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ORIGINAL RESEARCH

Evaluation of Nested Polymerase Chain Reaction targeting *hsp65* of *Mycobacterium tuberculosis* for the Detection of Organism in the Sputum Samples

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ABSTRACT

Introduction: The poor sensitivity of conventional smear microscopy and the delay in obtaining *Mycobacterium* culture results prevent the early diagnosis of *Myobacterium tuberculosis* (MTB). By using nucleic acid amplification techniques like polymerase chain reaction (PCR), one may be able to diagnose the disease on the day of arrival of specimen in the laboratory. The present study aimed to evaluate the applicability of the nested-PCR (nPCR) technique as a rapid and direct molecular method for the diagnosis of *M. tuberculosis* in sputum specimens of patients whose sputum smear was acid-fast bacilli (AFB) negative using heat shock protein (*hsp65*) as the gene target.

Materials and methods: Early morning sputum samples were collected in sterile containers respectively from about 40 suspected patients of pulmonary tuberculosis, attending the outpatient units of JSS Medical College and PKTB Hospital, Mysore and from 20 age and sex-matched healthy controls. Sputum samples were decontaminated by modified Petroff's method and DNA was isolated using QIAGEN DNA extraction kit. The nPCR was carried out for the detection of MTB using the target gene *hsp65.*

Results: Nested-PCR showed specific amplification (165bp) of *M. tuberculosis* in 18 out of 20 sputum AFB positive samples and 9 out of 20 AFB negative samples. None of the healthy controls showed any amplification with nPCR. The nPCR when compared to that of Ziehl–Neelsen staining had a sensitivity of 90%, specificity of 77.5%, positive predictive value (PPV) of 66.6%, and negative predictive value (NPV) of 93.9%. The percentage of false positive was 33.3% and percentage of false negative was 6.1%.

Conclusion: The detection of *M. tuberculosis* with nPCR in smear negative patients provides the bacteriological data 4 to 8 weeks earlier. A molecular approach, based on the amplification of *hsp65* gene by nPCR, showed that there is high probability of the disease being absent when the test is negative because of the high negative predictive value (NPV).

Keywords: Heat shock protein (*hsp65*), *Myobacterium tuberculosis*, Nested-polymerase chain reaction, Ziehl–Neelsen staining.

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INTRODUCTION

Tuberculosis (TB) remains a global health problem. In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320,000 deaths among HIV-positive people).[1] The number of TB deaths is unacceptably large given that most are preventable. The increasing number (advent) of AIDS patients has made the disease a major public health and about 21% of the world's TB-infected population is in India.[2,3] Although the pulmonary system is the most common location for TB, extra pulmonary TB (EPTB) disease, resulting from spread of *Myobacterium tuberculosis* (MTB) from lungs to other tissues and organs through bloodstream, occurs in more than 20% of immunocompromised patients. In India and other developing countries, lymph node TB is among a major form of EPTB caused by nontuberculous mycobacteria species.

A rapid and an early diagnosis of TB is thus an essential step to combat this disease. The conventional methods adopted for the diagnosis of TB include medical history, tuberculin skin test, chest x-rays, bacteriological examination by the acid-fast bacilli (AFB) and mycobacterial culture. Sputum smear microscopy with Ziehl–Neelsen (ZN) staining is the most widely used rapid method in most laboratories for diagnosing TB under the Revised National Tuberculosis Control Program. The poor sensitivity of conventional smear microscopy and the delay in obtaining *Mycobacterium* culture results prevent the early diagnosis of TB. The difficulties of identifying mycobacteria in patient specimens emphasize the need for rapid and sensitive diagnostic methods for TB.

Recently, molecular tests based on nucleic acid amplification (NAA) technologies, such as polymerase

chain reaction (PCR) have been shown to be a useful tool for the detection of infectious pathogens, such as MTB. The advantage of this method is that it can provide results within 1 day of collection of specimens, with a high degree of sensitivity and specificity.[4] Also, PCR is able to detect less than 10 bacilli per mL from a variety of clinical specimens.[5,6] However, despite being promising, the sensitivity of these methods is sometimes low mainly in samples with negative AFB smear.[7] For smear and culture negative specimens, PCR is the only currently available method that can provide bacteriological diagnosis. The development of PCR assays has used different genomic targets, such as *IS6110*, *16S rDNA*, *rpoB*, *recA*, and heat shock protein (*hsp65*), which have shown discrepant results in sensitivity.

The gene encoding the 65-kDa *hsp65* has been reported as an ideal target for *M. tuberculosis* detection by PCR as it is estimated to be highly conserved among mycobacterial species and present in all mycobacteria. It is more variable than the *16S rRNA* gene sequence and for this reason it is a potential tool for the identification of genetically related species of mycobacteria.

Although conventional PCR is very useful for detection of *M. tuberculosis*, a variation of PCR, known as nested-PCR (nPCR) uses two sets of oligonucleotide primers in subsequent reactions, in which the amplification product of the first reaction is used as a template for the second reaction. This technique has been proposed for the detection of *M. tuberculosis* in cases requiring high specificity and sensitivity.[8-11]

The purpose of this study is to evaluate the applicability of the nested-PCR technique as a rapid and direct molecular method for the diagnosis of *M. tuberculosis* in sputum samples of patients using *hsp65* as the gene target. The results of the nPCR were then compared with the results of the conventional method to evaluate the efficacy of nPCR for the diagnosis of MTB in clinical samples.

MATERIALS AND METHODS

Source of the Data

All adult patients who were clinically suspected of pulmonary TB as per World Health Organization guidelines attending JSS Hospital/PKTB Hospital, Mysore form the source of material. Early morning expectorated sputum samples were collected in wide mouthed disposable sterile containers from each suspected patient of pulmonary TB. Ethical committee approval was obtained before the commencement of the study. A well-informed and written consent was obtained from each patient.

Type of Study

A pilot study.

Sample Size

A total of 20 confirmed (sputum AFB positive by ZN staining) pulmonary TB patients and 20 suspected (sputum AFB negative by ZN staining) pulmonary TB patients, and 20 healthy nontubercular controls without any symptoms of MTB and negative ZN staining were included in the study.

Inclusion Criteria

- The patients were selected based on the presence of clinical symptoms leading to suspect TB, such as prolonged cough, fever, without apparent cause, weight loss, and night sweat.
- The diagnosis of TB was made on gold standard criteria employed by the health services in the diagnostic definition of TB, such as the combination of epidemiological, clinical and laboratory data (chest x-ray, tuberculin test, sputum smear), and patient history.
- Based on sputum AFB results, they were divided into two groups: Group 1: Confirmed pulmonary tuberculosis and Group 2: Suspected pulmonary tuberculosis.

Exclusion Criteria

- Patients who were already on anti-TB treatment, immunocompromised, or under corticosteroid-based medication were excluded from the study.

Method of Sample Collection

A total of 5 to 10 mL of early morning sputum samples were collected from 40 patients included in the study group during the period from November 2013 to October 2014. All the samples were smeared and screened with conventional microbiological test, such as Ziehl–Neelsen acid fast staining for recording smear positivity.

Isolation of DNA from Sputum Samples

Majority of sputum specimens submitted to the laboratory are contaminated by more rapidly growing normal flora. These overgrow before tubercle bacilli start to grow. Therefore, specimens must be submitted to harsh digestion and decontamination procedure that liquefies organic debris and eliminates unwanted normal flora.[12]

All currently available digesting/decontaminating agents are to some extent toxic to tubercle bacilli. Therefore

to ensure survival of maximum number of bacilli in specimen, decontamination procedure must be precisely followed. As a general rule, contamination rate of 2 to 3% is acceptable in laboratories that process fresh specimens. A laboratory which experiences no contamination is probably using a method that kills too many of tubercle bacilli.[12]

Modified Petroff's Method

The sputum samples were digested and decontaminated by the aseptic addition of equal volume of sterile N-acetyl-L-cysteine- 2% NaOH – 1.5% sodium citrate solution in a 15 mL centrifuge tube. The cap was tightened and then incubated at room temperature for 30 minutes, centrifuged at 10,000 rpm for 25 minutes. The supernatant was discarded slowly into a container with 5% phenol solution. The pellet was washed twice with phosphate buffered saline pH7.6 or sterile distilled water. The supernatant was discarded and the pellet was resuspended in 0.5 mL of sterile distilled water and the mixture was transferred to a microcentrifuge tube and 40 µL (20 mg/mL) of proteinase K and 8 µL RNase (100 mg/mL) was added, mixed by vortex followed by the addition of equal volume of lysis buffer and vortexed. The mixture was transferred to the QIAamp Mini spin column. Further, DNA was extracted by following the procedure mentioned in the DNA extraction kit (QIAGEN, Germany). The DNA sample eluted was stored in –20°C until use.

Measurement of DNA Concentration

The spectrophotometric analysis and agarose gel electrophoresis was used to reveal the concentration and the purity of the genomic DNA. Elution buffer was used to dilute samples and to calibrate the spectrophotometer and measure the absorbance at 260, 280, and 320 nm using quartz microcuvette. The amount of ultraviolet (UV) radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. For pure sample of DNA, the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) is between 1.6 and 1.9. 10 µL of each DNA sample was loaded onto 1% agarose gel and the result was documented.

Nested-PCR Amplification of *hsp65* Gene and Detection of Mycobacterial DNA

Following extraction, a nPCR for the detection of MTB was performed from DNA samples. Oligonucleotide primers (Eurofin) were synthesized and primary PCR was performed utilizing a pair of oligonucleotide primers, TB-1(5'-GAGATCGAGCTGGAGGATCC-3') and TB-2(5'-AGCTGCAGCCCAAAAGGTGTT-3'). For primary PCR, 5 µL of stored DNA (150 ng) was used. The DNA was amplified in 50 µL of reaction mixture, which was composed of 10× PCR buffer (10 mMTrisHCl [pH8.3], 50 mm KCL, 2.5 mm $MgCl_2$), 2.5 mm each of deoxynucleoside triphosphates (i.e., deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate) (Himedia) and 50 picomol of 0.3 mm oligonucleotide primers, TB-1 and TB-2. Primary PCR was completed by adding 2.5 U Taq DNA polymerase (Himedia) to this mixture. The samples were subjected to 40 amplification cycles consisting of 1.5 minutes denaturation at 95°C, 2 minutes annealing at 60°C, and 2 minutes extension at 72°C and with a final extension at 72°C for 10 minutes. The PCR reaction cycles were performed within an automated Thermal Cycler (Master Cycler Gradient, Eppendorff). For the nPCR of tuberculosis, the 10 µL of primarily amplified DNA product was used as a template and the same procedure as primary PCR was performed, except for the utilization of a second pair of oligonucleotide primers. TB-3 (5'-CCATCGATCCGAGACCCTGCTCAAGGGC-3') and TB-4 (5'-TGCTCTAGACTCCTCGACGGTGATGACG-3'). DNA from the standard strain of MTB H37Rv was used as a positive control for the nPCR and the PCR solution without DNA template were used as negative controls.

Analysis of the Amplified Products/Gel Electrophoresis of Amplified DNA

An aliquot of 10 µL nested or second PCR products were analyzed by gel electrophoresis in 2% agarose gel prepared in Tris-Acetate ethylenediaminetetraacetic acid buffer pH8.6 containing 0.5 µg/mL of ethidium bromide. The gel was run at 100 volts and then visualized under UV transilluminator. Further, the gel was examined in a gel documentation system (S*yn*G*ene*) for a 165 bp product using standard molecular marker and the results were documented. Presence of a single band equivalent to 165 bp was taken as a positive result when negative control gave no reaction. All the false negative and doubtful results were retested.

Throughout the PCR processing, recommended stringent precautions were followed and the results were evaluated in the light of performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions. All the necessary clinical details were also taken. Known treated positive TB cases, failure cases, defaulter cases, and relapse cases were not considered for this study.

Determination of Sensitivity

To assess the sensitivity of the nPCR assay, all the DNA samples were diluted to get concentration ranging from 1 to 50 ng and each concentration was taken separately for testing by nPCR. It was observed that the nPCR was able to detect the MTB even at 30 ng DNA concentration.

Determination of Specificity

Primers amplified successfully MTB DNA to give a 165 bp product and there were no nonspecific amplified products in all the DNA samples tested. These results demonstrate the exquisite specificity of the amplified sequence of MTB.

RESULTS

The characteristics of the patients included in the study are shown in Table 1. Among the 20 AFB positive cases included in the study, 17 were males and 3 were females.

Table 1: Physical characteristics of the patients included in the study

	Mean	*Standard deviation*
Age (years)	42.70	15.647
Body weight (kg)	57.10	9.907
Height (cm)	160.98	7.238
Body mass index	22.047	3.6485
Sex (M/F)	48/12	

Among the 20 AFB negative patients included in the study 16 were males and 4 were females. Among the 20 healthy controls, 15 were males and 5 were females. The age of the patients recruited in the study ranged from 20 to 78 years. Among these, 10 were below 25, 34 between the age group 26 and 50, and 16 were above 50 years of age.

Nested-PCR showed specific amplification (165 bp) of MTB in 18 out of 20 sputum AFB positive samples (Fig. 1) and 9 out of 20 AFB negative samples (Fig. 2). In experiments for detection of lower limit of detection, the assay was able to detect the MTB even at 30 ng

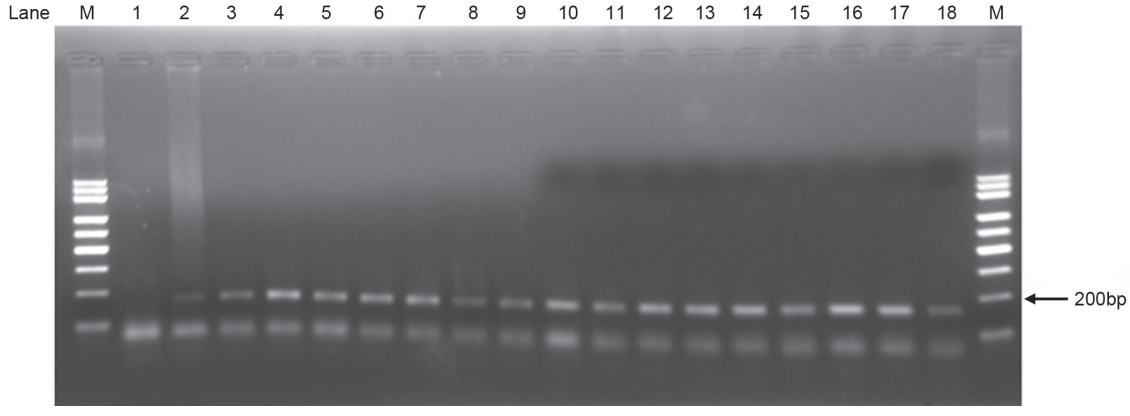

Fig. 1: Amplification products of PCR from MTB in sputum smear AFB positive samples. Polymerase chain reaction showing 165 bp target sequence amplified in case samples. Lane 1 = Negative control, Lane 2 = Positive control, Lane 3-18 = AFB positive cases, M = 100 bp DNA ladder

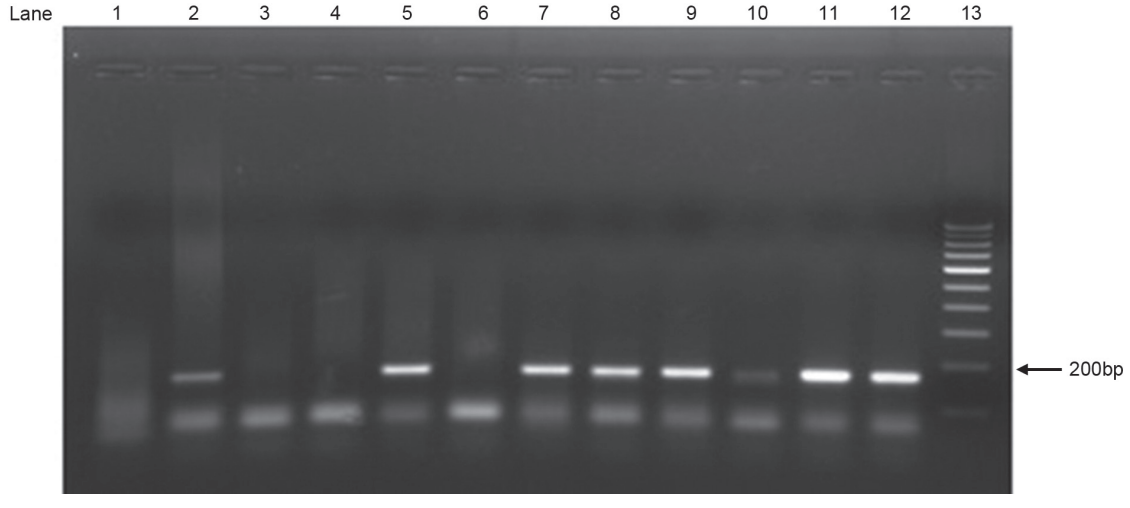

Fig. 2: Amplification products of PCR from MTB in sputum smear AFB negative samples. Polymerase chain reaction showing 165 bp target sequence amplified in case samples. Lane 1 = Negative control, Lane 2 = Positive control, Lane 3-12 = AFB negative cases, M = 100 bp DNA ladder

Table 2: Comparison of AFB staining and nPCR

	AFB positive (n = 20)	*AFB negative n = 40*	*Total*	*Chi-square test*	*p-value*
nPCR positive	18 (66.6%)	9 (33.3%)	27 (45%)	7.025	0.020
nPCR negative	2 (6.1%)	31 (93.9%)	33 (55%)		
Total	20 (33.3%)	40 (66.6%)	60 (100.0%)		

nPCR: Nested-polymerase chain reaction

DNA concentration. The results of the comparison of AFB staining and nPCR is shown in Table 2. Among the 20 healthy nontubercular controls included in the study, all the samples were negative for smear as well as nPCR. In this study, we have also evaluated the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of the nPCR against the most widely used ZN smear conventional method. The nPCR when compared to that of ZN staining had a sensitivity of 90%, specificity of 77.5%, PPV of 66.6%, and NPV of 93.9%. The percentage of false positive was 33.3% and percentage of false negative was 6.1%.

DISCUSSION

In India, most of the time the diagnosis of tuberculosis is primarily based on clinical features and gold standard methods, such as demonstration of AFB and culture of MTB. Since these gold standard methods fail to give results due to paucibacillary nature of the sample, NAA method to detect MTB in clinical specimens is increasingly used as a tool for TB diagnosis. Application of molecular methods in routine for diagnosing in developing country like ours depends on various factors like high cost and availability of skilled personnel to perform the test.

In the present study, 40 clinically suspected pulmonary TB cases admitted at JSS Medical College and PKTB Hospital, Mysore were studied along with 20 age and sex-matched healthy controls. DNA isolated from the sputum sample of 20 clinically suspected pulmonary TB whose sputum smear result was negative showed positive for TB in nine subjects by nPCR. However, the DNA isolated from 20 clinically suspected TB patients whose sputum smear result was positive for TB showed 18 positive by nPCR. The two subjects who showed a negative result by nPCR were reconfirmed and then reported. The negative result may be due to low copy number of bacilli or problem in digestion and decontamination of sputum sample. Also, the sputum is known to contain several inhibitors of PCR which makes successful amplification of mycobacterial DNA targets a challenge.[13]

It is widely accepted that nPCR is a new and rapid technique for the diagnosis of bacterial DNA with high specificity.[8-11] By applying the nPCR technique, the RAPID BAP-MTB assay was able to detect as little as 10 fg, or the equivalent of 1 to 20 copies of MTB complex genomic DNA.[14] Evaluation of nPCR targeting IS6110 of MTB using buffy coat of venous blood showed a sensitivity of 100% and a specificity of 95.1%. The lower limit of detection in that study was less than 1 genome copy per microliter.[15] The detection limit in whole blood using non-nested format was 20 cells per microliter.[16] In our study, the lower limit of detection in terms of DNA concentration was 30 ng. We have used *hsp65* as a target for the identification of MTB since several false positive results were reported questioning the specificity of IS6110 target.[17,18] Previously reported sensitivity of *hsp65* nPCR with restriction analysis compared to microscopy and culture was 100% (26/26 and 27/27 respectively). Specificity and PPV were 93.1% (94/101) and 78.8% compared with microscopy and 95.0% (95/100) and 84.4% compared with culture ($P < 0.05$) respectively.[19] Our study has shown a sensitivity of 90%, specificity of 77.5%, PPV of 66.6%, and NPV of 93.9%. Lower specificity shown in our study may be because the restriction analysis was not performed. Diagnosis of MTB using non-nested format of *hsp65* gene showed sensitivity of 81.13%, with specificity of 88.24%; the PPV and NPV were 95.56 and 60% respectively.[20] However, the use of nested format showed that there is high probability of the disease being absent when the test is negative because of the high NPV.

Small sample size is the limitation of this study. Also, we have compared the nPCR results with ZN staining and the clinical criteria of the patients and not with culture. Using clinical criteria as gold standard is somewhat flawed due to the low specificity of symptoms of TB in relation to other diseases.

Hence, we would like to further evaluate this method in a large group of patients using other clinical specimens, such as blood, urine, pleural fluid, bronchial aspirate, lymph node aspirate, and compare the results with ZN staining, clinical criteria as well as with culture.

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