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Diagnostic potential of IS6110 nested-PCR and *mpb*64 gene conventional PCR in diagnosis of *M.tuberculosis* in clinically suspected extra pulmonary tuberculosis cases: A hospital based study from Uttarakhand.

Sandeep Dabral[1],Tariq Masood[2], Anil Pandey[3], Deepak Juyal[4], R K Singh[5], *Rajeev Singh Kushwaha[6]

[1] Assistant Professor, Department of Biochemistry,
Soban Singh Jeena Government Institute of Medical Sciences & Research, Almora, Uttarakhand
[2]Professor & Head, Department of Biochemistry,
Shri Guru Ram Rai Institute of Medical and Health Sciences, and a Shri Mahant Indresh Hospital, Patel Nagar, Dehradun, Uttarakhand
[3] Associate Professor, Department of Dentistry,
Soban Singh Jeena Government Institute of Medical Sciences & Research, Almora, Uttarakhand
[4] Assistant Professor, Department of Microbiology,
Soban Singh Jeena Government Institute of Medical Sciences & Research, Almora, Uttarakhand
[5]Emeritus Professor,Department of Biochemistry,
TS Mishra Medical College and Hospital,Amausi Lucknow,Uttar Pradesh-226008
[6]Associate Professor, Department of Biochemistry,
Soban Singh Jeena Government Institute of Medical Sciences & Research, Almora , Uttarakhand,

** rajeevkushwaha27@gmail.com*

Abstract

Over the past few years, the incidence of extra pulmonary tuberculosis (EPTB) is on the rise. A high index of clinical suspicion coupled with early and accurate diagnosis, and prompt initiation of anti tubercular therapy is key to the better patient outcome. Use of molecular methods like polymerase chain reaction (PCR) ensures rapid and accurate detection of *Mycobacterium tuberculosis* in otherwise paucibacillary samples where the conventional diagnostic modalities are of little use.Evaluating the diagnostic potential of IS6110 nested PCR (nPCR) and mpb64 gene conventional PCR in diagnosis of EPTB.A total of 98 clinical specimens collected over a period of one year from various clinical departments were included in the study. After decontamination by NALC/NaOH method specimens were further processed by Direct fluorescent (DF) staining for acid fast bacilli (AFB) and two PCR assays: IS6110 nPCR and *mpb*64 gene conventional PCR for the detection of EPTB cases. Out of the total 98 clinical samples, 32 (31.4%) samples were found positive for IS6110 nPCR, whereas 24 (23.5%) samples were found positive for *mpb*64 PCR assay. 21 samples were positive for both the aforesaid targets. 11 samples were found positive for only IS6110 nPCR whereas three samples showed positive results for only *mpb*64 PCR assay. Only 8 (8.2%) samples were found positive by DF staining for AFB.Simultaneous use of two different target genes can improve the diagnostic yield of EPTB samples. Molecular methods like PCR facilitate the early and improved diagnosis of paucibacillary EPTB cases, which are otherwise difficult to diagnose with the available standard methods.

Keywords: Acid fast bacilli, Amplicons, Mycobacteria, Polymerase chain reaction, Tubercular lymphadenitis

INTRODUCTION

Tuberculosis (TB), primarily an airborne infectious disease (pulmonary TB) is caused by pathogenic bacterial species of Mycobacterium tuberculosis complex (MTBC), and accounts for nearly two million deaths worldwide every year. [1] TB has continued to plague mankind from the time immemorial, and despite of the availability of anti tubercular drugs since 1940s, it still remains the second most common infectious etiology of death after human immunodeficiency virus (HIV). [2] The burden of disability and death due to TB is immense, more so, in developing and low socio-economic regions of the world. [3] Presently, the WHO estimates that one third of the world's population is infected with Mycobacterium tuberculosis (MTB). [4] The six countries that stand out as having the highest number of incident cases in 2017 were India, China, Indonesia, Pakistan, Philippines and Nigeria. India alone accounts for 23% of the global TB incidence with an estimated 2.2 million cases reported in 2014. [4]

TB can be pulmonary or extra pulmonary TB (EPTB). Although the EPTB is less common but still it comprises one fifth of the total TB cases and is even more common among individuals with HIV/AIDS or other immunodeficiencies. Over the past few years there has been a continuous upsurge in the incidence of EPTB, particularly tubercular lymphadenitis (TBLN), the most common manifestation of EPTB, which accounts for approximately 20-40% of EPTB cases. [5,6] As the common symptoms for TB (fever, cough, weight loss, fatigue and night sweats) are absent in EPTB and patient often presents with enlarged lymph nodes (LN), it poses a diagnostic as well as therapeutic dilemma for clinicians. A high index of clinical suspicion coupled with fine needle aspiration cytology (FNAC), ZN-staining for acid-fast bacilli (AFB) and mycobacterial culture of the LN

aspirates/other clinical samples, play a vital role in the diagnosis of EPTB. [7] However the nonspecific/inconclusive cytological findings, poor sensitivity of the ZN staining (particularly in paucibacillary samples) often makes the diagnosis difficult. [8] Although mycobacterial culture is a gold standard method for definitive diagnosis, but the long turn around time (6-8 weeks) is a cause of concern. [9] Moreover both MTB as well as non-tubercular mycobacteria (NTM) can cause EPTB and, as the treatment modalities are different, it is important to differentiate tubercular EPTB from NTM-EPTB. [10] Therefore, in order to treat the patients effectively and efficiently early and accurate diagnosis is imperative, not only from patient's point of view but from public health perspective as well.

Since there are many problems associated with performance of conventional diagnostic methods, various molecular techniques like polymerase chain reaction (PCR), PCR based restriction fragment length polymorphism (PCR-RFLP) and PCR based DNA sequencing are developed for rapid, reliable and accurate detection of Mycobacteria in clinical specimens of EPTB. [11] PCR is currently the preferred method for identification of EPTB, as it is rapid and is quite sensitive for detection of bacteria in otherwise paucibacillary specimens. [12] There are several in-house nested PCR (nPCR) standardized for detection of MTB from Clinical specimens targeting *mpb*64, IS6110 and 16sRNA genes. *mpb*64 is a secretory mycobacterial antigen produced by certain mycobacterial strains while IS6110 is an insertion element present in single or multiple copies in MTBC isolates specifically, and hence is an important diagnostic tool in identification of MTBC species. The present study was done to evaluate the diagnostic potential of two different PCR assays (nPCR targeting IS6110 and a conventional PCR targeting *mpb*64 gene) in the diagnosis of MTBC among suspected EPTB patients.

MATERIALS AND METHODS

Study site and study population: The current study was conducted at Molecular Research Laboratory in the Department of Biochemistry, Shri Guru Ram Rai Institute of Medical and Health Sciences, and associated Shri Mahant Indresh Hospital, Patel Nagar, Dehradun, Uttarakhand. Over a period of one year (January 2015 to December 2015) a total of 98 clinical specimens (26 pus, 23 CSF, 15 biopsy, 12 ascitic fluid, 9 endometrial tissue and curetting, 8 pleural fluid, 3 urine and 2 synovial fluid) were collected from various departments of Shri Mahant Indresh Hospital. Patients >18 years of age, clinically suspected of EPTB on the basis of radiological imaging, biochemical parameters, histological/cytological findings and with no history of antitubercular drug intake, were included in the study. The study was approved by the Institutional Ethics Committee (IEC). The study protocol was explained to the participants and prior to their enrollment their written informed consent was obtained.

DNA extraction: Samples once received were subjected to decontamination by NALC/NaOH method and the DNA extraction was done by spin column based nucleic acid extraction method using DNASure Tissue Mini Kit (Nucleopore, G Brand, Genetix Biotech Asia Pvt. Ltd., New Delhi, India) as per the manufacturer's instructions. Briefly, 25mg of tissue or 1-2 ml of other samples were placed in a micro centrifuge tube (MCT). 180µl of lysis buffer and 25µl of proteinase K solutions were added to it and than vortexed. The MCTs were than incubated at 56°C for 1–3 h, with intermittent vortexing till complete lysis of the sample was obtained. Thereafter 200µl of lysis buffer 3 was added, vortexed and the MCTs were incubated at 70°C for 10 min. If any insoluble particles were still visible the MCTs were centrifuged for 5 min at 11,000 × *g*. Supernatant was transferred to a fresh MCT and 210µl of absolute ethanol was added and the tubes were vortexed vigorously. For each sample, one DNA sure mini kit column was placed in collection tube and the sample was transferred to the column. The collection tube along with the column was centrifuged for 1 min at 11,000 × *g*, the collection tube was than discarded and the column was placed into the new collection tube. Washing was done using the wash buffer twice, the flow-through collected in the collection tubes was discarded and each time the column was placed into the new collection tube. After washing the column was placed into1.5ml MCT and 100µl of pre-warmed elution buffer (70°C) was added to it and was incubated at room temperature for 1min and finally centrifuged at 11,000 × *g* for 1min. The extracted DNA was stored at -20°C until further use.

IS6110 Nested PCR: Following extraction, a nested PCR for the detection of MTBC was performed from the DNA samples. All the PCR reagents including Taq polymerase were procured from Qiagen (Hilden, Germany). The extracted DNA was amplified by two-step PCR assay as described previously by Shukla *et al*. [13] Briefly, in the first step, 220 bp DNA segment was amplified by external primers (listed in Table 1). The amplification was carried out in 12µl final volume, consisting of 8.22 µl amplification premix (reaction buffer with $MgCl_2$, dNTPs and *Mycobacterium tuberculosis* complex specific external primers) 0.33 µl Taq DNA polymerase, 0.5 µl uracil DNA glycosylase (UDG) and 3 µl DNA template. The PCR cycling conditions were as follows: After initial denaturation at 94°C for 5 min, 3 step cycling for 35 amplification cycles was performed, wherein each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min and primer extension at 72°C for 2 min. A final extension of 10 min at 72°C was applied. After complete amplification of 220 bp DNA segment, the amplified product was used as DNA template for amplification of 123 bp segment. 15 µl of master mix (buffer with $MgCL_2$, dNTPs and MTB specific internal primers; listed in Table 1) and first PCR product was added in PCR tube and amplification was carried out with PCR cycling conditions as follows: denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min and primer extension at 72°C for 2 min.

mpb64 gene conventional PCR: PCR assay using primers (listed in Table 1) targeting the 240 bp fragment of *mpb*64 gene sequence of MTBC was performed as described

previously by Parekh *et al*. [14] A reaction mixture of 25µl 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. 5µl of DNA template was added in the 25µl of master mix and the PCR cycling conditions were as follows: After initial denaturation at 94°C for 4 min, 3 step cycling for 40 amplification cycles was performed, wherein each cycle consisted of denaturation at 94°C for 30 sec, annealing at 60°C for 1 minute and primer extension at 72°C for 1 minute followed by final extension at 72°C for 7 minutes.

Detection of amplified products: The amplified products were stored at 4°C till the detection. The amplified PCR products, were to be run on 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide. 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. The stained gel was visualized under UV-light transilluminator (ULTRA LUM Electronic gel documentation system) for the presence of target base pairs (123 bp for IS6110 and 240 bp for *mpb*64) and the sizes of the amplicons were determined using DNA ladder of 100 bp as comparator, and then photographed. The samples showing the presence of targeted bands under ultraviolet transillumination were considered positive.

Quality Control: In every assay the tests were monitored for contamination using Nuclease free water as a negative control and DNA extracted from ATCC *M.tuberculosis* strain H37Rv as positive control.

All the specimens were also subjected to direct fluorescence (DF) staining for acid fast bacilli (AFB) using Auramine-Rhodamine staining and were examined under fluorescent microscope.

RESULTS

Out of the total 98 clinical samples, 32 (31.4%) samples were found IS6110 nPCR positive , whereas 24 (23.5%) were found positive for *mpb*64 PCR assay. When comparatively analyzed for both the molecular targets, it was observed that 21 samples were positive for both the targets i.e. IS6110 as well as *mpb*64 gene. 11 samples were found positive for only IS6110 nPCR and were negative for *mpb*64 PCR, whereas three samples showed positive results for only *mpb*64 PCR assay and were negative for IS6110 nPCR. **Table 2** depicts the specimen wise results of IS6110 nPCR and *mpb*64 conventional PCR assay compared with DF staining results for AFB. **Table 3** depicts the Comparative analysis IS6110 nPCR and *mpb*64 conventional PCR.

Table 1: The primers used for IS6110 nested PCR and *mpb*64 gene conventional PCR.

Target gene	Primers	Sequence	Amplicon Size
IS6110 (Outer Primers)	K J	5′-ATCGTGGAAGCGACCCGCCAGCCCAGGAT-3′ 5′-CGGGACCACCCGCGGCAAAGCCCGCAGGAC-3′	220 bp
IS6110 (Inner Primers)	MTB1 MTB2	5′-CCTGCGAGCGTAGGCGTCGG3′ 5′-CTCGTCCAGCGCCGCTTCGG3′	123 bp
*mpb*64	Mpb1 Mpb2	5′-TCCGCTGCCAGTCGTCTTCC3′ 5′-GTCCTCGCGAGTCTAGGCCA3′	240 bp

Table 2: Specimen wise results of IS6110 nPCR and *mpb*64 conventional PCR assay compared with DF staining results for AFB.

Type of specimen (no. of specimen); *n*=98	Positive PCR results for		DF staining for AFB; *n*=98	
	IS6110 nPCR; *n*=32	*mpb*64 gene; *n*=24	Positive	Negative
Pus (26)	11 (34.4%)	08 (33.3%)	05	21
CSF (23)	05 (15.6%)	04 (16.7%)	01	22
Biopsy (15)	08 (25.0%)	06 (25.0%)	01	14
Ascitic Fluid (12)	03 (9.4%)	03 (12.5%)	00	12
Endometrial tissue and Curetting (09)	04 (12.5%)	02 (8.3)	01	08
Pleural Fluid (08)	01 (3.1%)	01 (4.2%)	00	08
Urine (03)	nil (0%)	nil (0%)	00	03
Synovial Fluid (02)	nil (0%)	nil (0%)	00	02
Total (98)	32 (31.4%)	24 (23.5%)	08 (8.2%)	90 (91.8)

AFB: acid fast bacilli; CSF: cerebro spinal fluid; DF: direct fluorescent staining; nPCR: nested polymerase chain reaction; PCR: polymerase chain reaction

Table 3: Comparative analysis IS6110 nPCR and *mpb*64 conventional PCR. (*n*=98)

No. of specimens	IS6110 nPCR	mpb64 gene PCR	Percentage
21	+ve	+ve	21.4%
11	+ve	-ve	11.2%
03	-ve	+ve	3.1%
63	-ve	-ve	64.3%
Total positive with individual PCR	32 (31.4%)	24 (23.5%)	-

nPCR: nested polymerase chain reaction; PCR: polymerase chain reaction

DISCUSSION

EPTB is a manifestation of TB, involving organs other than lungs. The most common forms of EPTB include tubercular lymphadenitis (TBLN), genito-urinary TB, osteo-articular TB, renal TB and tubercular meningitis. Immunocompromised individuals, particularly the one with HIV/AIDS or other immunodeficiency disorders are at increased risk of acquiring the disease. The diagnosis of EPTB is always faced with multiple challenges, as collection of some specimens (pleural effusion, bone biopsy, CSF) require invasive/laborious procedures, moreover the EPTB samples oftenly are paucibacillary and can go undetected. Still worse is the fact that available, conventional diagnostic tools (AFB staining, histopathological/cytological investigations, microbiological culture) to detect EPTB are not sensitive enough to aid in early diagnosis. The clinical utility of detecting MTBC by molecular methods like PCR depends on its rapidity and accuracy in diagnosis of EPTB and has been evaluated in the current study. We have analyzed the accuracy of the two PCR assays: IS6110 nPCR and *mpb*64 conventional PCR in the diagnosis of EPTB and have also analyzed the comparative efficiency of these two commonly used PCR targets (IS6110 nPCR and *mpb*64) in India. IS6110 is an insertion element that is found exclusively within the MTBC, probably due to the lack of genetic exchange with other mycobacterial species. Rendering to its exclusivity with MTBC, IS6110 has become an important diagnostic tool in the differentiation of MTBC species from other mycobacteria. Moreover, its presence in multiple copies and at differing locations in the genome, IS6110 has been extensively used as a target gene in various epidemiological studies. *mpb*64 has also been found in large copy numbers in *M.tuberculosis*, *M.bovis* and some BCG strains of Indian subcontinent.

In order to minimize the inhibitors in the EPTB samples, the high pure extraction and purification kit was used in the present study, moreover the PCR product of the first reaction of PCR was used in nPCR to further reduce the concentration of remaining (if any) inhibitory substances. nPCR increases the sensitivity and specificity of an assay, also including UDG in premix further improved the sensitivity of IS6110 nPCR by preventing the chances of false positive results due to amplicon contamination. However, since false negative/positive results have been reported previously in nPCR, [15] employing another PCR assay, targeting *mpb*64 gene was used to improve the yield of diagnosis for EPTB. Among some of the members of MTBC, either IS6110 nPCR or *mpb*64 can be present in only few copy numbers and are unable to get amplified by PCR, hence targeting only a single gene can lead to false results. Hence from diagnostic point of view the use of dual targets is of utmost significance. Thus in the present study by employing two different target genes: IS6110 nPCR and *mpb*64 we also tried to get more positive results. Therese *et al*. from Tamil Nadu also concluded that simultaneous use of both the aforementioned target genes together on clinical specimens could attain 100% sensitivity. [16]

Our study results revealed that, nPCR targeting IS6110 gene showed more sensitivity in comparison to *mpb*64 gene PCR. Few previous studies also documented higher sensitivity of IS6110 target in EPTB samples when compared to other commonly used PCR targets. [17,18] A study by Singh *et al*. reported IS6110 (69.1%) as a better single target compared to *mpb*64 (48.2%). [19] Positivity rate for CSF samples was more or less same by using either of the target genes, a finding which was in concordance with the previous study by Sastry *et al*. who reported a high positivity rate (40.3%) for CSF samples. [20] Our study results also demonstrated that nPCR technique has higher sensitivity compared to conventional PCR method for detection of MTBC genes in EPTB samples, a finding which is in parallel to the study by Cruz *et al*. [21] Molecularly, the frequency of EPTB in our setting was determined as 31.4%, in contrast to the lower frequency obtained by DF staining (8.2%) for AFB. Data extrapolated from our study clearly indicates that molecular methods along with other conventional, clinical and radiological diagnostic modalities can clinch the early and accurate diagnosis in suspected EPTB cases. A multidimensional approach if followed by primary care physicians, microbiologists, pathologists, biochemists and radiologists will not only ensure effective and efficient patient care but will also be helpful in reducing the overall burden of TB.

CONCLUSION

In conclusion, we hereby report the rapid and accurate diagnosis of EPTB in clinical samples by targeting two different MTB genes. Although, the nPCR targeting the IS6110 gene of MTBC was found to be a better assay in comparison to *mpb*64 gene conventional PCR, but the simultaneous use of both the targets surely improves the diagnostic yield of EPTB samples. Molecular methods like PCR facilitate the early and improved diagnosis of paucibacillary EPTB cases, which are otherwise difficult to diagnose with the available standard methods. Although the variety of EPTB samples included in our study is an added advantage in deciding the clinical utility of PCR assay in various clinical settings, but the low sample size, especially when the individual sample types were analyzed separately, is a limitation of our study. More specimens in future studies will be included to investigate the efficiency of target genes in the diagnosis of EPTB at such sites.

Conflict of Interest: None

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