, 46.9%, 69.3%, and 39%, respectively (Table 3). The sensitivity of the nested PCRs on blood ( $P < 0.001$ ) and urine ( $P = 0.01$ ) were significant higher, and the sensitivity of the nested PCR on feces ( $P > 0.05$ ) was similar to the sensitivity of blood culture. Four patients with a clinical diagnosis of typhoid fever showed negative results in blood culture and the three PCRs on blood, urine, and stool. For five other patients with a clinical diagnosis of typhoid fever and negative results in blood culture and the PCR on blood, either no stool and urine samples were obtained or only a stool or a urine sample was obtained. These samples showed negative results in the PCR.

Stratification of the samples according to the duration of illness at first diagnosis showed that the Widal test showed negative results for samples collected during the first few days of illness (Table 4). The sensitivity of the Widal test increased with the duration of illness to 88.2% for serum samples collected more than nine days after the onset of the disease. The sensitivity of the PCRs was already high for samples collected at an early stage.

TABLE 3

Sensitivity of various laboratory methods for typhoid patients confirmed by blood culture, polymerase chain reaction (PCR) on blood, PCR on feces, and/or PCR on urine

| Assay                           | No. positive (sensitivity, 95% confidence interval)/no. tested |
|---------------------------------|--|
| Culture                         | 68 (61.8, 53–71)/110   |
| PCR on blood                    | 93 (84.5, 78–91)/110   |
| PCR on feces                    | 15 (46.9, 30–64)/32  |
| PCR on urine                    | 61 (69.3, 60–79)/88  |
| Widal test (titer $\geq$ 1:320) | 43 (39, 30–48)/110   |
| Widal test (titer $\geq$ 1:160) | 57 (51.8, 42–61)/110   |

## DISCUSSION

Typhoid fever is a major public health problem in many developing countries. Symptoms and signs of the disease are non-specific and laboratory testing is essential for diagnosis. Culture provides definite proof of typhoid fever. In the present group of 131 patients, typhoid fever was confirmed in 68 patients by blood culture and an additional 51 patients were diagnosed as having typhoid fever based on clinical signs and symptoms. Application of a nested PCR amplifying a segment of the flagellin gene of *S. typhi* in whole blood samples showed positive results for 67 (98.5%) culture-confirmed typhoid fever patients, as well as for 26 (51%) culture-negative patients diagnosed with typhoid fever. The sensitivity of the nested PCR appears to be higher than the sensitivity of blood culture performed on 5-mL blood samples.

The finding of positive PCR results for blood samples from culture-negative typhoid patients is consistent with the previous observations, which showed that the same nested PCR, as well as other PCRs for typhoid fever, may have higher sensitivities than blood culture.<sup>19–23</sup> Prakash and others used the same nested PCR but a different DNA extraction method and reported a sensitivity of 29.8% for blood culture and 92.8% for the nested PCR.<sup>20</sup> Hague and others reported a sensitivity of 14.5% for blood culture and 58.2% for the nested PCR.<sup>19</sup>

Patients with fever for 3–30 days were tested and it was suggested that the higher sensitivity of PCR compared with blood culture was the result of previous use of antibiotics and the average relatively long disease history that will have a negative effect on the recovery rate in culture. In our study, most of the typhoid patients were diagnosed at a relative early stage of illness and reported to have fever for less than 10 days. The sensitivity of the PCR on blood was 84.5% compared with 61.8% for blood culture and was highest (95%) for samples collected during the first five days of illness. The number of typhoid bacilli in the blood is usually low with approximately half of the patients having less than one colony-forming unit per milliliter of blood.<sup>24</sup>

The efficacy of blood culture decreases with the duration of illness, and use of antibiotics before collection of the blood sample for culture severely affects the isolation rate.<sup>25,26</sup> Antibiotics are freely available in Indonesia and many of the patients may have taken antibiotics before consultation. This possibility and the relatively small blood sample used for culturing could explain the relatively low culture rate. Only a small volume of blood could be collected from each patient because Indonesia patients are reluctant to donate blood. Patients infected with a multidrug-resistant *S. typhi* usually have a higher blood bacterial count and presumably a higher isolation rate.<sup>24</sup> However multidrug-resistant *S. typhi* has not been reported from Indonesia.

Our results confirm the notion that a large proportion of typhoid patients excrete the pathogen in their urine and or stool. Wain and others reported that 14.3% of stool cultures from typhoid patients were positive for the pathogen.<sup>24</sup> Our results indicate that the percentage of patients excreting the pathogen could be higher. *Salmonella typhi* DNA was detected in the urine or stool of 72.9% of the patients diagnosed with typhoid fever. In our study, more urine samples (65.6%) than stool samples (39.5%) were positive for the pathogen. The PCR on stool samples may have been less successful

TABLE 4  
Results of culture, polymerase chain reaction (PCR), and Widal test stratified according to duration of illness

| Duration of illness (days) | No. samples (%) positive in the following assays/no. tested |              |             |              |              |              |
|----------------------------|---|--------------|-------------|--------------|--------------|--------------|
|                            | Culture   | PCR          |             |              |              | Widal test   |
|                            |   | Blood        | Feces       | Urine        | Any PCR      |              |
| < 6                        | 31 (77.5)/40  | 38 (95)/40   | 5 (45.5)/11 | 22 (68.8)/32 | 40 (100)/40  | 0 (0)/40     |
| 6-9                        | 23 (37.1)/62  | 40 (64.5)/62 | 9 (42.9)/21 | 31 (67.4)/46 | 53 (85.5)/62 | 30 (48.4)/62 |
| > 9                        | 14 (82.4)/17  | 15 (88.2)/17 | 1 (16.7)/6  | 8 (53.3)/15  | 17 (100)/17  | 15 (88.2)/17 |

because of inhibitors present at higher concentrations in stool samples than in urine samples or because these inhibitors are more difficult to remove from feces.

Patients that excrete the bacteria are at high risk for spreading the disease. Strict sanitary precautions should be taken to avoid infection when in contact with patients who are recovering from this disease.<sup>4</sup> Our results indicate that the nested PCR may be used to detect *S. typhi* DNA in urine or stool from patients with typhoid fever. Further studies are needed to demonstrate that the detection of DNA in urine and stool reflects the presence of live bacteria. A previous study showed the presence of *S. typhi* antigen in urine samples in a high proportion of typhoid patients, which may be consistent with the presence of the bacterium in the urinary tract.<sup>27</sup> Since a large number of different pathogens may be present in the urine and stool of typhoid patients, the specificity of these antigen and DNA detection methods need further examination, and the presence of viable pathogen in urine samples from patients with typhoid fever should be confirmed by culture.<sup>28</sup>

A study in Vietnam showed that carriage of *S. typhi* in the stool is found predominantly in those typhoid patients with a higher bacterial count in the blood.<sup>24</sup> We have not determined the bacterial counts for the patients in our study group. Using the nested PCR, we found no difference in the percentage of culture-positive and culture-negative patients excreting the pathogen in their urine and or stool. Most likely, a large proportion of the culture-negative patients in our study had taken antibiotics before consultation, which affected the results of blood culture.

Blood culture and PCR on blood showed negative results in 25 patients with a clinical diagnosis of typhoid fever. The nested PCR with either a urine sample and or a fecal sample collected from 23 of these patients detected DNA of the pathogen in 16 of them. Blood culture, PCR on blood, PCR on urine, and PCR on feces confirmed the diagnosis of typhoid fever in 115 patients. It is noteworthy to mention that neither the PCR on urine nor the PCR on feces showed positive results in samples from the 12 patients with a diagnosis other than typhoid fever; all of these patients also had negative PCR results on blood. These results show that in addition to the PCR on blood, the nested PCRs on feces and or urine are useful as complementary tests in the diagnosis of typhoid fever, especially for patients suspected of having this disease and who have negative results in blood cultures.

Testing of urine may be preferred because collection of urine is easier to perform and the sensitivity of the PCR was good. The PCR on urine showed positive results for 13 urine samples collected from patients with a negative result for blood in the PCR. If the PCR on urine was used to comple-

ment the PCR on blood, the sensitivity would increase from 84.5% for the PCR on blood to 96.4% for the PCRs combined. The highly efficient GuSCN-silica method was used to obtain DNA from the stool and urine samples. The use of this extraction method could be useful because it efficiently removes substances from the sample that may inhibit the PCR.

Serologic analysis with the Widal test provides a result quickly but is of limited value because the sensitivity of this test (titer  $\geq 1:320$ ) based on the combined results of blood culture and the nested PCRs was only 34%. This value is lower than the sensitivity of 47.5% reported for the Widal test in our previous study,<sup>29</sup> and lower than the sensitivity of 47% reported at a titer of 1:400 in a study performed in Vietnam.<sup>30</sup> The low sensitivity is partly explained by the relative high proportion of patients tested at an early stage of the disease who presumably have not developed significant levels of specific agglutinating antibodies. The sensitivity of the Widal test increased with the duration of illness from 0% for samples collected less than six days after the onset of the disease to 48.4% for samples collected between day 6 and day 9 and to 88.2% for samples collected between day 9 and day 15.

Testing of paired serum samples increases the sensitivity of serologic analysis but is of less practical value for patients requiring immediate treatment.<sup>31</sup> Moreover, the specificity of the Widal test is relatively low. We previously reported a specificity of 84.4% for the Widal test (titer  $\geq 1:320$ ) for patients with clinical suspicion of typhoid fever in South Sulawesi.<sup>29</sup> In the present study, 33.3% of the patients with a diagnosis other than typhoid fever showed a positive result in this test. Although the specificity of the Widal test can be relatively high if a high cut-off value is selected, this value decreases considerable if a lower cut-off value is used because of high background levels of reactive antibodies in the population.<sup>30</sup>

Blood culture is relative insensitive and time-consuming, and the sensitivity and specificity of serologic analysis with the Widal test is too low to useful in diagnosis. The sensitivity of bone marrow culture is reported to be higher but this invasive method is not always practicable.<sup>32,33</sup> A nested PCR could be an attractive alternative. However, a nested PCR is relatively complicated to perform and is not suitable for use in routine clinical practice in laboratories in countries where typhoid fever is endemic. Also, in countries where *S. paratyphi* is a common cause of enteric fever, this method will not be useful. This study shows that there is a need for an improved diagnostic tool for typhoid fever that is adapted to the needs of resource poor clinical facilities in disease-endemic areas.

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