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Original Article

Microbiology Section

Evaluation of Nested PCR for Rapid Diagnosis of Pediatric Tuberculous Meningitis

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ABSTRACT

Introduction: Pediatric Tuberculous Meningitis (TBM) is a highly morbid, often fatal disease and its prompt diagnosis is fundamental to the proper management. Cerebrospinal fluid (CSF) is paucibacillary in such cases and conventional bacteriological methods for detecting *M.tuberculosis* has inadequate sensitivity, which limits their utility as diagnostic method. Molecular techniques, detecting DNA of *M.tuberculosis* in CSF, have better sensitivity and thus could be a potent tool for rapid diagnosis of TBM.

Aim: To evaluate Nested PCR protocol targeting 38 kDa gene for rapid detection of *M.tuberculosis* complex in clinically suspected cases of pediatric TBM.

Materials and Methods: In this prospective case control study was total of 109 subjects, age ranging from between 6 months and 12 years were included. Case group comprised of 75 clinically suspected cases and control group comprised of 34 children with non tubercular CNS infection or some non infectious CNS disorders. 1-3 ml of CSF was aseptically collected and assessed for *Mycobacterium tuberculosis* by centrifugation of CSF. Smear was prepared ZN staining, inoculation of LJ medium & BacT/alert bottles from the deposit was done on same day. A portion of deposit was kept at –20°C for DNA extraction and nested PCR was performed later.

Result: Among case group, there were 33 (44%) males and 42 (56%) females, with maximum number (33/75) of cases belonging to 6 months-4-year group. Nested PCR showed sensitivity and specificity of 89.3% and 97and respectively, for detection of *M.tuberculosis*. Sensitivity and specificity of BacT/Alert 3D, LJ Culture and ZN staining were 28%,8%, 2.67%and 100%,100%, 100% respectively.

Conclusion: Nested PCR targeting 38kDa gene (protein antigen b) proved to be highly sensitive and specific for the detection of *M.tuberculosis* in CSF in respect with the current gold standard, LJ culture. Nested PCR has the potential as an effective tool for early diagnosis of pediatric TBM.

Keywords: Automated liquid culture, Meningeal tuberculosis, Polymerase chain reaction

INTRODUCTION

Tuberculosis (TB) continues to be a formidable cause of mortality and morbidity throughout the world, especially so in developing nation like India. According to the World Health Organization, India is amongst the 22 high burden countries. The definite burden of pediatric tuberculosis is not known but is assumed to be 10% of total TB load is found in children. Globally, every year, about 1 million cases of pediatric TB are estimated to occur with more than 100,000 deaths, thus making TB amongst the top ten causes of childhood mortality [1].

Tuberculosis in children is the major health hazard, with neuro tuberculosis, especially Tuberculous meningitis (TBM), is the most dangerous extrapulmonary manifestation [2].

Timely diagnosis of TBM is vital for successful disease management as case fatality rate is very high and delayed treatment can lead to permanent neurological damage [3,4]. In developing countries, the highest incidence is noted in first three years of life [5].The diagnosis of TBM mainly relies on identification of *Mycobacterium tuberculosis* in cerebrospinal fluid (CSF) by either direct staining for Acid fast bacilli by Ziehl Neelsen stain (ZN staining) method or by culture. Direct staining, although a rapid method for diagnosis of TBM, has very low sensitivity (5-20%) as it requires minimum bacterial load of 104bacilli/ml and the CSF is paucibacillary in nature [6]. Culture has better sensitivity with detection limit of 10 bacilli/ml of the sample but requires prolong incubation of 3-6 weeks on solid medium. Automated mycobacterial liquid culture based methods have reduced the time to detection to 12-20 days and also increased rate of isolation [7-8]. BacT/Alert 3D detects colorimetric change in pH which occurs due to CO_2 production by growing mycobacteria [9].

Polymerase chain reaction (PCR) for diagnosis of TBM, targeting various *Mycobacterium tuberculosis* specific sequences such as MPB64, IS6110, 65kDa HSP has been described previously with sensitivities and specificities ranging from 63-100% and 38-100% respectively [10]. Nested Polymerase chain reaction (nPCR) protocols has been reported to have sensitivity over 1000 fold for rapid diagnosis of TBM when

compared to single round conventional PCRs [11]. The gene coding for 38 kDa protein is an important housekeeping gene of *M.tuberculosis* which is involved in phosphate transport and is highly specific for *M. tuberculosis* [12].

In our study, we evaluated the potential use of nPCR protocol based on amplification of 38kDa antigen (protein antigen b) of *M.tuberculosis* in comparison with BacT/Alert 3D, for rapid detection of *M.tuberculosis* in pediatric TBM cases.

MATERIALS AND METHODS

An observational prospective case control study was carried out in Department of Microbiology and Pediatrics at U.P. Rural Institute of Medical Sciences and Research, Saifai, Etawah, UP, during January 2014 to July 2015. The study was approved by the ethical committee of the institute. A written informed consent was obtained from the parents of the children.

A total of 109 patients were included in the study. The study group enrolled were drawn from the patients aged 6 months to 12 years, attending the indoor and OPD of Pediatrics Department and were classified into cases and control groups.

Case group consisted of 75 children who were clinically suspected of having Tuberculous Meningitis. The clinical criteria for the diagnosis of TBM, AIIMS, New Delhi was followed [13]. The clinical criteria for TBM included demonstration of Acid Fast Bacilli in CSF or fulfillment of the essential criteria that is CSF having predominant lymphocytic pleocytosis >50/mm^3, protein >60 mg/dl and sugar < 2/3rd of blood sugar along with supportive evidence including, history of fever for two weeks or more, positive family history of tuberculosis, positive Mantoux test (5TU) with induration ≥10 mm, radiological evidence of TB elsewhere in body, CECT scan showing evidence of basal exudates or CNS tuberculosis, generalized lymphadenopathy, isolation of AFB from gastric lavage or from other sites and histologically proven tubercular lymphadenitis.

Inclusion Criteria: All children with age group 6 months to 12 years and fulfilling clinical criteria of TBM were included. They were further categorized into definitive or confirmed cases and probable cases. Definitive cases were those which fulfilled essential criteria and demonstrated AFB in CSF either by ZN staining or by culture on LJ medium.

Probable cases included subjects that fulfilled essential criteria and at least two supportive criteria. A total of six definitive or confirmed cases and 69 probable cases of TBM were included in the study.

Control group consisted of 34 subjects that had history and findings suggestive of pyogenic meningitis or children with altered consciousness and convulsions due to cerebral malaria or having some non infectious neurological disorders like atypical febrile seizure and seizure disorders.

Exclusion Criteria: Infants <6 months of age or subjects already on Antitubercular therapy (ATT) were excluded.

Sample collection and processing

Approximately 2 ml of CSF, obtained by aseptic lumbar puncture from each subject, was collected in sterile vial. CSF was centrifuged at 3000 g for 15 minutes and the supernatant was discarded. Sediment was divided into two parts. One part was stored at -20°C for DNA isolation [14] and nPCR processing. Rest of the sediment was used for preparation of smear (ZN staining), culture on Lowenstein Jensen medium (LJ) and inoculation of BacT/Alert MP bottle. LJ slopes were incubated at 37°C for maximum of 8 weeks before declaring them negative for *M.tuberculosis*.

BacT/Alert bottles were loaded in BacT/Alert 3D system (bioMerieux, France). Instrument automatically monitors the bottle every 10 minutes and detects any change of color at the bottom of bottle and flags it as positive. All positive flagged bottles were unloaded, vortexed and 0.5 ml of fluid aspirated for confirmation of mycobacterial growth by ZN staining. AFB grown on LJ and BacT/Alert bottle was further identified as *Mycobacterium tuberculosis* by performing standard biochemical tests.

Nested PCR

The sediment of the centrifuged CSF was re-suspended in 50µl of sterile Distilled water. DNA extraction was carried out by combination of manual DNA extraction steps with automated extraction protocol of MagNA Pure Compact System® (Roche, Germany) for *Mycobacterium tuberculosis*. Briefly, the deposit was subjected to heat (95°C for 5 min), followed by freezing in ice (-80°C for 5 min) and thawing. Sample was further treated with 50µl of lysozyme, incubated at 37°C for 30 min and then processed as described in the MagNA Pure extraction protocol [15].

Following extraction, nPCR protocol was carried out as per method described by Miyazaki Y et al., [11]. Primers targeting the gene sequence that encoded the 38kDa protein (protein antigen b) of *Mycobacterium tuberculosis* were used as described by Sjobring et al., [16].

The sequence of TB PCR primer pairs for 1st and 2nd round of amplifications was:-

Forward Primer MT1: 5′ -ACCACCGAGCGGTTCGCCTGA-3′

Reverse Primer MT2: 5′ -GATCTGCGGGTCGTCCCAGGT-3′

Internal Forward Primer NF: 5′-TGACGTTGGCGGAGACCG-3′

Internal Reverse Primer NR: 5′-ATGGTGCCCTGGTACATG-3′

The first amplification reaction mixture consisted of 90µl of master mix (50mM KCl, 10mM Tris-HCl,1.5mM MgCl2, 0.3mM each of dNTP, 100 pmol each of primer MT1 and MT2, 2.5 u of Taq polymerase) and 10µl of target DNA. After the DNA was denatured at 95°C for 10 min, 2.5u of Taq polymerase was added and PCR performed under the following cycling conditions, denaturation at 94°C for 1 min, annealing at 63°C for 90 sec and extension at 72°C for 1 min. For the second PCR, 10µl from the first PCR was sampled and mixed with 90µl of freshly prepared reaction mixture (containing Primers

NF and NR). This was followed by same procedures used to obtain the first PCR product. Both cycles were run for 35 cycles. PCR products were analyzed by electrophoresis in 2% agarose gel, stained with ethidium bromide and documented by gel documentation system (Gel Doc XR+ System®, Bio-Rad, USA). For each amplification, a positive control containing DNA extracted from reference strain *M.tuberculosis* H37Rv and negative control containing molecular grade water (HiMedia, India) was run.

STATISTICAL ANALYSIS

The data was entered in Microsoft excel computer program. The analysis was done by using SPSS version 21. Chi square test was used to compare categorical dichotomous variables; Fisher Exact test, Mann-Whitney U non-parametric test was used to compare the continuous variables. The results were presented as mean+SD and percentages. The diagnostic performance of PCR was evaluated against that of clinical diagnosis as gold standard. Sensitivity, specificity, PPV, NPV and diagnostic accuracy were also calculated. The p-value <0.05 was considered significant.

RESULTS

Among 75 clinically suspected cases, 6(8.0%) belonged to Definite TBM group and 69 (92.0%) belonged to Probable TBM group. Both cases and controlled were aged and gender matched with p value of 1 by Fisher Exact test [Table/Fig-1]. The mean age of the clinically suspected cases was 5.4±3.5 years while that of control group was 4.3±3 years.

Sex		Male			Female		
Patient age group		6 months -4 years	5-8 years	9-12 years	6 months -4 years	5-8 years	9-12 years
Cases (n=75)	Definitive (n=6)	02	01	00	01	02	00
	Probable (n=69)	13	10	07	17	13	09
Control (n=34)		10	05	00	11	6	02
Total		48			61		

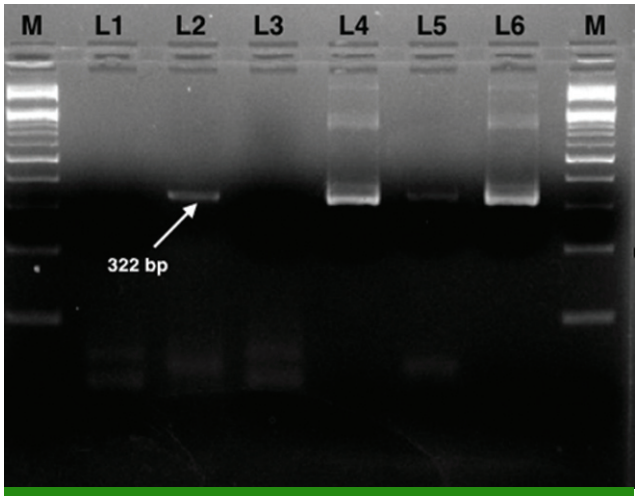
[Table/Fig-1]: Distribution of study group according to sex in various age group.

AFB was detected on direct smears in 2 of 75 (2.67%) samples. Both were seen in Definitive TBM cases. None of samples from control group were smear positive for AFB. Overall sensitivity, specificity, PPV and NPV of ZN microscopy was 2.67%, 100%, 100% and 31.8% respectively.

Out of total 75 cases, 6 CSF samples were positive by culture on LJ medium. CSF from control group was all culture negative. Overall, LJ culture had sensitivity, specificity, PPV, NPV of 8%, 100%, 100%and 33.01% respectively.

BacT/Alert 3D automated mycobacterial culture system yielded additional 15 positive cultures, with overall positivity being 28% (21/75). It had sensitivity, specificity, PPV and NPV of 28%, 100%, 100% and 38.64% respectively. The diagnostic accuracy of BacT/Alert 3 D (50.45%) was higher than LJ culture (36.69%). The mean time to detection in BacT/Alert 3D was 18.2 days while that of LJ culture was 32.3 days (p=0.0001)

Nested PCR was positive in 68 patients. *Mycobacterium tuberculosis* DNA containing samples gave amplicons of 419 bp and 322 bp respectively in first and second PCR. [Table/Fig-2] among nPCR positive cases, 6/6 (100% sensitive) were culture positive definitive cases, 61/69 were probable cases and 1 was in non TB control group. Overall, nPCR had sensitivity, specificity, PPV, NPV and diagnostic accuracy of 89.34%, 97.06%, 98.53%, 80.5% and 91.74% respectively [Table/Fig-3].



[Table/Fig-2]: Amplification of 322 bp product of *M.tuberculosis* by nested PCR. PCR products were analyzed by electrophoresis on 2% agarose gel.
M represents 100 bp DNA ladder, L1: Negative control, L2: Positive control DNA (*M.tuberculosis* strain H37Rv strain) L3: Non TB sample, L4,L5 andL6: Positive TBM cases(322 bp).

DISCUSSION

Tuberculous meningitis remains a diagnostic challenge in children due to its varied clinical presentation. The conventional "gold standard" based on bacteriological detection methods of *M.tuberculosis*, including the direct smear examination for acid-fast bacilli (AFB) and culture identification, is inadequate for early diagnosis, owing to the poor sensitivity or the long time required (4–8 weeks) for cultures [17,18].

In our study, the age group of children involved was 6 months to 12 years and maximum number of cases was in age group 6 months to 4 years (44%). The mean age of the case group was 5.4±3.5 years (mean ± SD). This is in concordance with various studies which had reported the majority of cases occur in age group less than 5 years with peak incidence between 3-5 years [19-21].The sex distribution among the cases (M:F ratio was 0.78) showed slight deviation towards the girls in cases group, which is contrary to the published data that

Test result	Case (n=75)		Control (n=34)	Sensitivity (95%CI)	Specificity (95%CI)	Diagnostic accuracy	PPV	NPV
	Definitive (n=6)	Probable (n=69)						
Nested PCR								
Positive	06	61	01	89.3% (80.0-95.3)	97.1% (84.6-99.9)	91.74%	98.5%	80.5%
Negative	00	08	33					
BacT/Alert 3D								
Positive	06	15	00	28% (18.2-39.5)	100% (89.7-100)	50.45%	100%	38.6%
Negative	00	54	34					
LJ Culture								
Positive	06	00	00	8% (3-16.6)	100% (89.7-100)	36.69%	100%	33%
Negative	00	69	34					
Microscopy								
Positive	02	00	00	2.6% (0.3-9.3)	100 (89.7-100)	33.02%	100%	31.8%
Negative	04	69	34					

[Table/Fig-3]: Microbiological tests among various subgroups of tuberculous meningitis (TBM) and healthy controls.

states more incidence among boys [20]. The reason may be as the study set up is in rural government tertiary health care institute, there is generalized neglection of the girls and the boys are generally taken to the private setup.

Among all test performed, microscopy had lowest sensitivity of 2.67%. This could be due the fact that CSF is paucibacillary in nature and atleast "10^4 bacilli/ml" are required in the specimen for smear positivity [22]. Various studies have documented similar sensitivities of the smear microscopy for TBM, which ranged from 5-20% [5,23]. Sensitivity of smear microscopy can be enhanced by collecting serial samples and centrifuging of large volumes (10–20 ml) of CSF for 30 minutes [24]. However, repeated collection of such large volume of CSF is practically not possible in pediatric patients.

The sensitivity of culture on LJ medium (8%) in our study was similar to that of Akash Roy et al., [25]. Other studies have also reported low isolation rates from CSF (2-8%) [23,26,27]. Enhanced culture positivity could be obtained by using sediments from larger volumes of CSF [28], however, it is to be noted that it is extremely difficult to obtain CSF from pediatric subjects in volumes reported to give reasonably good culture yield. Furthermore culture on LJ medium cannot be utilized in making rapid diagnosis as *M.tuberculosis* takes prolong time to form colonies. The BacT/Alert 3 D automated mycobacterial detection system showed an overall sensitivity of 28% which was definitely higher than that of LJ culture ($p < 0.001$). Both LJ culture and BacT/Alert 3 D had 100% specificity in TBM diagnosis. A higher sensitivity was found in Definitive (100%) subgroup and it decreased in Probable subgroup to 21.73%. The contamination rate of BacT/Alert 3D and the LJ culture were 5.8% and 7.2% respectively. Similar, reports have been documented in different studies [9,29].The mean detection time of BacT/Alert 3D in all 21 isolates was 18.2 days compared to 32.3 days taken by 6 isolates on the LJ medium. Various studies documented a mean detection time of 15-16 days for BacT/Alert 3D [9,30]. The early detection of the bacilli in these studies could be attributed to the inclusion of both pulmonary and extrapulmonary specimens. The earlier isolation of AFB by BacT/Alert 3D substantiates its role for an early start of antitubercular therapy (ATT) and also to perform the drug susceptibility test (DST) to detect Multidrug resistant or extensively drug resistant (MDR andXDR) *M.tuberuclosis* strains.

In present study, we followed the technique of in-house nested PCR as described by Miyazaki Y et al.,[11] using primers targeting 38kDa 'protein antigen b' gene for amplification. It can detect small amount of DNA as 10fg 2-3 organisms and is highly specific [4]. Our nested PCR showed overall sensitivity of 89.3%, which was significantly higher than that of LJ culture ($p<0.001$) and BacT/Alert 3D ($p <0.001$). However, the sensitivity decreased from 100% in definitive case group to 88.4% in probable case group. Our study is in concordance with Sharma K et al., [31] who, using 'protein antigen b' PCR primers, reported 90% and 81.7% sensitivity in confirmed and clinically suspected cases of TBM respectively. Similar, sensitivity of 90% was reported by Kulkarni SP et al., [4] in pediatric age group using 38kDa PCR primers. Our results were comparable to previous studies which showed 85-91% sensitivity [32-33] and differs from other studies which had a low sensitivity of 33-75% [23,26].Their low sensitivity could attribute to the fact that most studies have used, single step conventional PCR, different set of primers (IS6110, 65kDa, MBP64) and conventional methods of DNA extraction (CTAB-Phenol-chloroform method) which may not remove all PCR inhibitors, not properly lyse mycobacterium cell wall and release its DNA. We used nested PCR protocol along with automated DNA extraction method (MagNA pure compact). Magnetic glass based DNA extraction has been found superior to the conventional DNA extraction method [15]. There were 8 clinically suspected cases which could not be detected by

nPCR (false negative as per clinical criteria). This could be due to presence of PCR inhibitors, extremely low bacterial load and small amount of CSF tested.

The overall specificity of nested PCR in our study was 97.06%. A single false-positive results was obtained in spite of physical separation of areas used for sample processing, PCR-setup and analysis of amplification products. Other studies have also reported higher specificities of nPCR ranging from 96-100% [4,31,34]. False positivity could be due to cross contamination during initial processing or carry over contamination by amplicons. The assay specificity can be further improved by use of the dUTP–uracil glycosylase system and by conducting nPCR by single tube method.

The limitation of study is the non availability of 'Gold Standard' test for diagnosis of TBM. Traditional gold standard, the culture, has poor sensitivity and clinical assessment for diagnosis of TBM cannot be relied upon because of variable nature of its manifestations. Recent addition to the molecular diagnostic armamentarium for rapid diagnosis of TB is Xpert MTB/RIF, a cartridge-based real-time hemi-nested closed NAAT platform. However, this too has low sensitivity (46%) in diagnosing TBM [35]. Hence, further studies with larger sample size and using techniques with better sensitivity and specificity are required.

CONCLUSION

This study highlights the difficulty in confirming the clinical diagnosis of TBM by smear microscopy and culture. Real time PCR chemistries, although having shown equal or better sensitivity in diagnosis of TBM, the higher cost of equipment and the reagents are the limiting factors especially in resource constrained nations. Nested PCR targeting 38Kda gene can have significant impact in the rapid diagnosis of TBM, which is often missed when conventional methods are used or cause considerable delay in diagnosis, especially in pediatric TBM cases in which early diagnosis is essential for better outcome of the disease.

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