

## Enhanced detection rate of typhoid fever among clinically suspected patients in a tertiary referral hospital in Dhaka, Bangladesh using nested polymerase chain reaction technology

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### Abstract

A nested polymerase chain reaction (PCR) specific for *Salmonella enterica subspecies enteric* serovar Typhi was used for the detection of the pathogen in blood. This study was done during the period of March 2013 to February 2014. A total of 80 clinically suspected cases of typhoid fever were included in the study. Blood was collected from all participating individuals. Nested PCR targeting the flagellin gene (*fliC*) of *Salmonella* Typhi & blood culture were done for each of the cases. The positivity rate of PCR & blood culture was 70% & 20% respectively. The positivity rate of PCR was significantly higher than blood culture ( $P < 0.05$ ). With the nested PCR, *S. Typhi* DNAs were detected from blood specimens of 67.2% (43/64) patients among the suspected typhoid fever cases on the basis of clinical features but with negative cultures. We conclude that the PCR technique could be used as a novel diagnostic method of typhoid fever, particularly in culture-negative cases in an endemic country like Bangladesh.

**Keywords:** Typhoid fever, Nested polymerase chain reaction, Diagnosis.

### Introduction

Typhoid fever is an infectious disease in developing countries like Bangladesh. It is estimated that more than 21 million people suffer from typhoid fever annually with more than 216,000 death attributed to the disease.<sup>1</sup> Regions with high incidence of typhoid fever (> 100/1000.000 cases/year) include South Central Asia, South East Asia<sup>1</sup> because safe drinking water & sanitation are inadequate in this region<sup>2</sup>. Three countries Pakistan, India, Bangladesh-together account for about 85% of the world's typhoid cases.<sup>2</sup> Laboratory diagnosis of typhoid fever is a major challenge still now. Though several serological tests such as Widal, Typhidot, Tubex are available, blood culture remains the gold standard for diagnosis of typhoid fever. Blood culture may take 2-7 days & blood culture positivity wanes from 90% in the first week of fever to 25% after the third week.<sup>3</sup> Failure of blood culture may occur due to some factors like low bacterial load, prior antibiotic uptake.<sup>4</sup> Serological tests offer only indirect evidence of

infection. Their interpretation may be difficult owing to past exposure to infection, immunization with vaccines (Ty2 1a vaccine, Vi vaccine) or early antibiotic treatment.<sup>4</sup> Most of the physicians in Bangladesh have a tendency to prescribe antibiotics without micro biological confirmations. As a consequence, many strains of *S. Typhi* developed resistance to all the 3 primary antimicrobials-ampicillin, chloramphenicol, trimethoprim -sulfamethoxazole.<sup>2</sup> Quinolone resistance in *S. Typhi* has been recently described in various parts of Asia, possibly related to the widespread and indiscriminate use of quinolones.<sup>5</sup> So, accurate diagnosis of typhoid fever is needed not only for etiological diagnosis & clinical management but also for the prevention of development of multidrug resistant typhoid fever.

PCR has been emerged as a useful method for the diagnosis of many infectious diseases recently. The diagnostic value is significant specifically where there is a long cultivation period or low bacterial load.<sup>6</sup> PCR could be a reliable test in the diagnosis & management of typhoid fever. In case

of typhoid fever, nested PCR is able to detect as few as 10 bacilli in clinical specimens.<sup>7</sup> The purpose of the study was to optimize nested PCR in the laboratory and to assess whether the use of PCR could increase the detection rate of typhoid fever especially in case of culture negative cases due to prior antibiotic intake which is a common practice in Bangladesh.

## Materials and Methods

The study was carried out in the Department of Microbiology, Bangabandhu Sheikh Mujib Medical University, Dhaka, between March 2013 to February 2014. A total of 80 blood samples from patients clinically suspected of having typhoid fever were collected for both blood culture & PCR. The clinically suspected cases of typhoid fever were included based on continuous fever for more than 3 days, headache, anorexia, nausea, vomiting, abdominal discomfort with diarrhea, constipation, malaise, body ache. Patients with clinically suspected typhoid fever of both sexes, representing all age groups were included who came to Laboratory services of BSMMU for blood culture investigation, irrespective of their antibiotic treatment.

*Blood culture by conventional method:* Five ml of blood from each pediatric patient & 7 ml from each adult patient was drawn by vein puncture using disposable syringes with all aseptic precautions. The collected blood was ejected immediately into the conventional blood culture bottle, 3 ml in pediatric bottle and 5 ml in adult bottle. Two ml of blood was kept in a EDTA tube for PCR & stored at -20°C until DNA extraction. Inoculated blood culture bottle was incubated at 37°C aerobically. Blind subculture was done from the blood culture bottle after 24 hours, 48 hours, 72 hours of incubation on blood agar and McConkey agar media. The inoculated plates were incubated for 24 hours at 37°C aerobically. Culture negative bottles were reincubated for up to 7 days before discarded. Suspected colonies on subculture were identified as *S. Typhi* by colony morphology, Gram staining, biochemical tests.<sup>8</sup>

*Extraction of DNA from blood sample:* DNA extraction from blood was done by a method described earlier<sup>9</sup> with some modifications. From previously collected 2 ml blood in EDTA tube, one ml was taken in a sterile test tube and mixed

with five ml RBC lysis buffer ( Qiagen, Germany) and incubated for 15 minutes at room temperature. Then it was centrifuged at 4,000g for five min & supernatant was discarded. One ml 0.2% Triton-X was added to the pellet & transferred into 1.5 ml eppendorf tube, vortexed, and incubated for 10 minutes at room temperature, followed by centrifugation at 13,000g for 10 minutes. The supernatant was discarded; the pellet was mixed with 0.2% Triton X-100 again, vortexed & incubated for 10 minutes at room temperature, followed by centrifugation at 13,000g for 10 minutes.

The procedure was repeated until white pellet was formed. Then the pellet was washed with 1 ml of nuclease-free water. Finally, the pellet was resuspended in 30 µl of nuclease-free water, boiled for 10 minutes at 99°C, then cooled in ice for 5 minutes and again boiled for 3 minutes followed by centrifugation for 3 minutes at 13,000g. Supernatant was taken in 1.5 ml eppendorf tube. The suspension containing DNA was used as a template for PCR & stored at -20°C for use.

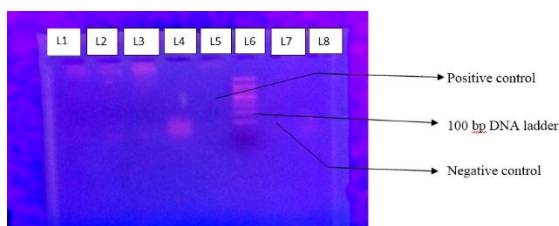
*DNA extraction from culture:* A single colony was suspended in 100 µl of double- distilled water in 0.2 ml PCR tube. Tube was placed in PCR machine, where incubated at 95°C for 10 min, and cooled to 25°C (Prefixed). The PCR tube was then taken out & centrifuged at 16,000g for 30 sec & clear supernatant was used as the template for PCR<sup>10</sup> which was diluted at 1: 200 in double-distilled water, and the diluted template was stored at -20°C which was used as a positive control later.

*Primers:* Primers which target the flagellin gene of *S Typhi* designed by Song et al were used for PCR. Primers synthesized on Jena Bioscience, Germany. Oligonucleotides ST1 (5'- ACT GCT AAA ACC ACT ACT-3') and ST2 (5'- TTAACGCAGTAAAGAGAG-3') were used for regular PCR to amplify a 458 bp fragment. For nested PCR, oligonucleotides ST3 (5'- ACT GCT AAA ACC ACT ACT -3') and ST4 (5'- TGGAGACTTCGGTCGCGTAG-3') were used to amplify a 343 bp fragment.

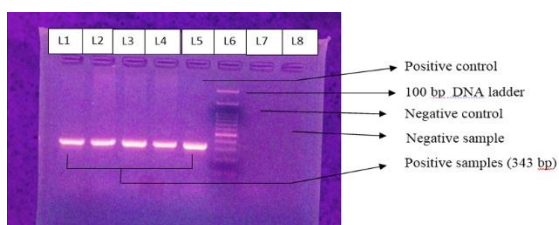
*Application in thermal cycler:* For regular PCR, 10 µL master buffer contained PCR buffer, MgCl<sub>2</sub>, deoxynucleoside triphosphate obtained

from Texas Biogene Inc. USA, 1.5 µl of each primer, 0.2 µl of Taq *polymease*, 2 µL of template were taken in a 0.2 ml PCR tube. Using a thermal cycler (Applied biosystem 2720), the reaction mixture was subjected to 40 cycles of 1 minute each at 94°C, 55°C and 72°C, followed by heating at 72°C for 10 minutes. For nested PCR, 1:5 dilution of amplified product of first round was used as template. The tube was kept in thermal cycler and allowed to run for 30 cycles as well as the same protocol of the first round PCR.

**Detection of PCR product:** Following nested PCR, 10µL of amplified product was fractionated electrophoretically in 2% agarose gel containing 0.5 µg of ethidium bromide per ml. A positive control representing diluted suspension containing DNA (1:200) from isolate of *S Typhi*, and a negative control without any DNA were also included in each lot. A 100bp DNA ladder was included to determine the size of the fragments.



**Fig. 1:** Amplification of 458 bp product of flagellin gene (*fliC-d*) of *S. Typhi* after first round of PCR (Lane 1,2,3,4,8: Samples from suspected typhoid fever cases; Lane 5: Positive control containing diluted suspension of *S.Typhi* DNA; Lane 6: 100 bp DNA ladder; Lane 7: Negative control).



**Fig. 2:** Amplification of 343 bp product of flagellin gene (*fliC-d*) of *S. Typhi* after second round of PCR (Nested PCR) (Lane 1,2,3,4 : Positive samples; Lane 5: Positive control containing diluted suspension of *S.Typhi* DNA; Lane 6: 100 bp DNA ladder; Lane 7: Negative control. Lane 8: Negative sample).

**Statistical analysis:** After collection of all data were checked, edited and analyzed by using computer based SPSS (Statistical Package of Social Science) software, p value < 0.05 was taken

as minimal level of significance. Diagnostic statistics, viz. sensitivity, specificity, positive & negative predictive values & likelihood ratios (LR+, LR-) were calculated taking blood culture as a gold standard.

## Result

From the 80 clinically suspected cases, 16 (20%) cases were blood culture positive for *Salmonella* species and the remaining 64 (80%) cases were negative for *Salmonella* & other organisms. Among 80 blood samples subjected to PCR from the suspected cases, 56 (70%) showed positive results for *S. Typhi*. Among the total 16 *Salmonella* isolates, 13 (81.3%) were *S. Typhi* & 3(18.7%) were *S. Paratyphi A*.

To calculate the diagnostic validity of PCR method for the diagnosis of typhoid fever taking blood culture as a gold standard, it was found that 13 (16.3%) cases which were blood culture positive for *S Typhi* were also PCR positive (Here, 3 *S Paratyphi A* cases were excluded from analysis). These 13 cases were considered as true positive cases. Among the culture negative cases, 43 (53.8%) cases were positive by PCR method. Therefore, these cases were regarded as false positive cases. The remaining 24(30%) culture negative cases were also negative by PCR method and these cases were considered as true negative cases. After calculation it was found that the sensitivity, specificity, positive & negative predictive values, positive & negative likelihood ratio of PCR were 100%, 35.8%, 23.2%, 100%, 1.6 & 0 respectively. (Table I).

**Table I:** Validity of PCR method for the diagnosis of typhoid fever (n=80)

PCR results	Blood culture results		Total
	Positive no (%)	Negative no (%)	
Positive	13(16.3)	43(53.8)	56(70)
Negative	0(0.0)	24(30)	24(30)
Total	13(16.3)	67(83.8)	80(100)

By observing the relationship between results of PCR & blood culture with duration of fever, it was found that among 36 cases who came with 3-7 days of fever, 27 (75%) cases were detected by PCR & 10 (27.8%) cases were detected by blood culture. Among 34 cases who came with 7-10 days of fever, 23 (67.6%) cases were detected by PCR whereas 3(8.8%) were blood culture positive

for *S. Typhi*. Out of 10 cases who came with fever more than 10 days, 6 (60%) were detected by PCR but none was positive for blood culture (Table-II).

**Table II:** Rate of *S. Typhi* detection by PCR & Blood culture method among the suspected typhoid fever cases in relation to duration of fever

Duration of fever (Days)	No. of cases	Detection by PCR	Detection by blood culture
		no. (%)	no. (%)
3-7	36	27 (75)	10 (27.8)
7-10	34	23(67.6)	3(8.8)
>10	10	6(60)	0(0.0)
Total	80	56(70)	13(16.3)*

\*Blood culture positive for *S. Typhi* were only included

Considering the relationship of antibiotic usage with the results of blood culture & PCR among the suspected cases it was found that none of the blood culture positive cases had taken antibiotic prior to sample collection. Majority of the culture negative cases gave history of taking different antibiotics for variable periods (Table-III).

**Table III:** Results of blood culture & PCR in relation to history of antibiotic intake.

History of antibiotic intake			No. of cases	No. (%) of Blood culture positive cases	No. (%) of PCR positive cases
Antibiotic	Dosage schedule	Duration of intake (Days)			
Ciprofloxacin	500 mg twice daily	3-6	12	0	11(91.7)
Levofloxacin	500 mg single	3-5	8	0	5(62.5)
Cefradine	250 mg Every 6 hourly	2-5	11	0	6(54.5)
Azithromycin	500 mg single	3-5	7	0	6(85.7)
Cotrimoxazole	1 double strength tablet twice daily	2-3	5	0	3(60)
Cefixime	200 mg single	2-4	7	0	4(57.1)

\*Fourteen cases who were culture negative had taken some kind of drugs but could not mention the names

## Discussion

The symptoms & signs of typhoid fever are nonspecific & may mimic the symptoms of other fevers including dengue, malaria, hepatitis in typhoid endemic regions<sup>11</sup>. Culture provide definite proof of typhoid fever. In present study, 16 out of 80 cases had positive blood culture for *Salmonella* species and an additional 64 cases were diagnosed as having typhoid fever based on clinical signs and symptoms. The reported positivity rate of blood culture is 14%-16.7% in

different studies done in Bangladesh.<sup>12-14</sup> The low culture-positivity rate of blood culture for *S. Typhi* in patients diagnosed clinically as suffering from typhoid fever in the community prompted us to look for an additional or alternative diagnostic tool. PCR is another test that has shown high sensitivity & specificity for diagnosis of typhoid fever in several studies. Several investigators have used different genes like flagellin gene,<sup>7</sup> *viaB* region<sup>15</sup> and no single method has yet been standardized for use in the clinical setting. In this study flagellin gene was used to detect *S. Typhi* by PCR, because the flagellin gene of *S. Typhi* has unique nucleotide sequences in hypervariable region VI which are different from those sequences in other strains of *Salmonella*.<sup>7</sup> Most of the studies used flagellin gene as a molecular technique for detection of *S. Typhi* in clinical specimens.<sup>7,16-19</sup> The alternative method where *ViaB* region is targeted can give false positive result due to the presence of this sequence in *S. Paratyphi C*.<sup>15</sup>

The sensitivity of PCR assay depends on target gene, primer sequence, type of DNA extraction procedures and type of PCR techniques<sup>20</sup>. Several different protocols were considered & that described by Nagarajan et al.<sup>9</sup> with some modification was selected. In this study, out of 80 suspected typhoid fever cases, flagellin gene (*fliC-d*) was detected by PCR in 56 (70%) cases which corresponds with the result of similar study done in Bangladesh where PCR was positive in 88.7% of suspected typhoid fever cases<sup>21</sup>. The reported positivity rate of PCR is 65%- 71.9% in different studies<sup>16,22</sup>.

Application of a nested PCR amplifying a segment of flagellin gene of *S. Typhi* in whole blood samples showed positive results in 67.2% (43/64) culture negative clinically diagnosed typhoid fever cases. The finding of positive PCR results for blood samples from culture-negative typhoid fever cases is consistent with the previous observations, which showed that PCR for typhoid fever might have higher sensitivities than blood culture<sup>16,18,19,23</sup>. PCR was found to be negative in 3 culture positive cases. These 3 cases were culture positive for *S. Paratyphi A*. This indicates that this PCR protocol is highly specific for *S. Typhi*.

In this study, PCR could diagnose 75% of the cases during the initial 7 days of illness. Only

27.8% of the blood cultures grew *S. Typhi* in this period. For patients presenting with fever for 7-10 days duration, PCR could detect 67.6% of the cases as compared to 8.8% positive blood culture cases. Even in the later stages of the diseases, 60% of cases were detected by PCR but no case was blood culture positive. Similar study done in India reported PCR could diagnose 76.2% cases during initial 1-5 days of illness, 61.9% during 6-10 days of illness and 53.8% during later stage of illness.<sup>23</sup> But they did not compare PCR results with blood culture results. This indicates that PCR can detect maximum number of typhoid fever cases in comparison to blood culture during any stage of disease. These values, though statistically insignificant, show that rate of *S. Typhi* detection by PCR decreases with increased duration of fever. The possible explanation might be intake of antibiotics in most of the cases. As a result the bacteria within blood might be dead due to the effect of antibiotics. The dead bacteria were subsequently cleared by the immune system which could not be detected by PCR.

Statistical analysis showed higher sensitivity & lower specificity. Despite these observations, it is possible to comment that PCR rarely miss the individual with typhoid fever. Again PCR can be used to exclude typhoid, as typhoid fever is one of the important causes of PUO (Pyrexia of unknown origin). From the positive & negative likelihood ratios it was found that this test had a diagnostic value.

**Conclusion:** According to the findings of this study it can be concluded that PCR assay can detect maximum number of typhoid fever cases mainly those who have history of prior antibiotic intake & those with problematic & varying clinical manifestations. Therefore, PCR assay is important for the reduction of mortality, morbidity & acquisition of the carrier state which helps in reducing transmission of typhoid fever.

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