Original Research Article

A Study on Comparison and Evaluation of Sensitivity and Specificity of Different Staining Methods with Conventional Polymerase Chain Reaction among Meningitis Cases - A Prospective Cross Sectional Study

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A B S T R A C T

A fast and accurate diagnosis is necessary to control and eliminate tuberculosis (TB). In India TB continues to be a serious public health problem. In this study, diagnostic tests on clinical samples from patients suspected to have TBM were performed and the sensitivity and specificity of the various techniques were compared. The main objective of the study was to compare various staining methods such as acid fast staining and fluorescent staining with conventional polymerase chain reaction and evaluate their sensitivity and specificity for detecting TBM. We have developed specific molecular method suitable for detection of mycobacterium tuberculosis bacteria in CSF of TBM patients. The present study was conducted over a period of one and a half year from March 2014 – August 2015, 100 CSF samples collected from clinically suspected cases of TBM from various wards and ICUs of Gandhi Hospital were tested with smear microscopy, acid fast staining, fluorescent staining and conventional polymerase chain reaction by using different molecular probes. The study results revealed that among 100 clinically suspected cases, 12 cases were confirmed TBM by PCR. The sensitivity and specificity of Ziehl Neelsen staining was 8.3% and 100% respectively. Positive predictive value (PPV) and Negative Predictive Value (NPV) of Ziehl Neelsen staining was 100% and 88.88% respectively when compared to PCR. One sample which was positive by Ziehl Neelsen staining was also positive by PCR. In addition to this, PCR could pick up 11 more cases which were negative by Ziehl Neelsen staining. Sensitivity and specificity of fluorescent staining was 16.6% and 100% respectively when compared to PCR. Positive predictive value and negative predictive values of fluorescent staining was 100% and 89.7% respectively when compared to PCR. Two samples that were positive by Fluorescent staining were also positive by PCR. In addition to this PCR could pick up 10 mores cases which were negative by Fluorescent staining. The p value was found to be significant statistically which is < 0.05. Among 100 clinically suspected cases of TBM, multiplex PCR was able to detect 12 cases where as uniplex PCR detected 2 cases. The study results can be used to improve existing TB diagnosis procedure as well as for comparing the effectiveness of the Polymerase chain reaction with staining methods. TBM is still a diagnostic problem, so we need accurate detection for diagnosis and treatment. PCR is simple, sensitive, reliable and most effective molecular method for detection of TBM in CSF of Tuberculosis patients.

Introduction

Tuberculosis has for centuries continued to be a public health problem of enormous importance, particularly in the developing world. In India, about 22 lakh cases of tuberculosis are added every year. The mortality rate is 5,00,000 per year, more than 1,000 per day, i.e., one every minute.

Tuberculous meningitis (TBM) is the most severe form of tuberculosis and causes substantial morbidity and mortality (Marais S, *et al.,* 2010). The early diagnosis of and prompt initiation of chemotherapy for TBM are crucial to a successful outcome. However, the early and accurate detection of *Mycobacterium tuberculosis* in the cerebrospinal fluid (CSF) of TBM patients still remains a challenge for clinicians, mainly due to the lack of rapid, efficient, and practical detection methods (Thwaites and Tran, 2005).

Currently, mycobacterial culture is the gold standard for detecting *M. tuberculosis*, but it is time-consuming and requires specialized safety procedures in laboratories (Mishra *et al.,* 2005; Radha Krishna *et al.,* 1990). Serological methods are convenient but lack sensitivity and specificity (Attallah *et al.,* 2003; El-Masry *et al.,* 2008). Although the PCR technique is rapid, it is costly for routine use in developing countries where most tuberculosis cases occur (Dora *et al.,* 2008; Lorino *et al.,* 1999; Pai *et al.,* 2003; Pal and Desai, 2007). Conventional smear microscopy with the Ziehl-Neelsen (ZN) stain is a rapid and practical method for detecting acid-fast bacilli (AFB), especially in low-income countries, due to its rapidity, low cost, and high positive predictive value for tuberculosis (Koch and Cote, 1965). However, the Ziehl-Neelsen method is severely handicapped by its low detection rate, ranging from 0 to 20% for CSF specimens (Trusov *et al.,* 2009;

Yeager *et al.,* 1967) one of the main reasons behind this is that *M. tuberculosis* can hardly be stained by acid-fast dyes once it enters the cells. Another important reason is that the Ziehl-Neelsen method requires a large volume of CSF for TBM diagnosis, as it is incapable of detecting bacilli that are fewer than 10,000 in number per slide or per ml of specimen (Trusov *et al.,* 2009; Yeager *et al.,* 1967). Therefore, it is important to develop an alternative, cost-effective method for detecting intracellular *M. tuberculosis*. Additionally, knowing which cell type is infected by *M. tuberculosis* in the CSF of TBM patients could help us to unravel new antituberculotic candidates (Gaspar *et al.,* 2008). Methods for the diagnosis of tuberculosis improved in recent years and several molecular techniques for its diagnosis have been introduced for clinical use. Molecular methods provide several advantages, including confirmation of the presence of *M. tuberculosis* within 1 to 3 day (lau *et al.,* 1990; Mishra *et al.,* 2005). Newer molecular techniques such as polymerase chain reaction (PCR), although rapid, are costly to be routinely used in developing countries where most TB cases occur (Goel *et al.,* 1990). To overcome these limitations with conventional methods, quicker, specific, and sensitive diagnostic methods are being sought for the diagnosis of TBM. Many gene amplification techniques for direct detection of *M. tuberculosis* are being attempted in research laboratories, with polymerase chain reaction (PCR) being one of them. Various species-specific primers are available for the detection of *M. tuberculosis*. The present study was conducted to find out the role of PCR using the primers (IS6110, MPB64, Protein B, and TRC4) in the diagnosis of TBM. Also there is no previous literature related to the comparison of Acid fast staining (AFB) smears and Fluorescent staining with conventional chain polymerase reaction. PCR using different primers had significant

advantage over the conventional staining methods to detect the presence of *M. tuberculosis* in specimens of clinically suspected cases of TBM.

Study centre

This study was carried out in the Department of Microbiology, Gandhi Medical College, Secunderabad, Telangana during the period March 2014 –August 2015. Ethical clearance was obtained from institutional ethical committee. Informed or written consent was taken from the patients or relatives and clinical and demographic data was obtained by structured proforma.

Materials and Methods

Collection of samples

100 CSF samples collected from clinically suspected cases of TBM from various wards and ICUs of Gandhi Hospital were processed.

Selection of cases was according to Thwaites *et al.,* [124] clinical entry criteria for Tuberculosis meningitis diagnosis which is defined as symptoms and signs of meningitis including one or more of the following:

Headache, meningeal irritability vomiting, fever, neck stiffness, convulsions, focal neurological deficits, altered consciousness and lethargy.

Study period

The present study was conducted over a period of one and a half year from March 2014 – August 2015.

Study type

Prospective Cross - sectional study.

Sample size

Type of sample- Cerebrospinal fluid (CSF) Sample size for the study was 100 clinically suspected cases of tuberculous meningitis.

Inclusion criteria

Headache, meningeal irritability, vomiting, fever, neck stiffness, convulsions, focal neurological deficits, altered consciousness and lethargy with any one of the following findings:

Biochemical - for CSF, proteins more than 60mg%, sugar < 45 mg% or $< 2/3^{rd}$ of blood sugar

Pathological- cell count more than 5-10/hpf, lymphocytic pieocytosis

Radiological -CT scan showing tubercular changes

Supportive evidence - family history of tuberculosis/positive tuberculin test.

HIV reactive cases

All age groups in both the sexes

Exclusion criteria

All cases which show presence of pus cells and bacteria in Gram staining

Trauma cases

Sample collection

CSF samples drawn aseptically by the treating physician were collected. One part was processed immediately and another part was stored in -20^0C for molecular methods. Gram staining, ZN staining, fluorescent staining, CSF analysis, and culture was done by

inoculation of a centrifuged CSF sample on chocolate agar and blood agar incubated at 37°C to rule out pyogenic meningitis.

Microscopical smears

For each sample, three smears were made from the centrifuged deposit on new glass slides. Smears were air dried and heat fixed. Gram staining, Ziehl Neelsen staining (ZN) and Fluorescent staining was done for each sample. The staining was done as per the standard protocols (Collee *et al.,* 2008; Cruickshank *et al.,* 1975).

Polymerase Chain Reaction

The main steps involved in PCR are DNA extraction, Amplification and Analysis of product.

DNA was extracted from the samples using Qiagen DNA Extraction mini kit (QIAGEN GERMANY).

In amplification, two types of PCR are performed Multiplex and Uniplex PCR (Kusum Sharma *et al.,* 2013), depending up on cycling conditions.

Multi targeted PCR (Multiplex PCR)

Designed to amplify IS6110, MPB64 and Protein antigen b was carried out using six primers in MTB complex with band sizes of 123bp for IS6110, 240bp for MPB64, 419bp for Protein antigen b (Kusum Sharma *et al.,* 2013). PCR Cycle was repeated for 40 cycles.

Uniplex PCR designed to amplify 173bp of TRC4 gene was carried out using two primers, in the genome of *M. tuberculosis.* Band size is 173bp. PCR cycle was repeated for 35 cycles (Ramya Barani *et al.,* 2012).

Working primers were reconstituted to 20pmol/ul, by adding 100ul of stock primers solution to 400ul of DEPC molecular grade water. Each reaction volume was 50μl of master mix.

Controls

Positive control: *M. tuberculosis*, H37Rv strain

Negative control: Reagent control without organisms.

Analysis of products:

The amplified PCR product was subjected to 2% agarose gel electrophoresis with ethidium bromide and bands were visualized under UV illuminator.

The bands were seen in Gel doc system at different levels

IS6110	at 123bp
MPB64	at 240bp
Protein b	at 419bp
TRC4	at 173bp

All the observations were noted and analysed.

Data analysis

All of the smear slides stained by the conventional or modified method were observed under oil immersion at a magnification of ×1,000. A total of 300 visual fields on each slide were observed, among which AFB-positive fields were counted by three experienced observers independently. All of the data were displayed as means ± standard errors of the means (SEM) and analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Results and Discussion

In the present study among 100 clinically suspected Tuberculous meningitis (TBM) cases, 12 cases were confirmed TBM cases by PCR (Table 1).

Among 100 clinically suspected TBM cases, 62% were males and 38 % were females with the male to female ratio of 1.63: 1.Most of the them (33%) belonged to age group 20-29 yrs. (Table 2)

In Confirmed TBM cases most of them belonged to age group of 20-29 yrs. 58% are males and 41.6% are females with male to female ratio of 1.4:1(Table 3).

Among 100 clinically suspected TBM cases, positivity for AFB by Ziehl-Neelsen staining was 1% (Table 4, Figure 4).

Among 100 clinically suspected TBM cases, detection of AFB by Fluorescence Microscopy was 2% (Table 5).

Sensitivity and specificity of Ziehl Neelsen staining was 8.3% and 100% respectively when compared to PCR.

Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Ziehl Neelsen staining was 100% and 88.88% respectively when compared to PCR.

One sample which was positive by Ziehl Neelsen staining was also positive by PCR. In addition to this PCR could pick up 11 more cases which were negative by Ziehl Neelsen staining (Table 6).

Sensitivity and specificity of Fluorescent staining was 16.6%% and100% respectively when compared to PCR. Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Fluorescent staining was 100% and 89.7 % respectively when compared to PCR.

Two samples that were positive by Fluorescent staining were also positive by PCR. In addition to this PCR could pick up 10 mores cases which were negative by Fluorescent staining. The p value was found to be significant statistically which is < 0.05 (Table 7).

Among 100 clinically suspected cases of TBM, multiplex PCR was able to detect 12 cases where as Uniplex PCR detected 2 cases (Table 8).

Out of the various genes (IS6110, mpb64, protein b, TRC4) screened among clinically suspected cases by multiplex and uniplex PCR, IS611O was detected in all the 12 samples.

Whereas mpb64, protein b were detected in 7 samples and TRC4 in 2 samples. IS6110 was found to be the sensitive gene which is detected in all the samples (Table 9).

According to Mann-Whitney U - test

The p value was found to be statistically significant ($p < 0.05$)

PCR is found to be more sensitive than ADA with significant p value (Table 10).

Tuberculosis meningitis (TBM) is a medical emergency with high mortality and morbidity that requires rapid detection of *Mycobacterium tuberculosis* for proper management because poor prognosis is directly related to delay in treatment.

To prevent the general increase in frequency and thus decreasing the mortality, new strategies for its control are needed such as multiple diagnostic modalities for rapid detection of *M. tuberculosis* to initiate early ATT, control transmission of TB, which has led to the assessment of several polymerase

chain reactions (PCR)-based procedures for detection of *M. tuberculosis* DNA in cerebrospinal fluid (CSF) samples (Desai *et al.,* 2002; Brienze, *et al.,* 2001; Deshpande *et al.,* 2007; Cheng *et al.,* 2004: Ho *et al.,* 2013).

Table.1 TBM Positives among clinically suspected cases (n=100)

	TBM positive	TBM negative	Total
Cases	12(12%)	88(88%)	100

Table.2 Clinically suspected TBM cases (n=100)

Age Group	Clinically suspected TBM cases(n=100)		
	Males (%)	Females (%)	Total No (n=100)
0 – 9 Yrs	7(7%)	0(0%)	7
10 – 19 Yrs	3(3%)	4(4%)	7
20 – 29 Yrs	19(19%)	14(14%)	33
30 – 39 Yrs	11(11%)	8(8%)	19
40 – 49 Yrs	6(6%)	5(5%)	11
50 – 59 Yrs	11(11%)	4(4%)	15
>60 Yrs	5 (5%)	3(3%)	8
Total	62(62%)	38(38%)	100

Table.3 Demonstration of AFB by Ziehl - Neelsen staining

Sample	No. of cases	No. of Positives
CSF	100	1(1%)
Controls	20	NIL

Table.4 Demonstration of AFB by Auramine phenol staining

Sample	No. of cases	No. of Positives
CSF	100	2(2%)

Table.5 Sensitivity of Ziehl Neelsen staining when compared with PCR

	PCR positive	PCR negative	Total
Positive by Ziehl Neelsen staining	01	00	01
Negative by Ziehl Neelsen staining	11	88	99
Total	12	88	100

Table.6 Sensitivity of Fluorescent staining when compared with PCR

	PCR positive	PCR negative	Total
Positive by Fluorescent staining.	02	00	02(2%)
Negative by Fluorescent staining.	10	88	98
Total	12	88	100

Table.7 Positivity of Multiplex PCR and Uniplex PCR among clinically suspected cases of TBM cases

MULTIPLEX PCR (IS6110,MPB64,Protein b)	UNIPLEX PCR(TRC4)
12 (100%)	2 (16.6%)

Table.8 Number of samples positive for individual genes among TBM positive cases

Samples positive for IS6110	Samples positive for MPB64	Samples Positive for	Samples positive for IS6110
12	7	7	2

Table.9 Clinical and laboratory parameters of patients with CSF specimen positive by PCR

S.No	Total WBC count	Lymphocytes	Protein	Sugar	Ada	Att outcome
1.	12	96	120	16	8	Improved
2.	40	80	34	28	12.4	Improved
3.	10	100	146	12	36	Improved
4.	5	80	144	54	8	Improved
5.	50	100	388	24	31	LAMA
6.	40	70	154	36	7	Expired
7.	6	100	24	88	8	Improved
8.	15	80	72	24	12	Expired
9.	5	95	76	20	10.4	Improved
10.	30	84	148	42	11.4	Improved
11.	6	70	22	56	6.1	Improved
12.	90	80	300	24	13	Improved

This study was undertaken to assess the utility of various diagnostic modalities for early diagnosis of tuberculosis meningitis. Also an attempt was made to compare the sensitivity of PCR with conventional diagnostic techniques for detection of MTB in CSF samples (Cheng *et al.,* 2004: Ho *et al.,* 2013). In the present study out of 100 suspected cases of TBM 12 were positive for *Mycobacterium tuberculosis* similar to previous studies (Aziz *et al.,* 2004). These results were correlated with other reports, 12.5% and 13.3% (Shastry *et al.,* 2013; Hooker *et al.,* 2003). Some other studies also showed low positivity of 1.7 % and 3.7% respectively (Shirani *et al.,* 2015). Whereas some previous studies reported high positivity of 41.6 %,

51.7% and 81.48 % respectively. (Shyam Chand Choudary *et al.,* 2015; Kusum Sharma *et al.,* 2010)

In the present study, male preponderance was observed however it was not significant statistically (p=0.78).

In the study Male to female ratio among clinically suspected cases of TBM was1.63: 1. These results were agreement with the findings of other studies 1:63:1 (Caws *et al.,* 2000). In a male dominated society, usually he is the earning member. As he goes out for work, he is more likely to come in contact with an active Tuberculosis case. Men are more likely to acquire habits like smoking and alcoholism which predispose to TB. In confirmed TBM cases, male preponderance was seen which was also observed (Shirani *et al.,* 2015).

In the present study, most common age group affected among clinically suspected TBM was 20-29 yrs. These results were similar with that of previous findings (Shyam Chand Choudary 2015; Shirani *et al.,* 2015). The reasons that make this age group vulnerable are, they are socially more active and are more exposed to an open case of TB than others. In some parts of the world HIV pandemic has contributed as people of this age group are sexually most active. In confirmed TBM cases, the most common age group affected is 20-29 yrs. In the present study, Ziehl Neelsen staining could detect 1% of cases among 100 clinically suspected TBM cases similar to the previous findings (Guyen *et al.,* 1996). Some other findings showed some variation in results, 1.81%, 2.85% (Kusuma Sharma *et al.,* 2015; Parija *et al.,* 2011).

The detection limit of microscopy is10^4 mycobacteria per milliliter whereas the majority of patients with TBM have fewer mycobacteria in their CSF samples and are thus missed as tuberculous meningitis cases. In the present study the sensitivity of ZN staining when compared to PCR is 8.3% which is nearer to previous findings, 7.72% sensitivity

respectively (Sajjid Iqbal *et al.,* 2011). In some studies conducted and showed higher sensitivity of 40.9%, while some studies reported lower sensitivity of 1.11% and 1% respectively (Kusum Sharma *et al.,* 2015; Guyen *et al.,* 1996).

This may be due to paucibacillary nature of specimens. PCR requires nearly 800 organisms/ml whereas Microscopy requires 10,000 bacilli per ml. CSF does contain few bacilli hence the low sensitivity.

In the present study the sensitivity of Auramine phenol staining when compared to PCR is 16.6% which is nearer to previous reports (Parija *et al.,* 2011), who reported 11.53% sensitivity respectively. In some studies conducted and reported a lower sensitivity of 4% (Shastry *et al.,* 2013).

Detection of AFB by Auramine phenol staining requires 10^4 organisms/ml like ZN staining, but Fluorescent microscopy is 10% more sensitive than ZN staining and more slides can be screened in lesser time.

Thus, PCR is a powerful and reliable technique for rapid diagnosis of *M. tuberculosis*, with a reported sensitivity of 55– 95% in culture positives but 100% in both smear- and culture-positive clinical specimens (Lau *et al.,* 1990). The usefulness of PCR in the diagnosis of TB by using a variety of unselected clinical specimens is not clear as studies have differed in techniques including lysing method and target nucleic acid to detect products as well as the number and type of samples used, making the reported sensitivities and specificities difficult to compare (Holodniy *et al.,* 1991; Schluger *et al.,* 1994). A recent study comparing four conventional techniques, FNA cytology, ZN staining, culture and lymph node biopsies, and TB PCR indicated 94.8%diagnosis but PCR was found to be highly sensitive (94.4%) though less specific (38.2%) (Singh *et al.,* 2000; Goel *et al.,* 2001). In the present study, PCR was found to be the most sensitive (89.5%) as well as specific

(86.1%) method for early diagnosis of tuberculosis.

In the Present study, among 100 clinically suspected cases of TBM, multiplex PCR (MPCR) was able to detect 12 cases where as Uniplex PCR detected 2 cases.

To increase diagnostic accuracy, multiplex PCR has been evaluated using three targets, i.e., IS6110, MPB-64 and protein 'b' together for diagnosis of *Mycobacterium tuberculosis* complex. Other studies which have evaluated two or three targets had put up separate PCRs for each reaction, which increased the cost of the test and also the chances of cross contamination. This method of using multiplex PCR for single PCR reaction reduced error, as well as cost, and increased the sensitivity of the test.

Previous studies reported, that multi plex PCR was shown to have good sensitivity and specificity. Multiplex PCR could potentially be used for patient care in endemic resource poor countries, where RT-PCR technologies is not feasible due to their cost and complexity. Multi plex PCR′s potentially improved sensitivity and specificity to diagnose TB as well as its easier application in resource poor areas, may hold the potential to give clinicians the tool they need to better diagnose and ultimately manage this deadly disease (Askin *et al.,* 2013).

Previous studies conducted at the Tuberculosis Research Centre have suggested that the sensitivity of PCR can be increased by using two sets of targets (IS6110 and TRC4) to detect tuberculous meningitis and tubercular pleuritis. Some previous studies suggested that the diagnosis of CNS tuberculosis requires not a screening examination but rather a definitive molecular procedure like IS6110 and mpb-64 gene characterization for accurate and confirm diagnosis of tubercular meningitis. It is shown that using multiple targets to detect TB on a single PCR reaction may increase the sensitivity of NAA tests for the diagnosis of TB (Singh *et al.,* 2015; Sharma *et al.,* 2013).

Out of the various genes (IS6110, mpb64, protein b, TRC4) screened among clinically suspected TBM cases by multiplex and uniplex PCR, 12 were confirmed by PCR, in which IS611O was detected in all the 12 samples, whereas MPB64, protein b were detected in 7 samples and TRC4 in 2 samples. IS6110 had shown100% positivity, whereas MPB64 and Protein b had shown a positivity of 58.33%, and TRC4 had shown a positivity of 16.6%.The reason for widely used IS6110 in PCR tests is the presence of its multiple copies in *M. tuberculosis* complex genome, which is believed to confer higher sensitivity.

MPB64 is the most specific sequence for the diagnosis of the MTB complex by PCR, because of lowest number of false positive results obtained (10%) in comparison to IS6110 (62%) and heat shock protein (38%) The MPB64 gene codes for the MPB64 protein and is a 240bp region (nucleotides 460–700).

Various studies done and the results were shown that IS6110 is the most sensitive gene, a member of IS3 family found in almost all members of the *M. tuberculosis* complex and had multiple copies more or less randomly distributed throughout the genome, with copy numbers ranging from 10 to 26 copies, made it an ideal target for PCR (Raffi *et al.,* 2007; Maurry *et al.,* 2012).

The overall aim of this study was to evaluate rapid, sensitive and specific tests like ZN microscopy, Fluorescent microscopy and PCR for diagnosis of Tuberculosis meningitis. TB PCR was found to be highly sensitive, specific and rapid method for early diagnosis of TBM. Although conventional diagnostic techniques remain the method of choice in regions with low-resource settings, PCR may be employed in cases with strong clinical suspicion and equivocal results, especially at an early stage of the disease, for better diagnosis, management and treatment. Given the acute need for simple, efficient and practical method for detecting *M. tuberculosis* within the cells and from small

CSF sample. In the present study finally we concluded that, this study shown low sensitivity of Ziehl Neelsen and Fluorescence staining, compared with conventional PCR. Detection of IS6110 PCR was found to be most sensitive, valuable and reliable tool in diagnosing TBM. Therefore, this method will be of tremendous value in improving both the diagnosis and treatment of tuberculosis.

Acknowledgement

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