EARLY DIAGNOSIS OF ENTERIC FEVER BY POLYMERASE CHAIN REACTION

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ABSTRACT
A Polymerase chain reaction (PCR) specific for Salmonella typhi was used for the detection of the pathogen in blood samples from 50 patients with clinical suspicion of typhoid fever. The sensitivity and specificity of blood culture and the PCR with blood was 75.47% and 33.33% respectively. The sensitivity of the PCR with blood (P < 0.001) showed positive results for 40 (80%) of 50 typhoid patients with 10(20%) negative results. These results showed that the PCR with blood is a sensitive method for the diagnosis of typhoid fever and PCR is an gold standard for the flagellin gene detection.

INTRODUCTION
Enteric fever is potentially life-threatening illness mainly caused by the bacteria Salmonella enterica subspecies enterica serotype Typhi(S. typhi)¹. Worldover 17 million people are affected annually by typhoid fever and 600,000 succumb to it. In India, the incidence of culture proven typhoid fever is 980 cases/100,000 population/year.² Typhoid fever continues to be undated in the developing countries of Africa, Asia and Latin America where proper sanitary facilities still remain a remote possibility.¹

Enteric Fever is a severe systemic, Gram negative bacterial infection caused by several serovars of Salmonella enterica subspecies enterica including S.Typhi and Paratyphi serotypes A (most common), B and C. It is characterized by high fever and a myriad of other non-specific features including abdominal pain, constipation, headache, myalgia, arthralgia, cough, lymphadenopathy and rash.³ This bacterium is faeco-orally transmitted,S. typhi and paratyphiA,B,C cause enteric fever in humans. Symptoms are non-specific and overlap with those of other febrile illness like malaria, dengue, rickettsiosis, leptospirosis and meliodosis.

Diagnosis of Enteric Fever can be detected by isolation of bacteria, detection of specific antibodies, antigens and nucleic acid. Isolation of the causative organism remains the most effective diagnostic method in suspected typhoid fever and blood has been the main sample for culture for Salmonella serovarTyphi Since 1900. The sensitivity of blood culture is highest in the first week of the illness and reduces with advancing illness. However, blood culture is time-consuming and takes at least 2 to 5 days until the identification of the organism. Blood culture is positive in the first week but its utility is restricted by the very low numbers of bacteria in blood sample and prior antibiotic therapy. As a consequence, blood culture can detect only 40%-45% of cases, and even if antibiotic treatment has not been administered, the rate of detection is not more than 70%⁴. Blood culture are universally practiced in diagnostic procedures, because other methods are either invasive, or have failed to prove their utility or they are expensive, but Molecular diagnosis techniques target the pathogen itself so they are useful in early detection of disease⁵. PCR is the gold standard method for the diagnosis of a number of infectious diseases .The sensitivity and specificity of different cut-off titers of Widal test have also been evaluated using PCR as Gold standard test⁶. In typhoid fever it is an effective tool because it can be used even in cases where antibiotic therapy has been started or pathogen load is very low⁷. Hence, an attempt was made to detect flagellin gene asearly diagnosis of Enteric Fever by Polymerase Chain Reaction.

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**Aims and Objectives**

Diagnosis of enteric fever by detection of flagellin gene in Blood sample by PCR as early as day one fever.

**MATERIALS AND METHODS**

This is a Hospital based prospective study carried out for a period one year. Clinically suspected patients of Enteric fever from pediatric department were included and Febrile patients diagnosed with other causes of fever and from other departments were excluded from the study. From clinically suspected patients 5ml blood (pediatric patients) was collected aseptically for blood culture.

Blood sample for culture was collected and dispensed with great care to avoid contamination of the specimen and culture medium. 70% ethanol was used and the area was cleansed about 50 mm in diameter then allowed to air-dry. Then, 2% tincture of iodine was used and disinfected in circular action and the area was swabbed beginning at the point where the needle enters the vein. Iodine was allowed to dry on the skin for at least 1 minute. Then, using a sterile needle nearly 2-3ml of blood from young children was collected. The collected blood was immediately transferred into blood culture bottle for automated identification through Bact alert.

Once the presence of bacteria was identified, 0.1-0.2μl of the blood was subcultured onto the culture media (Blood agar and MacConkey agar) for bacterial growth and isolation. The respective plates were incubated overnight at 37ºC in aerobic environment and was observed for the growth of Salmonella spp after incubation.

**On Blood agar** Colonies were seen about 2-3 mm in diameter, greyish white with smooth convex surface & entire edge. No pigmentation was observed.

**MacConkey agar** : non- lactose fermenting. Colourless, smooth, shiny & translucent colonies were grown. The growth was then subjected for the Species level identification using biochemical reactions such as Indole, Citarate utilization test, Urease test and Triple sugar iron agar test for confirmation of the organism. Identification was carried out by Vetek 2 Compact System as per the CLSI guidelines.

DNA extraction was carried out using DNA extraction kit (Helini Biomolecules Pvt. Ltd- Cat no.2003) as per the manufacturer’s protocol. Detection of Flagellin gene in Salmonella Typhi was detected using HELINI Salmonella typhi PCR kit – Flagellin gene [Cat no. 8213] as per the manufacturer protocol.

**The final 25μl of the PCR mixture contains**

- **Positive control tubes**: 10μl of Red master mix, 5μl of primer and 10μl of positive control.
- **Negative control tubes**: 10μl of Red master mix, 5μl of primer and 10μl of double distilled water.
- **Endogenous control tube**: 10μl of Red master mix, 5μl of Endogenous primer and 10μl of Extracted DNA.
- **Sample tubes**: 10μl of Red master mix, 5μl of primer and 10μl of Extracted DNA.

The test always performed along with positive, negative and endogenous controls for standardizing the results. PCR vials were centrifuged briefly before placing into thermal cycle, to bring down drops to bottom of the tube. Conventional PCR was used to detect 300bp length of Flagellin gene which includes 36 cycles under the following conditions: Initial denaturation 96ºC for 5min, Denaturation 95ºC for 30 sec, Annealing 58ºC for 30 sec, Extension 72ºC for 30 sec and Final extension 72ºC for 5min.

The PCR products were visualized by gel electrophoresis by preparing 2% agarose gel and loaded entire PCR product along with 300bp DNA ladder stained with ethidium bromide and the bands were compared with the positive control band and Endogenous control band.

**RESULT AND INTERPRETATION**

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Endogenous Control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative typhi</td>
<td>Positive Salmonella Typhi (flagellin gene) 300bp</td>
<td>Positive</td>
<td>S.typhi DNA detected</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>S.typhi DNA not detected or beyond detection sensitivity</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Experiment fail</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Experiment fail</td>
</tr>
</tbody>
</table>

*Experiment fail* indicates no band detected.

Statistical analysis was done using Sensitivity, specificity, Positive predictive value, Negative predictive value, Likelihood ratio for positive result and Likelihood ratio for negative result. The difference, association and correlation are expressed statistically significant at p-value less than 0.005.

**RESULT**

In the present study, a total of 50 Pediatrics whole blood samples were collected from clinically suspected cases of Enteric fever. The collected samples were processed for blood culture as per standard protocol. All samples were further subjected for Genotypic Detection of Flagellin gene by conventional PCR method.

Out of 50 samples, 33 (66%) samples were collected from male patients and 17 (34%) samples were from female patients. Majority of the samples were from the age group 1-5years and 6-10years with 19(38%) each and 12(24%) were from 11-14years. Maximum number of samples were collected from Pediatric ward with 49(98%), followed by PICU with 01(2%) respectively with P value = 0.000 (very Highly significant).

All the 50 blood samples collected, were subjected to Blood culture. In which, 31 samples yielded Salmonella spp. growth. Among them 31(62%) positive isolates, 26 (52%) were Salmonella Typhi and 5(10%) were Salmonella Paratyphi A. None of the samples showed positive for Salmonella Paratyphi B and rest 19 samples showed negative result for Blood culture with P value = 0.003 (very Highly significant)

Polymerase chain reaction was carried out for all 50 samples collected for the detection of flagellin gene. In which 40(80%) were positive and 10(20%) were negative by gene detection. Nine samples showed negative result for blood culture, but which were positive by PCR detection.
### DISCUSSION

Enteric fever is a major systemic health problem in developing countries, including India. As the clinical picture of typhoid fever is often unspecific, misdiagnosis and insufficient or inadequate treatment are potential risks associated from blood during the first week of illness but can also be isolated during the second or third week of illness, during the first week of antimicrobial therapy and during clinical relapse. Since it is difficult to obtain bone marrow specimens, microbiologic culture of a blood sample is considered to be the current state for the diagnosis of typhoid fever even though its sensitivity may be as low as 40%.

Culture may take up to seven days and requires a well equipped laboratory, which is often not available in areas with endemic typhoid fever. As a result, diagnosis may be delayed or overlooked and patients without typhoid fever may receive unnecessary and inappropriate antimicrobial treatment.

In the present study out of 50 samples, 33 (66%) samples were collected from male patients and 17 (34%) samples were collected from female patients. By A R Nateghian et al., showed that 21(46.7%) were male patients and 24(53.3%) female patients which was comparably high rate when compared to our study. Rudreshet al. conducted a study in 50 samples, out of which 32(64%) were isolated from males patients and 18(36%) from females patients. The male:female ratio was approximately similar to our study.

In our study, total 50 patients samples were collected, among which 40(80%) were positive for PCR and 10(20%) were negative. Similar to our study, Zainabkhudhuret al., conducted a study in 50 patients, among which 33(66%) were positive and 17(34%) were negative which was lower compared to our study.

Another study by Zainabkhudhuret al., conducted a study in 50 cases, in which blood culture 26(52%) were positive and 24(48%) were negative which was lower compared to our study.

In our study, 50 samples were collected, in which blood culture 31(62%) were positive and 19(38%) were negative. Whereas, 40(80%) were positive and 10(20%) were negative by conventional PCR method respectively. Nine samples showed negative result for blood culture, which was positive by PCR detection for flagellin gene. Similar to our study, S Khan et al. conducted a study in 80 patients, 40(50%) samples which had grown S. Typhi, and 52(65%) were positive for PCR.

Typhoid fever continues to be a major public health problem worldwide. In the areas of endemi city, the standard blood culture method cannot be relied upon for diagnosis due of expertise laboratories. There is a need for early diagnostic method for the better management of the disease in the areas of endemi city. In our study, we got nine samples which was negative for blood culture was positive for Polymerase chain reaction for flagellin gene. Hence, PCR is a gold standard method and has greatest diagnostic value for the detection of S. typhi among all the diagnostic tests used. So, it is beneficial to use molecular methods in early stages for diagnosis of Enteric Fever.

### References

7. Alireza Nateghian Soudabeh Hoseini, Mohsen Sadeghi Zahraa, Behnamfar: Evaluation of a PCR technique in a blood samples of suspicious cases to systemic

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Table showing Sensitivity, Specificity, Positive predictive value, Negative predictive value of Flagellin gene with Blood culture:

<table>
<thead>
<tr>
<th>Blood culture</th>
<th>Flagellin gene Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (N=50)</td>
<td>40(80%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>75.47%</td>
</tr>
<tr>
<td>Specificity</td>
<td>33.33%</td>
</tr>
<tr>
<td>Positive predictive value (PPV)</td>
<td>80.00%</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td>27.78%</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>1.13</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.74</td>
</tr>
<tr>
<td>Disease prevalence</td>
<td>77.94%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>66.18%</td>
</tr>
</tbody>
</table>

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