CUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Genomic Mutations in *rpoB, katG,* and *inhA* Genes Among Multi-Drug Resistant *Mycobacterium tuberculosis* Complex Strains Isolated from Cukurova Region

Omer Ali Namiq AL-JAF

Biotechnology Department

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This Doctorate Thesis was evaluated by the following Jury Members on 27/03/2024 and was approved by unanimity of votes.

Jury : Prof. Dr. Sadık DİNÇER (Advisor) …………

: Prof. Dr. Burhan ARIKAN …………

: Prof. Dr. Mutlu Nisa ÜNALDI CORAL …………

: Prof. Dr. Gökhan CORAL …………

: Assoc. Prof. Esin Gülnaz CANLI …………

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Thesis Number:

Prof. Dr. Sadık DİNÇER
Director
Institute of Natural and Applied Sciences

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Advisor: Prof. Dr. Sadık DİNÇER

Department of biotechnology

ABSTRACT

Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), which is resistant to at least rifampicin (RIF) and isoniazid (INH), is a major threat to public health due to the greater complexity, cost, and side effects of treatment. Resistance to RIF and INH generally occurs by mutations in the *rpoB* and *KatG* genes, the *inhA* promoter, and the *oxyR-ahpC* intergenic regions. The development of modern genomics provides an effective method to understand the molecular mechanism of drug resistance and diagnose drug-resistant *M. tuberculosis*. We used whole-genome sequencing (WGS) to find changes in 4 genes or intergenic regions in 70 clinical *M. tuberculosis* strains from the Cukurova Region of Turkey. The strains included 40 MDR-TB and 30 pan-susceptible isolates. We then looked at how these changes were related to resistance to RIF and INH. The results show that, compared to the phenotypic proportional method, the sensitivity and specificity of WGS for resistance detection were 77.5 and 100.0% for RIF (based on mutations in *rpoB*), 80% and 100%, 30% and 100%, and 12.5% and 93.3% for INH (based on mutations in *katG*, *inhA*, and *oxyR-ahpC*, respectively). WGS data also showed that mutations in the *inhA* promoter increased only 5% sensitivity for INH based on mutations in *katG*. Also, mutations in the *oxyR-ahpC* intergenic regions decreased by 6.3% specificity for INH based on mutations in the *katG* gene and *inhA* promotor. The codons S450L (52.5%), H445T (7.5%), A435V (7.5%), L430P (5%), L430L (2.5%), and 494 (2.5%) of RIF-resistant isolates exhibited the highest rate of mutation within the *rpoB* gene. The parts of the *katG* gene that changed the most were codons S315T (52.5%), S315A (2.5%), G316S (7.5%), L336P (5%), S309T (5%), T328C (2.5%), A311P (2.5%), and in one strain, both T328C, and S315A (2.5%); the positions C-15T (25%), G-47C (2.5%), and G-64C (2.5%) of the *inhA* promoter; and the positions G-88A (5%), G-48A (2.5%), and T17C (5%) of *oxyR-ahpC* among INH-resistant isolates.

Keywords: *Mycobacterium tuberculosis* complex, DNA sequence analysis, Mutation, *rpoB*, *katG*, *inhA* and *oxyR-ahpC*.

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

DOKTORA TEZİ

Çukurova Bölgesinden İzole Edilen Çoklu İlaca Dirençli *Mycobacterium tuberculosis* Kompleks Suşlarında *rpoB*, *katG* ve *inhA* Genlerindeki Genomik Mutasyonlar

Omer Ali Namiq AL-JAF

Danışman: Prof. Dr. Sadık DİNÇER

Biotechnology Anabilim Dalı

ÖZ

En azından rifampisin (RIF) ve izoniazide (INH) dirençli olan çok ilaca dirençli *Mycobacterium tuberculosis* (MDR-TB), tedavinin daha karmaşık olması, maliyeti ve yan etkileri nedeniyle halk sağlığı için büyük bir tehdittir. RIF ve INH'ye karşı direnç genellikle *rpoB* ve *KatG* genlerinde, *inhA* promotöründe ve *oxyR-ahpC* intergenik bölgelerinde gen mutasyonları ile ortaya çıkarmaktadır. Modern genomik bilimin gelişimi, ilaç direncinin moleküler mekanizmasını anlamak ve ilaca dirençli *M. tuberculosis*'i teşhis etmek için bize etkili bir yöntem sunmaktadır. Türkiye'nin Çukurova Bölgesi'nden 70 klinik *M. tuberculosis* suşunda 4 gen veya intergenik bölgedeki değişiklikleri bulmak için tüm genom dizileme (WGS) kullanılmıştır. Suşlar 40 MDR-TB ve 30 pan-duyarlı izolat içermekteydi. Daha sonra bu değişikliklerin RIF ve INH'ye karşı dirençle ilişkisi incelenmiştir. Sonuçlar, fenotipik oransal yöntemle karşılaştırıldığında, direnç tespiti için WGS'nin duyarlılığı ve özgüllüğünün RIF için %77,5 ve %100,0 (*rpoB*'deki mutasyonlara dayalı olarak), INH için %80 ve %100, %30 ve %100 ve %12,5 ve %93,3 (sırasıyla *katG*, *inhA* ve *oxyR-ahpC*'deki mutasyonlara dayalı olarak) olduğu görülmektedir. WGS verileri ayrıca *inhA* promotöründeki mutasyonların *katG* deki mutasyonlara bağlı olarak INH için duyarlılığı yalnızca %5 artırdığını göstermiştir. Ayrıca, *oxyR-ahpC* intergenik bölgelerindeki mutasyonlar, *katG* geni ve *inhA* promotöründeki mutasyonlara bağlı olarak INH için özgüllüğü %6,3 oranında azaltmıştır. RIF dirençli izolatların S450L (%52,5), H445T (%7,5), A435V (%7,5), L430P (%5), L430L (%2,5) ve 494 (%2,5) kodonları *rpoB* geninde en yüksek mutasyon oranını sergilemiştir. *katG* geninin en çok değişen kısımları S315T (%52,5), S315A (%2,5), G316S (%7,5), L336P (%5), S309T (%5), T328C (%2,5), A311P (%2,5) kodonları ve bir suşta hem T328C hem de S315A (%2. 5); *inhA* promotörünün C-15T (%25), G-47C (%2,5) ve G-64C (%2,5) pozisyonları; ve INH dirençli izolatlar arasında *oxyR-ahpC*'nin G-88A (%5), G-48A (%2,5) ve T17C (%5) pozisyonları.

Anahtar Kelimeler: *Mycobacterium tuberculosis* kompleksi, DNA dizi analizi, Mutasyon, *rpoB*, *katG*, *inhA* ve *oxyR-ahpC*.

GENİŞLETİLMİŞ ÖZET

M. tuberculosis bakterisinin neden olduğu tüberküloz (TB), tanı yöntemleri ve tedavi seçeneklerinin varlığına rağmen dünya çapında oldukça yaygın ve ciddi bir bulaşıcı hastalıktır. Bu hastalık dünya çapında yaklaşık 10 milyon kişiyi etkilemektedir ve 2019 yılında ölüm oranı 1,4 milyon olarak belirlenmiştir.

Çok ilaca dirençli *M. tuberculosis* (ÇİD-TB), TB tedavisinde en sık kullanılan iki ilk basamak ilaç olan rifampisin (RIF) ve izoniazide (INH) dirençli bir tüberküloz türüdür. ÇİD-TB'nin ortaya çıkışı, önemli bir küresel halk sağlığı tehlikesi oluşturmaktadır. 2019 yılında dünya genelinde yaklaşık 465.000 ÇİD ve rifampisine dirençli (RR) TB vakası bildirilmiştir. Yeni vakaların %3,3'üne ÇİD/RR TB teşhisi konulurken, daha önce tedavi görmüş hastaların %13'üne ÇİD/RR TB teşhisi konulmuştur. Ayrıca, yeni vakaların %18'inde ve daha önce tedavi görmüş vakaların %17,4'ünde INH dirençli TB tespit edilmiştir.

ÇİD-TB'nin hızlı tespiti ve kesin olarak tanımlanması, ilaca dirençli suşların gelişimini ve bulaşmasını azaltmak için etkin bir şekilde tedavi uygulayarak hem mortalite hem de morbidite oranlarını azaltmak için çok önemlidir.

Fenotipik İlaç Duyarlılık Testi (DST) yöntemi, ilaç direncinin belirlenmesinde en güvenilir ve doğru teknik olarak kabul edilmektedir. Bununla birlikte, bu süreç zaman alıcıdır ve tüberküloz dışı mikobakteriyel hastalıkların neden olduğu bakteriyel kontaminasyon ve çapraz enfeksiyon olasılığını artırmaktadır. Bu çalışmada Polimeraz Zincir Reaksiyonu (PCR) ve DNA dizileme gibi iki hızlı moleküler tanı tekniği seçilmiştir.

İlaca dirençli genlerin bulunduğu yerlerde çeşitlilik vardır ve tedavi planlarını daha etkili hale getirmek için ÇİD-TB'nin mutasyon modellerini bulmak çok önemlidir. İlaçların nasıl çalışmadığı ve RIF ve izoniazid INH'nin en yaygın mutasyon kodonları hakkında daha fazla bilgi edinmemiz gerekiyor. RIF, bir anti-TB ilacı olarak oldukça verimlidir. Bu maddenin etki mekanizması, mRNA'nın uzamasını etkili bir şekilde önleyen RNA polimerazın β-alt birimine bağlanmayı içerir.

M. tuberculosis'te RIF'e karşı direnç oluşumu, *rpoB* genindeki genetik mutasyonlarla bağlantılıdır. RIF direncini belirleyen bölge (RRDR) olarak bilinen bir bölge, *M. tuberculosis* izolatlarını rifampisine dirençli hale getiren mutasyon kodonlarının yaklaşık %96'sına ev sahipliği yapmaktadır.

Bu bölge 81 baz çiftini kapsamakta ve 426 (507) ila 452 (533) arasındaki kodonları içermektedir. Bu kodonlardan 450, 445 ve 435, %70 ila %95 arasında değişen en önemli direnci sergilemiştir. İzoniazid, *M. tuberculosis* enfeksiyonlarını tedavi etmek için kullanılan bir diğer güçlü ilaçtır.

Bu tedaviye direnç, *katG, inhA, oxyR, kasA, ahpC* ve *ndh* dahil olmak üzere çeşitli genlerdeki mutasyonlardan kaynaklanır. INH direncinin birincil kaynağı, katalaz-peroksidaz enzimini kodlayan *katG* genindeki bir mutasyondur. Bu enzim mikolik asit üretiminde çok önemli bir rol oynar. INH dirençli suşların %50 ila %95'inin *katG* geninin 315 kodon pozisyonunda *katG*315 olarak adlandırılan bir mutasyona sahip olduğu bildirilmiştir.

Bu çalışmada 70 (40 ÇİD-TB suşu ve 30 tam ilaç duyarlı) MTBC suşu arasında ilaç direnci mutasyonları incelenmiştir. Bu çalışma Çukurova Bölgesi'nde *rpoB, katG, inhA* promotor ve *oxyR-ahpC* intergenik bölgelerindeki mutasyon paternlerini aramayı amaçlamıştır. Fenotipik yöntemler, tedavi hızlı bir şekilde başladığında duyarlılığı belirlemek için çok yavaştır. Bu ÇİD-TB ilaçları için moleküler DST'nin, ilaç direncini nasıl etkilediklerine göre doğru bir şekilde gruplandırılması gerekir, böylece anlaşılabilir ve tedavi planlarının yapılmasına yardımcı olmak için kullanılabilir.

M. tuberculosis suşları, Çukurova Üniversitesi Tropikal Hastalıklar Araştırma ve Uygulama Merkezi, Sağlık Bakanlığı Halk Sağlığı Kurumu ve Adana Bölge Tüberküloz Laboratuvarı'na Türkiye'nin güneyindeki Çukurova Bölgesi'ndeki sekiz ilden gönderilen tüberküloz şüphelilerinin klinik örneklerinden izole edilmiştir. Fenotipik analiz için Bactec MGIT 960 TB sistemi kullanılmıştır. Genomik DNA, BACTEC-MGIT 960 üzerinde ekilen kültürlerden Mickle mekanik yöntemleri ile izole edilmiştir.

Tablo (3.2) ve (3.3) RIF ve INH direnç genleri için primerleri ve PCR karışımını detaylandırmaktadır. PCR, 95°C'de 5 dakika boyunca ilk denatürasyonu, ardından *rpoB* (450 bp), *katG* (500 bp), *inhA* promotörü (248 bp) için 30 döngü ve *oxyR-ahpC* intergenik bölgesi (293 bp) için 35 döngüyü içermiştir. İlk üç gen için bağlanma 30 saniye boyunca 60°C'de, uzatma 30 saniye boyunca 72°C'de ve son uzatma 5 dakika boyunca 72°C'de yapılmıştır. *oxyR-ahpC* amplifikasyonu 94°C'de 5 dakika denatürasyon, 60°C'de 45 saniye bağlanma ve 72°C'de 1 dakika uzama ve 72°C'de 7 dakika son uzama ile sona ermiştir. Amplikonlar bir agaroz jel üzerinde analiz edilmiş ve hemen sekanslanmış veya -20°C'de saklanmıştır. Dizilemede bir Sanger kiti, BigDye Terminator döngü kiti ve ABI Prism 3130xl Genetik DNA Analizörü kullanılmıştır. Primer seçimi literatüre veya özel tasarıma dayanıyordu. Tablo (3.3) bağlanma sıcaklıklarını ve primer dizilerini belirtmektedir.

BACTEC MGIT 960 sistemi gibi otomatik kültür yöntemleri 10 ila 15 gün içinde sonuç vermektedir. Şekil 4.2, 40 ÇİD-TB izolatının ilaç duyarlılık profillerini göstermektedir; bunların %75'i (30/40) yalnızca INH ve RIF'e, %12,5'i (5/40) INH, RIF ve STR'ye, %2,5'i (1/40) INH, RIF ve EMB'ye ve %10'u (4/40) tüm MGIT (SIRE) setlerine dirençlidir.

ABI PRISM 3130xl Genetik Analiz Cihazı, 70 izolattan elde edilen 280 PCR ürününün DNA dizilerini incelemek için kullanılmıştır. Örnekler elektroforezden geçirilmiş ve elde edilen kromatogramlar pik analizi için incelenmiştir. Nükleik asit ve çevrilmiş dizilere BioEdit yazılımı kullanılarak bakılmıştır. CLUSTAL W ile hizalandılar ve PubMed GenBank'tan referans dizilerle karşılaştırıldılar (*rpoB*: 888164, *katG*: 885638, *inhA* promotor or f*abG1*: 886551, *oxyR-ahpC*: 885717). Mutasyonlar GENEDOC'ta hizalama ve kırpma yoluyla tespit edilmiştir (bkz. Şekil 4.8, 4.10, 4.12 ve 4.14.).

En fazla ilaç direnci taşıyan mutasyon *rpoB* Ser450Leu olmuştur. 40 ÇİD-TB izolatı arasında 31 suş *rpoB* mutasyonlarına sahipti. Pan-duyarlı 30 izolat arasında hiçbir direnç mutasyonu karakterize edilmemiştir. RIF'e karşı fenotipik direnci öngörmede *rpoB*'nin duyarlılığı ve özgüllüğü sırasıyla %77,5 ve %100'dür.

Her bir *rpoB* His445Tyr, Asp435Val mutasyonuna sahip üç (%7,5) izolat, *rpoB* Leu430Pro (%5) mutasyonuna sahip iki izolat, *rpoB* Leu430Leu (%2,5) mutasyonuna sahip bir izolat ve RRDR dışında bulunan *rpoB* Leu494Leu (%2,5) mutasyonuna sahip bir izolat ve *rpoB* geninde herhangi bir mutasyon bulunmayan fenotipik olarak dirençli dokuz suş vardı.

katG geninde, analiz edilen ÇİD-TB izolatlarının %80'inde mutasyonlar bulunmuştur. Yirmi iki (%55) izolatta 315 kodonunda mutasyon vardı (21 Ser315Thr ve 1 Ser315Asn). Üç suşta (%7,5, 3/40) Gly316Ser mutasyonu, iki suşta (%5, 2/40) Leu336Pro mutasyonu, iki suşta (%5, 2/40) Ser309Thr mutasyonu, bir suşta (%2. 5, 1/40) Trp328Cys mutasyonuna, bir suşta (%2,5, 1/40) Asn311Phe mutasyonuna ve bir suşta (%2,5, 1/40) *katG* geninde hem Trp328Cys hem de Ser315Asn mutasyonlarına sahiptir. *katG* geninde herhangi bir mutasyon bulunmayan sekiz fenotipik dirençli suş bulunmaktadır.

Primer, 248 nükleotidlik *inhA* operon geninin promotör bölgesini (82 amino aside eşdeğer) çoğaltmıştır. Bu lokusun dizilendiği 40 ÇİD-TB suşunun onunda *inhA* promotöründe %25'lik bir değişiklik (*inhA*-15 C-to-T) vardı. Ayrıca, izolatların %2,5'inde ikinci bir mutasyon (*inhA*-47 G-to-C) ve *inhA*-64 izolatların %2,5'inde mutasyon sergilemiştir (bkz. Tablo 4.4).

Primerle çoğaltılmış 293 nükleotid parçası elde edilmiştir. Kırk üçü *ahpC*'den, 106'sı intergenik bölgeden ve 144'ü *oxyR* geninden geliyordu. 40 ÇİD-TB izolatının %87,5'inde mutasyon görülmemiştir. Beşinde (%12,5) üç farklı kodonda mutasyon vardı (ikisi G-88A'da, biri G-48A'da ve ikisi T17C'de). Duyarlı izolatların %6,7'sini oluşturan iki izolat, *oxyR-ahpC* yapısal geninde mutasyon sergilemiştir.

WGS tahminlerinin doğruluğunu artırmak amacıyla, daha duyarlı olabilecek sık mutasyon kombinasyonlarını keşfetmek için bir bootstrap yöntemi kullandık. *rpoB* S450L ve *katG* S315T kombinasyonunun ÇİD fenotipini tahmin etmek için öngörücü duyarlılığa sahip olduğu belirlenmiştir. Daha fazla mutasyon bölgesi dahil edildiğinde öngörücü duyarlılığın arttığı bulunmuştur. *rpoB* S450L, *rpoB* H445A/P, *katG* S315T ve *inhA*-15 kombinasyonu maksimum tahmin hassasiyetini vermiştir.

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SYMBOLS AND ABBREVIATIONS

A:	Alanine
ACP:	Acyl Carrier Protein
AFB:	Acid-Fast Bacilli
AG:	Arabinogalactan
ahpC:	Alkyl Hydroperoxide Reductase Subunit
Asp:	Asparatate
BCG:	Bacille Calmette-Guérin
CDC:	Centres for Disease Control and Prevention
CRISPR:	Clustered Regularly Interspaced Short Palindromic Repeat
Cys:	Cysteine
DGTS:	Directly Observed Treatment
DLM:	Delamanid
DNA:	Deoxyribonucleic acid
EMB:	Ethambutol
ETB:	Ethambutol
Gly:	Glycine
HBCs:	High Burden Countries
His:	Histidine
HIV:	Human Immunodeficiency Virus
INH:	Isoniazid
IS:	Insertion Sequence
katG:	Catalase-Peroxidase
Leu:	Leucine
LAM:	Lipoarabinomannan
LJ:	Lowenstein-Jensen
LM:	Lipomannan
LPA:	Line Probe Assays
mAGP:	Mycolyl-Arabinogalactan-Peptidoglycan
MAs:	Mycolic Acids
MDR-TB:	Multidrug-Resistant Tuberculosis
MICs:	Minimal Inhibitory Concentrations
MTBC:	Mycobacterium Tuberculosis Complex
NTM:	Non-Tuberculosis Mycobacteria
OBR:	Optimized Background Regimen
PAS:	Para-Amino Salicylic Acid

PG:	Peptidoglycan
PIMs:	Phosphatidylinositol Mannosides
PPD:	Purified Protein Derivative
Pre-XDR-TB:	Pre-Extensively Drug-Resistant Tuberculosis
Pro:	Proline
PZA:	Pyrazinamide
RIF:	Rifampicin
RR-TB:	Rifampicin-Resistance Tuberculosis
Ser:	Serine
SCC:	Short-Course Chemotherapy
SDGs:	Sustainable Development Goals
SNPs:	Single Nucleotide Polymorphism
STR:	Streptomycin
Thr:	Threonine
TB:	Tuberculosis
Tyr:	Tyrosine
UHC:	Universal Health Coverage
UI:	Uncertainty Interval
Val:	Valine
WGS:	Whole Genome Sequencing
WHO:	World Health Organization

1. INTRODUCTION

Tuberculosis (TB) is one of the infectious diseases caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), a species of the genus *Mycobacterium*, and affects millions of humans worldwide. Tuberculosis is one of the oldest known diseases in humans. Robert Koch, a German physician and microbiologist, originally discovered tuberculosis's etiology and infectious nature in immobile, non-sporulating, and non-encapsulated. *M. tuberculosis* is a strictly aerobic species; hence, it multiplies significantly in the lungs and air cavities. Its reproduction, taking 14 to 20 hours, is slow compared to other pathogenic bacteria, explaining the gradual progression of the disease and the effectiveness of medication when properly administered. *M. tuberculosis* can reduce its metabolism and multiplication, remaining latent for an extended period, which aligns with clinical findings of disease reactivation years after the initial infection (Kumar et al., 2024).

In 2020, the WHO estimated that one-quarter of the world's population has a TB infection, which means TB bacteria have infected people but are not (yet) ill with the disease and cannot transmit it (Organization, 2022a). Approximately 10% of these latently infected individuals will eventually develop active infection. *M. tuberculosis* can be transmitted by an individual with active pulmonary disease through droplet aerosol. However, patients with smears positive for acid-fast bacilli (AFB) have a high risk of transmitting the disease (Palomino, 2009).

Although developments in the diagnosis and treatment of tuberculosis led to a significant decrease in the incidence of infection and mortality rates in developed countries in the mid-20th century, increasing Acquired Immunodeficiency Syndrome (AIDS) cases and the emergence of multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) strains at the end of the century caused tuberculosis to come to the fore again (Tuncer, 2016).

For diagnosis and therapy, mycobacteria can be categorized into several primary groups: non-tuberculosis mycobacteria (NTM), which includes all other mycobacteria; the *Mycobacterium tuberculosis* complex (MTBC), which causes TB and *M. leprae*; which causes leprosy. NTMs are often spread throughout the environment and are not harmful to humans. The genus *Mycobacterium* includes more than 140 species, of which about 50 are now thought to represent the etiological agents of human disease, excluding *M. leprae* and MTBC.

Two common NTM species that typically affect immunocompromised patients are *M. avium* and *M. intracellulare*. *M. ulcerans* is also NTM and causes Buruli ulcer, a necrotizing skin disease with the highest incidence in West Africa (Cinicola et al., 2024; Van Ingen et al., 2013).

Slow-growing, closely related mycobacteria constitute the MTBC. It typically consists of *M. tuberculosis*, the agent responsible for most cases of tuberculosis in humans; *M. africanum* and *M. canetti*, which are human TB agents in Africa; *M. microti*, which is a TB agent in voles; and *M. bovis*, which is responsible for infecting a wide range of mammalian species, including people and cattle. *M. pinnipedii* and *M. caprae* are further species that cause tuberculosis in animals (Chernick, 2004).

The high degree of similarity (>99% nucleotide sequence identity) at the Deoxyribonucleic acid (DNA) level has confirmed the MTBC member's near relatedness. Multilocus sequence typing, one of the primary methods used to investigate bacterial genetic variation, is not very useful in the case of the MTBC due to its low sequence variation compared to other bacteria. As a result, nothing is known about the ecology and evolution of the MTBC. By indexing MTBC variety over the entire genome, recent developments in next-generation sequencing have circumvented these constraints and allowed for in-depth examinations of the evolutionary processes underlying this variation (Chernick, 2004; Gagneux, 2018; Niemann et al., 2000).

Researchers have chosen to keep the traditional species designations of these bacteria rather than lumping them together despite their close genetic kinship due to certain behavioral traits, such as varying host preferences. Cole et al. reported the first whole genome sequence of *M. tuberculosis* in 1998. It belonged to the laboratory strain H37Rv (Cole et al., 1998). The genome consists of 4,411,532 base pairs (bp) and has a high guanine (G) and cytosine (C) content (65%). About 4,000 genes were identified in the most recent reannotation (Camus et al., 2002).

There have been reports of mobile genetic components, such as plasmids, in certain mycobacterial species but not in *M. tuberculosis*. However, numerous mycobacterial species, including *M. tuberculosis*, have been found to possess IS and other repeating components. For molecular epidemiology, particular emphasis has been given to IS6110 and the clustered regularly interspaced short palindromic repeat (CRISPR) sequences; the former is a crucial component of the bacterial adaptive immune system. The CRISPR system is utilized for spoligotyping, and IS6110 is employed for restriction fragment length polymorphism analysis (Kent et al., 1995; Wiedenheft et al., 2012).

In response to the growing prevalence of tuberculosis, particularly in low-income nations, the World Health Organization (WHO) issued a worldwide health alert on the disease in 1993 and the dramatic impact of the HIV epidemic in Africa on the number of TB cases and deaths prompted WHO to declare a global TB emergency, prompting all countries to implement a Directly Observed Therapy (DGT) strategy for the care and control of TB and thus take action to achieve TB eradication. Although this strategy has achieved a significant reduction in the incidence of TB and the death rates caused by it, the increase in the number of people infected with HIV and the increasing global concerns about the emergence and spread of MDR-TB and XDR-TB strains have made the WHO take TB globally and revealed the need to deal with scale more effectively. Increasingly developing drug resistance, poverty, irregular migration, malnutrition, and income inequality among MTBC types have led to inadequate implementation of eradication programs recommended by WHO, especially in underdeveloped and developing countries (Çevirme, 2017; ÖZGÜR, 2019).

Over the past decade, the global community has focused its attention on twenty-two nations with a high burden of TB, commonly known as TB-high burden countries (HBCs). In 2011, these countries accounted for nearly 82% of all tuberculosis infections worldwide (Espinal & Raviglione, 2021; Organization & Staff, 2013).

As a result, TB presents a significantly higher risk to global public health, especially in low-income nations. Individuals residing in regions with a high prevalence of TB or having proximity to individuals with pulmonary TB, as well as those with compromised immune systems, are at the highest risk of contracting TB (Sia & Wieland, 2011). Tuberculosis is a prevalent disease among impoverished populations worldwide. Practitioners and public health officials are encountering difficulties in managing TB due to its intricate entanglement within the socio-economic framework and cultural structure of society. This elucidates the reason why TB ranks as the seventh most prevalent cause of mortality on a global scale. The primary factors contributing to the inability to discover successful remedies include human immunodeficiency virus (HIV) infection, TB epidemics, and delays in both diagnosis and appropriate treatment (Lönnroth et al., 2010).

In 2010, there were an estimated 8.0 million incident cases of TB, 1.0 million TB-related fatalities among HIV-negative patients, and roughly 0.4 million TB-related deaths among HIV-positive patients (Organization, 2011).

Geographically speaking, the global distribution of tuberculosis cases in 2020 revealed that (43%) of cases were in Southeast Asian nations, (25%) in Africa, (18%) in Western Pacific nations, (8.3%) in the eastern Mediterranean states, (3.0%) in North and South America, and (2.3%) in European nations. Eight countries account for two-thirds of the total global TB cases. These 30 countries with the highest burden of TB represent 86% of all estimated incident cases worldwide. The countries with the highest TB burden are India (26%), China (8.5%), Indonesia (8.4%), the Philippines (6.0%), Pakistan (5.8%), Nigeria (4.6%), Bangladesh (3.6%), and South Africa (3.3%) (Organization, 2022a).

The intensity of national tuberculosis epidemics varies significantly among countries, as indicated by the annual number of incident cases per 100,000 people (Figure 1.1). In 2020, 57 countries experienced a low prevalence of TB, with less than 10 cases per 100,000 individuals annually. These countries were mainly located in the WHO European and American regions, with a few also found in the WHO Eastern Mediterranean and Western Pacific regions. Among the thirty countries with the highest burden of TB, most had an incidence rate of 150 to 400 cases per 100,000 individuals. However, the Democratic People's Republic of Korea, Gabon, Lesotho, the Philippines, South Africa, and the Central African Republic had an incidence rate above 500 cases per 100,000 individuals (Organization, 2022a).

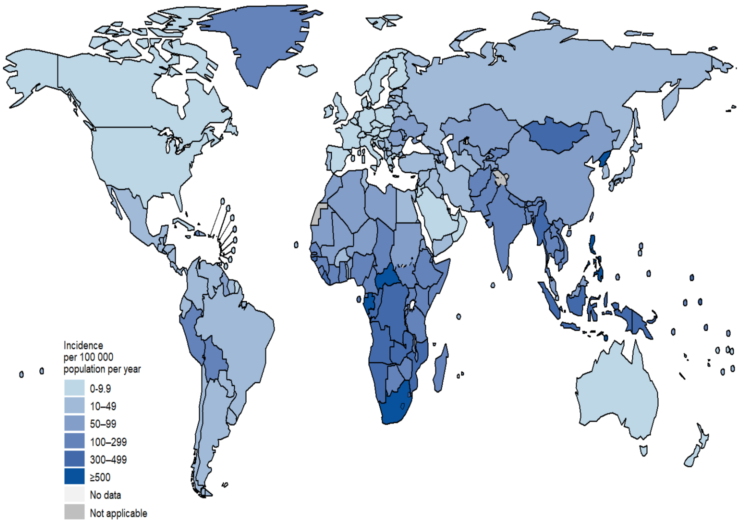


Figure 1.1. Estimated TB incidence rates, 2020

Through their endorsement of the Sustainable Development Goals (SDGs) at the United Nations General Assembly in 2015 and the WHO End TB Strategy (2016–2035) at the World Health Assembly in 2014, all WHO and UN member nations have agreed upon global targets and milestones for reducing the burden of TB disease, measured in terms of TB incidence and the number of TB fatalities (Çevirme, 2017; Özgür, 2019).

The initial target of the End TB Strategy is to achieve a 20% reduction in the TB incidence rate from 2015 to 2020. The incidence rate measures the number of new and recurring cases of TB per 100,000 individuals each year. The upcoming milestone in 2025 represents a reduction of 50% compared to the year 2015. The objectives for subsequent years are to achieve reductions of 80% and 90% by 2030 and 2035, respectively. SDG aims to eliminate the global tuberculosis pandemic by 2030, monitoring advancements by measuring the incidence of tuberculosis cases per 100,000 individuals yearly (Sotgiu et al., 2017; Organization, 2022a).

To meet the goals and milestones, the TB incidence rate had to decrease by 4-5% per year by 2020, then accelerate to 10% annually by 2025, and finally average 17% annually from 2025 to 2035. When setting the milestones and targets, we determined that providing TB prevention, diagnostic, and treatment services is crucial in the context of progress toward Universal Health Coverage (UHC). It is also essential to take multisectoral actions to address the broader social and economic factors contributing to TB. A new vaccine must be developed by 2025 to meet these requirements, a crucial technological advancement (Floyd et al., 2018).

Tuberculosis affects individuals of all age groups and both genders. During the year 2020, the prevalence of tuberculosis was highest among adult males, who constituted 56% of all cases. Adult females accounted for 33% of cases, while children made up 11%. Prevalence survey data indicates that men are more likely to be afflicted by TB disease compared to women. Additionally, there are more gaps in identifying and reporting cases of TB among men, which corresponds to the higher percentage of TB cases observed in this community.

An HIV-positive individual was involved in 8% of all TB cases. In 2020, the global tuberculosis-related mortality among HIV-negative adults consisted of 53% men, 32% women, and 15% children (under 15 years old). The higher proportion of children receiving a share more significant than the expected 11% suggests limited availability of diagnosis and treatment for this group. Of the HIV-positive individuals who died from TB, 50% were male, 40% were female, and 9.8% were children (Pedraz et al., 2024).

The WHO states that the global estimates of TB occurrence in 2020 should be considered tentative. The WHO estimates that 2020 tuberculosis will affect 10 million or more individuals worldwide, as in previous years. The best estimate is 9.9 million (with a 95% uncertainty range of 8.9–11 million), which is equivalent to 127 cases (with a UI of 114–140) per 100,000 people (as shown in Table 1.1) (Organization, 2022a).

Table 1.1. The WHO produces estimates of the burden of TB in 2020 in consultation with countries

	Number	(Rate per 100 000population)
Total TB incidence	9 870 000 (8 880 000-10 900 000)	127 (114-140)
HIV-positive TB incidence	787 000 (701 000-879 000)	10 (9-11)
HIV-negative TB mortality	1 280 000 (1 210 000-1 360 000)	17 (16-18)
HIV-positive TB mortality	214 000 (187 000-242 000)	2.7 (2.4-3.1)

The first information about the epidemiological state of tuberculosis in Turkey dates back to 1950. From 204/100.000 in 1950 to 8.8/100.000 in 1980 and 1.6/100.000 in 2000, there was a decline in tuberculosis mortality. The incidence of tuberculosis decreased to 24/100.000 in 2002 from 177/100.000 in 1960's values. In Türkiye, the estimated rates of tuberculosis prevalence in 2014 were 22/100.000, the incidence was 18/100.000, and mortality was 0.61/100.000 (Pala et al., 2016).

Members of the General Assembly made a political commitment to treat 40 million cases of tuberculosis (TB) between 2018 and 2022, including children and those with drug-resistant strains of the disease. This promise was made during the assembly's high-level discussion on the disease. Between 2018 and 2021, 26.3 million people received TB treatment, equivalent to 66% of the five-year cumulative target (Figure 1.2.). Globally, 43% of the 1.5 million people who need to be treated for MDR/RR-TB over five years have been treated. Although the percentage of children with MDR/RR-TB who received treatment remained extremely low, the total number of children who received TB therapy was 1.9 million, or 54% of the five-year cumulative target of 3.5 million (Millington et al., 2023).

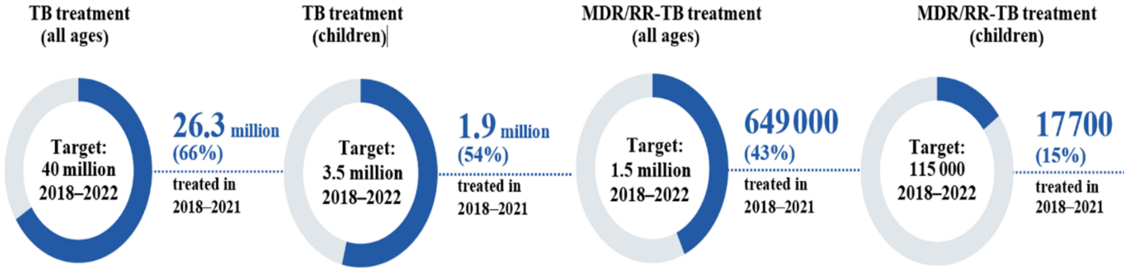


Figure 1.2. Global progress in the number of people treated for tuberculosis, 2018–2021

Resistance to antimicrobials is a natural phenomenon, but resistance to anti-TB medications in *M. tuberculosis* is a product of human activity. Anti-TB drugs don't work on *M. tuberculosis* because they pick out drug-resistance mutations that happen on their own, usually because of inadequate or incomplete treatment. Approximately 35 years ago, Michael Iseman pointed to the phenomenon of 'unconscious genetic engineering' about ineffective tuberculosis control programs and issued a cautionary warning about a potentially disastrous situation that was imminent. According to his statement, We are unwittingly turning a highly treatable infection into a life-threatening disease that is extremely expensive to treat."(Ahmed et al., 2016; Iseman, 1985).

To successfully manage TB, it is necessary to determine the specific *Mycobacterium* species responsible for the disease and conduct drug susceptibility testing (DST) to assess their patterns of resistance to drugs. The etiological agent of TB is a collection of species and subspecies closely connected at the genetic level. This group is known as the MTBC (Yeboah-Manu et al., 2016). *M. tuberculosis* and *M. africanum* are humans' primary etiological agents responsible for tuberculosis. Species within the MTBC exhibit about 99.9% similarity in terms of their gene sequence (Gagneux et al., 2006). Nevertheless, they display variations in their range of hosts, geographical distribution, ability to cause disease, drug susceptibility, and specific phenotypes (Larsen et al., 2002).

Jorgen Lehmann discovered para-aminosalicylic acid (PAS) in 1943, while Gerhard Domagk discovered thiosemicarbazone during wartime in Germany, which concluded in 1945. These medications were the initial ones capable of efficiently treating tuberculosis. Both were underwhelming, exhibiting just bacteriostatic properties. In 1947, scientists discovered streptomycin (STR), establishing it as the initial efficacious antibiotic and bactericidal agent against *M. tuberculosis* (Hayman, 1984; Daniel, 2006).

The development of resistance to anti-TB drugs was detected shortly after their introduction in 1947 (streptomycin). Based on clinical, epidemiological, and laboratory data, several patterns of treatment resistance have arisen over the past 40 years, with a growing number of TB strains experiencing this. Drug resistance must be tested using fast molecular testing and sequencing technologies and bacteriological confirmation of tuberculosis (Migliori et al., 2007; Gandhi et al., 2010).

The tuberculosis pandemic persists due to the absence of adequate tools, which hinders humanity's ability to overcome it. The Bacille Calmette-Guérin (BCG) vaccine, used to prevent TB, is a weakened form of the *M. bovis* bacterium, closely related to the *M. tuberculosis* bacterium. Initially given more than a century ago, BCG protects against severe types of tuberculosis in children, but its effectiveness in preventing pulmonary disease in adults is limited (Lange et al., 2022).

The treatment protocol for drug-susceptible tuberculosis is successful, although it can be complex. The WHO recommends taking rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA) every day for two months, followed by daily INH and RIF for another four months. This is the primary treatment method. The long duration, the combination of many medications, and the adverse effects increase the challenges faced by patients in completing their anti-tuberculosis regimen, leading to failure of therapy and the development of drug resistance. Furthermore, drug resistance exacerbates the situation by necessitating the administration of antibiotics with higher rates of adverse reactions, which patients must take for up to 18 months. Hence, it is unsurprising that *M. tuberculosis* poses the most significant threat to global health in the present era (Mekonnen & Azagew, 2018).

The TB pandemic is notable in terms of its temporal and geographic range despite the genetic similarity of the bacteria to other disease-causing microbes. Genetic variety enables viruses to effectively overcome challenges posed by antimicrobial medications and the host's immune system during infection. *M. tuberculosis*, on the other hand, has genetic monomorphism because it doesn't change much and doesn't do much horizontal gene transfer or recombination. This implies that the genetic diversity present in *M. tuberculosis* plays a crucial role in facilitating the species' exceptional ability to cause diseases (Allué-Guardia et al., 2021).

Healthcare providers utilize INH as a primary medicine for the treatment of both latent infections and active tuberculosis. The emergence of INH-resistant *M. tuberculosis* strains has led to the rapid development of MDR-TB (*M. tuberculosis* strains resistant to INH and RIF). Hence, the transmission of MDR-TB presents a worldwide obstacle to the treatment and prevention of tuberculosis. Timely molecular diagnosis is essential in tuberculosis treatment to minimize the transmission of resistant strains and the development of further drug resistance. However, further investigation into the factors contributing to drug-resistant tuberculosis and identifying new indicators are necessary to diagnose this condition (Gegia et al., 2017).

The *katG* gene produces the enzyme catalase-peroxidase, which activates the prodrug INH. Finding the *katG* deletion in clinical INH-resistant isolates was the first step in figuring out how *M. tuberculosis*'s genes cause it to resist INH. INH resistance is associated with various genes in alternative metabolic networks and pathways. Activated INH produces several compounds that contribute to the synthesis of mycolic acids. The enzymes involved are β-ketoacyl ACP synthase (encoded by kasA), NADH-dependent enoyl ACP synthase (encoded by *inhA*), acetyl-CoA carboxylase (accD6), and malonyl-CoA acyl carrier protein (ACP) transacylase (fabD). Consequently, *M. tuberculosis* strains that possess these genetic modifications may develop resistance to INH. In addition, researchers have identified 15 new structural genes and regulatory regions that contribute to NH resistance. Researchers have discovered alterations in the intergenic regulatory region between *ahpC-oxyR* and the *ahpC* gene, which encodes the alkyl hydroperoxide reductase subunit C, in isolates resistant to INH (Unissa et al., 2016).

Although multiple genes cause *M. tuberculosis* resistance to INH, data suggests frequent mutations are primarily localized in the *katG*, *inhA*, and *ahpC-oxyR* regulatory areas. Due to the challenge of concurrently obtaining all genetic sequences linked to INH resistance in a collection of isolates, the knowledge of further genetic variants is likewise restricted; this results in a significant deficiency in the progression of the accumulation of diverse genetic changes that lead to the development of INH resistance during TB treatment (Zhao et al., 2012). We analyzed the mutation pattern of four known genes in clinical *M. tuberculosis* isolates using whole genome sequencing (WGS). For this investigation, 40 MDR isolates and 30 susceptible isolates were chosen.

Many MDR-TB strains have been linked to INH and RIF mono-resistance development. This is because the choice of medication used to treat TB has a lasting effect on the bacteria. The extensive variability of these mutations presents an opportunity to examine the relationship between mutations and the extent of phenotypic resistance. This aims to identify the mutations linked to resistance against RIF and INH in MDR-TB isolates. The study of phenotypic drug resistance levels in these isolates showed how cumulative gene changes affect *M. tuberculosis* strains during mono-resistance development in people with MDR-TB. Finding high levels of mutations linked to drug resistance helps to prove new molecular methods for finding drug resistance to RIF and INH (Hazbón et al., 2006).

Currently, the WHO classifies drug-resistant tuberculosis cases into five separate categories. The many forms of tuberculosis include INH-resistant tuberculosis, RIF-resistant tuberculosis (RR-TB), multidrug-resistant TB (MDR-TB), pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB). MDR-TB refers to the coexistence of resistance to isoniazid and rifampicin, which are the two most effective main drugs employed in the treatment of tuberculosis.

The coexistence of resistance to INH and RIF, the two most effective first-line drugs used in tuberculosis treatment, is of clinical importance, as they are the two most effective primary antituberculosis agents. Pre-XDR-TB is a kind of MDR-TB caused by TB bacteria that are resistant to INH, RIF, and either a fluoroquinolone or a second-line injectable (amikacin, kanamycin, and capreomycin). XDR-TB is an uncommon form of MDR-TB that is caused by TB bacteria that have developed resistance to INH and RIF, as well as to fluoroquinolone and a second-line injectable antibiotic (amikacin, kanamycin, and capreomycin). Alternatively, XDR-TB can be caused by TB bacteria that are resistant to INH, RIF, fluoroquinolones, and either linezolid or bedaquiline (Organization, 2022a).

On a global scale, the percentage of cases of MDR-TB or RR-TB among newly identified tuberculosis cases in the past ten years is approximately 3-4%, whereas it is 18-21% among those who have been previously treated for TB. This rate is estimated to be around 350–400 thousand people in 2020, and most of them are pulmonary TB cases. Out of the total global count of 5.8 million individuals who were newly diagnosed with TB and officially registered as TB cases in 2020, 4.8 million people (82%) had pulmonary TB. Out of the total of 4.8 million, 59% were confirmed through bacteriological analysis, slightly higher than the 57% recorded in 2019. Of these bacteriologically diagnosed cases, 71% (2.1/3.0 million) were tested for rifampicin resistance, of which 157,903 TB was found to be drug resistant. Of these, 132 222 MDR/RR-TB cases and 25 681 pre-XDR-TB or XDR-TB cases were reported. Compared to the 201,997 cases of resistant TB identified in 2019, a decrease of approximately 22% was observed (Londt, 2022).

A global survey conducted by the WHO and the U.S. Centres for Disease Control and Prevention (CDC) has identified the presence of XDR-TB in every part of the world. Bhargava et al. (2011) demonstrated that the countries with the highest occurrence of XDR-TB worldwide are the former Soviet Union, China, and India. XDR-TB infections present significant treatment challenges and are characterized by high mortality rates (Bhargava et al., 2011).

MDR-TB was diagnosed in a significant majority of the 465,000 incident cases of RR-TB in 2019, accounting for 78% (362,700 cases). India, China, and the Russian Federation accounted for the largest share of the global burden of RR-TB cases, with 27%, 14%, and 8%, respectively. In 2019, there was a 10% rise in the number of identified and reported cases of MDR/RR-TB compared to 2018, with a total of 206,030 cases (which accounts for 44% of the expected 465,000 cases). Twelve thousand three hundred fifty cases, equivalent to 6% of the total, were diagnosed with XDR-TB among those notified with MDR/RR-TB (G. WHO, 2020).

Detecting the MTBC is crucial for comprehending, managing, and eradicating TB. It holds significant value in bolstering the One Health strategy. However, due to influencing factors such as different pretreatment methods for various sample types, typical reagents, and sample collection methods, standardized and highly accurate methods for detecting MTBC cases are needed. The existing techniques for detecting MTBC fall mainly into four categories: microscopic examination, culture-based methods, molecular biology methods, and whole genome sequencing (WGS) methods (Khatri et al., 2012).

Alongside previous challenges, a novel issue for TB management appeared in early 2020: the COVID-19 pandemic. The COVID-19 pandemic response caused a decrease of 21% in global TB case notifications in 2020 compared to 2019 due to disruptions to essential health services and the reallocation of human and financial support from TB to COVID-19. The COVID-19 epidemic risks reversing the progress achieved in recent years. Current estimates suggest that TB worldwide will experience a similar number of new cases and fatalities in 2021, as observed in 2013 and 2016, respectively. This indicates a setback of at least 5 to 8 years in the efforts to control TB. Experts predict that there will be an increase of 6.3 million tuberculosis cases and 1.4 million tuberculosis-related deaths by the year 2025 (Organization, 2021).

This study investigated the mutations associated with resistance to rifampicin and isoniazid in multidrug-resistant tuberculosis isolates. We looked at the levels of phenotypic drug resistance in these isolates to show how gene mutations affect the evolution of *M. tuberculosis* strains, especially in people who have MDR-TB, where resistance to rifampicin and isoniazid builds up over time. Detecting elevated amounts of mutations (*rpoB, katG, inhA, and oxyR-ahpC*) linked to resistance aids in developing dependable molecular techniques for identifying MDR-TB.

2. PRELIMINARY WORK

2.1. History of Tuberculosis

Tuberculosis is a well-known old disease caused by the *Mycobacterium* genus. *Mycobacterium* is an ancient genus that originated more than 150 million years ago, as suggested by one scenario (Hayman, 1984). Using contemporary molecular genetics tools and MTBC genome sequencing, researchers investigated the origin of mycobacteria during the Jurassic era. Gutierrez and colleagues identified the ancient precursor of MTBC as having emerged in East Africa approximately 3 million years ago. They deduced that this TB-causing microorganism began infecting people during that period (Gutierrez et al., 2005).

Furthermore, it was discovered that all contemporary members of MTBC, such as *Mycobacterium africanum* (*M. africanum*), *Mycobacterium canettii* (*M. canetti*i), and *Mycobacterium bovis* (*M. bovis*) but not including *M. tuberculosis* were linked with common African progenitor between 15,000 to 35,000 years ago (Brosch et al., 2002; Gutierrez et al., 2005). The present variants of MTBC trace their origins back to a common ancestor around 15,000 to 20,000 years ago (Sreevatsan et al., 1997). Nevertheless, the strains of MTBC analyzed in the research are composed of six primary clades and families, all originating from East Africa. However, their distributional range varies worldwide (Gagneux et al., 2006).

The MTBC DNA has been identified in Egyptian mummies, indicating the existence of an ancient illness. Archeologically, as well as in Egypt, some evidence of TB was also identified in America (Nerlich et al., 1997). In ancient Greece, the TB was referred to as "phthisis," whereas in Rome, it was termed "tabes," and in Hebrew, it was known as "schachepheth." During the 1700s, TB acquired the term "the white plague" because of the whiteness shown by its effects. In 1800, the term "consumption" was used to refer to the disease, and eventually, Schonely referred to it as "TB." During this point, TB was also referred to as the "Captain of all these men of death." Individuals were together trying to unravel the enigma of TB, including pursuing its etiology, diagnosis, and therapy (Arriaza et al., 1995).

The story spanned an extended duration, with a collective desire among all participants to become victorious in the competition. The remarkable event occurred on March 24, 1882, when Hermann Heinrich Robert Koch presented his renowned work "Die Aetiologie der Tuberculose" to the Berlin Physiological Society. In this publication, Koch discovered the TB bacilli and proposed his well-known Koch postulates. These postulates served as the fundamental basis for establishing most infectious etiologic hypotheses. These postulates demonstrated that specific microbes are the etiological agents of various diseases. Koch was awarded the Nobel Prize in 1905 for his significant contributions to microbiology and his pivotal role in unraveling the enigma of TB. (Daniel, 2006).

M. bovis, which causes TB in cattle, is believed to be the precursor of *M. tuberculosis* (Hoagland et al., 2016). Members of the MTBC exhibit a DNA sequence similarity of over 99.9%, but they can still be differentiated from one another based on Single Nucleotide Polymorphism (SNPs) (Brosch et al., 2002). Sreevatsan and his colleagues conducted a study on the evolution of MTBC and discovered that *M. bovis* evolved simultaneously with *M. tuberculosis*, as determined by identifying SNPs (Sreevatsan et al., 1997).

However, another study provides strong evidence that they evolved independently from another member of MTBC, most likely *M. canetti*i. This study analyzes the deletions and insertions in the MTBC genome (Brosch et al., 2002). The concept of a sanatorium was initially proposed in 1859 to offer patients with TB a dedicated treatment facility (Daniel, 2006; Frieden et al., 1995).

The first sanatorium was established in the rural Silesian Mountain settlement of Gobersdorf by Her-man Brehmer. It offered many amenities, including nourishing food, ample rest, and beneficial physical activity. Charles Mantoux was the first scientist in 1908 to administer tuberculin through intracutaneous injection. Florence Seibert introduced Purified Protein Derivative (PPD) in a series of research investigations conducted at the Phipps Institute of the University of Pennsylvania in 1930. The study examined up to 3000 TB patients in a hospital setting. The findings of this study indicated that fewer than 1% of participants did not respond (Palmer & Bates, 1952).

The utilization of the BCG vaccine coincided with the introduction of STR and other antibiotics in the 1950s to 1960s, resulting in a decline of TB in most regions worldwide. In the 1970s, with treatment methods and vaccination programs being introduced in the developed western countries, the elimination of TB was close to being achieved, which led to a loosening in TB control programs. In the late 1980s, TB emerged again as a serious threat to public health due to factors that are related to a high TB burden, including the HIV pandemic, homelessness and poverty, increased uncontrolled migration, and an increase in the incidence of TB in Europe, as in the rest of the world, have brought TB back to the attention of public health authorities (Valadas & Antunes, 2005).

Unfortunately, the effectiveness of pharmacological therapy for tuberculosis has indirectly led to the creation of a new pattern of drug resistance. Patients received the freedom to bring their medicines home without any supervision. The drugs were provided individually, and patients did not consume all the prescribed medication. A combination of inadequate adherence to treatment protocols and medical supervision might contribute to MDR (Veldsman, 2010). To achieve a cure, patients must adhere to long-term treatment with high-quality medications, specifically INH and RIF. Non-compliance during the 1990s led to an increased prevalence of TB resistance to the most effective drugs, INH and RIF, despite the challenges of guaranteeing adherence (Valadas & Antunes, 2005).

Also, the agent that causes tuberculosis is becoming less sensitive to anti-tuberculosis drugs, and MDR-TB and XDR-TB strains are spreading. This has limited treatment options and made the disease more likely to spread around the world. This resulted in the development of the MDR-TB strains (Girard et al., 2005). In 1994, the WHO designated TB as a worldwide emergency. Consequently, the WHO supported efforts to advance research and promote the development of quick diagnostic techniques (Valadas & Antunes, 2005; Yakici, 2013; Kerantzas & Jacobs Jr, 2017).

2.2. Taxonomical Classification

Lehmann and Neumann initiated the taxonomy of mycobacteria in 1896 by introducing the genus *Mycobacterium*. Scientists classified this genus within the family Mycobacteriaceae, order Actinomycetales, and class Actinomycetes, including *M. tuberculosis* and *M. leprae*. *Mycobacterium* is the sole genus within the family Mycobacteriaceae, but there was a suggestion to incorporate Nocardia and Rhodococcus into this family (Goodfellow & Magee, 1998).

Scientists have recognized *M. tuberculosis*, *M. bovis*, and *M. leprae* as the only human pathogens for years. In 1935, Pinner introduced the term "atypical acid-fast microorganisms" to differentiate isolates that caused human diseases but could be distinguished from *M. tuberculosis* based on their appearance, pigmentation, and virulence in animals (Pinner, 1935).

In 1965, Runyon conducted the primary research and categorization of the taxonomy of *Mycobacterium* and applied the same term “atypical” that Pinner had used to describe clinical isolates that did not appear to be human tubercle bacilli (Runyon, 1965). Mycobacteria that are not classified as tuberculosis complexes or leprosy have been referred to using various terms, such as "atypical mycobacteria" and "mycobacteria other than tuberculosis" (MOTT), “environmental mycobacteria,” or "non-tuberculous mycobacteria” (NTM) (Wolinsky, 1979).

The Runyon classification categorizes mycobacteria, except those in the MTBC and non-cultivable mycobacteria (*M. leprae*), into four classes based on their growth rates and pigmentation qualities: Pigment synthesis occurs exclusively in the absence of light or solely following exposure to light. The Runyon classification of NTM is a guideline for choosing the appropriate identification tests.

Group I: *Slow-growing bacteria* that exhibit photochromogenic properties: Cultures that are actively growing exhibit the development of a yellow pigment when exposed to light but do not create pigment when kept in darkness. Visible growth in cultures such as *M. kansasii* and *M. marinum* typically takes 2–6 weeks of incubation.

Group II: *Slow-growing bacteria* that exhibit scotochromogenic properties. These bacteria generate color under both illuminated and unilluminated circumstances. Visible growth of cultures such as *M. scrofulaceum, M. Gordon*, and *M. sugar* typically develops after an incubation period of 2–6 weeks.

Group III consists of *slow-growing bacteria* that do not produce pigments. This group includes both potentially pathogenic and non-pathogenic organisms. Most organisms, such as *M. avium* intracellular, *M. xenopi*, and *M. terrae*, lack pigmentation and have slow growth rates.

Group IV consists of organisms that exhibit rapid growth, which is defined as the ability to increase within a time frame of 2 to 7 days. They can have pigmentation or lack pigmentation. Colonies of *M. fortuitum complex*, *M. peregrinum*, *M. abscessus*, and *M. chelonae* often have a smooth texture, although rough variations can be observed. Based on the most recent nomenclature, the genus *Mycobacterium* consists of about 100 officially identified species (Euzéby & Tindall, 2004).

The MTBC comprises the following species: *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*i, *M. microti*, *M. caprae*, and *M. pinnipedii*. These are the primary causative agents of tuberculosis in both humans and animals. The species can be differentiated and identified based on variations in their biochemical features and molecular traits. However, it can also infect animals that have direct contact with humans. *M. bovis* is the causative agent of tuberculosis in domestic and wild animals, such as cattle and goats. Moreover, it possesses the capacity to induce tuberculosis in people (Wayne, 1986). Bovine TB continues to be a severe illness in numerous nations worldwide, resulting in substantial economic losses (Niemann et al., 2000).

Table 2.1 shows the MTBC species and their hosts

Mycobacterium sp.	Host
M. tuberculosis	Human TB
M. bovis, M. bovis BCG	TB in cattle and human TB
M. africanum	TB predominant in Africa
M. canettii	TB variant in a Somali patient
M. microti	TB from small rodents
M. caprae	TB in goats
M. pinnipedii	TB in seals

M. africanum is primarily found in various regions of Africa. *M. africanum* comprises up to 60% of the isolates acquired from individuals with pulmonary tuberculosis in some areas (Haas et al., 1997; Aranaz et al., 1999). *M. canetti*i, a recently identified bacterium, can induce TB in humans. However, the number of isolated *M. canetti*i strains remains limited (G Canetti et al., 1963; Kamerbeek et al., 1997). *M. microti* mainly induces TB in small rodents, such as voles. However, its significance concerning TB in humans has been uncertain until now (Wells, 1937; Wells & Robb-Smith, 1946; Van Soolingen, 2001). *M. caprae* is responsible for TB in animals and, to a limited extent, in people in certain European nations. *M. caprae* was initially identified in goats and cattle (Aranaz et al., 1999).

Table 2.2. Taxonomy of *M. tuberculosis*

Kingdom	*Bacteria*
Phylum	*Actinomycetota*
Class	*Actinomycetia*
Order	*Mycobacteriales*
Family	*Mycobacteriaceae*
Genus	*Mycobacterium*
Species	*Tuberculosis*

2.3. General Characteristics of Mycobacteria

Mycobacteria are aerobic bacteria that are non-spore-forming, non-motile, and have a slightly curved or smooth bacilli shape. They typically have a width of 0.2–0.6 µm and a length of 1–10 µm. The physical characteristics of this organism can vary based on its growth on solid media. For instance, certain species may appear as shorter cocci-bacilli or curved rods when cultivated on artificial media (Kaur G et al., 2019).

The morphological variations in *M. tuberculosis* can be divided into two categories: i) commonly observed shapes such as rods, V or Y-shapes, branched structures, or buds (Figure 2.1. A), and ii) less frequently observed forms that appear under stressful or unfavorable environmental conditions, including round, oval, ultra-virus-like, spore-like, and cell wall-defiant or L-forms (Figure 2.1. B) (Velayati & Farnia, 2012).

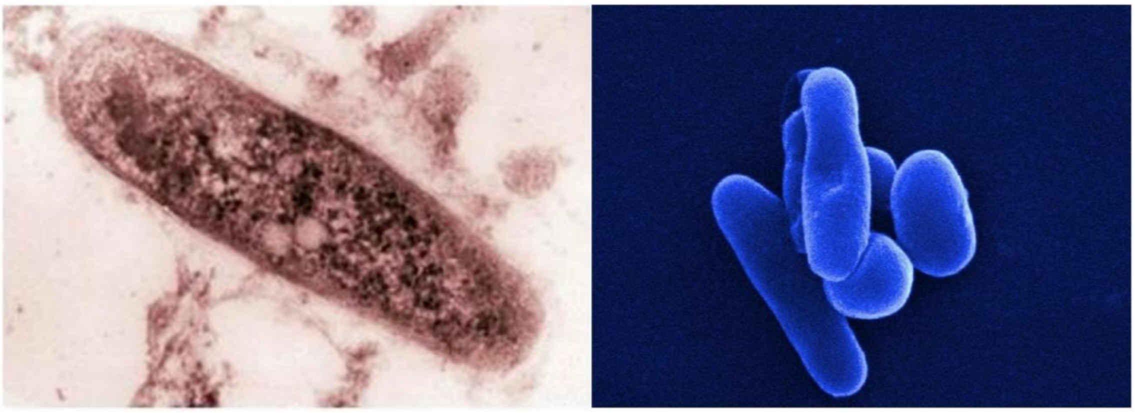


Figure 2.1. displays the physical forms of *M. tuberculosis*. (A) thin-section transmission electron micrograph of *M. tuberculosis*. (B) A scanning electron microscope reveals the morphological diversity of *M. tuberculosis* throughout the exponential growth phase (Velayati & Farnia, 2012)

M. tuberculosis is an organism that requires oxygen for its survival and growth. In individuals with TB, *M. tuberculosis* consistently inhabits the well-oxygenated upper lobes of the lungs. The bacterium is a parasite that can live inside cells, particularly macrophages. It has a relatively slow reproduction rate, taking 15–20 hours to complete one generation. This is significantly longer than other bacteria, such as Escherichia coli, which may divide every 20 minutes (Kaur et al., 2019). Mycobacteria have an unusually high guanine and cytosine (G+C) content (61–71 mol%) in their genomic desoxyribonucleic acid (DNA) and probably the highest lipid content in their cell walls among bacteria. They are classified in the family Mycobacteriaceae of the order Actinomycetales.

They don't stain readily, but if stained, they cannot be decolorized easily, even with a slight acid concentration, so they are also called acid-fast bacilli (AFB). The growth is generally slow. Tubercle bacilli stained with the Ziehl Neelson method using carbol fuschin or fluorescent dyes like rhodamine and auramine O.(Köksal & Yaman, 2003; Palomino, 2007).

Mycobacteria and other closely related genera (Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus, and Dietzia) show some phenotypic similarities due to similar cell wall compounds and structures (Rastogi et al., 2001).

2.3.1. Mycobacterial Cell Wall

The cell wall of *M. tuberculosis* possesses distinct characteristics that differentiate it from other prokaryotes. Robert Koch initially identified the distinctive cell wall of *M. tuberculosis* and recognized its significance in the physiology of mycobacteria. Due to its limited permeability, it confers inherent resistance to numerous antimicrobial drugs and is potentially attributed to multiple efflux pumps. The cell wall serves multiple crucial functions, including determining the bacteria's morphology and protecting it against adverse external pressures (Maitra et al., 2019).

M. tuberculosis exhibits a unique cell surface layer primarily consisting of mycolic acid, a lipid-based substance. This attribute makes the cells resistant to Gram staining, thus requiring acid-fast detection methods instead. *M. tuberculosis* demonstrates primarily an aerobic metabolism and requires substantial amounts of oxygen. *M. tuberculosis* is predominantly a pathogen that invades the mammalian respiratory system, particularly the lungs, leading to the onset of tuberculosis (Hochberg & Ryan, 2004).

The cellular structure is known as the cell wall. *M. tuberculosis* has a thickness of approximately 35 nanometers, significantly more than most other bacteria. The cell wall comprises two protective layer sections with distinct chemical compositions: the upper and lower segments. The cell wall contains peptidoglycan (PG), is covalently connected to arabinogalactan (AG), and is located beyond the membrane. Mycolic acids (MAs) have long meromycolate and short α-chains, to which the AG is attached.

The mycelial-arabinogalactan-peptidoglycan (mAGP) complex is the cell wall core. The upper section contains lipids that consist of longer fatty acids that complement shorter α-chains, as well as lipids that consist of shorter fatty acids that complement longer chains. Figure 2 illustrates the coexistence of many components, including cell wall proteins, phosphatidylinositol mannosides (PIMs), phthiocerol-containing lipids, lipomannan (LM), and lipoarabinomannan (LAM) (Brennan, 2003).

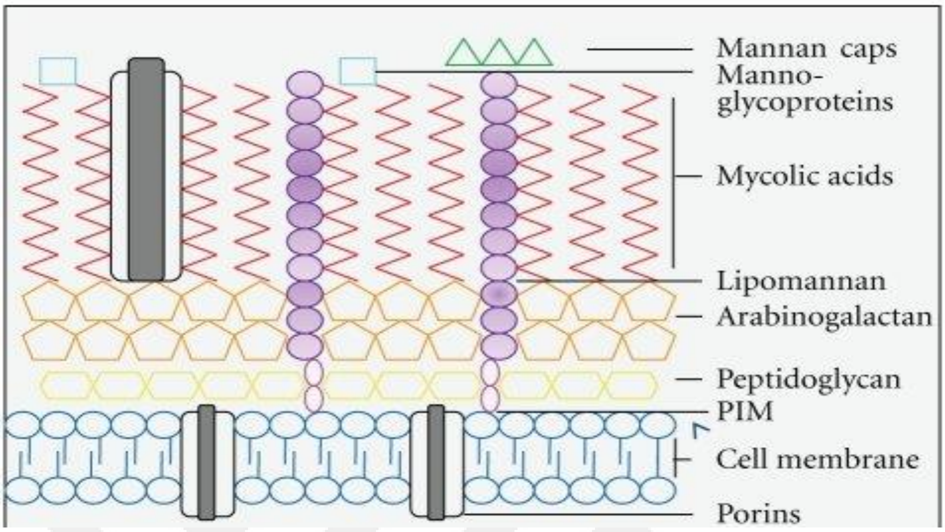


Figure 2.2. Illustrates the composition and arrangement of the cell wall in *M. tuberculosis*. This diagram shows the critical constituents of the cell wall and their respective distributions. The PG layer forms the inner layer and covalent bonds with the AG layer. The outer membrane consists of MAs, glycolipids such as (mannose-capped) lipomannan, and mannoglycoproteins. (https://www.researchgate.net/figure/The-structure-of-the *Mycobacterium-tuberculosis*-cell-wall-This-figure-shows-a-schematic_fig1_51156793)

The peptidoglycan in mycobacteria is located outside of the mycobacterial inner membrane and plays a crucial role in cell growth and cell-to-cell communication, triggering the host immune response. The peptidoglycan of *M. tuberculosis* contains a high percentage of cross-linked peptides, 70%–80%, compared to 30%–40% in *E. coli* (Alderwick et al., 2015; Maitra et al., 2019).

The investigation of the structure and characteristics of MAs membranes is a significant research topic, primarily because of their crucial function in safeguarding mycobacteria. Mycolic acids have been discovered to enhance *M. tuberculosis*'s resilience to chemical degradation and dehydration while reducing the efficacy of hydrophilic antibiotics and biocides. The presence of mycolic acids enables mycobacteria to proliferate inside macrophages, thereby evading detection by the host immune system.

Researchers investigated the membranes' structures, compositions, and characteristics. The analysis was based on the time and initial composition of the membranes. The structural composition has been demonstrated to significantly influence the thickness and density and, consequently, the permeability and strength of the membrane (Steshin et al., 2024).

Mycolic acids regulate the bacterium's permeability, acid-fast staining, viability, and pathogenicity. Mycobacteria possess the most renowned MAs esters. Trehalose dimycolates (TDMs) and trehalose monomycolates (TMMs) constitute the widely recognized esters known as MAs. The serpentine cording is responsible for the cord factor's toxicity against mammalian cells and its role as an inhibitor of polymorphonuclear leukocyte (PMN) migration. The most abundant production of the cord factor occurs in virulent strains of *M. tuberculosis*. The pathogenicity of *M. tuberculosis* culture has been linked to developing an elongated cord-like structure on agar or in a liquid medium, resulting from the aggregation and intertwining of lengthy chains of bacteria. The growth of the cord is attributed to the presence of a glycolipid known as the cord factor (trehalose 6, 6 dimycolate) on the cell surface (Madigan et al., 2006; Minnikin et al., 2015).

2.3.2. Genomic Structure of *M. tuberculosis*

In the past, investigations into drug resistance in *M. tuberculosis* mostly centered around examining genetic changes in the genes responsible for encoding antibiotic targets. These modifications increase the drugs' minimum inhibitory concentrations (MICs) above a certain level. Researchers have made progress in understanding how genetic differences affect *M. tuberculosis*. They have identified genomic sites that connect to pharmacological properties, such as small increases in MIC and tolerance. These characteristics facilitate the development of drug resistance and the subsequent inefficacy of treatment. The genome of *M. tuberculosis* provides essential information into the biology of the species and the presence of variation in genes. This knowledge forms the basis for comprehending disease progression, the immune responses implicated, and the evolutionary adaptations of *M. tuberculosis* (Fleischmann et al., 2002).

Cole et al. provided annotations for the *M. tuberculosis* H37Rv genome sequence. It is the second-largest genome sequence now readily available, following E. coli (Cole et al., 1998). The complete sequencing of the genome of the *M. tuberculosis* laboratory strain H37Rv has been accomplished. The genome has 4,411,532 base pairs and exhibits a mean Guanine (G) and Cytosine (C) content of 65.6 mol%. The organism possesses approximately 4000 genes, of which 50 are responsible for encoding functional RNA molecules (Cole, 2002; Fleischmann et al., 2002).

An essential revelation of the genomic sequence was identifying and characterizing the repeat elements found in *M. tuberculosis*. Because they may vary from strain to strain, repeat elements help type strains using molecular techniques. *M. tuberculosis* H37Rv harbored sixteen copies of IS6110 and eight copies of the more stable repeat element IS1081 (McAdam et al., 1990; Thierry et al., 1990).

The MTBC members are under clonal change and have similar genomes, with >99.9% identity over homologous regions. Horizontal gene transfers, significant recombination, and large variations in gene synteny are absent. Genetic differences between MTBC genomes are due to single nucleotide polymorphisms (SNPs) and minor indels (insertions and deletions). Despite this high degree of similarity, the different species and lineages of the MTBC acquired adaptive traits throughout evolution to differentially survive in various hosts and environments and to modulate virulence (Soler-Camargo et al., 2022).

The gain and loss of genes can only be estimated by assessing the genetic content of individual strains at the population level. Therefore, pan-genome analysis is essential for estimating the diversity of the genetic repertoire of defined bacterial groups. Most MTBC comparative genomics studies focus on evaluating SNPs and indels. Studies analyzing the genetic content of MTBC have considered strains of *M. tuberculosis*, *M. africanum*, *M. microti*, and *M. bovis*. Other MTBC species were not comparatively analyzed within the complex. There are also conflicting reports on the openness of *M. tuberculosis* or MTBC pan-genome (Silva-Pereira et al., 2024).

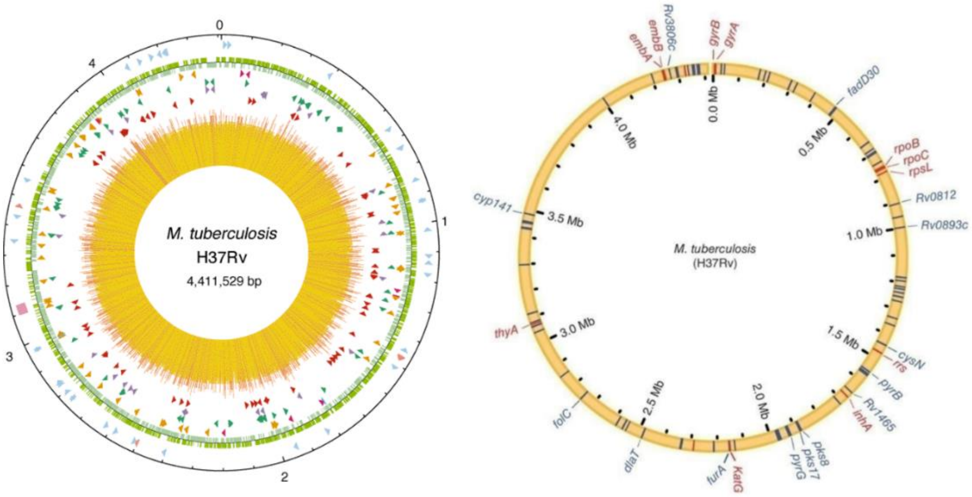


Figure 2.3. Circular map of the chromosome of *M. tuberculosis* H37Rv (Cole et al., 1998)

The genome contains a substantial proportion of repetitive DNA, such as insertion sequences (IS), along with recently emerged multigene families and duplicated housekeeping genes. Over 51% of the genes in *M. tuberculosis* appear to have been created by gene duplication or domain shuffling processes. Insertion sequences and prophase (phi Rv 1, phi Rv 2) constitute 3.4% of the genome. There are 56 IS elements belonging to the widely recognized IS3, IS5, IS21, IS30, IS110, IS256, and IS1535. In addition, thirty-two other types of repeat elements were found in the genome. The discovery of new, repetitive, polymorphic DNA elements of *M. tuberculosis* may facilitate novel approaches for the molecular typing of *M. tuberculosis*. A second significant discovery was the identification of numerous regulatory genes. Thirteen RNA polymerase sigma factors were found. Sigma factors are the promoters that recognize the subunit of bacterial RNA polymerase and are used for conditional gene expression in bacteria. This suggests that *M. tuberculosis* has a rich repertoire of adaptive strategies mediated by these transcription factors. This abundance of regulatory proteins indicates that *M. tuberculosis* is a highly adaptive pathogen (Manabe & Bishai, 2000). The H37Rv and H37Ra strains of *M. tuberculosis* have been extensively utilized as standard strains for investigating the virulence and pathogenesis of the bacterium on a global scale since the 1940s. H37Ra is employed as an adjuvant to enhance the immunogenicity of the

BCG vaccine during immunization. The genome of H37Ra has a significant degree of similarity to that of H37Rv in terms of DNA content and arrangement (Zheng et al., 2008).

2.4. Pathogenesis of *M. tuberculosis*

Tuberculosis aerosols spread throughout the environment and start the TB infection process. Disseminating a quantity of one to 10 bacilli in the air significantly increases the chance of transmission. The alveolar macrophage cells in the patient's lung engulf the bacilli through phagocytosis before infiltrating the underlying epithelium. In this context, monocytes originating from the adjacent blood vessels initiate the formation of a granuloma as the immune system endeavors to combat the disease.

The bacteria's virulence and host macrophages' ability to kill them determine the probability of inhaled tubercle bacilli causing infection. *M. tuberculosis* does not cause a fast immune response due to its absence of endotoxins or exotoxins. Infected macrophages secrete cytokines and chemokines, which recruit other phagocytic cells and assemble into a nodular granuloma called a tubercle complex. The slow growth of tubercle bacilli, dividing every 25–32 hours, occurs within the macrophages due to the failure of alveolar macrophage defense. Figure 2.3 shows that the granuloma comprises infected macrophages gathered in the middle and foamy macrophages, mononuclear phagocytes, and lymphocytes around the edges.

Tubercle infiltrates the lymph nodes responsible for drainage and progresses into lymphadenopathy, forming lung lesions known as Ghon. The area is known as the Ghon focus and is characterized by immune cells, dead bacteria, and other inflammatory components. Over time, the immune system may successfully confine the infection to the Ghon focus, inhibiting the dissemination of the bacteria to other body regions (Society, 2003; Hill et al., 2004).

The result is the development of a fibrous capsule that contains a high quantity of foamy macrophages. The creation of characteristic caseous debris (necrotic tissue like cheese) at the center of the granuloma is thought to be caused by these microorganisms. The caseous center, although initially contained immunologically, tends to undergo liquefaction and cavitation, releasing many *M. tuberculosis* bacilli into the airway.

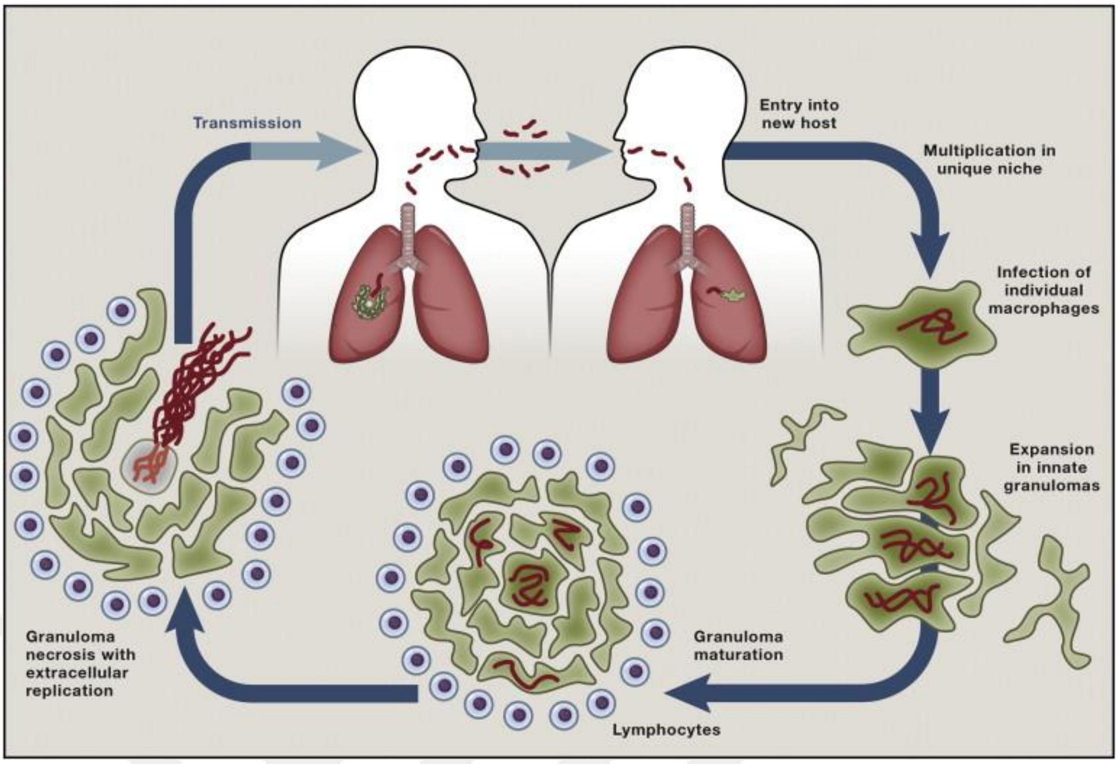


Figure 2.4. Pathogenic Life Cycle of *M. tuberculosis* (Cambier et al., 2014)

This is a characteristic that clearly distinguishes TB from other diseases. The cycle concludes when the compromised lungs generate a cough that again carries the extremely contagious droplet nuclei accountable for transmission. The lymphatic system can disseminate infected macrophages to multiple structural sites, such as the lungs, lymph nodes, kidneys, epiphyses of the long bones, and other locations. Immunocompromised individuals, such as those with AIDS, may have infected macrophages present in their bloodstream. After three to eight weeks, even though there is a high occurrence of infection, there are no immediate signs or symptoms except for a positive tuberculosis skin test (TST) (Wani, 2013).

People with strong cell-mediated immunity make granulomas with the help of T-cells and macrophages. These granulomas stop *M. tuberculosis* from spreading and multiplying, leading to latent tuberculosis infection. About 10% of individuals with latent tuberculosis have the potential to develop an active illness. The disease progresses fast from hilar or mediastinal lymph nodes to bronchial cavitation, leading to pneumonia, particularly in youngsters, older adults, and those with AIDS. Children specifically exhibit localization of caseous material in this area, which is seen in acute miliary tuberculosis or tuberculosis meningitis. Patients aged 30 to 65 exhibit a more favorable prognosis than individuals in the categories of children, adolescents, young adults, and the elderly. This is attributed to their lower likelihood of experiencing tissue necrosis. Hypersensitivity typically arises 3 to 8 weeks after infection, indicating the activation of cellular immunity and the control of the infection. At present, a skin test reaction will produce a positive outcome, indicating the existence of a latent infection (Dannenberg Jr & Rook, 1994; Society, 2003).

2.4.1. Transmission of *M. tuberculosis*

The transfer of Tubercle bacilli from an infected individual to a non-infected individual is mainly caused by inhaling tiny particles called droplet nuclei, which have a diameter ranging from 1 to 5 microns. When an infected individual sneezes, coughs, shouts, or sings, they release an infectious droplet into the environment. It is widely recognized that a sneeze can generate approximately 40,000 droplets, whereas a cough can create up to 3,000 droplets.

The droplets can stay airborne for extended periods, depending on their size and surroundings. *M. tuberculosis* transmits tuberculosis, an airborne infectious respiratory disease. Transmission occurs when an individual breathes in droplet nuclei that contain *M. tuberculosis*. These droplets then migrate from the nasal channel to the upper respiratory tract and eventually reach the bronchi and alveoli of the lungs. It is important to note that not every infected person will develop the disease. Several elements influence the transmission's ability to infect a person or the likelihood of transmission. The four primary elements accountable for this are infectiousness, environment, susceptibility, and exposure. The infection rate is based on the individual's immune system. Environmental factors, including the closeness, frequency, and length of exposure, influence the concentration of *M. tuberculosis*.

These mycobacteria are microorganisms that infect the cells of people and animals; formerly, *M. bovis* was transmitted to individuals via milk before the discovery and adoption of milk pasteurization (C. O. WHO, 2020). *M. caprae* is recognized for inducing TB infection in livestock. At the same time, *M. pinnipedii* can transmit the infection to humans through sea lions, and *M. microti* can be transmitted through pet animals. Additionally, there are characteristic MTBC species commonly found in people, such as *M. africanum* and *M. canetti*i. However, *M. tuberculosis* is the most widespread strain of the MTBC in humans and continues to impact a significant portion of the population today (Jagielski et al., 2016).

Furthermore, individuals can inhale them through sputum induction, aerosol treatments, and when handling or manipulating secretions in the laboratory (Society, 2003). Transmission is contingent upon the quantity and virulence of bacilli, the degree of room ventilation, the extent of UV radiation exposure to bacilli, and the immunological status of the individual inhaling the droplets. As a result, effective TB screening, careful room design, adherence to safety protocols, and prompt treatment of active cases can stop the spread of TB (Nardell, 2016).

Tracing people who have had intimate contact with TB patients can effectively stop transmission in nations with low incidence rates. Meanwhile, countries with a high prevalence of infections, which are primarily low and middle-income countries, are facing a burden of active cases without adequate resources to conduct comprehensive investigations and trace persons in close contact (Morrison et al., 2008; Shi & Sugawara, 2013).

2.4.2. Risk Factors for *M. tuberculosis*.

Specific risk factors that elevate the probability of an individual acquiring MTBC have been found. These risk factors encompass proximity to an individual with active tuberculosis, residing or working in densely populated or inadequately ventilated settings, and compromised immune systems, such as those of individuals who are HIV positive or who utilize corticosteroids or immunosuppressive medications (Society, 2003; Friedland, 2009). HIV-positive individuals have a 100-fold higher risk of *M. tuberculosis* infection compared to those who are HIV-negative. Additional notable risk factors involve people with diabetes, smoking, drug abuse, and alcohol abuse. Socioeconomic determinants, such as malnutrition, are linked to health insurance (Davies & Pai, 2008; C. O. WHO, 2020). Furthermore, *M. tuberculosis* is associated with various human diseases, such as lung cancer, systemic lupus erythematosus (SLE), and obesity (Chai et al., 2018).

2.4.3. Virulence Factors of the *Mycobacterium tuberculosis* Complex

The virulence of MTBC species has evolved mechanisms to evade or influence the immune response in their favor. Certain bacteria can persist in a latent state within the granuloma for several decades without causing active clinical symptoms. During any immunosuppressive state, latent bacteria have the potential to reactivate, reproduce, and disseminate throughout the lungs and other bodily tissues. Most of these virulence genes encode enzymes that participate in various lipid processes, cell surface proteins, regulators, and proteins of signal transduction systems. Another notable group focuses on the capacity of mycobacteria to stay alive inside the host's macrophage environment. Mycobacteria do not possess conventional virulence factors, such as toxins, commonly seen in other bacterial infections (Chugh et al., 2024).

The virulence factors have been categorized based on their function, molecular characteristics, or cellular localization metabolism of lipids and fatty acids, particularly the breakdown of cholesterol. Cell envelope proteins encompass various types, such as cell wall proteins, secretion systems, and lipoproteins. Proteins that hinder the activity of antimicrobial agents in macrophages, namely those involved in responding to oxidative and nitrosative stressors, stopping the movement of phagosomes, and preventing apoptosis, are also protein kinases. Gene expression regulators encompass mechanisms such as two-component systems, transcriptional regulators, and sigma factors. There are two categories of metal-transporter proteins: exporters and importers. This includes proteases like metalloproteases (Silva-Pereira et al., 2024).

The Early Secreted Antigenic Target of 6 kDa Protein (ESAT-6), which is the primary factor responsible for the virulence of *M. tuberculosis*, is mainly released into the bloodstream and sputum of individuals with TB. It has been recognized as a biomarker that can be used to diagnose TB quickly. A recombinant BCG vaccine that secretes ESAT-6 protein increases the immune response against TB in mice, and the N-terminally formylated ESAT-6 protein, either alone or in conjunction with anti-TB medicines, improves the therapeutic impact against experimental TB moderately. Furthermore, ESAT-6 plays a role in various immunological responses such as T-cell differentiation, antigen presentation, apoptosis, autophagy, and necrosis (Mir & Sharma, 2022; Sun et al., 2024).

Toll-like receptors (TLRs) are a subset of pattern recognition receptors (PRRs), essential protein molecules that contribute to the functioning of the innate immune system. The macrophages have TLR1, RTL2, TLR3, TLR4, TLR7, and TLR9. TLR2 and TLR4 play a role in promoting inflammatory responses and the infection produced by *M. tuberculosis*. Macrophages experienced TLR2-induced inflammation, which promoted the progression of hepatic fibrosis. TLR4 inhibition can alleviate inflammation in human hepatocellular carcinoma cells (Sun et al., 2024).

2.4.4. Type of Tuberculosis and Symptoms

Tuberculosis can infect any part of the body and causes symptoms that are widespread and not unique to any organ. The prevalent symptoms include sudden weight loss, fever, decreased appetite, and nocturnal sweat. TB infection can be categorized based on the specific organs affected. The two types of TB are Pulmonary Tuberculosis and Extrapulmonary Tuberculosis (Lee, 2015).

2.4.4.a. Pulmonary Tuberculosis

Pulmonary tuberculosis refers to active TB, specifically within the lungs. The most prevalent clinical presentation is a cough accompanied by sputum. Haemoptysis, the coughing up of blood, can be found in individuals with chronic diseases and related symptoms. Pleural pain may be present, primarily caused by inflammation of the lung parenchyma close to the pleural surface (Karkhanis & Joshi, 2012).

Abnormalities in chest X-rays are highly prevalent. TB that arises from the internal activation of a latent infection typically leads to abnormalities in the upper lobes of the lungs, with the occurrence of cavities being frequent. Bronchopneumonia may develop in the later stages of an illness when contaminated material spreads across the airways, forming patches in different lung areas. Possible symptoms include a persistent cough lasting over two weeks, fatigue and fever, unintended weight loss, night sweats, chills, and difficulty breathing (Stevic & Odalovic, 2020).

2.4.4.b. Extrapulmonary Tuberculosis

TB can affect organs other than the lungs, resulting in a condition known as TB lymphadenitis. This frequently occurs as a TB type that affects organs outside the lungs. Lymph nodes, such as the cervical lymph node or any other lymph node, can get infected with this condition. The primary indication is the enlarged lymph node, accompanied by other typical signs of infection. Another manifestation of TB is skeletal tuberculosis, which occurs when the infection travels from the lungs or lymph nodes to the bones. This can have an impact on the spine and joints as well.

The symptoms associated with this condition include intense dorsalgia, rigidity, inflamed abscesses, and skeletal malformations. Additional manifestations of TB outside the lungs include miliary tuberculosis, genitourinary tuberculosis, tuberculous pleural effusion, TB peritonitis, TB pericarditis, and uncommon cases of cutaneous tuberculosis (Lee, 2015).

2.5. Global and Türkiye Epidemiology of Tuberculosis

There is notable variance in the geographic distribution of the MTBC species. *M. tuberculosis* is the primary causative agent of tuberculosis in Europe and America, while *M. africanum* is frequently observed in African individuals with tuberculosis, notably in West Africa. The TB epidemic in Africa primarily arises from localized epidemics, including distinct lineages of the *M. tuberculosis* bacterium (Niemann et al., 2000; Chihota et al., 2018). *M. tuberculosis* in these locations could have been brought from either Europe or Asia and has disseminated through pastoralism, mining, and warfare. *M. tuberculosis* lineages display a phylogeographical structure, indicating that specific lineages are strongly linked to particular geographic regions and prefer to infect individuals from these places. Turkey serves as a geographical and anthropological bridge connecting Europe and Asia. It is an ancient region of human habitation situated in the western section of Eurasia (Comas et al., 1996; Yeboah-Manu et al., 2016)

Efforts to understand the genetic structure of the MTBC population and the species present in specific geographic regions are becoming more relevant for controlling TB. Different lineages of *M. tuberculosis* have been analyzed and recorded using geospatial methods. Recent genomic research has shown significant genetic variation among the other members and genotypes of the MTBC. This variation could have significant consequences for creating and using new TB vaccines and diagnostic tools (Yeboah-Manu et al., 2016; Chihota et al., 2018; Kapona, 2019).

Although the fight against TB in Turkey has a history spanning more than a century, the systematic efforts for the control and eradication of TB have been hampered by the 2007 TB eradication guidelines adopted by the WHO in 2007. National Tuberculosis Control Program" aligns with the strategy to stop TB and with the same standards. The Ministry of Health provides the planning, infrastructure, budget, and human resources required to achieve this goal. Within the scope of this program, medicines are supplied and distributed from health institutions to all citizens with TB or who are in contact, regardless of any social security. In addition, the data of each diagnosed TB patient registered at dispensaries and health institutions is collected, analyzed, and reported. The data obtained are published in national tuberculosis reports. At the same time, these data are entered into the "World Health Organization Tuberculosis Network" and contribute to preparing the Global Tuberculosis Reports.

According to the 2018 report by the WHO, Turkey's TB prevalence is reported as 0.18%, the estimated incidence rate is 17/100,000, and the estimated mortality rate is 0.53 per thousand. The report states that 3.3% of the cases detected in Turkey are new cases, while 14% have previously received TB treatment. Treatment success rates in new and relapsed cases were reported at 87% in 2016, while previously treated cases had a success rate of 51%.

In the Turkey 2019 Report, published in 2020 by the Turkish public health institution, 12,046 TB patients were registered in health institutions in 2017, and 68 of these cases were found to be HIV+. The total case rate was determined as 14.9/100,000 according to WHO in 2016; 211 of the TB cases seen were MDR-TB, 9 were XDR-TB, while 191 of the cases seen in 2017 were MDR-TB and 2 were XDR-TB (Başkanlığı, 2020; Organization, 2020).

In the global tuberculosis report published by the WHO in 2021, the incidence of TB in Turkey was reported at 18 per 100,000, and the estimated mortality rate was 1.1 per 100,000. In the same report, the number of new and relapse cases reported from Turkey in 2021 was 9047, and 41% of these cases were female, 55% were male, and 4% were children (0–14 years old). It was determined that 65% of the cases reported in the relevant report were pulmonary tuberculosis cases, and 35% were extrapulmonary tuberculosis cases (Organization, 2022a).

The proportions of previously treated cases among the total patients were determined as follows: 6.0% (744) relapse cases, 1.3% (167) return after treatment abandonment, 0.5% (62) treatment failure cases, and 0.02% (2) chronic cases. Among the patients, 61.3% (7,616) had pulmonary involvement, 33.6% (4,169) had extrapulmonary organ involvement, and 5.1% (632) showed both pulmonary and extrapulmonary involvement. When the results of drug sensitivity tests were analyzed for 6,037 patients, it was found that 19.2% (1,161/6,037) developed resistance to at least one drug. The highest resistance rate was observed against INH, with 11.9% (720/6,037), and 33% (200/6,028) of these were found to be MDR-TB. The rate of MDR-TB was 2.1% (115 cases) in new cases and 14.2% (85 cases) in previously treated cases. Among MDR-TB cases, 9 had XDR-TB, and it was determined that only one of these cases was born in Turkey, while the remaining eight were born in other countries.

The social dimension of TB is often undervalued in numerous studies. The sex ratio of men to women indicates a much higher proportion of men (3 times more) than women who are affected by *M. tuberculosis*. In addition, the global rates of resistance to drugs in men are twice as high as those in women. The age group of 17–39 years comprises 80% of the strains investigated, indicating a particular susceptibility of young males to TB (Talarico et al., 2005)

2.6. Tuberculosis and HIV Infection

A rise in HIV transmission has played a substantial role in the global increase in tuberculosis cases. The primary and most significant risk factor for TB is HIV, resulting in an estimated 703,000 individuals being co-infected with both TB and HIV (Organization, 2022a). The concurrent presence of TB and HIV infection has significantly complicated and hindered the effectiveness of the TB control effort. Tuberculosis has emerged as the primary cause of mortality among those who have HIV/AIDS, HIV infection being the most influential factor in the progression of latent TB infection to active TB. The death rate for those with both HIV and TB infections is five times higher than that for individuals with TB infection alone, as is the likelihood of developing drug-resistant strains of mycobacteria (Davies & Pai, 2008).

It is expected that places with a high incidence of HIV infection will experience a significant global rise in TB rates. Individuals infected with HIV have a substantially higher rate of reactivation (10% per year) compared to those without HIV infection. Furthermore, individuals with HIV who also have tuberculosis suffer a reduced lifespan and a more rapid progression to AIDS compared to those without tuberculosis. This underscores the criticality of immediately diagnosing and treating tuberculosis (Valadas & Antunes, 2005). The mortality rate of TB/HIV co-infection is highly high globally, with a mortality rate of 33% among individuals who are HIV-positive among TB cases (Organization, 2009).

Immune reconstitution inflammatory syndrome (IRIS) is being more recognized in poor nations as a significant consequence resulting from the concurrent use of anti-retroviral (ARV) and anti-TB medications (Meintjes et al., 2009). Immune reconstitution inflammatory syndrome manifests in 8–43% of individuals who start antiretroviral therapy while undergoing tuberculosis treatment (Meintjes et al., 2009). It is understood that tuberculosis IRIS is caused by the inhibition of viral reproduction induced by ARV treatment and the subsequent restoration of the immune system, which leads to the reawakening of tuberculosis-specific immunological reactions. Predictive factors for developing IRIS include a low CD4 cell count, early initiation of ARV treatment, and extrapulmonary TB (Friedland, 2009). A study conducted by Baba and colleagues (2008) determined the correlation between a very low CD4 count (median of 59 cells/µl) and the diagnosis of patients with TB and HIV co-infection. The study's results indicated that adenosine deaminase activity can be a sensitive marker for patients with a low count. This is an essential finding for countries with a high burden of TB and HIV co-infection (Baba et al., 2008). As the HIV infection advances and the CD4 count decreases, the occurrence of extra-pulmonary illness becomes more frequent. (Davies & Pai, 2008; Friedland, 2009).

Emerging research indicates a potential correlation between HIV and MDR-TB. The prevalence of MDR-TB has been extensively reported in healthcare facilities and other communal environments among HIV-positive individuals (Gandhi et al., 2006). Several reports on drug resistance in TB have found a strong correlation between being HIV-positive and having MDR-TB in Latvia and the Donetsk Oblast of Ukraine. Moreover, in Lithuania, TB patients who were HIV-positive had 8.4 times greater chances of carrying MDR-TB strains compared to TB patients whose HIV status was not known. The initial findings of a survey carried out in Mozambique in 2007 have also discovered a noteworthy correlation between HIV and MDR-TB. (Control, 1991; Organization, 2010).

2.7. Prevention of *Mycobacterium tuberculosis* Complex Infections

Vaccination strategies are a crucial component of preventing MTBC infections. The Bacillus Calmette-Guérin (BCG) vaccine is the single authorized vaccine for TB prevention and is given to babies in countries with a high disease prevalence. Nevertheless, its effectiveness in preventing adult pulmonary tuberculosis is restricted (Skeiky & Sadoff, 2006). Therefore, research is ongoing to develop more effective vaccines, including pre-exposure prophylactic vaccines and post-exposure therapeutic vaccines. These vaccines are specifically engineered to stimulate the immune system to identify and attack MTBC, preventing the development of active TB disease (Qu et al., 2021).

Infection control measures are crucial for preventing the transmission of MTBC infections. These measures include implementing respiratory protection programs, training healthcare workers in proper infection control procedures, and accommodating patients with pulmonary TB in unfavorable pressure or respiratory isolation rooms (Kuyinu et al., 2016). Additionally, environmental and respiratory protective measures, such as proper ventilation and protective equipment, effectively decrease the risk of spreading TB in healthcare settings. Administrative measures, such as screening and isolating patients with suspected or confirmed TB, are also critical in preventing the spread of MTBC infections (Lai et al., 2023).

Public health interventions are necessary to combat the global burden of TB. These interventions include targeted screening and treatment of high-risk populations, such as HIV-positive persons, healthcare workers, and individuals in congregate settings. Public health should also focus on improving TB diagnostic and treatment facility availability, promoting treatment adherence, and addressing social determinants of health contributing to TB transmission (Shamputa et al., 2023).

Furthermore, there is a need for epidemiological research and targeted screening and public health interventions for TB in sewage. These efforts can help reduce the incidence of TB and prevent the emergence of drug-resistant strains of MTBC. Overall, a comprehensive approach that includes vaccination strategies, infection control measures, and public health interventions is necessary to prevent the spread of MTBC infections and combat the global TB epidemic (Heldal et al., 2003; Ngqinayo, 2019).

2.8. Main Drugs Used in Tuberculosis Treatment and Resistance Mechanisms

An important strategy to manage TB is to prevent the transmission of the disease by identifying people who have had contact with TB patients and administering suitable therapy. Treatment options for TB were limited until the mid-1940s, when streptomycin (STR), para-aminosalicylic acid (PAS), and isoniazid (INH) were discovered. Subsequently, the introduction of rifampicin (RIF), ethambutol (ETB), and pyrazinamide (PZA) facilitated the availability of "short-course chemotherapy (SCC)" (Sharma & Mohan, 2003).

People with active TB harbor TB bacilli and exhibit signs and symptoms of the disease. These bacilli can be identified using laboratory tests. Furthermore, these people can transmit the disease to others through droplet aerosols generated by coughing, sneezing, and talking (Guerra-Assunção et al., 2015); during the latent stage of TB, an individual harbors a minimal quantity of TB bacilli. The immune system effectively regulates these microorganisms, preventing any manifestation of signs or symptoms in the host. Furthermore, individuals with latent TB may have unremarkable findings on chest X-rays and yield negative results on laboratory TB testing. Individuals are determined to be infected with latent TB when they have a positive result on a skin test. (Yew et al., 2018).

The therapy for tuberculosis produced by active *M. tuberculosis* consists of two stages. The first stage, referred to as the "intensive phase," has a duration of two months and necessitates the administration of four main drugs: RIF, INH, PZA, and (EMB) or STR. The second phase, lasting four months, is referred to as the "continuation phase" and involves the administration of two drugs: INH and RIF. If the strain is susceptible, adhere to the World Health Organization's guidelines and administer a six-month course of first-line medication treatment (Dye et al., 1999). These medications exhibit lower toxicities and increased potency. Consistent adherence to the medication regimen for six months is crucial for eradicating TB bacilli. However, discontinuing or skipping doses of the medication during therapy can potentially result in the development of antibiotic resistance. Treatment should be administered by a qualified professional, particularly in cases where the disease has developed resistance or when dealing with extrapulmonary tuberculosis.

Efficient treatment protocols required the inclusion of second-line drugs, such as those mentioned below: aminoglycosides (kanamycin or amikacin) and fluoroquinolones, such as moxifloxacin, levofloxacin or gatifloxacin, and polypeptides (capreomycin). Second-line medications are frequently employed to treat cases of MDR-TB and XDR-TB. These drugs are generally more toxic and less effective compared to first-line drugs (Jassal & Bishai, 2009; Bansal et al., 2018).

The emergence of drug resistance in TB, which has become a substantial issue since the early 1990s, frequently occurs due to incorrect treatment techniques. Resistance to anti-TB drugs can develop through various mechanisms, including decreased permeability, increased efflux mechanisms, enzymatic inactivation, changes in antibiotic targets, and reduced activity of enzymes that activate prodrugs. These mechanisms can be categorized into five categories (Zhang et al., 2004).

TB resistance mechanisms can occur naturally (intrinsically) or acquirely. Natural resistance refers to resistance that does not result from genetic changes, while acquired resistance arises due to genetic material mutation or transfer. Many natural resistance mechanisms exist in *M. tuberculosis.* The unique structure of the TB bacillus reduces the efficacy of anti-TB medications, as its lipid-rich cell wall inhibits drug absorption and serves as a mechanism that hinders the host's immune response. It develops physiological adaptations to various settings encountered during infection and, through entering a dormant state, creates resistance to drugs that disrupt the cellular processes required for growth (Gold & Nathan, 2017). Some natural resistance mechanisms in *M. tuberculosis* include having a highly hydrophobic cell surface that provides permeability for some antibiotics, secreting certain enzymes like beta-lactamases to render penicillin ineffective, and possessing efflux pumps that are effective against tetracycline, fluoroquinolones, or aminoglycosides (Hartman et al., 2017).

In the case of acquired resistance, *M. tuberculosis* mostly depends on single or multiple changes in the genetic code (nucleotide polymorphisms), as well as insertions and deletions in specific genes responsible for the effectiveness of drugs. This contrasts with many bacterial infections, which often acquire resistance through horizontal gene transfer. Table 2.3 presents these findings. Researchers have a comprehensive understanding of the relationship between genetics and phenotype in certain instances of resistance to anti-TB treatment. Cross-reactions between anti-tuberculosis medications belonging to the same or separate categories are possible. For example, alterations in the *gyrA* and *gyrB* genes can lead to fluoroquinolone resistance. In contrast, modifications in the *rpoB* gene that cause resistance to RIF can also confer resistance to other rifamycins (Hoagland et al., 2016; Chiaradia et al., 2017; Schön et al., 2017).

Table 2.3. Resistance-conferring mutations are usually found in the *M. tuberculosis* genes, which the WHO classifies along with drugs used for TB treatment (Papaventsis et al., 2017; Koch et al., 2018)

Drug	Mechanism of action	Genes associated with resistance
First-line oral drugs		
Rifampicin	Inhibition of RNA synthesis	*rpoB, rpoA, rpoC*
Isoniazid	Inhibition of mycolic acid synthesis	*katG, inhA, oxyR-ahpC, fabD, fabG1, kasA, accD, oxyR, ndh, fadE24, nat, kasA, mabA, p_inhA, accD6, efpA, fpbC, Rv1592C, Rv1772, Rv2242*
Ethambutol	Inhibition of arabinogalactan biosynthesis	*embA, embB, embC, embR, manB, PPE49, rmlD, manB, iniA, iniB, iniC, Rv3124,*
Pyrazinamide	Disruption of energy homeostasis; inhibition of trans-translation and coenzyme A biosynthesis	*pncA, panD, rpsA*
Group A: Fluoroquinolonesd		
Levofloxacin	Inhibition of DNA synthesis	*gyrA, gyrB*
Moxifloxacin	Inhibition of DNA synthesis	*gyrA, gyrB*
Gatifloxacin	Inhibition of DNA synthesis	*gyrA, gyrB*
Group B: second-line injectable drugs		
Kanamycin	Inhibition of protein synthesis	*rrs, eis, whiB7, tlyA*
Amikacin	Inhibition of protein synthesis	*rrs, eis, gidB, tlyA, whiB7*
Capreomycin	Inhibition of protein synthesis	*rrs, eis, tlyA, gidB*
Streptomycin	Inhibition of protein synthesis	*rpsL, rrs, gidB*
Group C: other second-line drugs		
Clofazimine	Disruption of energy metabolism	*Rv0678*
Linezolid	Inhibition of protein synthesis	*trl, rpIC*
Cycloserine	Inhibition of peptidoglycan biosynthesis	*alr, ddl, cycA*
Terizidone	Inhibition of peptidoglycan biosynthesis	*rpoC, alr, ddl, cycA*
Ethionamide	Inhibition of mycolic acid biosynthesis	*ethA, ethR, p_inhA, inhA, fabG1*
Prothionamide	Inhibition of mycolic acid biosynthesis	*p_ethA, ethA*
Group D: other additional drugs		
Para-aminosalicylic acid	inhibition of folic acid and thymine nucleotide metabolism	*thyA, folC, dfrA, ribB, ribD*
Delamanid	Inhibition of mycolic acid biosynthesis	*Ddn, fdg1*
Thioacetazone	Inhibition of mycolic acid biosynthesis	*EthA*
Bedaquiline	Inhibition of ATP synthesis	*atpE, Rv0678*
Imipenem, Meropenem	Inhibition of cell wall biosynthesis	*Rv2421c-Rv2422*

2.8.1. First Line Anti-TB Drugs

2.8.1.a. Streptomycin

Streptomycin (STR), an antibiotic from the aminoglycoside class, was extracted from the bacteria *Streptomyces griseus*. It was the initial medication used for the treatment of tuberculosis. It interacts directly with the 16S rRNA small ribosomal subunit and interferes with translational processes, suppressing protein synthesis (Zhang et al., 2005). Mutations in the genes responsible for encoding 16S ribosomal RNA (*rrs*) and ribosomal protein S12 (*rpsL*) are associated with high-level resistance to STR in *M. tuberculosis* (Gygli et al., 2017). Around 65-75% of strains exhibiting resistance to STR show mutations in the *rpsL* or *rrs* genes, which are directly linked to the emergence of resistance. Exploring additional loci or alternative mechanisms accountable for STR resistance was imperative. Later on, it was discovered that changes in the gene that codes for a 7-methylguanosine (m7G) methyltransferase (*gidB*), which is responsible for producing the 16S rRNA, led to a reduced level of resistance to STR. Both susceptible and resistant strains of bacteria have been found to exhibit mutations in the *gidB* gene (Johnson et al., 2006; Palomino & Martin, 2014).

Most point mutations in STR-resistant isolates occur in the *rpsL* gene. The predominant mutation occurs at codon 43, where an AAG→AGG alteration leads to a replacement from Lys→Arg. Less frequently, a substitution of AAG→ACG (Lys→Thr) is found (Musser, 1995). Point mutations can also be found in codon 88, leading to amino acid changes from Lysine to Arginine (AAG→AGG) or Lysine to Glutamine (AAG→CAG). In their study, Sreevatsan et al. (1996) analyzed 78 isolates that displayed resistance to STR and 61 isolates that exhibited sensitivity to STR. The objective was to examine the mutations in the *rpsL* and rrs genes and ascertain the prevalence of these variants among patients from different regions around the globe. *rrs* mutations were identified in either the 915 or 530 regions in 8% of the isolates that exhibited resistance. Furthermore, 54% of the isolates resistant to STR had mutations in either codon 43 or codon 88 of the *rpsL* gene. Some isolates exhibited distinctive combinations of mutations, including changes such as Lys43Arg, Lys88Arg, Val93Met, and Arg9His (Sreevatsan et al., 1996)

2.8.1.b. Isoniazid

Isoniazid, also known as isonicotinic acid hydrazide (INH), was launched in 1952 as a crucial first-line medication for TB treatment alongside RIF (Bernstein et al., 1952). INH has a bacteriostatic effect within 24 hours of treatment, subsequently changing to a bactericidal effect. Current short-course chemotherapy for TB is mainly based on this approach, which is also commonly used to treat latent *M. tuberculosis* infection (LTBI) to prevent the development of active disease and subsequent transmission of TB (Hu et al., 2010). INH resistance is often an initial stage in the evolution of MDR-TB strains. The development of INH resistance in *M. tuberculosis* is caused by mutations in many genes, the most common of which are *katG*, *inhA*, and *ahpC* (Dye & Espinal, 2001; Hu et al., 2010).

The *katG* gene creates the catalase-peroxidase *KatG* enzyme. This enzyme helps INH become active by changing it from a prodrug to its active form. The isonicotyl radical is a highly reactive chemical species that bonds with the nicotinamide group of nicotinamide adenine dinucleotide (NAD+). This results in the formation of an INH-NAD adduct. In *M. tuberculosis*, the enoyl carrier protein reductase *inhA* is believed to be the main target of INH. The enzyme *inhA*, known as enoyl acyl carrier protein reductase (ENR), plays a vital role in the type II fatty acid synthesis pathway of *M. tuberculosis*. INH-NAD adducts inhibit the activity of *inhA*; this inhibition hinders the production of MA and leads to the destruction of bacterial cells (Oliveira et al., 2006; Wang et al., 2011; Rickman et al., 2013).

Mutations in the *inhA* and *katG* genes are the primary causes of INH resistance in *M. tuberculosis*. The molecular weight of *katG* is 80 kDa, and its gene length is 2,223 bp, whereas *inhA* has a molecular weight of 28 kDa and a gene length of 810 bp. (Bollela et al., 2016; Hameed et al., 2018). Mutations in these genes largely contribute to isoniazid resistance mechanisms. Both INH-resistant and INH-sensitive strains showed genetic alterations in *Rv0340-0343*, *efpA*, *fadE24*, and *kasA*. Within the *katG* mutations, it was noted that a significant number of mutants exhibited a loss of catalase activity, but not all mutants displayed this characteristic. The precise contribution of catalase to INH resistance has been uncertain for an extended period. The hypothesis posited that the decrease in catalase-peroxidase activity suggested that the enzyme functioned as an activator for INH. Since *katG* is the only enzyme that can activate isoniazid, loss of function by preventing activation may lead to resistance. When functional *katG* was transformed into isoniazid-resistant strains of *M. smegmatis*, antibiotic susceptibility was restored (Vilchèze & Jacobs Jr, 2019).

The *katG* Ser315Thr (S315T) mutation is the predominant mutation found in INH-resistant bacteria, representing 50-95% of the clinical isolates resistant to INH (Ando et al., 2010; Laurenzo & Mousa, 2011).

The incidence of the *KatG* S315T mutation varies among different geographic locations in the resistant strains. Wojtyczka et al. (2004) examined the *KatG* S315T mutation prevalence in Poland, accounting for 30% of INH-resistant isolates. The *KatG* S315T mutation is responsible for 93.6% of INH resistance isolates in Russia. Previously, there were known mycobacterial isolates that entirely lacked the *katG* gene. (Mokrousov et al., 2002).

The most prevalent type of gene mutation is a transversion mutation occurring in the *katG* gene and the *inhA* promoter region. No deletions or insertions have been detected in the *katG* gene in any isolates. The bacteria are believed to strongly prefer amino acid substitution (S315T) in *katG*. The presence of the *katG* (S315T) alteration in *M. tuberculosis* isolates shows considerable global heterogeneity, especially with the prevalence of TB. Regions with a significant prevalence of tuberculosis, particularly with a predominance of Beijing and MDR-TB strains, have shown a higher frequency of this genetic alteration. In areas where TB is uncommon, the *katG* (S315T) substitution is minimal (Hu et al., 2010).

Molecular genetic analysis has revealed a two-gene operon that includes a continuous Open Reading Frame (ORP) known as fagG1 and *inhA*. The function of this operon is to synthesize mycolic acid and contribute to resistance against both INH and ethionamide (ETH). The protein synthesized by fagG1, often called *mabA*, exhibits the highest resemblance to the 3-oxoacyl-acyl carrier protein (ACP) reductase encoded by *fabG*. The *fabG*l and *inhA* enzymes have a role in mycolic acid biosynthesis. (Larsen et al., 2002).

Studies conducted in Bangladesh and Nepal have found that 10% and 34% of cases with resistance to INH show mutations in the *inhA* promoter region. Drug resistance in *M. tuberculosis* can also occur through an overexpression mutation in the *inhA* promoter region. This genetic mutation results in the excessive production of *inhA*, which subsequently confers resistance to INH using a mechanism (Barnard et al., 2012; Rahim et al., 2012).

Further examination of INH-resistant *M. tuberculosis* isolates has uncovered many alterations in the intergenic region between the *oxyR* and *ahpC* genes, commonly called the *ahpC* promoter sequence. The *ahpC* gene encodes alkyl hydroperoxide reductase, a pivotal enzyme in the physiological response to oxidative stress. Increasing *ahpC* activity is recommended to counteract the decline in catalase-peroxidase activity in the context of lowering organic peroxides. Distinct modifications were explicitly identified in this region, exclusively in isolates resistant to INH (Kelley et al., 1997).

Western blot analysis revealed that several of these variants exhibited increased transcriptional activity. Nevertheless, frequent mutations in *ahpC* are present in both INH susceptible and resistant isolates, indicating their polymorphic nature. Additional mutations are infrequent throughout a wide range of INH-resistance strains, most of which are found in catalase-negative INH-resistant isolates. These facts indicate that these mutations seem compensatory and are likely selected after a decrease in catalase-peroxidase activity caused by *katG* alterations resulting from INH therapy. No direct evidence suggests that *ahpC* is responsible for INH resistance (Baker et al., 2005).

2.8.1.c. Rifampicin

Rifampicin (RIF), a derivative of rifamycin, was released in 1972 as a medication used to treat TB. This antibiotic is highly effective against TB and, when combined with INH, is a critical component of a treatment plan for MDR-TB. RIF exhibits activity against actively proliferating and metabolically inactive (non-proliferating) bacilli. RIF is a lipophilic medication that can permeate cells and accumulate in leukocytes, inhibiting intracellular bacteria. This offers a therapeutic benefit for treating intracellular pathogens and chronic granulomatous disorders. Rifampicin exerts its mechanism of action in *M. tuberculosis* by blocking the elongation of messenger RNA (mRNA) by binding to the β-subunit of RNA polymerase. Most *M. tuberculosis* strains resistant to RIF have genetic mutations in the *rpoB* gene, which encodes the β-subunit of RNA polymerase. Hence, there are structural changes that reduce the drug's binding ability and lead to the development of resistance (Telenti et al., 1993; Blanchard, 1996)

The gene length of *rpoB* is 3519 bp, and changes inferred by *rpoB* mutations can direct RIF resistance (Jagielski et al., 2018b). DNA transcription into mRNA is catalyzed by *rpoB,* and the substrate is tetraribonucleoside triphosphate. The *rpoB* gene of RIF-resistant *M. tuberculosis* isolates showed an 81 bp mutation in codons 507 (426) to 533 (452) in approximately 95% of cases. The section in question is also known as the rifampicin resistance-determining region (RRDR) or hotspot region (Ullah et al., 2016).

Most research has indicated that mutations in codons 531(450), 526(445), and 516(435) of the *rpoB* gene are the most prevalent mutations linked to rifampicin resistance. Although occurring less frequently, several studies have also documented the presence of mutations outside the RRDR of *rpoB*. The presence of mutations in the *rpoB* gene has been determined to be the primary reason for developing resistance to additional rifamycins. Particular mutations occurring in specific codons (such as 518 or 529) have been associated with a restricted level of resistance to rifampicin. However, the connection between these genetic alterations and the ability to withstand other rifamycins, such as rifalazil or rifabutin, remains unclear (Caws et al., 2006). Nevertheless, it is crucial for TB patients requiring antiretroviral therapy, as rifabutin exhibits lower efficacy in inducing the cytochrome P450 CYP3A oxidative enzyme. However, monoresistance to RIF is rare, but RIF-resistant isolates are often resistant to other medicines, especially INH. RIF-resistance is a crucial determinant of MDR-TB (Burman & Jones, 2001).

Recent genomic sequencing analyses have discovered that strains of bacteria resistant to RIF due to mutations in the *rpoB* gene also develop further mutations in the *rpoA* and *rpoC* genes, which encode the α and β' subunits of RNA polymerase. These compensatory alterations are believed to be accountable for restoring susceptibility to RIF-resistant strains in living organisms (Almeida Da Silva & Palomino, 2011).

2.8.1.d. Ethambutol

Ethambutol (EMB), an effective and particular synthetic antibiotic used in combination with isoniazid in the treatment of TB, was first used in TB treatment in 1996 and is currently one of the first-line antibiotics in the TB treatment regime. EMB exhibits a bacteriostatic action by limiting bacilli growth through interference with the biosynthesis production of arabinogalactan in the cell wall. The *embCAB* genes of *M. tuberculosis* form an operon and are responsible for encoding arabinosyl transferase, which plays a crucial role in synthesizing arabinogalactan. This process ultimately leads to the buildup of D-arabinofuranosyl-P-decaprenol (Mikusova et al., 1995).

The established resistance mechanism to EMB is associated with mutations in the *embB* gene, with mutations at position *embB306* being the most common in most conducted research. Nevertheless, specific investigations have also identified mutations in *embB306* in isolates susceptible to EMB. Furthermore, a comprehensive investigation involving many *M. tuberculosis* isolates revealed that mutations in *embB306* were not directly linked to resistance against EMB. However, these changes increased the possibility of developing resistance against several medications and being transmitted (Hazbón et al., 2005).

Allelic exchange experiments have demonstrated that specific mutations, such as Met306Val, Phe330Val, and Thr63Ile, result in EMB resistance. Conversely, other amino acid substitutions have minimal or no impact on EMB resistance (Safi et al., 2008). Subsequently, the researchers have documented those mutations in the genes responsible for the decaprenylphosphoryl-B-d-arabinose (DPA) biosynthetic and utilization pathway, namely *Rv3806c* and *Rv3792*, in conjunction with mutations in *embB* and *embC*, result in the accumulation of various minimum inhibitory concentrations (MICs) of EMB. The specific type and quantity of mutations determine the range of MICs observed (Safi et al., 2013).

These findings could potentially impact the accurate identification of EMB resistance using existing molecular techniques. *EmbB306* mutations result in varying levels of ethambutol resistance and are necessary but insufficient to confer high-level resistance to EMB. Approximately 30% of strains are resistant to EMB without exhibiting any mutation in *embB*, indicating the necessity to identify alternative drug resistance pathways to this medication (Palomino & Martin, 2014).

2.8.1.e. Pyrazinamide

Pyrazinamide (PZA) was introduced into the tuberculosis treatment regimen in the early 1950s and is currently an essential component of the conventional first therapy for this disease. PZA is a nicotinamide derivative, and its inclusion has reduced the treatment time to six months. PZA is known for suppressing the growth of partially latent bacteria that live in acidic conditions, such as those found in tuberculosis lesions. As a result, it is believed that PZA is most successful in treating areas of active inflammation where the tissue environment is acidic (Zhang & Mitchison, 2003). PZA, like INH, is a prodrug that requires activation of its active form, pyrazinoic acid, through the action of a bacterial enzyme known as Pyrazinamidase to impact *M. tuberculosis*. The *pncA* gene in bacteria is responsible for encoding the enzyme Pyrazinamidase. When this enzyme is absent, it leads to resistance to PZA. The suggested mechanism of PZA involves the transformation of PZA to pyrazinoic acid, which disturbs the energy production in the bacterial membrane and inhibits the transport of substances across the membrane. PZA is transported into the bacterial cell through passive diffusion. Once inside, it converts to pyrazinoic acid and is eliminated from the cell by a weak efflux pump. Under acidic conditions, the pyrazinoic acid in its protonated form would be absorbed back into the cell and accumulate inside. This is because of a poorly functioning efflux pump, leading to damage to the cell (Shi et al., 2011).

The investigation was conducted on PZA-resistant strains that did not have mutations in *pncA* but did have mutations in *rpsA*, which identified the ribosomal protein (*rpsA*) as the suggested target. Increased levels of *rpsA* resulted in elevated resistance to PZA, and it was verified that pyrazinoic acid binds to *rpsA*. Although the concept of *rpsA* mutations being the target of PZA is highly interesting, the inability to carry out allelic transfers in specific investigations makes it challenging to establish this as the situation definitively. The prevalence of mutations in the *pncA* gene is most significant among strains that exhibit resistance to PZA. However, these mutations are dispersed randomly across the gene, most occurring in a 561-base pair section within the ORF (open reading frame) or an 82-base pair segment of its assumed promoter. Only a few studies have shown the existence of PZA-resistant strains that offer no alterations in the *pncA* gene. These findings indicate that the resistance could be linked to mutations in an undiscovered regulatory gene (Juréen et al., 2008).

2.8.2. Second-Line Anti-TB Drugs

The WHO recommends that the following drugs be classified as second-line therapy for TB treatment. The following antibiotics are included: polypeptides (capreomycin, viomycin, and enviomycin), aminoglycosides (kanamycin and amikacin), fluoroquinolones (ofloxacin, ciprofloxacin, and gatifloxacin), D-cycloserine and thioamides (ethionamide), and prothionamides (Organization, 2001). Unfortunately, second-line medications possess an inherently higher level of toxicity and worse efficacy compared to first-line drugs. MDR-TB management primarily employs second-line medications, extending the overall treatment period by 6 to 9 months (Cheng et al., 2004).

2.8.2.a. Fluoroquinolones

Fluoroquinolones, including ciprofloxacin and ofloxacin, are synthetic compounds derived from nalidixic acid. These medications are currently employed as second-line treatments for MDR-TB. Nalidixic acid was initially found as a byproduct of the antimalarial drug chloroquine. Moxifloxacin and gatifloxacin, which belong to the newer generation of quinolones, are now undergoing clinical studies and are recommended as the first-line antibiotics for TB treatment to reduce the treatment period. Fluoroquinolones exert their effects by inhibiting the activity of topoisomerase II (DNA gyrase) and topoisomerase IV, which are essential enzymes for the survival of bacteria. The *gyrA, gyrB, parC,* and *parE* genes encode these proteins. The activity of fluoroquinolones in *M. tuberculosis* depends on suppressing the type II topoisomerase (DNA gyrase) enzyme, the only enzyme present in *M. tuberculosis*. (Carlos Palomino & Martin, 2013).

The Type II topoisomerase is a protein compound with four subunits: two α and two β. These subunits are produced by the genes *gyrA* and *gyrB*, respectively. The primary function of this complex is to facilitate the process of supercoiling DNA. Chromosomal mutations in the quinolone resistance-determining region of *gyrA* or *gyrB* are the primary cause of fluoroquinolone resistance in *M. tuberculosis*. Prevalent mutations occur at locations 90 and 94 of the *gyrA* gene, although mutations at sites 74, 88, and 91 have also been documented (Sun et al., 2008).

2.8.2.b. Kanamycin, Capreomycin, Amikacin, Viomycin

Kanamycin and amikacin belong to the class of aminoglycosides, whereas capreomycin and viomycin are classified as cyclic peptide antibiotics. All four antibiotics have the same mode of action, which involves blocking protein synthesis. All four are second-line medications used in the therapy of MDR-TB (Von Groll et al., 2009).

2.8.2.c. Ethionamide

Ethionamide is a derivative of isonicotinic acid structurally very similar to isoniazid. Additionally, it is a prodrug that necessitates activation by a monooxygenase enzyme expressed by the *ethA* gene. The NAD-inhibiting enoyl-ACP reductase enzyme binds with mycolic acid to generate an adduct, inhibiting mycolic acid production (Brossier et al., 2011).

2.8.2.d. Para-Amino Salicylic Acid

Para-amino salicylic acid (PAS), along with isoniazid and streptomycin, is today considered the preferred second-line medicine for treating MDR-TB, despite being one of the earliest drugs used for this purpose. Until recently, the complete characterization of its mode of action was unclear. Due to its similarity to para-aminobenzoic acid, it has been proposed that it functions by interfering with the production of folate (Zhao et al., 2014).

2.8.2.e. Cycloserine

Cycloserine is an orally administered bacteriostatic medication used as a second-line treatment for MDR-TB. It is a compound with a structure similar to D-alanine and inhibits its activity. The D-alanine ligase enzyme acts as an inhibitor of peptidoglycan production. Additionally, it can impede the activity of D-alanine racemase (AlrA), an enzyme necessary for transforming L-alanine into D-alanine. (Chen et al., 2012).

2.8.2.f. Thioacetazone

Thioacetazone is a former medication that was utilized in the management of TB because of its advantageous effectiveness against *M. tuberculosis* in laboratory settings and its low price. However, it has toxicity issues, particularly in patients who are co-infected with HIV. It is classified as a WHO group 5 medicine and functions by blocking the formation of mycolic acid (Grzegorzewicz et al., 2012).

2.8.3. New Anti-TB Drugs

2.8.3.a. Bedaquiline

Bedaquiline (BDQ), previously referred to as TMC207 or R207910, is a novel antibiotic that falls under the category of diarylquinolines. It has targeted efficacy against *M. tuberculosis* and demonstrated in vitro effectiveness against other non-tuberculous mycobacteria. The treatment exhibited efficacy against *M. tuberculosis* both in vitro and in vivo. Subsequently, it underwent clinical assessment for its effectiveness against drug-susceptible and MDR-TB (Goulooze et al., 2015). By the end of 2015, 50 nations reported obtaining BDQ to treat over 2500 cases. According to estimates from the WHO, by the end of 2017, 68 countries said that they had implemented or started utilizing BDQ to treat MDR-TB and XDR-TB (Organization & Staff, 2013).

BDQ is known to be closely associated with fluoroquinolones. However, unlike fluoroquinolones, BDQ does not exhibit any inhibitory actions on DNA gyrase, and its mode of action is distinct. BDQ is the sole anti-TB medication regulated by the FDA that explicitly targets adenosine triphosphate (ATP) by blocking the mechanism responsible for proton pumping. ATP is generated by ATP synthase and is an essential molecule for both prokaryotic and eukaryotic cells, including mycobacteria, in various states such as extracellular or intracellular, replicating or non-replicating, and active or dormant. ATP synthase, an essential enzyme in the inner membrane of mycobacterial mitochondria, can provide energy to power the catabolic and anabolic activities of proliferating mycobacterial cells (Lohrasbi et al., 2018).

BDQ specifically inhibits ATP synthase in mycobacteria. It is essential to mention that the ATP synthase complex in humans has a sensitivity to BDQ that is 20,000 times lower than that of *M. tuberculosis*. This suggests that it is unlikely for BDQ to cause toxicity or interact with the human ATP synthase based on its target. The analysis of BDQ-resistant mutants of *M. tuberculosis* and *M. smegmatis* identified the mechanism by which BDQ works. Compared to the susceptible strains, the single genetic mutation identified in these mutants was found in the *atpE* gene through genome sequencing. This gene is responsible for encoding the C subunit of the F0 component of the ATP synthase. The mycobacterial cell relies on a complex structure to produce ATP, which is essential for its functioning. BDQ prefers this structure more highly than mitochondrial ATP synthase (Masuku et al., 2020).

The most common mutation observed in BDQ-resistant mutants in the *atpE* gene is A63P, although I66M has also been detected. The latter introduces a change that hinders the appropriate attachment of bedaquiline to its intended target. However, a study investigating the resistance mechanisms to BDQ in *M. tuberculosis* revealed that only 15 out of 53 mutants resistant to the drug had mutations in the *atpE* gene. The remaining 38 strains did not exhibit any mutations in *atpE* or the F0 or F1 operons, indicating that alternative resistance pathways may still be available (Khoshnood et al., 2021).

2.8.3.b. Delamanid

Delamanid (DLM), formerly OPC-67683, is a compound derived from nitro-dihydro-imidazooxazole that exhibits efficacy against *M. tuberculosis*. DLM showed strong efficacy against drug-susceptible and drug-resistant *M. tuberculosis* throughout in vitro and in vivo studies. When contrasted with rifampicin, it demonstrates early solid bactericidal activity. A recent study showed that DLM effectively eradicates dormant Mycobacteria both in vitro and in vivo. OPC-67683 is a compound that prevents the production of specific types of MA, namely methoxy-mycolic and keto-mycolic acid.

In contrast, isoniazid inhibits the production of all subclasses of MA, which are vital components of the *M. tuberculosis* cell envelope. DLM is now undergoing clinical study. Recently, a clinical test against MDR-TB demonstrated its safety and efficacy. (Bhat et al., 2017; Vilchèze, 2020).

The WHO released interim recommendations for the administration of DLM in October 2014. The Phase III trial evaluating the safety and effectiveness of DLM as part of an optimized background regimen (OBR) for treating MDR-TB in adults has recently concluded its follow-up stage (Bhat et al., 2017).

2.9. Sample Collection and Processing.

To make a complete and accurate microbiological diagnosis of mycobacteria, the clinical sample should be taken from the appropriate place, at the right time, in sufficient quantity, and processed under proper laboratory conditions. Most samples sent to the mycobacteriology lab come from the respiratory system. These include sputum that has been sucked up or forced up, gastric fluid that has been fasted, bronchoalveolar lavage, transtracheal aspiration, bronchial brushing, and laryngeal swab samples. On the other hand, urine, gastric aspirates, tissues, biopsy samples, and typically sterile bodily fluids, including cerebrospinal fluid and pleural and pericardial aspirates, are additional samples. Cultures performed from sputum samples sent with suspicion of mycobacteria may be positive on the first day and negative on the next day. Therefore, for the isolation of mycobacteria, samples should be sent for three consecutive days at 24-hour intervals. All samples should be sent to the laboratory immediately and processed immediately (Procop et al., 2017).

These specimens contain mycobacteria, which causes TB, and many other microorganisms in the flora. Accordingly, if cultivation is made from the samples taken for culture since the average growth time of mycobacteria is 15-20 days, the flora bacteria that grow faster cover the medium's surface and prevent mycobacteria's growth and visualization. Therefore, decontamination, homogenization, and concentration procedures are performed to eliminate the bacteria in the contaminated samples before culture, reveal the bacilli hidden between the mucosa and epithelium in the samples, and ensure homogenous distribution of the bacilli in the sample.

Following the application of the decontamination agent, the acid or alkaline solution is neutralized, and the mixture is subjected to high-speed centrifugation to concentrate the mycobacteria. Samples sent for mycobacteria cultures, such as cerebrospinal fluid (CSF), synovial fluid, and other sterile body fluids under normal conditions, do not need to be decontaminated before the procedure. Today, the most widely used method in routine mycobacteriology laboratories is the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. In this method, NALC is used as a mucolytic to release mycobacteria in mucus, NaOH is used as a decontaminant and concentration solution, and sodium citrate is used to prevent the inactivation of NALC by binding heavy metal ions that may be present in the clinical sample. After adding an amount of NALC-NaOH equal to the volume of the sample and mixing it thoroughly with vortex, it waits for 15 minutes. After this time, neutralization is achieved with phosphate buffer. After centrifugation at 3000 g for 15 minutes to concentrate, preparations are prepared from the precipitate obtained and cultured in appropriate media (Özkütük et al., 2014).

2.10. Methods for Detection of Drug Susceptibility Tests

Resistance can be detected through special laboratory tests that assess the bacteria's susceptibility to the drug or determine the presence of resistance. Rifampicin resistance can be detected using phenotypic or genotypic methods, either alone or in combination with resistance to other anti-tuberculosis drugs. Molecular testing techniques such as polymerase chain reaction (PCR) and line probe assays (LPA) are increasingly used to identify genetic variants contributing to drug resistance. Colorimetric methods using redox indicators to detect drug resistance are also available. These methods can detect drug-resistant TB early, allowing healthcare providers to adjust treatment plans and prevent further spread (Gill et al., 2022).

For *M. tuberculosis*, phenotypic DST includes three traditional methods: proportion, resistance ratio, and absolute concentration. These culture-based conventional methods use Middlebrook 7H10 and 7H11 agar and Lowenstein-Jensen (LJ) agar, an egg-based medium. Culture-based methods are well-standardized and still widely used in many countries (G_ Canetti et al., 1963). They are also recognized as "gold standard" methods by the WHO. However, these methods are time-consuming due to the incubation period of 3–4 weeks for TB bacteria, and they require advanced laboratory conditions due to the risk of transmission. High biosafety standards and trained personnel should implement phenotypic DST (Organization, 2018).

To manage the treatment of patients correctly and to use antibiotics rationally, it is necessary to develop a more rapid treatment program, high and specific sensitivity tests to manage the treatment of patients correctly and use rational antibiotics and shortened ongoing anti-TB treatment regimens thought to increase the success of resistant tuberculosis and latent tuberculosis cases. The principle of susceptibility testing is based on the correlation between the clinical response to an antimicrobial agent and the in vitro test result. If more than 1% of bacilli are drug-resistant in vivo, treatment with anti-tuberculosis drugs is not clinically useful. A suitable method should be able to determine the proportion of bacteria susceptible and resistant to a particular drug (OK, 2010).

2.10.1. Conventional Phenotypic Methods

In these tests, bacterial colonies grown in a suitable medium, either by classical or rapid culture methods, are used. Standardization of susceptibility tests, according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST), is essential because there are differences in the media used to detect the resistance of MTBC species to anti-tuberculosis drugs, the amount of inoculum, the minimum concentration of the drug, and the resistance criteria used. These difficulties are related to classical cultures, such as absolute concentration, resistance ratio, and proportion methods; radiometric (BACTEC), automated (MGIT) systems, and colorimetric methods using alamar blue as an indicator dye, and rapid culture-based susceptibility tests (Heifets, 2000).

2.10.1.a. Proportion method (PR method)

The PR method is considered the "gold standard" for *M. tuberculosis* DST, and resistance is calculated by culturing on agar with different drug concentrations. This method is labor-intensive and has a long incubation period, making it unsuitable for rapid diagnosis of drug-resistant tuberculosis. Despite this, the PR method is still used due to its accuracy and reproducibility. In the PR method, the drug-containing and drug-free control mediums are inoculated with the same number of bacteria. The proportion of resistant colonies was calculated by dividing the number of colonies growing on drug-containing media by the total number of colonies growing on drug-free media (Hall et al., 2011).

This is a quantitative test in which several dilutions of a standardized inoculum are inoculated onto (drug-free) and drug-containing control agar media. Compare the reduction in growth in media containing the active ingredient versus media without the active ingredient. An isolate was considered clinically resistant if growth at a critical concentration of the drug was more significant than 1%. It cannot be performed in liquid media as it is a quantitative test, except for BACTEC, which quantifies growth (Palomino et al., 2008).

2.10.1.b. Absolute Concentration method

The absolute concentration method determines drug susceptibility by observing growth or metabolic inhibition in culture media containing antituberculosis drugs and is used to determine the MIC of test strains. Testing is usually performed on Middlebrook's 7H10 medium. The method uses a range of concentrations of anti-tuberculosis drugs that are semi-automatically dispensed into a 25-well plate. Determine the concentration of active ingredient that inhibits 99% of bacterial growth and compare the results to known active ingredient concentrations (van Klingeren et al., 2007; Rampersad et al., 2018).

A solution of mycobacteria containing $2x10^3$ or $1x10^4$ colony forming units/ml (cfu/ml) is inoculated on media without antibiotics and with a certain amount of antibiotic added. The inoculum amount should not exceed 100.000 bacilli. After a 4-week incubation period, the test can be evaluated. Observing more than 20 colonies on the medicated medium means the bacteria resist that drug. The disadvantages of the method are the precise preparation of the cultivation sample and the high error rate (Tansel, 2003).

2.10.1.c. Resistance Ratio method (RR method)

The RR method is performed in solid media (egg or Middlebrook agar) or liquid media (Middlebrook or Dubos broth). This process compares the resistance of an unknown *M. tuberculosis* strain (the test organism) to that of a standard lab strain, H37Rv, or preferably three wild strains, using its model resistance as a control. In this technique, a set of parallel test tubes containing a twofold dilution of the test drug is then inoculated with a standard inoculum of the test and reference strains and incubated at 37°C for four weeks. Tubes with 20 or more colonies are considered positive and require further testing. Isolates with a resistance ratio of 2 or lower were considered susceptible, whereas isolates with a resistance ratio of 8 or higher were considered resistant (Kim, 2005).

2.10.1.d. Radiometric Culture System (BACTEC 460-Becton Dickinson):

In the system recommended by WHO and CLSI, mycobacterial samples are inoculated into bottles containing Middlebrook 7H9 liquid medium. Patient samples in the BACTEC 460 system are spiked into a liquid culture medium containing radiocarbon-14. When mycobacteria grow and metabolize carbon-14, they release radioactive carbon dioxide, which the system detects. The system continuously monitors samples for the presence of radioactive carbon dioxide, providing faster and more accurate results than traditional solid culture methods. The BACTEC 460 system has been shown to have high sensitivity and specificity in detecting mycobacteria (Panicker et al., 2010; Pai et al., 2014).

The device calculates the released carbon dioxide as a growth index (GI). A sensitivity test should be performed if the GI value reaches between 500 and 800. Inoculate the 0.1 ml bottle containing the growing strain with Middlebrook 7H9 medium containing the drug to be tested for antibiotic susceptibility. Inoculate the control medium with one percent of the drug medium and measure and report the daily growth index for both media. This method can be used for drug susceptibility testing of first- and second-line anti-tuberculosis drugs, and results can be obtained within 5-7 days. However, disadvantages of this method include excessive labor, the inability to determine the rate of drug-resistant bacteria, and radioactive waste. The BACTEC 460 system offers several advantages over traditional solid culture methods, including reduced detection time and improved mycobacterial recovery. However, it has some limitations, including the need for radioactive materials and specialized equipment and longer turnaround times for sensitivity testing than other automated systems. Despite these limitations, the reliability and rapid detection capabilities of the BACTEC 460 system have been extensively proven, making it an essential tool for TB diagnosis and treatment (Aggarwal et al., 2008; Organization, 2022b).

2.10.1.e. Fluorescent Culture System (BACTECMGIT 960-Becton Dickinson)

Becton Dickinson's BACTEC MGIT 960 system is a diagnostic technique for detecting *M. tuberculosis*. This system is an entirely automated approach for liquid culture and susceptibility testing of mycobacteria. The BACTECMGIT 960 system utilizes the fluorescence emitted by an oxygen sensor to identify the proliferation of mycobacteria in a culture (Liao & Lainhart, 2017).

Many consider the BACTEC MGIT 960 TB system comparable to the BACTEC 460 TB system for retrieving and examining *M. tuberculosis* and its susceptibility testing. The technique relies on the utilization of altered Middlebrook 7H9 broth in 7-ml plastic tubes equipped with screw caps and fluorescent technology. The presence of dissolved oxygen in the broth initially suppresses the fluorescence. However, when the mycobacteria and other microbes increase, they consume the oxygen, leading to the indicator fluorescing when exposed to a 365 nm UV lamp.

The MGIT 960 kit conducts qualitative drug susceptibility testing within 4 to 19 days of completion. The test relies on the proliferation of mycobacterial complexes in test tubes containing the medication, in contrast to control tubes lacking the drug. The MGIT 960 device continuously monitors and analyses the tubes every hour to detect any augmentation in fluorescence. The device algorithms use comparisons of these data in test and control tubes to calculate outcomes that are automatically analyzed and reported as either resistant or sensitive. Otherwise, oxygen consumption does not occur when growth does not occur, so the ultraviolet (UV) light sent to the environment does not cause fluorescence because the fluorescent substance is not released. Thus, it is understood that mycobacteria do not grow (Güney, 2011).

The BD BACTECMGIT 960 system has many advantages and disadvantages. One of the advantages is that it is a fully automated system that can meet the needs of growth, detection, and drug susceptibility testing for medium and large numbers of mycobacteria. Additionally, the BD BACTEC MGIT 960 SIRE kit is a qualitative test based on the growth of *M. tuberculosis* isolates in drug-containing tubes. The BACTEC MGIT 960 system also has shorter detection times than others, such as the BACTEC 460 system. However, one of the disadvantages of the BACTECMGIT 960 system is that it requires specialized equipment and training, making it less easy to use in resource-limited environments (Pai et al., 2014; Liao & Lainhart, 2017).

Another advantage of the BACTECMGIT 960 system is that it can be used for drug susceptibility testing of *M. tuberculosis* to various anti-tuberculosis drugs such as pyrazinamide, bedaquiline and delamanid. In addition, the BACTECMGIT 960 system was compared with other diagnostic culture methods and was found to have good reproducibility and diagnostic effects. This system has become an essential tool for diagnosing *M. tuberculosis*, especially in settings where sample volumes are large and rapid and accurate results are required (Garrigó et al., 2007; Dlamini et al., 2017).

2.10.2. Molecular Methods

The traditional method for detecting drug resistance in *M. tuberculosis* strains is based on culture isolation followed by DST of clinical isolates. Due to the long generation time of *M. tuberculosis*, these methods are very time-consuming, and susceptibility testing can take weeks to perform. Using time-saving methods with high specificity and sensitivity to detect and identify pathogens and determine drug susceptibility, particularly in cases of drug-resistant TB, is critical to ensuring that these patients receive appropriate treatment and are not contagious.

Molecular diagnosis of tuberculosis drug resistance is based on identifying the most common mutations in genes associated with developing drug resistance in *M. tuberculosis* strains found directly in clinical samples or in cultures grown in vitro. These mutations occur in genes encoding antibiotic targets or in genes encoding enzymes for the conversion of pre-antibiotic substances and lead to the development of drug resistance in mycobacteria. Determining drug resistance using these molecular methods involves two steps: first, PCR amplification of the DNA fragment of the gene of interest is performed using specially designed primers targeting the drug resistance gene, and then hybridization or sequencing is performed to identify the genes through the amplified genes. Specific mutations or DNA sequence analysis (Lawn & Nicol, 2011).

Several commercial and laboratory-developed molecular methods have been developed to detect drug resistance in mycobacteria. Xpert MTB/RIF (Xpert), Linear Probe Assay (LPA), and DNA sequence analysis methods (also recommended by WHO) enable simultaneous detection of MTBC species and drug susceptibility in pulmonary and extrapulmonary samples (Chakravorty et al., 2017; Horne et al., 2019).

2.10.2.a. GeneXpert MTB/RIF assay

The GeneXpert MTB/RIF test has proven to be a valuable tool for the early detection of TB, which can be challenging. A meta-analysis was conducted to evaluate the effectiveness of Xpert in diagnosing different types of TB using multiple samples. WHO recommends the use of the Xpert MTB/RIF assay for rapid diagnosis of TB and detection of rifampicin resistance and approved molecular diagnostic test for the simultaneous detection of *M. tuberculosis* and the mutations responsible for approximately 95% of RIF-resistance in pulmonary and extrapulmonary clinical samples (Zong et al., 2019; Gong et al., 2023).

The GeneXpert test is an automated, kit-based, semi-nested real-time PCR system that uses five nucleic acid probes (A-E) targeting the 81 bp rifampicin resistance-determining region

(RRDR) of the *rpoB* gene. These targets are specific and complementary, bind to different target sequences within the gene in the same multiplex reaction, and are labeled with different colored fluorophores to detect MTBC and mutations leading to RIF resistance. Five different probes correspond to sequences A (codons 507-511), B (codons 512-518), C (codons 518-523), D (codons 523-529), and E (codon 529-533) of the hybrid resistance determination region (RRDR) of *rpoB* gene, as shown in Figure 2.5 (Uddin et al., 2020).

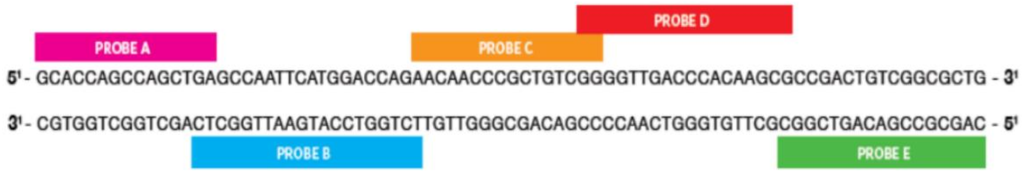


Figure 2.5. *rpoB* gene 81 bp RIF-resistance determining region and Xpert MTB/RIF probes

The GeneXpert MTB/RIF assay has revolutionized TB diagnosis by significantly reducing the time required for results compared to traditional culture-based methods. It can provide accurate results within two hours, enabling rapid initiation of appropriate patient treatment. Additionally, the assay's ability to detect rifampicin resistance helps identify cases of MDR-TB early, enabling timely intervention and appropriate management of patients.

It's important to note that while the GeneXpert MTB/RIF assay is highly reliable and widely used, it is not a substitute for comprehensive diagnostic evaluation and clinical judgment. It should be used with other clinical and laboratory findings to diagnose accurately and determine the most appropriate treatment strategy for patients suspected of having TB (Hernandez et al., 2021).

2.10.2.b. GenoType MTBDRplus

Line probe assays are another molecular method for detecting genetic mutations in *M. tuberculosis*. Line probe assays detect specific genetic mutations using DNA probes immobilized on a membrane strip. These probes bind to complementary DNA sequences in the sample, allowing for the detection of specific genetic mutations. Line probe assays have several advantages over traditional methods, including high sensitivity and specificity, rapid turnaround time, and the ability to detect multiple mutations simultaneously. Line probe assays can find changes in *M. tuberculosis* that make it resistant to drugs. These changes can be in the *rpoB*, *katG*, and *inhA* genes.

A DNA line probe technology called Genotype MTBDRplus (LPA; Hain Life Sciences, Germany) can directly find MTBC members in clinical specimens or cultures. It does this by using reverse hybridization and a multiplex PCR amplification step. It can also find gene mutations that simultaneously cause RIF and INH resistance (Hillemann et al., 2007).

To identify resistance to RIF, it is necessary to analyze the *rpoB* gene, which codes for the beta subunit of RNA polymerase. To identify INH resistance, it is essential to analyze the promoter regions of the *katG* gene, which encodes catalase-peroxidase, and the *inhA* gene, which encodes NADH-enoyl-ACP reductase (Chetty et al., 2017).

Amplicons amplified by multiplex PCR are reverse hybridized with membrane-bound probes overlapping the RRDR of the *rpoB* gene, eight of which are sensitive wild-type WT probes and four of which contain Asp516Val, His526Tyr, His526Asp, and Ser531Leu mutations in the *rpoB* gene.

The presence of RIF and INH resistance is thought to be due to the lack of hybridization in the wild-type (WT) probe and the existence of DNA signal bands that form due to hybridization to probes representing specific mutations (Figure 2.6).

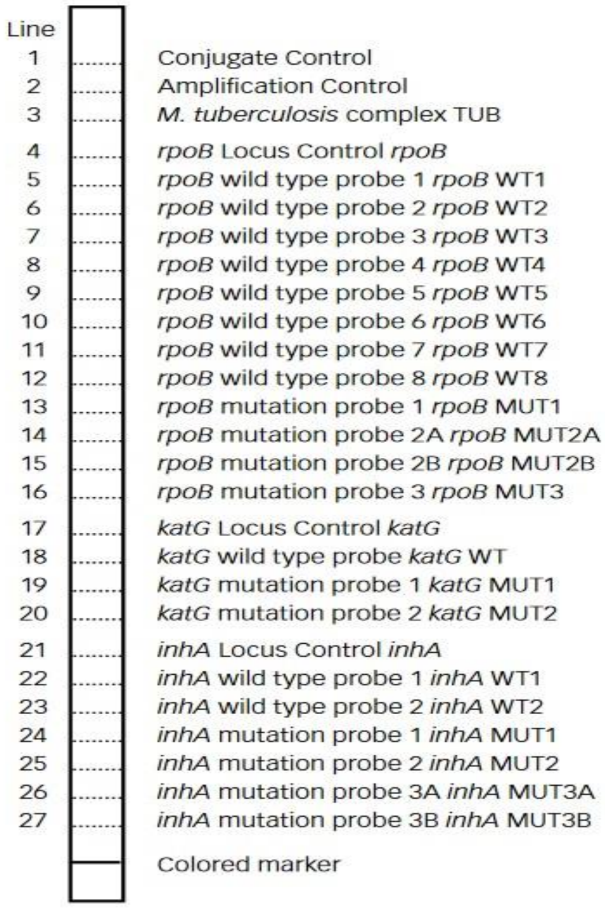


Figure 2.6. GenoType MTBDR plus FL-LPA strip to determine wild-type genes and mutations responsible for resistance to Rifampicin and Isoniazid; *Source: GLI LPA Guidance Document*

The MTBDRplus test, which has good sensitivity and specificity with a short response time of 48 hours compared to conventional methods, can be a helpful tool for rapidly diagnosing MDR-TB. In studies in which the GenoType MTBDRplus test was compared with phenotypic drug susceptibility tests, it was determined that this test had a sensitivity and specificity for detection of resistance to RIF of 98-100% and 95-97.3% to INH of 86-91.9% and 90-98.4% respectively, in comparison with the phenotypic DST (Bai et al., 2016; Ahmed et al., 2017).

2.10.2.c. DNA Sequencing

Whole-genome sequencing (WGS) is a highly effective molecular technique to identify mutations in *M. tuberculosis*. WGS involves the complete sequencing of the entire genome of *M. tuberculosis*, enabling the identification of all genetic alterations, even previously unidentified ones. WGS has several advantages over other methods, including detecting mutations associated with drug resistance and virulence and tracking the spread of TB outbreaks. WGS can also provide information on the genetic diversity of *M. tuberculosis*, which can aid in developing more effective TB control strategies. However, WGS is still relatively expensive and requires specialized laboratory facilities and expertise.

Single nucleotide polymorphism (SNP) is a prevalent genetic variation in the *M. tuberculosis* genome, happening approximately once per 3 kilobases (kb) of DNA sequence. However, it is worth noting that SNP is the predominant type of genetic variation, along with insertions and deletions. Hence, SNP-based typing might be considered the most reliable and accurate method for molecular typing in *M. tuberculosis* (Comas et al., 2010).

The genome of *M. tuberculosis* exhibits a notable degree of conservation, demonstrated by the limited number of SNPs observed compared to the reference strain H37Rv. The utilization of molecular clock data derived from SNP analyses, which reveal the occurrence of 0.3 to 0.5 SNPs per genome per year, has facilitated the ability to forecast epidemiological connections among the cases. Nevertheless, it is essential to consider the available epidemiological data when interpreting this, as variations in SNPs can be influenced by factors such as the timing of sample collection, the prevalence of tuberculosis, and the genetic similarities across strains in a specific geographic region (Walker et al., 2013).

The WGS process includes amplifying the complete set of genetic material and comparing it to the standard (wild type) genetic material to detect any alterations (mutations) in a specific gene structure. WGS is becoming more and more prevalent in the clinical diagnosis of tuberculosis and the examination of MTBC. WGS has enabled numerous research to investigate tuberculosis, allowing for a more comprehensive understanding of the disease by examining the complete genome sequence. This approach has significantly enhanced the accuracy of molecular epidemiology and contact tracing, surpassing the limitations of analyzing only a few fragments (Takiff & Feo, 2015; Gautam et al., 2018).

Quick and precise identification of drug susceptibility in *M. tuberculosis* isolates is essential to inhibit the spreading of MDR-TB within the community and guarantee efficient treatment. The conventional techniques for detecting *M. tuberculosis* and assessing drug susceptibility in the laboratory are time-consuming, making the global fight against TB more challenging (Clark et al., 2019). Also, there has been an increased use of fast phenotypic approaches and molecular testing, such as the GeneX-pert MTB/RIF assay (Cepheid, CA, USA) and GenoType MTBDRplus (Hain Lifescience, Nehren, Germany), to identify drug susceptibility and facilitate identification. However, these tests have disadvantages, including the inability to detect all resistance-conferring mutations and the need for culture before use (Coll et al., 2014).

Performing genomic sequence analysis of *M. tuberculosis* isolates and detecting mutations related to drug resistance has overcome several significant challenges associated with traditional phenotypic tests and eliminated the limitations of probe-based molecular tests. Multiple gene shadows, or WGS, have provided great convenience in determining the drug resistance profiles, genetic diversity, and transmission dynamics of MTBC members (Satta et al., 2018; Katale et al., 2020).

DNA polymerase replicates a specific section of template DNA during the Sanger sequencing procedure. The concept of Sanger or chain termination sequencing has been put out, explaining how the reaction produces fragments of different lengths ending in fluorescent nucleotides. Sequencing reactions necessitate synthesizing numerous components to create fragments of various lengths. These substances are mixed within a small tube. Sequencing primers or short oligonucleotide primers (about 20 bp) that are complementary to the starting site of the target DNA are required. The primer supplies a 3'-hydroxyl group (-OH) that DNA polymerase uses to initiate the synthesis of complementary DNA strands, guided by the target DNA sequence.

During the sequencing reaction, DNA polymerase replicates the target DNA by synthesizing a complementary strand using a collection of deoxyribonucleoside triphosphates (dNTPs) consisting of four bases (dATP, dTTP, dGTP, and dCTP). These nucleotides have a hydroxyl (-OH) group attached to the 3' carbon of the deoxyribose sugar ring. DNA polymerase links the incoming nucleotides to the chain, starting at the 3' hydroxyl of the primer. The linkage between nucleotides occurs when the phosphate group connected to the 5'-carbon of the incoming nucleotide is bonded to the 3'-hydroxyl group of the DNA chain that is being formed. DNA is synthesized in the 5' to 3' orientation. As the reaction continues, the hydrogen ion (H+) on the 3' hydroxyl group (OH) and the two outer phosphate groups from the dNTP are freed. These two outer phosphates are called pyrophosphates after they are released.

Cycle sequencing, also known as Sanger or chain-termination sequencing procedures, necessitates the generation of template copies that have a lesser length than the original. Consequently, these reactions incorporate an extra component in the pool of dNTPs to terminate DNA synthesis. A group of dideoxynucleoside triphosphates (ddNTPs), including ddATP, ddTTP, ddCTP, and ddGTP, can be used for all four bases, but they lack the necessary hydroxyl group on the 3' carbon. If a ddNTP is introduced into a developing DNA strand during the process of DNA synthesis, it prevents further elongation by not providing a 3'-hydroxyl group. DNA polymerase is unable to incorporate more nucleotides. Each ddNTP, apart from lacking a 3’-OH group, also comes with a distinct fluorophore that emits a specific wavelength of light when stimulated by a laser. Each base in DNA has a specific fluorophore associated with it. Consequently, the emission of light in a particular color or wavelength indicates the identity of the most recent base added by DNA polymerase during extension (Clark et al., 2019).

The ratio of ddNTPs to dNTPs is carefully determined to ensure that DNA polymerase incorporates ddNTPs frequently enough to generate subsets of fragments with a difference of one nucleotide in length. However, it is also ensured that the copies are not predominantly full-length compared to the template. The term "chain termination sequencing" is derived from this procedure. The sequencing reaction includes buffers and ions, which optimize the ingredients' performance, including DNA polymerase, dNTPs, ddNTPs, primer, and the template DNA sample (Reinhard et al., 2019).

Due to the various limitations of conventional methods used in the laboratory diagnosis of TB, nucleic acid amplification tests (NAATs) have emerged as an alternative or complementary approach to traditional microbiological identification. Nucleic acid amplification-based systems such as classical PCR, Nested-PCR, reverse-transcription (RT)-PCR, and TB loop-mediated isothermal amplification (LAMP) utilize molecular probes that can hybridize with specific regions of MTBC strains' nucleic acids. These systems can directly detect MTBC organisms' IS6110 insertion sequence, 16S rRNA, and/or 23S rRNA from both smear-positive and smear-negative clinical samples. In the species-level identification of mycobacteria, these systems target specific gene regions such as 16S rRNA, *hsp65*, *rpoB*, and many others. They exhibit nearly 100% sensitivity and specificity in the presence of $1x10^5$ CFU/ml of bacteria in clinical samples, providing better and faster results than microscopic diagnosis and culture. In a study, a 100% correlation was found between IS6110-based PCR and the BACTEC MGIT 960 system, and the authors recommended using both methods as a combined protocol for routine clinical diagnosis. In smear-negative specimens, NAAT systems were found to have high specificity but low sensitivity (Das et al., 2020).

2.10.2.d. RFLP

Insertion sequences (IS) are mobile genetic components that are distributed throughout the genomes of numerous bacterial species and typically have a size smaller than 2.5 kb. Unlike transposons, IS elements are characterized by carrying solely the genetic material required for their transposition and control. In contrast, transposons, such as antibiotic resistance, can have additional genes that encode phenotypic markers. The transposition of IS elements frequently results in gene disruptions that can cause significant polar effects. Additionally, in certain instances, it can induce the activation or alteration of nearby genes by affecting the regulatory sequences, such as promoters and protein-binding regions. Thierry et al. initially reported the discovery of IS6110, a 1,355-base pair element belonging to the IS3 family. This element is exclusive to the MTBC when it is complete. The IS6110 elements appear sporadically across the genome, with varying copy numbers ranging from clones that lack any IS6110 elements to those with 26 copies (Thierry et al., 1990).

The copy numbers and integration sites of IS6110 vary among strains. These variations form the basis of the genetic polymorphism commonly used in strain differentiation. While some strains lacking IS6110 copies have been identified, it has been determined that clinical isolates of *M. tuberculosis* can possess varying numbers of IS6110 copies, ranging from 1 to 25, in their genomes. In 1993, van Embden proposed a standardized approach to performing IS6110-based Southern blot hybridization analysis. The recommendation was based on utilizing a widely used restriction endonuclease called PvuII. This enzyme cuts the IS6110 at a single asymmetric site, resulting in reasonably sized fragments of the *M. tuberculosis* chromosome fragments, A hybridization probe specific to the right side of IS6110, by which Every hybridizing band corresponds to a PvuII-PvuII chromosomal fragment containing a single IS6110 insertion and standardized molecular weight markers (Van Embden et al., 1993).

Epidemiological research has utilized a range of molecular biology approaches that rely on IS6110 polymorphism, known for its high stability. An example of such a method is the IS6110-based Restriction Fragment Length Polymorphism (RFLP) method, which is regulated for conducting IS6110 Southern blot hybridization analysis. Different researchers identified copies of the insertion sequence in MTBC DNA, leading to the development of IS6110 RFLP. According to the recommendations of Van Embden et al., this method involves the following steps: PvuII, a restriction endonuclease, cleaves the IS6110 regions found in the genome of *M. tuberculosis*. Subsequently, the fragments are segregated based on their size using agarose gel electrophoresis. The IS6110 probe is employed in Southern blot hybridization to generate distinct DNA fingerprints specific to each species. Chemiluminescent kits are utilized for the visualization of DNA fragments. The observed bands in the tested strains indicate differences in the quantity and location of IS6110 elements within the genomes of *M. tuberculosis* isolates (Gagneux, 2017).

Although IS6110 RFLP has high discriminatory power, like other genotyping methods, it has some limitations. These limitations include the lengthy and time-consuming procedures, the requirement for a substantial amount of DNA, the difficulty in comparing banding patterns that can still be relatively complex despite standardization, and the need for more significant amounts of DNA compared to other methods. These limitations have made it challenging for the technique to find widespread use. There are also *M. tuberculosis* sublineages that do not have the IS6110 element or have fewer than five copies, which can make the method less helpful in telling them apart. These factors have encouraged researchers to explore PCR-based alternative genotyping methods (Dekhil et al., 2018).

2.10.2.e. SNP analysis

The extensive comparative genomic analysis of *M. tuberculosis* has shown high similarity in DNA sequences (99%) among MTBC members. This means that nucleotide-level variations can be used as a useful molecular marker to differentiate clinical isolates and study the evolutionary relationships of clinical strains. Both nonsynonymous single-nucleotide polymorphisms (nsSNPs) and synonymous single-nucleotide polymorphisms (SNPs) offer valuable data for comparing different isolates of *M. tuberculosis*.

Typically, nsSNPs can lead to an alteration in the amino acid sequence as a result of either internal or external factors. Hence, nsSNPs occurring in genetic loci associated with drug resistance can result in the development of phenotypic drug resistance. Consequently, the resistance of *M. tuberculosis* to antituberculosis medications is predominantly related to genetic changes, such as point mutations, deletions, or minor duplications, occurring in specific regions of the chromosomes. nsSNPs occurring in genes that confer drug resistance can provide valuable insights into the characteristics and transmission of resistance within and between populations (Zhang et al., 2004).

Conversely, the SNP variations, regarded as functionally benign, do not alter the amino acid content. These neutral mutations in structural or housekeeping genes can be a foundation for investigating genetic drift and evolutionary relationships across mycobacterial populations. These three primary groups are PGG1 (gyrA95 ACC [Thr] *katG*463 CTC [Leu]), PGG2 (gyrA95 ACC [Thr] *katG*463 CGG [Arg]), and PGG3 (gyrA95 AGC [Ser] *katG*463 CGG [Arg]) (Gutacker et al., 2006).

Prior researchers have employed comparable sSNP studies to determine the evolutionary structure of *M. tuberculosis* groups, yielding predominantly similar results. While these studies have provided additional information into the evolutionary relationship of clinical isolates, they also offer a comprehensive framework to investigate whether distinct lineages exhibit varying epidemiological characteristics within their populations. Moreover, SNP analysis can be customized to focus on numerous significant genetic variations using a single platform. These variations can include phylogenetic grouping, virulence, drug resistance, and other markers that provide valuable information for epidemiological studies (Mathema et al., 2006).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Ethics Approval of Research

The study exclusively utilized isolates regularly collected from the Tropical Diseases Research and Application Center in Adana / Turkey, which was approved by the Ethical Review Committee in Çukurova University/College of Medicine.

3.1.2. Study Design

This study was a retrospective cross-sectional analysis that involved retrieving all archived RIF-resistant and INH-resistant MTBC isolates from routine TB diagnosis at the Çukurova University Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory. The isolates were obtained from eight different cities (Adana, Hatay, Kilis, Osmaniye, Kahramanmaraş, Mersin, Gaziantep, and Adıyaman) in the Çukurova Region of Turkey. The retrieval was conducted between January 2021 and June 2022 for further analysis. Only the initial multidrug-resistant tuberculosis (MDR-TB) and drug-sensitive tuberculosis (DS-TB) strain from each patient was considered while eliminating any additional isolates from the same patient. The demographic data, including gender and age, were incomplete and excluded from the analysis.

3.1.3. Selection of *M. tuberculosis* Complex Clinical Isolates

This study included 40 strains of MDR-TB and 30 strains of DS-TB obtained from clinical samples of individuals suspected of tuberculosis. From January 2021 to October 2022, we dispatched the samples to the Çukurova University Tropical Disease Research and Application Centre, Ministry of Health, Public Health Institution, and Adana Regional Tuberculosis Laboratory from eight distinct cities in the Çukurova Region of Turkey. All the isolates were confirmed as MTBC using the Bactec MGIT 960 TB system, which is considered the most reliable method. Additionally, we did thorough isolation profiles. We eliminated duplicate specimens from our investigation. Each isolate was individually assessed for resistance to the first-line medications (rifampicin, isoniazid, streptomycin, and ethambutol). Of the total, 30 isolates demonstrated sensitivity to all primary drugs. Conversely, 40 isolates were classified as MDR-TB, meaning they were resistant to at least isoniazid and rifampicin. The research, including pathogenic *M. tuberculosis,* was conducted by skilled and experienced researchers at the University Teaching Hospital (UTH) in a Biosafety Level 3 facility. The *M. tuberculosis* reference strain H37Rv (ATCC 27294) was used as a control in each batch of drug susceptibility testing (DST) conducted in this study, including in the phenotypic and genotypic susceptibility detection processes.

3.1.4. Materials and Kits Used in Experiments:

Bactec MGIT Tube 100, Bactec MGIT OADC Supplement, Bactec MGIT PANTA, Isoniazid (INH), Rifampicin (RIF), Streptomycin (STR), Ethambutol (ETM) were purchased from Becton Dickinson (BD) and Each solution was made fresh on the day of the experiment.

exoSAP (Applied Biosystems™), Sephadex (Sigma-Aldrich), BigDye Terminator v3.1 Cycle sequencing kit USA,10µl Filtered Pipette Tip, Biopointe Scientific, USA, 200µl Filtered Pipette Tip.

3.1.5. Devices Used in Experiments

Bactec MGIT 960 Liquid Culture System (BD) in the USA, conventional PCR device, electrophoresis tank, Biospectrum 500 Bioimaging System-UV gel imaging device, UVP, UK, refrigerated microcentrifuge, heat block, oven, autoclave, +4 °C refrigerator, -20 °C freezer, Class-2 Biosafety cabinet (SCANLAF), and an AB HITACHI 3130*xl* Genetic Analyzer.

3.2. Methods

3.2.1. Phenotypic Drug Susceptibility Testing Method

We conducted antibiotic susceptibility testing on all clinical isolates. We performed the MGIT culture (Becton Dickinson, USA) using commercially available kits according to the manufacturer's directions. We marked every MGIT tube with the study code and the date of the examined sample. The MGIT system incorporates a fluorescent sensor that measures the oxygen consumption and fluorescence of the culture broth. Microorganisms in the specimens metabolize nutrients, consume oxygen, and produce fluorescent molecules detected by the sensor. This allows for continuous monitoring and real-time detection of bacterial growth. The test relies on identifying oxygen consumption by the mycobacteria in the MGIT™ (Mycobacterial Growth Indicator Tube) medium and comparing their growth between media with and without antibiotics. The equipment analyzes the growth results and classifies them as susceptible, resistant, or error, requiring a test repeat in cases of error. The equipment obtained phenotypic DST findings for the first line and PZA within 4 to 13 days and 21 days, respectively. DST supplements and MGIT 960-based antimicrobial testing are also used to examine strains (Becton, Dickinson, and Company). The manufacturer's guidelines prepared the inoculum. The tubes contained INH (0.1 g/ml), RIF (1.0 g/ml), EMB (5.0 g/ml), STR (1.0 g/ml) (Springer, Lucke et al. 2009), and each batch of DST. In this investigation of DST, we used the *M. tuberculosis* reference strain H37Rv (ATCC 27294) as a control.

3.2.1.a. Equipment and Materials for First-Line DST:

The BACTEC MGIT 960 SIRE kit (catalog number 245119 and BD catalog number GDF 106028) includes individual vials of lyophilized drugs such as RIF, STR, INH, and EMB. Additionally, it contains eight vials of SIRE Supplement, allowing for roughly 40 tests per drug per kit. The quantities of RIF, STR, INH, and EMB are 322.0 µg, 322.0 µg, 33.2 µg, and 1660.0 µg, respectively. Mix each medication in the BACTEC MGIT SIRE kit with 4 ml of sterile distilled water to achieve the desired stock concentration (Table 1). Do not proceed until the growth supplement has been introduced to the MGIT medium. The Becton Dickson SIRE supplement was accessible, but the BBL OADC growth supplement (catalog number: 245116) was not employed and was replaced with an equivalent amount (800µl).

Table 3.1. Reconstitution volumes and final concentrations for first-line anti-TB

Drug Lyophilized	Dilution Amount by distilled water	Concentration of drugs after reconstitution	Volume added to MGIT tube	Final concentration in MGIT tube
STR (332 µg)	4 ml	83 µg /ml	100 µl	1.0 µg/ml
INH (33,2 µg)	4 ml	8.3 µg /ml	100 µl	0.1 µg/ml
RIF (332 µg)	4 ml	83 µg /ml	100 µl	1.0 µg/ml
EMB (1660µg)	4 ml	415 µg /ml	100 µl	5.0 µg/ml

Stock solution and solvent: To produce and sterilize solutions of anti-TB agents, we made the required dilutions. This was done to ensure that when 0.1 ml of the anti-TB agent solution is mixed with 7 ml of MGIT medium, the desired test concentration is achieved, which depends on the volume of the medium in milliliters.

The dilution factor for the MGIT medium can be calculated by adding 7.0 ml of medium, 0.8 ml of SIRE Supplement, and 0.5 ml of inoculum, resulting in a total volume of 8.3 ml. 0.1 ml of the drug solutions were added to 8.3 ml of the medium, resulting in a dilution ratio of 1:83.

3.2.1.b. Preparation of MGIT Tube for Culture

The Mycobacteria Growth Indicator Tubes (MGIT) consist of 7 ml of modified 7H9 Middlebrook media containing silicon-embedded, oxygen-quenched fluorescence sensors positioned at the base of the tube. This oxygen-quenched fluorescent sensor is called fluorochrome, tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate. To perform the anti-susceptibility test, approximately 0.8 ml of the OADC supplement (Becton Dickinson), containing Oleic Acid, Albumin, Dextrose, and Catalase, was introduced to the MGIT tubes. We took a 0.1 ml consumption of the antibiotics Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, and Azlocillin (PANTA) to prevent contamination and suppress the growth of microorganisms that are not mycobacteria. Before sample inoculation, it was introduced into the MGIT tube. The reagents utilized in the MGIT960 apparatus were procured from Becton, Dickinson (BD), and Company, USA (Siddiqi & Rüsch-Gerdes, 2006).

3.2.1.c. Inoculation of Culture Sediment to MGIT Tube

Inoculation suspensions were prepared following the manufacturers' instructions for preparation in MGIT culture. Undiluted positive cultures prepared the organism suspension on days 1–2. On days 3–5, the cultures were diluted by a factor of 1:5 using a sterile saline solution. This was achieved by adding 1.0 ml of the suspension to 4.0 ml of sterile saline. Thoroughly mix the ingredients and utilize them as the sample to assess the susceptibility of drugs. Assign a distinct label to five MGIT tubes for every test culture. Assign one label for GC (growth control, without medicine), one for STR, one for INH, one for RIF, and one for EMB. Add 0.8 ml of BACTEC 960 SIRE Supplement to each MGIT tube using an aseptic technique. Exclusively utilize the MGIT SIRE Supplement. Add 0.1 ml of a correctly prepared STR medication to the tube labeled with STR using an aseptic technique. Likewise, more medications should be introduced into the remaining labeled tubes. Precisely measuring the appropriate dosage of medicine is crucial for each tube. Avoid introducing any substances into the GC tube.

Using a pipette, add 0.5 ml of the 1:5 diluted culture suspension (inoculum) into each tube, retaining the medication and ensuring aseptic conditions. To establish control, begin by diluting the test culture suspension at a ratio of 1:100. This can be achieved by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline. Thoroughly mix by inverting the tube 5–6 times. Utilize this diluted mixture to introduce 0.5 ml into the growth control tube.

We carefully closed the tubes and then inverted them three times to ensure thorough mixing of the components. Upon scanning the barcode on each tube, we proceeded to insert it into the BACTEC equipment. The apparatus consistently maintains a temperature of 37 ºC ± 1 ºC, the ideal development temperature for *M. tuberculosis*, for 28 days.

The device records and analyses the inputted susceptibility test set. Upon completion of the test (within a timeframe of 4 to 21 days), the instrument will promptly indicate the availability of the results. Perform a scan of the susceptibility set and provide a report. The instrument printout displays the susceptibility data for each medication. The results are categorized as qualitative, indicating susceptibility (S), resistance (R), or indeterminacy (X). The instrument analyses results when the growth unit (GU) in the growth control reaches 400. A resistance result is evaluated when the GU of the drug-containing tubes exceeds 100 and the GU of the growth control tube reaches 400. A drug-containing tube is termed susceptible if its GU value is less than or equal to 100.

Errors or indeterminate (X) results may arise due to specific situations that can impact the test, such as the control's GU exceeding 400 within a timeframe of fewer than four days. Under such circumstances, it is necessary to repeat the test using a pure, actively proliferating culture verified to be MTBC. Some drug-resistant strains may not produce results within 13 days using the standard inoculum because they develop slowly in the medium. To obtain measurable results, the concentration of the culture solution should be increased by reducing its dilution in such a scenario. According to the European Committee for Antimicrobial Susceptibility Testing

(ECAST), the epidemiological cutoff value (ECOFF) is the MIC value that determines the highest level of susceptibility for the wild-type population (Ängeby K 2012).

Figure 3.1. BACTEC MGIT 960 Device, Tropical Disease Research and Application Centre, Cukurova University

Quality control of drug susceptibility testing is of the utmost importance. Utilise *M. tuberculosis* H37Rv (ATCC number 27294) as a quality control strain susceptible to all anti-tuberculosis medications. There is no need to include a strain resistant to a drug, as most resistant strains obtained from ATCC and other culture collections are already highly resistant and do not offer any additional advantages for quality control purposes. The test process for quality control organisms is the same as described for clinical isolates. The inoculum should originate from a recently cultivated culture in the MGIT medium or on an LJ slant.

3.2.1.d. Culturing on Lowenstein-Jensen (LJ) agar:

After the decontamination, homogenization, and concentration processes, the routine primary culture method using LJ agar was employed to inoculate 0.3 ml of the prepared samples. Subsequently, these samples were incubated at 37°C for 6–8 weeks (an average of 42 days). One notable distinction between the growth observed on LJ agar and MGIT is that LJ agar, being a solid agar medium, can more accurately reflect the chromatic properties of the bacteria, a common feature rarely observed in liquid media.

3.2.2. Immunochromatographic TB Ag MPT64 Card Test

The MPT64 card, created by Becton Dickinson in the USA, is a basic immunochromatographic test called the BD MGITTM TBc ID test. This test uses anti-MPT 64 antibodies to detect 23 kDa proteins in the cell structure of MTBC species. It can rapidly differentiate between MTBC and NTM in as little as 15 minutes. We extracted 0.1 ml of cultivated mycobacterial colonies from the MGIT medium during the test and introduced them into the MPT64 card. Subsequently, we allowed it to incubate for 15 minutes. If only the control line is visible after this timeframe, it signifies the existence of non-tuberculosis mycobacterial (NTM) species.

A positive result for MTBC species was shown by the presence of two pink-red lines, one in the test zone (T) and the other in the control zone (C) (Figure 3.2). We assessed the TBc ID test's effectiveness in detecting and identifying MTBC directly from positive MGIT 960 cultures (Barouni et al., 2012).

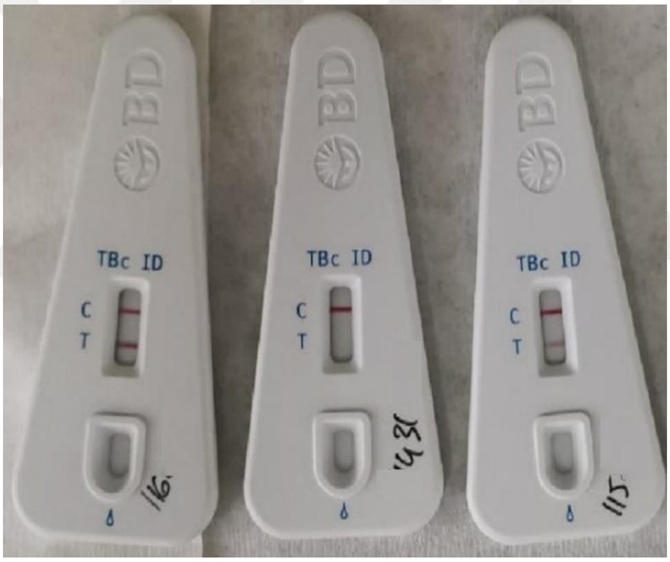


Figure 3.2. Identification of MTBC by ID TBc test from BACTEC MGIT 960 positive cultures. Left, positive result (strong); Middle, negative result; Right, positive result (weak)

3.2.3. Genotypic Drug Susceptibility Analysis

The tuberculosis genomes have mutations in the gene regions associated with resistance to RIF and INH. Using the Sanger DNA sequencing method, we examined 70 strains of MTBC isolates, consisting of 30 strains totally DS-TB and 40 MDR-TB strains. The process involved DNA isolation, PCR amplification, detection of the resulting amplicons on an agarose gel, and Sanger sequencing. Subsequently, we assessed the obtained data.

3.2.3.a. DNA extraction by Mickle Mechanical methods and Sequence analysis

Genomic DNA was isolated from cultures cultivated on BACTEC-MGIT 960.

1. A 0.5 ml liquid sample was deposited in microcentrifuge tubes with a volume of 1.5 ml, ensuring sterility.
2. The bacteria were killed by incubating them at 95 °C for 30 minutes.
3. Spin at 15000xG for 15 minutes and discard the supernatant.
4. 500 µl of 1xTE buffer was added.
5. Spin at 15000xG for 15 minutes and discard the supernatant.
6. 250 µl of 1xTE buffer was added to the tubes.
7. Fifty to one hundred micrograms of SIGMA acid-washed glass beads were added to the tube.
8. Two minutes of cell fragmentation in a Mickle (Mickle tissue disintegrator) device
9. After 10 minutes of spinning at 15000xG, the supernatant was transferred to a fresh microcentrifuge tube.
10. The isolated DNA was stored at -20°C.

3.2.3.b. Conventional PCR Amplifications

The *rpoB* genes of each isolate were sequenced to detect RIF-resistance, whereas the *inhA*, *katG*, and *oxyR-ahpC* genes were sequenced to detect INH-resistance. Table (3.2) presents the primers employed for amplifying and sequencing the genes associated with resistance to RIF and INH. Table (3.3) presents the PCR mixture necessary for a single PCR experiment. The PCR amplification was conducted under the ordinary conditions outlined below: The procedure begins with an initial denaturation step at 95°C for 5 minutes. This is followed by 30 cycles of denaturation at 95°C for 30 seconds. For three specific genes (*rpoB*, *inhA*, and *katG*), the annealing step is carried out at 60°C for 30 seconds. Subsequently, an extension step is performed at 72°C for 30 seconds. A final extension is conducted at 72°C for 5 minutes. The amplification of the *oxyR-ahpC* gene was performed through 35 cycles. This involved an initial denaturation phase at 94°C for 5 minutes, followed by denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 1 minute. The process concluded with a final elongation step at 72°C for 7 minutes. The amplicons produced by the PCR were analyzed following their placement on an agarose gel. The amplicons were either sequenced immediately or stored at -20 °C for future analysis. The DNA sequences of the PCR products were determined using a Sanger sequencing kit, a BigDye Terminator cycle sequencing kit, and an ABI Prism 3130 Genetic DNA Analyzer. Primer selection has been based on existing literature or a custom design for each gene. The table labeled 3.3 contains the precise temperatures at which annealing occurred and the specific sequences of the primers employed for amplification. The findings were examined using the Basic Local Alignment Search Tool (BLAST), available at http://www.ncbi.nlm.nih.gov/BLAST. We performed a sequence alignment with the H37Rv *M. tuberculosis* genome.

Table 3. 2. The primer sequences used for PCR

Antimicrobial agent	Target Gene	Primer Sequence (5' to 3')	Amplicon size base pair
Rifampicin	*rpoB _F*	ACCGACGACATCGACCACTT	450
	rpoB _R	GTACGGCGTTTCGATGAACC	
Isoniazid	*katG_F*	AATCGATGGGCTTCAAGACG	500
	katG_R	CTCGTAGCCGTACAGGATCTCG	
	inhA_F	CCTCGCTGCCCAGAAAGGGA	248
	inhA_R	ATCCCCCGGTTTCCTCCGGT	
	ahpC_F	GAGACCGGCTTCCGACCACC	293
	ahpC_R	GCTGGTAGGCGGGGAATTGAT	

Table 3.3. A PCR mixture is required for a single reaction

PCR master mix	12.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
Water	6 µl
DNA	5 µl
Total	25 µl

Table 3. 4. Genes of *rpoB*, *inhA*, and *katG* have been amplified as follows

Reaction steps		Temperature (°C)	Time	Number of cycles
Step1	Initial Denaturation	95	5m	1
Step2	Denaturation	95	30s	35
	Primer Annealing	60	40s	
	Extension	72	40s	
Step3	Final Extension	72	5m	1
	Hold	4	∞	∞

Table 3.5. The gene of *oxyR-ahpC* has been amplified as follows

Reaction steps		Temperature (°C)	Time	Number of cycles
Step1	Initial Denaturation	94	5m	1
Step2	Denaturation	94	45s	35
	Primer Annealing	60	45s	
	Extension	72	1m	
Step3	Final Extension	72	7m	1
	Hold	4	∞	∞

3.2.3.c. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method that separates DNA fragments of nucleic acid-based on their size. When an electrical current is supplied, smaller fragments exhibit more incredible migration speed as negatively charged DNA flows through the agarose gel's pores toward the gel's positively charged end. The produced bands can be observed by illuminating them with ultraviolet (UV) light.

A 1.5% agarose solution was created by dissolving 1.5g of agarose in 100 ml of 1X TAE buffer. Heat the agarose in a beaker until it becomes transparent and accessible to any agarose particles. The bottle was chilled by exposing it to flowing tap water until it reached a temperature of 50°C. Subsequently, 2 µL of either ethidium bromide or gel red dye was introduced into the bottle and swirled for approximately 45 seconds. The solutions were then poured slowly to prevent the creation of any bubbles and ensure proper gel rigidity with the comb. The gel was applied and allowed to remain for approximately 30 minutes. Once the gel had cooled down, a solution of 1X TAE was carefully poured over the gel in the tank, and the comb was subsequently extracted from the gel. A volume of 5 µL of ladder was loaded for the initial well, coupled with a further 2 µL of loading dye. We added approximately 5 µL of sample and 2 µL of loading dye to the remaining wells. The gel was electrophoresed at a voltage of 120 V for 25–35 minutes. The bands' progress was monitored by withdrawing the gel and placing it on a mid-wave UV transilluminator to observe the result.

3.2.3.d. The Sanger DNA Sequencing Analysis of *rpoB*, *inhA*, *katG*, and *oxyR-ahpC*

Automation in sequence analysis technologies allows for rapid study of DNA. The reactions for these methods (dye-terminator sequencing) happened in a single tube with four ddNTPs labeled with dyes that give off light at different wavelengths. This made laser reading possible. We used Sanger sequencing to look at the *rpoB* (246 bases), *katG* (500 bases), *inhA* promotor (*fabG*1) (248 bases), and *oxyR-ahpC* (293 bases) gene areas of *M. tuberculosis*. The Sanger DNA sequence analysis on the PCR data was conducted using a BigDye Terminator Cycle Sequencing kit and an ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The data was analyzed using a crucial local alignment search tool at http://www.ncbi.nlm.nih.gov/BLAST. We used the genome of *M. tuberculosis* H37Rv (GenBank accession no. AL123456.3) for this evaluation.

3.2.3.e. Purification of PCR Products

Following acquiring PCR products, we purified them using the "ExoSAP-ITTM" kit according to the manufacturer's guidelines. The procedure entailed combining 2µl of the enzyme with 5µl of the PCR product, followed by incubation at 37°C for 30 minutes. Subsequently, the mixture was maintained at 80°C for 15 minutes.

3.2.3.f. Cycle Sequence PCR Amplification

We are employing the ADS SupreDye™ v3.1 Cycle Sequencing Kit for both standard templates and templates with high-GC content. The ADS SupreDye™ v3.1 Cycle Sequencing Kit provides enhanced durability, consistent peak heights, and extended read durations. Compatible with all ABI genetic analyzers. The kit includes the following components: SupreDye, a premix ready for sequencing reactions, pGEM control DNA, M13 control primer, and 5x sequencing buffer.

The sequencing procedure is conducted after the appropriate preparation and quantification of the DNA template. This preparation approach differs from conventional PCR techniques that use two primers to amplify both DNA strands exponentially. The sequencing reaction employs one primer to establish a base pairing with a single template strand. The process involves linearly replicating the complementary DNA template strand via Sanger's dideoxy chain-termination technique. A series of denaturation, annealing, and amplification cycles accomplish this.

During this procedure, a minute portion of the four distinct types of fluorescently labeled dideoxynucleotides (ddCTPs, ddATPs, ddTTPs, and ddGTPs) is introduced as terminators to compete against their corresponding deoxyribonucleotides (dNTPs) for integration into synthesized DNA fragments. DNA synthesis ceases elongation when the ddNTPs substitute for the usual dNTPs. Genetic analyzers or sequencers employ capillary electrophoresis to differentiate DNA fragments synthesized with a single nucleotide variation. The instrument software carries out base-calling by identifying fluorescent signals using lasers. The reaction components are presented in Table 3.6, whereas the reaction conditions are displayed in Table 3.7.

Table 3.6. Components of the " ADS SupreDye™ v3.1 Cycle Sequencing Kit"

Reagents	Concentration	Volume
SupreDye reaction ready premix (Rxns-100)	5x	1µl
Sequencing Buffer	5x	2µl
Primer	4pmol	2µl
dH2O	-	3µl
Pure PCR product	-	2µl
final volume	-	10µl

Table 3.7. Temperatures and times are applied in the "Cycle sequencing" reaction.

Reaction steps	Temperature (°C)	Time	Number of cycles
initial denaturation	96	1min	1
Denaturation	96	10s	25
Primer annealing	50	5s	
Extension	60	4min	
Final extension	4	∞	∞

3.2.3.g. Cleaning and Loading the Sequencer with Purified PCR Products Derived from a Cycle Sequence:

Before loading into the sequencing machine or post-sequencing reaction, dye terminator removal or "clean-ups" can be done by gel filtration columns. Cycle-sequence products required further purification using the Sephadex spin column technique. To purify the amplicons, we followed this approach and loaded them onto columns made from a solution of 14 ml of water and 1 g of Sephadex chemical.

1- We placed empty spin columns into 1.5-ml collection tubes.
2- 700 µl of Sephadex mixture was placed into each column.
3- Put the tube into centrifugation at 4500 rpm for 2 minutes. Draining the fluid from the collection tube had no detrimental effects on the Sephadex column.
4- The column was reinserted into the collecting tube, and the entire sequence product of the cycle was added to the center of the column.

5- The pure cycle sequencing product was collected by centrifuging the sample for 3 minutes at 4500 rpm and then put into an ABI Prism 3130 sequence instrument for analysis according to the manufacturer's instructions, as shown in Figure 3.5.
6- The result was interpreted using Sequencing Analysis 5.3.1, a software developed by Applied Biosystems.

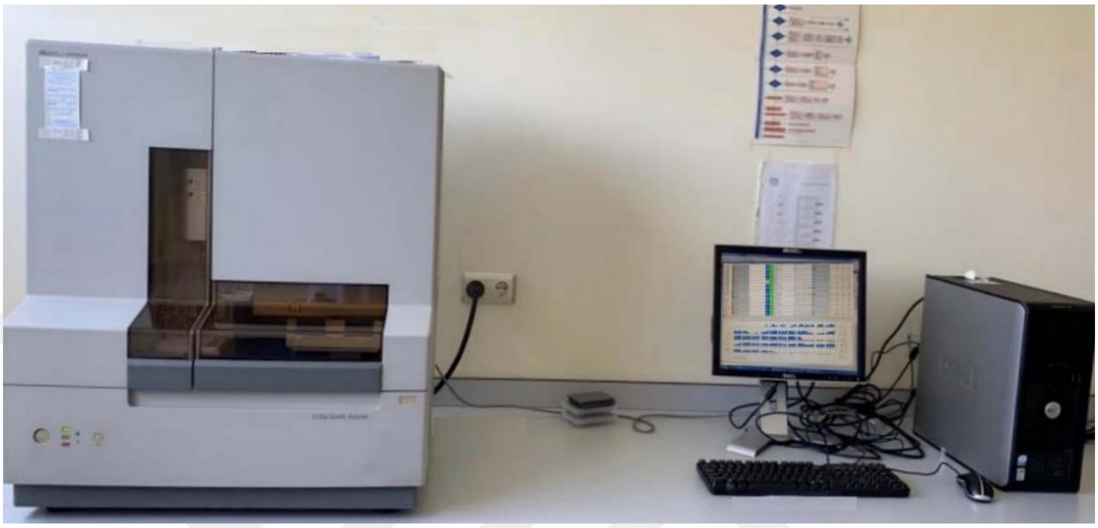


Figure 3.3. Applied Biosystems 3130*xl* Genetic Analyzer

4. RESULTS AND DISCUSSION

4.1. Phenotypic Identification and Susceptibility Testing of MTBC

The Çukurova University Tropical Diseases Research and Application Centre, Ministry of Health Public Health Institution, and Adana Regional Tuberculosis Laboratory received clinical specimens from eight cities in southern Turkey (Adana, Gaziantep, Antakya, Mersin, Osmaniye, Kahramanmaraş, Kilis, and Adıyaman) for potential tuberculosis cases. From these samples, a selection of 76 strains was made. Due to cultural failure or contamination, four strains were deleted from the study, and two duplicate samples were excluded.

The Bactec MGIT 960 TB system, widely regarded as the most reliable method for this task, verified that all 70 isolates were part of the MTBC. We subsequently employed the susceptibility test using the SIRE sets to determine the efficacy of first-line medicines against MTBC. Thirty of the total isolates demonstrated sensitivities to MGIT medicines: STR, INH, RIF, and EMB. We discovered that the remaining forty isolates were MDR-TB, indicating their resistance to both INH and RIF, at the very least. (see Figure 4.1).

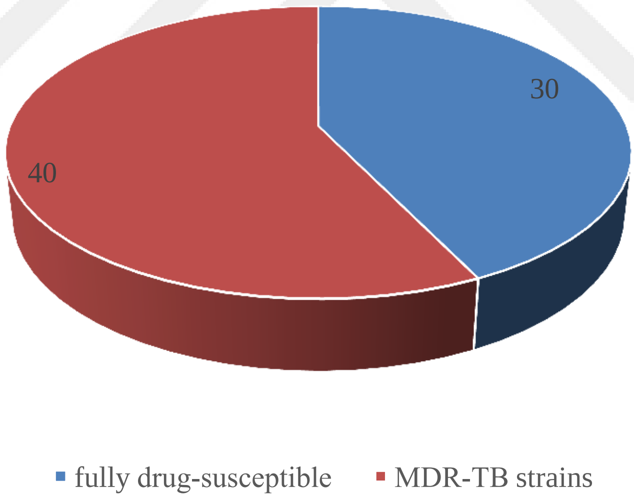


Figure 4.1. Distribution of MTBC isolates

The MDR-TB diagnosis is only confirmed through culture and sensitivity testing. The Löwenstein-Jensen medium method provides results in 30 to 60 days, which is excessively long. Automated culture methods for mycobacteria, such as the BACTEC MGIT 960 system, can provide results in 10 to 15 days. Diagnosing resistance through mutation in specific genes in cultured Bacillus DNA is also costly. However, it has allowed the identification of gene regions with a higher incidence of mutations and promoted the development of rapid diagnostic methods through molecular biology techniques.

The drug susceptibility profiles of these 40 MDR-TB isolates are shown in Figure 4.2. Of these 40 MDR-TB isolates, 75% (30/40) were resistant to only INH and RIF. 12.5% (5/40) are resistant to INH, RIF, and STR. INH, RIF, and EMB resistance was observed in 2.5% (1/40) of the samples, while 10% (4/40) exhibited resistance to all MGIT (SIRE) sets, including STR, INH, RIF, and EMB.

Table 4.1. The drug susceptibility testing (DST) profiles of MDR-TB strains

No. of Strains	**Susceptibility Profiles**				**Percentage (%)**
	Rif	Inh	Str	Emb	
30	R	R	S	S	75
5	R	R	R	S	12.5
4	R	R	R	R	10
1	R	R	S	R	2.5

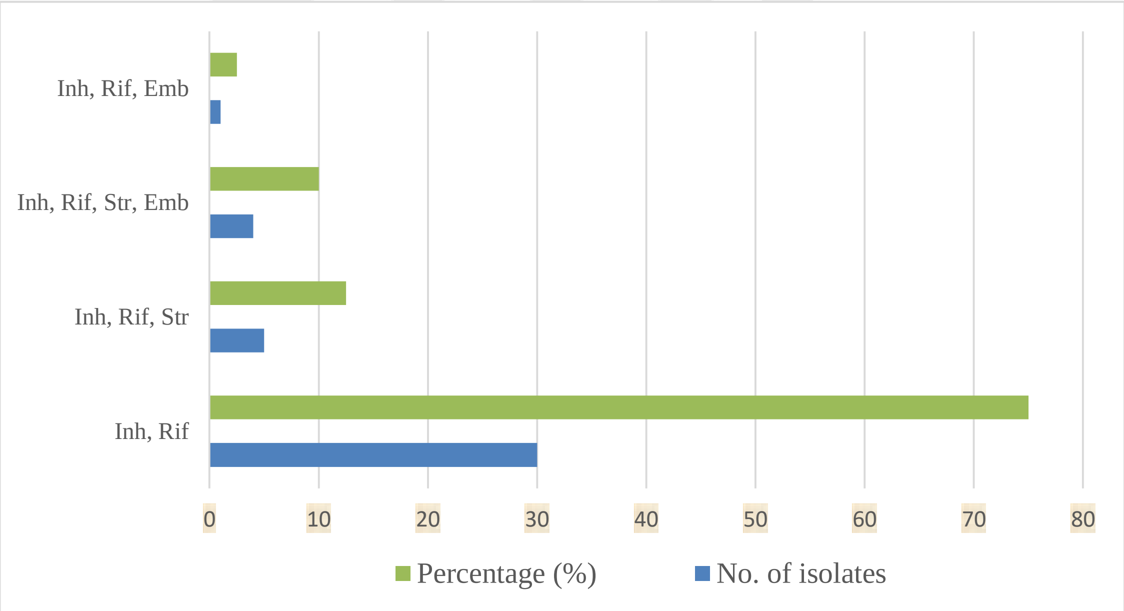


Figure 4.2. The drug susceptibility testing profiles of MDR-TB strains

The Bactec MGIT 960 TB system, widely recognized as the most reliable method for this objective, verified that all isolates were classified as MTBC. Following the confirmation of the isolates, we proceeded to do susceptibility tests on each of them. We conducted individual tests on all isolates to determine their resistance to first-line tuberculosis medications, such as isoniazid, rifampin, streptomycin, and ethambutol. All first-line drugs were fully susceptible to treatment in 30 isolates. Nevertheless, 40 isolates demonstrated resistance to both INH and RIF, confirming the presence of MDR-TB (refer to Figure 4.2).

4.2. Differentiation of MTBC by Multiplex PCR

We performed a molecular identification process using genomic DNA extracted from seventy clinical isolates of MTBC, including both sensitive and resistant strains obtained from sputum samples. This protocol entailed PCR amplifying the *rpoB*, *inhA*, *katG*, and *oxyR-ahpC* genes. PCR was performed using primers specific to the genus to achieve this objective. All amplified isolates utilized the previously mentioned genes to validate the identification of *M. tuberculosis*. The specific primer sets specified earlier in Table 3.2 (Materials and Methods section) were employed for this purpose.

4.2.1. Analysis of *rpoB* Gene PCR Results

A polymerase chain reaction was done on all 70 *M. tuberculosis* isolates for the gene *rpoB* using specific primers, as listed in Table 3.2. We confirmed the amplified product by running it on a gel and determined that the molecular size of the amplicon was 450 bp.

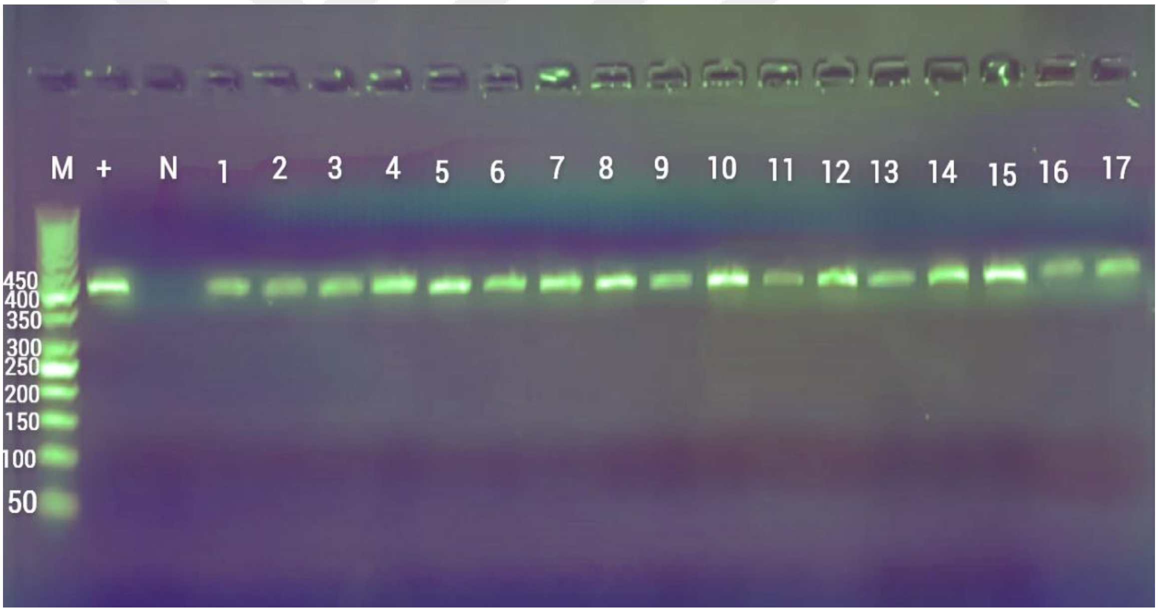


Figure 4.3. displays an agarose gel electrophoresis picture of the *rpoB* locus. The PCR products were examined using a 1.5% agarose gel and seen using a 0.01% ethidium bromide solution. We developed specific primers to generate a 450-base pair fragment of the *rpoB* gene. Column M contains a 50 bp DNA molecular weight standard ladder. The (+) column is the positive control, while the (N) column is the negative control. Columns 1-17 contain 450 base pair amplification products

4.2.2. Analysis of *katG* Gene PCR Results

We conducted a PCR on all 70 *M. tuberculosis* isolates to amplify the gene *katG* using the particular primers specified in Table 3.2. We subjected the amplified product to gel electrophoresis to validate the findings and ascertained that the amplicon possessed a molecular size of 500 bp.

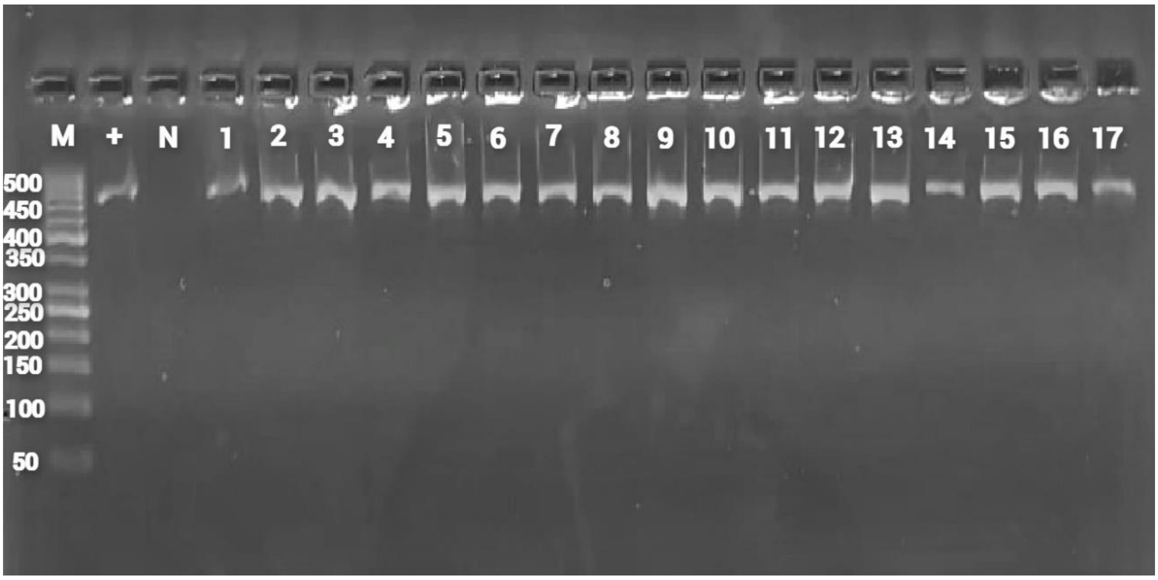


Figure 4.4. Displays a picture of agarose gel electrophoresis of the *katG* locus. We examined the PCR products using a 1.5% agarose gel and visualized them with a 0.01% ethidium bromide solution. We specifically designed primers to generate a 500-bp fragment of the *katG* gene. Column M contains a 50-base pair DNA molecular weight standard ladder. The (+) column is the positive control, while the (N) column is the negative control. Columns 1–17 contain 500 base pair amplification products

4.2.3. Analysis of *inhA* Gene PCR Results

All 70 *M. tuberculosis* isolates were subjected to a PCR to amplify the gene *inhA*. As shown in Table 3.2, specific primers were used for this purpose. The amplification was verified using gel electrophoresis, which revealed that the amplicon had a molecular size of 248 bp.

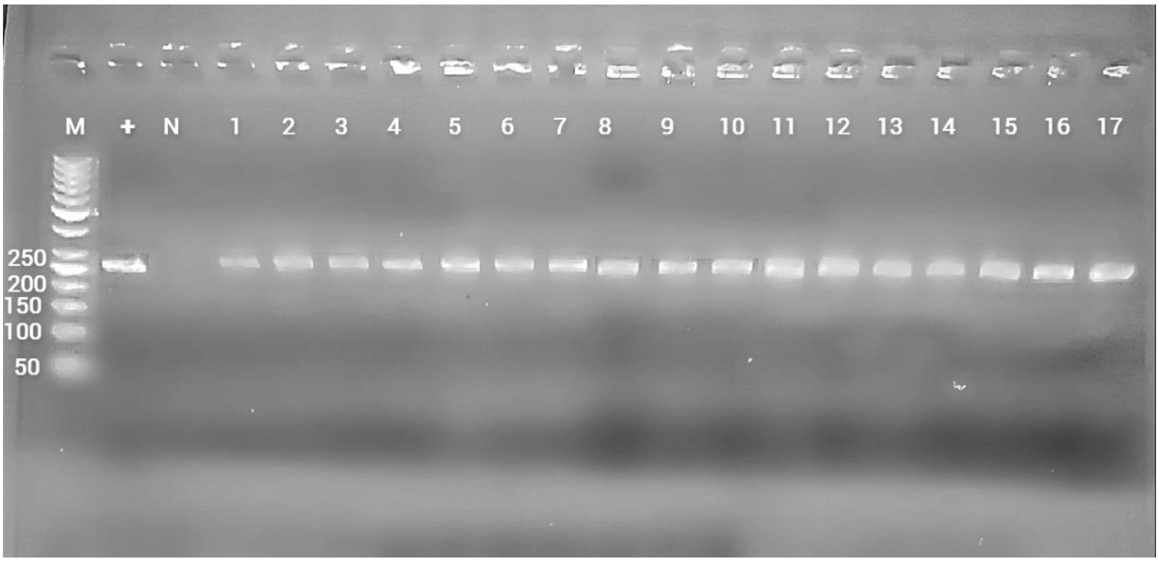


Figure 4.5. Displays the results of PCR experiments used to identify the *inhA* gene of *M. tuberculosis*. The assays were conducted using 1.5% Agarose gel electrophoresis. Column M has a 50 base pair DNA size marker, the positive control is denoted by (+), the negative control is denoted by N, and columns 1-17 contain amplification products of the *inhA* gene with a size of 248 bp.

4.2.4. Analysis of *oxyR-ahpC* Gene PCR Results

A PCR was performed on all 70 *M. tuberculosis* isolates to amplify the *oxyR-ahpC* gene. Table 3.3 demonstrates the utilization of specific primers for this objective. The amplification was confirmed using gel electrophoresis, which indicated that the amplicon had a molecular size of 293 bP.

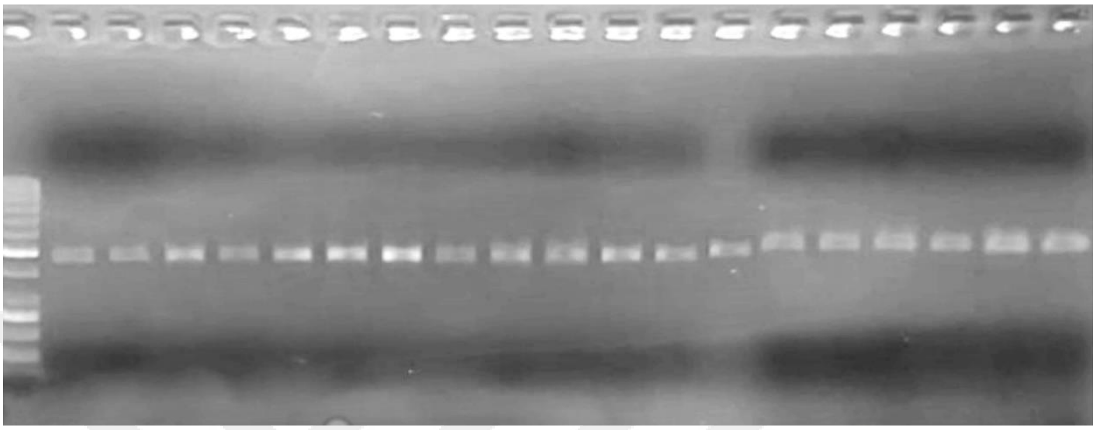


Figure 4.6. Presents the results of PCR experiments to identify the *oxyR-ahpC* gene in *M. tuberculosis*. The experiments were performed with 1.5% Agarose gel electrophoresis. Column M has a 50 base pair DNA size marker, the positive control is indicated by (+), and columns 1-18 contain amplification products of the *oxyR-ahpC* gene with a size of 293 bp

4.3. Mutation Analysis.

To detect mutations in the *rpoB*, *katG*, *inhA* regulatory region (*fabG*1), and *oxyR-ahpC* intergenic region, DNA sequence analysis was conducted for each of the 280 PCR products from 70 isolates. The DNA sequence analysis was performed on an automated DNA sequencer, the "ABI PRISM 3130*xl* Genetic Analyzer" (Applied Biosystems, Foster City, CA, USA).

The samples were analyzed using electrophoresis, and the resulting data were acquired as chromatograms. We thoroughly examined the chromatogram peaks of the forward sequences for every sample. The genes' nucleic acid and translated sequences were analyzed using the BioEdit software. The sequences were aligned using CLUSTAL W and then visualized as a graphical view using the BioEdit program. Next, we aligned the consensus sequences for each isolate with reference sequences obtained from PubMed GenBank. These reference sequences include 888164 (*rpoB*), 885638 (*katG*), 886551 (*fabG*1) in the *inhA* regulatory region, and 885717 in the *oxyR-ahpC* intergenic regions. We found mutations in the *rpoB*, *katG*, *fabG*1, and *oxyR-ahpC* intergenic regions by aligning them in CLUSTAL W and trimming them in GENEDOC (see Figures 4.8, 4.10, 4.12, and 4.14).

We selected putative or verified resistance genes and promoter areas associated with drug resistance. We examined all the mutations in these genes and promoter areas and compared them to the pan-susceptible reference genome (H37Rv, accession number: NC_000962.2). This analysis focused on identifying single nucleotide polymorphisms (SNPs) in promoter regions, changes in amino acids within genes, and deletions and insertions. Subsequently, we investigated the genetic alterations associated with the development of drug resistance. We compared the phenotypic and genotypic results to determine the specificity and sensitivity of each gene or area under examination.

4.3.1. Distribution of Mutations of *rpoB* Gene among 40 MDR-TB Isolates

RIF-resistant *M. tuberculosis* strains often exhibit mutations in the 81-base pair portion of the *rpoB* gene known as the RRDR. We conducted a genetic investigation of 70 isolates of *M. tuberculosis*, consisting of 40 MDR-TB and 30 DS-TB isolates. We examined the nucleotide sequences of their *rpoB* genes using Sanger sequencing.

The sequence alignment results revealed several notable alterations that are believed to be directly associated with medication resistance. Genetic variations were identified in the 450 bp segment of the *rpoB* gene that was amplified and sequenced. These variations were found in 31 strains (77.5%, 31/40) of the MDR-TB isolates that also had mutations in this area. Among 40 MDR-TB isolates, the most prevalent mutation type was *rpoB* Ser450Leu, observed in 21 isolates (52.5%, 21/40) in RRDR. Following codon 445, there were three isolates (7.5%, 3/40) with a *rpoB* His445Tyr mutation. Also, at codon 435, there were three isolates (7.5%, 3/40) with a *rpoB* Asp435Val mutation. Some of the 40 MDR-TB isolates had different mutations. One isolate (2.5%, 1/40) had a *rpoB* Leu430pro mutation, two isolates (5%, 2/40) had a *rpoB* Leu430 Leu mutation (nucleotide changes CTG-CTA but no effect on the protein's amino acid sequence), and one isolate (2.5%, 1/40) had a *rpoB* Leu494 Leu (CTG-CTT) silent mutation.

Additionally, there are phenotypically resistant strains with no mutations in the *rpoB* gene. Among the 40 MDR-TB strains, nine are phenotypically resistant without any mutations, and all 30 pan-sensitivity strains also have no mutations. WGS could account for 77.5% (31/40) of phenotypically MDR-TB strains. Jagielski et al. (2018), Jagielski et al. (2019), and Haratiasl et al. (2020) conducted studies that obtained some results similar to ours.

Table 4.2. Distribution of mutations of *rpoB* gene among 40 MDR-TB isolates

No. of isolates	Percentage distribution (100)	Location	Nucleotide change	Codon change	Amino acid location	Amino acid change	Pattern
21	52.5%	1349	C-T	TCG-TTG	450	Ser-Leu	C1349T
3	7.5%	1333	C-T	CAC-TAC	445	His-Tyr	C1333T
3	7.5%	1304	A-T	GAC-GTC	435	Asp-Val	A1304T
1	2.5%	1289	T-C	CTG-CCG	430	Leu-Pro	T1289C
2	5%	1290	G-A	CTG-CTA	430	-Leu-Leu	G1290A
1	2.5%	1482	G-T	CTG-CTT	494	-Leu-Leu	G1482T
9	22.5%	-	-	-	-	-	-

We studied genetic variants that provide resistance in patients with pulmonary tuberculosis. All clinical isolates tested for MDR-TB exhibited clinical resistance to rifampicin. We subjected the 450-bp amplified area to sequencing, encompassing a variable region from codon 420 to 500. We identified six distinct mutations in the *rpoB* gene in MDR-TB strains, two of which were determined to be silent.

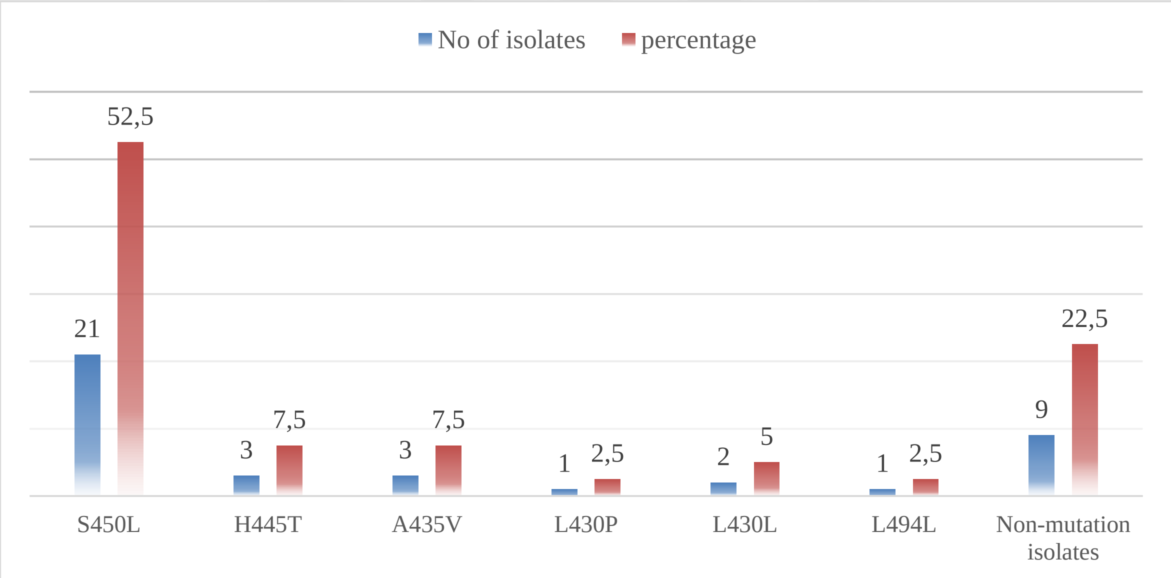
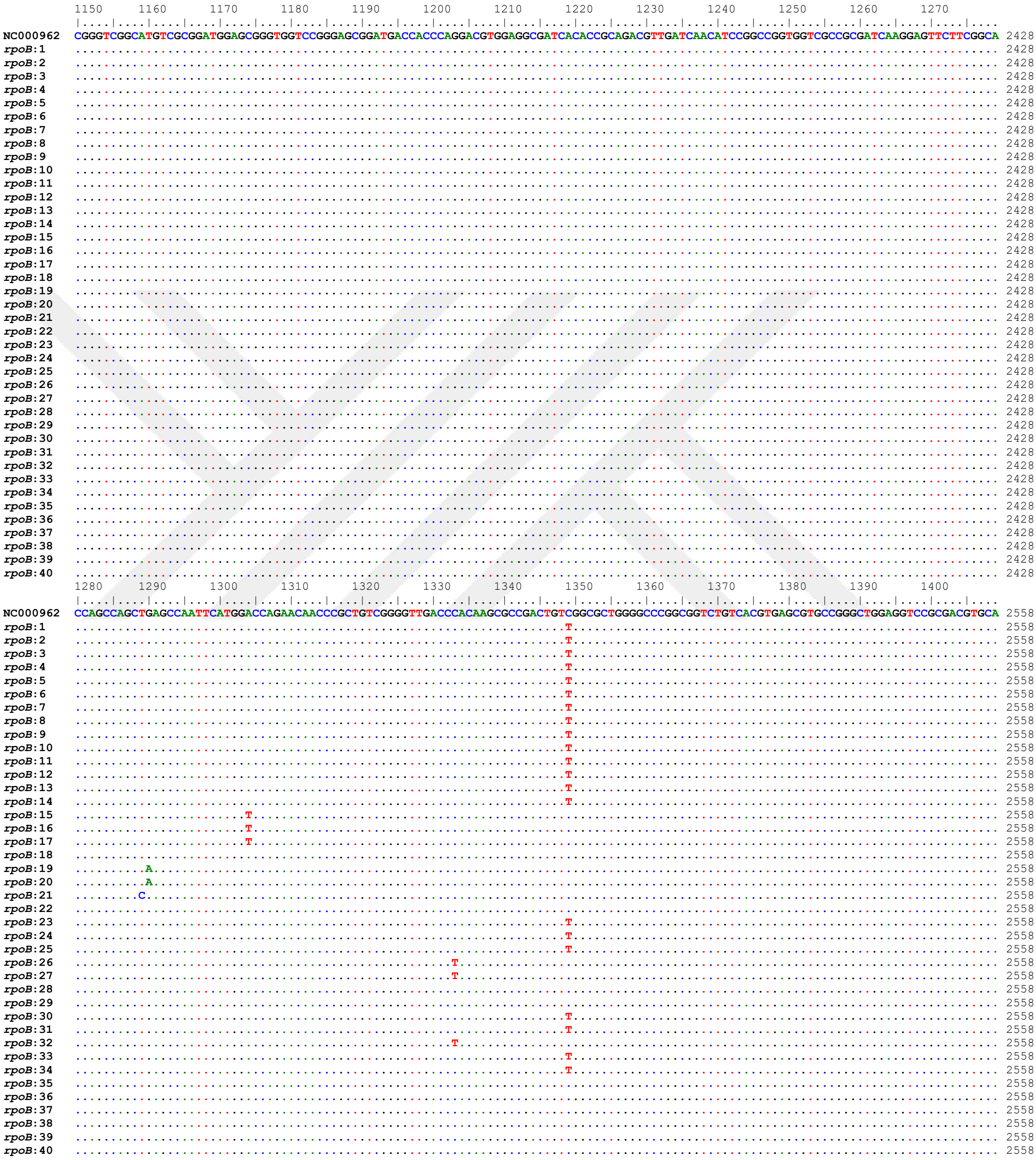


Figure 4.7. The distribution of the isolates was studied according to the mutations found in the *rpoB* gene

Multiple studies have shown that codon 450 in the *rpoB* gene is the most commonly mutated. The primary mutation observed in this codon is Ser450Leu (C1349T). This mutation is the most prevalent in globally occurring RIF-resistant strains, although its rates vary across different geographic areas. The mutation has a high prevalence in Kazakhstan (80.9%), Taiwan (66.7%), Italy (59.5%), and China (59.1%). It is similarly prevalent in South Korea (53.1%), Brazil (52.4%), Bangladesh (52.3%), and Australia (52%). However, it is somewhat less frequent in Spain (42.6%), Russia (41.5%), Mexico (40.4%), Vietnam (37.8%), and Hungary (Jagielski et al., 2018a). The codons most affected in our investigation were codon 450, observed in 52.5% of all MDR-TB strains, followed by codon 445 and codon 435, each at 7.5%. Codons 435 and 445 are the most frequently mutated *rpoB* codons in RIF-resistant strains, following codon 450. The mutation frequencies at these codons fall between 1.1 to 20.4% and 6.8 to 32%, respectively (Jagielski et al., 2018a; Jabbar et al., 2019).



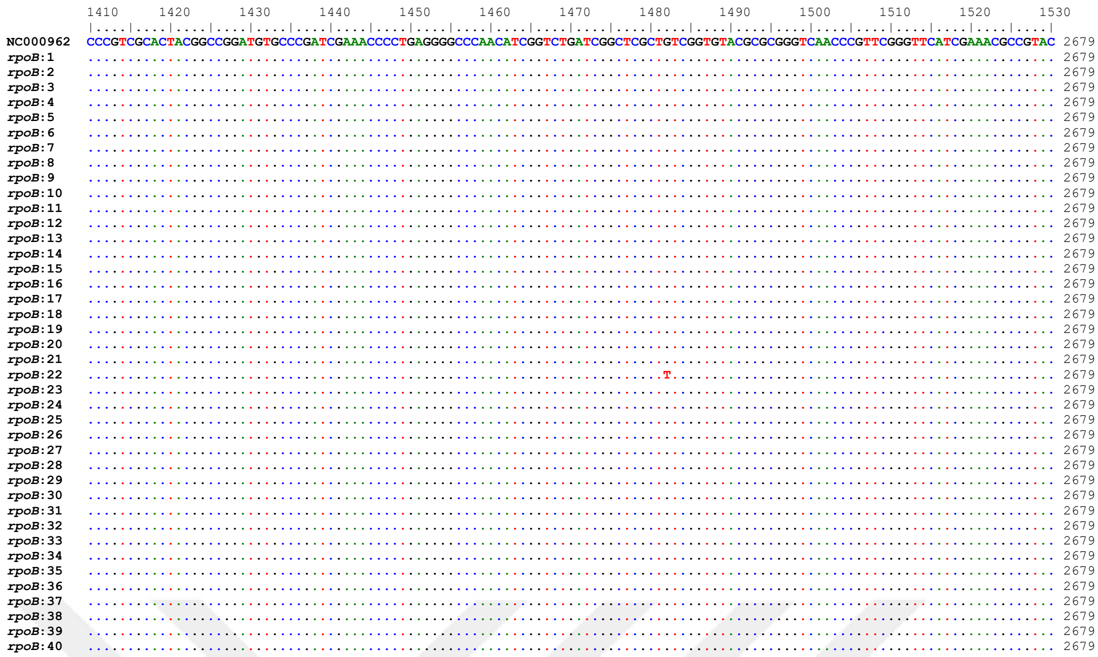


Figure 4.8. DNA sequences of the *rpoB* gene region for MDR-TB isolates

Changes in the mutability of *rpoB* codons and the frequency of specific *rpoB* mutations are caused by the fitness costs that come with the harmful effects that these mutations might have on the functions of RNA polymerase (RNAP) and DNA transcription. The Ser450Leu mutation provides an exemplary demonstration of this. We demonstrated the substitutions in the strains. Strains possessing this change exhibited equivalent fitness to other *rpoB* mutants, indicating no disadvantage in terms of fitness.

These mutations have a selection advantage, which allows them to persist and propagate more effectively throughout human populations. Hence, the clinical prevalence of specific *rpoB* mutations directly corresponds to their overall fitness. The link between resistance to INH and low-cost resistance mutations, such as *katG* Ser315Thr, also occurs. Strains exhibiting MDR-TB and XDR-TB phenotypes specifically anticipate this specific change, as the buildup of mutations associated with resistance would often compromise the life of the bacteria. An observation that supports this is the notably elevated prevalence of the *rpoB* Ser450Leu mutation among XDR-TB (Lau et al., 2011).

The disparities in the *rpoB* mutation patterns between the RIF-monoresistant and MDR-TB strains remain unknown. Previous research examining these two groups found a consistent finding: the Ser450Leu mutation was the predominant mutation in both RIF-monoresistant and MDR strains, with His445Tyr as the second most prevalent mutation. While the first mutation was more common in MDR strains (58.4% compared to 36.4% or 64.7% versus 57.1%), the second mutation was more widespread among RIF-monoresistant bacteria (36.4% versus 13.4%, or 28.6% versus 0%). Nevertheless, the substantial disparity in the proportions of the two strain groups may introduce a considerable bias to our findings (Jagielski et al., 2018a).

Our investigation revealed five alterations in the codons detected inside the RRDR areas. This limited number of changes could be due to insufficient samples and a low mutation rate. Small sample sizes are inadequate for identifying complete mutations. Four of the identified mutations related to those mutations have been previously documented. In contrast to two mutations, namely Leu430Leu (silent) and Leu494Leu (silent), additional research is still necessary to determine if these mutations can be considered indicators of drug resistance.

In a study by Lau et al. (2011), specific mutations were found to be natural polymorphisms that are valuable for studying the evolution of the genome. These mutations were observed at higher frequencies in resistant and sensitive strains, indicating they were not responsible for conferring drug resistance (Lau et al., 2011).

Freitas (2009) analyzed 120 MDR-TB strains in his study. Thirteen were resistant to RIF and did not show mutations (10.8%). When RIF resistance is combined with the absence of mutations in the RRDR region of the *rpoB* gene, it is often associated with mutations in other codons outside this region, such as 146, 25, or the presence of another resistance mechanism. Specific oligonucleotides were designed for a more comprehensive region of the *rpoB* gene for these 13 samples, and only a silent mutation was found, indicating the involvement of another resistance mechanism other than mutation (Freitas, 2009).

In their study, Khademi et al. (2017) discovered that MDR-TB, which is resistant to INH and RIF, constituted 5% of new tuberculosis cases and 26.6% of cases when treatment was repeated in the Middle East. This suggests an increasing pattern. The mutation has been observed in over 95% of RIF-resistant bacteria in the 81-bp RRDR region, specifically in codons 507 (426) to 533 (452). The codons 531 (450), 526 (445), and 516 (435) exhibit the most frequent alterations (Khademi et al., 2017). Haratiasl et al. found mutations at codons 531 (450) (56.3%), 533 (452) (12.5%), 526 (445) (6.3%), and 516 (435) (6.3%) of the *rpoB* gene in their 2020 study. The predominant mutation observed was the substitution of Serine at position 531 (450) with Leucine. The prevalence of RIF resistance-causing mutations can differ among drug-resistant *M. tuberculosis* isolates depending on the geographical location (Haratiasl et al., 2020).

In 10.7% of cases, the study conducted in the Russian Federation involving 412 MDR-TB strains and mass spectrometry analysis observed no mutation. Another possibility for this, in addition to the ones described above, is heteroresistance, which is defined as the coexistence of bacteria resistant to and sensitive to TB drugs in the same patient. It is a pre-stage to overall resistance and can occur for two reasons: superinfection with two different strains or the transformation of one strain into two, one resistant and one sensitive, due to the patient's non-adherence to treatment. Culturing a suspected strain of heteroresistance in a medium with RIF selects the resistant variant and allows for the correct diagnosis of mutations (Afanas' ev et al., 2007).

4.3.2. Gene Mutations Conferring Resistance to Isoniazid

Structurally, the drug isoniazid is simple, yet *M. tuberculosis* has a highly complex resistance mechanism against it. This is attributed to the involvement of numerous genes, including *katG*, *inhA* ORF, *fabG*, *inhA* regulatory region, *oxyR-ahpC* intergenic region, *ahpC* ORF, and various other genes. The current study aimed to elucidate the correlation between different types of combination mutations and the corresponding phenotypic resistance. Using a WGS platform, we were able to discover alterations in these genes for each individual in the collection at the same time. Using these findings, we determined the impact of these genetic alterations on the development of INH resistance in MDR-TB strains (Liu et al., 2018).

To study resistance to INH due to mutations in these three genes, we analyzed 70 *M. tuberculosis* strains. Of these, 40 are MDR-TB, and 30 are sensitive to all four first-line drugs. Our study focused on regions that exhibit different patterns concerning the numbers and types of mutations. Therefore, we can conclude that the mutation responsible for resistance is either in one of these studied gene regions, another region of the same gene, or another gene related to drug resistance.

katG was associated with the most frequent mutation in INH-resistance isolates, with 75%–90% of mutations occurring in codon 315. This was followed by *inhA* (8%–20% in the *inhA* promoter), *ahpC*, and its promoter (6%–13%). In 10% to 25% of INH-resistant strains, the absence of mutations in the studied loci suggests that resistance may have been caused by other loci not examined here or by different resistance mechanisms, such as efflux pumps.

4.3.2.a. Distribution of Mutations of *katG* Gene among 40 MDR -TB Isolates

We employed sequence alignment to compare the sequenced loci and their translated counterparts among isolates and a wild-type strain of *M. tuberculosis* to determine the intra-sequence variation. The predominant *katG* mutation was identified in the active site, particularly at the INH binding site. There is a strong link between the *katG* gene and resistance to INH because of the Ser315Thr residue mutation. Amplification and sequencing determined that 22 out of 40 strains (55%) in the 500-bp segment of the *katG* gene at codon 315 had mutations. Three strains (7.5%, 3/40) had the Gly316Ser mutation, two strains (5%, 2/40) had the Leu336Pro mutation, two strains (5%, 2/40) had the Ser309Thr mutation, one strain (2.5%, 1/40) had the Trp328Cys mutation, one strain (2.5%, 1/40) had the Asn311Phe mutation, and one strain (2.5%, 1/40) had both the Trp328Cys and Ser315Asn mutations in the *katG* gene.

Table 4.3. Distribution of mutations of *katG* gene among 40 MDR-TB isolates

No. of isolates	Percentage distribution (100)	Location	Nucleotide change	Codon change	Amino acid location	Amino acid change	Pattern
21	52.5%	944	G-C	AGC-ACC	315	Ser-Thr	G944C
1	2.5%	944	G-A	AGC-AAC	315	Ser-Asn	G944A
3	7.5	948	G-A	GGC-AGC	316	Gly-Ser	G946A
2	5%	1007	T-C	CTG-CCG	336	Leu-Pro	T1007C
2	5%	898	T-G	GGT-GCT	309	Ser-Thr	T898G
1	2.5%	984	G-T	TGG-TGT	328	Trp-Cys	G984T
1	2.5%	984 944	G-T G-A	TGG-TGT AGC-AAC	328 315	Trp-Cys Ser-Asn	G984T G944A
1	2.5%	931,932	GA-TT	GAC-TTC	311	Asn-Phe	G931T A932T
8	20%	-	-	-	-	-	-

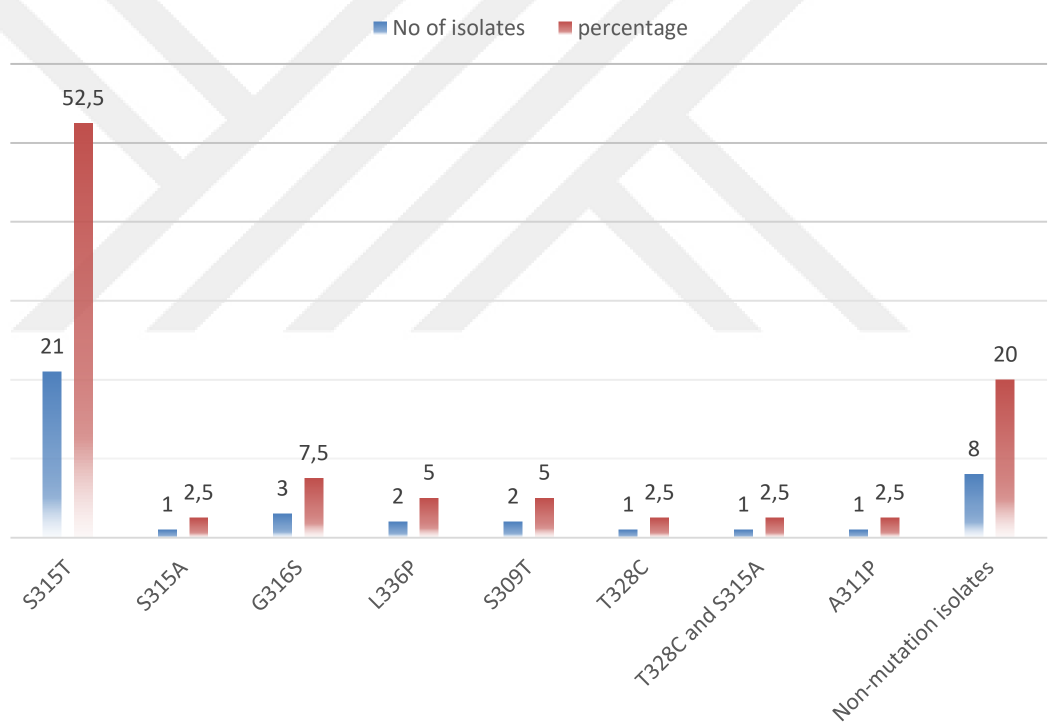


Figure 4.9. The distribution of the isolates was studied according to the mutations found in the *katG* gene

Our results for the *katG* Ser315 mutation were similar to the earlier reported rates of patients diagnosed with MDR-TB in Korea (49.1%), the Netherlands (55%), Kuwait (65%), Morocco (68.6%), and Russia (76.9%) (Ilyas et al., 2023; Rudeeaneksin et al., 2023)

Prior published data has shown that *katG* 315 mutations, particularly Ser315Thr, are the primary factor behind INH resistance, with a prevalence ranging from 42% to over 90% in various locations worldwide (Liu et al., 2018).

An analysis of the *katG* gene in this study found the Ser315Thr (AGC-ACC) mutation in 21 of the 40 INH-resistant isolates. This is 52.5% of all MDR-TB. The study specifically examined mutations between codon 193 and codon 370 of the *katG* gene. The mutation Ser315 was detected most often in the study and was linked to resistance to INH.

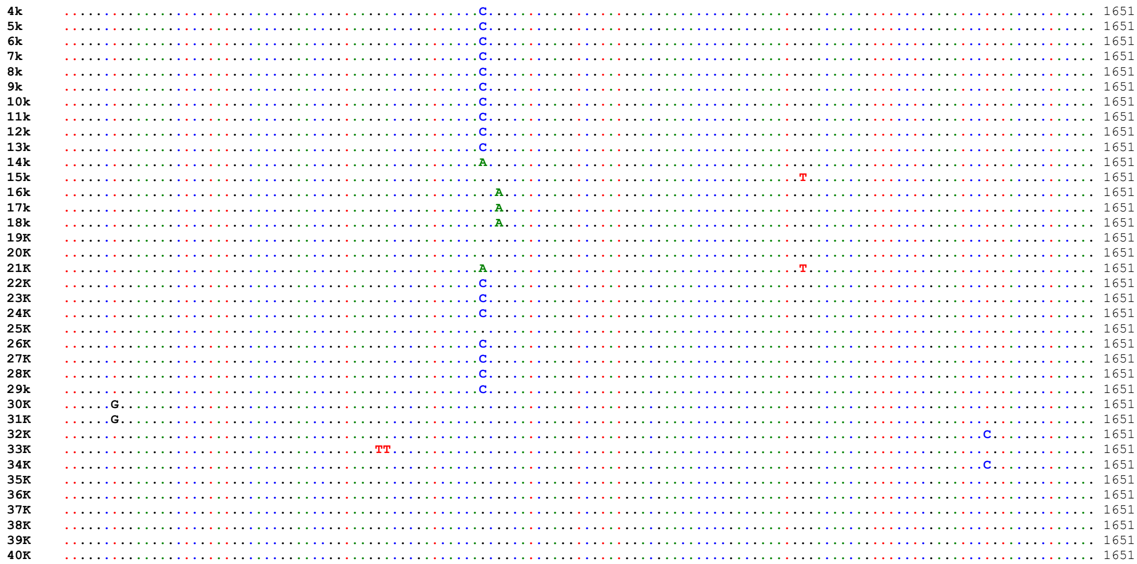


Figure 4.10. Continued: DNA sequences of the *katG* gene region for all MDR-TB isolates

This substitution causes a reduction in the size of the access path to the heme group. This reduces the likelihood of INH binding and activating and decreases the possibility of NAD-INH adduct formation. Remarkably, this alteration partially preserves the *katG* catalase-peroxidase function. Additional mutations, such as the Leu336Pro substitution, result in protein instability, causing the protein to unfold and rendering it unable to activate INH or catalyze its reaction.

The systematic review by Nguyen et al. (2023) analyzed 118 articles and examined 11,411 *M. tuberculosis* isolates from 49 countries. The review revealed that 64% of the observed phenotypic INH resistance was linked to the *katG*315 mutation. Various investigations have indicated that 54.5% of isolates in Japan possess the *katG*315 mutation. Among the 125 clinical isolates of *M. tuberculosis* in Iran, 34 strains demonstrated resistance to INH, while INH sensitivity was observed in 91 strains. *katG*315 mutations were found in 46% of the 34 isoniazid-resistant strains (Nguyen et al., 2023).

The mutations associated with resistance may vary based on geographical locations, including within the same country. This variability offers valuable information about the spread and distribution of tuberculosis. Among the 50 strains of TB resistant to INH, 74% (37/50) showed the presence of the *katG* mutation. A study in Tamil Nadu and Puducherry revealed that 71.0% of isolates resistant to isoniazid (INH) mutations were located in the *katG* region. The prevalence of these mutations was 11.8% among the 15,438 isolates (Kumar et al., 2023).

This substitution is frequently observed in MDR-TB and XDR-TB strains and is linked to varying levels of resistance to INH, ranging from intermediate to high. In addition, *M. tuberculosis* strains with mutations at codon 315 in the *katG* gene retain their ability to cause disease. Several investigations examining the S315T mutation and other variations of *katG* have shown that changes in the activity of *M. tuberculosis* proteins are closely associated with modifications in the protein structure (Unissa et al., 2018).

M. tuberculosis may exhibit susceptibility to high doses of INH. Research findings indicate that 64% of the 70 samples from India, 62% of the 79 samples from Spain, and 53% of the 67 samples from northern Mexico have this mutation. Partially concurring, additional studies have revealed that 67% of 37 samples obtained from the northern region of Mexico exhibit this genetic alteration, alongside 69% of 105 samples from Poland and 72% of 69 samples from Brazil. In another investigation, the mutation frequency of codon 315 demonstrated no variation; all 92 isolates from Kazakhstan had the mutation, amounting to 100%. Hence, examining this mutation within a regional framework rather than a global comparison is imperative (Zenteno-Cuevas et al., 2009).

Of the 43 strains, 93% had mutations in the katG gene. The mutations at codon 315 were the most commonly reported, occurring in 34 strains, which accounted for 74% of the total. Few mutations were observed at other codons: specifically, four strains had mutations at codon 463, 2 strains had mutations at codon 131, and two additional strains had mutations at codon 234 (Jagielski et al., 2013). Otkun et al. (2003) conducted a study in Edirne, a city in northwest Turkey, where they discovered that the Ser315Thr mutation in the *katG* gene was present in 56.5% of the strains (52 out of 92).

Aktaş's study revealed notable disparities between the group of isolates with MDR strains (78.9%) and the group of isolates with INH mono-resistant strains (25%) among the isolates exhibiting mutations at codon 315 of the *katG* gene (Aktas et al., 2005).

Researchers conducted another investigation examining the genetic mutations of 82 *M. tuberculosis* strains resistant to INH. Researchers collected the strains from various parts of Vietnam and analyzed them using sequence analysis. Five distinct mutations were detected in the *katG* gene, with the mutation at codon 315 being the most prevalent (76.83%). The researchers found that the amino acid change Ser315Thr was the most prevalent, occurring in 73.1% of cases. Lower rates were seen for the Ser315Asn, Gly309Cys, and Lys327Ile modifications (Minh et al., 2012). The catalase-peroxidase enzyme, expressed by the *katG* gene, transforms INH into its biologically active state. Mutations in *katG*, specifically at codon 315, result in significant resistance to INH, making it ineffective for treatment (Aktas et al., 2005; Rudeeaneksin et al., 2023).

In their study, Kandler et al. (2018) utilized WGS to examine 52 strains of INH-resistant *M. tuberculosis*. They discovered five previously unidentified mutations in the *katG* gene, specifically substitutes of residues Trp121Gln, Trp161Arg, and Leu415Pro, as well as a stop codon at position Ala402 and a deletion at position 480 (Kandler et al., 2018). Islam et al. (2019) conducted an extensive susceptibility test on 206 clinical isolates, evaluating the effectiveness of 10 drugs, including INH, RIF, and EMB. *katG* was shown to have numerous new variants associated with INH resistance, including C20R, G33V, W91stop, W91R, P92S, G125S, Q127P, D142G, L147P, G111S, S211G, G279V, A312P, H417Q, Q461P, G466R, G490S, V581G, V431A, L436P, N508D, E607A, and N660D (Islam et al., 2019).

WGS was utilized to predict drug resistance in 137 multi-drug resistant tuberculosis (MDR-TB) isolates from Shanghai and 78 isolates from Russia. This study also discovered a new *katG* mutation, S17G, that is relevant to isoniazid (INH) resistance. The N-terminal region of *katG* plays a role in interdomain contacts, which is crucial for dimerization (Wang et al., 2022). The mutations S17G, C20R, and G33V can potentially lead to instability in the process of protein dimerization. Studies have indicated that residues 278 to 312 form a loop that could play a role in the binding site of INH. The mutation G279V is anticipated to reduce the affinity of INH binding while leaving the *katG* function unaffected. Further research is required to classify these new mutations as providing resistance and identify the resistance mechanism. However, the WGS approach has effectively predicted changes that give resistance to *M. tuberculosis* (Kandler et al., 2018).

4.3.2.b. Distribution of Mutations in the *inhA* Promoter Region among 40 MDR-TB Isolates.

Resistance to isoniazid is linked not only to *katG* gene mutations but also to mutations in the *inhA* gene and its promoter. We determined the DNA sequence of the *inhA* gene for 30 INH-susceptible *M. tuberculosis* isolates and 40 INH-resistant isolates. We found resistance-associated mutations within the *inhA* gene in 12 (30%) of the 40 MDR-TB isolates, while all DS-TB isolates had the wild-type sequence.

The primer of 248 nucleotides amplified the promoter region (equivalent to 82 amino acids) of the *inhA* operon gene. So, we completely sequenced this locus in all 40 MDR-TB strains and found that ten had a 25% change (*inhA*-15 C-to-T) in the *inhA* promoter region. We found a second mutation (*inhA*-47 G-to-C) in the *inhA* promoter region in 2.5% of isolates that were not susceptible. We also found mutations in the *inhA* promoter region (*inhA*-64, G-to-C) in 2.5% of the isolates, as indicated in Table 4.4.

Previous reports indicate that the mutation at −15 (C→T) in the *inhA* promoter region is the most prevalent in the *inhA* gene. Still, unlike *katG*, other *inhA* promoter mutations do not typically co-occur with *inhA* -15. Including mutations at *inhA*-47 and *inhA*-64 as resistance markers identified an additional 2 (5%) phenotypically resistant isolates.

Table 4.4. Distribution of *inhA* promoter mutations among 40 MDR-TB isolates

No. of isolates	Percentage distribution (100)	Location	Nucleotide change	Pattern
10	25%	-15	C-T	C-15T
1	2.5%	-47	G-C	G-64C
1	2.5%	-64	G-C	G-47C
28	70%	-	-	-

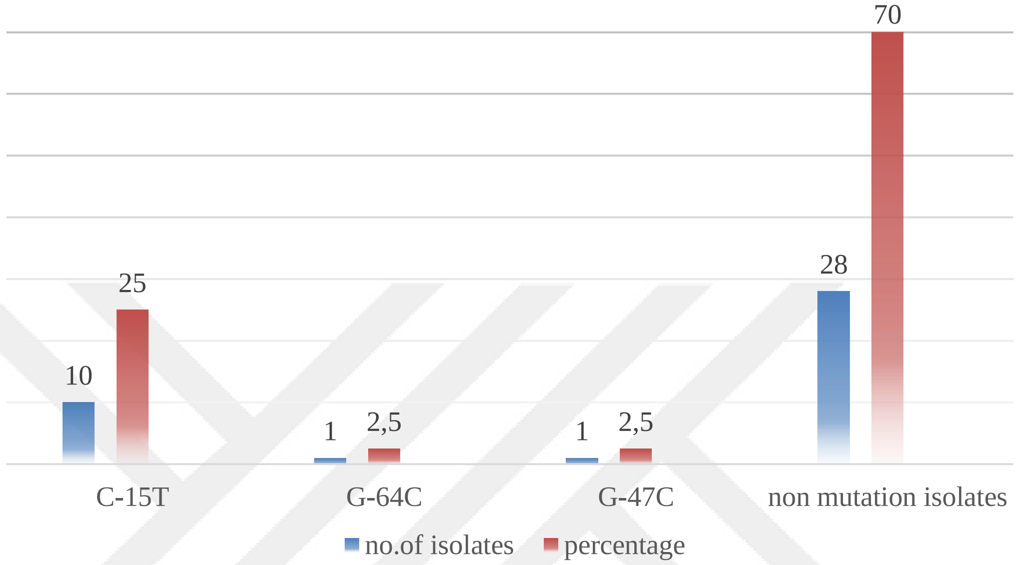


Figure 4.11. The distribution of the MDR-TB isolates was studied according to the mutations found in the *inhA* promotor

Mutations in the *inhA* gene can result in resistance to INH through changes in the drug's target. Drug targets exhibit a high degree of conservation. The occurrence of *inhA* structural gene mutations, which account for approximately 30% of cases and primarily occur at two polymorphic locations, is insignificant in clinical isolates. However, *M. tuberculosis* follows an alternative route. Mutations are introduced in the *inhA* promoter region when the drug target is overexpressed. Consequently, this results in decreased levels of resistance.

```
                0         10        20        30        40        50        60        70        80
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
H37Rv       TGCGAAAGTTCCCGCCGGAAATCGCAGCCACGTTACGCTCGTGGACATACCGATTTCGGCCCGGCCGCGGCGAGACGATAGGTTGTCGGG  70
1           ..........................................................................................  70
2           ..........................................................................................  70
3           ..........................................................................................  70
4           ..........................................................................................  70
5           ..........................................................................................  70
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39          ..........................................................................................  70
40          ..........................................................................................  70

                  90        100       110       120       130       140       150       160
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
H37Rv       GTGACTGCCACAGCCACTGAAGGGGCCAAACCCCCATTCGTATCCCGTTCAGTCCTGGTTACCGGAGGAAACCGGGGGAT  150
1           ................................................................................  150
2           ................................................................................  150
3           ................................................................................  150
4           ...................................C............................................  150
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36          ................................................................................  150
37          ................................................................................  150
38          ................................................................................  150
39          ................................................................................  150
40          ................................................................................  150
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Figure 4.12. Continued: DNA sequences of the *inhA* promotor region for all MDR-TB isolates

Within the scope of this investigation, we found that 25% of the isolates exhibiting resistance contained a -15 C>T mutation in the promoter region. An investigation has documented an average incidence rate of about 19%, which agrees with our findings, suggesting that this mutation provides resistance. The mutation −15 C>T alone confers a state of low-level resistance. However, if it matches a mutation in *katG*, mainly S315T, it will result in high resistance. Only two isolates that carried the -15 C>T mutation did not have any mutations in the *katG* gene. Further research is required to explore the potential synergistic effects of mutations in other regions of the isolates with *inhA* promoter mutations (Bakhtiyariniya et al., 2022).

A study by Wan et al. found that isolates resistant to INH had low mutation rates in *inhA* and *ahpC* coding areas. Additional investigations into the phenotypic consequences of these mutations are necessary to establish their correlation with INH resistance. An additional four genes (*kasA, efpA, ndh, iniA, iniB*, and *iniC*) have a high incidence of mutations in INH-resistant *M. tuberculosis*. According to Wan et al., the mutation frequencies for *ndh, iniA, iniB,* and *iniC* were 12.6%, 3.3%, 24%, and 5.5%, respectively (Wan et al., 2020).

When explicitly examining mutations in the *inhA* promoter, a C-15T substitution was detected in 51 out of the 66 isolates (77%), all exhibiting a low INH resistance state. In 2015, Cambau et al. found that 83% of isolates with mutations in the *inhA* promoter had low-level resistance, with a MIC ranging from 0.1 µg/mL to 1 µg/mL. Additionally, they observed that 93% of isolates with the *katG* S315T mutation had intermediate-level resistance, with a MIC ranging from 3 µg/mL to 10 µg/mL (Cambau et al., 2015).

Previous studies primarily identified the mutation in *inhA*-15 in all strains, regardless of their sensitivity or resistance to isoniazid. However, they did not specifically examine mutations in *katG*315 and *inhA*-15 as separate entities. The prevalence of *inhA*-15 mutations exhibited a range of frequencies: 2.7%, 8%, 20%, 22.9%, 31.6%, and 37.8%. Three distinct investigations, conducted by Sajduda (2004), Yang (2005), and Feuerriegel (2009), examined the correlation between alterations in *inhA*-15 and *katG*315 and the development of resistance to isoniazid (Setareh et al., 2009).

Among the 40 MDR-TB strains examined in this investigation, 10 (25%) had mutations in *inhA*-15. Five strains had concurrent mutations in *inhA*-15 and *katG*315. Prior research has established that mutations in the *inhA* promoter cause low-level resistance to isoniazid. Therefore, when considering the epidemiology and prevalence of isoniazid resistance, the importance lies more in mutations in *katG*315, which are highly significant in resistance to isoniazid at a high level.

4.3.2.c. Distribution of Mutations in the *oxyR-ahpC* Intergenic Region among 40 MDR-TB Isolates.

The amplified primer had a total of 293 nucleotide fragments. There were 43 of the *ahpC* gene, 106 from the intergenic region, and 144 from the oxyR pseudogene. Among the 40 MDR-TB isolates, 35 (87.5%) had no mutations in this specific area. Out of the total isolates, five (12.5%)

had mutations detected at three polymorphic locations. Three isolates were resistant, making up 7.5% of the total. These isolates had changes in the *oxyR-ahpC* intergenic region, specifically in the *ahpC* promoter. Among these isolates, two (5%) had a replacement of (G to A) at position -88, while the remaining isolate (2.5%) had a (G to A) mutation at position -48. In the *ahpC* structural gene, the isolates changed two nucleotides (T to C) at position 17 upstream of the *ahpC*. Further mutations in *katG* accompanied every nucleotide variation inside the intergenic region of *oxyR-ahpC*. Two isolates, accounting for 6.7% of the susceptible isolates, exhibited mutations in the *oxyR-ahpC* structural gene.

Table 4.5. Distribution of mutations of *oxyR-ahpC* gene among 40 MDR-TB isolates

No. of isolates	Percentage distribution (100)	Location	Nucleotide change	Pattern
2	5%	-88	G-A	G-88A
1	2.5%	-48	G-A	G-48A
2	5%	17	T-C	T17C
35	87.5%	-	-	-

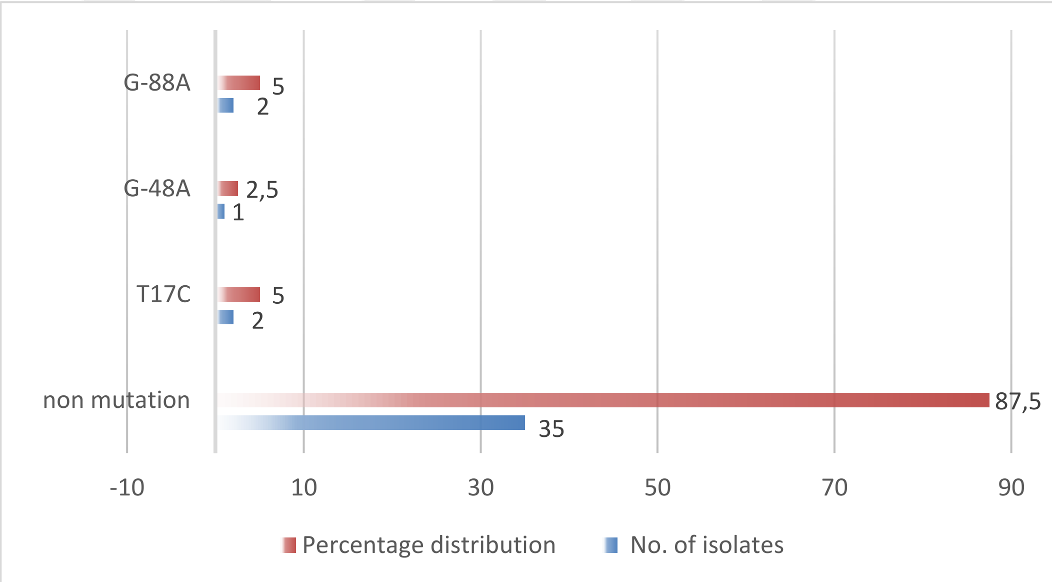

Figure 4.13. The distribution of the MDR-TB isolates was studied according to the mutations found in the *oxyR-ahpC* intergenic region

Earlier research found that between 4.8% and 24.2% of the isolates resistant to INH had genetic changes in the *oxyR-ahpC* region (Cardoso et al., 2004; Zhang et al., 2005). The study by Liu et al. (2018) found that 18.6% of isolates resistant to INH had mutations in the central region of the *ahpC* promoter. This study, on the other hand, found fewer mutations in the *oxyR-ahpC* area (Liu et al., 2018)

All five isolates in our investigation exhibited an extra mutation in the *katG* gene. Genetic and biochemical investigations have demonstrated that specific mutations in the promoter region *ahpC* lead to increased production of *ahpC* and compensate for the decrease in catalase activity caused by *katG* mutations (Zhang et al., 2005).

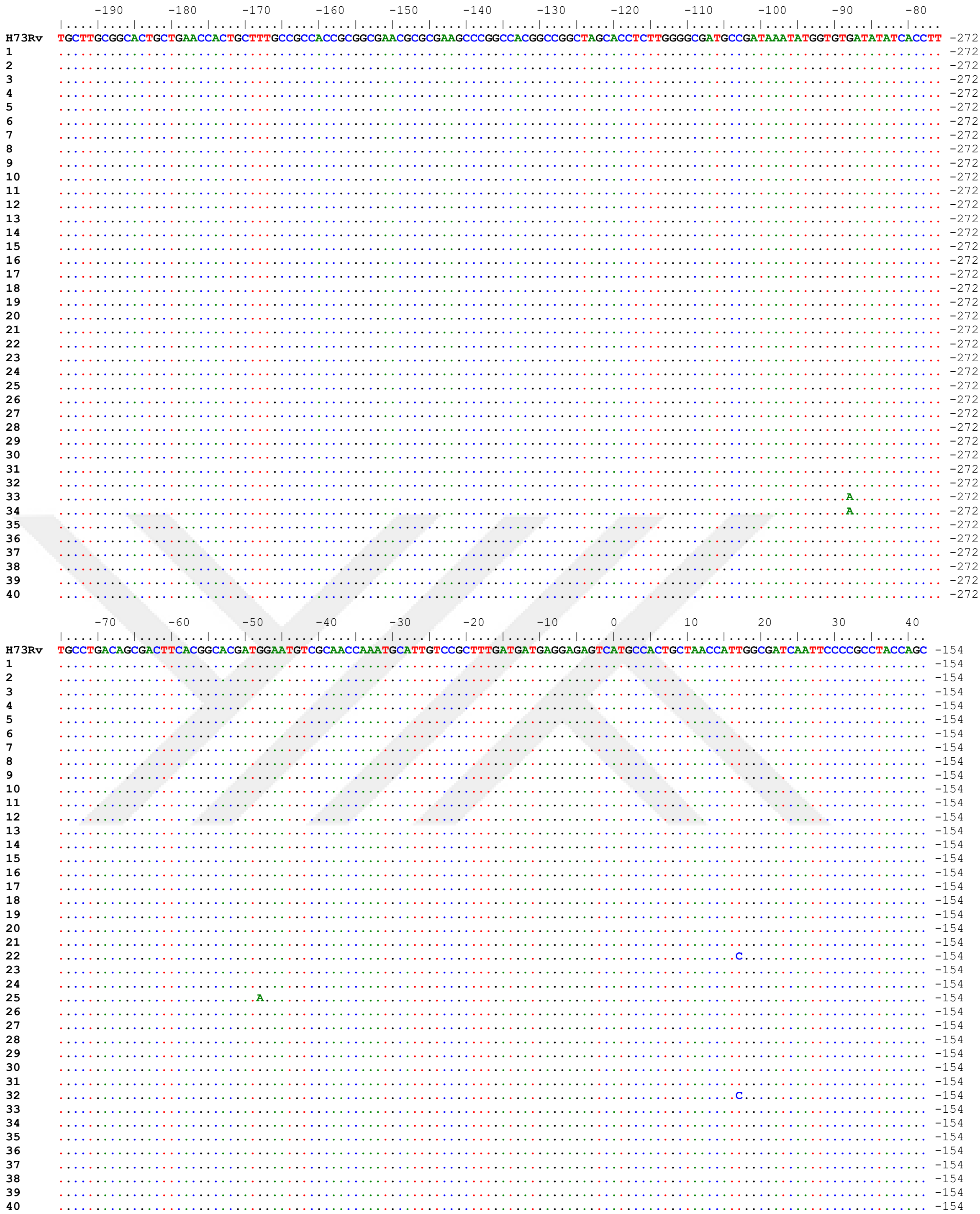

Figure 4.14. DNA sequences of the *oxyR-ahpC* intergenic region for all MDR-TB isolates

Previous research has also identified two mutations (-88 G>A and *ahpC* − 48 G>A) in isolates susceptible to INH (Laurent et al., 2020). The −88G>A polymorphism and other alterations in the *ahpC* promoter are unlikely to have contributed to INH-resistanceM. These mutations may not provide any fitness benefit and should not be used to predict isoniazid resistance. Additional mutations in the *ahpC* promoter region are present in isolates susceptible to or resistant to isoniazid (Laurent et al., 2020).

A prior investigation has established that mutations in *oxyR-ahpC* are compensatory alterations resulting from a decline in catalase-peroxidase activity. *ahpC*-oxyR mutations are infrequent due to the scarcity of data from earlier detections. Most of the MDR-TB isolates had mutations in this regulatory area, and their MIC readings were higher than 6.4 mg/L, showing they were very resistant. These isolates had *oxyR-ahpC* mutations within the region, ranging from −48 to −54 (Liu et al., 2018).

The acquisition of these compensatory changes can contribute to the survival of bacteria and the development of transmissible resistant strains, which threaten public health. The mutations identified in the *oxyR-ahpC* gene complex were not limited to resistant isolates. The reduced fitness of INH-resistant isolates may hinder their prevalence among the *M. tuberculosis* population in the province (Gygli et al., 2017).

4.3.3. Sensitivity, Specificity, and Overall Accuracy of gDST Results to Predict Resistance Against RIF and INH.

As described in Table 4.6, we investigated further the sensitivity and specificity of whole-genome sequencing DST for RIF and INH in predicting drug resistances, as well as the overall agreement (proportion of resistant and susceptible strains), in comparison to the phenotypic standard method BACTEC™ MGIT 960. The results of our study showed that the sensitivity and specificity for detecting resistance to RIF were 77.5% (95% CI: 70.24% to 84.76%) and 100% (95% CI: 92.97% to 100%), respectively, among 40 MDR-TB and 30 pan-sensitivity isolates. This resulted in an overall agreement of 88.75% (95% CI: 81.61% to 92.38%). The mutations in *rpoB* Ser450Leu, Asp435Val, His445Tyr, Leu430Pro, and Leu430Leu (silent mutations) caused five discordant resistant results.

The INH resistance prediction exhibited a sensitivity of 85% (95% CI: 77.04% to 92.96%) and a specificity of 93.3% (95% CI: 86.27% to 100%). The total concordance of the diagnosis resistant to INH was 89.2 (95% CI: 81.66% to 96.48%), as indicated in Table 4.6.

Table 4.6. Sensitivity, specificity, and overall accuracy of gDST results to predict resistance against RIF and INH

		Phenotypic DST Result using BACTEC™ MGIT 960			
		RIF		INH	
		R	S	R	S
Genotypic DST using Sanger Sequencing	R	31	0	34	2
	S	9	30	6	28
	total	40	30	40	30
Sensitivity (95% CI)		77.5% (70.24% to 84.76%)		85% (77.04% to 92.96%)	
Specificity (95% CI)		100% (92.97% to 100%)		93.3% (86.27% to 100%)	
Overall agreement, (95% CI)		88.75% (81.61% to 92.38%)		89.15% (81.66% to 96.48%)	

Table 4.7. The evaluation is based on the WGS analysis of four drug-resistant associated genes or regions and phenotypic drug susceptibility testing

Drugs	Genes	Number of isolates (%) carried mutations& in resistant strains	Number of isolates (%) carried mutations& in susceptible strains	Sensitivity (%)	Specificity (%)
RIF	*rpoB*	31 (77.5)	0(0.0)	77.5	100
INH	*katG*	32 (80)	0(0.0)	80	100
	inhA promotor	12 (30)	0(0.0)	30	100
	oxyR-ahpC	5 (12.5)	2 (6.7)	12.5	93.3

Based on the WGS results, 32 MDR-TB isolates and 0 DS-TB isolates have changes in *katG*, 12 MDR-TB isolates and 0 DS-TB isolates have changes in the *inhA* promoter, and 5 MDR-TB isolates and 2 DS-TB isolates have changes in the *oxyR-ahpC* intergenic region (see Table 4.7). For further details, 80% of MDR-TB isolates exhibit *katG* mutations. Including *katG* mutations in conjunction with those in the *inhA* promoter resulted in a modest improvement in sensitivity, increasing it from 80% to 85%. However, there was no further effect on specificity, as shown in Figure 4.14. The sensitivity remained unchanged when mutations were introduced in the *oxyR-ahpC* intergenic region, *katG*, and *inhA* promoter. However, as depicted in Figure 4.14, the specificity decreased from 100% to 93.6%.

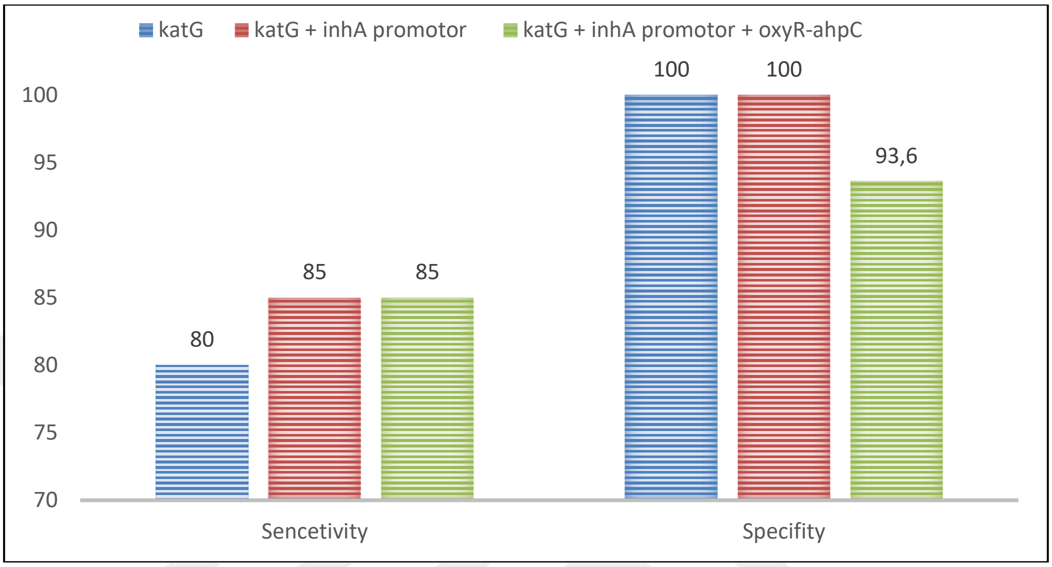

Figure 4.15. The sensitivity and specificity of sequencing *katG*, the *inhA* promoter, and the *oxyR-ahpC* intergenic region for diagnosing MDR-TB and DS-TB

4.3.4. Cumulative Mutation Frequencies

Initially, we evaluated the cumulative frequency of multiple or co-occurring mutations linked to MDR-TB by individual genes and then collectively across several gene areas (Table 4.8). Thirty-two isolates showed resistance due to numerous mutations in *katG*, specifically at codon positions 309 to 336. 55% of the INH-resistant isolates in this subset exhibited the mutation *katG*315. The addition of mutations at codons 309, 311, 316, 328, and 336 as markers for INH resistance increased the total cumulative frequency to 80%. In this subset, the cumulative frequency *inhA*-15 was 10 (25%). Unlike *katG*, additional mutations in the *inhA* promoter do not typically occur with *inhA*-15. Mutations at *inhA*-47 and *inhA*-64 identified 2 (5%) other resistant isolates, and the *inhA* cumulative frequency became 12 (30%). Five isolates that showed resistance in appearance were examined for mutations in the *oxyR-ahpC* intergenic region. We did not find any co-occurring mutations in the *oxyR-ahpC* intergenic region. Three separate mutations at positions -88, -48, and 17 in the *oxyR-ahpC* intergenic region caused 12.5% of the resistant isolates in this sample to have *oxyR-ahpC* mutations. The occurrence rates of mutations in the *katG*, *inhA*, and *oxyR-ahpC* intergenic areas are 80%, 30%, and 12.5%, respectively, with a cumulative frequency of 85%.

Twenty isolates of INH resistance have a unique mutation: *katG*315 connects 35% (14/40; 13 *katG* S315T and one *katG* S315A) of the phenotypic isoniazid resistance. This mutation's dominance is believed to stem from its low or non-existent fitness cost, enabling its propagation without negative selection pressure. Unique mutations in the *katG*non315 (4 *katG*non315) gene occur infrequently, at around 10%. A unique mutation in the *inhA* promoter region of 5% wild-type *katG* comes from the *inhA*-15 mutation. This mutation is often used to find INH resistance. In contrast, no single mutation occurred in the *oxyR-ahpC* intergenic region. 12.5% of phenotypically resistant isolates had mutations at positions -88, -48, and 17 in the *ahpC-oxyR* intergenic region.

Combined gene mutations, 14 out of 40 INH resistance isolates had co-occurring mutations in INH-resistant genes. Only nine isolates of *katG*315 (5 *katG* S315T+*inhA*-15; 2 *katG* S315T+ *inhA*-47 or *inhA*-64; 1 *katG* S315T+*oxyR-ahpC*; and one *katG* S315T with *katG* T328C) had combined mutations in the *inhA* promoter and/or the *oxyR-ahpC* intergenic region. The last five INH-resistant isolates had changes in *katG*non315: two had *katG*nonS315T+*inhA*-15 mutations, two had *katG*nonS315T+*oxyR-ahpC* mutations, and one had *katG*nonS315T+*inhA*-15+*oxyR-ahpC* mutations. These changes were combined with changes in the *inhA* promoter and/or the *oxyR-ahpC* intergenic region. Of the 8 INH resistance isolates with the wild-type *katG* gene, there are 2 (wild-type *katG*+*inhA*-15) mutations with the *inhA* promoter. Besides that, there were 6 (wild-type katG) INH-resistant strains. The wild-type of 3 sequenced genes was linked to INH resistance.

We used the bootstrap method to detect combinations of frequent mutations that could be more responsive. We found the prevalent mutation combinations that cause the MDR-TB phenotype at *katG* and INH resistance-linked genes. Out of the 40 isolates examined for MDR-TB, the most frequent mutations were *rpoB*S450L and *katG*S315T, present in 17 (42.5%) MDR-TB isolates at combinations 1, 6, and 7. Four MDR-TB isolates lacked any identified INH or RIF-linked gene mutations.

Table 4.8. Combined mutation frequencies on *rpoB*, *katG*, *inhA*, and *oxyR-ahpC*

Combinations	**Drug**	**Combined gene mutations**	**No. (%)**
Combination-1	RIF+INH	*rpoB* S450L, *katG* S315T and 1 *katG* S315A	14 (35)
Combination-2	RIF+INH	*rpoB* A435V and *katG*nonS315T	3 (7.5)
Combination-3	RIF+INH	*rpoB* L430L and Wild types *katG*	2 (5)
Combination-4	RIF+INH	*rpoB* L430P, *katG* S315T and *katG* T328C	1 (2.5)
Combination-5	RIF+INH	*rpoB* L494L, *katG* S315T and *oxyR-ahpC*	1 (2.5)
Combination-6	RIF+INH	*rpoB* S450L, *katG* S315T and *inhA*-15	2 (5)
Combination-7	RIF+INH	*rpoB* S450L, *katG* S315T, *inhA*-15 and *oxyR-ahpC*	1 (2.5)
Combination-8	RIF+INH	*rpoB* H445T +*katG* S315T+*inhA*-15	2 (5)
Combination-9	RIF+INH	Wild types *rpoB*, *katG* S315T+ *inhA*-47 or *inhA*-64	2 (5)
Combination-10	RIF+INH	*rpoB* S450L, *katG*nonS315T and *inhA*-15	2 (5)
Combination-11	RIF+INH	*rpoB* H445T, *katG*nonS315T, *inhA*-15 and *oxyR-ahpC*	1 (2.5)
Combination-12	RIF+INH	*rpoB* S450L, *katG*nonS315T and *oxyR-ahpC*	2 (5)
Combination-13	RIF+INH	Wild types *rpoB*, Wild types *katG*, and *inhA*-15	2 (5)
Combination-14	RIF+INH	Wild types *rpoB* and Wild types *katG*	4 (10)
Combination-15	RIF+INH	Wild types *rpoB* and *katG*nonS315T	1 (2.5)

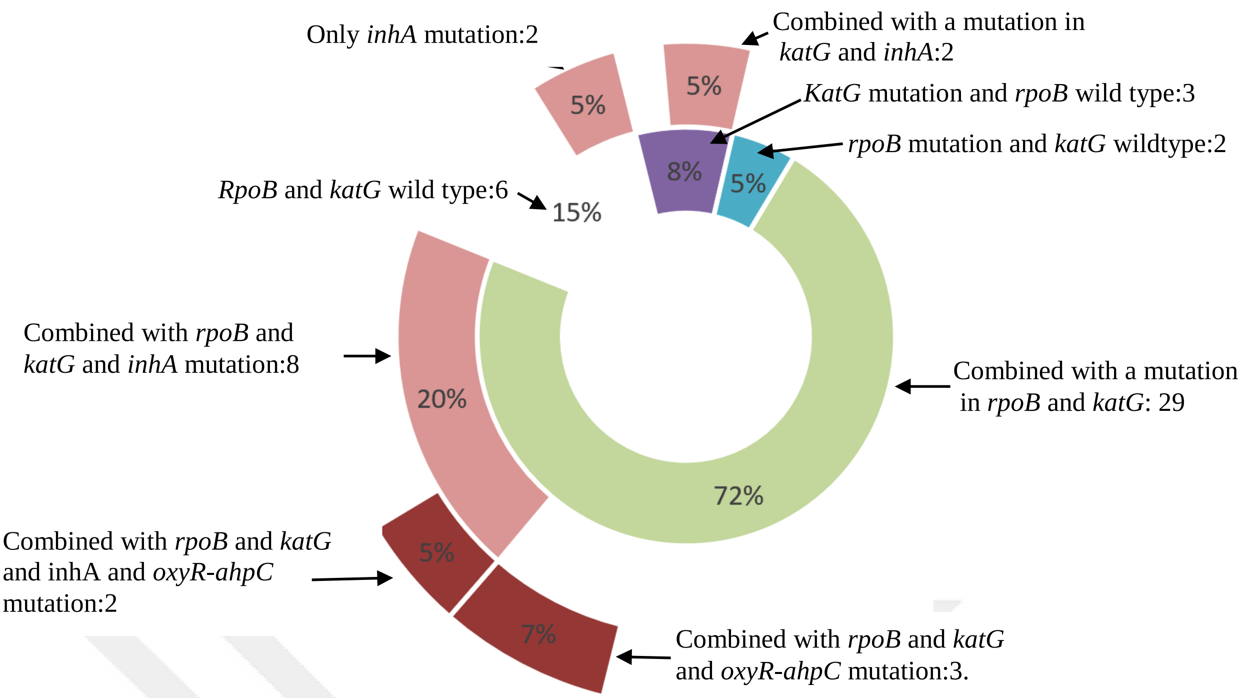

Figure 4.16. Several isolates have phenotypically MDR-TB carrying *rpoB*, *katG*, *inhA*, and *oxyR-ahpC* combined mutations with different combination groups

Study limitations One problem with the study is that changes in genes like *katG*, *inhA*, *oxyR-ahpC*, *ndh*, *iniABC*, *efpA*, *fadE*, *furA*, *Rv1592c*, *Rv1772*, and *kasA* could make the bacteria resistant to isoniazid. We found a mutation in the *katG*, *inhA*, and *oxyR-ahpC* genes. The mutation in the *katG* gene resulted in high-level resistance, especially in the *katG*315 codon. Moreover, increasing the sample size might enhance the validity of our results. Since we concentrated on sequencing the most common mutation locations, we could not determine the whole genes of *rpoB* and *katG*. Hence, subsequent research should involve sequencing the whole *rpoB* and *katG* genes for more precise outcomes.

5. CONCLUSION AND SUGGESTION

This study characterized mutations found in four genes of *M. tuberculosis* isolates from the Cukurova region and compared them with those from other areas or nations. Ultimately, while the culture-based phenotypic susceptibility test is considered the gold standard method for identifying drug resistance in MTBC, our study revealed that WGS demonstrates excellent sensitivity and specificity to first-line anti-TB medications. These novel mutations and common mutations can serve as a guide for clinical microbiology laboratory diagnostic techniques in detecting drug-resistant TB.

The bootstrap method showed that *rpoB* S450L, *rpoB* H445T, and *rpoB*A435V for RIF were the most important mutations for predicting the MDR-TB phenotype. We have identified three silent mutations: two (L430L) and one located outside the RRDR area at codon 494 (L494L). This research will aid in studying resistance mechanisms and creating new diagnostic tests. At least 77.5% of our *M. tuberculosis* isolates with MDR-TB phenotypes should be identifiable using molecular diagnostics that analyze these mutations at *the rpoB* gene.

The *katG* gene was characterized by a predominance of point mutations, mainly at codon S315T, *katG* G316S, and mutations in the other regions of the *katG* gene at codons 309, 311, 328, and 336 are not common. Confirming that the area of the *inhA* C-15T promoter is a reliable marker for resistance to INH. The use of *oxyR-ahpC* intergenic region mutations to predict INH resistance phenotypes is widely debated. Mutations in the *ahpC* promoter are seen in isolates susceptible to INH. Therefore, understanding INH resistance when there is an *ahpC* promoter mutation without an associated *katG* mutation is difficult. Our analysis found that almost 85% of MDR-TB isolates had mutations in the *katG* gene and the *inhA* promoter.

Furthermore, the remaining four isolates were phenotypically MDR-TB retrieved from our collection. In a few cases, the phenotypic susceptibility results did not match the genotypic results, suggesting mutations may have arisen in unexamined regions of *rpoB*, *katG*, *inhA*, and *ahpC-oxyR*, mutations in other genes related to RIF and INH resistance not investigated in this study, or the presence of alternative resistance mechanisms like efflux pumps.

The effectiveness of molecular diagnostic tests for drug-resistant TB depends on the prevalence of mutations in different regions and the variety of changes the tests can detect. Both of these issues impact the highest level of sensitivity that fast molecular tests can reach.

Summary and recondemnation from this study: Drug resistance is a significant challenge in global healthcare systems, with the spread of MDR-TB strains playing a crucial role in this problem. Exploring new targets for developing innovative medications that can fight the disease locally and globally is essential.

Which genetic polymorphisms in RIF and INH-resistant bacteria are associated with the prevalence of genetic changes causing *M. tuberculosis* drug resistance in various geographic regions? Detecting INH resistance was challenging due to mutations being linked to several genes. Our research only examined a restricted scope of each *katG, inhA*, and *oxyR-ahpC*.

Additional research is required to fully comprehend the genetic variations in *M. tuberculosis* resistance to medicines and to establish the incidence of resistant mutations among *M. tuberculosis* isolates in various geographic locations in Turkey.

We have confirmed that the region of the *katG* gene with codon 315 and the *inhA*-15 promoter is a reliable indicator of resistance to INH. Additionally, we have identified another significant section of this gene for further research in this area.

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CURRICULUM VITAE

Omer Ali Namiq AL-JAF, After completing my primary, secondary, and high school education, I earned a bachelor's degree from Sulaimani University, Faculty of Science, Department of Biology, in 2008–2009. In 2015, I earned a master's degree at Kahramanmaras Sutcu Imam University, Institute of Bioengineering and Science, Department of Medical Microbiology. In 2019, I started my Ph.D. at Cukurova University, Institute of Science and Technology, Department of Biotechnology.