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Comparative evaluation of IS6110 and protein antigen b PCR in cerebrospinal fluid for rapid diagnosis of tuberculous meningitis in children

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Abstract

Introduction. Childhood tuberculosis meningitis is a severe form of tuberculosis with high morbidity and mortality. The diagnosis is frequently missed and delayed due to lack of sensitive tests like acid-fast bacilli (AFB) smear and delayed results by culture.

Aims. To compare the role of IS6110 and protein antigen b PCR in cerebrospinal fluid (CSF) for rapid diagnosis of tuberculous meningitis (TBM) in children.

Methodology. Forty-five cases of TBM and 20 controls were enrolled in this prospective study.

Results. The mean ages of cases and controls were 4.2±0.5 years and 4.5±0.7 years, respectively. In the TBM group, two-thirds of the children were <4 years of age, and 62% were males. Sensitivities of AFB smear examination, Löwenstein–Jensen (LJ) medium and bactenecin (BACTEC) culture in cases were 4.4, 0 and 2.2%, respectively. The protein antigen b PCR was most sensitive as it was positive in 35 (77.8%) of TBM patients; IS6110 PCR was positive in 27 (60%) patients. Both PCR-based tests had higher positivity than conventional tests and BACTEC culture. No significant difference was seen between the PCR tests. Excellent agreement was observed between both PCR-based tests as they were concordant for 26 positive samples and 35 negative samples.

Conclusion. Protein b PCR is a sensitive and rapid method for the diagnosis of TBM (sensitivity 77.8%). Both PCRs were more sensitive than smear, LJ and BACTEC. The specificity of both PCR was 100%.

INTRODUCTION

Childhood tuberculosis has been a neglected disease resulting in significant morbidity and mortality in young children, especially in developing countries [1]. Children below 15 years of age constitute 15% of the total burden of tuberculosis in many developing regions [2]. Central nervous system (CNS) tuberculosis accounts for 5% of all extra-pulmonary tuberculosis cases. Tuberculous meningitis (TBM) is the most severe form of the disease, with 6–45% mortality and 19–87% morbidity [3–5].

Diagnosis of TBM in children from resource-limited settings is primarily based on the clinical manifestations, cerebrospinal fluid (CSF) analysis, neuroimaging characteristics, and response to anti-tuberculous therapy (ATT) [6]. Atypical clinical presentations, a paucibacillary disease in children, long turn-over time and low yield (8–10%) of routine cultures, and lack of a rapid, sensitive diagnostic test delay the diagnosis and institution of ATT [7]. Assays based on CSF adenosine deaminase (ADA), mycobacterial antigens and antibodies lack sensitivity and specificity. Hence, molecular techniques using PCR are gaining popularity due to their improved sensitivity (50–90%) and specificity (60–100%) [8, 9]. However, these tests are not routinely available in many developing countries, and there is a shortage of

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Abbreviations: ADA, adenosine deaminase; AFB, acid fast bacilli; ATT, anti-tuberculous therapy; CSF, cerebrospinal fluid; LJ culture, Löwenstein–Jensen medium; Pab, protein antigen B; PCR, polymerase chain reaction; PEM, protein energy malnutrition; TBM, tuberculous meningitis; TLC, total leucocyte count.

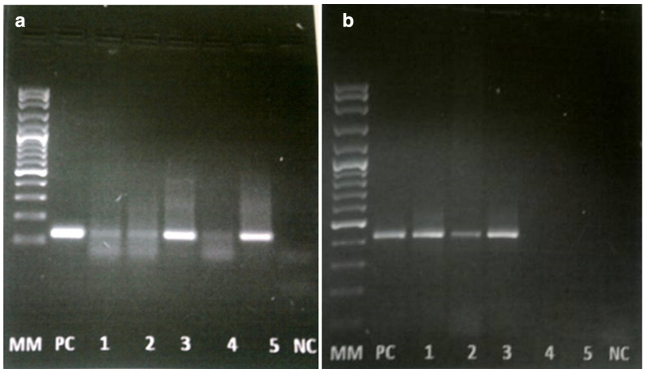

Fig. 1. (a) Gel photograph of protein antigen b PCR (lanes 1, 2, 3, 4, 5=positive sample) (b) Gel photograph of IS 6110 PCR (lanes 1, 2, 3=positive samples, lanes 4, 5=negative samples). MM, molecular marker, PC, positive control, NC, negative control.

clinical studies using PCR-based diagnostic tests in the CSF of children from these regions.

Additionally, the majority of the available studies have utilized the IS6110 sequence of *Mycobacterium tuberculosis*. However, nearly 40% of isolates in the Indian population either lack IS6110 or demonstrate very low copy numbers [10]. Another 38 kDa protein antigen b (Pab) specific for virulent *M. tuberculosis* has been used for the detection of bacilli in body specimens [11, 12]. Still, there is scanty information on the diagnostic utility of this antigen from developing countries. Hence, we conducted this study to evaluate the role of Pab-based PCR in CSF for the rapid diagnosis of TBM in children and to compare it with IS6110-based PCR and conventional diagnostic tests [smear for acid-fast bacilli (AFB), culture and bactenecin (BACTEC)].

METHODOLOGY

We conducted a prospective study over 1 year at a tertiary-care pediatric hospital in North India. The Institutional Ethics Committee approved the study. We enrolled 50 consecutive children between the ages 3 months and 12 years with a clinical suspicion of TBM (fever of >7 days duration associated with either change in sensorium, behaviour, seizures or focal neurological abnormalities), suggestive neuroimaging and CSF (cells >20/mm3 with more than 50% lymphocytes, >50 mg dl^{-1} proteins, blood: CSF glucose ratio <0.6). An informed, written consent of parents or assent from the patient if applicable was taken. Twenty-six consecutive children with non-TBM or non-infectious CNS illness undergoing a diagnostic lumbar puncture were enrolled as controls. All children with a previous diagnosis of TBM, ongoing ATT for >2 weeks before admission or refusal to participate in the study were excluded. We used a pre-structured proforma to note the clinical data [13]. A CSF sample between 500 µl–1 ml was centrifuged, and the deposit was used for smear formation, cultures (LJ medium and BACTEC) and PCR using standard techniques [14] in each patient. Relevant diagnostic tests from extraneural sites (gastric aspirate for AFB, fine-needle aspiration cytology or biopsy of specific areas) were done as and when required. The final diagnosis was concluded, once reports of all investigations were available, and the response to treatment after 2–3 weeks was assessed. Children were grouped into *TBM cases* (cases confirmed by demonstration of *M. tuberculosis* by staining and culture as well as cases with high clinical suspicion and response to treatment, but negative smear and culture) and *non-TBM controls*.

Procedure for TB-PCR

DNA was extracted using a commercially available Qiagen DNA extraction kit (Qiagen Company, Germany). The initial step is to keep the preliminary processed materials at 80 °C for 10 min for the inactivation of possible mycobacteria. The material was then processed as per the guidelines of the manufacturer of the kit to obtain the DNA. Two types of PCRs were performed on extracted DNA samples using two different primers in order to amplify two different regions of DNA. One primer was used to amplify a 419 bp sequence coding for the Pab antigen gene as per protocol [15]. DNA amplification of the 123 bp IS6110 insertion element was done by two oligonucleotide primers as below [14]:

Forward Primer Pabf**: 5′-ACCACCGAGCGGTTCG CCTGA-3′**

Reversed primer Pabr: **5′-GATCTGCGGGTCGTCC CAGGT-3′**

Forward primer IS6110f: **5′- CCT GCG AGC GTA GGC GTC GG-3′**

Reversed primer IS6110r: **5′CTC GTC CAG CGC CGC TTC GG-3′**

In each test, the results were compared with positive and negative control (Fig. 1). The positive control was DNA H_{37} RV strain, and negative control was double-distilled water. After DNA amplification, the samples were run on 1.5% agarose gel electrophoresis stained with ethidium bromide. The stained gel was examined under ultraviolet light to look for DNA bands of 419 bp and 123 bp using a 100 bp DNA ladder.

Quantitative variables are represented using measures of central tendency like mean and standard deviation for normally distributed data and median and inter-quartile range for skewed data. Qualitative or categorical variables were reported as frequencies and proportions. Proportions were analysed using Chi-squared or Fisher's exact test wherever applicable. To see the agreement between different procedures, the kappa test of the agreement was used. All statistical tests were two-sided and performed at a significance level of α=0.05. The study was approved by the institutional ethical committee.

RESULTS

Of the total 50 newly suspected cases of TBM, five patients left against medical advice. Hence, 45 cases of TBM were available

for analysis. Of these, three cases (7%) were confirmed by positive stain and/or culture, and 42 cases (93%) were diagnosed based on clinical criteria and response to treatment. The control group comprised children with acute bacterial meningitis (*n*=5), partially treated meningitis (*n*=4), viral meningoencephalitis (*n*=3), acute febrile encephalopathy (*n*=3), chronic meningitis (*n*=3), retroviral infection with meningitis (*n*=3), hypocalcemic seizures (*n*=3), febrile seizures (*n*=1) and leptospirosis (*n*=1). The demographic and clinical details of the patients are presented in Table 1.

Pab-PCR was able to detect mycobacterial DNA in 35 patients compared to IS6110-PCR, which showed positive results in 27 patients. None of these tests were positive in the control group. Both the PCR techniques showed higher sensitivity than the gold standard (BACTEC culture, *P*=0.001). However, no significant difference was observed when both PCRs were compared with each other (*P*=0.335). Various microbiological investigations and their validity are presented in Table 2. For 61 samples (out of 71 samples), both probes gave consistent results with 26 positive and 35 negative samples. So, there was a concordance between the results of two PCR among 61 of the 71 samples. Thus, we found an excellent agreement between the two methods. Pab-PCR was positive in 9 (20.5%) patients who were negative by IS6110 PCR while there was only one patient in whom IS6110 PCR was positive, and Pab-PCR came negative (Fig. 2).

A positive correlation was identified between ADA and PCR positivity; with increasing ADA values, the chances of a positive PCR (IS6110 correlation co-efficient 0.5, *P*=0.007, Pab correlation co-efficient 0.69, *P*=0.000). There was also a significant positive correlation between Pab-PCR positivity and nutritional status (correlation co-efficient 0.39, *P*=0.008); patients with TBM with grade 3 malnutrition had a higher Pab-PCR positivity.

DISCUSSION

TBM is the most common cause of chronic meningitis in developing countries, including India. Rapid diagnosis and early institution of ATT can be life-saving in children. Hence, looking at the burden of TBM in our setting and the low sensitivities of clinical and routine microbiological testing, we aimed to evaluate the role of specific CSF PCR-based tests and to compare their efficacy with routine staining and culture methods. As similar studies are not available from our region, our study provides essential clinical and practical information towards enhancing the diagnosis of TBM by molecular tests in children from developing countries.

We observed that the sensitivities of routine microbiological tests used in our setting were low (ZN smear 4.4%, LJ medium 0% and BACTEC culture 2.2%, *n*=45) in children with TBM. All these tests were negative in controls (specificity 100%). Low sensitivity of ZN smear (1.4 to <10%) and LJ culture (14.3%) has been noted in clinical series from India [16, 17]. The reduced sensitivity of conventional modalities in diagnosing TBM is most likely due to the paucibacillary nature of CSF in children with TBM. Centrifugation of larger volumes (>10 ml) of cerebrospinal fluid for 30 min and smear examination from the deposit of as many as four serial samples has been shown to enhance the detection rate of AFB, but large samples are difficult to obtain in pediatric patients. We also tried to increase the sensitivity by centrifugation of the available CSF samples and the use of deposit for testing. However, the poor sensitivity and longer processing time make conventional diagnostic modalities an inferior choice for rapid diagnosis of TBM in the emergency room. On the other hand, Pab and IS6110-PCR had higher sensitivity (77.8 and 60%, respectively) and 100% specificity in our study. Additionally, each PCR test had statistically significant higher positivity than ZN smear, LJ and BACTEC culture. This is in accordance with the previous studies, which have found PCR to be more sensitive than conventional modalities [3, 15, 16]. However, no significant difference in the sensitivity was observed when both PCRs were compared with each other, as noticed in prior studies [18, 19]. In contrast to our research, IS6110-PCR has shown higher positivity (83%) compared to 38kDa-PCR (74.2%) in one study, although the difference was not statistically significant [19].

In our study, Pab-PCR was positive in all three confirmed TBM patients, (one culture-positive and two AFB smear proven). Using this as the gold standard, the Pab-PCR showed an overall sensitivity of 100%. Additionally, the Pab-PCR detected the presence of mycobacterial DNA in 71% of the children, which were classified as suspected TBM, which were not detected by AFB smear and culture. Only a handful of studies have evaluated Pab-PCR in diagnosing TBM. Sharma *et al.* reported Pab-PCR to be 82.8% sensitive, which was significantly higher than culture (14.3%) and ZN-staining (1.4%) and 100% specific [16]. Kulkarni *et al.* also observed a high sensitivity (90%) and specificity (100%) of 38 kDa-based PCR in 30 patients with TBM, but the target for amplification in this study was 340 bp nucleotide sequence located within the 38 kDa protein gene [3]. Negi *et al.* reported sensitivities of Pab-PCR, BACTEC, LJ medium and ZN smear to be nearly 74, 53, 47 and 35%, respectively, in 168 patients of suspected pulmonary and extra-pulmonary TB [15]. In a recently published systematic review, the pooled sensitivity and specificity of in-house nucleic acid amplification tests were 68% (38–88%) and 98% (95–100%), respectively [20]. The use of CSF 'filtrates' rather than 'sediments' may increase the sensitivity of PCR from 53–88% [21]. Our study thus shows that Pab-PCR is a sensitive, specific and rapid diagnostic method in children with suspected TBM in resource-limited settings and has better diagnostic utility than conventional microbiological investigations.

Nearly half of the patients with TBM were undernourished, and these patients had higher PCR-Pab positivity (correlation co-efficient 0.39, *P*=0.008). This indicates that malnutrition may selectively compromise portions of the cell-mediated response that are important for containing and restricting TB [22]. A history of exposure to adults with active tuberculosis has been noted in 40–70% of pediatric cases and forms an essential clinical clue towards the diagnosis of TBM in

Table 1. Demographic and clinical profile of patients in the study

	TBM cases *N*=45	Non-TBM controls *N*=26
Mean age (years)	4.24±0.56	4.58±0.74
Age distribution		
0.25–4 years	30 (66.7%)	13 (59%)
4–8 years	7 (15.6%)	7 (26.9%)
8–12 years	8 (17.8%)	6 (23.1%)
Sex distribution		
Males (%)	28 (62%)	19 (73%)
Nutrition status		
Normal	23 (51.1%)	10 (38.5%)
PEM grade 1	6 (13.3%)	8 (30.8%)
PEM grade 2	10 (22.2%)	4 (15.4%)
PEM grade 3	6 (13.3%)	4 (15.4%)
Clinical characteristics at presentation, *n* (%)		
Fever	45 (100%)	26 (100%)
Altered sensorium	26 (58%)	12 (46%)
Seizures	26 (58%)	14 (54%)
Vomiting	26 (58%)	14 (54%)
Headache	16 (36%)	6 (23%)
Anorexia	2 (4.4%)	2 (7.6%)
Excessive crying	6 (13.3%)	4 (15.4%)
Extra-neural complaints	11 (24.4%)	10 (38.4%)
Clinical examination, *n* (%)		
Raised intracranial pressure	29 (64%)	6 (23%)
Neck rigidity	27 (60%)	12 (46%)
Cranial nerve palsy		
7th	11 (24.4%)	1 (4%)
6th	4 (8.8%)	–
3rd	1 (2.2%)	–
Bulging anterior fontanelle	12/16 open AF (75%)	3/8 open AF (38%)
Bilateral disc edema	8 (18%)	2(8%)
Hemiparesis	4 (9%)	2(8%)
History of contact with tuberculosis	19 (42.2%)	4(15.4%)
Immunization status, *n* (%)		
Immunized	26 (58%)	15(58%)
Unimmunized	10 (22%)	4(15%)
Incompletely immunized	9 (20%)	7(27%)
Mantoux test, *n* (%)		
Positive	29 (64%)	2 (8%)
Could not be read	–	1 (4%)

Continued

Table 1. Continued

	TBM cases *N*=45	Non-TBM controls *N*=26
Neuroimaging characteristics, *n* (%)		
Hydrocephalus	35 (77%)	12 (46%)
Basal exudates	23 (51%)	3 (11.5%)
Infarcts	6 (13%)	2 (8%)
Tuberculomas	10 (22%)	--
Normal	3 (6.6%)	8 (31%)
Characteristics of CSF		
Mean TLC (cells/mm3)	82.89 (0–480)	56.54 (0–500)
Mean Protein (mg dl^{-1})	192.32 (12–3100)	64.12 (13–226)
Mean Glucose (mg dl^{-1})	48.07 (9–130)	57.77 (12–217)
Mean ADA activity (IU l^{-1}) (P <0.001)	15.85±2.97 (range 2–62, median 10.5) *n*=26/45	4.43±0.571 (range 2–6, median 5) *n*=7/26

ADA, adenosine deaminase; PEM, protein energy malnutrition; TLC, total leucocyte count; TBM, tuberculous meningitis.

children, especially in developing countries [23–25]. A similar proportion of our patients with TBM (42%) had contact with an adult case and helped in early clinical suspicion. Subsequent Mantoux positivity was seen in 64% of children with TBM and only 8% of children in the non-TBM group. This is comparable to previous long-term studies, where a higher incidence of up to 78% has been noted [26].

The classic neuroimaging characteristics of TBM noted on contrast-enhanced CT scans were hydrocephalus (77%), basal exudates (51%), tuberculomas (22%) and infarcts (13%). Although the high incidence may be due to the referral bias of a tertiary care center, it is similar to that reported in the literature [23, 27]. In a child with sub-acute to chronic febrile illness, the presence of these radiological features helps in

Table 2. Results of AFB smear, LJ medium culture, BACTEC and PCR (Pab and IS6110) in children with and without TBM

	TBM *N*=45	Control *N*=26	Sensitivity	Specificity	Positive predictive value	Negative predictive value
AFB Smear						
Positive	2	2	4.4%	100%	100%	37.6
Negative	43	26				
LJ culture						
Positive	0	0	0%	100%	100%	36.6
Negative	45	26				
BACTEC						
Positive	1	1	2.2%	100%	100%	37.1
Negative	44	26				
IS6110 PCR						
Positive	27	0	60%	100%	100%	59.09
Negative	18	26				
Pab-PCR						
Positive	35	0	77.8%	100%	100%	72.2
Negative	10					

AFB, acid fast bacilli; LJ culture, Löwenstein–Jensen medium; Pab, protein antigen B.

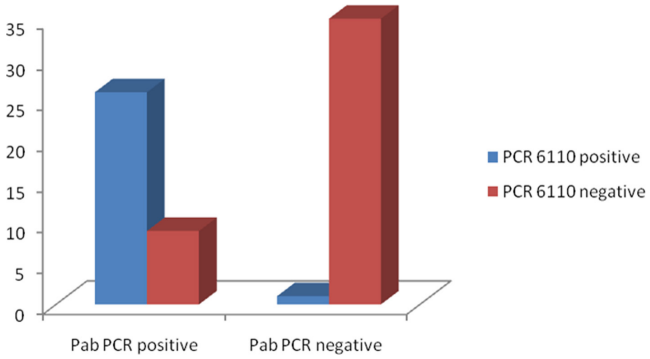

Fig. 2. Agreement between protein antigen b and IS6110 PCR result.

early suspicion of TBM in the emergency room, and thus ATT can be initiated while awaiting bacteriological confirmation. The sensitivities and specificities for CSF-ADA levels have been reported as 89 and 92% respectively for values >5 IU l^{-1}, and 82 and 83% respectively for values>11.4 IU l^{-1} in diagnosing children with TBM [28, 29]. Our study also showed that the mean CSF-ADA value in children with TBM was significantly higher than the non-TBM group (15.8±2.9 IU l^{-1} vs. 4.4±0.5 IU l^{-1}), although CSF-ADA could not be done in all the study patients. Although CSF-ADA activity seems useful as a rapid diagnostic test in the resource-limited settings, yet the variable cut-offs impede a universal application. A microbiological diagnosis should always be sought, especially in children with atypical features.

In recent years, there have been major technological breakthroughs in the nucleic acid amplification testing methodology with the invention of Xpert MTB/RIF and next-generation sequencing. Xpert MTB/RIF is a cartridge-based system based on real-time PCR. It has the advantage of it being a closed-loop system reducing the risk of contamination and the likelihood of false-positive [30]. In a recently published systematic review on Xpert MTB/RIF, the pooled sensitivity was 62% (59–66%), and pooled specificity was 98% [31]. TB/RIF Ultra is an advancement in the technique of Xpert with the addition of two different amplification targets. In a study by Donovan *et al.*, the sensitivities of Xpert MTB/RIF Ultra and Xpert MTB/RIF was 38.9 and 22.9% in HIV negative tuberculous meningitis [32]. In a study by Yan *et al.*, the sensitivity of metagenomic next-generation sequencing in the diagnosis of TBM was 84%, compared to 24.4 and 40% with Mycobacterium PCR and Xpert MTB/RIF [33]. As is observed from the literature, the sensitivities of PCR-based tests have been low. The challenge in the future would be to test multiple additional targets on a cartridge-based platform to give rapid real-time results, which may help in early initiation of anti-tubercular therapy. Though the results of metagenomic next-generation sequencing are encouraging, the test is unlikely to help in early initiation of anti-tubercular therapy, but may aid in stopping the anti-tubercular therapy in negative cases.

The limitation of this study is that there is no diagnostic test that can serve as a gold standard for comparison. The sensitivity of Pab-PCR in detecting TBM was 78% in our study; hence, many TBM patients can still be missed. More extensive studies with multiplex PCRs based on the organism characteristics in specific regions are needed to improve the sensitivity and diagnostic yield of TBM in children in developing countries.

CONCLUSION

PCR-based molecular techniques have great potential to improve the clinician's ability to diagnose TBM. The sensitivity, specificity and rapidity of the PCR test shown in our study should encourage the use of this method in the routine diagnosis of tuberculous meningitis.

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Author contributions
S.A. literature search, figures, study design, data collection, data analysis, data interpretation and writing. A.G.S., S.D., A.K. literature search, figures, study design, data analysis, data interpretation and manuscript writing. KS: literature search, study design, data analysis, microbiological data analysis, interpretation and writing. P.D.S. literature search, figures, study design, data analysis, data interpretation and writing, supervised the entire process and will act as guarantor.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was approved by the Institutional Ethics Committee reference.

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