

## Original Article

# Molecular Diagnosis of Drug-Resistant Tuberculosis

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**ABSTRACT**

**Objective:** The worldwide emergence and spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* has adverse effects on tuberculosis (TB) control programs. The goal of this paper is to describe the advances made in the understanding of the molecular basis of *M. tuberculosis* resistance to first-line anti-TB drugs, and to discuss the potential of molecular methods in early diagnosis of drug-resistant TB.

**Methods:** Molecular methods such as DNA sequencing, polymerase chain reaction, DNA hybridization and restriction fragment length polymorphism have been used to identify/detect mutations in gene-encoding proteins or

rRNA which are targets for the first-line anti-TB drugs.

**Results:** High level resistance to rifampin (RIF), isoniazid (INH), pyrazinamide (PZA), ethambutol (EMB), and streptomycin (STR) is caused by mutations in *rpoB*, *katG*, *pncA*, *embB* and *rpsL/rrs* genes, respectively. The most common mutations conferring high level resistance to RIF, INH, EMB and STR have been identified in *rpoB*, *katG*, *embB* and *rpsL* genes, respectively.

**Conclusion:** Molecular methods to detect the most frequent mutations in the gene encoding functions that are targets for first-line anti-TB drugs have provided encouraging results for early diagnosis of MDR-TB.

KEYWORDS: molecular diagnosis, multidrug resistance, tuberculosis

**INTRODUCTION**

*Mycobacterium tuberculosis*, the causative agent of human tuberculosis (TB) infects one of every three people in the world. In Asia, it infects one of every two people. Every year, about two million people in the world die of TB and eight million new cases occur. TB is the leading cause of mortality due to a single infectious agent. It kills 250,000 children every year and accounts for 26% of all avoidable adult deaths worldwide<sup>[1]</sup>. Considering the magnitude of the problem, the World Health Organization (WHO) declared TB as a 'Global Emergency'.

The above situation has emerged despite the fact that TB is usually a curable disease. The threat of TB is assuming alarming proportions with the dramatic worldwide increase in isolation of *Mycobacterium tuberculosis* strains resistant to commonly used (first-line) anti-TB drugs. This phenomenon is occurring in both immuno-competent and immuno-compromised patients<sup>[2]</sup>. With the increase in human immunodeficiency virus (HIV) epidemic, and the high risk of HIV-infected individuals to develop TB, the prevalence of MDR-TB is almost certain to rise in most countries of the world.

**CHEMOTHERAPY AND DRUG RESISTANCE IN TUBERCULOSIS**

The most commonly used (first-line) anti-TB drugs are isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), streptomycin (STR) and ethambutol (EMB). The directly observed therapy - short course (DOTS) for the treatment of TB, advocated by the World Health Organization (WHO), includes an initial daily dose of INH, RIF, PZA and either STR or EMB for two months. This regimen is then followed for the next four months by a daily dose of INH and RIF<sup>[3,4]</sup>. The drugs such as ethionamide (ETA), cycloserine, p-aminosalicylic acid, thioacetazone, kanamycin, capreomycin, amikacin and fluoroquinolones are used as second-line or alternative agents to treat TB when the bacilli are resistant to first-line drugs.

Clinical isolates of *M. tuberculosis* resistant to one or more first-line drugs are increasingly being isolated throughout the world. Resistance to drugs evolves primarily due to three main reasons.

1. The drug or the gene target is modified in resistant strains such that the drug is no longer effective in killing the organism.
2. The gene product, whose activity is being inhibited by the drug, is overproduced by the bacteria (titration mechanism).

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3. Changes have occurred at the level of entry of the drug into microbial cells such that the intracellular concentration of the drug does not reach the minimum inhibitory concentration (MIC) that is required for efficient killing of pathogenic microorganisms<sup>[5]</sup>.

The mechanism of resistance to antibiotics may involve chromosomal mutations or expression of a latent chromosomal gene as a result of exposure to the drug. Alternatively, the resistance could also be acquired by exchange of genetic material through plasmid-mediated transformation/conjugation or bacteriophage mediated transduction. Substantial progress has been made in our understanding of the molecular basis of drug-resistance in *M. tuberculosis* in the last decade. The resistance in a great majority of clinical *M. tuberculosis* strains to antimycobacterial agents is due to modification of the drug target brought about by spontaneous mutations (mis-sense or nonsense point mutations and/or small deletions or insertions) in key target genes<sup>[6]</sup>. Deletion of the entire gene of the drug target or acquisition of new genes is rare. The frequency of spontaneous generation of mutants occurs at the rate of  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . The bacterial load in a well-developed cavity in pulmonary TB is quite high ( $1 \times 10^9$ ) ensuring that drug-resistant strains will also be present. Though multiple drug therapy is designed to virtually eliminate the emergence of drug-resistant strains, selective growth of drug-resistant mutants occurs when inappropriate drug regimens (due to inadequate therapy) suppress the growth of susceptible strains but permit multiplication of resistant strains<sup>[7,8]</sup>. The long duration of the therapy and poor patient compliance add further to the emergence of drug resistance.

Drug resistant TB was first observed in 1948, subsequent to the first trials of streptomycin for TB treatment<sup>[9]</sup>. In a survey of 35 countries for drug-resistant TB, 12.6% *M. tuberculosis* isolates were found to be resistant to at least one first-line drug (primary resistance) and 2.2% were resistant to two of the most effective and commonly used anti-TB drugs, INH and RIF<sup>[10]</sup>. The rates of secondary (acquired) resistance were much higher. Secondary drug resistance occurs when patients who had the disease with the drug sensitive bacilli, become resistant to drugs due to inappropriate therapy. In a global survey carried out between 1985 and 1995, the incidence of primary resistance to first-line anti-TB drugs were isoniazid (4.1%), streptomycin (3.5%), rifampin (0.2%) and ethambutol (0.1%)<sup>[2]</sup>. However, the rates of secondary resistance were much higher than those of primary resistance. Multiple drug resistant (MDR)-TB strains are described as those strains that are resistant to at

least INH and RIF. The MDR-TB strains evolve due to sequential accumulation of individual mutations in genes that encode targets for the individual antimycobacterial agents (see below) and not due to spontaneous acquisition of multi-drug resistance genes enblock as seen in other systems<sup>[5,6]</sup>. Many outbreaks by multi-drug resistant (MDR) strains of *M. tuberculosis* have been reported from nearly all parts of the globe<sup>[2,10]</sup>. The fatality rate in patients infected with MDR-TB strains varies from 50 to 80% with maximum fatality occurring between 4 to 15 weeks after routine diagnosis<sup>[10]</sup>. The MDR-TB treatment is difficult and very expensive, costing up to 1000 times more than TB with susceptible strains in the United States of America (USA). Outbreaks of MDR-TB among patients with HIV infection have been associated with rapid progression of the disease, high mortality and very expensive treatment<sup>[11]</sup>.

The emergence of drug-resistant strains of *M. tuberculosis* means that the drug susceptibility testing should be performed before initiating therapy. The Center for Disease Control (CDC), USA, has recommended that expeditious identification of *M. tuberculosis* and rapid and accurate drug-susceptibility testing for all front-line anti-TB drugs should be essential steps for effective control of TB. Growing the pathogen in the presence of various concentrations of the drug performs the conventional susceptibility testing of pathogenic microorganisms to antibiotics, after the primary culture has been obtained. Genotypic drug susceptibility testing by molecular methods detects either the mutations conferring drug resistance in the drug target or acquisition of drug modifying or inactivating genes in drug-resistant strains. The genotypic testing may thus provide results faster and unambiguously than phenotypic drug susceptibility testing.

#### IDENTIFICATION OF DRUG-RESISTANT *M. TUBERCULOSIS*: CONVENTIONAL METHODS

The conventional (agar proportion) method of antimycobacterial drug susceptibility testing requires growing *M. tuberculosis* on medium in the presence and absence of the drug for 2 to 3 weeks<sup>[12]</sup>. A more rapid method, BACTEC TB 460 system (Becton Dickinson Microbiology System, Cockeysville, MD) still requires 4 to 12 days of incubation before results are available to the clinician. This method involves the measurement of  $14\text{CO}_2$  produced by *M. tuberculosis* growing in liquid Middlebrook 7H12 medium containing  $14\text{C}$  labeled palmitic acid with or without the anti-TB drug<sup>[13]</sup>. The rate and amount of  $14\text{CO}_2$  produced is directly proportional to the rate and growth of the

bacilli. More recently, non-radioactive BACTEC system has become available. However, the BACTEC system is costly and requires several days before test results are available. Molecular methods, if feasible, offer the advantage of determining the genetic basis of resistance to various anti-TB drugs, thus providing susceptibility results rapidly and unambiguously.

## MOLECULAR METHODS

The progress made in understanding the genetic basis of drug resistance to the first-line antimycobacterial agents has resulted in the development of molecular methods for rapidly determining the susceptibility profiles of clinical isolates of *M. tuberculosis*<sup>[6]</sup>. The target genes that are mutated in drug-resistant strains are listed in Table 1. The mechanism of resistance and the molecular methods to detect the resistance against the first-line anti-TB drugs are described below.

### Rifampin:

Rifampin (RIF) is a semi-synthetic derivative of rifamycin and is used as a first-line antimycobacterial drug. The drug is a key component of anti-TB therapy due to its highly effective bactericidal action<sup>[3]</sup>. RIF binds to the b-subunit of ribonucleic acid (RNA) polymerase (*rpoB*), the enzyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity<sup>[14]</sup>. The molecular diagnosis of rifampin resistance is most suitable because the studies have shown that the drug resistance is due to mutations in a small region of the *rpoB* gene. The molecular approaches that have been utilized include direct automated sequencing of the amplified target region of the *rpoB* gene, amplification of the target region by polymerase chain reaction (PCR) followed by single stranded conformation polymorphism (SSCP) analysis and a commercially available test based on amplification of the target region by PCR followed by line probe hybridization to specific

oligonucleotide probes (INNO-LiPA Rif. TB kit, Innogenetics, Zwijnaarde, Belgium)<sup>[15-17]</sup>. The resistant strains carry mutations within *rpoB* gene encoding the b-subunit of RNAPolymerase<sup>[15]</sup>. Since resistance to rifampin alone is rather rare, determination of rifampin resistance is a useful surrogate marker for MDR-TB<sup>[6,7]</sup>. The data available from many studies have indicated that nearly 95% of epidemiologically unrelated rifampin-resistant clinical *M. tuberculosis* isolates carry 35 distinct point mutations or small insertions/deletions located within 81-bp core region (rifampin resistance determining region, RRDR) of *rpoB* (codons 507-533) encoding 27 amino acids<sup>[6]</sup> (Table 1). There is a strong correlation of specific amino acid substitutions and minimum inhibitory concentration (MIC) of the drug. Typically mis-sense mutations in codons 513, 526 and/or 531 result in high level rifampin resistance whereas amino acid changes at position 514 or 533 usually result in low level rifampin resistance<sup>[18]</sup>. Earlier studies indicated that 45% of isolates had mis-sense mutations in codon 531 (S531) while 35% of resistant strains had codon 526 (H526) alterations resulting in amino acid replacements<sup>[19]</sup>. These mutations were absent in susceptible organisms. However, more detailed studies carried out from different parts of the globe have shown that the frequency of specific mutations varies significantly among various ethnic populations and geographical locations. The mutations at codon H526 are identified in majority (43%) of clinical isolates from New York compared to mutations at codon S531 being present in 53%, 75% and 65% of clinical isolates from Greece, St. Petersburg, Russia and patients of Middle Eastern origin, respectively<sup>[6,20-23]</sup> (Table 2). However, the mechanism of resistance was not identified in nearly 5% of rifampin-resistant clinical isolates as no mutations were detected in the 81 bp core region or elsewhere in the *rpoB* gene<sup>[6]</sup>. Since these isolates had confirmed rifampin resistance by standard susceptibility testing, this suggests a fraction of

**Table 1**  
Gene targets involved in drug-resistant strains of *M. tuberculosis*

Anti-TB Agent*	Gene Target(s)	Encoded Product	Target Region	Level of Resistance
RIF	<i>rpoB</i>	b-Subunit of RNAPolymerase	81 bp RRDR	High
INH	<i>katG</i>	Catalase/oxidase	Entire gene	High
INH/ETA	<i>inhA</i>	Enoyl-ACP reductase	Regulatory region	Low
INH	<i>kasA</i>	b-Ketoacyl ACP synthase	Many codons	Low
PZA	<i>pncA</i>	Pyrazinamidase	Many codons	High
EMB	<i>embB</i>	Arabinosyl transferase	Few codons in ERDR	High
STR	<i>rrs</i>	16S rRNA	Two core regions	Low
STR	<i>rpsL</i>	Ribosomal S12 Protein	Codons 43 and 48	High

\* Anti-TB agents include RIF, rifampin; INH, isoniazid; ETA, ethionamide; PZA, pyrazinamide; EMB, ethambutol and STR, streptomycin

**Table 2**

Frequency of the most common mutations in gene targets conferring high level resistance to first-line anti-TB drugs in drug-resistant strains of *M. tuberculosis*

Anti-TB Agents	Gene Target	Region Target	Frequency (%) in Resistant Strains	Most Common Mutation	Frequency (%) in Resistant Strains
RIF	<i>rpoB</i>	RRDR	95	Codon 531	30 to 75
INH	<i>KatG</i>	Entire gene	60 to 95	S315T	60 to 95
PZA	<i>pncA</i>	Entire gene	68 to 97	No Specific Mutation	
STR	<i>rpsL</i>	Codons 43, 88	40 to 95	K43R	40 to 90
EMB	<i>embB</i>	ERDR	47 to 94	Codon 306	47 to 89

rifampin resistant strains will not be detected by rapid molecular methods compared to the conventional phenotypic drug susceptibility testing.

The commercially available line probe assay kit for the detection of rifampin resistance provides results within two days. The assay is easy to perform but remains relatively expensive. Since mutations external to the core region and mutations in other genes causing rifampin resistance cannot be detected by the line probe kit (nearly 5% of the total rifampin resistant strains), negative results with this assay do not rule out rifampin resistance. However, the kit may still serve an important role as a rapid and convenient screen for rifampin resistance with negative cases still being diagnosed by the conventional method. This suggests that genotypic testing will not replace phenotypic susceptibility testing, but it can provide results faster in positive cases thus saving time. Conventional testing will still be required in cases that are negative by this approach.

#### Isoniazid:

Isoniazid is a synthetic, first-line drug that is used mainly to treat infections by *M. tuberculosis* complex members (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) as all other mycobacteria and other prokaryotes are resistant to isoniazid. The susceptible strains of *M. tuberculosis* have MIC of less than 0.05 µg/ml. The molecular basis of resistance to isoniazid is more complex and is caused by a variety of mutations in four different genes of *M. tuberculosis* i.e. *katG* encoding catalase peroxidase, *inhA* encoding the enoyl acyl carrier protein (ACP) reductase, *kasA* encoding b-ketoacyl ACP synthase and *ahpC* encoding alkyl-hydroperoxide reductase<sup>[6,24-27]</sup> (Table 1). Even then, nearly 5-10% of isoniazid-resistant *M. tuberculosis* isolates do not have an identifiable mutation<sup>[6]</sup>. Thus, a reliable clinical application of genotypic testing for isoniazid resistance seems unlikely due to the diversity of these mutations. The genetic and biochemical evidence suggests that mutations in

*ahpC* are not directly involved in isoniazid resistance but represent compensatory alterations occurring as a consequence of loss of catalase/peroxidase activity<sup>[6]</sup>. The mutations in *inhA* occur in the upstream regulatory region that result in increased *inhA* protein expression, thereby elevating the drug target levels and producing isoniazid resistance via titration mechanism leading to low level of resistance<sup>[6,25,26]</sup>. Low level isoniazid-resistant *M. tuberculosis* isolates have also been identified carrying mis-sense mutations in the *inhA* structural gene that decrease binding affinity of the enzyme for reduced nicotinamide adenine dinucleotide (NADH)<sup>[6]</sup>. This prevents mycolic acid biosynthesis.

Clinically relevant high level resistance to isoniazid is mainly due to small deletions/insertions or mis-sense/nonsense mutations within *katG* gene<sup>[6,24-26]</sup>. The most common genetic alterations within the *katG* gene in isoniazid-resistant strains involve amino acid changes in codons 315 and 463<sup>[6,24-26]</sup>. Though mutation R463L was found in a great majority of isoniazid-resistant strains from North America, the role of this mutation in conferring resistance to isoniazid is not well established. Introduction of this mutation *in vitro* in *M. tuberculosis* did not alter the peroxidase activity and was shown to confer only low level of resistance to isoniazid (MIC <1 µg/ml)<sup>[28]</sup>. More detailed analyses have shown that nearly 10-20% of *M. tuberculosis* isolates from USA, Central America and Europe contain leucine (Leu) at codon 463 while the great majority of organisms recovered from patients in China and Southeast Asia contain Leu at this position<sup>[6]</sup>. It is clear that striking geographic differences exist in the frequency of occurrence of arginine (Arg) or Leu at codon 463. Lee et al<sup>[29]</sup> analyzed 63 isoniazid-resistant and 50 isoniazid-sensitive isolates of *M. tuberculosis* from patients in Singapore. These authors reported the presence of R463L mutation in 73% of the isoniazid-resistant and 56% of the isoniazid-sensitive strains. Some of the isoniazid susceptible strains in several other studies have

also been shown to contain Leu at codon 463 [26,30,authors unpublished data]. Further, all isolates of *M. bovis*, *M. microti* and *M. africanum* (other members of the *M. tuberculosis* complex) that are fully susceptible to isoniazid have been shown to carry Leu at codon 463<sup>[29]</sup>. These data suggest R463L substitution in *katG* gene may not confer clinically significant resistance to isoniazid.

The mutation S315T at codon position 315 within the *katG* gene has directly been shown to confer high level of resistance to isoniazid<sup>[31]</sup>. The S315T mutation is accompanied by the loss of *Msp* I restriction enzyme site at this position. Thus, the mis-sense mutation can be readily detected by differential restriction patterns of PCR amplified fragments (PCR-RFLP). The occurrence and prevalence of this specific mutation in isoniazid-resistant clinical *M. tuberculosis* isolates varies considerably according to geographical location and ethnic background of the infected individual<sup>[24,25,30,32]</sup> (Table 2). Thus, screening for this genetic alteration in the *katG* gene may help to provide a rapid screening method for the detection of isoniazid-resistant *M. tuberculosis* isolates provided a high prevalence of this specific mutation could be established from a given geographical location.

#### Ethionamide:

Ethionamide is a structural analog of isoniazid that is used as a second-line drug against TB. Ethionamide also inhibits mycolic acid biosynthesis<sup>[6,33]</sup>. For some strains, low level isoniazid resistance is accompanied by co-acquisition of ethionamide resistance suggesting that both isoniazid and ethionamide share the same molecular target. Low level isoniazid and ethionamide resistance was shown in clinical *M. tuberculosis* isolates due to mutations in the upstream regulatory region that result in increased *inhA* protein expression, thereby elevating the drug target levels and producing isoniazid and ethionamide resistance via titration mechanism<sup>[5,6]</sup>.

#### Pyrazinamide:

Pyrazinamide is a structural analog of nicotinamide that is used as a first-line anti-TB drug. This drug is postulated to kill semi-dormant mycobacteria in an acidic environment such as those of phagolysosomes<sup>[34]</sup>. The pyrazinamide is converted to pyrazinoic acid, the active drug, by the mycobacterial enzyme pyrazinamidase encoded by *pncA* and pyrazinamide-resistant *M. tuberculosis* strains lack the enzyme activity. The susceptible clinical *M. tuberculosis* isolates were found to have identical wild-type sequence while 75-95% of pyrazinamide-resistant isolates had a

point mutation (mis-sense/nonsense or insertion/deletion) spread over the entire length of the *pncA* gene<sup>[35-37]</sup> (Table 1). The diversity of mutations makes it unlikely that a suitable molecular method could be devised for rapidly determining the resistance to pyrazinamide in clinical *M. tuberculosis* isolates<sup>[6,35-37]</sup>. Lack of *pncA* mutations in up to 25% of clinical isolates in some studies point towards the existence of at least one additional gene participating in pyrazinamide resistance<sup>[6,36]</sup>.

#### Streptomycin:

Streptomycin is an aminoglycoside antibiotic that is used as a first-line drug to treat TB. The drug binds to 16S rRNA inhibiting translational initiation and affects translational fidelity during protein synthesis<sup>[38]</sup>. Mutations associated with streptomycin resistance in *M. tuberculosis* have been identified in the 16S rRNA gene (*rrs*) and *rpsL* gene encoding ribosomal protein S12<sup>[39-41]</sup> (Table 1). Since *M. tuberculosis* genome has a single gene copy of *rrn* genes<sup>[42]</sup>, single nucleotide changes can potentially produce antibiotic resistance. Point mutations in the *rrs* gene are located in two loops centered around nucleotides 530 and 915 that interact with the ribosomal S12 protein<sup>[39,43]</sup>. Point mutations producing high level streptomycin resistance occur in *rpsL* gene, mostly involving codon 43 and less frequently in codon 88<sup>[40,43]</sup> (Table 2). Low level of resistance to streptomycin may also occur due to cell permeability changes, production of aminoglycoside modifying enzymes or alterations in other ribosomal molecules as some of the resistant isolates with MIC of 10 µg/ml have wild type *rrs* and *rpsL* genes<sup>[41,43]</sup>. Since only about 65-75% of streptomycin-resistant isolates carry mutations in *rrs* or *rpsL* genes, other molecular mechanisms of streptomycin resistance exist, thus efficient molecular diagnosis of streptomycin resistance in *M. tuberculosis* seems unlikely at this stage<sup>[41,43]</sup>.

#### Ethambutol:

Ethambutol (EMB) (a structural analog of arabinose) is a first-line anti-TB drug that also inhibits the incorporation of mycolic acids into mycobacterial cell wall<sup>[44]</sup>. Genetic and biochemical studies have shown that resistance to ethambutol is mediated by mutations in *embB*, one of the three genes encoded by *embCAB* operon<sup>[45]</sup>. The *embB* gene encodes an arabinosyl transferase, an integral membrane protein with 12 transmembrane domains that is inhibited by the drug<sup>[45]</sup>. DNA sequence analyses of *embB* gene from ethambutol-resistant strains have shown that EMB-resistance determining region (ERDR) in *embB* is located in the cytoplasmic loop of the membrane spanning

domain<sup>[45,46]</sup>. Nearly 50-70% of ethambutol-resistant strains contain mis-sense mutations within ERDR of *embB* gene with majority (47-60%) of strains carrying alterations at codon 306<sup>[45,46]</sup> (Table 2). However, the molecular basis of EMB resistance involves other targets as well since nearly 30% of ethambutol-resistant strains lack mutations in the ERDR of *embB*<sup>[45,46]</sup>.

## CONCLUSIONS

- Though strategies to detect MDR-TB are urgently needed, the sheer multiplicity of genetic loci that must be targeted to screen for drug resistance, renders molecular diagnosis tedious, labor intensive and with the exception of rifampin, lacks sensitivity for a broader approach.
- Since rifampin resistance is generally a marker for MDR-TB, reference laboratories should consider having the ability to test for rifampin resistance directly from specimens and the line probe assay strategy has the advantages of relatively reliable performance and commercial availability.
- The detection of S315T mutation may be performed as a rapid screening method for identifying isoniazid-resistant clinical isolates provided a high prevalence of this mutation is established from a given geographical location/ethnic group.
- All strategies for molecular diagnosis suffer from the fact that the molecular basis of resistance is not understood for all the resistant organisms for any of the antimycobacterial agents.

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