DNA AMPLIFICATION OF A REPETITIVE SEQUENCE - IS 6110 IN THE EARLY DIAGNOSIS OF EXTRA PULMONARY TUBERCULOSIS

Lt Col KK LAHIRI ^{*}, Brig YK GOORHA ⁺

ABSTRACT

DNA amplification by Polymerase Chain Reaction (PCR) of a repetitive sequence specific for *Mycobacterium tuberculosis*, from clinical samples of extra pulmonary origin were evaluated. The 123 base pair fragment of the insertion element IS 6110 in *Mycobacterium tuberculosis* was amplified. A total of 50 samples were analysed by PCR and compared with culture on Lowenstein-Jensen medium (LJ) and the clinical findings of the patient. Out of the total 26 samples were positive by PCR, while only seven grew the bacilli in culture. 24 samples were negative by PCR and culture. All the seven samples that grew the bacilli on culture were positive by PCR. In remaining 19 cases that were positive by PCR but did not grow the bacilli clinical features, radiological findings and Mantoux test were strongly suggestive of *M. tuberculosis*. All the amplification negative cases had no positive evidence of tuberculosis but were being followed up. When correlated with culture and clinical history the sensitivity of PCR for the diagnosis of active tuberculosis was 100%. However, the specificity was only 55.8% as culture on LJ (Gold Standard) was positive in only 7 samples out of 26 samples that were positive by PCR.

MJAFI 2001; 57 : 12-15

KEY WORDS : DNA Amplification; IS 6110; *Mycobacterium tuberculosis*.

Introduction

Tuberculosis is being reported with increasing frequency in the developing world and has been a major cause of morbidity and mortality. With the pandemic of AIDS and development of drug resistant strains of *Mycobacterium tuberculosis* it poses a major health problem [1]. The lack of a sensitive, specific and rapid method for the early diagnosis of the disease poses an obstacle in initiating early therapy [2]. Conventional diagnostic tests like smear examination for acid fast bacilli and culture identification have serious limitations of sensitivity and the long time taken for growth of the bacilli (6-8 weeks) [3]. The epoch making discovery of DNA amplification by polymerase chain reaction has altered the entire approach to identification of pathogens which grow with difficulty or take a long time to grow [4]. This has enabled early detection of mycobacterial DNA directly from clinical specimens, especially of extra-pulmonary origin which are paucibacillary. Direct detection of mycobacterial DNA in clinical specimens using polymerase chain reaction (PCR) is now considered as a method of choice for diagnosis mainly due to the specificity, sensitivity and rapidity that the technique offers [5]. In cases of extra pulmonary tuberculosis like in the CNS, genitourinary tract and abdomen, the bacterial load is much less as compared to the pulmonary form of the disease and hence a con-

firmed diagnosis continues to remain an enigma in most cases [6]. Polymerase chain reaction using insertion sequence IS6110 as the target, has a potential to overcome limitations of conventional methods and to be established as a rapid, sensitive technique for detecting DNA of *Mycobacterium tuberculosis* in various clinical specimens [7]. In the PCR laboratory set up in our institution, we studied 50 samples from cases clinically suspected to suffer from extra pulmonary tuberculosis by PCR technique and conventional methods.

Material and Methods

Specimens from 50 patients clinically diagnosed to suffer from extra pulmonary tuberculosis were tested. A performa giving details of history, clinical findings radiological and haematological findings, result of Mantoux test was evaluated before undertaking the procedure. All specimens were processed for direct smear examination and cultured on LJ medium by standard methods. Except for CSF and joint fluid, decontamination of specimens was carried out using NaOH-n-acetyl-L-Cystiene method [8]. To avoid false positivity arising from cross contamination, DNA extraction and PCR were carried out in a separate laboratory away from the culture laboratory. A bio safety laminar air flow hood and separate set of pipettes were reserved for PCR [9].

Extraction of DNA

200ul of sample was mixed with 200ul of lysis buffer (5.3 M Guanidine Isothiocyanate, 10mMDiethylribol, 1%Tween 20,0.3 M Sodium Acetate, 50mM Sodium citrate) and the mixture was incubated at 65°C for 10 min, 50ul of glass mixing powder was added to the solution and incubated at room temperature for 1 min with

^{*}Classified Specialist (Path & Micro), Command Hospital (Western Command), Chandimandir, ⁺Commandant, AFMSD, Delhi 110 010.

Fig. 1 : Bands of amplified DNA seen on 2% agarose gel electrophoresis

M : DNA molecular size markers. Arrow indicates 123 base pair fragment. Lanes 1-9 show the electrophoretic pattern of various clinical samples. (+) : indicate positive control. (-) : indicates negative control. Lanes 1,2,4,5 show positive samples.

occasional shaking. Thereafter it was centrifuged at 1600 for 1 min. Supernant was discarded and the matrix resuspended in wash buffer (50% ethanol, 10mM Tris HCL, 100mM NaCl). Bound DNA was eluted by periodic mixing in 100ul of 10mM Tris Hcl. for PCR. The sequence was as follows :

Primers : The insertion sequence IS 6110 was the target for PCR reaction. Primers T4,T5 were selected for amplification of a 123 base pair nucleotide sequence in IS6110. The oligonucleotides were custom made by Bangalore Genie Ltd and used

Primer 1 : T4-5' CCT GCG AGC GTA GGC GTC GG3'

Primer 2 : T5-5' CTC GTC CAG CGC CGC TTC GG3'

Amplification of DNA :

The reaction buffers dNTPs and Taq polymerase enzymes were all purchased from Bangalore Genie Ltd. PCR reaction in 50 µl (1 vol. Reaction master mixture contained the following : 10X Reaction buffer - 5 µl per sample, dNTP's (200 µM each)-3 µl (each ATGC), Taq polymerase (1 unit/sample), Primers T4-1 µg/sample, T5-1 µg/sample). Make up volume to 50 µl with distilled water.

Amplification was carried out on a Cambridge Techno Thermocycler with a heated lid. The hot start protocol for amplification thereafter was carried out for 35 cycles:

90°C-1 minute (denaturation)

60°C-1 minute (annealing)

72°C - 1 minute (extension)

Extra extension was carried out at 72°C for 10 minutes after running 35 cycles. The amplification products were analysed on 2% agarose gel. The bands were visualised by staining with ethidium bromide under ultraviolet light. Positive and negative controls were run with each batch of sample analysed.

The following precautions were taken to avoid false negative cases in the study [10] :

i) Use of guanidine isothiocyanate in lysis buffer during pre treatment of samples for DNA extraction.

ii) Testing for several specimens-three days samples were pooled in case of urine.

iii) All specimens were concentrated by centrifugation before proceeding for analysis.

Results

A total of 50 samples of various specimens obtained from patients suspected to have extra pulmonary tuberculosis were analysed for DNA amplification. The various clinical samples analysed are as shown in Table 1.

TABLE 1

Result of clinical samples evaluated for mycobacterial DNA by PCR and culture method

Sample	Total no.	Only PCR positive	PCR positive culture positive	PCR negative culture negative
Urine	07	04	02	01
CSF	15	08	01	06
Pleural fluid	11	04	02	05
Ascitic fluid	11	02	Nil	09
Pus	02	Nil	02	Nil
Joint fluid	02	Nil	Nil	02
Lymphnode aspirate	02	01	Nil	01
Total	50	19	07	24

24 samples were negative by both methods of detection, ie:PCR and culture. None of the samples were positive for acid fast bacilli on direct ZN staining. Of the 26 PCR positive cases, 7 were positive on culture, while 19 cases did not yield any growth on culture. All the samples negative for DNA amplification did not grow *M. tuberculosis* on culture (Table 2).

TABLE 2

Comparison of microbiological and PCR methods in detection of mycobacterial infections (n = 50)

Microbiological diagnosis	PCR diagnosis	No. of patients (%)
Negative	Negative	24 (48%)
Positive	Positive	07 (14%)
Negative	Positive	19 (38%)
Positive	Negative	Nil

Of the 19 PCR positive, culture negative samples, 8 were from CSF, 4 each from urine and pleural fluid, 2 from ascitic fluid and 1 from lymph node aspirate. In all these cases tuberculosis was a very strong possibility, clinically and radiologically. In case of CSF specimens the patients had presence of meningeal syndrome with lymphocytosis and hypoglycorrhachia. Patients whose urine samples were evaluated had sterile pyuria with characteristic intravenous urography and endoscopy findings. The histopathology report confirmed tuberculosis in case of lymph node aspirate. The ascitic fluid evaluated showed predominance of lymphocytes. All cases showed clinical improvement with antituberculous therapy and were being followed up.

Discussion

In this study we have attempted to investigate the relevance of PCR technology in cases of extra pulmonary tuberculosis where empirical antitubercular therapy is initiated on the basis of clinical profile, radio-

logical findings and a positive tuberculin skin test (Mantoux). Pao et al and Brisson et al have established the significance of PCR as a more sensitive technique than the traditional bacteriological culture [11,12].

In this study the high degree of sensitivity is apparent in the actual clinical setting, since PCR detected 26 out of the 56 cases (52%) studied, of which culture was positive in only seven cases (26.9%). A significant number of PCR positive-19/43 (44%) but culture and ZN negative samples were seen from specimens of different sites. Kolk et al have found significant number of PCR positive-culture negative samples from different sites [13]. False positive cases need to be excluded in such a study due to cross contamination. However strong clinical suspicion and improvement on follow up with anti tuberculous therapy were good indicators that these cases had extra pulmonary tuberculosis.

A confirmed diagnosis of tuberculous meningitis is always difficult due to low bacterial load and in most cases direct microscopy and culture is usually negative. In such cases, clinical findings and CSF examination report determine whether anti tuberculous therapy is to be instituted or not. We found that 8 of the 15 CSF samples were positive by PCR, while only one sample was culture positive (Table-1). PCR of this sample showed a more intense band as compared to the culture negative only PCR positive samples. Similar observations were made by Kolk et al in their study of CSF samples, indicating that PCR is able to detect a greater Mycobacterial load in culture positive samples [13]. Rodrigues et al studied 41 CSF samples of which 13 were positive for DNA amplification while only 3 samples grew the bacilli on culture in LJ medium [14]. Similar low sensitivity for CSF has been reported by Shanker et al and Albright et al [15,16].

Of 11 samples from pleural fluid 6 were positive by PCR of which two grew bacilli on culture, thus demonstrating fairly good sensitivity and specificity. Samples of pus showed 100% sensitivity and specificity since both samples that grew bacilli on culture were also positive by PCR. In case of lymph node aspirate although culture did not grow the bacilli, classical granulomas were seen on histopathology and PCR result was positive.

7 urine samples analysed showed good results as 4 of them were PCR positive, while 2 samples were PCR and culture positive. Only 1 case was negative by both the tests. Kolk et al in their study on urine samples from 6 cases, grew bacilli on culture in 2 cases that were also PCR positive but had 4 cases

which were negative by culture and PCR [13]. All cases positive by PCR had associated findings on their IVP, cystoscopy and clinical features to suggest genito-urinary tuberculosis. The patients subsequently improved with anti tuberculous therapy. The case that was negative by culture and PCR was detected to have obstructive uropathy and improved with surgical intervention and antibiotics.

11 samples of ascitic fluid were evaluated and showed predominance of lymphocytes. These patients were suspected to suffer from intestinal tuberculosis on the basis of their clinical presentation, barium studies and other parameters. Of these only 2 were positive by PCR while none grew the bacilli on culture. Although PCR negative and culture positive results have been reported, none were found in this study, but it brings forth the limitations of PCR and the importance of a representative sample for the procedure [13].

The specificity of PCR technique is controlled by the DNA segment chosen for amplification. We used IS-6110 an IS-like element of *M. tuberculosis* complex which is a repetitive sequence found repeated 10-16 times in the chromosome. Among the genetic markers, IS-6110 shows maximum heterogeneous polymorphic patterns between different clinical isolates of *M. tuberculosis*. The sequence of the element is highly conserved in various isolates. The heterogeneity in finger printing pattern reflects difference in copy number and sites of insertion, which could be due to duplication and/or movement of the element, yet the laboratory investigations suggest that IS6110 patterns are relatively stable [17]. Studies by Anderson et al and Rodrigues et al using IS6110 as the target sequence found no difficulty in detecting DNA of *M. tuberculosis* [14]. However although most strains of *M. tuberculosis* have between 8-15 copies, Sahadevan et al and Van Soolingen et al have reported that some strains have only one copy and others lack IS6110 completely [18]. In such cases PCR will give false negative results. Recent studies have also shown IS6110 homologues being present in multiple copies in mycobacteria other than tuberculosis causing mycobacteria, hence caution must be used in designing and evaluating PCR tests based on this element.

The value of DNA amplification by PCR as a diagnostic tool in tuberculosis infections lies in its sensitivity. This will enable us to diagnose many more cases missed by the traditional methods because of their low sensitivity. Clinical co-relation is of utmost importance when a test based on DNA amplification is analysed without support from any other confirmed investigation. It is known that extra pulmonary sites of

M. tuberculosis infection have a very low yield of bacilli and in this study the significance of PCR as a laboratory diagnostic tool is highlighted. It is the method of choice, as it provides reliable, rapid and accurate results in such cases thereby reducing the number of cases being treated empirically [19]. Refinement of PCR technique by using specific DNA probes for the analysis of amplified products, hybridisation assay, digestion of amplicons prior to PCR and following strict laboratory protocols to prevent cross contamination will make this procedure an important technique for the future in the early diagnosis of extra pulmonary tuberculosis.

Acknowledgement

We would like to acknowledge the help of Dr. TN Dhole, Additional Professor, Dept of Microbiology, SGPGI, Lucknow and Dr. Ranjana Srivastva, Scientist E, Microbiology Division CDRI, Lucknow for all their whole hearted help and guidance in establishing PCR laboratory at CPL (CC), Lucknow.

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