EARLY DETECTION OF TYPHOID BY POLYMERASE CHAIN REACTION

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Background: Typhoid is a common problem in developing countries. Cultivation of bacteria and serology (especially Widal test) give unacceptable levels of false-negative and false-positive results, respectively.
Patients and Methods: In this study, a recently introduced polymerase chain reaction-based technique (which has 100% specificity for *Salmonella typhi*) was compared with blood culture and Widal test during the first week of illness of 82 suspected cases of typhoid.
Results: The respective figures of positivity for PCR, blood culture and Widal test were 71.95%, 34.1%, and 36.5%. A control group of 20 healthy persons gave figures of 0%, 0%, and 33.3%, respectively.
Conclusion: We conclude that this PCR-based technique is not only absolutely specific, but also very sensitive and, therefore, much superior to blood culture and Widal test for the early diagnosis of typhoid.
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Typhoid is a major health problem throughout the world. Globally, there are more than 21 million cases of typhoid each year, with more than 700,000 deaths.[1] It is especially prevalent in developing countries such as Pakistan. Early detection of the disease is very important for its control, but unfortunately, definitive diagnostic procedures are not available.

Different techniques are used for the diagnosis of typhoid, including blood culture, bone marrow culture, rectal swab culture, urine culture, rose-spot culture,[2-4] duodenal string culture,[5,6] Widal test,[7,8] ELISA,[9] and immunofluorescence.[10] Widal test and blood culture remain the only universally practiced diagnostic procedures, because other methods are either invasive, have failed to prove their utility, or are expensive.

Widal test is quite sensitive but has become highly nonspecific. In a study in Peru,[7] O and H antibodies were found to be raised in 30% and 75%, respectively, of the normal population. In another study in Pakistan,[11] Widal test was positive in 28% of the general population. Another shortcoming of the Widal test is that it becomes positive only in the second week of illness, so its value for early detection of the disease is limited.

Blood culture is positive in the first week but its utility is restricted by the very low numbers of bacteria causing severe disease (which may be less than 10/mL). 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%.[12] Molecular techniques, as is the case in blood culture, target the pathogen itself (not antibodies produced against it), so they can be useful for the early detection of the disease. Hybridization by using DNA probes was the first molecular biology technique used for the diagnosis of typhoid fever. It is specific but its sensitivity is poor.[13,14] The advent of PCR technology has provided unparalleled sensitivity and specificity for the diagnosis of typhoid.[15,16]

We thought that PCR might be an excellent tool for the early diagnosis of typhoid. Clinically suspected cases of typhoid who had had fever for less than three days and had not received any anti-typhoid therapy were included in this study. PCR (by targeting the flagellin gene), blood culture and Widal test were performed on samples from these cases to evaluate their relative utility, specificity and sensitivity.

Materials and Methods

Criteria for Selection of Patients

Patients included in this study had fever for less than 3 days, had had no anti-typhoid treatment, or any condition suggestive of typhoid (soft enlarged spleen, headache, and abdominal discomfort with diarrhea). Members of both sexes representing all ages were included. The control group constituted 25 healthy individuals with no recent history of fever.

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Bacterial Strains and Cultivation

Two pure strains of *Salmonella typhi* (STK-1 and STK-2) provided by the Department of Microbiology, University of Karachi, were used for the standardization of PCR conditions and as reference strains. Two mL of blood were inoculated into a culture bottle containing 16 mL of trypticase soy broth (Difco) with 0.02% SPS (sodium polyanethanol sulfonate), and incubated at 37ºC for 72 hours. From the broth culture, biochemical identification was done by conventional procedures,[17] and confirmation was carried out by PCR. Widal test was performed by tube method according to the manufacturer's instructions, using reagents provided by Biosystems (Barcelona, Spain).

Extraction of DNA from Bacterial Cultures

Brain heart infusion broth was inoculated with stock culture, and incubation was done at 37ºC for 24 hours. After centrifugation of 3 mL of culture at 5000 rpm for one minute, the supernatant was removed and the pellet was resuspended in 360 µL of TE buffer (pH 8.0) containing 0.1% proteinase K, and kept at 37ºC for 30 minutes. It was followed by the addition of 10% SDS and incubation at 65ºC for one hour. DNA was extracted by conventional phenol/chloroform method, followed by RNase treatment for the removal of contaminating RNA. Its purity was checked by measurement of A_{260} and A_{280} and agarose gel electrophoresis.

Extraction of DNA from Blood Samples

One mL of blood containing 20 mM potassium EDTA as anticoagulant was centrifuged at 10,000 rpm for 5 minutes. Plasma was separated for serology. One mL of lysis buffer (0.2% Triton X100 in Tris HCl pH 8.0) was added to the pellet. The mixture was gently aspirated several times to effect hemolysis. The tube was centrifuged at 12,000 rpm for 6 minutes, the supernatant was discarded, and the procedure was repeated once. The pellet was washed once with distilled water. After the removal of the supernatant, the pellet was resuspended in 20-30 µL of distilled water. The tubes were sealed, kept in boiling water for 20 minutes, and brought back to room temperature before being used as a template for PCR.

Contamination Precautions

Cultures, DNA extraction and PCRs were performed in separate rooms, using different sets of pipettes and tips. For further safety, all the reagents used in the PCR were aliquoted, so that an aliquot was used only once and then discarded.

Primers

First reported by Song et al.[16] and modified by Frankel,[18] primers which target the flagellin gene of *Salmonella typhi* were synthesized on Pharmacia LKB, Gene Assembler Special. Oligonucleotides ST1 (5'-TATGCCGCTACATATGATGAG-3') and ST2 (5'-TTAACGCAGTAAAGAGAG-3'), which were used for regular PCR to amplify a 495 bp fragment, corresponded to nucleotides 1036-1056 and 1513-1530, respectively, in the flagellin gene of *Salmonella typhi*. For nested PCR, oligonucleotides ST3 (5'-ACTGCTAAAACCACTACT-3') and ST4 (5'-TGGAGACTTCGGTCGCGTAG-3') were used to amplify a 363 bp fragment corresponding to nucleotide 1072-1089 and 1416-1435, respectively, of the same gene.

PCR Conditions

For regular PCR, 100 µL DNA amplification mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 150 pmol of each primer, 95 nmol of each dNTP, 1 µL of *Ampli Taq*, 20 µL of template, and distilled water to make up the volume. Using a thermal cycler (Perkin-Elmer Gene Amp PCR system 2400), the reaction mixture was subjected to 30 cycles of 1 minute each at 94ºC, 63ºC and 72ºC, followed by heating at 72ºC for 7 minutes. For nested PCR, conditions were the same, except that a 1:5 dilution of amplified product was used as template.

Detection of PCR Products

A reaction mixture of 10 µL was fractionated electrophoretically in 2.5% agarose gel containing 0.5 µg of ethidium bromide per mL, and was photographed by using Eagle Eye (Strategene). PCR was performed in duplicate for each sample. A positive control representing 5 bacteria/mL of *Salmonella typhi,* and a negative control without any DNA were also included in each lot.

Results

Determination of Sensitivity of PCR

DNA was extracted from the culture of the reference strain STK-1 and STK-2 by phenol/chloroform method, and serial dilutions were made in distilled water. Each dilution was used as template for PCR. The calculations were made according to the recommendations of Song et al.[16] The results showed that regular PCR had a sensitivity of 10^5 bacteria/mL, whereas nested PCR could even detect 5 bacteria/mL.

Comparison of PCR, Blood Culture and Widal Test in Healthy Persons

This group had had no history of fever for the last six months. It was used as a negative control. PCR and blood culture were negative in all 25 cases. Widal test (with a titer of more than 1:160 for *Salmonella typhi* O antigen) was positive in as many as 11 (44%) persons. The results are given in Table 1.

Comparison of PCR, Blood Culture and Widal Test in Suspected Cases of Typhoid

The patients included in this group represented both

TABLE 1. *Comparative study of PCR, blood culture and Widal test in healthy individuals.*

Cases	PCR	Blood culture	Widal
11	–	–	+
14	–	–	–
25	0	0	9 (44%)

Positive Widal test is a titer of 1:160 or above for *Salmonella typhi* O antibody.

TABLE 2. *Comparative study of PCR, blood culture and Widal test for early diagnosis of typhoid.*

Cases	PCR	Blood culture	Widal
12	+	+	+
15	+	+	–
13	+	–	+
19	+	–	–
01	–	+	+
04	–	–	+
18	–	–	–
82	59 (71.9%)	28 (34.1)	30 (36.5%)

Positive Widal test is a titer of 1:160 or above for *Salmonella typhi* O antibody.

TABLE 3. *Comparison of PCR, blood culture and Widal test for different groups of study.*

	PCR (%)	Blood culture (%)	Widal (%)
Control group I (healthy persons)	0	0	44.0
Typhoid patients	71.9	34.1	36.5

sexes and all ages. They had suspected typhoid fever of less than three days' duration and had not taken any anti-typhoid drug. The results are given in Table 2. Out of 82 cases, 12 (14.6%) were positive for all three parameters; 15 (18.2%) were positive for PCR and blood culture only; and 13 (15.8%) showed positivity for PCR and Widal test but not blood culture. Only PCR was positive in 19 cases (23.2%), whereas only Widal test was positive in four patients (4.9%). One (1.2%) patient was negative for PCR but positive for both blood culture and Widal test. There were 18 (21.9%) cases which were negative for all three parameters.

Discussion

Typhoid fever is one of the most common infectious diseases in developing countries. Early and definitive diagnosis of the disease is not only important in relieving patients' suffering, but also critical in avoiding fatal complications such as perforation of the intestines. It also makes possible specific treatment at an early stage, which leads to the rapid elimination of the pathogen. Otherwise, the patient's excreta, especially stool, becomes a constant source of spread of the disease.

Although various diagnostic techniques have been used, Widal test and blood culture are the most favored methods. Widal test is not a candidate for early detection of the disease because specific antibodies take at least one week to reach detectable levels. Beside these shortcomings, its value is further diminished by its nonspecificity. It is particularly unreliable with single titers.[8]

Blood culture has the promise of diagnosis in the first week and is very specific, but its sensitivity is poor due to various factors. The most important factor is the very few numbers of bacteria needed to cause severe infection, which can be as low as 10/mL.[19,20] Hence, positive culture yields are very low and elude definitive diagnosis. Other limiting factors, beside the bacteriostatic effect of antibiotics (already administered before the culture sample is taken), may be the nature of culture medium employed, the time of blood collection, the host's immune response system, and the intracellular characteristics of *Salmonella typhi*.[6,21]

Although detection of *Salmonella typhi* by using labeled probes is 99% specific,[14] its sensitivity is poor because it cannot detect less than 500 bacteria/mL.[13] The recent introduction of PCR techniques[15,16] offers highly specific, sensitive and reasonably quick diagnostic modality. Even 1-5 bacteria/mL can be detected with absolute specificity within 1-2 days.

We decided to check this theoretical promise of PCR in the actual situation and compare it with Widal test and blood culture. Widal test was performed as described above. Although different liquid culture media have been used for liberal growth of *Salmonella typhi*, TSB with SPS remains the best among non-commercial media for routine blood culture.[22] For polymerase chain reaction, we preferred to target the flagellin gene because its hypervariable region VI is unique for *Salmonella typhi*, and its amplification provides 100% specificity.[16,18] The alternative method in which the Vi gene is targeted can give false-positive results because of the presence of *Salmonella paratyphi C*.[15] The sensitivity of the latter has been reported to be more (5 bacteria/mL) than the former (10 bacteria/mL). The sensitivity of our regular PCR was tenfold more than that reported by Song et al.,[16] and we were able to improve the sensitivity of nested PCR to less than 5 bacteria/mL. The results are given in Tables 1 and 2. Widal test results were insignificantly different from the healthy group (36.5% and 44%, respectively).

We conclude that PCR is much superior to both blood culture and Widal test. It has great discriminating value due to its very high sensitivity and specificity. Therefore, it can be of singular importance for the detection of early cases of typhoid, which is not only important for the treatment of patients but is also necessary for control of the disease. Due to the need for extensive infrastructure and specialized skills, the PCR facility cannot be made available everywhere, especially in developing countries. Nevertheless, due to its high sensitivity and specificity, as demonstrated in this study, it needs to be made available to everyone by establishing collection centers which can send samples to a few specialized reference laboratories built in larger cities. Another factor to be considered is the relatively high cost, which is almost twice that of blood culture. In fact, due to the rapid and definitive diagnosis, the patient is saved from hospital admission, loss of working days, unnecessary expenditure on unrelated and misdirected treatment which may be many times more than the cost of PCR. It is believed that in the long run, it will turn out to be less expensive for both the patient and the community. Furthermore, due to rapid diagnosis and definitive treatment, the patient's physical suffering is reduced to a minimum.

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