**Early Diagnosis of Typhoid By PCR For FliC-d Gene of Salmonella Typhi in Patients Taking Antibiotics**

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**ABSTRACT**

**Objective:** To compare PCR (Polymerase Chain Reaction) with blood culture, typhi-dot and Widal test for the diagnosis of typhoid in patients taking antibiotics.

**Study Design:** Cross-sectional, comparative study.

**Place and Duration of Study:** National University of Sciences and Technology, Islamabad, Pakistan, from April 2013 to August 2014.

**Methodology:** One hundred and five patients were included in the study. Blood was collected and inoculated into tryptone soya broth for culture. Any growth obtained was identified by API 20 E and confirmed by *Salmonella* anti-sera. Typhi-dot and Widal test were also done on all the samples. DNA extraction was done and PCR was carried out.

**Results:** Among the 105 patients, 79 (75.2%) were males and 26 (24.8%) were females, with mean age of 20.64 ± 14 years. Typhi-dot was positive in 58 (55.2%) and negative in 47 (44.8%) patients. Blood widal test was positive in 27 (25.7%) and negative in 78 (74.3%) patients. *Salmonella* Typhi was positive on blood culture in only one (1%) patient. PCR for *Salmonella* Typhi was positive in 102 (97.1%) and negative in 3 (2.9%) patients. Positive cases detected by PCR were significantly higher as compared to Typhi-dot (p < 0.001), blood Widal test (p < 0.001) and blood culture (p < 0.001).

**Conclusion:** Positivity rate of PCR was significantly higher as compared to blood culture, Typhi-dot or Widal test for diagnosing typhoid in patients who were already taking antibiotics.

**Key Words:** Early diagnosis. PCR. Antibiotics. Serological tests. *Salmonella Typhi*.

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**INTRODUCTION**

Typhoid fever continues to be present globally causing > 27 million new cases every year and resulting in approximately 2,00,000 deaths.1,2 It is endemic in countries where there is non-availability of clean drinking water. Almost 80% of typhoid infections occur in developing countries in Asia,3 with thousands of cases occurring all round the year and affecting people of all ages. Ninety percent of these infections are caused by *Salmonella* Typhi.4 The symptoms of typhoid appear insidiously, are diverse and with non-specific signs, hence, cannot be diagnosed only clinically.5 Early detection of infection is required to prevent its spread, moreover, wrong diagnosis and delay in treatment may result in life threatening complications.3 Blood culture remains the gold standard for the diagnosis of typhoid, however, the procedure is laborious and requires several days for isolation and identification and is not being performed by many laboratories. Moreover, a negative blood culture is obtained even in established cases of typhoid if the patient has taken an antibiotic.6 Widal test is not rapid and typhi-dot has low sensitivity and specificity to be of any use for the diagnosis of typhoid.2,4 Over the past several years molecular methods have been developed to overcome the problems associated with diagnosing typhoid. The molecular methods give a reliable and early diagnosis of typhoid, with acceptable sensitivity and specificity when compared with culture positive typhoid cases and controls.2,7-9 The number of bacteria circulating in blood in patients of typhoid fever is small; some studies have claimed that as few as 10 bacteria per milliliter of blood could be detected by PCR and can be employed even in patients who have been given antibiotics.10

Because of the failure to obtain a positive blood culture in patients who have already taken an antibiotic, this study was carried out to compare the diagnosis of typhoid by detecting FliC-d gene of *Salmonella* Typhi by PCR with diagnosis by blood culture, typhi-dot and Widal test. If found sensitive the molecular method may be recommended for routine use for the diagnosis of typhoid and appropriate therapy can be started earlier.

**METHODOLOGY**

This cross-sectional study was conducted at Microbiology Department, Army Medical College Rawalpindi, National University of Science and Technology, Islamabad, from July 2013 to August 2014. One hundred and five patients with clinical diagnosis of typhoid fever were included in this study. Samples were collected by non-probability purposive sampling after taking informed written consent of patients or one of the parents in case of a minor.
of children. The approval of the Ethical Committee of the Institution was obtained before the study was started. Patients suffering from fever diagnosed as typhoid fever clinically, who were taking or had taken antibiotics, were inducted. Patients having fever with localizing signs and confirmed to be other than typhoid on clinical ground or results of laboratory investigations, and duplicate sample of the same patient, were excluded.

Eight milliliters of blood were collected aseptically from adults and 5 ml from paediatric patients. Two ml of the sample was added to a plain tube with anticoagulant for DNA extraction and 3 - 5 ml was inoculated into tryptone-soya broth with Ox bile for culture in a ratio of 1:10 and incubated at 37°C. The bottles were screened for growth by subculture on blood agar and MacConkey agar after 24 and 72 hours. Any growth obtained was identified by API 20 E and confirmed by Salmonella antisera. Typhi-dot test (CTK Biotech, Germany) and Widal test (Omega, UK) were also done on all the samples.

PCR was done on blood collected in EDTA from the patients. PCR standardization was done with pure cultures of known Salmonella Typhi isolate. DNA extraction from blood was done using Purelink Genomic DNA kit. One hundred and 80 µl of blood containing potassium EDTA as anticoagulant was taken in a microcentrifuge tube, 20 µl Protein kinase and 20 µl RNase were added to the tube, mixed gently by vortexing and incubated at room temperature for 2 minutes. Two hundred µl of Purelink Genomic lysis buffer (1% Triton-X 100 in Tris-HCL, pH 8.0) was added and mixed gently to completely lyse the RBCs. Two hundred µl ethanol was added to the lysate. Centrifugation was done at 10,000 x g for 1 minute at room temperature. Binding, washing and elution of DNA was done according to manufacturer's instructions to get purified DNA and was used as template to perform PCR.10

The primers used were targeted against Flit-c-d gene of Salmonella Typhi and obtained from Genbank with Nucleotide sequence accession number: L21912: H-for (ACTCAGGCTTCCGTAACGC) and Hd-rev (GGCTAGTATTGTCCTTATCGG). The PCR was carried out in ependorf tube with 2.5 µl PCR buffer, 1.5 µl MgCl2, 0.8 µL dNTP, 0.5 µl each of forward and reverse primers, 0.5 µl Taq polymerase and 20 µl of DNA template. The mixture was centrifuged for 3 minutes.

Following steps performed for amplification of DNA were initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 40 minutes and a final extension at 72°C for 5 minutes.

The amplified product was fractionated electrophoretically at 80 V for 40 minutes using 2% agarose gel containing 0.5 µgram of ethidium bromide. The amplified PCR product was loaded into the well of the electrophoresis well after mixing with bromothymol blue. A DNA ladder of 100 bp (base pairs) was also loaded into one of the wells to determine the size of the fragments. Electrophoresis was performed in an electrophoresis unit. Gel documentation system (Bio Rad, USA) was used to document the gels and was photographed by a UV trans-illuminator.

The statistical software SPSS version 20 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Quantitative variables like age was described as mean and standard deviation while qualitative variables like gender, fever, coated tongue, palpable liver and spleen was described through percentages and frequencies. Chi-square test was applied to compare the positive cases detected through PCR, blood culture, Widal test and typhi-dot. A p-value < 0.05 was considered as significant.

RESULTS

Among the 105 patients who were included in the study, 79 (75.2%) were males and 26 (24.8%) were females with mean age of 20.64 ± 14 years. All (100%) the patients were taking antibiotics, 23 patients (21.9%) orally and 82 (78.1%) parentally for the present illness. Ninety one (86.7%) patients were taking antibiotics for up to 5 days, 12 (11.4%) patients for up to 10 days and only 2 (1.9%) patients were taking antibiotics for more than 10 days. Liver and spleen were palpable in 16 (15.2%) and 4 (3.8%) patients respectively. Coated tongue was present in 35 (33.3%) patients only.

![Figure 1: Electropherogram of an ethidium bromide stained 2% agarose gel showing the amplified product of Flit-c-d gene of Salmonella Typhi consisting of 350 bp.](image1)

![Figure 2: Comparison of diagnosis of typhoid by PCR with other tests (n = 105).](image2)
Typhi-dot was positive in 58 (55.2%) and negative in 47 (44.8%) patients. Blood Widal was positive in 27 (25.7%) and negative in 78 (74.3%) patients.

PCR for FliC-d gene of Salmonella Typhi was done on all the samples. The amplified product detected at the end of the procedure consisted of 350 bp. FliC-d gene of Salmonella Typhi (Figure 1) and was positive in 102 (97.1%) and negative in 3 (2.9%) patients. Salmonella was not detected in 3 (2.9%) samples from symptomatic patients (Figure 2). Positive cases detected by PCR were significantly higher as compared to typhi-dot (p < 0.001), Widal test (p < 0.001) and blood culture test (p < 0.001).

**DISCUSSION**

According to WHO guidelines the mainstay of diagnosing typhoid is the blood culture and the definitive diagnosis is established only when a positive culture is obtained,11 but several days are required for the isolation and identification of the pathogenic organism. Moreover, performing blood culture requires expertise and many laboratories are inadequately equipped to undertake this method of diagnosis. The bacteria have to be present in sufficient numbers before they can be cultured. An early diagnosis and prompt treatment are mandatory not only for management of the patient but also for the prevention of the spread of the organism by chronic typhoid carriers to the community.12,13 The sensitivity of blood culture is reported to be 40 - 45% in the 1st week of illness whereas other studies have reported a sensitivity of 45 - 70% in regions where antibiotic usage is restricted by the Health Authorities.10 In the third world countries, and in Pakistan as well, where over-the-counter antibiotics are freely available, most of the patients have already taken antibiotics by the time they report to specialized healthcare facility, further lowering the number of viable bacteria and resulting in negative blood cultures in almost 100% of patients. In most of the studies the sensitivity of blood culture for patients on antibiotics is not mentioned as these patients are always excluded from the study, hence making blood culture method for diagnosing typhoid elusive in our set up. In this study, the sensitivity of blood culture was only 1% and the diagnosis could not be confirmed in 99% patients by blood culture alone, indicating that blood culture was not an adequate gold standard.

Ali et al. performed multiplex PCR for the rapid detection of major Salmonella serotypes including Salmonella Typhi and the results showed that all the reference and clinical isolates were identified accurately. No cross reactivity with other Enterobacterial strains was identified.7

Khan et al. carried out a study using nested PCR for the detection of Salmonella Typhi on culture negative cases of suspected typhoid fever. He also compared the results of PCR with blood culture and Widal test and found that PCR was 100% sensitive.10

Nga et al. carried out a study in Nepal in suspected cases of typhoid fever on blood and bone marrow samples from patients who had not taken any antimicrobials. In 100 culture-confirmed cases of typhoid, PCR could detect Salmonella Typhi gene in all the samples. Moreover, none of the 50 culture negative blood specimens and samples from 25 patients having some other cause of bacteremia yielded the target DNA, showing a specificity of 100%.11

In a study carried out by Pratap et al. nested PCR targeting sta A gene, a member of the fimbrial gene family specific to Salmonella Typhi detection sensitivity equal to FliC-d gene of Salmonella Typhi was documented.12

Commercial serological tests like Tubex and Typhi-dot are being used in different countries with a positivity rate of upto 70 - 79%.14,15 In this study, however, the positivity rate of Typhi-dot was much lower. Other serological tests (Enterocheck WB) which also detect IgM antibodies to Salmonella Typhi have been defined which can detect up to 85.5% suspected cases of typhoid.16

Widal test is a non-specific serological test and is not useful for the diagnosis of typhoid in areas where the residents already have high levels of anti-typhoid antibodies and hence diagnosis by single test using Widal test is not effective.10,17 Due to the problems encountered to make a definitive diagnosis of typhoid, a reliable method needs to be devised. PCR has been found to be a reliable method for diagnosing various infectious diseases. In cases of typhoid, it can be very useful when the organism load is low as well as for detecting non-viable pathogens when the patient is taking antibiotics.11,18 The PCR takes less than 8 hours to complete rather than several days which may be

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**Table I:** Age distribution of the patients.

<table>
<thead>
<tr>
<th>Age</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 10 years</td>
<td>44</td>
<td>41.9</td>
</tr>
<tr>
<td>10 - 20 years</td>
<td>10</td>
<td>9.5</td>
</tr>
<tr>
<td>20 - 30 years</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>≥ 30 year</td>
<td>9</td>
<td>8.6</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table II:** Comparison of results obtained by blood culture, widal test, typhi-dot and PCR for the diagnosis of typhoid (n=105).

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive (%)</th>
<th>Negative (%)</th>
<th>p-value (compared to PCR results)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>102 (97.1)</td>
<td>3 (2.9)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Typhi-dot</td>
<td>58 (55.2)</td>
<td>47 (44.8)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Widal test</td>
<td>27 (25.7)</td>
<td>78 (74.3)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Blood culture</td>
<td>1 (1)</td>
<td>104 (99)</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>

**Highly significant**
taken for conventional culture, with additional benefit of detecting DNA from dead organisms which are un-cultivable. PCR detects the genes from the dead organisms and this study determines the same as all the patients had been taking antibiotics. In this study, 97.1% of suspected cases of typhoid were detected by PCR. The results of this study also show that PCR could also reliably detect organisms viable or otherwise even after treatment for several days. Hence, in our set up where antibiotics are frequently being taken indiscriminately by patients, PCR can be a valuable procedure for the diagnosis of typhoid. Most of the studies document that PCR can be useful for culture negative patients, but it can be used for the diagnosis of typhoid right away without having to do blood culture.

Ox bile 2.4% added to the media is said to cause lysis of RBCs, hence releasing the organisms and improving the positivity of detection by culture as well as PCR. In this study, however, no difference was observed for detecting the organisms by PCR in the media used with and without Ox bile.

In this study, conventional PCR was used for detecting FliC-d gene present in Salmonella Typhi only. However, multiplex PCR assay can be used for the simultaneous detection of multiple genes of different Salmonella species in blood as well as in other specimens.

Molecular assays although very sensitive are expensive, the method is laborious and most of the laboratories in endemic areas are not capable of performing this test. However, given the extent of the problem and the difficulty in diagnosis, the authorities should further evaluate this procedure and develop an in-house method to incorporate it into routine microbiology laboratory. Interference due to the presence of human DNA in blood is another problem. This difficulty has been overcome by performing PCR on urine specimens which are easy to obtain in large amounts. PCR could detect more number of cases of suspected typhoid, however, as the majority of these samples had a negative blood culture so there was no standard test against which the PCR results could be interpreted.

Large scale hospital-based studies are required to compare the results obtained by PCR with the blood culture positivity rate ensuring, however, that patients have not taken any antibiotics at the time of collection of samples.

**CONCLUSION**

Positivity rate of PCR was significantly higher as compared to blood culture, Typhi-dot or Widal test for diagnosing typhoid in patients who were already taking antibiotics.

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**REFERENCES**


