ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Khaoula BALGOUTHI

MOLECULAR GENETIC DIFFERENCES OF HIGH AND LOW DOSE ISONIAZID RESISTANCE IN MULTI DRUG MYCOBACTERIUM TUBERCULOSIS

DEPARTMENT OF BIOTECHNOLOGY

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FEN BİLİMLERİ ENSTİTÜSÜ

ÇOKLU İLAÇ DIRENÇLI *MYCOBACTERIUM TUBERCULOSIS*’DE YÜKSEK VE DÜŞÜK İSONIAZID DIRENCININ MOLEKÜLER GENETIK FARKLILIKLARI

Khaoula BALGOUTHI

YÜKSEK LİSANS TEZİ

BİYOTEKNOLOJİ ANABİLİM DALI

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DANIŞMAN	ÜYE	ÜYE

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ABSTRACT

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Tuberculosis is one of the top ten causes of death worldwide. Isoniazid (INH) is an one of the important major chemotherapeutic and prophylactic drug against TB and it is key component of global tuberculosis (TB) control programs which we cannot afford to lose. INH prophylaxis reduces the risk of developing TB (suspicious and latent). Also it is cheap, effective and has a low rate of adverse events that cannot be substituted by an equally positive alternative.

INH resistance to is typically caused by mutations katG and inhA promoter regions. katG mutations confer high-level isoniazid resistance while inhA mutations confer low-level resistance to isoniazid.

In this study, we investigated which genetic mutations predict the level of phenotypic isoniazid resistance. For this purpose, phenotypic resistance of 91 INH resistant isolates was determined at 3 different drug levels and four gene regions associated with INH drug resistance, including katG, furAlG, fabGl-pro inhA and inhA, were amplified and sequenced.

As a result of this study, we found Mutations in the promoter region of inhA tend to result in low-level, katG Ser315Thr mutations moderate and the combination of katG Ser315Thr and inhA c-15t tend to high level phenotypic resistance. No clear association was found between mutations other than these and isoniazid the level resistance.

Key words: Tuberculosis, *M. tuberculosis*, INH-resistance, mutations, anti-tuberculosis drugs

ÖZ

YÜKSEKLİSANS TEZİ

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Danışman : Prof. Dr. Fatih KÖKSAL
Yıl: 2019, Sayfa: 51
Jüri : Prof. Dr. Fatih KÖKSAL
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: Prof.Dr.Nizami DURAN

TB günümüzde dünyada en fazla ölüme yol açan 10 ölüm nedeni arasındadır. Izoniazid (INH), kaybetmeyi göze alamayacağımız en önemli kemoterapötik ve profilaktik ilaçlardan biridir ve global tüberküloz (TB) kontrol programlarının ana bileşenidir. INH profilaksisi TB gelişmesi riskini azaltır (şüpheli ve latent). Ayrıca, ucuz, etkili ve yan etkisi düşük olan alternatifi olmayan bir ilaçtır.

INH direncine tipik olarak katG ve inhA promotör bölgelerinde mutasyonlar neden olur. katG mutasyonları yüksek seviyeli izoniazid direnci sağlarken, inhA mutasyonları düşük seviyeli isoniazid direnci sağlar.

Bu çalışmada, INH direncine neden olan genetik mutasyonların, fenotipik izoniazid direnci seviyesi ile ilişkisini inceledik. Bu amaçla, 91 INH dirençli izolatın 3 farklı ilaç düzeyinde fenotipik direnci belirlenmiş ve aynı zamanda KatG, furAlG, fabGl-pro inhA ve inhA dahil olmak üzere INH ilaç direnci ile ilişkili dört gen bölgesi, PCR ile amplifiye edilerek çoğaltılmış ve bu bölgelere DNA sekans çalışması yapılarak mutasyonlarının ileç direnç seviyeleri incelenmiştir.

Çalışmanın sonucunda; inhA'nın promotör bölgesindeki mutasyonların düşük seviyeli, katG Ser315Thr mutasyonlarının orta derecede ve katG Ser315Thr ve inhA c-15t kombinasyonunun yüksek seviyede fenotipik direnç gösterme eğiliminde olduğunu bulduk. Bunlardan başka mutasyonlar ile izoniazid seviye direnci arasında net bir ilişki bulunamamıştır.

Anahtar Kelimeler: Tüberküloz, *M. tuberculosis*, INH Direnç, mutasyonlar, anti-tüberküloz

EXPANDED ABSTRACT

Tuberculosis is one of the top ten causes of death worldwide. Isoniazid (INH) is an one of the important major chemotherapeutic and prophylactic drug against TB and it is key component of global tuberculosis (TB) control programs which we cannot afford to lose. INH prophylaxis reduces the risk of developing TB (suspicious and latent). Also it is cheap, effective and has a low rate of adverse events that cannot be substituted by an equally positive alternative.

INH resistance to is typically caused by mutations katG and inhA promoter regions. katG mutations confer high-level isoniazid resistance while inhA mutations confer low-level resistance to isoniazid.

In this study, we investigated which genetic mutations predict the level of phenotypic isoniazid resistance. For this purpose, phenotypic resistance of 91 INH resistant isolates was determined at 3 different drug levels and four gene regions associated with INH drug resistance, including KatG, furAlG, fabGl-pro inhA and inhA, were amplified and sequenced.

M. tuberculosis clinical isolates including INH-resistance strains were selected. Strains were randomly selected among the MDR tuberculosis isolates which found phenotypicly drug resistance tests were performed at the Cukurova University Tropical Disease Research and Application Center, Adana Regional Tuberculosis Laboratory.

All clinical isolates, H37Rv were tested for drug susceptibility. Strains were analyzed by MGIT 960 Based Antimicrobial Testing (Becton, Dickinson and Company). The medium contained INH (0.4g/ml). Resistance is expressed as the percentage of colonies that grow on critical concentrations of the substances. The interpretation will be based on the usual criteria for resistance.

Mycobacterium tuberculosis isolates from multiple drug resistant patients show a particular set of mutations in drug targets conferringresistance. 91 M. tuberculosis clinical isolates from Cukurova University Tropical Disease Research and Application Center, Adana Regional Tuberculosis Laboratory , including INH-resistance strains were selected to investigate the mutations in the katG ,inhA genes fragment including almost every mutation sites previously published was amplified by PCR and the mutations in these genes fragments were detected by DNA sequencing.

Based on the sequencing results, the mutations were analyzed. We recently reported 5 novel mutations in katG (T. Mori et T. Kirikae. 2007). Including these mutations, 280 different mutations in katG were found in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) when articles were searched by the keywords “katG,” “mutation,” and “tuberculosis.”Also, in 2003 , six mutations in the upstream region of the fabG1-inhA operon, including C−15T, and seven in inhA cause INH resistance were funded by Riccardi et al (Riccardi, and D. Ghisotti. 2003).

In our study, we found an additional 26 novel mutations in katG. Two was a deletion C, insertion G, while the other 24 caused amino acid substitutions. These mutations and amino acid substitutions are shown in Table 17. Furthermore, several novel mutations were detected in the present study: one in fabG1 (Deletion A, C-T]), one in inhA (C126T, deletion A), and three in the intergenic region of thefurA-katG operon (G−226A, insertion A, and G224A).

As a result of this study, we found Mutations in the promoter region of inhA tend to result in low-level, katG Ser315Thr mutations moderate and the combination of katG Ser315Thr and inhA c-15t tend to high level phenotypic resistance. No clear association was found between mutations other than these and isoniazid the level resistance.

GENİŞLETİLMİŞ ÖZET

Tüberküloz, Dünyada genelinde ölüme yol açan ilk on neden arasında yer almaktadır. Isoniazid (INH), tüberküloz tedavisinde en önemli kemoterapötik ve profilaktik ilaçlardan ve kaybetmeyi göze alamayacağımız küresel tüberküloz (TB) kontrol programlarının temel bileşenlerinden biridir. İsoniazidin dahil edildiği profilaktik tedavi, tüberkuloz şüpheli ve latent hastalarda infeksiyon gelişim riskini azaltır.Ayrıca, ucuz, etkin ve yan etkilerinin düşük olması nedeni ile daha iyi bir alternatifi bulunmamaktadır.

INH direncine genel olarak katG ve inhA promotör bölgelerinde mutasyonlar neden olur. Yaygın olarak katG mutasyonları yüksek izoniazid direncinden sorumlu iken, inhA mutasyonları düşük isoniazid direncine sebep olur. Bu çalışmada, meydana genetik mutasyon çeşitlerinin fenotipik izoniazid direncinde ilaç konsantrasyon seviyeleri ile muhtemel ilişkisini araştırdık.

Bu amaçla, fenotipik olarak INH dirençli 91 izolat, 3 farklı ilaç konsantrasyonunda değerlendirildi ve KatG, furAlG, fabGl-pro inhA ve inhA dahil olmak üzere INH ilaç direnci ile ilişkili dört gen bölgesi çoğaltılarak, DNA dizi analizi gerçekleştirildi.

Suşlar, Adana Bölge Tüberküloz Laboratuvarı Çukurova Üniversitesi Tropikal Hastalık Araştırma ve Uygulama Merkezi'nde fenotipik olarak antibiyotik duyarlılık testleri yapılan ve MDR olarak belirlenen izolatlar arasından rastgele seçildi.

Tüm klinik izolatlar ve H37Rv, antibiyotik duyarlılıkları açısından test edildi.

Suşlar, MGIT 960 Tabanlı Antimikrobiyal Testler (Becton, Dickinson ve Company) ile analiz edildi. Direnç, izoniazidin kritik konsantrasyonlarında üreyen kolonilerin yüzdesi olarak ifade edildi. Yorum normal direnç kriterlerine gore yapıldı. Çoklu ilac dirençli hastalara ait *Mycobacterium tuberculosis* izolatları, direnç sağlayan ilaç hedeflerinde belirli bir mutasyon dizisi gösterir.

KatG, InhA gen bölgelerinde meydana gelen daha önce yayınlanmış mutasyonları araştırmak amacı ile Çukurova Üniversitesi Tropikal Hastalık Araştırma ve Uygulama Merkezi, Adana Bölgesel Tüberküloz Laboratuvarında izole edilen INH dirençli 91 *M. tuberculosis* klinik izolata ait DNA’lar PCR ile çoğaltıldı ve bu genlerdeki mutasyonlar DNA dizilimi ile tespit edildi.

Dizi analizi sonuçlarına dayanarak, mutasyonlar analiz edildi ve katG'de 5 yeni mutasyon bildirildi (T. Mori et T. Kirikae. 2007).

Bu mutasyonlar dahil olmak üzere, makaleler katG, mutasyon ve tüberküloz anahtar kelimeleri ile PubMed'de (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) arandığında katG'deki 280 farklı mutasyon bulundu. Ayrıca, 2003 yılında, INH direncine neden olan C15T içeren fabG1-inhA’da altı ve inhA gen bölgesinde yedi mutasyon Riccardi ve arkadaşları tarafından belirlendi (Riccardi ve D. Ghisotti. 2003).

Çalışmamızda katG' geninde 26 adet farklı mutasyon bulduk. Tablo 17'de gösterilmiştir. Ayrıca, bu çalışmada birkaç yeni mutasyon tespit edildi: fabG1'de (A delesyonu, C-T]), inhA'da (C126T, A delesyonu) ve üçü furA-katG operonunun intergenikte bölgesinde (G − 226A, A insersiyonu ve G224A).

Bu çalışmanın sonucu olarak, inhA'nın promotör bölgesindeki mutasyonların düşük seviyeli, katG Ser315Thr mutasyonlarının orta düzeyde ve katG Ser315Thr ve inhA c-15t kombinasyonunun yüksek seviyede fenotipik direnç gösterme eğiliminde olduğunu bulduk. Bunlardan başka mutasyonlar ile izoniazid seviye direnci arasında net bir ilişki bulunamamıştır.

Bu çalışmada amacımız; Çukurova Bölgesinde izole edilen konvansiyonel fenotipik yöntemlerle düşük doz ve yüksek doz izoniazid direnci gösteren *Mycobacterium tuberculosis* kompleks (MTK) suşlarında bu direnç seviye farklılıklara sebep olan mutasyonları tespit etmektir..

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LIST OF SYMBOLS AND ABBREVIATONS

MTB	: *Mycobacterium tuberculosis*
MDR-TB	: Multidrug-resistant tuberculosis
°C	: Degree Celcius
WHO	: World Health Organization
TB	: Tuberculosis
PCR	: Real-time quantitative PCR
ETH	: Ethionamid
XDR TB	: Extensively drug-resistant tuberculosis
HIV	: Human immunodeficiency
INH	: Isoniazid
MDG	: Millennium Development Goals
DST	: Molecular phenotypes
InhA	: Inhibin Subunit Alpha
KatG	: Catalase peroxidase
APC	: Antigen presentation cells
CFPs	: *M.tuberculosis* culture filtrate proteins (CFPs)
SodA	: Superoxide dismutase
HspX	: The crystalline protein homolog or the 16-kDa protein
Mas	: Mycocerosic acids
FbpA	: Mycolyl-transferase enzymes
HbhA	: A heparin-binding hemagglutin protein
AhpC	: Alkyl hydroperoxide reductase

1. INTRODUCTION

Tuberculosis has been known to mankind since ancient times. Earlier this disease was called by many names including consumption (because of severe weight loss and the way the infection appeared to "absorb" the patient), phthisis pulmonaris and white plague (due to extreme pallor seen among these infected).

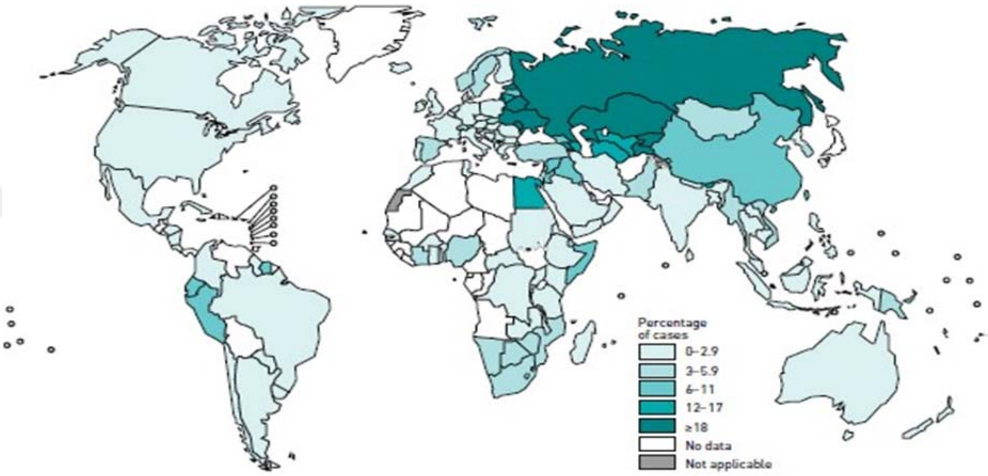
Even today after the development of advanced methods of critical examination, diagnosis and treatment for the disease, one-third of the world's population has been exposed to and is infected with the organism. Numbers are over 90% in the developing world.

With the advent of HIV infection there is a dramatic reappearance of TB with over 8 million new cases every year worldwide and more than 2 million people dying from it. In the 19th century, tuberculosis was known as "pilot of all men of death". It is still true today.

In the eighteenth century in western Europe, tuberculosis reached its peak with a prevalence as high as 900 deaths per 100,000. Badly ventilated and overloaded housing, primitive hygiene, malnutrition and other risk factors led to the increase. White condition plague appeared around this time.

Tuberculosis spared no region of Europe during the 18th and 19th centuries. In turn, witches, fairies, malnutrition and unsanitary air are put in the dock. In the nineteenth century, the medical profession seemed to better understand the workings of the disease and eventually establish links between pulmonary tuberculosis, scrofula and skin lesions. In 1882, Robert Koch discovered the bacillus of tuberculosis and confirmed the high degree of contagion of this infection. By building on a better understanding of the disease, the medical community is developing preventive measures and better adapted treatment methods. This discovery also allows the development of vaccines and drugs against tuberculosis. Robert Koch receives the Nobel Prize in 1905.

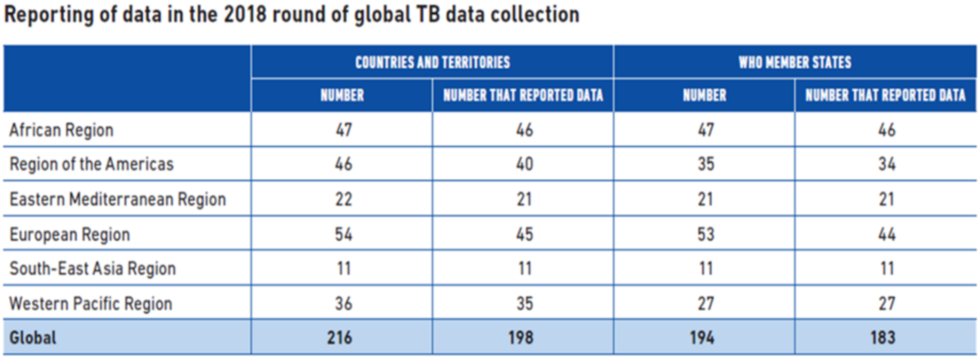
Even today after the development of advanced methods of critical examination, diagnosis and treatment for the disease, one-third of the world's population has been exposed to and is infected with the organism. Numbers are over 90% in the developing world. (Dye et al., 2005)(table 1).



ᵃ Figures are based on the most recent year for which data have been reported, which varies among countries. Data cover the period 2002–2018.

Figure 1.1Percentage of TB cases with MDR 2002-2018

Table 1.1. Estimated WHO Regional for 2018



Reporting of data in the 2018 round of global TB data collection

	COUNTRIES AND TERRITORIES		WHO MEMBER STATES	
	NUMBER	NUMBER THAT REPORTED DATA	NUMBER	NUMBER THAT REPORTED DATA
African Region	47	46	47	46
Region of the Americas	46	40	35	34
Eastern Mediterranean Region	22	21	21	21
European Region	54	45	53	44
South-East Asia Region	11	11	11	11
Western Pacific Region	36	35	27	27
Global	**216**	**198**	**194**	**183**

Aims

INH is one of the cornerstones of anti-tuberculosis treatment, as it exhibits mycobactericidal activity by inhibiting mycolic acid biosynthesis. A randomised clinical trial has reported on the benefits of adding high-dose INH systematically to a standard MDR-TB regimen. Recognition of INH resistance patterns and the frequency of katG and inhA mutations in different geographic areas may help to guide decision making about standardization of treatment regimens or individualized treatment, mainly in the case of MDR- or XDR-TB, at the same time in these settings the number of effective available drugs is limited. INH mutations varied geographically; molecular DST can be used to guide and accelerate our decision making in the use of low or high doses of INH (Clinical microbiology reviews July, 2003).

The question: Are the reported clinical and laboratory mutations the only viable options for INH resistance arising from mutation in inhA and katG or can novel mutations be isolated?

Objectives:

1. Broad objective: The systematic linking of isoniazid resistance data with genomic mutations.
2. Specific objective: the combination of inhA promoter region and katG mutations with the highest-level resistance.

2. LITERATURE REVIEW

2.1. Biology of the TB disease

Firstly Tuberculosis bacilli or the causative organism of tuberculosis were explained by Robert Koch in 1882. He proved that the only layer of protein in the body made it difficult to conceive earlier until a specific soiling called Zeihl Neelson's soiling has been discovered (Koch, 1882). Moreover, *Mycobacterial* species includes MTB, *M. bovis*, *M. africanum*, the latter being commonly found in immune compromised patients (Forrellad et al., 2012).

Mycobacterium spp. are a small aerobic non-motile bacillus with a protective waxy mycolic cell wall that protects it from adverse environmental condition (Veyron-Churletet al., 2010). When stained with auramine rhodamine (fluorochrome stains), and observed under microscopy, the mycolic acid accounts for its fluorescence characteristics, appearing yellow against a black background (Figure 2), which scientist have used as a diagnostic tool in the identification of mycobacterium in clinical specimens.

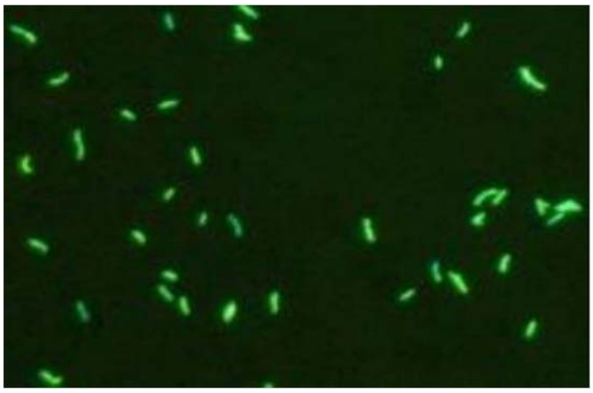
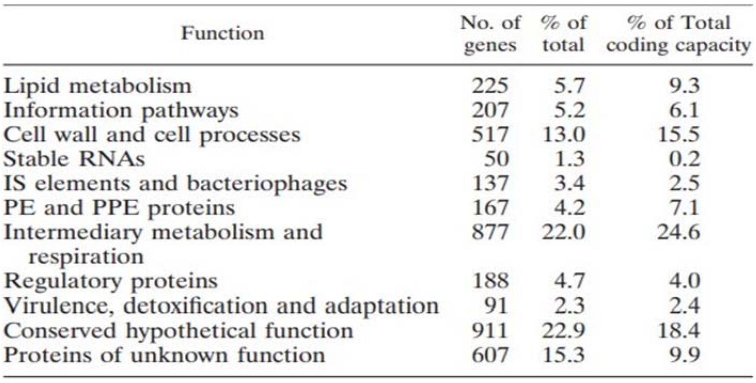


Figure 2.1. Fluorescence microscopy of *Mycobacterium tuberculosis* adapted from slide share acid fast staining in tuberculosis (Principles practices and application Dr.T.V Rao).

2.2. The *M. tuberculosis* genome

The DNA of *M. tuberculosis* has more than 99.9% identity with that of other members of the tubercle bacilli complex: *M. bovis,* the agent of bovine tuberculosis; *M. bovis* BCG, the vaccine strain derived from *M. bovis*; *M. africanum*, a pathogen of African origin; *M. canettii*, an atypical tuberculosis bacillus in humans and *M. microti*, responsible for tuberculosis in some rodents. Knowledge of the genetics of mycobacteria has advanced a lot in recent years thanks to the development of new tools (Pelicic et al., 1997, Braunstein et al., 2002) and decryption of the *M. tuberculosis* H37Rv genome sequence (Cole et al., 1998). As a result, and contrary to the situation encountered in the 1970s and 1980s, mycobacteria are among the most genetically characterized bacteria. Currently, we have the complete sequence of *M. tuberculosis* H37Rv (Cole et al., 1998). These genetic data (Figure 3) have, moreover, brought key elements in the understanding of the molecular mechanisms put into play by *M. tuberculosis* to infect the host but also in the protective effect of the BCG vaccine .

Table 2.1. General classification of *M.tuberculosis* genes (Nature 393:537–544)



Function	No. of genes	% of total	% of Total coding capacity
Lipid metabolism	225	5.7	9.3
Information pathways	207	5.2	6.1
Cell wall and cell processes	517	13.0	15.5
Stable RNAs	50	1.3	0.2
IS elements and bacteriophages	137	3.4	2.5
PE and PPE proteins	167	4.2	7.1
Intermediary metabolism and respiration	877	22.0	24.6
Regulatory proteins	188	4.7	4.0
Virulence, detoxification and adaptation	91	2.3	2.4
Conserved hypothetical function	911	22.9	18.4
Proteins of unknown function	607	15.3	9.9

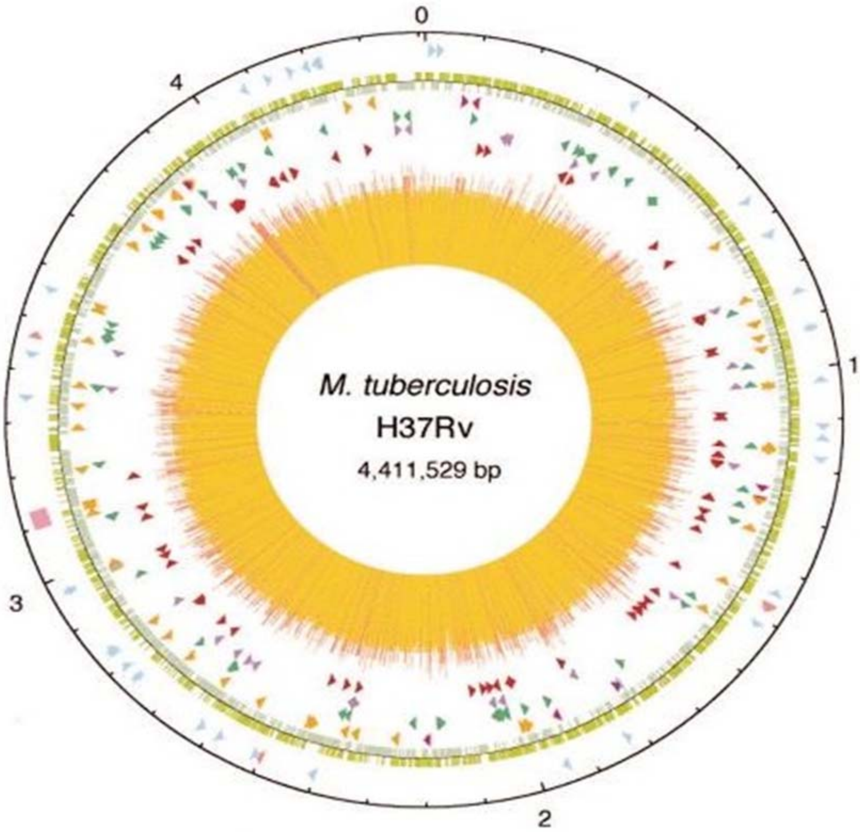


Figure 2.2. Circuler map of the chromosome of *M.tuberculosis* H37Rv (genolist.pasteur.fr)

In the case of *M. tuberculosis*, sequencing and systematic genome analysis of the H37Rv strain were undertaken in a collaborative work between the Institute Pasteur in Paris and the Sanger Center in Hinxton (Cambridge, Great Britain). Genome sequencing revealed that the *M. tuberculosis* chromosome is circular and contains 4,411,529 base pairs with a GC base percentage of approximately 65.6.

In addition, bioinformatic analysis led to the prediction that 3924 genes encode proteins. The function is partially or completely elucidated for respectively 20% and 40% of these genes, while no information could be obtained for the remaining 40% (Cole et al., 1998, Camus et al., 2002).

The genome analysis of *M. tuberculosis* has also identified several new previously unknown gene families, such as the genes encoding PE and EPP proteins, which occupy 10% of the genome. These proteins are characterized by typical proline-glutamic acid or proline-proline-glutamic acid units, located in the amino-terminal portion, and by central and carboxy-terminal regions repetitive, very rich in glycine for PE and in asparagine for PEP.

The function of these proteins is currently unknown, but their abundance suggests that they play an important role in the biology of M. tuberculosis (Brennan and Delogu, 2002). PE and EPP proteins are therefore considered as potentially interesting targets for the development of new therapies. International Center for Public Health, Newark, New Jersey 07103-3535).

2.2.1. Pathogenesis of pulmonary tuberculosis

To successfully cause disease, *Mycobacterium tuberculosis* must have a strategy on how to reside and multiply in the host, without recognition by host immune system in order to avoid elimination. But also, to survive, the *Mycobacterium* has armed itself with numerous immune evasion strategies (Figure 4). One such mechanism is the modulation of antigen presentation cells (APC), and preventing its interaction with the T helper cell (Korbel et al., 2008). Despite its ability to determine virulence, the mycolic acid is part of lipid *Mycobacterial* cell wall alongside cord factor and wax-D (Favrot & Ronning, 2012; Moody, 2009). they confer different survival mechanisms and enable the bacteria to cause disease. The cell wall too has hydrophilic property that resists antibiotics, alkaline and acidic compounds known to be injurious to the cell (Barkanet al., 2009).

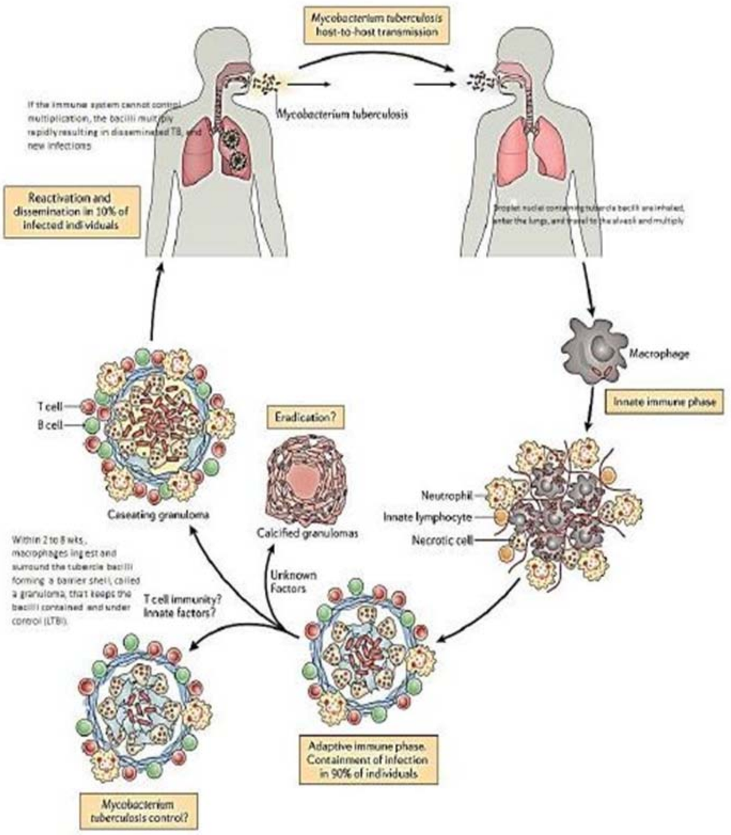


Figure 2.3. Pathogenesis of Pulmonary tuberculosis (Nature Reviews Microbiology)

2.3. M. Tuberculosis virulence factors

Unlike other pathogenic bacteria, *M. tuberculosis* does not have a classical virulence factor, toxin type. In humans, *M. tuberculosis* uses the respiratory tract as the main route of entry. It resists degradation by alveolar macrophages, in which it is able to multiply (Russell, 2001).

One of the crucial elements for the survival of *M. tuberculosis* in macrophage phagosomes is its ability to reduce acidification, which has the effect of blocking or delaying the fusion of the phagosome with the lysosome (Goren et al., 1976). Anes et al., 2003). At the molecular level, this phenomenon is not clearly elucidated, the determination of the mechanisms involved is one of the major issues of research in this area (Russell, 2001).

The answer immune system controls the severity of *M. tuberculosis* infection. This immune response involves the Th1 pathway, which recruits dendritic cells, macrophages, and CD4 and CD8-like lymphocytes (Kaufmann, 2001). The molecular basis of pathogenicity is therefore multifactorial, involving both the bacterium and the host.

2.4. TB Treatment

Antibiotics were used against tuberculosis for the first time in 1944 after the discovery of streptomycin. The use of this single agent has led to antibiotic resistance which is still a major problem.

Better results followed the development of PAS (Para-aminosalicyclic acid). PAS was a different streptomycin oral agent. Then more effective treatments like INH (isoniazid) came in 1950 and treatment with rifampicin followed. Currently, there are fewer than 20 agents with activity against mycobacteria.

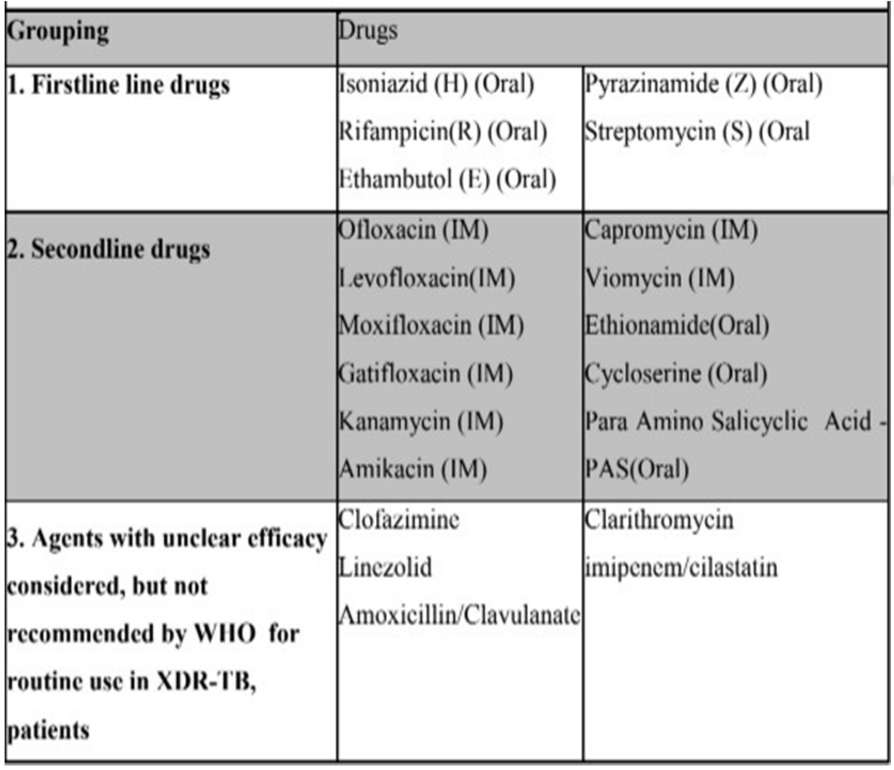
In effect for several decades, the former second-line treatment lasted 24 months and included an intensive 8-month phase with Kanamycin (KAN, Prothionamide (PTH), Ofloxacin (OFX), PZA and Cycloserine or EMB. The 16-month continuation phase was done with PZA, Levofloxacin (LFX), PTH, and para-amino-salicylic acid (PAS). Since May 2016, WHO has recommended the new therapeutic regime proposed by the International Union for the Fight against Tuberculosis. It is a short treatment lasting 9 months which includes an intensive 4-month phase with KAN, Clofazimine (CFZ), Moxifloxacin (MFX), EMB, a high dose of INH, PZA and PTH, followed by the continuation phase (5 months) with CFZ, MFX, EMB and PZA (www.who.int/tb).

Apart from the apparent duration of treatment, the two types of chemotherapy are distinguished from each other by their therapeutic performances on several aspects.

The previous 24-month treatment has favorable therapeutic outcomes not often exceeding 55% and is associated with more than 20% relapse /failure rates, and the selection of resistant mutant bacilli is much higher important . In contrast, the treatment recommended by the Union achieves a constant cure rate of more than 85%, it is associated with a very low relapse failure rate 3, 10, 137 compared to the old 24-month treatment.

In addition, this treatment would not contribute to the selection of mutant bacilli resistant to FQs and injectable ATBs: Amikacyne (AMK) -Kanamycin (KAN) -Capréomycin (CAP) in contrast to standard treatment of 24 months.

Table 2.2 Drugs recommended for management of pulmonary tuberculosis (From Tuberculosis chemotherapy, Joshi Jyotsna and modified with reference notes)



Grouping	Drugs	
1. Firstline line drugs	Isoniazid (H) (Oral) Rifampicin(R) (Oral) Ethambutol (E) (Oral)	Pyrazinamide (Z) (Oral) Streptomycin (S) (Oral
2. Secondline drugs	Ofloxacin (IM) Levofloxacin(IM) Moxifloxacin (IM) Gatifloxacin (IM) Kanamycin (IM) Amikacin (IM)	Capromycin (IM) Viomycin (IM) Ethionamide(Oral) Cycloserine (Oral) Para Amino Salicyclic Acid -PAS(Oral)
3. Agents with unclear efficacy considered, but not recommended by WHO for routine use in XDR-TB, patients	Clofazimine Linezolid Amoxicillin/Clavulanate	Clarithromycin imipenem/cilastatin

2.5. Tb drug resistance

The relationship between drug resistance in *M. tuberculosis* strains and their virulence and transmissibility has not been totally investigated. However, drug resistance in *Mycobacterium tuberculosis* arises from spontaneous chromosomal mutations at low frequencies required for antibiotic action (Somoskovi et al., 2001). To survive the micro-environmental stress, the bacteria acquire new genes, or delete certain genes (mutate) that expose them to harmful effects of the drugs, and thereafter produce off- springs that can proliferate in such adverse conditions set by drug stress (Zhang & Yew, 2009).

Multi Drug Resistance TB: *M. tuberculosis* that is resistant at least to INH and RMP.

Extensive Drug Resistance: *M. tuberculosis* resistant to ISONIAZID, rifampin, fluoroquinolones, and injectable agents. Spontaneous mutation within a crucial structural gene conferring mycobacteria the ability to thrive in a hostile drug environment is not without risk.

In fact, according to the genetic background of mutant cells (MDR or XDR), most mutations cause very heterogeneous energy cost "fitness costs" - generation time longer than normal (24-38 hours) in absence of ATB and lower multiplication in a population containing wild-type bacilli. The accumulation of mutations at the origin of the state of MDR and XDR could have resulted in a lower transmission of TB-MDR and XDR however, given the epidemiology of TB MDR and XDR in the world, it is clear that this is not the case. Indeed, *M. tuberculosis* mutant cells develop two types of adaptations that result in the decrease of the "fitness cost" of mutations associated with resistance. (Jason et al., 2008).

Molecular mechanisms of drug resistance have been demonstrated for the major first- and second-line drugs, including rifampicin, isoniazid, pyrazinamide, ethambutol, the injectable fluoroquinolone (Ofloxacin gatifloxacin levofloxacin), capreomycin, viomycin, and amino glycoside kanamycin amikacin (Velayati et al., 2009). As well as that anti TB drug isoniazid, for instance, is an essential drug used in TB treatment, and together with rifampicin can serve as prophylactic agents in latent TB cases (Surendra et al., 2014).

2.6. Isoniazid (INH)

INH is a bactericidal anti-TB drug that inhibits bacterial wall synthesis. Two mechanisms of action have been described: the first concerns the action of INH on the catalase-peroxidase enzyme encoded by the katG gene. The oxidation of INH by this enzyme results in the formation of an active metabolite leading to cell death. The second mechanism concerns the inhibition of the synthesis of the inhA protein encoded by the inhA gene. This protein plays an important role in the synthesis of mycolic acids, major components of the wall of mycobacteria. (Vilchèze and Jacobs, 2007)

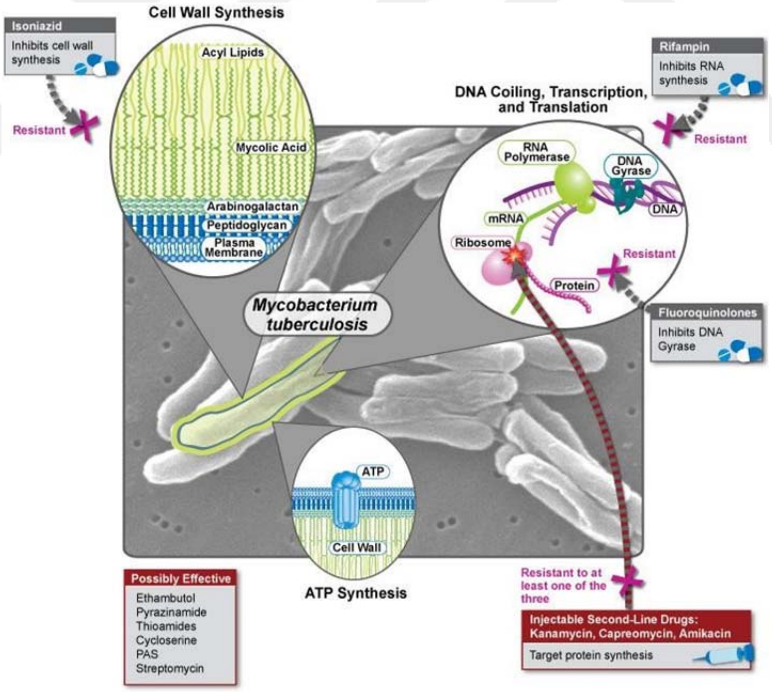


Figure 2.4. The action of drugs Isoniazid is a prodrug and must be activated by a bacterial catalase-peroxidase enzyme in *Mycobacterium tuberculosis* called KatG. (Wahab et al., 2009).

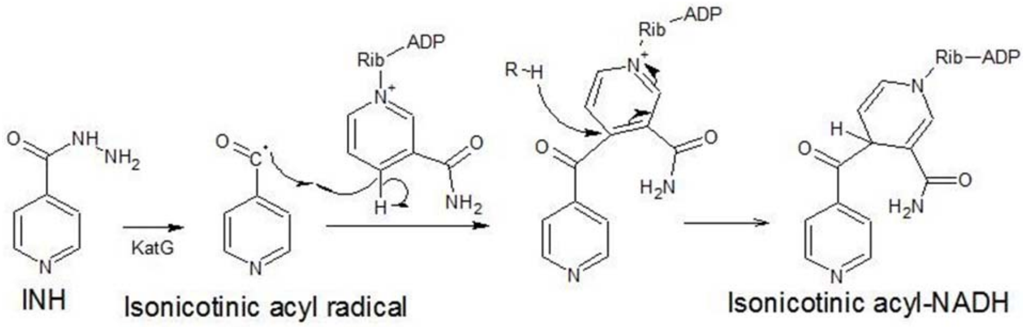


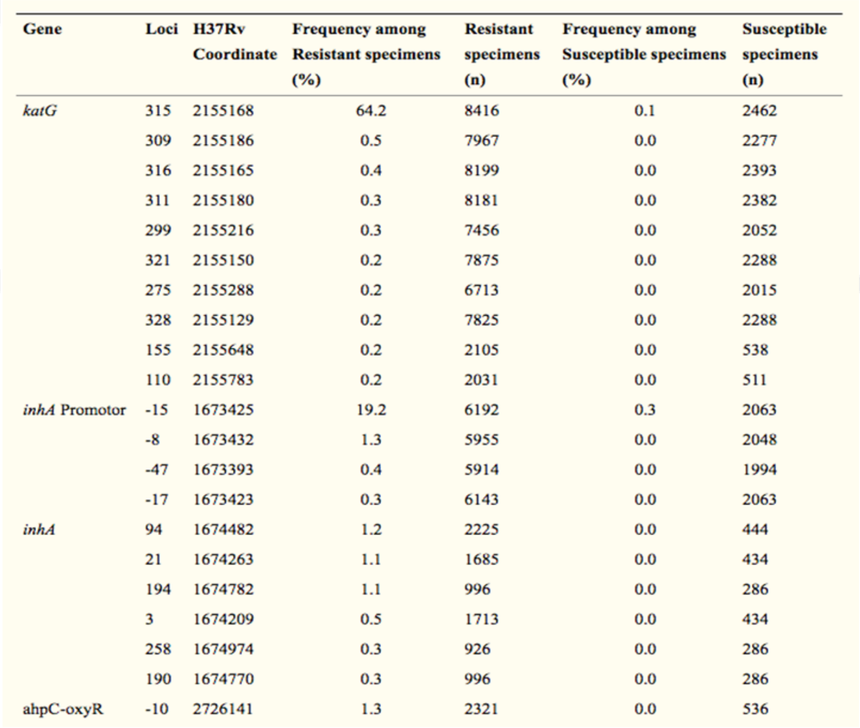
Figure 2.5. Mechanism of action for INH

Regrettably, within the first year of its use, resistance to INH was identified in clinical isolates (Steenken et al., 1952). INH resistance may arise through different genetic mutations commonly found in the katG, inhA and ahpC gene loci, the drug target sites (table 4). To be effective against the bacilli, INH requires activation by catalase peroxidase coded by the katG gene, mutation which has been reported to confer resistance to the drug (Isakova, 2008). But other studies have also reported resistance to INH due to mutation in bacterial inhA gene (Tsenget al., 2013).

In 2006, Oliveira explained firstly the mutations within inhA decrease the affinity of the protein for the NAD-INH adduct via either structural changes that alter the architecture of active site or mutations within the active site itself (Oliveira et al, 2006) and secondly the lack of katG gene consequently represses gene code for catalase and peroxidase release, and this result in inactivation of the pro-drug, with limited toxic effect.

Adjacent to the katG gene locus of the mycobacterial cell is ahpC gene locus that is responsible for production of alkylhydroperoxidase. Also this enzyme is necessary for protecting the cell wall against the oxidative stress exerted by catalase, and as well as other environmental stress produced by the host immune defense, particularly phagocytic cells (macrophages) (Flynn, 2013). In the same time missense reading or deletion of katG gene further leads to over expression of alkylhydroperoxidase, which protects organisms from destructive effects of the drug on the cell wall fatty acid (Catherineetal. 2007).

Table 2.3 Common mutations of drug resistance



Gene	**Loci**	**H37Rv Coordinate**	**Frequency among Resistant specimens (%)**	**Resistant specimens (n)**	**Frequency among Susceptible specimens (%)**	**Susceptible specimens (n)**
katG	315	2155168	64.2	8416	0.1	2462
	309	2155186	0.5	7967	0.0	2277
	316	2155165	0.4	8199	0.0	2393
	311	2155180	0.3	8181	0.0	2382
	299	2155216	0.3	7456	0.0	2052
	321	2155150	0.2	7875	0.0	2288
	275	2155288	0.2	6713	0.0	2015
	328	2155129	0.2	7825	0.0	2288
	155	2155648	0.2	2105	0.0	538
	110	2155783	0.2	2031	0.0	511
inhA Promotor	-15	1673425	19.2	6192	0.3	2063
	-8	1673432	1.3	5955	0.0	2048
	-47	1673393	0.4	5914	0.0	1994
	-17	1673423	0.3	6143	0.0	2063
inhA	94	1674482	1.2	2225	0.0	444
	21	1674263	1.1	1685	0.0	434
	194	1674782	1.1	996	0.0	286
	3	1674209	0.5	1713	0.0	434
	258	1674974	0.3	926	0.0	286
	190	1674770	0.3	996	0.0	286
ahpC-oxyR	-10	2726141	1.3	2321	0.0	536

What is interesting that the resistance to INH is predominantly associated with the amino acid substitution; Ser315Thr in katG (in roughly 70% of INH-resistant strains) and the −15 C-to-T mutation in the inhA promoter (in 15 to 35% of INH-resistant strains) (Hiroki Ando et al 2010).

3. MATERIALS AND METHODS

3.1. Materials

Figure 3.1. Displayed BACTEC MGIT 960 system

3.1.1. The MGIT system

For more than 14 years, BD BACTEC ™ MGIT ™ 960 has become the reference technology in France and worldwide for the detection and antibiogram of mycobacteria1, offering:

- ✓ A system specifically dedicated to mycobacteria and fully automated.
- ✓ A detection in liquid medium and antibiogram on the same automaton.
- ✓ Automated 1st and 2nd level antibiograms

- ✓ Reliable and fast results thanks to early detection of growth.
- ✓ Simplicity of use.
- ✓ Safety thanks to the use of plastic tubes with screw caps.

Principle of detection :

The BD BACTEC ™ MGIT ™ instrument uses non-radiometric, fluorescence-based technology. The microorganisms present in the samples metabolize the nutrients and oxygen present in the culture. The tubes contain a fluorescent detector that reacts according to the oxygen concentration of the culture medium. The photosensitive detectors of the instrument measure the intensity of the fluorescence, which corresponds to the quantity of oxygen consumed by the micro-organisms.

Detection by the instrument of microorganisms growing in the culture medium is the result of reading these fluorescence measurements.

BD BACTEC ™ MGIT ™ technology is designed to reduce the time needed between receiving a sample and getting results. (the Global Tuberculosis Community Advisory Board).

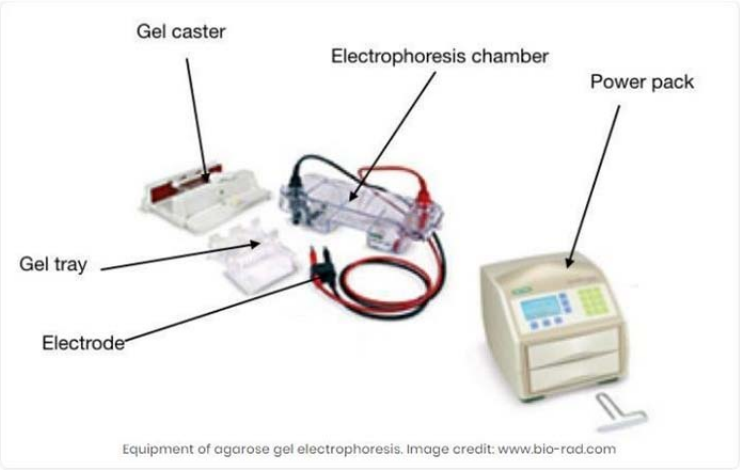
Detecting the susceptibility of *Mycobacterium tuberculosis* to streptomycin, isoniazid, rifampicin, pyrazinamide and ethambutol from solid or liquid culture. The test is based on the comparison of the growth of *Mycobacterium tuberculosis* in a tube containing an antibiotic and that obtained in an antibiotic-free tube by means of algorithms interpreted by the BD BACTEC ™ MGIT ™ 3 system (the Global Tuberculosis Community Advisory Board).

3.1.2. PCR Thermocyclers

Figure 3.2. Thermocyclers

The Applied Biosystems StepOne ™ real-time PCR system (StepOne ™ system) is based on the principle of polymerase chain reaction (PCR).
PCR reagents based on the principle of fluorescence to ensure:

- ✓ Quantitative detection of target nucleic acid sequences (targets) by real-time analysis.
- ✓ Qualitative detection of target nucleic acid sequences (targets) through end-point analysis and fusion curve analysis(biocompare.com).



Equipment of agarose gel electrophoresis. Image credit: www.bio-rad.com

Figure 3.3. Agarose electrophoresis

3.1.3. Agarose gel electrophoresis

Agarose electrophoresis is an important technique in genomic research. It separates biological molecules based on their size and charge. Influence of the negatively charged DNA molecules migrate towards the positive charge under the constant current, also the separation depends on the mass and charge of DNA. The DNA molecules are forced to move through the agarose gel pores (geneticeducation.co.in).

3.1.4. ABI Prism 3730 automated DNA sequencer (ABI Prism)

It uses a capillary electrophoresis system that allows a high speed of migration (obtaining 750bases in 2h30) and a good automation (200 sequences in 30 hours without human intervention).

An articulated plate called autosampler supports the 96-well plates containing the purified sequence reactions, the buffer and water reservoirs required respectively for the electrophoretic migration, rinsing and disposal of waste. The plate moves in two dimensions (x, y) and thus ensures the transfer of the sixteen capillaries between the different constituents.

Figure 3.4 ABI Prism 3730 automated DNA sequencer

3.2. Methods

3.2.1. Sample collection

To investigate katG and inhA genes mutations in *M. tuberculosis*, and identify the characteristics and the distribution of mutations, *M. tuberculosis* clinical isolates including INH-resistance strains were selected. Strains were randomly selected among the MDR tuberculosis isolates which found phenotypically drug resistance tests were performed at the Cukurova University Tropical Disease Research and Application Center, Adana Regional Tuberculosis Laboratory.

3.2.2Drug susceptibility testing

All clinical isolates, H37Rv were tested for drug susceptibility. Strains were analyzed by MGIT 960 Based Antimicrobial Testing (Becton, Dickinson and Company). The medium contained INH (0.4g/ml). Resistance is expressed as the percentage of colonies that grow on critical concentrations of the substances. The interpretation will be based on the usual criteria for resistance.

3.2.3. DNA sample preparation, extraction from bacterial cells:

The *M. tuberculosis* strains were cultured on the MGIT system medium at 37°C constant temperature incubator for 3 to 4 weeks.

1. 500 (µl) of The bacterial suspension were collected from the liquid culture and subsequently heating at 80°C to 100°C for 30 minutes to kill and lyse the cells (van der Zanden et al., 2002).
2. the mixture was centrifuged for 15 min at 15,000 × g .
3. 0.5ml of 1xTE buffer was added.
4. the mixture was centrifuged for 15 min at 15,000 × g
5. 0.25 ml of 1xTE buffer was added.
6. 50-100µl of Glass beads (SIGMA acid washed) were added to the tube.
7. 2 minutes in the Mickle tissue disintegrator.
8. In the end the aqueous supernatant was carefully transferred to a fresh tube. The lysates were stored at -20°C until further use.

3.2.4. PCR amplification:

5 microliters (µl) of the lysates obtained from the cultured M. tuberculosis strains were added to 12.5µl of PCR mixture which included 1.0µl dNTP (1mM), 1.5µl 10×Buffer, 3.0µl Betain (5M), 0.9µl MgCl2 (1.5mM), 0.25µl primer L (5µM), 0.25µl primer R (5µM), 0.3µl DNA polymerase (3U), and µl distilled water, with a final 25 µl total volume.

The primers are :

No.	Gene	Primer sequence (5′→3′)	Amplicon size (bp)
1	furA	5′-GCTCATCGGAACATACGAAG-3′	129
2	katG	5′-TGCTGCGGCGGGTTGTGGTTGATCGGCGG-3′	50
3	fabG1	5′-TTCGTAGGGCGTCAATACAC-3′	200
4	inhA	5′-CCGAACGACAGCAGCAGGAC-3′	40

PCR amplification process:

katG

5 minutes at 95°C for DNA denaturation; 35 cycles of:
1 minute at 94°C for DNA denaturation,
1 minute at 56°C for primer annealing
1 minute at 72°C for primer extension; followed by a last extension cycle of 10 minutes at 72°C.

inhA

15 minutes at 95°C for DNA denaturation; 40 cycles of:30 second at 98°C for DNA denaturation, 1 minute at 54°C for primer annealing 30 second at 72°C for primer extension;

furA-katG

5 minutes at 95°C for DNA denaturation; 35 cycles of:
1 minute at 94°C for DNA denaturation, 1 minute at 56°C for primer annealing
1 minute at 72°C for primer extension; followed by a last extension cycle of 10 minutes at 72°C.

fabG1-inhA

15 minutes at 95°C for DNA denaturation; 40 cycles of: 30 second at 98°C for DNA denaturation, 1 minute at 54°C for primer annealing 30 second at 72°C for primer extension;

PCR products were analyzed by 2% agarose gel electrophoresis at 120 V for 40 min (15-cm gel) in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), A 100bp DNA ladder was used as size markers and the results were recorded photographically. Next there were kept at 4°C until further analysis.

3.2.5. DNA sequencing

PCR products were used as templates for direct DNA sequencing. DNA sequences were compared with the H37Rv sequence using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan).

3.2.5.1. Purification by ExoSAP®

ExoSAP-IT™ PCR Product Cleanup Reagent is used for enzymatic cleanup of amplified PCR product. It contains two hydrolytic enzymes, exonuclease I (Exo) which degrades single-stranded DNAs and shrimp alkaline phosphatase (SAP) which hydrolyzes free dNTPs, and in excess without

interference with downstream applications. The enzyme is active at 37 ° C and inactive at 80 ° C. Single strand fragments less than 100 bp are thus degraded.
5µL of PCR product were mixed with 2µL of ExoSAP in 0.2mL PCR tubes. Next Incubation of the mixture in the PCR machine with the program as following: 37°C for 30min to allow the enzyme to work, 80°C for 15min to denature the enzyme, and a final step of 10°C until to take the tube out of the machine.

3.2.5.2. Run the sequencing reaction by bigdye® Terminator v 3. 1 cycle sequencing

According to Sanger's reaction based on the random incorporation of dideoxynucleotide chain switches (ddNTP) also present in the reaction medium, each of which is labeled with a fluorophore whose emission spectrum is specific. A spectral analysis will differentiate the different fluorochromes, associate the corresponding base and thus define the nucleotide sequence of the initial DNA strand. The synthesized DNA fragments carry this terminal fluorophore. They are called elongation terminators or"BigDyeTerminators" or "Dye-labeled terminator" (Faculty of Medicine And Pharmacy Fees Thesis N067/15). The BigDye Terminator (BDT) technology uses a resonance energy transfer system (FRET) between two fluorochromes attached to the same ddNTP and linked together. The first is a fluorescein (6 carboxyfluoresin) called fluorochrome donor, common to all four ddNTPs. The second is a dichlororhodamine (dRhodamine) which plays the role of fluorochrome acceptor (Figure 10). The donor fluorochrome is excited by an argon laser beam emitting at 488 nm and 514.5 nm. Its fluorescence energy emitted (515- 520 nm) is captured integrally by the acceptor fluorochrome which is excited in turn. The acceptor fluorochrome or dichlororhodamine is different for each type of ddNTP.

The spectrum of fluorescence emitted will be specific to each type of ddNTPs (Faculty Of Medicine And Pharmacy Fees Thesis N067/15).

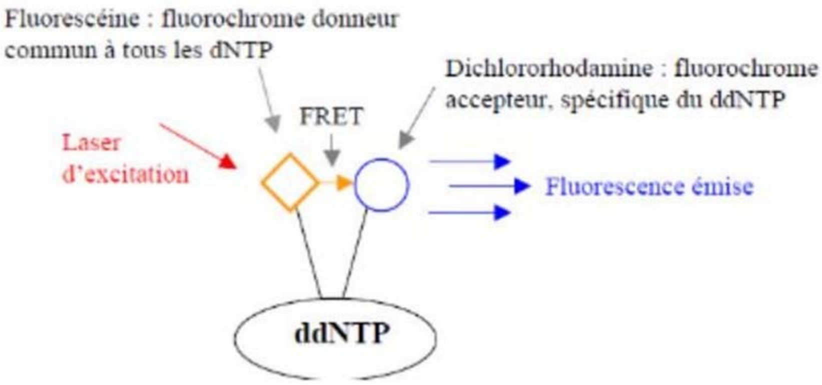


Figure 3.5 Mechanism of fluorescence by the technique of resonance energy transfer.

PCR the following:

-5 L purified PCR product

-1.0 µl of the diluted sequencing primer (whichever one we used in the above PCR reaction)

-4.0 uL of the BigDye Ready Reaction Termination Mix

30 cycles of amplification are carried out on the thermocycler under conditions specific to the sequence reaction. A cycle corresponds to:

-A step of denaturing the DNA at 95° C for 10 seconds to obtain the DNA in single-stranded form.

- A hybridization step at 50° C. for 5 seconds (average hybridization temperature for the majority of the primers used).

- A step of elongation of the DNA with Taq polymerase at 60° C for 4 minutes. This low temperature slows the Taq and will shift the balance to allow a better incorporation of ddNTP. Once the thermocycling step has been performed, it is necessary to purify the sequence reaction.

3.2.5.3. Cleaning PCR products by sephadex spin-column

This step is intended to purify by gel filtration the sequence reaction products. The Sephadex® G50 resin used makes it possible to de-salt the samples and eliminate unincorporated nucleotides and excess PCR primers. The exclusion limit of the G50 resin is about 20 bases. The 96-well MultiScreen HV is used as an inert carrier for G50 resin. To prepare new sephadex: 5 g of sephadex G-50 powder was added to 75 ml of ddH2O, hydration of the sephadex solution for at least 45 min before using.

1. Empty spin columns were placed in old 1.5 ml collecting tubes.
2. Sephadex solution was swirled to suspend. Next each column was filled with 800 µl of sephadex sol.
3. The water was allowed to drain (~5 min) from columns until sephadex becomes visible.
4. While columns are draining, 1.5 ml eppendorf tubes were labeled.
5. Centrifuge collecting tubes with columnswere centrifuged inside at 3,800 rpm for 2 min.
6. Column was carefully removed and placed them in labeled eppendorf tubes.
7. PCR sample was transferred carefully to the appropriate column.
8. Collection tubes were placed with colums inside in the centrifuge so that the hinged lid of the tube faces the center of the rotor and the plastic nub of the column faces out, next Centrifugation at 3.8 rpm for 2 min.
9. Columns and collection tubes can be reused after cleaning by spraying the inside vigorously with distilled water or boiling them for a few min using a microwave.

3.2.5.4. Determination of the sequence

The plate is placed in the sequencer which is an automaton capillary electrophoresis. The latter initiates an electrical flow of ions through a capillary, which causes the migration of DNA fragments. Once at the detection site, the four fluorochromes of the terminal ddNTPs will be excited. Following this excitation, each fluorochrome will emit light at a different wavelength, which will be detected and converted into sequence by the analysis software (Figure11) Faculty of Medicine And Pharmacy Fees Thesis N067/15).

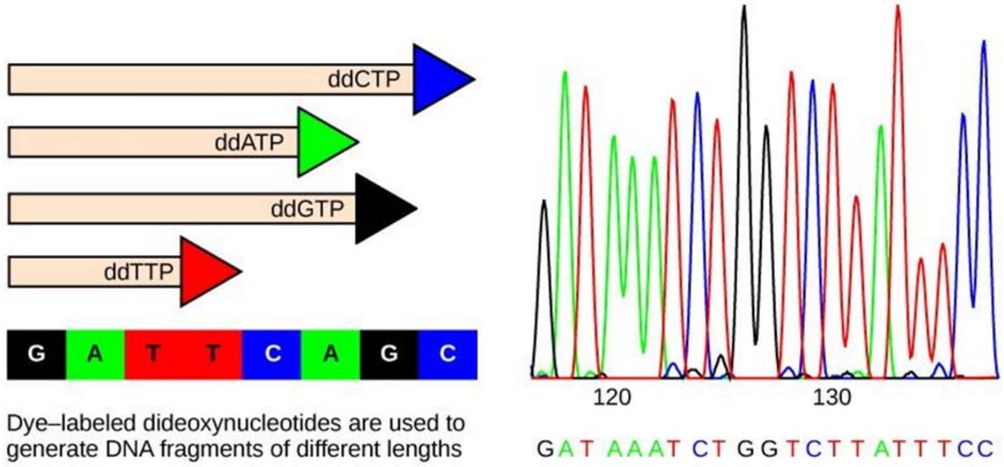


Figure 3.6 Frederick Sanger’s dideoxy chain termination method (lumen learning)

4. RESULT

The findings of the study were organized in various sections to outline the numerical values, correlations between the demographic and clinical data with the resistance patterns of Mycobacterium tuberculosis (MTB), for the first line drugs anti-tuberculosis isoniazid.

4.1. Susceptibility testing

As shown in Table 5 among 91 Inh^r isolates, 65 (70%) were to susceptible INH at 0.4 µg/ml , the remaining 26 (30%) were resistant to INH at 0.4 µg/ml. Among the 91 isolates, 33 were resistant to INH but susceptible to other antituberculosis drugs. 42 were multidrug-resistant (MDR) isolates and five 16 were extensively drug resistant (XDR).

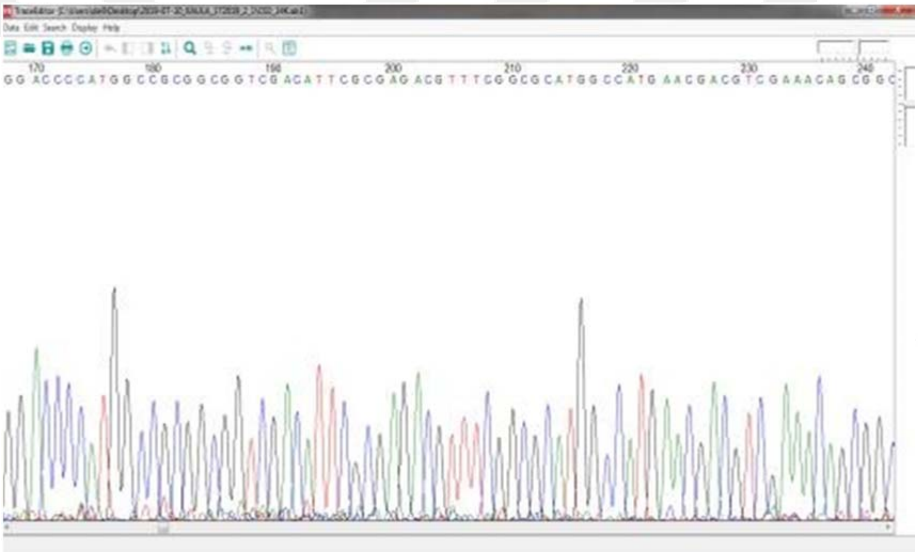
Table4.1. Drug susceptibility profiles

Strain	**Susceptibility pattern**	**No. of isolates**	**INH (0.4)** Resistant	**INH (0.4)** Susceptible
M. tuberculosis	Inh^r	24	7	17
	Inh^r , Rif^r	20	3	17
	Inh^r , Rif^r, Emb^r	18	8	10
	Inh^r, Emb^r,	3	1	2
	Inh^r, Str^r, Rif^r	4	2	2
	Inh^r, Str^r	4	1	3
	Inh^r, Str^r, Emb^r,	2	1	1
	XDR/ totally drug resistant	16	3	13

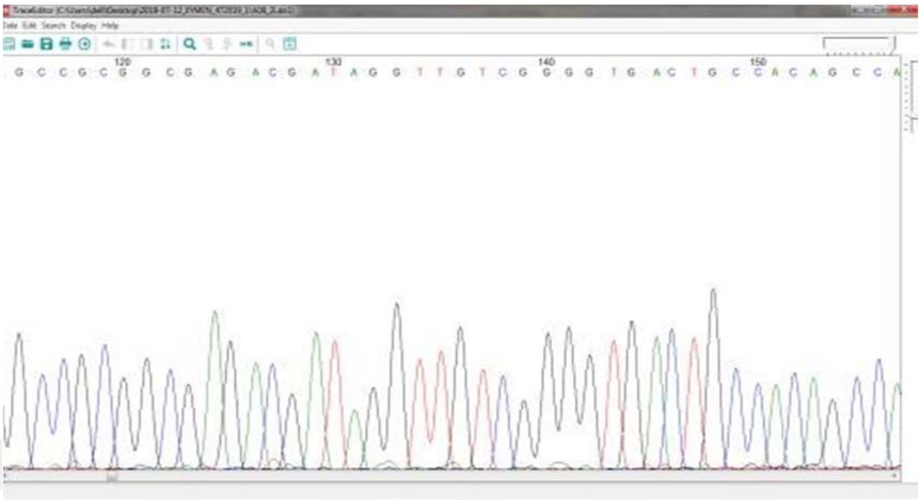
4.2. Electrophoretic mobility of the amplification products

Amplification products from PCR of *M. tuberculosis* H37Rv were separated by electrophoresis.

The resulting DNA sequences were analyzed using the basic local alignment search tool (http://www.ncbi.nih.gov/BLAST), the H37Rv genome sequence and the computer software Molecular Evolutionary Genetics Analysis (MEGA) for conducting statistical analysis of molecular evolution (figure 16). (http://genolist.pasteur.fr/TubercuList/)

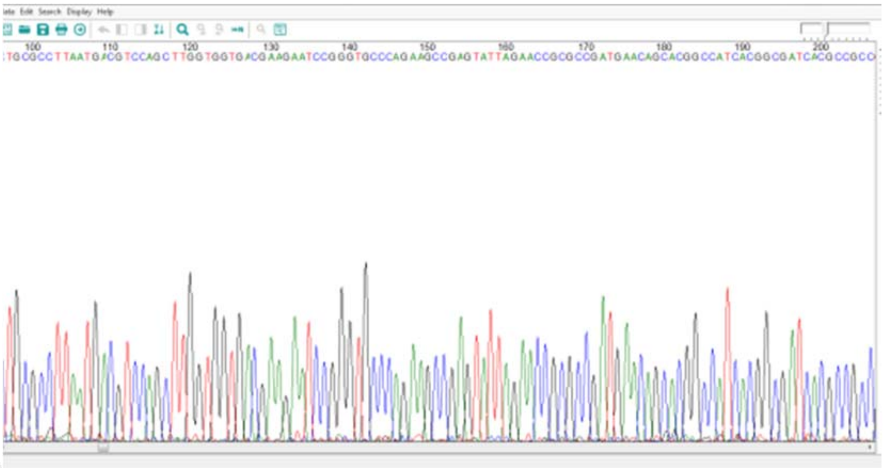


katG



inhA

furA-katG



fabG1-inhA

Figure 4.1The four fluorochromes of the terminal ddNTPs

Table4.2. Gene Mutations

Genes	Mutations	
	Nucleotide	Amino acid
katG	Deletion G28/58A/33C	Lys 48 gln Glu49 gly Ser403 thr
	C461G	glu49 gly lys48 gln ser403 thr
	Deletion C25	ser 388 thr pro 225 thr
	Deletion A51	lys 48 gln ser 403 thr pro 240 thr
	Deletion C29	phe 457 trp ser 392 thr
	Deletion C27	pro 226 thr
	Insertion G 465 Deletion C 29	pro 228 thr
	Delection C 29	glu 471 lys pro 371 gln
	Deletion G23 Deletion C28	ser 390 thr pro 227thr * phe 457 trp
	C335G	Met 213 thr Leu 371 val
	Deletion C21 Insertion G467	ser 386 thr phe 457 trp
	Deletion C25	ile 388 ser
	G392C	ser 389thr
	Deletion C25 Insertion C443	ser 389 thr
	Insertion A 222	Val145Leu
	insertionA220 G224A	Val221Leu

furA-katG	Deletion A83 /125T	ala 154 gly thr 167 pro thr 168pro lys 169 ser leu 170 trp asp 171 thr val 172 ser ile 173 leu lys 174 arg gln 176arg ala 177 pro
	Insertion A212 G215A	Val212Leu thr56 gly thr 55 cys glu 54 gly
	Insertion A 216 G219A	Val 216 Leu thr 270 gly glu 268 trp
	Insertion A 223	Val 220Leu glu 62 gly thr 63 cys thr 64 gly
	Insertion A 221 G224A	Thr 270 gly Val221Leu
	Insertion A218 G221A	Val 218 Leu
inhA	C126T	Met151 val thr 137 met
	Deletion A 64	Met153val
	C122T	Met153val
	Deletion A24A25 A40A54A62	Ala 166 val Gly 167 cys val 169 ser
	Insertion A 43	Met 50 val
fabG1-inhA	C126T	Met51 val thr 137 met
	Deletion A 29 Deletion A 68	Met 51 val
	Deletion A25 Deletion A 33	Met153 val thr 140 met

5. DISCUSSION

This study is the first study to be conducted in Turkey in such a comprehensive manner according to the literature data, in the same time to provide insight into the molecular basis of drug resistance and distrubition of the wild strains circulating in a high prevalent setting.

Mycobacterium tuberculosis isolates from multiple drug resistant patients show a particular set of mutations in drug targets conferring resistance.

91 *of M. tuberculosis* clinical isolates from Cukurova University Tropical Disease Research and Application Center, Adana Regional Tuberculosis Laboratory , including INH-resistance strains were selected to investigate the mutations in the *katG ,inhA* genes fragment including almost every mutation sites previously published was amplified by PCR and the mutations in these genes fragments were detected by DNA sequencing. Based on the sequencing results, the mutations were analyzed.

We recently reported 5 novel mutations in katG (T. Mori et T. Kirikae**.** 2007). Including these mutations, 280 different mutations in katG were found in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) when articles were searched by the keywords ‘katG’, ‘mutation’, and ‘tuberculosis’. Also, in 2003, 6 mutations in the upstream region of the fabG1-inhA operon, including C−15T, and seven in inhA cause INH resistance were funded by Riccardi et al **(**Riccardi, and D. Ghisotti. 2003).

In our study, we found an additional 26 novel mutations in katG. Two was a deletion C, insertion G, while the other 24 caused amino acid substitutions. These mutations and amino acid substitutions are shown in Table 17. Furthermore, several novel mutations were detected in the present study: one in fabG1 (Deletion A, C-T]), one in inhA (C126T, deletion A), and three in the intergenic region of the furA-katG operon (G−226A, insertion A, and G224A).

Apparently, elsewhere that these mutations in furA and the intergenic region are associated with INH resistance induced by downregulation of katG expression (H. Ando and T. Kirikae, unpublished results), and those in fabG1 are also associated with INH resistance induced by upregulation of inhA expression (Ando et al., unpublished).

In a recent multinational study, katG (Ser315Thr) was identified as the harbinger mutation that is most frequently associated with future MDR occurrence and risks subsequent clonal spread (Manson AL, Nature genetics. 2017).

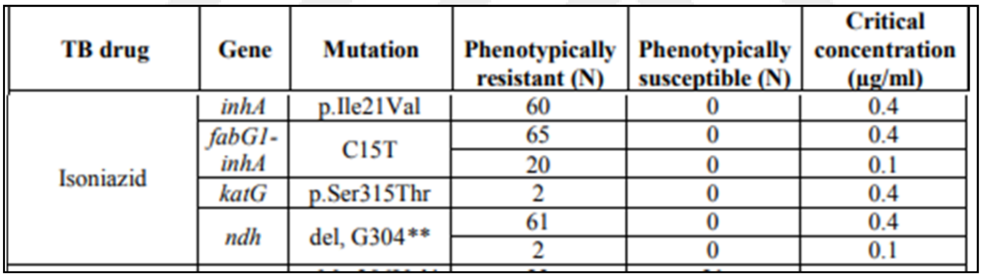
A first point, the prevalences of the KatG (S315T) mutation in *M. tuberculosis* strains from around the world differ, especially with regard to the prevalence of tuberculosis.

In regions where the prevalence of tuberculosis is low or intermediate, the mutation has been reported relatively infrequently: it occurred in 26% to 30% of 95 isolates from Singapore (Lee, A. S.et al 1999) and Madrid (Piatek et al 2000) and rarely in isolates from Scotland (Fang et al 1999) and Finland (Marttilaet al 1996**).** Dissimilarity the S315T mutation accounted for INH resistance in 52% to 64% of strains in Africa (Victor et al 1996), 79% in Peru (Escalante et al 1998), 91% in Russia (Marttila et al 1998), and 58% in New York (Piatek et al 2000).

In the second place, Gagneux et al (2006) reported that the strains carrying the katG (S315T) or inhA promoter mutation were more likely to spread than those carrying other mutations; our results were consistent with these previous findings. Also strains with fabG1 (G609A) (L203L) and furA (C41T) (A14V) mutations were also more likely to spread in Japan.

Some studies demonstrated that the S315 mutation is known to confer high-level INH resistance **(**Pym et al 2002**,** Saint-Joanis et al 1999**,** Wengenack et al 1997). Next, katG is a functional homodimer, and each monomer is composed of two domains that are mainly α-helical. The N-terminal domain contains a heme binding site, whereas the C-terminal domain lacks this feature (Wilming et al 2001). The high-level INH resistance-associated mutations D419H and M420T are located in the region connecting the N-terminal and C-terminal domains .In addition the interdomain interactions between the N-terminal and C-terminal domains of the two monomers are essential for forming the functional homodimer (Bertrand et al 2004**).** So the changes in the interdomain interactions due to the D419H and M420T mutations may result in loss of enzymatic activities of katG.

Other result, In 2018, from The University of Queensland Australia , Beijing strains (table 7) possessing signature fabG1-inhA (C-15T) mutation that confers low-level isoniazid resistance with ethionamide cross-resistance has been observed in South Africa (Marais BJ Journal of clinical microbiology 2006, BMC genomics. 2010 (table 7). From another country, from Portugal, another strain (LAM family) had cross-resistance to high level isoniazid and ethionamide resistance which was attributed to the double inhA mutations (C-15T and Ile21Val) (Machado D, The Journal of antimicrobial chemotherapy. 2013).



TB drug	**Gene**	**Mutation**	**Phenotypically resistant (N)**	**Phenotypically susceptible (N)**	**Critical concentration (µg/ml)**
Isoniazid	*inhA*	p.Ile21Val	60	0	0.4
	fabG1-inhA	C15T	65	0	0.4
			20	0	0.1
	katG	p.Ser315Thr	2	0	0.4
	ndh	del, G304**	61	0	0.4
			2	0	0.1

Figure 4.2 Drug resistance mutations and phenotypic drug resistance observed among Beijing sublineage strains 2018

In 2006 the double accumulation of mutations which were reported by PLoS computational biology may confer high-level isoniazid resistance without the fitness cost associated with katG mutations, supporting successful clonal expansion

and acquisition of additional resistance conferring mutations to other drugs (PLoS computational biology,2016).

Several recent reports have examined relative frequencies of katG and inhA promoter mutations in INH-resistant isolates and have shown considerable geographic variation, with 0.8% frequency of inhA (without katG) mutation in

Ethiopia [13], 4% in Poland [14], 9.6% in Chonqing, China [15], and 22% in the Philippines [16]. In Saudi Arabia, a study of isolates from 9 regional centers found that inhA mutations were more common in Southeast Asian immigrants compared to both the autochthonous population as well as African immigrants [17]. A study from the Western and Eastern Capes of South Africa found that nearly 40% of MDR-TB isolates carried an inhA promoter mutation without concurrent katG mutation [18], but there is considerable genetic diversity of TB throughout South Africa and this precludes extrapolating results between provinces [19].

The frequency of inhA promoter mutations increased as the overall drug-resistance of the isolate increased, from MDR-TB to pre-XDR-TB to XDR-TB, but this could be due to increased clonality from transmitted strains. We have previously shown that TB strain diversity (as measured by spoligotyping) decreases with increasing drug-resistance in KwaZulu-Natal [20].

This study, through the systematic linking of isoniazid resistance level with genomic mutations, suggests that the combination of well-characterized, easily detectable mutations can reliably predict excessively high resistance of M. tuberculosis to isoniazid.

We found that the combination of inhA promoter region and katG mutations is associated with the high-level resistance, exceeding peak level concentrations of the drug even at the highest doses in clinical use.

Our results are largely in agreement with the associations between known resistance-conferring mutations and the level of phenotypic resistance described in the literature, typically tested at lower concentrations.

Moreover, further research is required to investigate whether high-dose isoniazid indeed contributes to successful outcome in patients with low- or moderate-level resistance, caused by a katG or inhA mutation alone.

Suggestions;

- INH is one of the cornerstones of anti-tuberculosis treatment, as it exhibits mycobactericidal activity by inhibiting mycolic acid biosynthesis
- A randomised clinical trial has reported on the benefits of adding high-dose INH systematically to a standard MDR-TB regimen.
- Recognition of INH resistance patterns and the frequency of katG and inhA mutations in different geographic areas may help to guide decision making about standardisation of treatment regimens or individualised treatment, mainly in the case of MDR- or XDR-TB, as in these settings the number of effective available drugs is limited.
- INH mutations varied geographically; molecular DST can be used to guide and accelerate decision making in the use of low or high doses of INH.

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