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# Application of Smear Microscopy, Culture and Polymerase Chain Reaction for Diagnosis of Tuberculous Meningitis in Puducherry

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## ABSTRACT

*Tuberculous meningitis, a serious manifestation of extra pulmonary tuberculosis, needs early diagnosis and treatment for better clinical outcome. Conventional methods are time consuming and less sensitive. This study compares the use of Ziehl Neelsen smear microscopy, culture on Lowenstein Jensen medium & Mycobacterium Growth Indicator Tube and polymerase chain reaction. Cerebrospinal fluid collected from 43 patients with clinical suspicion of TBM and five patients with non-infectious meningitis as controls were included in the study. Nested PCR targeting IS6110 gene was carried out for 19 randomized CSF. Analysis of the results was made to assess the role of multiple techniques in the diagnosis of TBM. All CSF including the controls were negative by microscopy and culture. A total of 7 among the 19 CSF tested (36.8%) were positive in TB PCR. PCR plays a crucial role in the diagnosis of TBM where smear and culture remains negative but caution has to be exercised while interpreting the results correlating with clinical features.*

**Key words:** Tuberculous Meningitis; CSF; MGIT960; IS6110 PCR; M. tuberculosis

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## INTRODUCTION

Tuberculous meningitis (TBM) is the common form of chronic central nervous system infection. It accounts for 1% of all cases of tuberculosis and 5-10% among the extra pulmonary tuberculosis (EPTB) cases [1]. Among children, it develops typically 3-6 months after the primary infection. TBM often presents with nonspecific clinical symptoms, which makes early diagnosis difficult. In India, mortality due to TBM is estimated to be 1.5 per 100,000 populations [2]. Confirmatory diagnosis of TBM depends upon the isolation of *M. tuberculosis* from the CSF specimen. Conventional methods like smear microscopy can detect Acid Fast Bacilli (AFB) only when the load is above $10^5$ bacteria / ml. The "gold standard" technique culture cannot detect *M. tuberculosis* in CSF and considered less sensitive and time consuming [3]. Delay in diagnosis and treatment leads to poor prognosis and development of sequelae in around 25% of cases [4]. Serological tests which detect antigen and antibodies against *M. tuberculosis* in CSF were not recommended routinely for TBM diagnosis [5]. Today molecular techniques which amplify specific target regions of *M.tuberculosis* genome such as polymerase chain reaction are found to be rapid with high sensitivity and specificity. Researchers have targeted various regions like IS6110 region, protein antigen B, devR, 38KD protein, 65kDa, MPB64 and TRC4 gene for the diagnosis of TBM from CSF [3-9]. Due to the repetitive nature, high amplification efficiency and its presence only in *M. tuberculosis* complex (MTBC) PCR targeting IS6110 region is routinely employed for TBM diagnosis [5]. Though researchers applied several techniques, until today there is no single rapid technique that can detect *M. tuberculosis* in TBM patients with high sensitivity. Hence, in this study, results from a combination of tests like Ziehl Neelsen (ZN) smear microscopy,

culture on LJ & MGIT and IS6110 PCR were analyzed for the diagnosis of TBM.

## MATERIAL AND METHODS

Institutional Human Ethical Committee (IHEC) has approved this research and the work was carried out in compliance with the principles of the Declaration of Helsinki. CSF samples were collected after obtaining written informed consent from patients or from relatives in case of unstable patient. Between June 2011 and October 2014, 43 CSF specimens collected from patients with clinical suspicion of TB Meningitis were included. Pulmonary tuberculosis patients who are confirmed bacteriologically and/or clinically/radiologically and patients treated with ATT (Anti-tuberculous treatment) drugs for any forms of tuberculosis were excluded from our study. Following sterile precautions, CSF was transferred to sterile screw capped falcon centrifuge tubes in a Type 2B Bio safety cabinet and centrifuged at 3500 rpm for 15 minutes. The supernatant was discarded and the deposit was used for smear and inoculation on LJ and MGIT960. After inoculation, LJ slants and MGIT tubes were incubated and monitored for the presence of AFB growth up to eight and six weeks respectively. As control, five CSF samples from non-infectious meningitis were included. A small portion of the sample was preserved at -20°C separately, for performing TB PCR. IS6110 PCR was carried out for 19 CSF only using Amplification Reagent Set for Mycobacterium tuberculosis, Bangalore Genei, Bangalore, India. DNA from CSF was extracted using guidelines recommended by the manufacturer for nPCR. This is single–tube nested PCR with two – step sequential assay. In the first step, 220 bp Insertion sequence (IS6110) was amplified and in second step nested primers were added to the amplicon to further amplify the 123 bp region. Gel electrophoresis was carried out and amplified products were observed using gel documentation system (BIO-RAD, Hercules, CA, USA). Positive and negative controls provided in the kit were included and monitored to avoid false positivity and carry over contamination.

## RESULTS

All 43 CSF samples from clinically suspected TBM patients and five controls were uniformly negative in smear and culture (LJ and MGIT). Seven among the randomly selected 19 samples were positive in TB PCR (36.8%). Among the CSF positive cases 85.7%, cases were male and 14.3% cases were female. All the control samples were negative by PCR.

## DISCUSSION

Clinical diagnosis of TBM is a challenge because of widely varying clinical presentation between patients. Since the diagnosis cannot be made based on clinical features alone, TBM is often diagnosed empirically after brain damage. Hence prompt diagnosis is always supported by demonstration of bacilli from CSF along with other laboratory parameters and neuroimaging techniques. Because CSF profile of TBM mimics both infectious and non-infectious meningitis demonstration of bacilli from CSF confirms the diagnosis.

Ziehl Neelsen smear microscopy is economical but is less sensitive in case of paucibacillary specimens particularly body fluids. AFB microscopy needs $10^4$–$10^6$ bacilli/mL for smear positivity and hence most of the time it turns out to be negative making the diagnosis difficult [3]. As acid fast smears of CSF are usually negative, treatment of TB meningitis depends upon high index of clinical suspicion and rapid molecular methods rather than smear and culture. Review conducted by Kusum *et al* and other authors [3, 10-12] report sensitivity ranging from 0– 27.3% and specificity of 100% for CSF microscopy. According to Feng *et al* [12] patients positive for AFB in CSF microscopy will have significant evidence of lung tuberculosis on chest radiograph and have higher numbers of CSF white cells particularly neutrophils. In our study we have excluded pulmonary tuberculosis patients. Thwaites *et al* [13] observed that the diagnostic performance could be increased by including at least 6 ml of CSF and screening for at least 30 minutes. Another study from China [14] reported that pre-treatment of CSF leucocytes with triton prior to ZN staining will increase the sensitivity. Quantity of most of our CSF samples referred to Microbiology was between 2 to 3 ml only and this is an important factor for smear and culture negativity.

In developing countries like India, conventional LJ culture is used routinely for isolation and it consumes 4-6 weeks for culture positivity. Most of the researchers reported LJ CSF culture positivity between 0-17percent [3, 10, 15-17]. In our study all CSF specimens were negative by culture. Venkataswamy *et al* [18] reported a little higher percentage of 39% from LJ. They also reported that 7% of isolates recovered from CSF had grown

exclusively in LJ and not in liquid system. This is explained as some isolates were unable to metabolize the palmitic acid in BACTEC 12B medium (radiometric system) or due to absence of some growth promoting factors in the BACTEC 12B that are present in the egg-based medium. Thus they stressed the need for inclusion of LJ media for isolation, as no single medium could recover all the culture positive specimens. Fluormetric systems include BacT/ALERT and MGIT960. Liquid culture system is sensitive and rapid compared to solid media. Sastry *et al* [15] reported sensitivity and specificity of 25.8% and 100% employing BacT/ALERT for isolation. Few authors have reported CSF positivity ranging from 5.4% to 27.4% in MGIT960 [19, 20]. In our study, all specimens were negative using the MGIT automated system. This could be perhaps due to our samples did not contain the optimal quantity (6 ml). According to Venkataswamy *et al* [18], increasing the duration of incubation up to 10 weeks during culture might increase the sensitivity. Thus there is a need for a diagnostic technique with short turnaround time, high sensitivity and specificity.

Today molecular techniques like PCR are widely used in the diagnosis of TBM. Research works targeting IS6110 region by PCR reported positivity ranging from 8.5% to 80.5% [21, 6]. Iqbal *et al* [17] observed that, while 58.5% of the CSF was positive by PCR only 7.3% and 17% were positive by smear and LJ respectively. In our study 36.8% samples were positive by PCR targeting IS6110 region, whereas all were negative by smear, LJ and MGIT culture showing the superiority of PCR over culture. However, PCR positivity should always be interpreted with caution, since TB PCR is yet to be approved by Indian FDA as a diagnostic test. Sattar *et al* [4] and Narayanan *et al* [6] demonstrated the increased positivity from CSF for TRC4 primer compared to IS6110 region. Sattar *et al* [4] reported 83.33% positivity while using TRC4 primers and 66.67% with IS6110 primer, whereas Narayanan *et al* [6] reported 91% by TRC4 and 80.5% positive by IS6110 PCR, among the culture positive CSF samples. Thus compared to IS6110, TRC4 primer is superior in diagnosing tuberculous meningitis because single copy of IS6110 region is present in 40% of *M. tuberculosis* and even absent in 4% of the strains from South India [22]. Thus Mycobacteriologists have recommended that detection of several target genes will increase sensitivity and eliminate false positive and false negative results [6, 9]. Various studies have reported overall sensitivity and specificity ranging between 32-100% and 38-100% respectively for IS6110 PCR [5]. In some cases of TB meningitis, PCR could be a valuable tool. Narayanan *et al* [6] has insisted the need for research in PCR to make it very specific, simple and cost effective. Unfortunately, until today there is no rapid and sensitive single technique which could help in diagnosing TBM and hence always has to rely on multiple modalities for diagnosis.

**Limitations of the study**

1. Only small volume of CSF samples (1-2ml) were received. Larger volume upto 6ml could have yielded additional positivity.

2. PCR targeting a single common gene IS6110 was carried out using commercial nested PCR. Inclusion of multiple genes targets like TRC4, GlcB or HspX Antigens or devR DNA MPB64would have increased our positivity [4, 8-10].

## CONCLUSION

Diagnosis of TBM by conventional smear microscopy and culture on LJ and automated MGIT960 is less sensitive and time consuming, hence the dependence on molecular techniques. The results of molecular techniques are to be interpreted with clinical correlation, since Indian FDA has not yet approved TB-PCR for diagnostic purpose.

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**Conflicts of interest**

Authors have no conflicts of interest to disclose.

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