COMPARATIVE EVALUATION OF CONVENTIONAL METHODS AND NESTED POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF EXTRA-PULMONARY TUBERCULOSIS

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ABSTRACT

Background: The conventional techniques like smear microscopy and culture suffer from low sensitivity for diagnosis of extra-pulmonary TB (EPTB). Polymerase Chain Reaction (PCR) is presently seen as a promising alternative to conventional techniques. This study evaluates IS6110 sequence based nested PCR (nPCR) for the detection of *Mycobacterium tuberculosis* (MTB) DNA directly from clinical samples of extra-pulmonary origin.

Material and Methods: A total of 120 extra-pulmonary samples from the patients with history suggestive of tuberculosis were taken. All the samples were processed for Ziehl-Neelsan staining for acid fast bacilli (AFB), culture and PCR with primers targeting 123bp fragment of IS6110 of MTB complex.

Results: A significant difference was seen in the sensitivities of conventional methods and PCR ($p<0.05$). Out of these 120 samples 14 (11.7%) were positive by smear, culture was positive in 16 (13.3%) samples. Either smear or culture positive 18 (15%) and 39 (32.5%) were positive by PCR.

Conclusions: The nPCR assay targeting IS6110 gene sequence is a rapid and sensitive diagnostic technique for detection of *M. tuberculosis* genome in clinically suspected extra-pulmonary tuberculosis specimens, as compared to the conventional techniques.

Key words: *Extra-pulmonary tuberculosis, Mycobacterium tuberculosis, nested polymerase chain reaction, IS6110 sequence.*

INTRODUCTION

Tuberculosis (TB) is one of the major air borne infectious bacterial diseases. While pulmonary tuberculosis is the most common presentation, extra-pulmonary tuberculosis (EPTB) has emerged as an important clinical problem. EPTB constitutes about 15 to 20 per cent of all cases of tuberculosis in immunocompetent patients and accounts for more than 50 per cent of the cases in HIV-positive individuals. [1]

In developing countries the diagnosis of EPTB with conventional diagnostic tools is a major challenge. Direct smears microscopy lack sensitivity and cultures are time consuming, susceptible to contamination problems and give frequent negative results in paucibacillary specimens as in case of EPTB. Smear for AFB is reported to be positive in less than 10 to 37% of patients and mycobacterial culture is reported positive in variable proportion (12 to 80%) in different body fluids. [1] Lack of standardization in methodology among laboratories, is another major limitation of the conventional methods.

As an alternative to these classical methods, new nucleic acid-based technologies showed promises as more rapid, sensitive and specific means of detection and identification of mycobacteria. [2,3] Some reports have evaluated the role of polymerase chain reaction (PCR) in the diagnosis of EPTB, with high sensitivity and specificity using various primers to amplify targets like IS6110, 65 Kda, TRC4, devR etc. [4-8]

The present study was undertaken to evaluate the role of nested Polymerase Chain Reaction (nPCR) using IS6110 in the diagnosis of extra-pulmonary TB in comparison to the conventional methods.

MATERIAL AND METHODS

The study was carried out in the Department of Microbiology from September 2009 to May 2011. One hundred and twenty, non-repeated clinical samples from the patients with suspected tuberculosis, were obtained. The samples included 32 ascitic fluid samples, 30 pleural fluid, 14 CSF, 10 synovial fluid, 10 endometrial biopsy, 8

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urine, 8 pus and 8 lymphnode aspirate. All the necessary clinical details were also taken.

Inclusion criteria:

All new suspected cases of tuberculosis of either sex and all age groups having a strong clinical or radiological evidence of tuberculosis and not receiving anti tubercular treatment for more than four weeks were included in the study.

Exclusion criteria:

Known treated positive TB cases, failure cases, defaulter cases, relapse cases were not considered for this study.

Processing of samples

All the samples were apportioned for conventional bacteriological techniques and for PCR procedure. For every clinical sample, two smears one direct and other after concentration were prepared. Concentration was done by standard methods depending on the nature of samples. [9] Pus samples were processed by N-acetyl-L-cysteine NaOH (NALC-NaOH) method, fluid samples were centrifuged at 3000 rpm for 30 minutes and tissue biopsies were homogenized. Lymph node aspirates were taken aseptically and were processed directly. The deposits obtained after concentration were processed by Ziehl-Neelsen staining for AFB, culture on Lowenstein-Jensen (LJ) medium and PCR.

Culture for *M. tuberculosis* was carried as per the standard bacteriological procedure. [10] Briefly, a portion of the deposit after digestion was inoculated directly on to Lowenstein-Jensen (LJ) medium. These were examined daily for first week for detection of growth of rapidly growing Mycobacteria or any contamination. Thereafter the cultures were examined weekly up to 8 weeks, after which they were discarded. The growth was confirmed to be that of *Mycobacterium tuberculosis*, based on slow growth rate, absence of pigmentation and Niacin test positivity.

Extraction of DNA from clinical samples

A portion of material obtained after concentration was then further processed for DNA extraction using commercially available kit, Amplification Reagent Set for *Mycobacterium tuberculosis,* Bangalore Genei, Bangalore, India. The guidelines of the manufacturer of the kit were followed.

***Nested polymerase chain reaction (nPCR)*:**

Single tube nPCR using primers targeting IS6110 gene sequence of *Mycobacterium tuberculosis* was performed using PCR thermal cycler. Nested primers (synthesized by Bangalore Genei, Bangalore, India) included primers for 220 bp IS region of *MTB-complex* DNA sequence which was amplified in first round of amplification. In second step nested primers were added to further amplify a 123 bp amplification product.

Gel electrophoresis of amplified DNA

Amplified DNA was subjected to electrophoresis in 2.5% agarose gel containing ethidium bromide. The gel was run at 110 volts and then visualized under UV transilluminator. The results were documented.

Throughout the PCR processing, recommended stringent precautions were followed and the results were evaluated in the light of the performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions.

Statistical analysis: The sensitivity of the conventional tests and PCR assay was calculated keeping culture as gold standard and the significance of difference was determined by proportion test; probability value of 0.05 was taken as significant value ($p < 0.05$). The study was approved by ethical committee of the institute.

RESULTS

1) Microbiological study:

A total of 120 clinical samples were processed for bacteriological studies with conventional methods, direct microscopy by Ziehl-Neelsen (ZN) staining and culture on Lowenstein-Jensen (LJ) medium. Four smears were positive for AFB with ZN stain before concentration and 14 were positive after concentration (Table 1). Out of 120 samples processed for culture on LJ medium 16 samples were found AFB positive (Table 1). Fourteen smears which were positive by direct and after concentration microscopy showed growth on LJ medium except two samples of ascitic fluid in which no growth was obtained till 8 weeks of incubation. However four additional samples which were negative with direct microscopy showed growth on LJ medium.

2) PCR

PCR was performed on all samples using IS6110 primers

specific for *M. tuberculosis* complex. Out of 120 samples 39 samples were found to be PCR positive (Table1). All the samples which were positive by either smear or culture were also positive by PCR (Table 2). Twenty-five additional samples were positive with PCR.

Highest positivity was shown by PCR ie. 32.5% followed by culture on LJ medium 13.33% and least with ZN stained direct microscopy 11.67%.

DISCUSSION

Accurate and early diagnosis of tuberculosis is important for its effective management. Direct microscopy provides rapid diagnosis, but it requires sufficient number of acid fast bacteria to be present (10^4 organisms/ml) in the specimen.

Extra-pulmonary tuberculosis encounters many problems like the pauci-bacillary nature of the samples, inadequate sample amount or volume, non uniform distribution of microorganism etc. All these limitations reflect in the poor contribution of conventional bacteriological techniques in the establishment of diagnosis of EPTB. This has stimulated the application of PCR in the laboratory diagnosis of EPTB.

PCR, a rapid diagnostic technique has better positivity rate than smear microscopy with high degree of specificity for identification of *Mycobacterium tuberculosis* complex, directly in clinical specimen. In our study PCR results were obtained in 24-48 hours, which may prove to be helpful in early starting of treatment in suspected cases and avoiding many doubts and trial therapies.

There are various method of rapid diagnosis of tuberculosis PCR with a large number of gene target and many primers are available to amplify targets like IS6110, 65 Kda, TRC4, devR etc. Our PCR assay was based on the amplification of a fragment of the IS6110, which is specific for the *M. tuberculosis* complex. [13] The amplification of IS6110 insertion sequence, which belongs to IS3 family and is found in almost all members of the *M. tuberculosis* complex. Most strains of *M. tuberculosis* carry 10-15 copies of IS6110; this characteristic helps to increase sensitivity of PCR over that obtained in amplification of a single DNA sequence.

In our study, out of 120 samples, conventional bacteriological techniques were positive in 18 (15%) samples (Smear positive 11.67% and culture positive 13.33%) where as with PCR positive samples were 39 (32.5%). This difference was found to be statistically significant ($p = 0.015$). In our study, PCR showed 100% positivity as all samples found positive by conventional techniques were also positive with PCR. Earlier Indian studies also documented increased positivity by PCR targeting IS6110 gene sequence in samples of EPTB.

Tiwari *et al* (2003) showed 62% of total positivity rate among EPTB samples and detection of *M. tuberculosis* DNA in 57% of AFB negative EPTB samples. [11]

A study by Kesarwani RC *et al* (2004) also reported that PCR assay targeting IS6110 sequence was more sensitive in case of EPTB. [12]

A study by Negi *et al* (2007) on comparison of different PCR protocols, targeting different gene sequences of *M. tuberculosis*, showed higher positivity (77%) in PCR targeting IS6110 compared to other targets like 65kDa (75%), 38 kDa (72%) and 85B protein(73%). Among samples found negative by conventional techniques, PCR targeting IS6110 has shown higher positivity (83%) than PCR for other targets. Also the methodology of PCR for IS6110 has been widely carried out in different technical set ups and has been proven to be simple and reproducible, compared to methodologies for PCR targeting other gene sequences [6].

This finding was also supported by another study by B Sekar *et al* (2008) which also showed that the assay targeting IS6110 sequence were more sensitive [1].

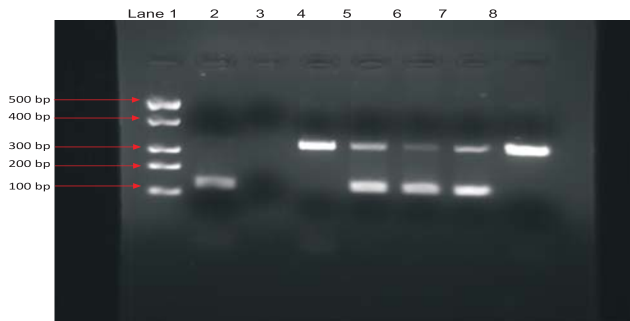
However the absence or the presence of fewer copies of target sequence IS6110, in some strains of *Mycobacterium tuberculosis* related to geographical origin has been reported in the studies by Das *et al* (1995) and D.S Chauhan *et al* (2007). [13,14] The isolates from geographical areas like Indian subcontinent contain less copies of IS6110 as compared with 8-15 usually found in strains from most developed countries. Since the number of copies of target sequence is an important determinant of PCR sensitivity; it would be lower in the strains having only a few copies of this insertion sequence. As India accounts for a large proportion of TB cases it has become necessary to evaluate PCR protocol based on other genes of MTB in developing countries.

Samples	Smear pos/samples investigated	Culture pos/ samples investigated	Smear/culture pos/samples investigated	PCR pos/ samples investigated
Ascitic fluid	4/32	2/32	4/32	8/32
Pleural fluid	2/30	2/30	2/30	8/30
CSF	0/14	0/14	0/14	0/14
Synovial fluid	0/10	0/10	0/10	4/10
Endometrial biopsy	0/10	0/10	0/10	1/10
Urine	2/8	2/8	2/8	4/8
Pus	2/8	4/8	4/8	6/8
Lymphnode aspirates	4/8	6/8	6/8	8/8
Total	14/120	16/120	18/120	39/120

Table 1: Results of conventional bacteriological tests and PCR

Direct Smear Microscopy	Growth on LJ medium		PCR	
	Positive	Negative	Positive	Negative
Positive 14	12	2	14	0
Negative 106	4	102	25	81
Total 120	16	104	39	81

Table 2 : Comparison of results of direct microscopy, culture and PCR (N=120)



Result of PCR :
Lane 1 DNA molecular weight marker (500-100bp above downwards)
Lane 2 Positive control with band at 123bp
Lane 3 Negative control with no band
Lane 4 Sample 1 showing band at 340bp only, result is negative
Lane 5 Sample 2 showing band at 340bp and 123bp, result is positive
Lane 6 Sample 3 showing band at 340bp and 123bp, result is positive
Lane 7 Sample 4 showing band at 340bp and 123bp, result is positive
Lane 8 Sample 5 showing band at 340bp only, result is negative

CONCLUSIONS

To conclude nPCR assay targeting IS6110 is highly useful in the establishment of diagnosis of EPTB where there is strong clinical suspicion but conventional techniques are negative. The rapidity, high sensitivity and simplicity of nPCR targeting IS6110 gene sequence, may even compensate the higher cost of the test compared with less sensitive conventional tests. However in view of isolates having few or no copies of IS6110, the role of other methods like multiplex PCR using other target gene sequences also needs to be investigated.

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