

Original Article

TRC4 Gene Based PCR Assay in Diagnosis of Tuberculous Meningitis

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Abstract

The prevalence of Tuberculous meningitis remains largely underestimated due to nonspecific clinical manifestation at early stages. A total of 20 CSF samples were studied from clinically suspected cases of tuberculous meningitis. All the samples were processed for ZN staining, TB culture on LJ media and TB-PCR using IS6110 and TRC4 primers by standard protocols. Of the total 20 CSF samples, 12 cases were diagnosed as TB meningitis. Most of the tuberculous meningitis cases were found positive by PCR using TRC4 primers (83.33%) followed by IS6110 primer (66.67%) and culture on L-J media (8.33%). None were found positive by ZN staining. TB-PCR using TRC4 primer showed higher positivity than using IS6110 in detecting tuberculous meningitis, since some strains of MTB may lack the IS6110 element in their genome. PCR assay using TRC4 primer is superior in diagnosing tuberculous meningitis. This study was aimed to evaluate the diagnostic potentials of CSF polymerase chain reaction (PCR) by using TRC4 and IS6110 primers. Further the results were also compared with culture on Lowenstein-Jensen (LJ) media and AFB smear by Zeihl-Nelson (ZN) staining.

Key words: Tuberculosis (TB), TRC4, Meningitis

Introduction:

Tuberculosis (TB) is the major cause of death worldwide and is due to a single pathogen¹. Mortality in TB occurs mainly due to the neural form of TB, namely, TB meningitis. Delay in diagnosis and so in the start of effective treatment results in poor prognosis and sequelae in up to 25% of cases². The prevalence of TB meningitis remains largely underestimated because clinical manifestations are nonspecific in early stages of the disease and bacteriologic confirmation is available only for a small proportion of patients. Also, clinical diagnosis of TB meningitis is difficult due to its varied clinical presentations. Further, routinely used tests employed for clinical diagnosis of TB meningitis are inadequate.

PCR is currently the most sensitive and rapid method to detect extra-pulmonary Mycobacterium tuberculosis^{3, 4, 5}. We used as a new primers TRC4 which is a conserved repetitive element with specificity for M. tuberculosis complex.

The aim of this study was to compare the efficiency of a PCR with a target chosen from this cloned fragment with that of a PCR with the widely used IS6110 sequence in detecting M. tuberculosis in cerebrospinal fluid (CSF) samples from patients suspected with tuberculous meningitis.

MATERIAL METHODS

Study population:

The present study was approved by the Institutional Review Board, basic science faculty, Bangabandhu Sheikh Mujib Medical University. A total of 20 cerebrospinal fluid (CSF) samples collected from clinically suspected cases of tubercular meningitis of all age and sex group which were sent to Microbiology and Immunology Department, Bangabandhu Sheikh Mujib Medical University between March 2013 to February 2014 for laboratory investigation of Mycobacterium tuberculosis, were enrolled in the present study.

All the samples were processed for direct microscopy using conventional ZN staining, TB culture by Lowenstein Jensen (L-J) media and PCR by IS6110 and TRC4 primers. Other supporting investigations were collected from patient case files. Out of the 20 patients, 12 were diagnosed as tubercular meningitis based on the following diagnostic criteria⁶.

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- 1) Positive tuberculin test and ADA value ($\geq 10U/L$) in CSF with lymphocytic pleocytosis, protein level $\geq 80mg/dl$ and /or glucose ≤ 2.5 mmol) with or without cobweb appearance or clot.
- 2) Detection of AFB by microscopy or isolation of mycobacteria by culture from CSF samples.
- 3) Positive tuberculin test and / or high ESR and clear response to anti-TB drugs.

A positive to treatment response was defined as the improvement of clinical and / or imaging findings after two months of treatment.

Sample collection and processing:

5 ml CSF was withdrawn from patients using lumbar puncture by attending physician. 1 ml was sent to clinical pathology for routine biochemical (glucose, protein) test and cytology⁷. Four ml were used for Gram stain and routine bacteriological culture, L-J culture, ADA, AFB smear for Z-N stain and PCR assay.

PCR assay

Mycobacterial DNA extraction from the samples were done by Qiagen kit and followed by conventional PCR for the IS6110 and TRC4 gene specific for *M.tuberculosis*. The eluted DNA can be stored at -20°C until use in PCR. The PCR mastermix was prepared according to standard protocol of the kit which contained primer pairs, DNA polymerase, buffer containing dNTPs, MgCl₂. This 15µl of mastermix is mixed with 5µl of sample's nucleic acid. The needed product was proliferated using primers IS6110 insertion element regions including

5'- CCT GCG AGC GTA GGC GTC GG- 3'

5'- CTC GTC CAG CGC CGC TTC GG-3' and

using primers TRC4 insertion element regions including

5' - GAC AAC GAC GTG CGC CTA CT - 3'

5' - GAC CGA ATT AGC GTA GCT CC - 3'

The PCR conditions for DNA amplification were an initial denaturation step of 94°C for 15 min, followed by 40 cycles of 94°C for 05 min, 62°C for 15 min, 72°C for 15 min. and a final extension step at 72°C for 10 min. The final products assessed by gel electrophoresis were 123bp for IS6110 and 173bp for TRC4.

STATISTICAL ANALYSIS:

After collection all data were checked and edited. Then all data were analysed by using computer based SPSS (Statistical Package of Social Science) software version: 21.00 with a 95% confidence interval (95% CI). The results of individual

tests were compared by Chi square (X^2) test. p value < 0.05 was taken as minimum level of significance. Significance was calculated by Z-test of proportion.

RESULT

A total of 20 patients clinically suspected of having tubercular meningitis were enrolled in this study. Among them; 12 patients were finally diagnosed as tuberculous meningitis and 8 were non-TB cases. The mean age of the study subjects was 34.17 ± 1.42 years with age ranging from 1- 75 years. Out of 20 patients 11 (55%) were males and 9 (45%) were females. The major presenting clinical features included fever, malaise, weight loss, irritability, convulsion, neck stiffness, clouding of consciousness and anorexia. Out of 12 tuberculous meningitis cases, 7(58.33%) were tuberculin positive and mean ESR was 87.46 ± 2.34 . ADA values were found $\geq 10U/L$ in all 20 CSF samples. All the 12 TB meningitis patients responded to anti-tuberculous treatment.

Among the twelve TB meningitis cases PCR assay using TRC4 primer showed significantly higher positivity 10 (83.33%) cases in comparison with PCR assay using IS6110 primer that was positive in 8(66.67%) cases. Culture that was positive in only one (8.33%) case. None were positive by AFB smear. (Table-I)

Table - I: Results of AFB smears, mycobacterial culture & PCR assay among Tuberculous meningitis cases (n=20)

Tests	Number of positive case	Percentages
AFB smear	0*	0
Mycobacterial culture	1**	8.33
PCR assay by IS6110	8	66.67
PCR assay by TRC4	10	83.33

*p < 0.05, compared between AFB smear and PCR

**p < 0.005, compared between Mycobacterial culture and PCR

Table-II: PCR results using two probes among study population (n=33)

	PCR primers			
	IS6110 Primers		TRC4 Primers	
	Positive	Negative	Positive	Negative
TB meningitis (n=12)	8	4	10	2
Non-TB (n=8)	1	7	0	8
Total 20	9	11	10	10

Of the 12 diagnostically positive TB meningitis samples, eight were (66.67%) positive by PCR using IS6110 probes, whereas ten (83.33%) were positive by PCR using TRC4 probes (Table-II). Among the eight non TB meningitis, 5

cases were viral meningitis and 3 cases were malignant hypertension. Of the 8 non-TB samples, a case of viral meningitis showed positive (1.14%) by using IS6110 probes.

DISCUSSION:

The diagnosis of tuberculous meningitis poses a special challenge as it is often missed or misdiagnosed due to its atypical presentations. Due to the poor performance of conventional microbiological techniques, the diagnosis is largely based on clinical suspicion and non-microbiological parameters in CSF specimens. As a result, a number of cases having atypical clinical presentation, remain undiagnosed and even worse, untreated and leaves clinician with a long delay without an affirmative answer.

In this study, diagnostic efficacy of PCR was evaluated in relation to the conventional techniques. A total of 20 suspected tuberculous meningitis cases were included in this study. Among them 12 patients were finally diagnosed as tuberculous meningitis and 8 were non-TB cases. Clinical samples collected from this suspected tubercular meningitis cases were subjected to AFB smear, AFB culture and PCR. To assess a better target for PCR amplification, two sets of *Mycobacterium tuberculosis* complex specific primers, namely IS6110 and TRC4 were used.

Out of the 12 tuberculous meningitis cases in this study, only 8.33% were found positive by L-J medium culture. None of the CSF sample was positive by AFB smear examination. The lower positivity of AFB smear and culture in the present study is comparable with that of another study where the positivity of AFB smear and culture was 3% and 4% in CSF respectively⁸. This low yield of AFB smear and culture may be due to the paucibacillary nature of CSF samples. Furthermore, inadequate volume of samples for a number of tests, like cytology, biochemistry, microbiology and PCR, gives low positive result⁹. AFB smear negativity might be due to the low concentration of mycobacteria in those samples i.e. below the detection limit of 10000 organism/ml¹⁰. Culture negativity might be explained by the absence of viable mycobacteria in the samples. In this study 83.33% PCR positivity was found in CSF which is comparable with a study in India where CSF sample was found 91% PCR positive¹¹. All the PCR positive with bacteriological negative cases were responded to anti tuberculous therapy (ATT) with significant clinical improvement. In patients particularly with clinical and radiological features suggestive of active tuberculosis, a negative AFB culture cannot exclude a clinical diagnosis of tuberculosis, as demonstrated in our patients¹².

Of 8 non-TB samples, a case of viral meningitis was false positive by PCR assay using IS6110 primer. It can be explained by that, in country like Bangladesh where the

prevalence of tuberculosis is very high, the possibility of infection with tuberculosis may coexist¹³. This could be also due to cross contamination during tube to tube transfer¹⁴.

The two samples from patients who were clinically positive and responded to antituberculous therapy were negative by PCR using both probe. This false negativity of PCR test can be explained either by sampling errors (non-uniform distribution of microorganisms), low bacterial load, inefficient extraction of DNA, or the presence of PCR inhibitors¹⁵. Monitoring and accurate evaluation of PCR inhibitors is possible only if an internal control is included during DNA amplification; which was the main limitation in this study¹⁶.

In the present study the oligonucleotide primers derived from IS6110, and TRC4 gene, were successfully used to amplify a 123bp and 173bp DNA sequence in *M. tuberculosis* complex. TRC4 gene-based PCR showed highest (83.33%) positivity and IS6110 showed 66.67% positivity. As mentioned earlier, IS6110 is an excellent target of PCR in detecting *M tuberculosis* because of its repetitive sequence in the genome¹⁷. But in this study four cases of tuberculous meningitis were found to be negative by IS6110. These are false negatives because; among these four cases one case was positive by bacteriological test and three cases were responded to antituberculous treatment subsequently. Other studies in India and in other geographical regions of the world have also reported similar results^{11,18,19}. Reason of this may be due to absence or low copy number of IS6110 in genome of some isolates of *Mycobacterium tuberculosis* mostly reported from south-east India, Tanzania, Malaysia, Oman and Denmark^{17,19}. There are no such reports from Bangladesh. But the genotyping analysis of the available oligotype data suggests that IS6110 low copy number strains belong to the TbDI+/EAI lineage, which have recently been reported to be in the range from 25% to 50% in Bangladesh and Singapore^{6,20,21}. PCR assay based on TRC4 primer in this study showed lowest negativity in only two cases among the tubercular meningitis samples. Narayanan et al (2001)²³ described TRC4 as an insertion like sequence that was present in all *M. tuberculosis* containing specimens and clinical isolates examined in their laboratory. We found that using primer from a repetitive element like TRC4 can be an ideal target for PCR to identify *M tuberculosis* from clinical specimens in TB meningitis, especially to detect carrying no copy or single of IS6110 which is comparable to this study. There is limited data available about the performance of 173 bpTRC4 primer based PCR assay in Bangladesh which necessitates its further evaluation. Research also needed to simplify and convert PCR into a cost effective technique that can be set even in an unsophisticated laboratory of a developing country like Bangladesh.

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