Research Article (Open access)

Amplification of *rpoB, kat G & mab A (fab G1)- inh A Promotor* DNA Sequences by PCR in Multiple Drug Resistance Tuberculosis

Bhanu Mehta[1], Afreen Siddiquie[1], Rakhi Kaushik[1], Rahul Bisht[1], Narotam Sharma[1]*,

[1]Central Molecular Research Laboratory, Department of Biochemistry, SGRR Institute of Medical & Health Sciences, Dehradun, India

ABSTRACT- Multiple Drug resistance (MDR) tuberculosis timely diagnose is of utmost clinical relevance and needs to be diagnose at initial stages for the proper treatment. The current study was done to detect the several genes for MDR tuberculosis (TB) in clinical isolates by molecular tools. 60 clinical isolates were collected and subjected for AFB smear preparation, Nested PCR (IS6110) for *Mycobacterium tuberculosis* complex detection and MDR TB PCR targeting *rpoB, kat G, mab A promoter.* 12 came positive for AFB smears, out of which 08 were pulmonary and 04 were extra pulmonary. Nested PCR targeting *IS6110* gene was amplified at 123 base pairs with 340 base pairs as IC (internal control) was seen in 25 cases which include 19 pulmonary and 6 extra pulmonary. The Positive TB PCR specimens were subjected for MDRTB PCR Only 06 cases yielded, an amplicon of 315 bp confirming the *rpo*B gene resistance for resistance for rifampcin drug. In any of the 06 positives none of the other resistance gene other than *rpo*B was amplified. Targeting multiple genes at once, additional information will be gained from a single test run that otherwise would require several times the reagents and more time to perform. Current study signifies the usage of quick, cost effective, DNA sequences based method for MDR TB detection where disease will be diagnosed earlier and hence treatment would be started at an early stage.

Keywords: Multiple drug resistance, amplicon, Polymerase chain reaction, Nested PCR, Rifampicin.

----------IJLSSR----------

INTRODUCTION

Tuberculosis is a contagious disease caused by various strains of *Mycobacterium tuberculosis* (MTB). In India each year about 2 million people develop active disease and up to half a million die. About .5 million HIV patients and about 1.8 million of these are co-infected with tuberculosis [1]. The bacteria that cause tuberculosis (TB) can develop resistance to the antimicrobial drugs used to cure the disease. Multidrug-resistant tuberculosis (MDR-TB) is TB that does not respond to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. Inappropriate or incorrect use of antimicrobial drugs, or use of ineffective formulations of drugs (e.g. use of single drugs, poor quality medicines or bad storage conditions), and premature treatment interruption can cause drug resistance, which can then be transmitted, especially in crowded settings such as prisons and hospitals [2-3]. Treatment options are limited and expensive, recommended medicines are not always available, and patients experience many adverse effects from the drugs.

Address for Correspondence:
Dr. Narotam Sharma, Scientist,
Central Molecular Research Laboratory,
Biochemistry Department
Shri Guru Ram Rai Institute of Medical and Health Sciences, Patel Nagar
Dehradun-248001(Uttarakhand), India
Email: sharmanarotam5@gmail.com

Received: 03 August 2015/Revised: 14 August 2015/Accepted: 26 August 2015

In some cases even more severe drug-resistant tuberculosis ay develop. The genetic basis of resistance to most anti-TB drugs is established. Resistance to rifampicin results from missense mutatations in the *rpo*B gene, which encodes the β subunit of RNA polymerase [4]. Rifampicin specifically binds to the β subunit and prevents early steps of transcription that leads to the bacterial death. However, mutation in *rpo*B gene results in resistance by decreasing rifampicin (RIF) binding affinity. Mutation leading to resistance of *M. tuberculosis* to rifampicin is rare and occurs at a rate of 10^{10} per cell division with an estimated prevalence of 1 in 10^8 cells in drug free environment [5]. However, it rapidly results in the selection of mutants that are resistant to other anti-TB drugs. Most commonly, it exists in conjunction with mutations in *kat* G. The rate of mutation for isoniazid is 10^8 resulting in resistance in 1 out of 10^6 bacilli [6]. A missense mutation of the *inh*A gene which encodes an enzyme involved in the mycolic acid biosynthetic pathway also causes resistance. About 20-34% resistant isolates have mutations in the promoter region of *inh* A, either alone or in combination with *kat* G. Isoniazid resistance following *inh* A mutation alone is rare. Mutations have been found in the *ahp* C promoter region of approximately 10% of isoniazid resistant isolates; however these mutations have always been found to occur in association with mutations in *kat* G. The rate of mutation for isoniazid is 10^8 resulting in resistance in 1 out of 10^6 bacilli [7]. Pyrazinamide is also a pro-drug that can be coverted into an active form presumably by pyrazinamidase enzyme of susceptible organisms. The target for the active drug is not fully known. However, mutations in

the gene *pnc* A, encoding for the enzyme pyrazinamide is the major causes of pyrazinamide resistance. Between 72% and 98% of pyrazinamide resistance in clinical isolates is correlated with mutations scattered throughout the 558 bp *pnc* A coding region and 11 promoter regions. The rate of mutation for pyrazinamide is 10^3 with a probability of resistance 1 out of 10^6 bacilli [8]. The identification of specific mutations responsible for drug resistance has facilitated the development of novel, rapid molecular tools for Drug Susceptibility Test (DST). The detection of RIF resistance is traditionally used as a predictor of MDR-TB– its positive predictive value is a function of the sensitivity and specificity of RIF resistance testing and the prevalence of MDR and non-MDR RIF resistance, which is highest among previously treated cases in settings with high MDR prevalence and low non-MDR RIF resistance [9-10]. Thus, the present study was carried out to study various genes responsible for multiple drug resistant tuberculosis which includes; genes sequences: *rpoB, katG, mabA*(fabG-1)*inh-A* promoter and were amplified by PCR (multiplexing) and drug resistance pattern were studied from clinical isolates.

MATERIALS AND METHODS

Specimen collection

A total of 60 Clinical specimens which includes Pulmonary such as Sputum, Pleural fluid, Bronchiolar alveolar lavage, bronchial secretions and extra pulmonary specimens such as Pus, Urine, Semen, Tissue, Endometrial biopsy, Cerebrospinal Fluid were considered for the study. Specimens were collected from patients attending Out Patient Department (OPDs) and In Patient Departments (IPDs) of different Departments of Shri Mahant Indresh Hospital, Dehradun, Uttarakhand, India. The current study was approved by institutional ethical clearance body and written consent was taken from patients. All the specimens were subjected for different parameters like Acid fast bacilli smear preparation, Nested Polymerase Chain reaction (N-PCR) and multiplex PCR targeting *rpoB, kat G & mab A(fab G1)- inh A* Promotor DNA Sequences.

The Nucleic Acid (DNA) was extracted from the clinical specimen by Spin Column based Nucleic Acid Extraction method (Genetix) and the template was used for the N-PCR and multiplex PCR.

Nested PCR for the detection of *Mycobacterium tuberculosis* complex targeting *IS6110* gene

Further Nested PCR was performed on the DNA template isolated from various specimens. 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 seq- euence 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 [11-12]. Nested PCR. An amplimer 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 Fig. 1).

PCR for Amplification of *rpo*B, *kat G & mab* A *(fab G1)- inh A Promotor* DNA Sequences by PCR in Multiple Drug Resistance tuberculosis

Multiplex PCR involving amplification of *rpoB, kat G & mab A(fab G1)–inh A* Promotor DNA sequences [13] were done for 25 nested TB PCR positive cases for which master mix was prepared using 5 µl ,10X PCR buffer, 5 µl, deoxynucleotides (2 mM) 5µl magnesium chloride (25 mM), 0.5 µl primers (25 µM) , 0.25µl Taq DNA polymerase (5 unit/µl) & nuclease free water was added (6.75µl) to make the total volume upto 25µl. Add 25µl of template DNA amplification was done on Benchtop 9600 thermocycler, Germany involving following parameters; initial Denaturation at 94°C for 5 minutes followed by 35 repetitive cycles of denaturation at 94°C for 30 seconds, annealing at 55.5°C for 30 seconds & elongation at 72°C for 30 seconds with final elongation at 72°C for 7 minutes. After completion of amplification post amplification was done using 1.6 % agarose gel electrophoresis. The PCR will yield an amplicon of 315 b.p, 2223 b.p and 1362 b.p respectively for *rpoB, kat G and mab A promotor* respectively (figure 2). Primers used are tabulated in table 1.

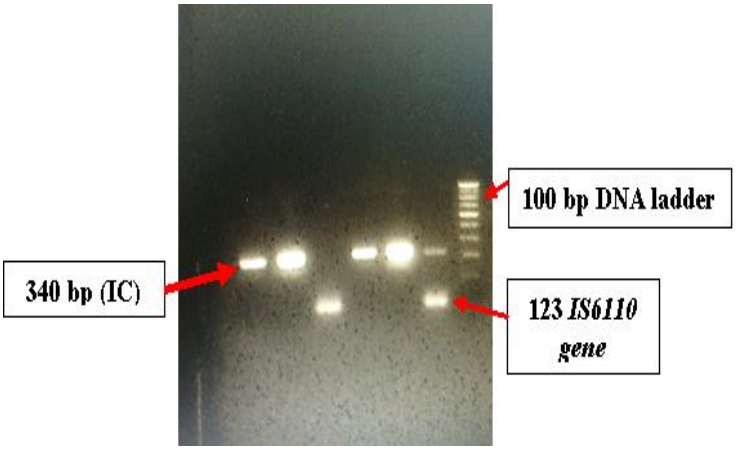

Fig. 1: Gel image for Nested TB PCR

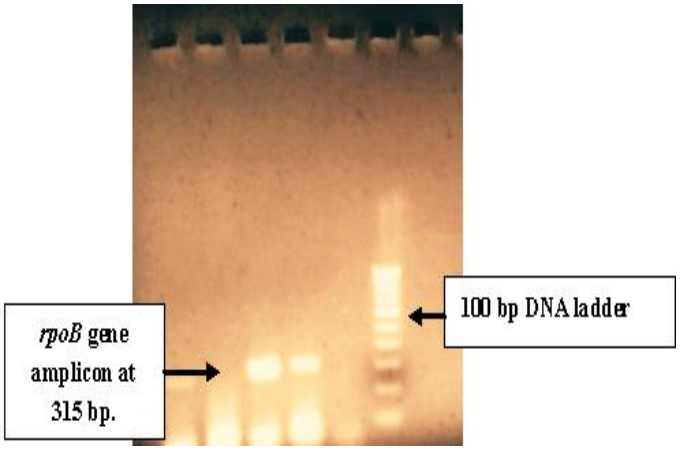

Fig. 2: Agarose Gel image for *rpo*B gene in MDR PCR

RESULTS

Sixty samples were collected from patients attending Out Patient Department (OPDs) and In Patient Departments (IPDs) of different Departments of Shri Mahant Indiresh (SMI) Hospital, Dehradun, Uttarakhand, India. Out of sixty clinical isolates collected for the purposed study, twelve came positive for AFB smears, out of which eight were pulmonary and four were extra pulmonary. Further the DNA from all the samples were isolated and subjected for N-PCR targeting *IS6110* gene and MDRTB PCR targeting *rpoB, kat G, mab A promotor* for N-PCR *IS6110* gene was amplified at 123 base pairs with 340 base pairs as IC (internal control) as shown in figure 1, it was seen that 25 cases which include 19 pulmonary and 6 extra pulmonary were TB PCR positive. The specimens were further subjected for MDR. TB PCR which will yield an amplicon of 315 b.p, 2223 b.p and 1362 b.p respectively for *rpoB,katG and mabA promoter* respectively. Only 06 cases yielded, an amplicon of 315 bp. confirming the *rpoB* gene resistance for resistance for rifampcin drug. In any of the 06 positives none of the other resistance gene other than *rpoB* was amplified (Table 2).

Table 1: Primers used to detect for MDR study in the study

Target Gene	Primer set (direction)	Nucleotide sequence	Positions	Product size (bp)
rpoB	PR1(forward) PR2 (reverse)	5-'CCGCGATCAAGGAGTTCTTC3' 5'-CCGCGATCAAGGAGTTCTTC-3'	1256–1275 1570–1551	315
katG	PR3(forward) PR4 (reverse)	5'GTGCCCGAGCAACACCCACCCATTAC AGAAAC-3' 5-TCAGCGCACGTCGAACCTGTCGAG-3'	1–32	2,223
mabA promo-tor	PR5(forward) PR6 (reverse)	5'-ACATACCTGCTGCGCAATTC-3' 5'-GCATACGAATACGCCGAGAT-3'	217 to 198	1,362

Table 2: Positivity rates for different diagnostics assays for tuberculosis

Assay	Positive	Negative	Positivity percentage	Negative percentage
Nested TB PCR	25(60)	35(60)	41.7%	58.3%
AFB smear	12(60)	48(60)	20%	80%
MDR TB PCR	06(25)	06(25)	24%	76%

DISCUSSION AND CONCLUSION

The resurgence of tuberculosis has been accompanied by high frequency of drug resistant strains from all over the world. In most TB patients drug resistance predominantly arises as a result of multiple interruptions of treatment [14]. To avoid these problems, fixed-dose combinations (FDCs) tablets are now recommended by WHO. However, in FDC formulations the bioavailability of the component drugs, and especially of *rifampicin*, may be reduced. Simple, rapid and inexpensive methods of detecting drug resistant tuberculosis are also essential for effective treatment. Multidrug-resistant tuberculosis (MDR-TB) caused by *Mycobacterium tuberculosis* resistant to both isoniazid and rifampicin with or without resistance to other drugs is among the most worrisome elements of the pandemic of antibiotic resistance. Globally, about three per cent of all newly diagnosed patients have MDR-TB. The proportion is higher in patients who have previously received anti tuberculosis treatment reflecting the failure of programmes designed to ensure complete cure of patients with tuberculosis. While host genetic factors may probably contribute, incomplete and inadequate treatment is the most important factor leading to the development of MDR-TB. The definitive diagnosis of MDR-TB is difficult in resource poor low income countries because of non-availability of reliable laboratory facilities. Efficiently run tuberculosis control programmes based on directly observed treatment, short-course (DOTS) policy is essential for preventing the emergence of MDR-TB [15,16]. Management of MDR-TB is a challenge which should be undertaken by experienced clinicians at centers equipped with reliable laboratory service for mycobacterial culture and *in vitro* sensitivity testing as it requires prolonged use of expensive second line drugs with a significant potential for toxicity. Judicious use of drugs, supervised individualized treatment, focused clinical, radiological and bacteriological follow up, use of surgery at the appropriate juncture are key factors in the successful management of these patients. In conclusion among the 60 samples, 12 were positive for AFB and 25 for TB PCR and out of them 6 for *rpoB* gene. Isolates resistant and partially resistant to *rifampicin* were found to be 24%. We standardized a multiplex In-house PCR based protocol for detection of Multidrug Resistance in *M. tuberculosis* from clinical specimens on molecular basis in our laboratory. The utility of

multiplex PCR is that it consists of multiple primer sets within a single PCR reaction to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information will be gained from a single test run that otherwise would require several times the reagents and more time to perform. This method will detect the very less number of infectious mycobacteria present in clinical specimens and hence the treatment will be started accordingly immediate after the diagnosis and detection. In the proposed study, we targeted *rpoB, katG and mabA promotor* gene for the detection of Multidrug Resistance in *M. tuberculosis* in the clinical specimens. The significance of the proposed study includes quick method, reduction in cost of test, use of DNA sequences for the detection of Multidrug Resistance in *M. tuberculosis* depends on the right choice of the target sequences. Disease will be diagnosed earlier and hence treatment would be started at the early infection [17].

ACKNOWLEDGEMENT

The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support, guidance and favor.

REFERENCES

[1] Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, *et al*. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* 2003; 163: 1009-21.

[2] Ramalingaswami V 1998. Opening address, p. 1-3. In RLSinghal & OP Sood (eds), *Drug Resistance:Mechanism and Management*, Proceedings of the Fourth Annual Ranbaxy Science Foundation Symposium Communicore Publishers, New Delhi, India.

[3] Miller, L. P., J. T. Crawford, and T. M. Shinnick. 1994. The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 38:805–811.

[4] Paramasivan CN. Status of drug resistance in tuberculosis after the introduction of rifampicin in India. *J Indian Med Assoc*. 2003; 10: 154-6.

[5] Heym B, Alzari PM, Honore N, Cole ST 1994. Missense mutations in the catalase-peroxidase gene, katG, are associated with isoniazid resistance in *Mycobacterium tuberculosis. Mol Microbiol 15*: 235- 245.

[6] Morris, S., G. H. Bai, P. Suffys, L. Portillo-Gomez, M. Fairchok, and D. Rouse. 1995. Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis*. J. Infect. Dis. 171:954–960.

[7] Lavender, C., M. Globan, A. Sievers, H. Billman-Jacobe, and J. Fyfe. 2005. Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* isolates collected in Australia. Antimicrob. Agents Chemother. 49:4068–4074.

[8] Banerjee, A., M. Sugantino, J. C. Sacchettini, and W. R. Jacobs, Jr. 1998. The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. Microbiology 144:2697–2704.

[9] Suhaila, H., Rahizan, I., Nik Marzuki S. and Noraziah M. Zin, 2008. The Usefulness of PCR Amplification for Direct Detection of M. tuberculosis DNA from Clinical Samples. Biotechnology 7(1): 100-105

[10] Dobner, P., S. Ru¨sch-Gerdes, G. Bretzel, K. Felsmann, M. Rifai, T. Lo¨scher, and H. Hinder. 1997. Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes *katG* and *inhA* for the prediction of isoniazid resistance. Int. J. Tuberc. Lung Dis. 1:365–369.

[11] Narotam Sharma, Amitabh Talwar, Satish C. Nautiyal, Rakhi Kaushik, Amanpreet Singh, Akhil Pratap Singh, Jivesh K. Shaw, Shilpi Dixit, and R. K. Singh, 2013. Nested-PCR and Uracil-N-Glycosylase-significant approach to prevent amplicon contamination in tuberculosis PCR performing laboratories. *Archives of Applied Science Research* 5(2): 177-179.

[12] Narotam Sharma, Mukesh Kumar, Natasha K Ahmed, Shivani Sailwal, Deepti Singh, Subhash Sharma, Danish Kalim, Application of Nested Polymerase Chain Reaction using IS6110 Molecular Target to Reveal Mycobacterium Tuberculosis DNA from Extra Pulmonary Tuberculosis Patient, *International Journal of Universal Pharmacy and Bio Sciences 2*(4)*: 2013, 43-49.*

[13] Sekiguchi, J. I., Miyoshi-Akiyama, T., Augustynowicz-Kopeć, E., Zwolska, Z., Kirikae, F., Toyota, E & Kirikae, T. (2007). Detection of multidrug resistance in Mycobacterium tuberculosis. *Journal of clinical microbiology*, *45*(1), 179-192.

[14] Siddiqi, N., M. Shamim, S. Hussain, R. K. Choudhary, N. Ahmed, Prachee, S. Banerjee, G. R. Savithri, M. Alam, N. Pathak, A. Amin, M. Hanief, V. M. Katoch, S. K. Sharma, and S. E. Hasnain 2002. Molecular characterization of multidrug-resistant isolates of Mycobacterium tuberculosis from patients in North India. Antimicrob. Agents Chemother. 46:443–450.

[15] Heym, B., P. M. Alzari, N. Honore, and S. T. Cole. 1995. Missense mutations in the catalase-peroxidase gene, katG, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol. Microbiol. 15:235–245.

[16] Fluit, A. C., M. R. Visser, and F. J. Schmitz. 2001. Molecular detection of antimicrobial resistance. Clin. Microbiol. Rev. 14:836–871.

[17] Leven, M. and Goossens H. 1997. Relevance of nucleic acid amplification techniques for diagnostic of respiratory transinfections in the clinical laboratory. Clinical Microbiology Review. 10:242.

