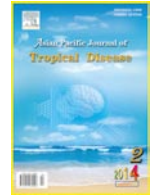




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Performance of newer and conventional diagnostic methods in detection of drug sensitive and resistant tuberculous meningitis

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ABSTRACT

Objective: To compare the performance of traditional and newer diagnostic methods for diagnosis of tuberculous meningitis (TBM) along with detection of drug resistant TBM.

Methods: Single cerebrospinal fluids sample from 281 suspected TBM patients was processed during August 2011 to July 2012 for acid fast bacilli (AFB) examination by Ziehl–Neelsen and auramine staining methods, AFB culture on Lowenstein–Jensen culture media, by microscope observation of drug susceptibility testing assay, and nucleic acid amplification tests by in-house conventional PCR and in-house real time PCR targeting IS6110 insertion sequence. All the isolates were subjected to drug susceptibility testing for isoniazid, rifampicin, ethambutol and streptomycin by 1% proportion method.

Results: The percentage positivity by AFB smear examination and culture on Lowenstein–Jensen media was 21% (59/281), 11.4% (32/281) respectively. The detection rate by conventional PCR was 33.1% (93/281). The real time PCR showed positivity rate of 44.8% (126/281). The extra detection by real time PCR was 11.7%. Only one isolate was multiple drug resistant and 22 (68.8%) were pan-susceptible. Remaining eight isolates showed either mono/poly drug resistant to first line antitubercular drugs.

Conclusions: Real time PCR is a more sensitive and rapid method if it is appropriately adopted in clinical practice for diagnosis of TBM. Prevalence of multiple drug resistance *Mycobacterium tuberculosis* causing TBM is low.

1. Introduction

The central nervous system tuberculosis is one of the devastating manifestations of extra pulmonary tuberculosis with high rate of morbidity and mortality. It accounts for approximately 1% of all cases of tuberculosis[1], presenting as tuberculous meningitis (TBM), arachnoiditis and tuberculoma with TBM being

the commonest. Unless rapidly diagnosed and treated, it leads to severe long term sequelae and high rate of mortality. Without treatment, death usually occurs within 5–8 weeks[2]. There are reports of high prevalence of drug resistant pulmonary tuberculosis, in India and other developing countries[3]. Drug resistant [especially multiple drug resistance (MDR)] tuberculosis is also difficult to treat. Not much data is available on drug resistant extra pulmonary tuberculosis, including TBM[4]. Since the complication, sequelae and mortality can be prevented by early diagnosis, knowing the prevalence of drug resistant TBM in this setting and early initiation of appropriate treatment, by application of the rapid

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and accurate diagnostic methods may prove useful to the patient in terms of better outcome and survival. Traditional staining methods used for diagnosis still hold good as they are easy to perform, rapid and inexpensive. However, their performance varies greatly with the observer and bacillary load. Conventional culture methods are time consuming. It takes approximately 8 to 12 weeks for the organism to grow in Lowenstein–Jensen (LJ) media. Newer automated methods are rapid, accurate but expensive and take about 5 to 42 d. Microscopic observation of drug susceptibility (MODS) testing assay, one of the liquid culture method for *Mycobacterium tuberculosis* (*M. tuberculosis*) growth and drug susceptibility testing detection is inexpensive and rapid[5]. PCR has gained popularity in recent years due to high clinical application along with substantial reduction in cost of reagents.

In the present study, we aimed to compare the performance of smear microscopy, culture methods including MODS and nucleic acid amplification methods targeting IS6110 insertion sequence in diagnosing TBM. Drug resistance patterns of mycobacterial positive isolates were studied.

2. Materials and methods

Cerebrospinal fluids (CSF) collected from the patients during August 2011 to July 2012 with suspicion of TBM were processed in bio-safety cabinet class II. All the samples irrespective of sample volume were centrifuged aseptically at 4 °C, 3000 r/min, for 10 min. CSF volume which was too low and could not be centrifuged was excluded from the study. Supernatant was discarded, except a small volume (approximately 100 µL), which was used for suspension of pellet. One loop full of sediment was used for each smear preparation and two loops full were used for inoculation on each LJ media bottle. Remaining sample was used for DNA extraction. Smear examination for acid fast bacilli (AFB) was done after Ziehl–Neelsen staining and auramine staining[6,7]. All samples were subjected to *M. tuberculosis* culture on LJ media with and without Para–Nitro Benzoic acid[8]. Drug susceptibility testing was done for isoniazid, rifampicin, ethambutol and streptomycin on all isolates by 1% proportion method[8]. Our laboratory is under external quality control for culture and first line drug susceptibility testing (provided by the National JALMA Institute for Leprosy and Other Mycobacterial Diseases,

Agra, India). MODS assay was done as previously described[5]. Early mycobacterial growth appears as small curved commas or spirals, and then progressed to thin cords later to more irregular serpentine cords. Any doubtful growth was confirmed by Ziehl–Neelsen staining. If two or more colonies (≥ 2 CFU) in drug free wells were detected then the result was considered positive. Growth in the drug free wells, but not in drug containing wells indicated susceptibility. Growth in the drug free wells as well as in drug containing wells indicated resistance.

2.1. DNA extraction

DNA extraction was done by phenol chloroform method[9]. The extracted DNA was amplified by conventional PCR and real time PCR methods. A positive and negative control was run in each batch of conventional and real time PCR.

2.2. Conventional PCR

Primers for IS6110 insertion segment amplification (synthesized by Bangalore, Genei, India) were used (forward primer: 5′CCT GCG AGC GTA GGC CTC GG3′ and reverse primer: 5′CTC GTC CAG CGC CGC TTC GG3′)[9]. Amplification reaction was set in a final volume of 25 µL. Total 5 µL of extracted DNA was added to 20 µL of PCR mixture. PCR mixture contained 25 mmol/L MgCl₂, 20 pmol of each primer, 10× *Taq* buffer, 10 mmol/L (each) dNTPs and 2.5 unit of *Taq* DNA polymerase. The reaction cycle was carried out for 35 times in thermal cycler (Quanta Biotech–96). Results were analyzed on 1.2% agarose gel electrophoresis. A sharp band of 123 bp of amplified DNA if visualized was considered to be positive[9].

2.3. Real time PCR

Same primers were used as in conventional PCR. The reaction was performed in a final volume of 10 µL containing 7 µL PCR master mix and 3 µL template DNA. [PCR master mix: SYBR Green master mix (SYBR Premix Ex *Taq*, Takara) 5 µL, nuclease free distilled water 1.78 µL, the target specific forward primer and reverse primer [0.11 µL (10 pmol/µL) each]]. The reaction was optimized in Light cycler II (Roche Diagnostics) to obtain the best amplification kinetics; cycle conditions were initial denaturation for 10 min at 95 °C followed by 40 cycles of denaturation for 10 seconds at 95 °C, annealing for

20 seconds at 63 °C and extension for 15 seconds at 72 °C. Melting curve kinetics were 95 °C for 2 seconds, 55 °C for 40 seconds then taken to 95 °C. Fluorescence was recorded continuously. Single sharp melting temperature peak at 89.0 °C was considered positive.

2.4. Controls

The extracted DNA from the *M. tuberculosis* standard strain H37Rv was used as the positive control and nuclease free water was used as negative control. A known culture positive sample and nuclease free water were used as positive and negative extraction controls with each run. Every amplification reaction included positive and negative controls along with positive and negative extraction controls.

2.5. Statistical analysis

Percentages were calculated wherever required. Percent positivity of each test was determined. Since this study did not have well characterized gold standards, and diagnostic accuracy of each test could not be ascertained.

3. Results

Total 281 CSF samples from equal number of TBM

suspects were tested. AFB smear examination by both Ziehl–Neelsen staining and auramine staining method detected 59 (21.0%) AFB positive samples. There was no discrepancy in Ziehl–Neelsen and auramine staining results. The culture on LJ media isolated *M. tuberculosis* from 32 (11.4%) samples and isolated mycobacteria other than tuberculosis from one (0.4%) sample. The contamination rate on LJ media was 2.1% (6 samples) (Figure 1). Total 35 (12.5%) AFB positive samples were negative on LJ media; however, 9 (3.2%) samples which were negative by AFB smear examination methods had shown the growth of *M. tuberculosis* on LJ media.

The molecular diagnostic methods detected all the positives detected by AFB smear examination methods, and culture methods. One isolate which was detected as mycobacteria other than tuberculosis on culture was not detected by tuberculosis–PCR. Conventional PCR detected 93 (33.1%) samples as positive. Extra 26 (9.3%) samples which were negative by AFB smear examination and culture were detected by conventional PCR. The real time PCR showed positivity rate of 44.8% (126/281). It has picked up all samples positive by AFB smear, culture and conventional PCR. Total 33 samples (11.7%), which were negative by all other methods, were detected by real time PCR (Figure 1).

Drug susceptibility testing was done on 32 isolates. A total of 22 (68.8%) isolates were pan susceptible (sensitive to all the 4 drugs). Only one (3.1%) isolate was MDR (both

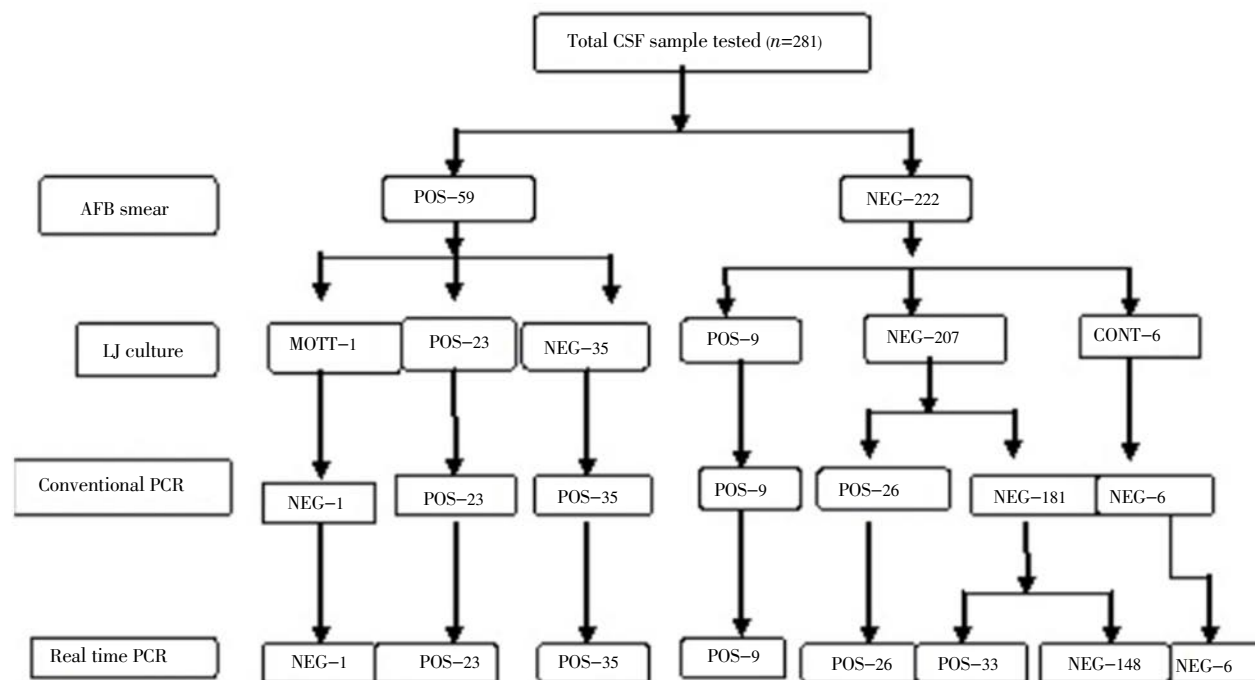


Figure 1. Flowchart of results obtained by various microbiological methods (AFB smear, LJ culture, Conventional PCR, Real time PCR) with CSF samples of TBM suspects. POS: Positive sample; NEG: Negative sample; CONT: Contaminated; MOTT: Mycobacteria other than tuberculosis.

isoniazid and rifampicin resistant). No mono rifampicin resistance was detected. The details of drug susceptibility testing are shown in Table 1.

Table 1

Resistance to first line anti-tubercular drugs in clinical isolates of *M. tuberculosis* from CSF of cases with clinical diagnosis of TBM.

Drugs	No. of positive isolates n=32 (%)
Pan sensitive	22 (68.8%)
Mono-R* to H	3 (9.4%)
Mono-R to R	Nil
Mono-R to E	2 (6.3%)
Mono-R to S	2 (6.3%)
R to H & E	1 (3.1%)
R to H & S	1 (3.1%)
H,R** & E	1 (3.1%)

*R: Resistance; **H, R: MDR isolate; H: Isoniazid; R: Rifampicin; E: Ethambutol; S: Streptomycin.

MODS assay was done only on 181 samples. Other samples could not be processed as the sample volume was low. *M. tuberculosis* was isolated from 13 samples (7.2%) and mycobacteria other than tuberculosis from one sample (0.6%). One (7.7%) was MDR, two (15.4%) showed monoresistance to isoniazid and 10 (79.9%) isolates were sensitive to both isoniazid and rifampicin. No discrepancy was noted between results of drug susceptibility testing with 1% proportion method and MODS assay.

4. Discussion

Laboratory confirmation of TBM was possible in 126 samples (44.8%) in our study. Only one case was MDR-TBM. MODS assay was less sensitive (7.2% positivity) than other modalities of testing, including cultures on LJ media (11.7% positivity). Stewart *et al.* detected 91/100 cases of TBM by Ziehl-Neelsen staining and all were subsequently confirmed by LJ culture^[10]. Simple measures like increased volume of CSF (at least 6 mL) and examination time at least 30 min can improve diagnostic performance^[11]. Various studies have reported detection rate of CSF samples from TBM patients by LJ culture ranging from 10.2% to 55.8%^[12]. Later conducted studies however, showed low positivity of approximately 4% in LJ culture^[6,13]. We reported higher positivity of AFB smear examination than culture. Culture supposedly provides higher positivity than smear examination. We accepted all the CSF samples irrespective of the volume of CSF. Excluding samples with low volume would have increased the culture yield. However, since CSF is a precious sample we decided not to exclude any samples on the basis of low volume. Culture positivity remains

low in case the patient is already on anti-tubercular treatment. We could not analyze the history of drug intake for each case. We also used solid culture method (LJ media). Using liquid media may have increased the culture positivity.

The percentage positivity was more by conventional PCR as compared with traditional methods. In our previous study it was shown that IS6110 is a reliable target sequence with high sensitivity and specificity, even in Indian wild strains of *M. tuberculosis*^[9]. In previous studies, PCR assays targeting the IS6110 insertion sequence revealed overall 70%–98% sensitivity and 80%–100% specificity for TBM diagnosis^[14]. However, a potential problem with using IS6110 target is that some strains from certain parts of the world lack the IS6110 insertion sequence^[15]. A study from South India suggested PCR using one target alone cannot detect all strains of *M. tuberculosis* but the use of more than two targets (IS6110 and TRC4) can improve detection of TBM and tubercular pleuritis^[16]. The maximum detection was possible by real time PCR along with certain advantages like real time PCR is more rapid than conventional PCR. Conventional PCR requires agarose gel electrophoresis, which may result in laboratory contamination, but post amplification gel electrophoresis is not required for real time PCR. Assay has increased sensitivity to detect even small amount of *M. tuberculosis* DNA with high positivity, sensitivity and specificity in extra pulmonary tuberculosis by real time PCR amplifying the same target (IS6110)^[17,18].

Published reports of MDR in TBM are very limited. In one of our recent publication^[19], similar results were seen although high prevalence of MDR in Indian pediatric meningitis cases was reported in other studies^[6]. A retrospective study conducted in South Africa showed 8.6% MDR in CSF isolates^[20]. High drug resistance in TBM (13% monoresistance, 32.6% polyresistance, 8.7% MDR) was reported from Vietnam^[21].

Evaluation of MODS in detection of drug resistance in pulmonary tuberculosis was done by our group and high sensitivity (92.7%) and specificity (98%) of detection of rifampicin resistance was observed^[22]. Other studies have shown low positivity and sensitivity of MODS^[23,24]. We found MODS to be less sensitive for detection of *M. tuberculosis*, however, the susceptibility data matched with that of 1% proportion method.

Real time PCR is a sensitive and rapid method if appropriately adopted in clinical practice for diagnosis of TBM. MDR-TBM is fortunately low in our setting; however, a continuous surveillance is needed.

Conflict of interest statement

We declare that we have no conflict of interest.

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