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# DIAGNOSTIC VALUE OF POLYMERASE CHAIN REACTION FOR THE RAPID DIAGNOSIS OF CLINICALLY SUSPECTED TUBERCULOUS MENINGITIS

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## ABSTRACT

To study the efficiency of Polymerase Chain Reaction for the diagnosis of clinically suspected tuberculous meningitis, a rapid molecular technique was established. The conventional bacteriological methods rarely detect *Mycobacterium tuberculosis* in CSF and are of limited use in diagnosis of tuberculous meningitis (TBM). This double blind study was, therefore, directed to the molecular analysis of CNS tuberculosis by PCR targeted for amplification of 123bp nucleotide sequence coding IS6110 insertion element. In this study, the efficiency of the Polymerase Chain Reaction (PCR), one of the most reliable and sensitive DNA-based assays, was compared with conventional Microscopy and Solid culture for the detection of *M.tuberculosis* in cerebrospinal fluid specimens from patients suspected of TBM. Of the 69 CSF specimens from highly-probable TBM patients based on clinical features, 51 were positive by PCR (78.46%), whereas microscopy and culture results were negative. Thus, PCR was found to be more sensitive than any other conventional method in the diagnosis of clinically suspected tubercular meningitis.

**KEY WORDS:** **CNS Tuberculosis, tuberculous meningitis (TBM), Polymerase Chain Reaction, *Mycobacterium tuberculosis***

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# INTRODUCTION

Tuberculosis (TB) is one of the major causes of infection worldwide. Extra pulmonary forms have been increasingly reported, accounting for 20 to 50% of all cases of TB in recent studies. Extra Pulmonary Tuberculosis (EPTB) remains a challenging diagnosis for both clinicians and microbiologists[1]. Tuberculosis of the Central Nervous System (CNS) is classically described as meningitis. However, altered mental status, including encephalitis, is within the spectrum of clinical manifestations. Because early treatment can dramatically improve outcomes, consideration of TB as a potential pathogen in CNS infections, including encephalitis, is vital[2]. The inhaled *M.tuberculosis* bacilli can localize in alternate sites, leading to Extra Pulmonary Tuberculosis (EPTB). Tuberculous involvement of the Central Nervous System (CNS) is an important and serious type of extra pulmonary tuberculosis. It has been estimated that approximately 10% of all patients with tuberculosis have CNS involvement. Fatality rates in developing countries have been reported to range from 44% to 69 %[3]. In fact, missed diagnosis and delayed treatment often results in serious long term debilitating complications. Moreover, the clinical response to antituberculosis therapy in all forms of neuro- TB is excellent, provided the diagnosis is made early; before an irreversible neurological defect occurs (delay in diagnosis is directly related to neurologic diagnosis sequelae in 20-25% of patients who do not receive early treatment). Clearly, prompt laboratory diagnosis is of vital importance. The great majority of patients with neuro-TB are diagnosed on the basis of clinical criteria, radiographic findings and laboratory investigation of the cerebrospinal fluid (CSF). Acid fast staining of CSF sediment is the most rapid and inexpensive method for the detection of mycobacteria, but this method requires >$10^4$ cells $ml^{-1}$ hence lacks sensitivity. Conventional methods like microscopy and culture, although considered as gold standards, are quite inadequate[4]. This diagnostic reference standard, isolation of *M.tuberculosis* from CSF samples, is insufficiently timely (it requires 2-6 weeks) to aid clinical judgment with respect to treatment and because of the paucibacillary state in the cerebrospinal fluid this method is insensitive if large amounts of CSF are not tested. PCR and molecular techniques show promise as tools for rapid diagnosis of pulmonary tuberculosis[5]. Diagnosis in the critical early stage of the disease is, therefore, often presumptive and a rapid diagnostic method like Polymerase Chain Reaction (PCR) is urgently needed[6]. In Indira Gandhi Government General Hospital and Post Graduate Research Institute, Puducherry, tuberculous meningitis is an important cause of meningitis despite diagnosis being still dependent on clinical and laboratory parameters, However, PCR results can be obtained within 48 hours of the receipt of the sample and its sensitivity and specificity far exceeds that of microscopy. Several studies have reported PCR sensitivity ranging between 50-91% and specificity ranging between 95-100%, using culture IS6110 primer was evaluated at Department of Microbiology, Intermediate Reference Laboratory,State TB Training and Demonstration Centre, Government Hospital for Chest Diseases for rapid and specific diagnosis of tuberculous meningitis.

# MATERIALS AND METHODS

### *CSF Samples processing*

A total of 69 CSF specimens were received from Department of Pediatrics ,Indira Gandhi Government General Hospital and Post Graduate Research Institute, Puducherry and all the specimens were processed at Department of Microbiology, State TB Training and Demonstration Centre, Intermediate Reference Laboratory, Government Hospital for Chest Diseases, Puducherry, South India, during the period from January 2006 to November 2010. All samples were concentrated by centrifuge at 3000xg for 15

minutes and decontaminated the pellets with 1ml of 5% $H_2SO_4$. After neutralization, the whole pellets were taken for ZN staining, FM staining, AFB Culture and PCR assays.*Staining*

### *Ziehl-Neelsen Method*

Take pellet portion of the deposited specimen and spread evenly onto central portion of the microscopic slide and fix the smear to the slide. Cover the slides with freshly filtered 1 % carbol fuchsin and heat underneath until steam rises from the stain and allow the hot carbol fuchsin to react for at least 5 minutes. Add 25% sulphuric acid to react for 2-4 minutes after water washing. Counter stain with 0.1% Methylene blue for 30 seconds and wash as before with water and slope the slides to air dry. Examine the slides under the Microscope to observe for the presence of tubercle bacilli[7].

### *Fluorescence Method*

Smear the specimen onto the centre portion of the slide and allow smears to air-dry for 15 minutes and fix the smear to the slide. Flood the slides with freshly filtered auramine-phenol. Let stand for 7-10 minutes. Decolorize by covering completely with acid-alcohol for 2 minutes, twice. Wash well with running water, as before to wash away the acid alcohol. Counter stain with 0.1% potassium permanganate for 30 seconds. Wash as before with water and slope the slides to air dry. Positive smears were graded into four degrees of positivity using the 20x, 25 x objectives along with 10 x eyepieces[7].

### *Specimen Processing for AFB Culture*

Approximately 1ml of 5% $H_2SO_4$ was added to each CSF sample. Mixed well and let stand for 15 minutes and filled the container with sterile distilled water and centrifuged at 3000 x g for 15 minutes. Discarded supernatant carefully in to a disinfectant bin containing 5% phenol without disturbing the deposit. Inoculated one slope each of LJ and LJ-P with one loopful of deposit for each slope. The inoculated media was placed in the 37 °C incubator[8].

### *Extraction target DNA from clinical samples*

DNA was extracted according to the CTAB-phenol chloroform extraction method. Briefly; 0.2ml of CSF was centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in 100 µl of TE buffer (Tris EDTA, pH7.4). The entire pellets were treated with 50 µl of lysozyme (10mg/ml) at 37°C for overnight incubation. Add 70 µl of 14% SDS and 6 µl Proteinase K (10mg/ml), mixed and incubated at 65° C for 15 minutes. After incubation, 10 µl of 5M NaCl and 80 µl of high salt CTAB buffer (containing 5M NaCl, 1.8%CTAB) was added and mixed followed by incubation at 65° C for 10 minutes. An approximate equal volume (0.7-0.8 ml) of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added, mixed thoroughly and centrifuged at 4°C for 10 minutes in a micro centrifuge at 12000rpm. The supernatant was separated and then mixed with 0.6 ml volume of ice cold isopropanol to get precipitate. The precipitated nucleic acids were washed with 70% ethanol, dried and re-suspended in 25 µl of 1X TE buffer [9].

### *PCR Amplification for Species Identification*

The target for the PCR assay was IS6110 insertion element specific to *M.tuberculosis* complex. The sequences of the one set of primers used were: Forward primer F 5'GTGAGGGCA TCGAGGTGG 3': R 5'CGTAGGCGTCG GTCACAAA 3'[10]. DNA amplification by PCR was performed in the total reaction volume of 50 µl with 2 µl of extracted DNA,25 µl of Master mix (eppendorf 2X),2 µl of each primer (10 pmol/µL). Amplification was carried out on a programmable Mastercycler gradient (eppendorf, USA). Initial denaturation at 95° C for 5 minutes was proceeded by 35 cycles each of denaturation (95° C for 1 min),annealing (57° C for 1 min) and extension (72° C for 1 min) followed by a final extension at 72° C for 10 minutes. The amplified product was electrophoresed into 1%agarose gel. The gels were stained with

ethidium bromide and visualized in a UV-transilluminator (Spectroline, France). The presence of a 123bp fragment indicate a positive test [9].

***Electropherogram Analysis of PCR Amplified Products***

DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature. 25 µL of dye concentration was added to DNA gel matrix, vortexed and transferred to spin filter and centrifuged at 2240 g for 15 minutes. The gel dye was allowed to settle at room temperature for 30 minutes. A new DNA chip was placed on the chip priming station. 9 µL of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it is held by the chip for 60 seconds. After 5 seconds the plunger was pulled back slowly to 1 mL position. The chip priming station was opened and 9 µL of gel dye was pipetted into the well marked G and 1 µL of ladder was added to the well labeled ladder. 5 µL of marker was pipetted into all 12 sample wells and in ladder well. 1 µL of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (Bioanalyzer-Agilent 2100) and the results were interpreted[11].

## RESULTS

In this retrospective study, overall 80 patients were analyzed by an IS6110 insertion elements targeted PCR to evaluate its diagnostic efficacy for rapid and specific diagnosis of CNS tuberculosis. Based on the clinical criteria, 69 CSF specimens and 11 negative control samples from the patients having non-tubercular lesions of the CNS included in the study were analyzed . A clear band of 123 base pairs (bp) was observed on a 2% agarose gel confirming the *M.tuberculosis* (Figure 1). Electropherogram analysis of the PCR-amplified product confirmed the molecular size (123 bp) of the products (Figure 2). Microscopic examination by ZN-staining indicated absence of AFB in all CSF samples (Table 2). These results are comparable to those reported in literature of only 0% to 10% of Ziehl Neelsen positive results [12] in TMB patients. CSF for AFB culture was also negative in all cases. The chance of growing mycobacterium becomes higher with the increase of sample volume. Only 2-3 ml of CSF per patients were available, and part of this volume, had to be utilized for ZN-staining and PCR, thus negative culture results in all the patients with CNS-TB could be attributed to sampling difficulties. *M.tuberculosis* specific IS6110 targeted sequence was detected in CSF sample from 51 out of 65 suspected TBM patients, whose microscopy and culture results were negative. There were no false positive results by PCR (out of 6 control cases none tested positive for PCR) and the specificity worked out to be 100% (Table 2). The sensitivity of the PCR as a diagnostic tool to detect CNS-TB worked out to be 78.0% for TBM and 50% for intracranial tuberculomas. Of the 41 pediatric CSF specimens of from highly-probable TBM patients (based on clinical features), 28 were positive by PCR (68%), whereas microscopy and culture results were negative (Table 1).

**Figure 1**
***PCR Amplification of IS6110 product. Lane 4: 100 bp DNA ladder, Lane 6: 123 bp PCR amplified product.***

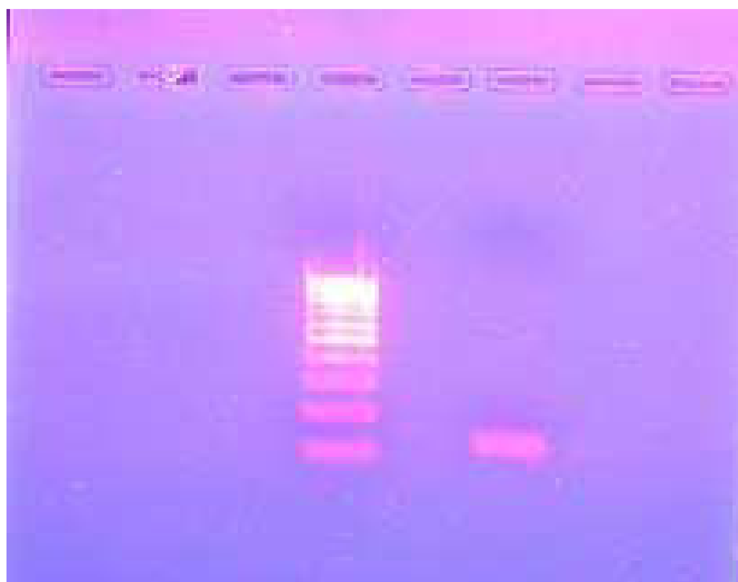

**Figure 2**
***Electropherogram Analysis of PCR Amplified Products. Peak 1: Lower Marker (15 bp), Peak 2: PCR amplified IS6110 product (123 bp) and Peak 3: Upper marker(1500 bp).***

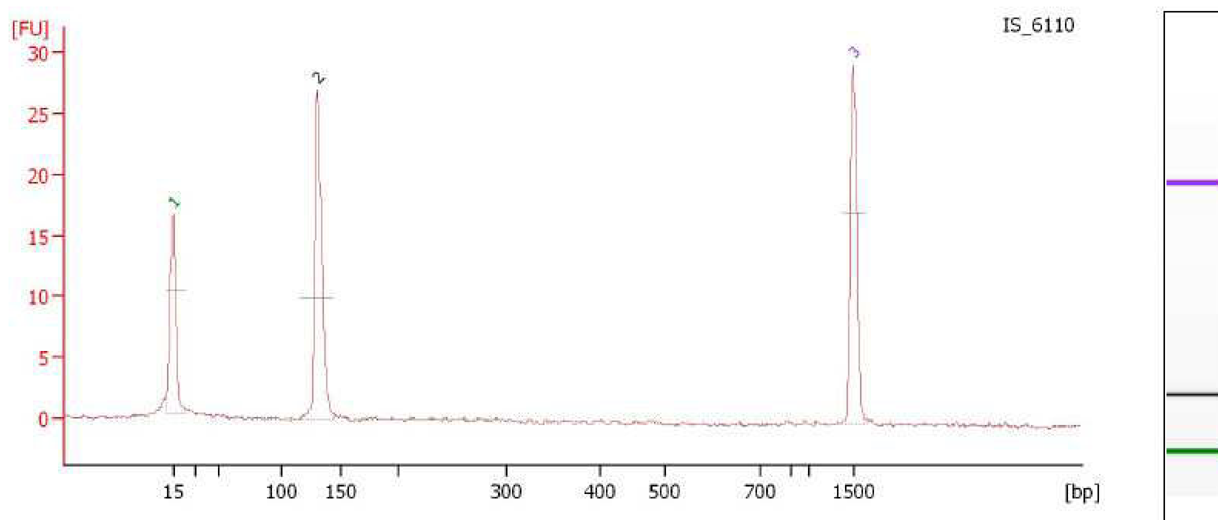

**Table 1**
***Demographic features of the study population***

| Group of patients | TBM | Intracranial tuberculoma |
|---|---|---|
| | (n=65) | (n=4) |
| Age in year | (2-60) | (19-27) |
| 00-10 years | 41 | 0 |
| 11-20 years | 8 | 3 |
| 21-30 years | 7 | 1 |
| 31-40 years | 6 | 0 |
| 41-50 years | 1 | 0 |
| 51-60 years | 2 | 0 |
| 61-70 years | 0 | 0 |
| Sex (Male / Female) | (38/27) | (4 / 0) |

**Table 2**
***Evaluation of PCR in CSF specimens from patients suspected of tuberculous meningitis (TBM).***

| Diagnostic Method | No. of suspected positive CSF Specimens | TBM(n=65) | | | Intracranial tuberculoma(n=4) | | |
|---|---|---|---|---|---|---|---|
| | | Positive | Sensitivity | Specificity | Positive | Sensitivity | Specificity |
| ZN Staining | 69 | None | 0% | 0% | None | 0% | 0% |
| FM Staining | 69 | None | 0% | 0% | None | 0% | 0% |
| AFB Culture | 69 | None | 0% | 0% | None | 0% | 0% |
| PCR assay | 69 | 51 | 78% | 100% | 2 | 50% | 100% |

# DISCUSSION

This study shows that the efficiency of PCR is significantly higher than microscopy and culture for the early diagnosis of TBM (Table 1). The accuracy of this study is demonstrated by the fact that the control group gave negative results. The repetitive nature of the target sequence amplified by the PCR described here probably contributes to the high sensitivity, and fewer than 10 bacilli can be detected by application of this PCR. The above-mentioned detection limit of this PCR, combined with the use of the best method of DNA extraction from clinical specimens, provide a powerful tool for the specific and rapid diagnosis of paucibacillary situations. In this study, the 78.46% positivity rate by PCR in a small proportion of patients suspected of TBM is remarkable. Since there are normally few bacilli in CSF specimens from TBM patients to be demonstrated by direct microscopy, and culture examination of CSF specimens from TBM patients takes several weeks, PCR will prove useful as an efficient technique for the rapid diagnosis of TBM,

even though conventional diagnostic methods are less expensive. PCR can specifically identify *M.tuberculosis* in a clinical specimen within 7-8 hours. The use of PCR thus saves valuable time in the early identification of mycobacteria and implementing effective treatment[13, 14]. Among the 65 cases considered either definitely TBM or intracranial tuberculoma, on the basis of clinical and laboratory findings, PCR proved to be a more sensitive method, detecting 51 out of TBM cases (78%), 2 out of 4 patients of intracranial tuberculmas (50%) whereas none were positive either by culture of staining (Table 2). Some studies report a relatively higher sensitivity of PCR for the diagnosis of TBM, ranging from 75-90%, but some authors had tested a very small number of patients[15] or had used a selected patient group[16]. An explanation for the lower sensitivity of PCR in our study could be attributed to the small volume of CSF (mean volume 200-300 ul) available for testing (after using for smear and culture) so that the sample could not be concentrated. The volume of sample is of great significance in PCR, especially in CNS-TB, due to frequent low number of bacteria in the CSF. Culture of CSF also requires larger volume and volume and when both culture and PCR have to be done, the minimum volume of CSF should be 2 ml. Another reason for low sensitivity of PCR may be the presence of PCR inhibitors in the CSF as well as poor lysis of *M.tuberculosis*[17]. Results of PCR are available with speed comparable to microscopy; sensitivity is higher than both microscopy and culture and the direct identification of the microorganism, as belonging to the *M.tuberculosis* complex is possible. Thus, the rapid availability of results obtained by PCR assays can be a considerable advantage to patient management in certain situations. More rapid and appropriate treatment can lead to fewer ultimate complications and hospitalization in patients who have the disease, and avoidance of unnecessary treatment and isolation of those who do not have TB. Similar pressure for more rapid diagnostic capabilities have driven the development of other molecular assays, particularly those for slow growing viruses and particularly for the diagnosis of infections of the central nervous system. As we know that tuberculoses meningitis (TBM) is a paucibacillary disease and if detected in the early state and treated can improve cure rate significantly. PCR represents a rapid and sensitive method for detection of mycobacterium DNA in early tuberculoses meningitis and may be a useful adjunct to diagnostic modalities in tuberculoses meningitis.

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