

Research Article

Comparative Analysis of *mpb-64* and Nested PCR targeting IS6110 for the Detection of Extra Pulmonary Tuberculosis

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Abstract

Introduction: Extra Pulmonary Tuberculosis can be seen in more than half of the patients with concurrent AIDS and tuberculosis. A high index of suspicion is necessary for timely diagnosis and prompt initiation of therapy. PCR has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms for which culture is too insensitive and paucibacillary nature of the pathogens in the specimens.

Aim: Evaluation of the efficacy of dual targets; *mpb-64* and Nested PCR targeting IS6110 for the detection of Extra Pulmonary Tuberculosis.

Place and Duration of Study: Done at Molecular Research Laboratory, Biochemistry Department, SGRRIM&HS, Dehradun, India, in between the duration, 2012-2013.

Methodology: A total of ninety clinical specimens, which includes; 14 pus, 32 CSF, 4 ascetic fluid, 6 biopsy, 10 blood, 3 urine, 6 endometrial tissues, 6 pleural fluids, 1 endometrial curetting, and 2 synovial fluid, collected from the various departments of SMI Hospital, Patel Nagar, Dehradun. Specimens were further processed for ZN staining, PCR utilizing; *mpb-64* and nested PCR (N-PCR) using IS6110 as a target for the detection of extra pulmonary tuberculosis cases.

Results: Out of 90 clinical specimens processed, 29 (32.2%) came N-PCR positive and 23 (25.5%) are *mpb64* TB PCR positive. When analyzed comparatively for both the molecular targets, it was seen that 20 samples came positive by both the targets i.e. IS6110 as well as *mpb64* gene, but 9 specimens alone came positive only by IS6110 NTPCR, and the same came negative for *mpb64* gene. 3 specimens showed positive result status for *mpb64* gene by conventional assay and at the same time they were negative for IS6110 gene.

Conclusion: An essential element in the management of extra pulmonary tuberculosis is the availability of rapid, sensitive and specific identification of the causative agent. The laboratory diagnosis is largely based on direct microscopy and culture for mycobacterium. Direct microscopic examination is used to detect acid-fast bacilli (AFB) & has very low sensitivity. The gold standard for isolation of mycobacterium is not only time-consuming but also has low sensitivity in case of extra pulmonary tuberculosis. TB PCR diagnostics kits must be prepared, targeting *mpb-64* as well as IS6110 as a targets to avoid false results.

Keywords: Extra-pulmonary samples, Copy Number, Molecular targets, Amplimers, Lymph node, Polymerase Chain Reaction.

1. Introduction

The extra-pulmonary Tuberculosis is much less common than pulmonary disease. However, in individuals with AIDS, extra-pulmonary tuberculosis predominates, particularly with lymph node involvements. Thus the diagnosis become very crucial for the detection of the same. AFB smears, serological tests, molecular tests and cultures can be used for the revealing of the pathogens. In terms of sensitivity, specificity, robustness and time, molecular diagnosis is increasing in Mycobacteriology laboratories. Microscopy with Acid Fast Bacilli (AFB) staining is the most rapid laboratory method for diagnosing tuberculosis. Its drawback is low specificity & sensitivity. Gold standard for laboratory diagnosis of tuberculosis is the isolation of M. Tuberculosis from clinical specimens by culturing. Culture is more specific & sensitive than staining & can detect as few as 10 organism/ml of digested and concentrated material but requires about 2-4 weeks to grow. Numerous targets have been studied as a tool for the detection of this disease. *IS6110* is an insertion element found exclusively within the members of the Mycobacterium Tuberculosis complex (MTBC), & because of this exclusivity, it has become an important diagnostic tool in the identification of MTBC species. *IS6110* is being used for the identification of TB using procedures such as RFLP and Spoligotyping for several years because this is a genetic insertion sequence (IS) or element that is found exclusively within the members of the Mycobacterium tuberculosis complex (MTBC). Some researchers have found *IS6110*-like elements in some other mycobacteria such as *M. smegmatis* is also present, but because *smegmatis* do not cause TB, its clinical importance is lesser¹.

Only small numbers of bacilli are needed to cause extra-pulmonary tuberculosis cases but PCR amplification for MTB complex in extra pulmonary samples like synovial fluid, CSF, pleural fluid and bone marrow renders the method capable of detecting such small numbers of bacilli in specimens which would otherwise be undetectable using other conventional methods for the diagnosis of tuberculosis. In the last decade, nucleic acid amplification-based techniques (NAAT's) have become accessible to the clinical mycobacteriology laboratory. PCR protocols amplifying a large variety of chromosomal DNA have concentrated on detection of both genus-specific and *M. tuberculosis* complex-specific DNA regions. The insertion element *IS6110* and the 16S rDNA are the most common targets used. Other regions used for amplification includes *rpoB* gene encoding the β -subunit of the RNA polymerase, the gene coding for the 32 kD protein, the *recA* gene, the *hsp65* gene, the *dnaJ* gene, the *sodA* gene and the 16S-23S rRNA internal transcriber spacer²⁻⁴. These tests are highly sensitive for clinical samples and studies have shown that sensitivity and specificity ranging as high as 90-100%. NAATs may be tested on any specimen thought to contain bacilli (blood, urine, cerebro spinal fluid (CSF) but there is even less sensitivity reported in extra pulmonary specimens. Sensitivity is improved when multiple sampling are tested, because not all samples necessarily contain detectable nucleic acid^{5,6}.

Most of in house PCR procedures achieve a sensitivity never matched by commercial systems but are often burdened by the high incidence of false positive results due to amplicon cross-contamination of specimens. To minimize the risk of specimen-to-specimen contamination, a physical separation of processes, equipment, and reagents is recommended. Enzyme contamination control systems such as uracil-N-glycosylase (UNG) can be incorporated into the PCR master mix as an added safeguard to sterilize amplified product that may be carried over to subsequent batches of tests⁹. Thus the present study focus on the comparative analysis of molecular markers; *IS6110* and *mpb64* with the utilization of Nested-PCR and Uracil-N-Glycosylase usage (to get rid of amplicon contamination), for the diagnosis of Mycobacterium Tuberculosis complex for extra pulmonary specimens.

2. Materials and Methods

The current research work was carried out at Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun, Uttarakhand. Clinical specimens which includes; 14 pus, 32 CSF, 4 ascetic fluid, 6 biopsy, 10 blood, 3 urine, 6 endometrial tissues, 6 pleural fluids, 1 endometrial curetting and 2 synovial fluid, were collected from various departments of Shri Mahant Indresh Hospital. Further the Nucleic Acid (DNA) was extracted from all the clinical specimens by spin column based Nucleic Acid Extraction method. Column-based nucleic acid purification is a solid phase extraction method to quickly purify nucleic acids. Further Nested PCR was performed on the DNA template isolated. This test is 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 Insertion sequence region of Mycobacterium tuberculosis 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 assay, false positive reactions that may be caused by previous amplicon contamination are prevented by the use of uracil DNA glycosylase (UDG) and dUTP instead of dTTP added in the premix. Nested PCR. An amplicon of size 123 bp is indicative

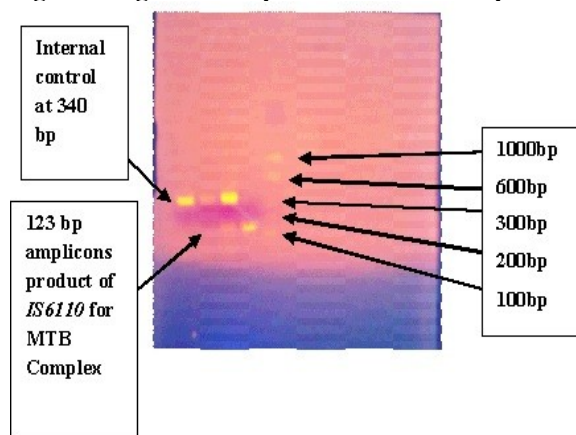
of infection with *Mycobacterium tuberculosis* complex. The amplification product of internal control DNA is 340 bp which is used for the validation of the results (as depicted in figure1). Primers utilized for the research work includes; IS6110 and *mpb64*. IS6110 using Nested primers, yielding, 123 base pairs amplicon of IS6110 repetitive sequence; forward primer, 5'-CCTGCGAGCGTAGGCGTCGGT-3' (P1) and reverse primers, 5'-CTCGTCCAGCGCCGCTTCGG-3' (P2). In case of *mpb-64* based PCR, amplification was carried out for all the templates including controls. PCR was performed for the amplification of gene *mpb64* using primers Forward primer P1-5'-TCCGCTGCCAGTCGTCTTCC-3' and Reverse primer P2 5'-GTCCTCGCGAGTCTAGGCCA-3'. A reaction mixture of 50 µl containing 10X PCR buffer (250 mM Tris Hcl, 500 mM KCl), 0.2 mM dNTPS, 25µM primers, Taq polymerase (3 units) and Mg²⁺ ions (25mM) as MgSO₄ was prepared. 25µl of DNA template was added in the 25µl of master mix and amplification was done in thermal cycler (Bench Top) for 40 cycles.

Cycling conditions include; initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 60°C for 1 minute and primer extension at 72° C for 1 minute were provided with final extension of PCR products at 72° C for 7 minutes. Electrophoresis was carried out at 100-150 Volt (5 to 8 V/cm for 20 cm gel) until the bands in the molecular weight marker were resolved. Gel was examined under UV light (302nm) on ULTRA LUM Electronic UV Trans illuminator gel documentation system for the presence of 240 bp PCR product and photographed. The sizes of the amplicons were determined using DNA ladder of 100 bp (Bangalore GENEI Cat # MBD13J). ZN smears for the examination of acid fast bacilli were also prepared and observed under oil immersion.

2.1 Detection of amplified products

Amplification of 240 base pairs DNA fragments against the 240 base pairs target sequence of *mpb-64* gene resolving on agarose gel equivalent to positive control and DNA ladder indicate the presence of MTB strain in clinical samples. Where as for N-PCR, 123 base pair amplicon for IS6110 gene was observed, including internal control appearing at 340 base pairs.

Figure 1. Agarose Gel picture for MTC amplimers.



3. Results

Out of 90 clinical specimens processed, it was analyzed that 29 (32.2%) specimens are N-PCR positive and 23 (25.5%) specimens are *mpb64* TB PCR positive. When analyzed comparatively for both the molecular targets, it was seen that 20 samples came positive for both the targets i.e. *IS6110* as well as *mpb64* gene. 9 specimens alone came positive only by *IS6110* Nested-PCR, and the same came negative for *mpb64* gene. 3 specimens showed positive result status for *mpb64* gene by conventional assay and at the same time they were negative for *IS6110* gene (as depicted in table 2). AFB positive smears were only observed in 5.5 % cases only (as tabulated in table 1).

When analyzed by age wise for positivity rate, higher percent was seen in between 11-40 years. Significantly it was seen that CSF came mostly from children were in age group ranging from 0-10, showing 33.3% positivity rate. Age groups 0-10, 11-20 showed positivity rates of 33.3% and 42.8% respectively, where specimens mostly includes CSF from infants and children.

Table no 1. Specimen wise results interpretation for mycobacterium tuberculosis complex detection by IS6110 and mpb64 genes as targets in PCR with comparative results with AFB smears.

Sr. No.	Type of Specimen	PCR Results Positive by N-PCR	PCR Results Positive by mpb64	AFB Results		Positivity rate AFB (%)
				Positive	Negative	
1.	Pus (14)	7 (50%)	4(4.4%)	4	10	4.4
2.	CSF (32)	16 (50%)	16(50%)	0	32	0
3.	Ascetic fluid (04)	1 (25%)	0(0%)	0	4	0
4.	Biopsy(06)	1(16.6%)	0(0%)	0	6	0
5.	Tissue (06)	1(16.6%)	0(0%)	0	6	0
6.	Blood (10)	1(10%)	0(0%)	0	10	0
7.	Urine (03)	1(33.3%)	0(0%)	1	2	1.1
8.	Endometrial Tissue (06)	1(16.6%)	0(0%)	0	6	0
9.	Pleural fluid(06)	0(0%)	2(2.2%)	0	6	0
10.	Endometrial curetting(01)	0(0%)	0(0%)	0	1	0
11.	Synovial fluid(02)	0(0%)	1(1.1%)	0	2	0
Total no. of Specimens		29(32.2%)	23(25.5%)	5(5.5%)	85 (94.5%)	5(5.5%)

Table no 2. Comparative results of IS6110 N-TB PCR and mpb64 TB PCR.

Number of specimens	N-PCR (IS6110)	TB PCR(mpb64)	%
20	+	+	22.2%
9	+	-	10%
3	-	+	3.3%
58	-	-	64.4%
Total positive with individual PCR	29(32.2%)	23(25.5%)	
Total positive with both PCR	32(35.5%)		

Table no 3. Age wise positivity distribution for extra pulmonary tuberculosis.

Age group (years)	Total no. of Specimens	PCR Positive	PCR Negative	Positivity (%)
0-10	12	4	8	33.3%
11-20	21	9	12	42.8%
21-30	24	8	16	33.3%
31-40	18	6	12	33.3%
41-50	8	1	7	12.5%
Above 50	7	1	6	14.2%
Total no. of Specimens	90	29	61	32.2%

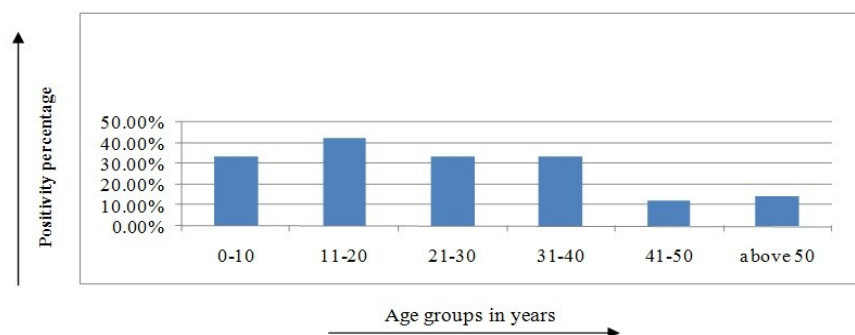
Fig.2. Age wise positivity distribution for extra pulmonary tuberculosis

Table no. 4. Comparative result of IS6110 and mpb64 genes.

Total no. of specimens	IS6110 (N-PCR)	<i>mpb- 64</i> MTC PCR
90	29 (32.2%)	23(25.5%)

4. Discussion and Conclusion

Extra Pulmonary Tuberculosis is a contagious bacterial infection that involves organs other than the lungs. Extra pulmonary tuberculosis is caused by the bacteria *Mycobacterium tuberculosis* (*M. tuberculosis*). Extra pulmonary tuberculosis affects people with weak immune system, diabetes, HIV, or malnourished people, very young or elderly, those undergoing prolonged treatment with Chemotherapy or cortisone. The most common forms of Extra pulmonary tuberculosis are:-Lymph node tuberculosis, pleural tuberculosis, osteo-articular tuberculosis, central nervous system tuberculosis, tuberculosis of other places. Amplification technologies offers the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity. Polymerase Chain Reaction has been found to be useful for rapid diagnosis of tuberculosis from variety of clinical specimens. Many laboratories around the globe are using primers designed from IS6110 sequence of *Mycobacterium* genome. IS6110 is an insertion sequence specific for *Mycobacterium tuberculosis*. Timely detection of various forms of extra pulmonary tuberculosis is of great importance for the proper treatment and management of the disease. Novel, rapid and cost effectiveness are the basic features of most of the PCR based techniques, but the technologies which can be either bio molecules, chemicals, antibodies or any recombinant proteins incorporating in the assays to prevent amplicons contamination will be an added advantage. As nested PCR it increases the sensitivity and specificity of an assay and when UNG is being utilized in pre-mix will make the assay a significant molecular diagnostic tool for *Mycobacterium tuberculosis* complex detection. Nested PCR for tuberculosis detection is a better tool when incorporated with an addition of UNG to prevent Amplicons contamination. False positive cases by amplicons contamination can be prevented by UNG and dUTP instead of dTTP⁶⁶.

The skill set required to adequately treat critically ill patients will also require knowledge of molecular biology for better diagnosis and treatment. The foundations of molecular biology and genetics are essential for the understanding of the mechanisms of disease. Correct, novel, significant molecular diagnostic tools are very important for all those laboratories performing routine diagnosis of tuberculosis in PCR based laboratories settings. In addition, particular emphasis should be applied to quality control and quality assurance programs in clinical laboratories which employ any new diagnostic approaches. Amplicons contamination detection and its prevention are of critical importance where the results interpretations are directly involved with patient's health. Acceptance and implementation of PCR in the diagnostic laboratory requires an understanding of its mechanics, meaning of results, the test's limitations, and being able to recognize problems and trouble-shoot them as they arise. IS6110 is an insertion element that is found exclusively within the MTBC; the assumption has been that this restriction is a result of the lack of genetic exchange with other Mycobacterial Species. A benefit of this exclusivity is that IS6110 has become an important diagnostic tool in the differentiation of MTBC species from other mycobacteria. Moreover, the element's presence in multiple copies, and at differing locations in the genome, has provided an excellent method by which strains can be genotyped; because of these characteristics, IS6110 has been used extensively for epidemiological studies. An essential element in the management of extra pulmonary tuberculosis is the availability of rapid, sensitive and specific identification of the causative agent. The laboratory diagnosis is largely based on direct microscopy and culture for mycobacterium. Direct microscopic examination is used to detect acid-fast bacilli (AFB) & has very low sensitivity. The gold standard for isolation of mycobacterium is not only time-consuming but also has low sensitivity in case of extra pulmonary tuberculosis. 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. *mpb-64* gene has also been found in large copy number in some of the members of *Mycobacterium tuberculosis*, *M. bovis* and some strains of BCG of the Indian subcontinent. Targeting only a single gene can leads to false results as in some of the members of *Mycobacterium tuberculosis* complex, either IS6110 or *mpb-64* can be in few copy number which can not be amplified by PCR. This the use of dual targets is of utmost significance for the molecular diagnostics assays designing as well as for studying the epidemiology of tuberculosis in a specific area.

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