

Development of Sequence Based Molecular Diagnostic Test to Evaluate MDR and XDR in *M. tuberculosis* Patients from Western India

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Abstract Globally, Tuberculosis is the most distress disease which remains as a major challenge from the standpoint of diagnosis, detection of drug resistance, and treatment. The increasing knowledge in molecular biology techniques has improved our understanding of the molecular mechanisms of drug resistance for the major first and second-line anti-tubercular drugs. Thus, rapid and accurate diagnosis of Tuberculosis would improve patient care and limit its transmission. This study is aimed to identify mutations in drug resistance genes of first and second line drugs against *Mycobacterium tuberculosis* by using polymerase chain reaction (PCR) and DNA sequencing methods. This study involves sputum samples of 20 patients diagnosed to have pulmonary tuberculosis. The genomic DNA from clinical isolates was isolated using simple boiling method. PCR amplification and DNA sequencing were performed for *katG*, *inhA*, *ndh*, *kasA*, *pncA*, *embB*, *rrs*, *rpsL*, *rpoB*, *gyrA*, *gyrB* and *thyA* genes. Sequence analysis was done using Bioedit, a bioinformatics tool. The isolates of *Mycobacterium tuberculosis* from western part of India were analyzed by DNA sequencing revealed total 29 mutations in first line drugs. Out of them, 5(25%) in *rpoB* at codon 516 and 531, 6(30%) in *katG* at codon 315, 8(40%) in *rpsL* at codon 43 & 88, 3(15%) in *embB* at codon 306 and 7(35%) in *pncA* at codon 195. No mutations in *inhA*, *ndh*, *kasA* and *rrs* genes were observed in our study. In the second line drugs, 2(10%) at codon 90 & 100% mutations at codon 95 in *gyrA* gene, and no mutation in *gyrB* and *thyA* genes were observed.

Keywords: *Mycobacterium tuberculosis*, PCR, DNA sequencing, MDR, XDR

1. Introduction

Tuberculosis (TB) is a bacterial infectious disease, caused by the obligate human pathogen, *Mycobacterium tuberculosis* (*M. tb*). Tuberculosis remains a vital life-threatening disease and posing the expanding global health crisis that mandates new therapeutic strategies. India contributes the highest number of new cases, accounting for 20% of the global burden, of which approximately 1.2% occur in persons with HIV [1]. The bacterium is transmitted via the respiratory route as a highly infectious aerosol with varying outcomes occurring from this initial *Mycobacterium tuberculosis* exposure. However, the majority of individuals infected with *M. tuberculosis* have a non-contagious, clinically-latent infection with an absence of clinical symptoms [2]. Latently-infected individuals have a 5-10% risk of developing reactivation TB disease during their lifetime [3], often due to immunosuppressive circumstances, with HIV infection being the greatest identified cause [4,5]. The history of TB is changed as the first drug with anti-mycobacterium activity was introduced. Streptomycin was exploited as a monotherapy and hence the first drug resistance got emerged [6]. Later on, several anti-mycobacterium drugs were discovered. However, the

resistance against all of them got developed by the strain of *M. tuberculosis*. Therefore, the tuberculosis that is resistant to at least isoniazid and rifampicin, the most powerful first line anti-TB drugs is called as Multi-Drug Resistance (MDR) strains. In addition to being MDR, the extensively drug resistance (XDR, resistance to MDR as well as to any member of the quinolone family and at least one of the second-line anti-TB drugs) was arisen subsequently by strains of *M. tb*. Therefore, it is necessitated to understand the mechanism of MDR and develop the potent therapeutics which will not easily predispose to the resistance by strains of *M. tb*. By virtue of the availability of the new information generated by decoding the complete genome sequence of *M. tb* and major progresses in the molecular biology tools we shall understand the specific gene mutations which are responsible for drug resistance [7,8].

A number of molecular assays are available to detect the presence of *M. tuberculosis* and to assess drug resistance pattern [9]. Line probe assay is a method which relies on the hybridization of amplicons to probes on a nitrocellulose strip. The assay detects 99% and 88% RIF and INH resistant strains respectively in direct sputum samples or culture isolates [10]. Although, assay has advantage of short time analysis, it has some limitations which need to improve in nucleic acid amplification test. The limitation of assay is its inability to carry out species

level identification of Mycobacterium and cannot detect novel mutations. Other major concern of the assay is possibility of cross contamination and mispairing of the DNA from another sources and may result in false positive results which cannot recommended as an accurate method.

The new method including PCR based sequencing has overcome the above limitations. This method has highest reproducibility and reliability as compared to line probe assay. This gives 100% specificity in the results and good interpretation helps in better understanding for the drug resistance and recommendation for the drug therapy. Polymerase Chain Reaction (PCR)-based sequencing is the key tool exploit for elucidating the genetic mechanisms of drug resistance in *M.tb*. However, the current knowledge of the techniques and diagnostic methods to screen gene mutations seems inadequate and hence there is a pressing need to improve the methodologies to explore the new candidate genes and detect novel mutations.

In the present study, we have focused on standardizing the rapid assay of PCR and sequencing for the detection of mutations in some of the candidate genes responsible for drug resistance in *M. tb*. The molecular aspects of drug resistance help to understand the molecular strategies to rapidly diagnose MDR-TB. The molecular aspects of drug resistance help to understand the molecular strategies to rapidly diagnose MDR-TB. We utilize PCR method for analyzing the drug sensitivity and mutation profile of these local *M. tuberculosis* isolates.

2. Materials and Methods

Sputum samples were received in a sterile container with prior consent of patient as per the guidelines of ethical committee of Jaslok Hospital and Research Center, Mumbai, India. Specimens were processed for acid fast staining to detect the presence of acid fast bacilli. Positive specimens were decontaminated by using equal amounts of *N*-acetyl-L-cysteine-sodium hydroxide solution (0.25g in 50 ml sodium hydroxide solution) and were incubated for 15 minutes. The solution was then diluted in phosphate buffer (pH 6.8) and centrifuged at 8,000rpm for 15min. The sediments obtained were stored in TE buffer (pH 8.0) at -20°C for subsequent PCR assay.

2.1. DNA Extraction

The genomic DNA was extracted from positive sputum samples by previously described simple boiling method [11]. The sediment obtained from decontamination procedure was washed three times in TE (Tris EDTA, pH 8.0) buffer by centrifugation at 12,000rpm for 10 minutes. The precipitation obtained was dissolved again in TE buffer(volume should be added according to the size of pellet)and boiled at 100°C for 15mins. The turbid supernatant containing DNA was used as a template for PCR amplification.

2.2. Polymerase Chain Reaction (PCR)

The extracted DNA was amplified with the oligonucleotide primers (listed in Table 1). The presence of regulatory gene targets of *M.tuberculosis* was identified by Polymerase Chain.

Table 1. shows primer sequences for 1st line drug resistant genes for *M. tuberculosis*

GENES	PRIMER SEQUENCES	ANNEALING (°C)	PRODUCT SIZE (bp)
rpoB Sense Antisense	TGGTCCGCTGCACGAGGGTCAGA CTCAGGGGTTTCGATCGGGCACAT	72	436
katG Sense Antisense	TGGCCGCGCGGTCGACATT GGTCAGTGCCAGCATCGTC	62	419
rrs Sense Antisense	TCACCATCGACGAAGGTCCG CTAGACGCGTCCTGTGCATG	62	569
rpsL Sense Antisense	GTCAAGACCGCGGCTCTGAA TTCTTGACACCCTGCGTATC	62	271
inhA Sense Antisense	AAACGGATTCTGGTTAGCGG CGGGTTGATGCCCATCCCGG	62	300
ndh Sense Antisense	GCAATGTCCAGGTAAGTGTG CCTTGGTCGAGTCGATGTG	62	386
pncA Sense Antisense	ATCAGCGACTACCTGGCCGA GATTGCCGACGTGTCCAGAC	62	180
embB Sense Antisense	CTGAAACTGCTGGCGATCAT GGTCTGGCAGGCGCATCC	63	414
kasA Sense Antisense	CCGTTGGCCCGATTGCTGGG CAGTAACGCCCGAAGGCAAG	68	470

Table 2. shows primer sequences for 2nd line drug resistant genes for *M. tuberculosis*

GENES	PRIMER SEQUENCES	ANNEALING (°C)	PRODUCT SIZE (bp)
gyrA Sense Antisense	CAGCTACATCGACTATGCGA GGCTTCGGTGTACCTCAT	58	318
gyrB Sense Antisense	CCACCGACATCGGTGGATT CTGCCACTGAGTTTGACA	62	330
thyA Sense Antisense	GCCCTGTCATGCGTTCTT ATGCGGATCGTAGTTCTTAC	58	427

Reaction. Each PCR contained 10µl of DNA template (undiluted or threefold-concentrated extract). For amplification of all genes (Table 1 & Table 2), PCR was performed in a final volume of 25µl containing 10 mM Tris-HCl (pH 8.3), 25 mM MgCl₂; 2mM deoxynucleoside triphosphate (Applied Biosystems); 10 pmoles of each primers (2µl), and 1.0 U of Taq DNA polymerase (Invitrogen, Paisley, UK) and PCR grade water to make final volume 25µl. The reaction mixture was then subjected to 40 cycles with run profiles: 95°C for 15min, 94°C for 1min, annealing according to genes (Table 1), and 72°C for 1min, followed by a final extension at 72°C for 10mins. All reactions were carried out on 2720 DNA Thermal Cycler (Applied Biosystems, USA). After PCR amplification, 10µl aliquot of the PCR product was electrophoresed for 1h through 2% agarose gel containing ethidium bromide dye, and the target band size of genes was visualized under UV illumination.

2.3. DNA Sequencing and Sequences Analysis

Before sequencing, PCR products were purified. Purified PCR products were used for pre-sequencing PCR with 5x sequencing buffer (Applied Biosystems, Warrington, UK), BigDye® Terminator v3.1 cycle (BDT) from Applied Biosystems, Foster city, CA, USA and 3.2 pmol/µl of either sense or antisense primer. PCR was carried out at 96°C for 1min. followed by 25 cycles of denaturation at 96°C for 10sec., annealing at 50°C for 5sec and elongation at 60°C for 4min. After pre-sequencing PCR, products were again cleaned and finally dissolved in Hi-Di™ Formamide (Applied Biosystems, Foster city, CA, USA). Amplicons were then loaded onto Micro Amp™ Optical 96-Well Reaction Plate (Applied Biosystems, Foster city, USA) and subjected to cycle sequencing using an automated Genetic Analyzer 3100 (Applied Biosystems, Foster city, CA, USA) which utilizes ABI's BigDye Terminator chemistry based on Sanger's sequencing method. Both Sense and antisense strand sequencing were carried out to confirm all mutations reported in this study. All post run analyses were performed using ClustalW, BioEdit Sequence Alignment Editor. Each sequence was compared with the previously published of respective genes sequences of strain H37Rv in the NCBI database.

3. Results

A diverse set of MDR and XDR clinical isolates of *M. tb* was selected for the investigation of genetic mutations. First we screened for the known mutations in *M. tb* genome that are notorious to confer resistance to the antibiotics in the population of western India. Total 5 healthy individuals were recruited as a control in our study. We employed PCR and sequencing methods to find out the mutation and we observed that all the mutations were in the coding region of drug resistance genes. The mutation and percentage in no. of isolates is shown in Table 3.

3.1. PCR Analysis

In the PCR assay of all the 12 genes of clinical isolates were amplified by using primers pairs for the respective genes. The size of fragment of each gene is determined by using molecular marker 100 bp (Fermentas, Thermo Scientific, USA). Figure 1 and Figure 2 depict the amplification of first line drugs and second line drugs gene fragments respectively.

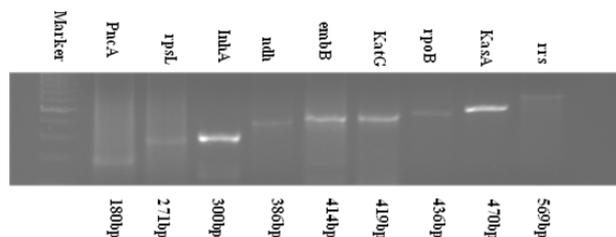


Figure 1. Amplification of first line drug gene

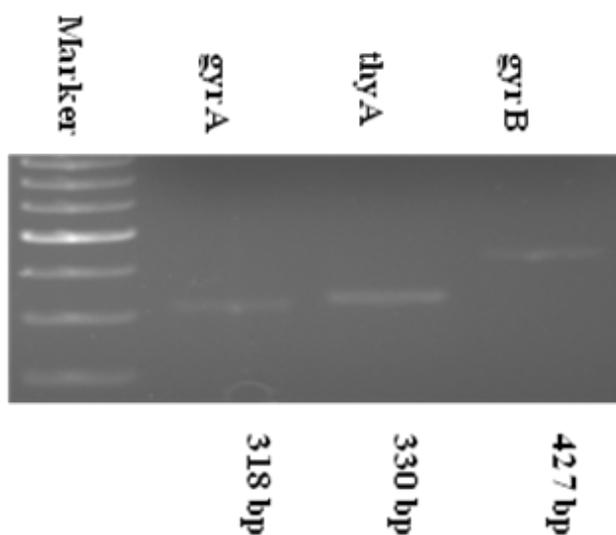


Figure 2. Amplification of second line drug genes

3.2. Identification of Mutation and Sequence Analysis

The first line drugs genes are rpoB, katG, ndh, inhA, kasA, rrs, rpsL, pncA and embB for the Rifampicin, Isoniazid, Streptomycin, Pyrazinamide and Ethambutanol respective antibiotics.

3.2.1. Rifampicin (RMP)

RMP is an important first-line drug for the treatment of TB. RMP interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. The sequencing of the 436 bp central region of the rpoB gene revealed two point mutations at two different codons. Across the rpoB gene, 2 (10%) isolates showed a base substitution at codon 516, out of them D516V (Figure 3b) was observed in one drug resistant isolate and D516G (Figure 3c) in another causing GAC→GTC and GAC→GGC substitutions respectively. There was no mutation in normal individual (Figure 3a). Second point mutation at rpoB was found at codon 531, in 3(15%) isolates causing TCG→TTG substitution. It is designated as S531L (Figure 4b). The overall 5 mutations were observed in rpoB gene in our population.

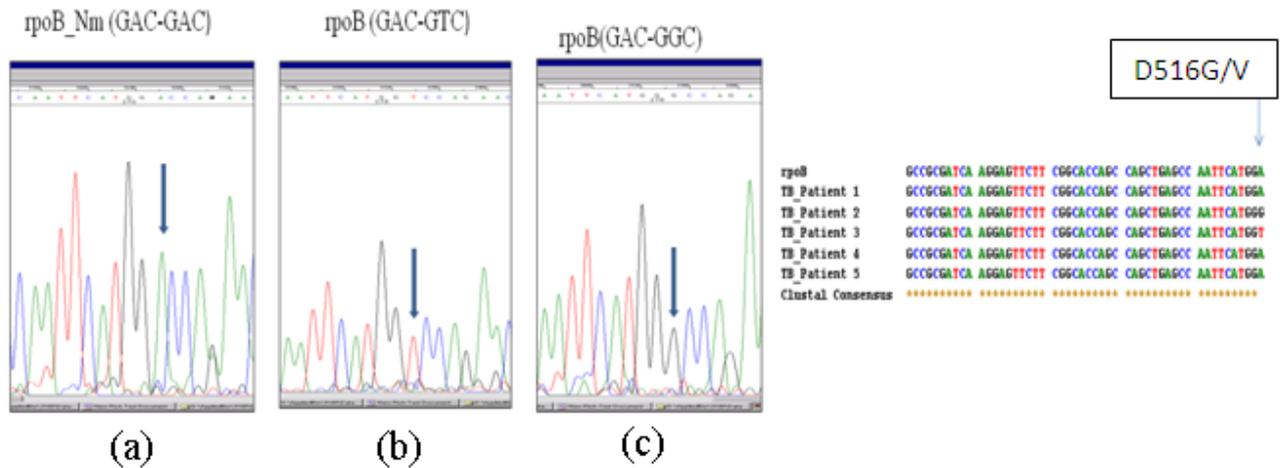


Figure 3. Electropherogram showing mutation *rpoB* gene D516G/V

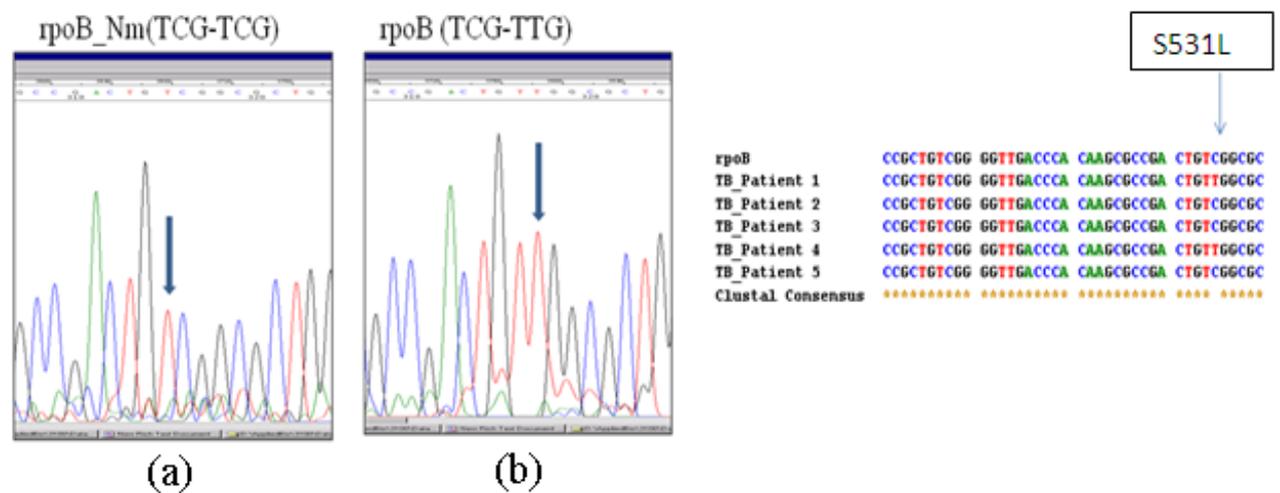


Figure 4. Electropherogram showing mutation in *rpoB* gene S531L

3.2.2. Isoniazid (INH)

INH is a prodrug that is activated by the catalase peroxidase enzyme (KatG) encoded by the *katG* gene. Mutation in *katG* is the main mechanism of INH resistance. The 414 bp fragment of *katG* when subjected to sequencing, we found S315T (Figure 5b) missense mutation in 6 (30%) isolates causing Serine to Threonine

amino acid change in the protein and AGC→ACC in the genome. There was no change in normal individual which is taken as positive control (Figure 5a). Interestingly, no mutations were observed in *inhA*, *kasA* and *ndh* promoter regions. In our population only *katG* shows mutation with high percentage.

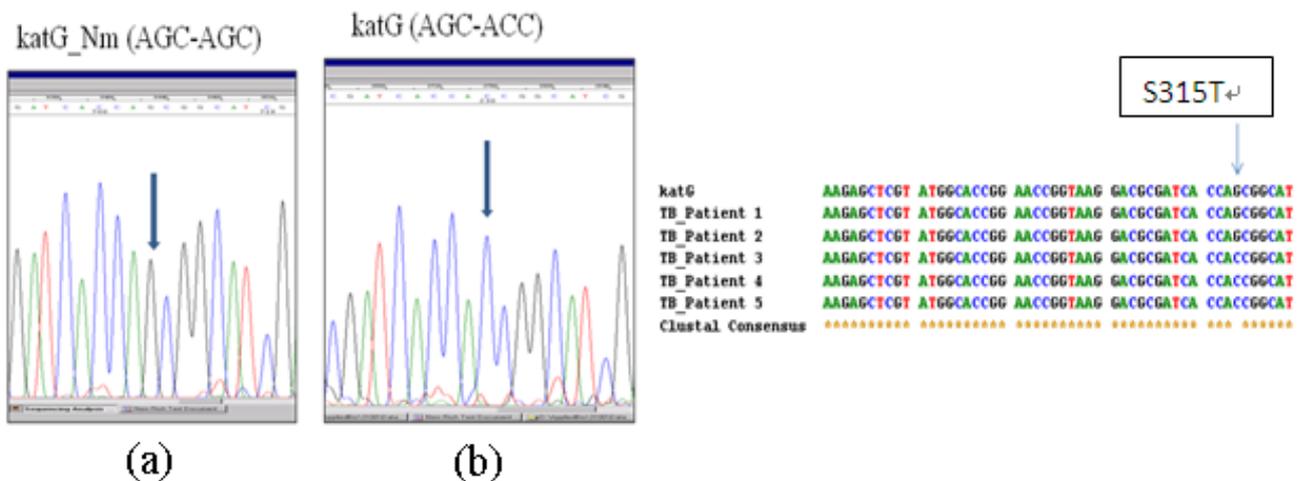


Figure 5. Electropherogram showing mutation in *katG* gene S315T

3.2.3. Aminoglycosides (Streptomycin SM)

SM is an aminoglycoside antibiotic that is active against a variety of bacterial species, including *M. tuberculosis*. SM inhibits protein synthesis by binding to the 30S subunit of bacterial ribosome, causing misreading of the mRNA message during translation. Mutations in *rpsL* and *rrs* are the major mechanism of SM resistance.

For Streptomycin resistance, *rpsL* showed two substitutions at codon 43 and 88 causing AAG→AGG responsible to substitute Lysine by Arginine (Figure 6b) in 8 (40%) isolates. There was no change in normal individual (Figure 6a). The *rrs* gene did not reveal any reported alterations.

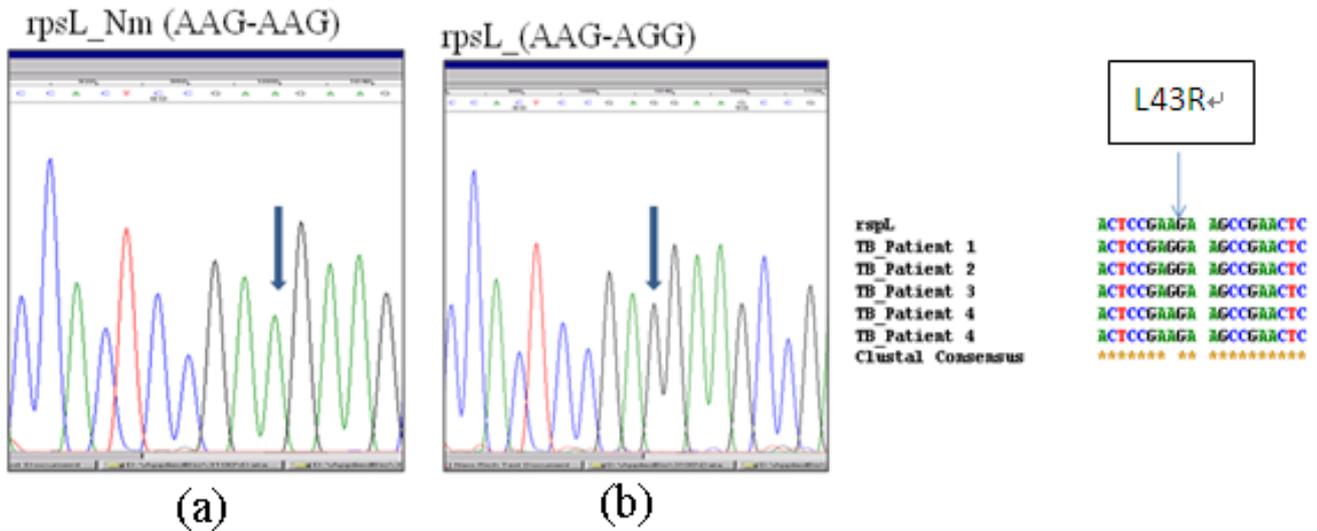


Figure 6. Electropherogram showing mutation in *rpsL* gene L43, 88R

3.2.4. Pyrazinamide (PZA)

PZA is a prodrug that requires conversion to its active form pyrazinoic acid (POA), by the pyrazinamidase/nicotinamidase enzyme encoded by the *pncA* gene of *M. tuberculosis*. The *pncA* mutations are highly diverse and

scattered along the gene, which is unique to PZA resistance. The most frequent mutation at *pncA*195 was detected in 7 (35%) of those 40 isolates, causing CCT→CTT/CAT (Figure 7b) substitution.

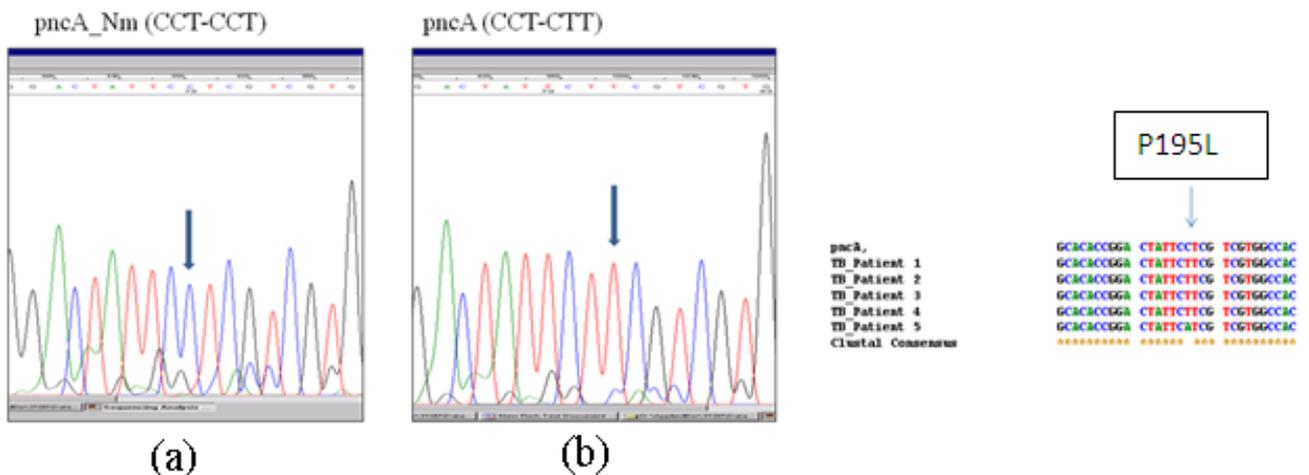


Figure 7. Electropherogram showing mutation in *pncA* gene P195L

3.2.5. Ethambutol (EMB)

EMB is a first line drugs that is used in combination along with RMP, INH and PZA to prevent the emergence of drug resistance. EMB interferes with the biosynthesis of cell wall arabinogalactan. Arabinosyl transferase encoded by *embB*, an enzyme involved in the synthesis of arabinogalactan, has been proposed as the target of EMB

in *M. tuberculosis* and *M. avium*. In *embB* gene, overall, mutations were observed in 3 (15%) isolates. The 2 different types of mutations in *embB* were observed at *embB*306 causing base change at 2 triplet positions ATG→GTG (Figure 8b) and ATG→ATC/ATA (Figure 8c/d).

The second line drugs genes *gyrA* and *gyrB* for fluoroquinolones and *thyA* for para amino salicylic acid.

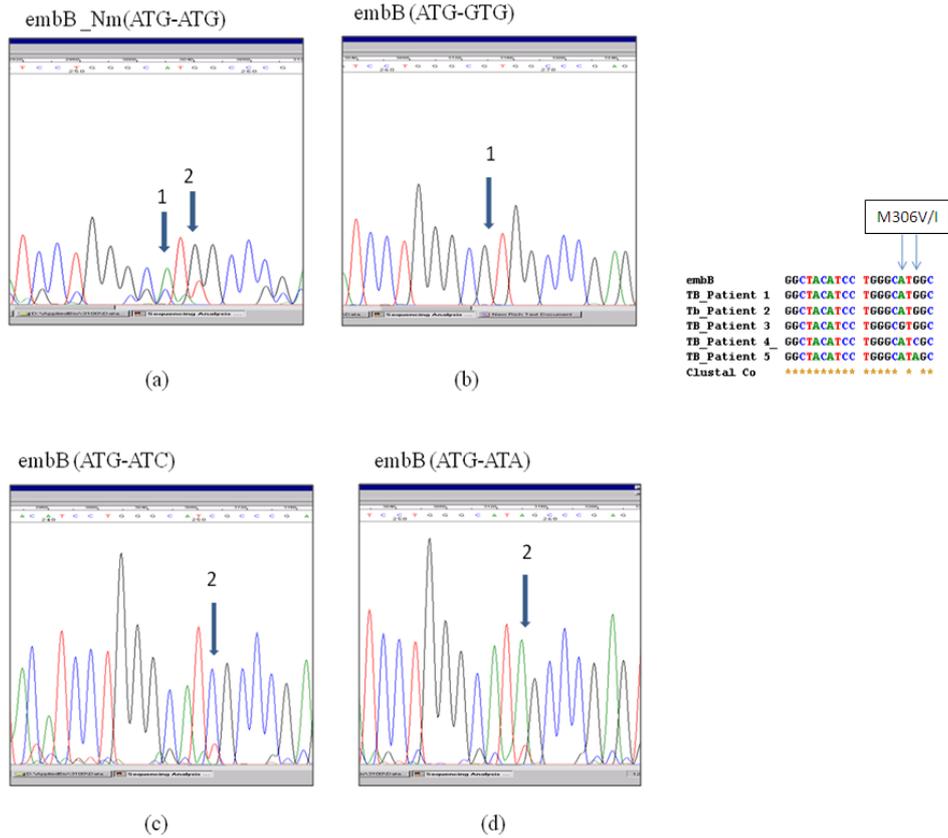


Figure 8. Electropherogram showing mutation in embB gene M306V/I

3.2.6. Fluoroquinolones (FQ's)

In most bacterial species, FQs inhibit DNA gyrase (topoisomerase II) and topoisomerase IV, resulting in microbial death. DNA gyrase is a tetrameric A2B2 protein. *M. tuberculosis* has respectively *gyrA* and *gyrB* encoding the A and B subunits. A conserved region, the quinolone-resistance-determining region (QRDR) of *gyrA* and *gyrB*,

has been found to be a most important area involved in the exhibition of FQ resistance in *M. tuberculosis*. In *gyrA* 2 (10%) isolates shows prominent mutation at codon 90 causing GCG to GTG (Figure 9b), all (100%) isolates were showing the mutation at codon 95 causing AGC to ACC (Figure 9c) and no mutation in *gyrB* gene. The *gyrA* gene mutation is high in our population.

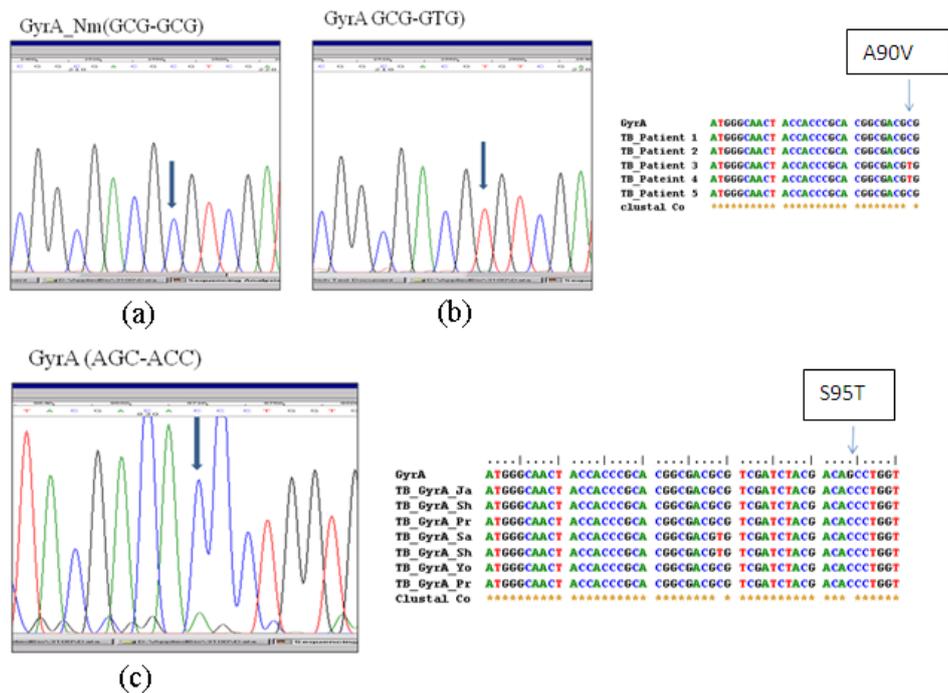


Figure 9. Electropherogram showing mutation in gyrA gene A90V and S95T

3.2.7. P-amino Salicylic Acid (PAS)

PAS is recommended with the combination of isoniazid and streptomycin. However, its mechanism of action is not clearly elucidated but recently an association of *p*-amino salicylic acid resistance with mutations in the *thyA*

gene that encodes thymidylate synthase A for thymine biosynthesis was shown. The common mutation in *thyA* gene is Thr202Ala was reported in many studies. No mutations in *thyA* gene were observed in our population.

Table 3. Mutations in the MDR and XDR genes in clinical isolates *M. tuberculosis*.

GENE	POSITION	MUTATION	AMINO ACID CHANGE	NO.(%) OF ISOLATES
rpoB	Coding(516,531)	GAC to GGC/GTC TCG to TTG	Asp-Gly/Val Ser-Leu	2(10) 3(15)
inhA	None	None	None	None
Ndh	None	None	None	None
katG	Coding(315)	AGC to ACC	Ser-Thr	6(30)
kasA	None	None	None	None
rsrL	Coding(43,88)	AAG to AGG	Lys-Arg	8(40)
Rrs	None	None	None	None
embB	Coding(306)	ATG to GTG ATG to ATC/ATA	Met-Val Met-Iso	3(15)
pncA	Coding(195)	CCT to CTT	Pro-Leu	7(35)
gyrA	Coding(90,95)	GCG to GTG AGC to ACC	Ala-Val Ser-Thr	2(10) All(100)
gyrB	None	None	None	None
thyA	None	None	None	None

The overall analysis of our mutation study reveals that alterations in *rpoB*, *katG*, *rsrL*, *pncA* and *gyrA* genes are with higher percentage (Table 3) in western part of India, which designates higher occurrence of this gene resistance in our population.

4. Discussion

Amongst the molecular methods available, the PCR directed DNA sequencing has proved its immense potential in the detection of alterations in the genome of *M.tb*. The precision and accuracy in exploring the genetic mutations is well exhibited by this method. The rapidly available information pertaining to alterations and drug susceptibility patterns reduces the spread of drug-resistant strains and even facilitate the rapid assortment of treatment regimens. Here, we employed PCR-combined DNA sequencing to detect drug resistance TB cases. In a cohort of 20 patients being investigated for tuberculosis, we have reported some of the mutations in the clinical isolates of *M.tb*.

Total 20 isolates from the western India showed a substitution(s) or a point mutation in genes of anti tubercular drugs. The majority of the mutations in the *rpoB* gene map to codon 516 and 531. Mani et al 2001 from India, found mutations most frequently at codon 531(56%) and codon 526 (19%) [12]. In a study from China, Lu et al., reported mutations at codon 531 (51.6%) [13]. Abdelaal et al. in Egypt, also found frequencies of mutations at codons 531 (45%), and 516 (20%) [14]. However, the frequency of *rpoB* mutations in our population is found to be different from other studies reported across the globe. Collectively 10% and 15% of the clinical isolates of *M.tb* are found mutated at *rpoB*516

and *rpoB*531 respectively. It indicates that, these mutations at 531 and 516 are common with a slightly varied occurrence across the geographical regions. The genetic mutations in isoniazid (INH) genes confer the resistance to the drug and impact on the clinical presentation of the disease. One of the targets for activated INH is the protein encoded by the *inhA* locus. Ramaswamy and Musser, Basso and Blanchard, identified 6 point mutations in *inhA* gene (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro) [15,16]. Recently, in an Indian study, Vadwai et al found 21.4% resistance frequency in *inhA* gene [17]. The other mechanism for the INH resistance is in the protein encoded by *ndh* locus. Rozwarski et al and Miesel et al. 1998 found prominent 10 mutations in *ndh* gene at codon 110 and 268 [18,19]. Lee et al and Piatek et al reveal 4 different substitutions in *kasA* at codon 66, 269, 312 and 413 [20,21]. Nusrath et al 2008, observed no mutation at *kasA* locus in South India [22]. Though the occurrence of this mutation was high in their study, we did not find any mutations at any parts of these genes in western Indian scenario. The Ser315Thr substitution is estimated to occur in 30-60% of INH resistant isolates [23]. In 2006 population genetics for isoniazid was studied throughout the globe, they revealed 49% mutation in *katG* gene in India isolates [24]. A recently Indian study, by Anamika et al found that the most common mutation in INH isolates is *katG* gene at codon 315 with the frequency 82.29% [25]. However, 30% of *katG* resistant clinical isolates of *M.tb* showed the frequency of *KatG*315 (Ser to Thr) in our study.

Ramaswamy and Musser, 1998 reported point mutations in *rrs* and *rpsL* genes in 65–67% of STR resistant isolates. Mutation in *rpsL* gene at codon 43 and

88 are associated with STR and in *rrs* at position 491,512,516 was highly observed [15]. In contrast, in our study 40% of the clinical isolates showed substitutions in *rpsL* gene but no alteration was found in *rrs* gene. Jureen et al in their study from Sweden observed many mutations for *pncA* gene [26], and in 2009, S S Prabhu et al from India had found many mutations in *pncA* gene at various codons [27], but we observed overall 35% frequency of the mutation at codon 195 of *pncA* gene. Mita et al 2008 from India had found reported and some novel mutation in *embB* [28]. Shinnick et al 2009 isolate mutants containing point mutation with 55% frequency at *embB* codon 306 [29]. Of the mutants, our result shows 15% mutation at position 306 with polymorphism.

The most common single nucleotide mutation sites were codons 94, 91, and 90 in *gyrA* and, in total, the relative frequencies of these codons were 56.8%, 6.3% and 25.3% respectively [30]. In a study from china, Cheng et al 2003 reported in all 138 isolates point mutation at codon 95 and Lau et al found in one strain a novel mutation at codon 95 [31,32]. Similarly, our study revealed 10% mutation frequency at codon 90 and in all 20(100%) isolates at codon 95 causing AGC to ACC (Ser to Thr). In *gyrB* gene the most common mutation was observed at codon 485 and 500 with 11.6% frequency in ofloxacin resistant strains [33]. However, no mutations were observed at *gyrB* in our study. The present study results clearly show that Thr202Ala in *thyA* is not involved in the development of resistance to PAS (Para amino salicylic) in *M. tuberculosis* complex isolates. There was no mutation in *thyA* in our PAS resistant strains in our study [30].

5. Conclusion

The genetic mutations among the MDR and XDR genes are being observed in a diverse set of clinical isolates of *Mycobacterium tuberculosis*, resulting in development of resistance against the newer drugs and ultimately responsible for exacerbation of the disease. We found substitutions and missense mutations among the genes against first and second line drugs. Out of them, 5 (25%) in *rpoB* at codon 516 and 531, 6 (30%) in *katG* at codon 315, 8 (40%) in *rpsL* at codon 43 & 88, 3 (15%) in *embB* at codon 306 and 7 (35%) in *pncA* at codon 195. No mutations in *inhA*, *ndh*, *kasA* and *rrs* genes were obtained in our study. In the second line drugs, 2(10%) at codon 90 & 100% mutations at codon 95 in *gyrA* gene, no mutation in *gyrB* and *thyA* gene was observed. We cogitate that the high level of genetic alterations in *Mycobacterium tuberculosis* may be one of the reasons for the emergence of MDR and XDR more rapidly. More-detailed molecular and epidemiological studies with strictly defined patient groups may further elucidate this proposition and help to formulate the new potent drugs to conquer on the battle of MDR and XDR.

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