

Application of nested Polymerase Chain Reaction for *Salmonella enterica* var. *typhi* in the diagnosis of Typhoid fever D1-4

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Abstrak

Demam enterik yang disebabkan oleh *Salmonella typhi* adalah masalah besar kesehatan masyarakat. Dibutuhkan segera pengembangan teknik molekuler untuk diagnosis cepat dan sensitif. Suatu teknik nested PCR telah dikembangkan untuk mendeteksi DNA *S. typhi* dalam specimen darah dari penderita demam tifoid melalui amplifikasi gen flagelin dH. Primer dirancang dari sekuens gen internal dari primer RK1 dan RK2 yang telah kami laporkan sebelumnya untuk mengamplifikasi fragmen 342 bp dari gen flagelin *S. typhi*. Nested PCR ini dapat mendeteksi 40 organisme *S. typhi*, yang telah dipastikan dengan pengenceran seri DNA. DNA *S. typhi* telah terdeteksi pada 11 dari 28 penderita tersangka demam enterik dengan biakan negatif; 4 terdeteksi dengan PCR tahap pertama, dan 7 dengan nested PCR. Nested PCR mempunyai harapan untuk dapat digunakan sebagai teknik diagnosis untuk kasus tersangka demam enterik dengan biakan negatif dan/atau pernah mendapat terapi antibiotika sebelumnya.

Abstract

Enteric fever caused by *Salmonella typhi* is a major public health problem. There is an immediate need for the development of molecular techniques for rapid sensitive diagnosis. A nested PCR was developed to detect *S. typhi* DNA in the blood specimens from patients with typhoid fever by amplification of the dH flagellin gene. Primers were designed from gene sequence internal to RK1 & RK2 primers reported previously by us to amplify a 342bp fragment of flagellin gene of *S. typhi*. Amplified products were analyzed by gel electrophoresis. The PCR was found to be specific for dH flagellin gene of *S. typhi*. The nested PCR could detect 40 organisms of *S. typhi* as determined by serial dilution of DNA. *S. typhi* DNA was detected from blood specimens of 11 patients out of 28 tested with suspected enteric fever who were culture negative; 4 by first round of PCR and 7 by nested PCR. Nested PCR holds promise to be used as a diagnostic technique for suspected enteric cases which are culture negative and/have received prior antibiotic therapy.

INTRODUCTION

Typhoid fever is still a major health problem in many countries of the world. The World Health Organization estimates approximately an incidence of 540 new cases/100,000 inhabitants in developing countries¹. Blood culture and the Widal test are the two procedures widely used for the diagnosis of typhoid fever. Blood culture can detect only 45-70% of patients with typhoid fever. Prior administration of antibiotics and a low number organisms could be the reasons for poor isolation rates^{2,4}. The last two decades witnessed controversies over the diagnostic utility of Widal. The uncertainty could be due to several factors like endemicity, antigenic cross reactivity with other agents, previous vaccination, non-availability of paired sera for the documentation of rising titres, poorly standardized reagents, diverse methods and criteria of interpretation⁵⁻⁶.

Typhoid fever is life threatening, typically requires treatment with a potentially toxic antibiotic and can be easily misdiagnosed as malaria in regions where these diseases are coendemic. For these reasons typhoid fever is in greatest need of specific, rapid and sensitive diagnosis.

Previously, a PCR assay which could detect *Salmonella typhi* DNA by amplification of the flagellin gene *S. typhi* in the blood of the typhoid patients has been reported⁷. We now report the development and evaluation of a nested PCR assay to detect *S. typhi* from the peripheral blood of patients who were both culture and first round PCR negative.

MATERIALS AND METHODS

Standardization of PCR

Primers RK1 & RK2 were designed to amplify a 486bp fragment of the flagellin gene of *S. typhi*. Standardization of the PCR assay was done as described previously⁷.

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Standardization of Nested PCR

From the sequence of the flagellin gene of *S. typhi* a pair of oligonucleotide primer which were nested to the RK1 & RK2 primers were synthesized. Oligonucleotides RK3 and RK4 which were used in the nested PCR to amplify a 342 kb fragment, correspond to nucleotides 1098 to 1121 and 1419 to 1440 respectively. To verify that primers used in this study were specific for *S. typhi*, the nested PCR was carried out with blood samples of healthy controls spiked with standard strain of *S. typhi* S901 (motile) and non Salmonella organisms. To investigate the sensitivity of the system, overnight culture of S901 was titrated by counting colonies on nutrient agar plates after 10 fold serial dilution of organisms ranging from 10^6 to 10^1 , to determine the minimal number of organisms detectable by nested PCR.

Blood specimens

Blood samples were obtained from 49 patients with clinically suspected typhoid fever attending our institution from January 94 to December 96. Blood from 22 normal healthy individuals was obtained to be used as negative controls. Blood specimens were also collected from 9 patients with other diseases which could mimic typhoid fever in terms of clinical infections.

Preparation of DNA from bacteria and blood specimens for PCR

Chromosomal DNA from Salmonella strains and other organisms was extracted by boiling method⁸. The DNA was isolated from 300µl of blood QIAamp blood isolation kit (Qiagen Inc, Chastworth, USA) or by Puregene isolation kit (Gentra systems, Minneapolis, USA) according to manufacturer's instructions.

PCR

The reaction mixture for the first round PCR contained 5µl of extracted DNA, 50 pmoles of each of the four deoxyribonucleotide triphosphate, 0.625 units of Taq DNA polymerase (Perkin Elmer, Rotkeuz, Switzerland), 2.5 mM and 1 x PCR buffer in a final volume of 25µl. Amplification in an automated DNA thermal cycler (MJ research Inc, Massachusetts, USA) consisted of 40 cycles at 94°C for 1 min (denaturation), 57°C for 1 min and 15 sec (annealing) and 72°C for 3 min (extension) and final extension of 6 min at 72°C. Amplified products (7.5µl) of the first PCR was transferred to a second reaction mixture (17.5µl) containing 50 pmoles (each) of RK3 and

RK4 for the nested PCR. The nested PCR was performed for 40 cycles at 94°C for 1 min, 68°C for 1 min and 15 sec and 72°C for 3 min.

Detection of PCR products

The DNA fragments of the flagellin gene of *S. typhi* amplified by the PCR were identified by agarose gel electrophoresis. Eight microlitres of the amplified products from both rounds of the PCR were electrophoresed on a 1.5% agarose gel for 60 min at a constant 100 V with TBE buffer (90 mM Tris-borate, 2 mM EDTA). Molecular size markers (Lambda Hind III, PhiX Hae III Bangalore Genei, Bangalore, India) were run concurrently along with standard strain S901, PCR products and negative control. The gels stained with ethidium bromide were examined under UV illuminator for the presence of 486bp or 342bp band.

RESULTS

Specificity of the PCR

PCR with RK1 & RK2 primers was found to be specific for dH flagellin gene amplifying the *S. typhi* specific band of 486bp as reported previously⁷.

With the nested PCR amplification products of 342bp were detected from blood samples spiked with *S. typhi* standard strain but not from those spiked with other organisms.

Sensitivity of the PCR

The minimum number of organisms detected with the single round of PCR was 3×10^2 . With the second round of PCR, even minimum of 40 organisms produced a visible amplification product of 342bp on the gel.

PCR with DNA from blood specimens of patients

Of 49 patients with clinically suspected typhoid fever, 21 samples were both culture and first round PCR positive for *S. typhi*. Another four samples were

Table 1. Results of nested PCR assay in culture and first PCR negative cases

	Blood Culture		Total
	Positive	Negative	
Nested PCR Positive	0	07	07
Nested PCR Negative	0	17	17

positive by PCR, but culture negative. No amplification was seen in patients that were culture positive for organisms other than *S. typhi* or in healthy controls. After the nested PCR, however, amplification products of 342bp were seen in an additional 7 cases of suspected typhoid fever (Figure 1) which were both culture and first round PCR negative (Table I). No amplification was seen in the control group and in patients with septicaemia with non *Salmonella* organisms.

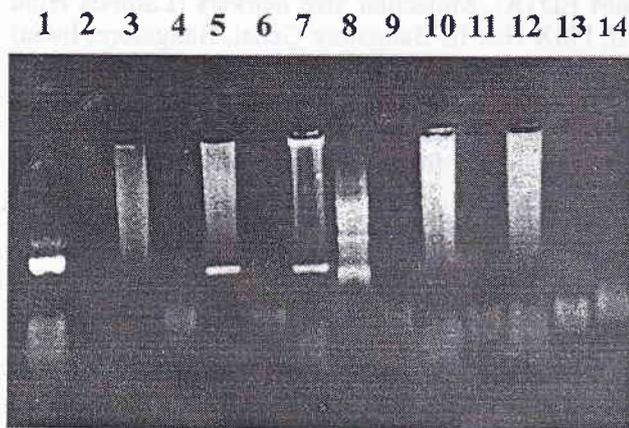


Figure 1. Amplification of *S. typhi* DNA from blood of patients with suspected typhoid fever.

Lane 1: positive control (*S.typhi* S901. Amplification product of single round PCR); Lanes 2,3 & 4: first round of PCR of 3 patients with suspected typhoid fever (no amplified product seen); Lanes 5,6,7 & 9: second round of PCR of positive control, and 3 patients with suspected typhoid fever (amplified product of 342bp seen in lane 7); Lane 8: λ Hae III digest marker; Lanes 10-13: DNA from blood of patients with non *Salmonella* bacteraemia; Lane 14: negative control (PCR mix).

DISCUSSION

The flagellin antigen of *S. typhi* (HI-d) is coded by a 1,530 bp DNA sequence^{9,10}. The 'd' antigen is also present in many *Salmonella* species other than *S. typhi*¹¹. The flagellin gene of *S. typhi* has unique nucleotide sequences in the hypervariable region of the gene⁹. The nucleotide sequence and predicted amino acid sequences of region VI (corresponding to nucleotide 969 to 1077) of the HI-d flagellin gene of *S. typhi* are different from those of *Salmonella muenchen*, which has also HI-d gene and nucleotide sequences highly homologous with *S. typhi*^{9,10}. These findings suggested that the PCR test, based on the unique sequence in flagellin gene of *S. typhi*, could be used to detect *S. typhi* specifically in the clinical specimens.

Our strategy was to develop the PCR technique with which amplified fragments of flagellin gene of *S. typhi* in the blood could be directly detected on the gel without the use of southern blot hybridization. For the practical use of PCR as a diagnostic test, the gel electrophoresis should be sufficient to detect amplification products without the aid of hybridization methods which take at least 2 days with the use of radiolabelled probes.

Isolation of *S. typhi* from blood and serological test viz. Widal are common methods of diagnosis of typhoid fever. Negative blood culture results because of low concentration of bacteria or previous antibiotic treatment in suspected cases of typhoid fever may lead to misdiagnosis and improper treatment and failure to form antibodies during the early stage of disease may lead to a negative Widal test.

PCR a highly sensitive method to detect very low quantities of infectious organisms shows promise for typhoid fever diagnosis. As shown in the sensitivity test for *S. typhi* DNA, the single round reaction is insufficient to detect a small number of organisms in clinical specimens. With the nested PCR it was possible to detect as few as 40 organisms of *S. typhi* compared with 3×10^2 bacteria by single round PCR. These results suggest that nested PCR is necessary to detect small number of organisms in actual blood samples. The specificity of PCR in clinical practice was confirmed by the results of PCR performed on DNA from blood samples of patients with other acute febrile diseases and healthy patients, which were consistently negative for *S. typhi* DNA.

The ultimate practical value of PCR in the clinical specimens is the detection of *S. typhi* DNA in the blood specimens from patients with suspected clinical findings but with negative cultures. In the study, the first round of PCR detected amplification products in 4 culture negative cases. Furthermore, nested PCR using RK3 & RK4 primers detected an additional 7 culture negative cases. All these 7 cases had fever of duration ranging from 2 weeks to 6 months and 3 of them had been treated with antibiotics before culture. The possibility of false positive results was minimized by meticulous handling of the materials and the simultaneous application of multiple negative controls.

Therefore, PCR is a rapid, sensitive and specific test for the early diagnosis of typhoid fever. Nested PCR can reinforce the clinical diagnosis of typhoid fever

in culture negative cases and can avoid unnecessary workup for cause of fever as well as improper treatment. It also holds promise in the diagnosis of complications of typhoid fever such as enteric encephalopathy and enteric perforation.

Acknowledgement

This study was supported by grant from DBT project BT/RD/9/11/94 Delhi. We acknowledge the help of Dr. Pawan Malhotra (Scientist, ICGEB) for his valuable suggestions. Technical assistance of Mr. Salek Chand, Mr Pooran Ram Arya and Mr. MM Khurana is acknowledged.

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Abstract: The objective of this study was to evaluate the diagnostic value of bone marrow culture in typhoid fever. The study was conducted in an endemic area of India. The results show that bone marrow culture is superior to other methods in the diagnosis of typhoid fever. The study concludes that bone marrow culture is the most reliable method for the diagnosis of typhoid fever. The study also shows that bone marrow culture is superior to other methods in the diagnosis of typhoid fever. The study concludes that bone marrow culture is the most reliable method for the diagnosis of typhoid fever.

INTRODUCTION
 The diagnosis of typhoid fever is a difficult task. The traditional method of diagnosis is by culture of the organism. However, the culture method is slow and often fails to detect the organism. The use of serological methods for the diagnosis of typhoid fever is also difficult because of the high degree of cross-reactivity between different serotypes of *Salmonella typhi*. The use of polymerase chain reaction (PCR) for the diagnosis of typhoid fever is a new and promising method. The objective of this study was to evaluate the diagnostic value of bone marrow culture in typhoid fever. The study was conducted in an endemic area of India. The results show that bone marrow culture is superior to other methods in the diagnosis of typhoid fever. The study concludes that bone marrow culture is the most reliable method for the diagnosis of typhoid fever.