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EFFICACY OF POLYMERASE CHAIN REACTION OVER ZIEHL-NEELSEN STAINING IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS

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ABSTRACT

Tuberculosis, one of the oldest diseases tormenting humankind, is especially rampant in developing countries like India, with a prevalence as high as 30%. Yet tuberculosis control has not been achieved due to lack of rapid identification methods. The objectives of this study were to examine microscopically all specimens after Ziehl-Neelsen staining, isolate genomic DNA from samples, perform PCR amplification using specific primers, and compare results obtained by both methods. Sixty clinical samples, which included 40 sputa and 10 samples each of pleural and ascitic fluid, from patients clinically suspected for tuberculosis were subjected to AFB staining and detection of *Mycobacterium tuberculosis* using PCR. Of the 40 cases, 16 were found positive by microscopy and 30 were found positive by PCR. Of the 20 extrapulmonary cases, 4 were found positive by microscopy and 16 were found positive by PCR, proving the superiority of PCR in the early detection of *Mycobacterium tuberculosis*.

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KEYWORDS

Ziehl-Neelsen Staining (ZN), Polymerase Chain Reaction (PCR), *Mycobacterium Tuberculosis* (Mtb), Acid-Fast Bacilli (AFB), Insertion sequence (Is)

INTRODUCTION

Tuberculosis is a major air borne infection, a disease with worldwide significance. The global mortality ranges from 1.6-2.2 million lives per year, with India harboring more than 30 % of cases[1]. Keeping in view the global prevalence of tuberculosis and its exacerbation, with the increasing incidence of drug resistance, there is a growing need to evolve a method to rapidly identify this organism. Early diagnosis is vital for the control of tuberculosis.

Traditionally, the diagnosis of tuberculosis is based on the Ziehl-Neelsen acid-fast Stain and culture of the organism, *Mycobacterium tuberculosis*. The ZN stain, although inexpensive and rapid, lacks sensitivity. Culture though it continues to be the gold standard, is time consuming (3-8 weeks)[2]. Clinical and therapeutic decisions have to be made earlier than the laboratory identification. Several studies have been done to detect *Mycobacterium tuberculosis* in respiratory and other clinical samples by amplifying different DNA sequences of *Mycobacterium tuberculosis* by polymerase chain reaction[3,4] . During the last decade, advances in understanding the genetic structure of mycobacterium have been made. Based on this, specific gene sequences, several gene probes and gene amplification systems for tuberculosis have been developed[5,6]. IS6110 is a 1,355 bp compound that is specific for *Mycobacterium tuberculosis* and is present in most strains at about 20 copies per genome[7]. IS6110 as the standard probe has proven to be a powerful molecular tool for epidemiological investigation, especially in outbreak situations[8]. A review of many studies indicates that most protocols use the repeat insertion sequence IS6110 as a target for amplification of *Mycobacterium tuberculosis*. The present study was carried out using a nested PCR method. We compared the results of the PCR with those of the conventional Ziehl-Neelsen stained acid-fast bacilli (AFB) microscopy.

MATERIALS AND METHODS

Sixty (60) clinical samples were obtained from patients with a strong clinical, radiological and histopathological evidence of tuberculosis attending two tertiary care hospitals and DOTS centers around these hospitals in a major city in India in the year, 2010. Necessary clinical details were obtained from the referring hospitals in the prescribed format.

(i) Clinical Specimens:

Clinical samples were selected from adult pulmonary and extrapulmonary TB cases in the age group of 20 to 60 years. All were initially screened by AFB smear examination. All specimens submitted for the diagnosis of TB from January to June 2010 were examined in parallel by conventional analysis, (ZN stain) and PCR.

(ii) Staining and Microscopic Exam:

Smears were made directly from sputum samples and after centrifugation from pleural and ascitic fluid specimens and Ziehl-Neelsen's staining was done on these smears using standard techniques. The smears were

examined microscopically for the presence of acid-fast bacilli.

(iii) Polymerase Chain Reaction:

The tests were done using GeNei™ (Bangalore, GeNei, India) amplification reagent set for *Mycobacterium tuberculosis*. The DNA was extracted and PCR was performed. The test was based on the principles of single tube nested PCR method, which is a powerful and sensitive diagnostic tool for the identification of *Mycobacterium tuberculosis* complex. This assay is a two-step sequential assay. In the first step; the IS region of Mtb complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product. In this test, false positive reactions that are caused by previous amplicon contamination were prevented by the use of uracil DNA glycosylase (UDG) and dUTP instead of dTTP. The highlights of this PCR technique were a fast and simple DNA extraction protocol, a fast result within 5.6 hours and the use of Hotstart Taq DNA polymerase for increased sensitivity.

(a) Sample Processing for DNA Extraction:

(1) Sputum

Sputum sample was mixed with equal volume of N-acetyl L-cysteine/5mg/ml solution (mucolytic) and centrifuged at 8000 rpm for 15 minutes. The pellet formed was suspended in 20-30 ml of 20 mM Tris buffer and centrifuged for 10 minutes. Pellet was re-suspended in 250 µl of lysis buffer I. If pellet was large, 1ml of lysis buffer I was added, mixed well, and 250 µl was taken for digestion with proteinase K.

(2) Pleural Fluid and Ascitic Fluid

The sample was centrifuged at 8000 rpm for 15 minutes and the pellet formed was suspended in 20-30 ml of 20 mM Tris buffer and centrifuged for 10 minutes. Pellet was again resuspended in 250 µl of lysis buffer I. If pellet was large, 1ml of lysis buffer I was added, mixed well, and 250 µl was taken for digestion with proteinase K.

(b) DNA Extraction

To 250 µl of the extracted material, 20 µl proteinase K was added, mixed by vortexing and incubated at 65°C for 30 minutes. The samples were then centrifuged at 10,000 rpm for 10 minutes. To 200 µl of supernatant, 200 µl of lysis buffer II was added, mixed by pulse vortexing for 15 seconds and incubated at 70°C for 10 minutes. 200 µl of 96-100% distilled ethanol was added and mixed thoroughly by vortexing. Spin column was kept in a 2 ml collection tube and sample ethanol mixture was added to the column and centrifuged at 8000 rpm for 3 minutes. Spin column was now kept in a new 2 ml collection tube, and 500 µl of wash buffer I was added and spun at 8000 rpm/3 min. 500 µl of wash buffer II was added to the spin column and spun at 14,000 rpm for 3 minutes and the flow through was discarded. Empty column was spun at same speed for 2 minutes. Spin column was placed in a new labeled 1.5 ml tube and 100 µl of pre-warmed (50°C) elution buffer was added. It was incubated at room temp for 5 minutes and centrifuged at 10,000 rpm for 1-2 minutes to elute the DNA.

(c) DNA Amplification

The PCR procedure is a nested PCR based on the amplification of the repeated insertion sequence IS*6110*. The outer primers corresponded to nucleotides 695 to 724 (5'-CGGGACCACCCGCGGCAAAGCCCGCAGGA C-3') and 885 to 914 (5'-CATCGTGGAAGCGACCCGCCAGCCCAGGAT -3') of the IS*6110* sequence and amplified a 220-bp fragment. The inner primers (primer IS1 [5' CCTGCGAGCGTAGGCGTCGG-3'] and primer IS2 [5'-CTCGTCCAGCGCCGCTTCGG-3']) amplified a 123-bp fragment.

(1) First Amplification

The first amplification master mix contained first amplification pre-mix (reaction buffer with $MgCl_2$, dNTPs and Mtb complex specific external primers) 8.2 µl; Hotstart Taq DNA polymerase 0.33 µl and uracil DNA glycosylase (UDG) 0.5 µl (final reaction volume 9 µl). To 9 µl of amplification master mix, 3 µl of sample DNA was added. 3 µl of positive control DNA was set up as a positive control vial. The last vial with no sample DNA or positive control DNA was the reagent control vial. The reaction cycle was carried out in a DNA thermal cycler (Biorad, California, USA) . The first amplification reaction profile consisted of a cycle of 22°C for 10 minutes and 94°C for 5 minutes – initial denaturation step; followed by 20 cycles at 94°C for 30 seconds (denaturation), 68°C for 1 minute (annealing) and 72°C for 1 minute (extension) and a final extension cycle at 70°C for 7 minutes. The vials were removed from the thermal cycler when samples reached 4°C and then centrifuged at 10,000 rpm for 30 seconds to avoid aerosol contamination.

(2) Nested or Second Amplification

The second amplification master mix contained second amplification pre-mix 14.7 µl (reaction buffer with $MgCl_2$, dNTPs, Mtb complex specific internal primers) and Hotstart Taq DNA polymerase 0.33 µl. No UDG was added. After the first PCR was completed, 15µl of second amplification master-mix was added to each tube and the second PCR was performed as follows: a cycle at 94°C for 5 minutes for initial denaturation followed by 30 cycles at 94°C for 30 seconds (denaturation), 68°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) and a cycle of final extension at 72°C for 7 minutes.

(d) Analysis of Amplified Products by Gel Electrophoresis

2.5% agarose gel was prepared by adding 2.5g of agarose to 100 ml of 1 x TAE (tris acetate EDTA) buffer and boiling. 10 µl of 10 mg/ml ethidium bromide dye solution was added for 100 ml of agar, cooled to 60°C and poured into the gel box (0.7 cm thick). The reservoir buffer was added and comb carefully removed, once gel solidified. 15 µl of sample mixed with 3 µl gel loading dye and 5 µl ready-to-use marker were loaded. The gel was run at 100-120 volts. The gel was removed and visualized using a mid wave UV transilluminator to read the final result. An amplification product of size 123 bp is indicative of infection with *Mycobacterium tuberculosis* complex. The amplification product of internal control DNA is 340 bp.

PCR amplification of Mtb DNA

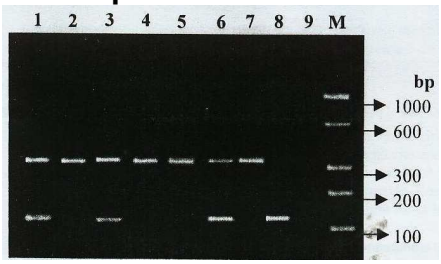


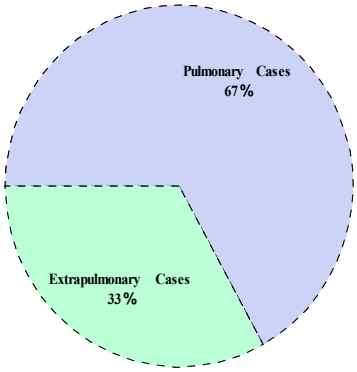
Figure 1

Positive: Band at 123 bp and 340 bp/band at only 123 bp. Lane 1, 3, 6, sample positive for Mtb. Lane 2,4,5,7, sample negative. Lane 8, positive control. Lane 9, negative control. Lane M, molecular weight marker.

RESULTS

A total of 60 cases were evaluated in the age group of 20-60 years. All 60 cases were clinically suspected to be suffering from tuberculosis. Sputum, pleural fluid or ascitic fluid were collected as a specimen for diagnosing tuberculosis. Of the 60 cases, 40 cases were pulmonary and 20 were extrapulmonary cases. The graph below shows the percentage of these cases - 40 pulmonary cases constitute a 67% of the total cases and 20 extra-pulmonary cases constitute a 33% of the total cases.

Graph 1
Distribution of pulmonary and extrapulmonary cases



Out of the 40 cases, 16 were found positive by microscopy and 30 were found positive by PCR giving a sensitivity of 40% for microscopy versus 75% for PCR. Table 1 shows the sensitivity of the different tests on pulmonary cases. This is a significant difference, with p-value = 0.00077. Out of the 20 extrapulmonary cases, 4 were found positive by microscopy and 16 were found positive by PCR giving a sensitivity of 20% for microscopy versus 80% for PCR which is represented in Table 2. This is a significant difference, with p-value = 0.000073.

Table 1
Sensitivity of Different Tests on Pulmonary Cases

	Result		Sensitivity
Tests	Positive	Negative	
Microscopy	16	24	40%
PCR	30	10	75%

Table 2
Sensitivity of Different Tests on Extrapulmonary Cases

	Result		Sensitivity
Tests	**Positive**	**Negative**	
Microscopy	4	16	20%
PCR	16	4	80%

DISCUSSION

Rapid detection of mycobacterium complex is important in patient management in terms of initiating appropriate therapy as well as in controlling the spread of this pathogen. AFB staining generally provides rapid evidence for the presence of mycobacteria in a clinical specimen. The detection of acid–fast bacilli in stained smears examined microscopically is the first bacteriologic evidence of the presence of mycobacteria in a clinical specimen. It is the easiest and quickest procedure that can be performed, and it provides the physician with a preliminary confirmation of the diagnosis also. Because it gives a quantitative estimation of the number of bacilli being excreted, the smear is of vital clinical and epidemiologic importance in assessing the patient's infectiousness. AFB staining is still the primary diagnostic test in most laboratories. Molecular tests based on nucleic acid amplification techniques have been available for the detection of *Mycobacterium tuberculosis* complex in a variety of clinical specimens[9-16]. TB is a major health problem in India. Amplification of Mtb-specific DNA sequences in clinical samples is the most sensitive and rapid method of detection available. PCR has been shown to be a sensitive (70% to 100%) and specific (> 90%) diagnostic test for both pulmonary and extrapulmonary tuberculosis in adults. The sensitivity and speed of PCR test in diagnosis of Mtb infection as shown in this study proves that it is a definitely superior modality of detection as compared to the conventional AFB staining, especially in cases of extrapulmonary TB where the mycobacteria are but rarely detected. PCR is the method of choice for the diagnosis of paucibacillary TB. Economic restraints constitute a great deterrent for the widespread and routine use of this technique. Efforts must be made to evaluate the utility of PCR for diagnosing TB in Indian conditions.

CONCLUSION

Molecular diagnosis of TB by PCR has great potential to improve the detection of the disease. Better detection by PCR because of its sensitivity will ensure early treatment and prevent further transmission of disease. However, more research is required to gain an insight into methods for improving sensitivity, specificity and reproducibility of this test, and to make it more user-friendly and cost-effective.

REFERENCES

1. World Health Organization (WHO). Global Tuberculosis Control. WHO Report 2001, Geneva Switzerland, WHO/CDS/TB/2001.287

2. Shenai S., C. Rodrigues, A. Almeida, and A. Mehta, Rapid Diagnosis of Tuberculosis using Transcription Mediated amplification. Indian Journal of Medical Microbiology, 19(4): 184-189, (2001).
3. Kox L.F.F. , D. Rhienthong, J. Van Leeuwen, S Van Heusden, S. Kuijper, and A. H. J. Kolk, A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. Journal of clinical Microbiology, 672-678, (1994).
4. Kesarwani R.C, Pandey Anjana, Misra Ashutosh, Singh Anupam Kumar, Polymerase chain reaction: its comparison with conventional techniques for diagnosis of extra-pulmonary tuberculosis diseases. Indian Journal of Surgery 66 (2): 84-88 (2004).
5. Van Soolingen, D, P.E.W. de Haas, P.W.H. Hermans, P.M.A. Groenen, and J.D.A. Van Embolen, Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbial, 31(8): 1987-1995, (1993).
6. Katoch V. M and Sharma V. D., Advances in the diagnosis of mycobacterial diseases. Indian Journal of Med Microbiology, 15: 49-55, (1997).
7. Cave, M.D., K.D. Eisenach, P.F. McDermott, J. H. Bates, and J.T. Crawford, IS-6110: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Mol. Cell. Probes, 5: Pages 73–80 (1991).
8. Thierry, D., M.D. Cave, K.D. Eisenbach, J.T. Crawford, J.H. Bates, B. Gicquel, and J.L. Guesdon. IS-6110, an IS-like element of *Mycobacterium tuberculosis* complex. Nucleic Acids Research, 18: 188, (1990).
9. Brisson-Noel, A., C. Aznar, C. Chureau, S. Nguyen, C. Pierre, M. Bartoli, R. Bonete, G. Pialoux, B. Gicquel, and G. Garrigue, Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. Lancet, 338:364-366, (1991).
10. Hance, A. J., B. Grandchamp, V. Levy-frebault, D. Lecossier, J.Rauzier, D. Bocart, and B.glcquel, Detection and identification of mycobacterium by amplification of mycobacterial DNA. Mol. Microbiol., 3:843-849, (1989).
11. Hermans, P. W. M., A. R. J. Schuitema, D. van Soolingen, C. P. H. J. Verstijnen, E. M. Blk, J. E. R. Thole, A. H. J. Kolk, and J. D. A van Embden, Specific detection of Mycobacterial tuberculosis complex strains by polymerase chain reactions. J. Clin. Microbiol., 28:1204-1213 (1990).
12. Longo, M. C., M. S. Berninger, and J. L. Hartley, Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene, 93:125-128, (1990).
13. McAdam, R. A., P. W. M. Hermans, D. van Soolingen, Z. F. Zainuddin, D. Catty , J. D. A. van Embden, and J. W. Dale. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. Mol. Microbiol., 4:1607-1613, (1990).
14. Pao, C. C., T. S. Benedict Yen, Jinn-Bang You, Juehn-Shin Maa, E. H. Fiss, and Chau-Hsiung Chang, Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. J. Clin. Microbiol., 28:1877-1880, (1990).
15. Persing, D. H., Polymerase chain reaction: trenches to benches. J.Clin. Microbiol., 29:1281-1285, (1991).
16. Thierry, D., A. Brisson-Noel, V. Levy-frebault, S. Nguyen, J. Guesdon, and B. Gicquel, Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. J. Clin. Microbiol., 28:2668-2673, (1990).