**CUKUROVA UNIVERSITY**
**INSTITUTE OF NATURAL AND APPLIED SCIENCES**

**PhD THESIS**

**Huda ALİ MOHAMEDALİ AHMED**

# BEDAQUILINE DELAMANID, AND LINEZOLID MINIMUM INHIBITORY CONCENTRATION DISTRIBUTIONS AND RESISTANCE-RELATED GENE MUTATIONS IN MULTIDRUG-RESISTANT TUBERCULOSIS STRAINS ISOLATED FROM SOUTH OF TURKEY

**DEPARTMENT OF BIOTECHNOLOGY**

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**ÇUKUROVA ÜNİVERSİTESİ**
**FEN BİLİMLERİ ENSTİTÜSÜ**

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We certify that the thesis titled above was reviewed and approved for the award of the degree of the Doctor of Philosophy by the board of jury on 30/01/2023

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**ABSTRACT**

**PhD THESIS**

**BEDAQUILINE DELAMANID, AND LINEZOLID MINIMUM INHIBITORY CONCENTRATION DISTRIBUTIONS AND RESISTANCE-RELATED GENE MUTATIONS IN MULTIDRUG-RESISTANT TUBERCULOSIS STRAINS ISOLATED FROM SOUTH OF TURKEY**

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Delamanid, bedaquiline, and linezolid are three newer drugs that have been approved for the treatment of MDR and XDR tuberculosis (TB). The use of drug susceptibility testing is essential for correct diagnosis and treatment with these medications, and may entail quicker molecular approaches. The present study's objective is to evaluate the MICs and mutations in genes linked to resistance of MDR and completely drug-susceptible *M. tuberculosis* strains isolated from clinical samples of tuberculosis suspects given to THUM from the Cukurova Region of Turkey. The minimum inhibitory concentrations (MICs) of delamanid, bedaquiline, and linezolid were determined with the use of a semiautomated MGIT 960 system, EpiCenter software, and a TB eXiST module. The PCR results of 71 MTB isolates, , were sequenced and aligned with those of *M. tuberculosis* H37Rv.The MICs for bedaquiline, delamanid, and linezolid ranged from ≤0.025 to 6.4 mg/L, 0.005 to 0.32 mg/L, and 0.125 to 2 mg/L respectively. Multiple mutations were detected in both drug-sensitive and -resistant strains. We did not identify specific mutations involved with bedaquiline, delamanid, or linezolid resistance. However Further research is required to establish the genetic mechanisms behind these drugs' resistance.



**Key words:** *Mycobacterium tuberculosis*, Delamanid, Bedaquiline, Linezolid

# ÖZ

## DOKTORA TEZİ

**ÇUKUROVA BÖLGESINDE PULMONER TÜBERKÜLOZLU HASTALARDAN IZOLE OLACAK ÇOKLU ILACA DIRENÇLI TÜBERKÜLOZ SUŞLARINDA BEDAQUILINE, DELAMANID VE LINEZOLID MINIMUM INHIBITÖR KONSANTRASYONLARININ VE DIRENÇLE ILIŞKILI GEN MUTASYONLARININ BELIRLENMESI**

**Huda ALİ MOHAMEDALİ AHMED**

**ÇUKUROVA ÜNİVERSİTESİ**
**FEN BİLİMLERİ ENSTİTÜSÜ**
**BİYOTEKNOLOJİ ANABİLİM DALI**

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Çok ilaca dirençli (MDR) ve yaygın ilaca dirençli (XDR) tüberkülozun tedavisindeki son gelişmeler, delamanid, bedakuilin ve linezolidin (TB) onaylanmasına yol açmıştır. Bu ilaçlarla doğru teşhis ve tedavi, ilaç duyarlılık testlerinin kullanılmasını gerektirir ve daha hızlı moleküler yöntemleri içerebilir.. Bu çalışmanın amacı, tüberküloz şüphelilerinin klinik örneklerinden izole edilen MDR ve tamamen ilaca duyarlı M. tuberculosis suşları arasında bu üç anti-TB ilacına dirençle ilgili minimum inhibitör konsantrasyonları (MİK) ve genlerdeki mutasyonları değerlendirmektir. Türkiye'nin Çukurova Bölgesi'ndeki sekiz farklı ilden THUM ye gonderilmektedir. Delamanid, bedaquiline ve linezolid MIC'leri, yarı otomatik bir MGIT 960 sistemi ve bir TB eXiST modülü ile birleştirilmiş EpiCenter yazılımı kullanılarak belirlendi. 71 MTB izolatının PCR sonuçları M. tuberculosis H37Rv'ninkilerle sıralandı ve hizalandı.Bedaquiline, delamanid ve linezolid için MIC'ler sırasıyla ≤0,025 ila 6,4 mg/L, 0,005 ila 0,32 mg/L ve 0,125 ila 2 mg/L aralığındadır. Hem ilaca duyarlı hem de dirençli suşlarda çoklu mutasyonlar tespit edildi. Bedakilin, delamanid veya linezolid direnci ile ilgili spesifik mutasyonları tanımlamadık. Bununla birlikte, bu ilaçların direncinin ardındaki genetik mekanizmaları belirlemek için daha fazla araştırmaya ihtiyaç vardır

**Anahtar Kelimeler:** *M. tuberculosis*, Delamanid, Bedakilin ve Linezolid

**EXPANDED ABSTRACT**

Despite the challenges associated with the development of novel TB medications, significant progress has been achieved in the worldwide anti-TB drug pipeline in recent years (Zumla, Nahid et al. 2013).

Several FDA-approved candidates have shown encouraging in vitro and clinical trial anti-TB efficacy(Zhang, Pang et al. 2014) For individuals with extremely drug-resistant TB, the use of linezolid (LZD), an established antibacterial agent, is on the rise (Lee, Lee et al. 2012). Patients with multidrug-resistant or extensively drug-resistant pulmonary tuberculosis may often be cured with LZD, as shown in a number of clinical investigations (Koh, Kang et al. 2012).

Bedaquiline (BDQ) and delamanid (DMD) are two novel drugs that, like LZD, Bedaquiline (BDQ) [authorized by the US Food and Drug Administration (FDA)] and delamanid (DEL) [approved by the European Medicines Agency (EMA)](Ginsberg 2011),have acquired prominence and look promising against drug-resistant TB (Gler MT and Vargas-Vasquez DE 2012).

Promising effectiveness against XDR-TB should be predicted since each of the two medications includes a mechanism of action that is distinct from that of other current treatments. Very few examples of acquired resistance to these medications have been reported in the scientific literature (Woods, Brown-Elliott et al. 2011).

In 2018, the World Health Organization (WHO) revised its recommendations for the management of multidrug-resistant TB based on a policy informed by evidence (Organization 2019).

Bedaquiline is now considered a cornerstone medicine in the new standard combination regimen for the treatment of rifampin-resistant tuberculosis, as outlined in the updated recommendations. As BDQ will now be employed in clinical practice, it is essential to identify any additional, non-target-based mechanisms that contribute to reduced sensitivity or resistance in order to design

suitable drug susceptibility testing procedures. However, the early success of bedaquiline is in danger due to the possible establishment of resistance due to poor adherence to TB therapy, relatively weak background regimens, and the prolonged half-life of the drug (Nguyen, Anthony et al. 2017).

The quick communication from the World Health Organization (WHO) suggesting for the prioritization of bedaquiline employment and its usage to replace injectable drugs has stoked a new interest in this substance (Ndjeka, Hughes et al. 2020).However, clinical case reports have previously demonstrated bedaquiline therapy failed (Bloemberg, Keller et al. 2015, Zimenkov, Nosova et al. 2017). Bedaquiline its mode of action, inhibition of ATP synthase, was found by whole genome sequencing of in vitro-selected resistant mutants (Andries, Verhasselt et al. 2005). Mycobacterial adenosine triphosphate (ATP) synthase is the target of BDQ because it has a proton pump expressed by the atpE gene (Andries, Verhasselt et al. 2005).

*M. tuberculosis* isolates acquire resistance to BDQ by spontaneous chromosomal mutation in the genes atpE and pepQ are among the current mechanisms of BDQ resistance (Nguyen, Anthony et al. 2017).

Cross-resistance between clofazimine and BDQ (Hartkoorn, Uplekar et al. 2014).is also accounted for by a mutation in the transcriptional regulator Rv0678 (transcriptional repressor of the MmpS5–MmpL5 efflux pump), which leads to an increase of the multi-substrate efflux pump Mmpl5.

Previous research has shown that BDQ's bactericidal action is low during the first week of chemotherapy (Koul, Vranckx et al. 2014).

Delamanid as a member of the nitro-dihydro imidazooxazole family of chemicals, delamanid inhibits the formation of methoxy- and keto-mycolic acids, which are found in the cell wall of *Mycobacteria*. (Matsumoto, Hashizume et al. 2006).

It is a prodrug that has to be converted into an active drug by the enzyme deazaflavin-dependent nitroreductase (Rv3547). DEL has been demonstrated to be

an effective inhibitor of both drug-sensitive and -resistant tuberculosis(Matsumoto, Hashizume et al. 2006). Primarily, it affects *M. tuberculosis complex* species, including actively reproducing and dormant forms. We require better delamanid drug susceptibility testing (DST) skills because the rate of spontaneous mutations imparting delamanid resistance, which is greater than the typical mutation rate of the *M. tuberculosis* genome (EMA 2013). One may develop resistance to DEL by carrying a mutation in any one of the five coenzyme F420 genes (ddn (Rv3547), fgd, fbiA, fbiB, and fbiC) (Singh, Manjunatha et al. 2008, EMA 2013).

Linezolid (LNZ) as a member of the oxazolidinone family of antibiotics, has shown promising bacteriostatic efficacy against drug-resistant Mycobacterium TB, including MDR and XDR strains, in both in vitro and animal tests (Cynamon, Klemens et al. 1999).

Linezolid's use in the treatment of drug-resistant tuberculosis has been validated by studies evaluating its safety and effectiveness. Linezolid interacts to the ribosome at the peptidyl-transferase center (Beckert, Hillemann et al. 2012). LNZ resistance has been shown to be conferred by mutations in 23S rRNA and in two ribosomal proteins, L3 and L4, in a wide range of pathogenic species (Beckert, Hillemann et al. 2012, Long and Vester 2012). Although numerous mutations in 23S rRNA and ribosomal L3 protein (rplC) have been found in in vitro produced or clinical LNZ-resistant isolates of *M. tuberculosis*, information of the mechanism of LNZ resistance remains restricted.

It is anticipated that TB patients would have a low level of resistance to the newly authorized drugs delamanid, bedaquiline, and linezolid. However, antibiotic resistance may develop without antimicrobial exposures in certain cases. As Rv0678 RAV was found in a patient's baseline isolate who had no known exposure to clofazimine or bedaquiline.

Drug susceptibility studies, especially quick molecular approaches, are so necessary for correct diagnosis and therapy. New drugs and effective drug combinations are urgently needed to reduce the time it takes to treat drug-resistant

tuberculosis. Therefore, studying MDR-TB isolates' susceptibility to bedaquiline, delamanid, and linezolid is important.

To the best of our knowledge and with significant consequences for future DR TB treatment administration, the following objectives have been identified in our study.

The aim of the current study was to evaluate Delamanid, Bedaquiline and Linezolid Minimum Inhibitory Concentration (MICs) Distributions and Resistance related Gene Mutations among MDR and fully drug-susceptible *M. tuberculosis* strains isolated from clinical samples of tuberculosis suspects sent to the Cukurova University ,Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory from eight different cities in the Cukurova Region of South Turkey.

This is the first study to assess the phenotypic and genotypic resistance of *M. tuberculosis* isolates to Delamanid, Bedaquiline, and Linezolid in treatment-naive patients in this region; nevertheless, the genetic basis of resistance to these three anti-TB medications remains largely unknown. Introduction of medications without sufficient diagnostics to detect drug resistance may lead to an increase in instances that are difficult to treat. Future effective use requires accurate phenotypic drug susceptibility testing and rapid molecular biology techniques.

The research will further enhance to identify resistance to Delamanid, Bedaquiline and Linezolid and support findings from the correlation analysis between mutations linked to resistant phenotypes because there is a noticeable shortage of appropriate data on resistance-related genetic variations. In addition, phenotypic procedures are too slow to offer a quick assessment of susceptibility status at the time treatment is initiated. In order to provide interpretable molecular DST for these three anti-TB drugs and to guide the formulation of treatment regimens, accurate categorization of genetic variations according to their connection with drug resistance is needed.

Delamanid, bedaquiline, and linezolid MICs were determined utilizing a semiautomated MGIT 960 system and EpiCenter software combined with a TB eXiST module.The PCR results of 71 MTB isolates, comprising fully drug-susceptible (n = 30) and multidrug-resistant (n = 41) strains, were sequenced and aligned with those of *M. tuberculosis* H37Rv. The MICs for bedaquiline, delamanid, and linezolid ranged from ≤0.025 to 6.4 mg/L, 0.005 to 0.32 mg/L, and 0.125 to 2 mg/L respectively. As a result of genotypic resistance tests bedaquiline for mutations associated with atpE gene linked with BDQ drug target site were 9 out of 71 (12.7 %) MDR-TB isolates carried a mutation located in the atpE gene. No mutations within the atpE gene were observed in these fully drug-susceptible isolates Multiple mutations were detected in both drug-sensitive and -resistant strains and all bedaquiline-resistant isolates were negative for Rv0678 mutations, indicating that other mechanisms of resistance to bedaquiline should be investigated. for delamanid, one non-silent mutation (Gly81Asp) in ddn was found in delamanid-susceptible strains, its involvement in drug resistance is yet unknown. We suggest that this is not the result of acquired resistance, because we used MDR and fully drug-susceptible strains that had probably not been exposed to delamanid, no mutations in the fbiA gene were identified in either sensitive or resistant strains to delamanid. for linezolid the MIC for the most resistant strains was between 1.6 and 2 mg/mL. Two genes (rplC and rrl) in 71 MTB had no mutations. Non-ribosomal mutation mechanisms may be responsible for LNZ resistance in *M. tuberculosis*, as the development of resistant isolates without mutations suggests. We did not identify specific mutations involved with bedaquiline, delamanid, or linezolid resistance. However Further research is required to establish the genetic mechanisms behind these drugs' resistance.

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**LIST OF SYMBOLS AND ABBREVIATONS**

AIDS : Acquired Immune Deficient Syndrome
BCG : Bacille Calmette-Guerin
bp : Base pair
°C : Celcius Degree
DMSO : Dimethyl sulfoxide
DNA : De*oxyR*ibonucleic Acid
DOTS : Directly Observed Therapy Short course
EMB : Ethambutol
ETH : Ethionamide
G+C : Guanine + cytocine
HIV : Human immunodeficiency
INH : Isoniazid
LJ : Lowenstein Jensen
MDR-TB : Multidrug-resistant tuberculosis
MGIT : Mycobacteria Growth Indicator Tube
MTB : *Mycobacterium tuberculosis*
MTBC : *Mycobacterium tuberculosis* complex
PCR : Polymerase Chain Reaction
PZA : Pyrazinamide
RIF : Rifampin/Rifampicin
RR-TB : Rifampicin-resistant tuberculosis
SNP : Single nucleotide polymorphism
TB : Tuberculosis
TE : Tris/EDTA
µg : Micrograms
µl : Microlitres

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|---|---|
| WHO | : World Health Organization |
| XDR TB | : Extensively drug-resistant tuberculosis |
| ZN | : Zielh Neelsen |
| MGIT | : Mycobacteria growth indicator tube |
| MIC | : Minimal inhibitory concentration |
| SNV | :Single-nucleotide variation |

# 1. INTRODUCTION

## 1.1. Global Tuberculosis History and Current Status

The ancient infectious illness tuberculosis (TB), caused by *Mycobacterium tuberculosis* and other closely related species, is responsible for an estimated two million to three million deaths yearly throughout the globe (Chan, Khadem et al. 2013, Fox and Menzies 2013, Nguyen, Cao et al. 2016).

*Mycobacterium tuberculosis* is the causative agent of tuberculosis, an infectious illness spread by the air (MTB). *Mycobacterium TB* has inherent efflux- and target-based mechanisms that allow it to acquire resistance to different medicines; this is a critical trait that undermines treatment effectiveness and affects morbidity and mortality. Multidrug-resistant tuberculosis (MDR-TB), which is TB that is resistant to at least isoniazid and rifampicin (Organization 2019) has become a significant impediment to controlling this illness globally. In 2006, a more severe type of drug-resistant TB, called extensively drug-resistant TB (XDR-TB), was first discovered in South Africa (Gandhi, Moll et al. 2006). These strains also show resistance to fluoroquinolones and at least one of three injectable second-line medicines frequently used to treat MDR-TB, making them even more difficult to treat than MDR-TB itself (Dheda, Gumbo et al. 2014).

In light of the bad prognosis for this group brought on by the high prevalence of drug-resistant strains of tuberculosis, XDR-TB has recently gained more attention from the medical establishment (Dheda, Gumbo et al. 2017). Multidrug-resistant tuberculosis (MDR-TB) , and more recently, extensively drug-resistant tuberculosis (XDR-TB) are on the rise and spreading at worrisome rates despite a general decrease in TB incidence (Van Heeswijk, Dannemann et al. 2014, Nguyen, Cao et al. 2016).

Epidemiological data show that tuberculosis is far more lethal than other infectious illnesses like AIDS and malaria. There was a 22% decrease in TB mortality globally between 2000 and 2015, yet it remains one of the top 10 causes

of death globally (Organization 2015). There were 6.3 million cases recorded globally in 2017, with an estimated 1.3 million fatalities (Organization 2013). Furthermore, TB has the greatest mortality rate of any infectious disease globally, even exceeding HIV/AIDS, and causing 1-5 million deaths in 2018 (Harding 2020) , furthermore, there have been about 600,000 new cases of MDR-TB recently, with an estimated 240,00 deaths due to MDR-TB in 2017 (Organization 2013). Recent WHO assessments indicated that in 2020, a turning point will be reached in Europe and seven other countries with severe loads (Kenya, Lesotho, Myanmar, Russia, South Africa, Tanzania, and Zimbabwe). In the WHO African Region, both incidence and death are falling quickly (Harding 2020).

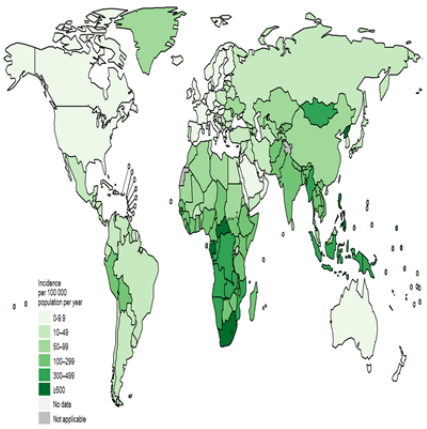


Figure 1.1. Estimated rates of TB incidence in 2021

It is projected that there were 484,000 new cases of MDR-TB in 2018, with an associated 214,000 fatalities worldwide. according to the World Health Organization (WHO) on the eve of the first turning point in 2020. MDR-TB incidence ranges from zero in certain countries to sixty-five in others, according to WHO data (Caminero 2003). Death rates from tuberculosis range from below five percent in certain countries to well over twenty percent in Africa (Organization 2013).

Nearly two-thirds of all cases of MDRTB have been documented in China, India, and Russia in recent years. There were almost 500,000 new cases of multidrug-resistant tuberculosis in 2018, although only about a third of those cases were treated. There were over 10.4 million new cases of tuberculosis in 2016, with an estimated 1.7 million fatalities due to tuberculosis therapy for multidrug-resistant tuberculosis (MDR-TB) has been difficult owing to the lengthy treatment time needed to cure this illness, limited therapeutic alternatives, and poor medication tolerability (Organization 2019, Gao, Gao et al. 2021).

Recent statistics from the World Health Organization (WHO) indicate that over 50% of MDR-TB patients worldwide have unsatisfactory treatment results (Organization 2019).

More chemotherapeutic measures are required as a result. Unfortunately, MDR and XDR-TB cannot be eliminated with the use of currently available anti-TB medications. Patient noncompliance is a major factor in the emergence of drug-resistant TB and is influenced by a number of factors, including the length of treatment, ineffectiveness of regimens, severity of side effects, centralization of drug-resistant TB services, and a lack of patient-centered care. For this reason, it is essential to find new medications and effective combinations to combat drug-resistant TB and reduce the length of time needed for therapy (Blumberg, Burman et al. 2003).

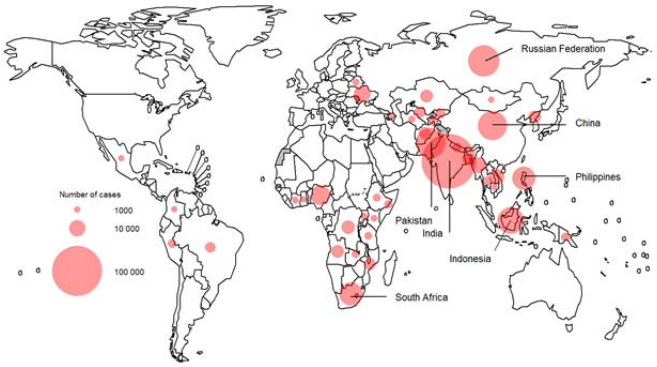


Figure 1.2. Estimated number of incident cases of MDR/RR-TB in 2021, for countries with at least 1000 incident cases. The seven countries with the highest burden in terms of numbers of MDR/RR-TB cases, and that accounted for two thirds of global MDR/RR-TB cases in 2021, are labelled.

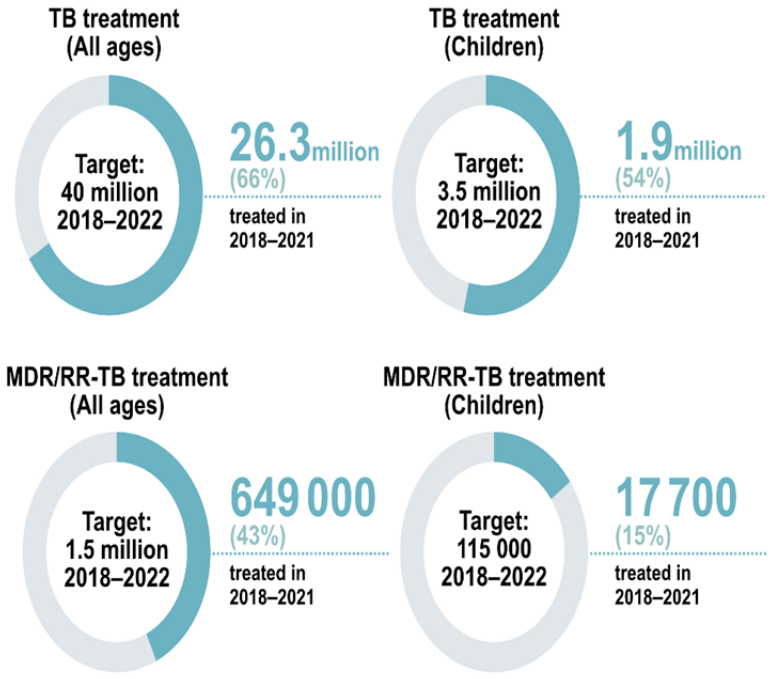


Figure 1.3. Global progress in the number of people treated for TB between 2018 and 2021, compared with cumulative targets set for 2018–2022 at the UN high-level meeting on TB.

Several promising compounds are being tested as part of clinical studies for tuberculosis treatment (Cole and Riccardi 2011). To combat the spread of drug-resistant tuberculosis, the Global Alliance for Drug TB Development (TB Alliance) is searching for novel combination regimens containing powerful compounds (Ginsberg 2011).

Despite the challenges associated with the development of novel TB medications, significant progress has been achieved in the worldwide anti-TB drug pipeline in recent years (Zumla, Nahid et al. 2013). Several FDA-approved candidates have shown encouraging in vitro and clinical trial anti-TB efficacy (Alcalá, Ruiz-Serrano et al. 2003, Huang, Liu et al. 2008, Zhang, Pang et al. 2014). For individuals with extremely drug-resistant TB, the use of linezolid (LZD), an established antibacterial agent, is on the rise (Villar, Sotgiu et al. 2011, Lee, Lee et al. 2012).

Patients with multidrug-resistant or extensively drug-resistant pulmonary tuberculosis may often be cured with LZD, as shown in a number of clinical investigations (Villar, Sotgiu et al. 2011, Koh, Kang et al. 2012). Bedaquiline (BDQ) and delamanid (DMD) are two novel drugs that, like LZD, Bedaquiline (BDQ) [authorized by the US Food and Drug Administration (FDA)] and delamanid (DEL) [approved by the European Medicines Agency (EMA)] (Ginsberg 2011), have acquired prominence and look promising against drug-resistant TB (Diacon, Pym et al. 2009, Gler, Skripconoka et al. 2012).

Promising effectiveness against XDR-TB should be predicted since each of the two medications includes a mechanism of action that is distinct from that of other current treatments. The literature reports only a small number of cases of acquired resistance to these drugs (Woods, Brown-Elliott et al. 2011). In 2018, the World Health Organization (WHO) revised its recommendations for the management of multidrug-resistant TB based on a policy informed by evidence (Organization 2019).

Bedaquiline is now considered a cornerstone medicine in the new standard combination regimen for the treatment of rifampin-resistant tuberculosis, as outlined in the updated recommendations. Bedaquiline will be used more often by TB control programs as the findings of research on its usage in all-oral shorter-course regimens for the treatment of rifampin-resistant TB continue to seem promising (Borisov, Dheda et al. 2017, Schnippel, Ndjeka et al. 2018, Honeyborne, Lipman et al. 2019).

Extensive pharmacovigilance to track side effects and the capacity to detect resistance in patients early in treatment should be prompted by the anticipated increase in bedaquiline usage (Akkerman, Aleksa et al. 2019). Bedaquiline its mode of action, inhibition of ATP synthase, was found by whole genome sequencing of in vitro-selected resistant mutants (Andries, Verhasselt et al. 2005). Mycobacterial adenosine triphosphate (ATP) synthase is the target of BDQ because it has a proton pump expressed by the atpE gene (Andries, Verhasselt et al. 2005).

*M. tuberculosis* isolates acquire resistance to BDQ by spontaneous chromosomal mutation in the genes atpE and pepQ are among the current mechanisms of BDQ resistance (Nguyen, Anthony et al. 2017).

Cross-resistance between clofazimine and BDQ (Hartkoorn, Uplekar et al. 2014) is also accounted for by a mutation in the transcriptional regulator Rv0678 (transcriptional repressor of the MmpS5–MmpL5 efflux pump), which leads to an increase of the multi-substrate efflux pump Mmpl5. Previous research has shown that BDQ's bactericidal action is low during the first week of chemotherapy (Koul, Vranckx et al. 2014).

The MIC of bedaquiline is increased, when resistance-associated variations (RAVs) RAVs are present in the atpE gene, which codes for a transmembrane protein of the ATP synthase, the drug's target. These RAVs have been isolated in vitro after exposure to bedaquiline. Two- to eightfold increases in bedaquiline MIC and two- to fourfold increases in clofazimine MIC are caused by

RAVs in Rv0678, a gene that regulates production of the MmpS5- MmpL5 efflux pump (Andries, Villellas et al. 2014).

It is crucial that bedaquiline treatment recommendations account for the frequency with which these Rv0678 RAVs are found in clinical isolates, as well as the effect they have on minimum inhibitory concentrations (MICs) and treatment results (Pym, Diacon et al. 2016). The first TB patient with multidrug-resistance and a BDQ-resistant isolate was reported in 2014. Cross-resistance to BDQ and CFZ (Rv0678 mutation) was found in isolates from a Tibetan patient who relapsed 24 months after therapy (Hoffmann, Kohl et al. 2016). If a CFZ-containing anti-TB regimen was previously used, then susceptibility to BDQ should be confirmed prior to initiating treatment (Riccardi, Giacomelli et al. 2020).

In 2019, a report appeared of a 50-year-old patient with extensively drug-resistant tuberculosis (XDR-TB) who had developed a strain of bacteria that was resistant to BDQ at a low dosage. The initial isolate was BDQ-resistant using the Mycobacterium Growth Indicator Tube (MGIT) (Polsfuss, Hofmann-Thiel et al. 2019). Ghodousi et al. analyzed 70 *Mycobacterium TB* strains from 30 patients in Pakistan who had previously been treated with regimens including BDQ. Five patients developed resistance to BDQ throughout therapy, and their MICs increased (from 0.125 to >0.5 mg/L), even though the strains at baseline were sensitive to the antibiotic.

Additionally, they looked at certain mutations in Rv0678 that cause high BDQ MICs in patients that did not respond to therapy (Ghodousi, Rizvi et al. 2019). Delamanid as a member of the nitro-dihydro imidazooxazole family of chemicals, delamanid inhibits the formation of methoxy- and keto-mycolic acids, which are found in the cell wall of mycobacteria (Matsumoto, Hashizume et al. 2006).

It is a prodrug that has to be converted into an active drug by the enzyme deazaflavin-dependent nitroreductase (Rv3547). DEL has been demonstrated to be an effective inhibitor of both drug-sensitive and -resistant tuberculosis (Matsumoto,

Hashizume et al. 2006). Primarily, it affects *M. tuberculosis complex* species, including actively reproducing and dormant forms. We require better delamanid drug susceptibility testing (DST) skills because the rate of spontaneous mutations imparting delamanid resistance, which is greater than the typical mutation rate of the *M. tuberculosis* genome (EMA 2013).

One may develop resistance to DEL by carrying a mutation in any one of the five coenzyme F420 genes (ddn (Rv3547), fgd, fbiA, fbiB, and fbiC) (Singh, Manjunatha et al. 2008, EMA 2013). A recent research revealed that the incidence of spontaneous mutations conferring resistance to DEL was high in in vitro settings, equivalent to that of isoniazid (INH) , but lower than that of rifampicin (RIF) and moxifloxacin (MFX) (EMA 2013).

DEL is not cross-resistant with any other first-line anti-TB drugs (Wells, Gupta et al. 2015). According to the 2017 WHO Global TB Report, 89 and 54 countries adopted BDQ and DLM, respectively (Cox, Brigden et al. 2018).

Rapid drug resistance development in DLM underlines the need for careful administration of these innovative medicines and shows the necessity of drug resistance monitoring. Acquired resistance to anti-TB drugs may be avoided by using DLM in combination with other potent anti-TB drugs (Diel, Hittel et al. 2015). Thus, it is essential to do in vitro combination studies since DEL monotherapy is likely to lead to resistance very quickly. When used alone, both BDQ and DEL are bactericidal, killing multidrug-resistant and extensively drug-resistant tuberculosis bacteria, DEL did not show any significant interactions with other antiretroviral medications (Mallikaarjun, Wells et al. 2016).

However, there is presently little information available on the possible adverse medication interactions when BDQ and DEL are used together. Similarly Frontline anti-TB medications had additive effects when combined with BDQ (Lechartier, Hartkoorn et al. 2012).

Human immunodeficiency virus (HIV) multidrug-resistant tuberculosis

(MDR-TB) co-infected individuals had increased exposure to BDQ while using the antiretroviral coformulation ritonavir-boosted lopinavir (LPV/r) (Brill, Svensson et al. 2017). In 2000, the FDA authorized linezolid for the treatment of different Gram-positive infections (Lee, Lee et al. 2012, Zhang, Pang et al. 2014). As a member of the oxazolidinone family of antibiotics, linezolid (LNZ) has shown promising bacteriostatic efficacy against drug-resistant *Mycobacterium* TB, including MDR and XDR strains, in both in vitro and animal tests (Cynamon, Klemens et al. 1999).

Linezolid's use in the treatment of drug-resistant tuberculosis has been validated by studies evaluating its safety and effectiveness. Linezolid interacts to the ribosome at the peptidyl-transferase center (Beckert, Hillemann et al. 2012). LNZ resistance has been shown to be conferred by mutations in 23S rRNA and in two ribosomal proteins, L3 and L4, in a wide range of pathogenic species (Beckert, Hillemann et al. 2012, Long and Vester 2012). Although numerous mutations in 23S rRNA and ribosomal L3 protein (rplC) have been found in in vitro produced or clinical LNZ-resistant isolates of *M. tuberculosis*, information of the mechanism of LNZ resistance remains restricted. Bedaquiline (Bdq), and linezolid (Lzd) were placed in Group A (medicines to be prioritized) by the World Health Organization (WHO) in August 2018, as part of their recommendations for treating multidrug- and RIF-resistant tuberculosis (MDR/RR-TB) (Organization 2018).

It is anticipated that TB patients would have a low level of resistance to the newly authorized drugs delamanid, bedaquiline, and linezolid.

However, antibiotic resistance may develop without antimicrobial exposures in certain cases. As Rv0678 RAV was found in a patient's baseline isolate who had no known exposure to clofazimine or bedaquiline. Drug susceptibility studies, especially quick molecular approaches, are so necessary for correct diagnosis and therapy. New drugs and effective drug combinations are urgently needed to reduce the time it takes to treat drug-resistant tuberculosis. Therefore, studying MDR-TB isolates' susceptibility to bedaquiline, delamanid,

and linezolid is important. To the best of our knowledge and with significant consequences for future DR TB treatment administration, the following objectives have been identified in our study.

### 1.2. Objective

1. The aim of the current study was to evaluate Delamanid, Bedaquiline and Linezolid Minimum Inhibitory Concentration (MICs) Distributions among MDR and fully drug-susceptible *M. tuberculosis* strains isolated from clinical samples of tuberculosis suspects sent to the Cukurova University, Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory from eight different cities in the Cukurova Region of South Turkey.
2. To investigate the mutations on six genes atpE, Rv0678, ddn, fbiA, rplC and rrl related to resistance of Bedaquiline, Delamanid and Linezolid among MDR and fully drug-susceptible *M. tuberculosis* strains.

# 2. LITERATURE REVIEW

## 2.1. History of Tuberculosis

Tuberculosis is a long-standing affliction. Disturbed throughout human history and expanded in ancient cities and reviewed, handled as if they were reviewed illnesses, but with a difficult time scale accepted explanations for the epidemic cycle (Hayman 1984, Xie, Chakravorty et al. 2017).

According to epidemiological findings, TB is more lethal than other infectious illnesses such as acquired immune deficiency syndrome (AIDS) and malaria. Although the global mortality TB decreased by 22% between 2000 and 2015, it remains one of the top 10 killers globally (Organization 2016).

*Mycrobacterium tuberculosis* claims to have killed more humans than any other microorganism. It is anticipated that the genus *Mycobacterium*150 million more existed a long time ago (Hayman 1984). TB has been found in human bones dating back thousands of years. *Mycobacterium tuberculosis,* a human disease with no significant environmental reservoir, has mastered the art of survival and has endured in human society from antiquity to the present (Adigun and Singh 2017). Historical writings in this illness use the terminology "consumption" and "phthisis" based on clinical findings (Ducati, Ruffino-Netto et al. 2006). Tuberculosis-related skeletal malformations have been found in Egyptian mummies as early as 2400 BC. Symptomatic Pott's lesions have been recorded, and comparable anomalies are shown vividly in early Egyptian art (Morse, Brothwell et al. 1964, Zimmerman 1979).

Tuberculosis affects not just the lungs, but also the bones, the central nervous system, and many other organs. However, this illness is primarily caused by the deposition of *M. tuberculosis*, which is already present on alveolar surface lungs' aerosol particles, so Tuberculosis has been a constant source of contention since ancient times (Smith 2003).

The ancients were aware of tuberculosis, and Hippocrates and Galen assumed that illness was infectious. In 1650, Sylvius characterized the tubercle, in 1720, Benjamin Marten developed the contagium theory, which offered an explanation based on the Germ Theory, describing the lesions as "wonderfully minute living organisms" in a new theory of Consumptions: More Particularly a Phthisis or Consumption of the Lungs. The term "animicula" was used by Marteen to explain the root of the problem (Daniel 1997, Aksu 2007).

In 1790, Benjamin Marten was the first to postulate that TB may be spread from person to person by contact with "certain specific kinds of animalcula (Doetsch 1978), and by 1819, Laennec was sure that the tubercle was the common cause in all types of the illness. Schoenlein coined the term "tuberculosis" in 1839.

The germ hypothesis of infectious illness proposed by Pasteur in 1862 stimulated the quest for infectious disease-causing organisms.

In 1865, Jean-Antoine Villemin (1827-1892) demonstrated via animal studies that TB could be transmitted from man or cow to rabbit or guinea-pig, and that the sputum of a tuberculous individual could infect a rabbit. Sir John Burdon Sanderson and Sir John Simon verified Villemin's conclusions (Sakula 1982). Cohnheim and Salamonsen effectively inoculated TB in 1877. Tappeiner was able to infect dogs with TB by exposing them to infected droplets inhaled from the anterior chamber of a rabbit's eye. The issue was no longer whether TB was present. Who would be the first to establish that a disease is caused by microorganism. In this period (1881-1882), Koch began his discovery for the tuberculosis bacillus (Sakula 1982).

Hansen isolated the first *Mycobacterium* almost one hundred years ago (1874). This was the leprosy bacillus, *Mycobacterium leprae*, which continues to defy all laboratory cultivation efforts to this day. Robert Koch found the tubercle bacillus, *M. tuberculosis*, eight years later (1882) (Velayati and Farnia 2012).

Hermann Heinrich Robert Koch's March 24, 1882 presentation to the Berlin Physiological Society, Die Aetiologie der Tuberculose, had a profound impact on the course of TB research and treatment (Daniel 1997, Daniel 2005, Perciaccante, Coralli et al. 2018).

Robert Koch discovered alcohol-methylene blue Weigert's 1875, staining technique did not readily stain tubercular lesions. Therefore, he implemented a novel technique for observing tubercular lesions with methylene blue and a solidified medium of coagulated bovine serum heated to 40°C (Weigert 1875, Ziehl 1882). Koch saw instantly that Ehrlich's staining technique (heat and acid decolorization) was better to his own.

Shortly afterwards, in May 1882, Ehrlich published the technique's specifics. Later, Ziehl substituted carbolic acid for aniline, while Neelsen proposed the use of sulphuric acid in place of nitric acid. Thus were created the "Ziehl-Neelsen" (ZN) stain and the "acid-alcohol fast bacillus" (AAFB) (Sakula 1982).

In 1882, when Koch initially characterized the tubercle bacillus, he believed that human and bovine bacilli were distinct organisms, confirming what Villemin had previously proved in 1865. Nonetheless, in his 1884 publication, Koch determined that the bacilli of human and bovine TB were same; nonetheless, despite the fact that bovine tuberculosis was transmissible to humans, he did not believe that this infection posed a significant threat to human health.

In 1898, Harvard professor Smith presented research demonstrating that human and bovine tubercle bacilli are distinct species. London hosted the Third International Tuberculosis Congress in 1901. Koch addressed at this Congress and acknowledged Theobald Smith's results, but maintained that TB in cattle posed no significant threat to humans and that specific preventive measures were unneeded (Sakula 1982).

Koch addressed the Tenth International Conference on the History of Ideas in 1890. International Health Conference in Berlin, which the tubercle bacillus was the source of the chemical he claimed to have isolated antibiotic-producing bacteria

that might "neutralize" dangerous those bacteria that are normally found in a live organism and perform this without harm to the physical form." tuberculin, and rapid administration by injections started being widely used to treat TB; They lost their credibility and effectiveness nearly as quickly as they were discarded. Koch administered 0.25cm$^3$ injection of his tuberculin, and saw that it was concentrated had got "an extremely severe case of ague temperature increase to 39.61 degrees Celsius" After some thought, he decided his tuberculin may be useful. Vets in Denmark quickly became experts at diagnostics bovine tuberculin testing (Koch 1891).

The first testing of Koch's tuberculin in 1907 by von Pirquet (to identify latent TB infections) was a forerunner to the intra-cutaneous application of PPD. Seibert created the modern PPD standard in the 1930s, and since then, the world has employed a PPD-S of five units to diagnose latent TB infections. Between 1910 and 1932, Bakteriyolojihane-Sahane was the first facility in the Ottoman Empire to produce and use tuberculin (Daniel 1997, Daniel 2005, Aksu 2007, Seber 2010).

In 1908, Charles Mantoux innovated intradermal administration of tuberculin using a cannulated needle and syringe, and in the 1930s, Florence Seibert initiated a series of experiments at the Phipps Institute of the University of Pennsylvania that resulted in the development of purified protein derivative (PPD) essentially in its current form (Daniel 2007). Since the tubercle bacillus was first identified, public health officials in the Americas and Europe have worked to control the disease. Similarly, in the Ottoman Empire, a detailed study was compiled by Cemiyet-i Tbbiye-i Sahane at the behest of Sultan Abdülhamid II to combat the widespread problem of spitting (Daniel 1997).

Patients with tuberculosis were placed in quarantine with a spitting container in hospitals and prisons. Military bases and schools instituted limit spitting policies in an effort to reduce the spread of tuberculosis, while hospitals set aside TB units as a precaution (Aksu 2007).

The TB epidemic in Europe and North America waned as a result of the research and development efforts of many people, including Villemin, Koch, von Pirquet, and others. Death rates started dropping in the early to mid-19th century (Daniel 2007).

Prior to the development of antibiotics, sanatoria were the only practical method of treatment, with the first one opening at Görbersdorf in the same year. Since the outcomes of patients treated in New York sanatoria were comparable to those of naturally-occurring patients who had no prior exposure to sanatoriums (Daniel 1997, Daniel 2005), its potential therapeutic impact has been challenged.

In 1905, the Ottoman Empire established the Hamidiye Etfal Hastane-i lisi, the world's first sanatorium specifically for children. Republican period efforts to create such houses continued, with the most notable sanatorium opening in 1925 on Heybeliada (Princes' Islands, Istanbul), surrounded by pine trees for fresh air (Aksu 2007, Seber 2010).

Until the 20th century, numerous nations practiced sanatorium treatment, and some hospitals had therapeutic open-air wards. In the majority of instances, however, only individuals with mild sickness benefited; the majority of those with serious problems died within a short period (Frith 2014).

In 1948, a remarkable initiative to eliminate TB was launched under the support of UNICEF and the Danish Red Cross Holm as its director. The strategy consisted of tuberculin testing followed by BCG vaccination of nonreactors. It originated in Poland, quickly expanded to other European nations, and eventually reached Ecuador. During the following three years, approximately 30 million people were screened for tuberculosis and nearly 14 million were vaccinated with BCG. In fact, it was the first disease control program undertaken by a World Health Organization institution (Comstock 1994).

In the United States, mobile X-ray teams tested millions of people beginning in 1947; however, as the disease's prevalence decreased, these teams were phased out in favor of dedicated TB clinics by the 1960s (Daniel 1997).

During the years 1966-1976, the whole population of Turkey, even those living in outlying villages, was checked for tuberculosis by similar mobile teams. Although it was reported in 1997 that tuberculosis cases were down, mobile teams continued to examine high-risk places including prisons and jails (Daniel 1997, Aksu 2007)

In 1922 to create a *Mycobacterium bovis* strain with low virulence, Albert Calmette and Camille Guérin at the Pasteur Institute in Lille subcultured the original strain 230 times. The first oral administration of a vaccine strain of Bacillus Calmette-Guérin (BCG) was made to a 3-day-old infant in 1919 (Daniel 1997).

In 1921 the efficacy of the BCG vaccination has been the subject of much controversy, although large-scale investigations have placed its effectiveness rate between 0 and 80% (Daniel 1997).

Vaccination is now only recommended for use in newborns, yet in Turkey it is commonly given to infants as young as 2 months old. With the development of chemotherapy thirty years later, the prognosis for tuberculous patients and the history of TB altered substantially, as predicted by Trudeau. Jorgen Lehmann's discovery of paraamino salicylic acid (PAS) in 1943 and Gerhard Domagk's discovery of thiosemicarbazone during World War II ending in Germany, in 1945, provided the first therapeutic drugs with effectiveness in the treatment of tuberculosis. However, both compounds were just bacteriostatic, which was a disappointment.

In 1944, Albert Schatz, Elizabeth Bugie, and Selman Waksman announced the discovery of streptomycin, the first antibiotic and efficient bactericidal against *M. tuberculosis.* Within a few months, it was utilized to treat a young lady with TB with significant results (Schatz, Bugle et al. 1944, Hinshaw and Feldman 1945, Ryan 1993). One female patient's health improved dramatically after being treated with the first efficient antibiotic, streptomycin, which was discovered in 1944 (Daniel 2005).

In 1952, the mycobactericidal medication isoniazid became available for oral administration, which made the condition manageable. Antibiotics like pyrazinamide (1954), ethambutol (1962), and rifampicin (1966) shortened the typical course of treatment to only 6 months (Daniel 2005, Seber 2010). Camlica Sanatorium in Turkey was the first to administer streptomycin (1947), following the World Health Organization's guidelines in 1969 (Çavuşoğlu 2014), combination rifampicin, isoniazid, pyrazinamide, and ethambutol therapy was used at Heybeliada Sanatorium (Çavuşoğlu 2014).

In 1974, the WHO Expert Committee on TB published its Ninth Report, which offered policy recommendations for tuberculosis control for the next two decades. It opposed radiographic screening and tuberculin testing and encouraged sputum microscopy for symptomatic and at-risk people. It favored ambulatory treatment and discouraged institutionalization of TB sufferers. It established a goal of 70–90% BCG vaccination coverage for all those less than 15–20 years old. Today, the WHO continues to implement TB control initiatives, but it no longer advises BCG immunization for anybody other than infants. In the United States, where BCG has not been utilized extensively, TB control initiatives prioritize the treatment of latently infected persons (Organization 1974).

Styblo and colleagues reported a decline in tuberculosis (TB) incidence in Europe and portions of Africa in 1978; however, the introduction of AIDS in the 1980s shifted the incidence over the next two decades. Subsequently, strict control procedures were implemented to monitor patients, and the Directly Observed Treatment Short Course (DOTS) offered fresh hope for eradication in Africa, with a substantial decline from 2005. Additionally, Turkey followed similar tactics, and in collaboration with the WHO, DOTS was initiated in 2002 in pilot areas, with national implementation to follow (Aksu 2007).

Currently, the infection incidence with multidrug-resistant tuberculosis (MDR-TB) in sub-Saharan Africa and nations of the former Soviet Union (FSU) is alarmingly high 28,29. In Turkey, 2012 studies suggest that only 2% of patients

have MDR; nonetheless, the transmission of the MDR-Beijing strain through FSU nations is commonly detected. Increasing human mobility necessitates worldwide collaboration for eradication. Even in the face of AIDS, the history of TB implies that the present wave will pass, although many may die in its path (Daniel 1997, Organization 2013, Gültekin, Boztaş et al. 2014). Also prevalent at the Ottoman Court was tuberculosis, which claimed the lives of Sultan Mahmut II (1785–1839), his mother, and his son. Ottoman documents exist as late as the in the 18th and 19th Centuries, tuberculosis was a significant concern across the Ottoman Empire (Barış 2002, Aksu 2007, Perciaccante, Coralli et al. 2018).

The sequencing of the genomes of many *M. tuberculosis* strains using splitting molecular genetics techniques has made it possible to more precisely date the origin of *Mycobacteria*. Researchers led by Dr. Gutierrez have found evidence that an ancestor of *Mycobacterium TB* was prevalent in East Africa as far back as 3 million years ago, raising the possibility that it affected early hominins at that time (Gutierrez, Brisse et al. 2005).

*Mycobacterium TB* complete genome in 1998, Published series of enhancements that increase expectations. Better treatments and vaccines to prevent TB (Cole, Brosch et al. 1998). *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium canettii*, as well as *Mycobacterium bovis*, are all current members of the *Mycobacterium TB* complex, and it is believed that they all descended from a common ancestor in Africa some 35,000-15,000 years ago.

Two to four of today's *Mycobacterium TB* strains have a common ancestor that lived between twenty thousand and fifteen thousand years ago (Kapur, Whittam et al. 1994, Brosch, Gordon et al. 2002, Gutierrez, Brisse et al. 2005).

There are now six primary lineages, or clades, of circulating strains, and all of them may be found in East Africa. Based on analyses of the known mutation rate of *M. tuberculosis*, it has been estimated that between 250 and 1000 years ago is when most of the current variety among these strains emerged (Hirsh, Tsolaki et al. 2004). Both groups' ancestors may be traced back to East Africa at the time.

human hosts for the tuberculin bacillus when it comes to sickness, archeological evidence in East Africa is generally scant (Cave and Demonstrator 1939, Morse 1964, Morse, Brothwell et al. 1964, Zimmerman 1979).

Currently, each strain of *Mycobacterium tuberculosis* that infects humans is derived from a separate progenitor. It produces six primary lines. West Africa is home to *M. africanum* strains, including lineages 5 and 6. Other lineages, however, exhibit major variations in their distribution. Strain 1 is an EAI [East African-Indian] spoligotype strain. The strains in this cluster are considered "ancient" *M. tuberculosis* strains. Ancestral strains of *M. tuberculosis* spread from India through migration developed into'modern' lineages 2 (Beijing), 3 CAS (Central Asian Indian), X, Haarlem, and LAM [Latino-American Mediterranean]. Lineage 4 was the prevalent strain in Turkey. Analysis of known *M. tuberculosis* mutation rates, indicates that strain differences emerged between 250 and 1000 years ago (Çavuşoğlu 2014).

In current history, research and innovation milestones in the diagnosis, treatment, and prevention of tuberculosis on a global scale have evolved with each passing day. Despite this, tuberculosis continues to be one of the fatal infectious illnesses affecting humans. As a result of the unique coronavirus pandemic, the number of tuberculosis (TB) cases observed and recorded in 2020 has decreased significantly. In recent years, the previously uninterrupted drop in the worldwide TB mortality rate was disrupted. The prognosis of individuals with tuberculosis has been seriously affected by interruptions in diagnosis and treatment (Yang, Han et al. 2022).

### 2.2. Tuberculosis infection prevention and control

It is assumed that *Mycobacterium* existed a hundred and fifty years ago. In 1875, Hansen discovered the first known representative of this group under the name *Bacillus leprae*, and thus The Bacillus Calmette Guerin vaccination has been shown to be one of the most effective TB preventatives. Multiple antibiotics with

varying concentration levels are used to treat TB. Vaccines against TB are being developed using a variety of splitting techniques in order to protect the body against these microorganisms (Khare, Khare et al. 2018).

In the early decades of the 20th century, Koch's "Tauruman" vaccine was commercialized. It was created utilizing human TB bacilli with attenuated virulence and surviving human tuberculosis bacilli. In 1930, 250 infants vaccinated with a BCG vaccination in Lübeck, Germany, were inadvertently infected with aggressive TB bacilli; 73 died of tuberculosis in the first year, and 135 were hospitalized (Martini, Besozzi et al. 2018).

Throughout the previous century, the BCG vaccination was utilized in several nations to protect children and adults against tuberculosis. However, its efficacy against pulmonary TB was inconsistent. To achieve long-lasting immunization, numerous formulations of new-generation TB vaccines are now being evaluated and are in various stages of clinical studies (Martin 2006).

In 2016, thirteen potential vaccines were evaluated in clinical trials, including candidates for the prevention of tuberculosis infection and candidates for the protection of tuberculosis illness in individuals with latent tuberculosis infection. These potential vaccines are designed not only to induce greater immune responses against MT, but also long-lasting responses, which will need the induction of memory T- and B-cell responses (Orme 2013).

Eight of the thirteen vaccines now in clinical development are subunit vaccines, and six of them include or express Ag85A or Ag85B proteins. The lack of variety in both the antigens contained in TB vaccines and the immunological responses generated by TB vaccine candidates is a significant obstacle to the development of TB vaccinations. To maximize the prospects for generating a viable candidate by 2025, both will need expansion (Fletcher and Schrager 2016).

Attenuated live vaccinations disrupt phagosome biology and intracellular host mechanisms, such as apoptosis and autophagy. Mucosal vaccination was proven to be better to parenteral immunization in recent investigations, and this

new mode of delivery is presently being investigated (Gengenbacher, Nieuwenhuizen et al. 2017). In recent years, a novel TB vaccine formulation has presented a fresh curative concept. This vaccine's efficacy is predicated on the premise that mycobacterial antigens accelerate bacterial death. RUTI is a non-live polyantigenic vaccine that may be used as a preventive vaccination in conjunction with brief intense antibiotic treatment; it is undergoing phase II testing (Gengenbacher, Nieuwenhuizen et al. 2017).

From preventive to treatment, five main steps are necessary. Urgent action is required to address the concerns of health system hurdles, diagnostic and therapeutic difficulties, and limited funds for care and research. Developing novel medications, enhancing diagnostics, and launching new TB vaccines are essential elements of a plan to battle tuberculosis and drug-resistant TB (Manjelievskaia, Erck et al. 2016).

In places where tuberculosis is endemic, children are more susceptible to drug-resistant TB; thus, a key objective is to enhance access to presently available vaccinations and treatment in order to halt the development of drug-resistant TB, particularly among children. According to the WHO Stop TB program, daily isoniazid therapy for individuals at high risk of active tuberculosis is an effective preventative intervention at both the individual and population levels. Other preventative actions are suggested in national programs and in the WHO stop TB program's recommendations, with the goal of providing universal access to TB diagnosis and treatment (Martini, Besozzi et al. 2018).

High-sensitivity interferon gamma-release assays have been developed as a result of the identification of *Mycobacterium tuberculosis*-specific immunodominant antigens. Despite substantial Despite their contribution to advancement, these methods should be enhanced. In order to expand their usage to nations with limited resources and to paucibacillary TB patients (Bull, Croft et al. 2016, Martini, Besozzi et al. 2018).

In recent decades, TB has decreased significantly in Western nations, and vaccination remains a selected treatment for susceptible populations in these regions.

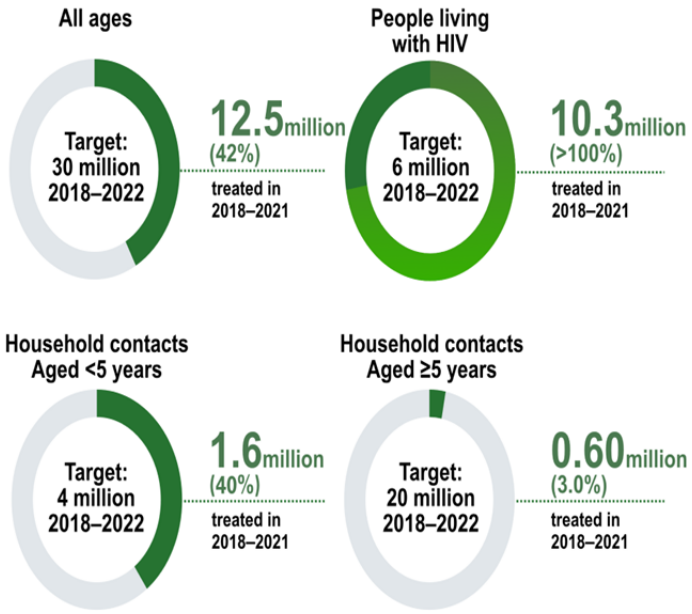


Figure 2.1. Global progress in provision of TB preventive treatment 2018–2021 compared with cumulative targets set for 2018–2022 at the UN high-level meeting on TB.

In countries where TB is very prevalent, the World Health Organization (WHO) continues to use mass vaccination to prevent the illness in children, despite the fact that this cannot break the "chain" of the disease, which is perpetuated by adults with advanced pulmonary forms. The World Health Organization (WHO) has implemented the TB end strategy, which is centered on the development of new instruments to detect, prevent, and cure tuberculosis more effectively, since 2012. In the future years, the difficulty will be to successfully translate creative findings into public health goals and socioeconomic initiatives for local TB control programs (Martini, Besozzi et al. 2018).

**2.3. Tuberculosis and HIV co-infection**

People with acute or latent Mycobacterium tuberculosis infection still had a higher risk of TB-related morbidity and mortality if they tested positive for HIV. Clinically evident TB risk is highest in the first year following HIV seroconversion and continues to climb over subsequent years of infection (Sonnenberg, Glynn et al. 2005). Becoming more prominent as immunosuppression worsens (Crampin, Floyd et al. 2002).

HIV-positive people have a 20-37% higher chance of contracting tuberculosis than HIV-negative people, and TB-HIV co-infection rates may reach 80% under particular conditions in sub-Saharan Africa (Granich, Akolo et al. 2010). It's unclear how much of an impact HIV has on TB transmission. Sputum bacterial load was reduced, lung cavities were reduced, and TB infectiousness was reduced in patients with TB complicated by HIV (Peters, Andrews et al. 2019).

As a result of its association with MTB, HIV increases the prevalence of active tuberculosis in the population and may contribute to the emergence of drug-resistant TB. Studies have shown that HIV may significantly alter the immune response and may hinder MTB's ability to selectively differentiate. For example, HIV affects the directionality of codon selection at celAb2, katG, and cyp138. Further, MTB may hasten HIV replication, promote active transcription, and affect HIV diversity. Patients are put in mortal danger due to HIV and MTB's ability to work together (Yang, Han et al. 2022). Co-infection with HIV was present in 15% of TB patients worldwide (1,2 million people) in 2015; this number ranged from 50% to 80% in parts of sub-Saharan Africa. One out of every three people with AIDS dies from tuberculosis, making it the leading cause of death among persons with HIV infection and AIDS (Martini, Besozzi et al. 2018).

With universal health care, patients would be assured of receiving the high-quality, comprehensive treatment they need for tuberculosis and HIV, reducing their risk of illness and death. Other interventions that help reduce mortality among PLWH include HIV/TB case detection and Isoniazid prophylaxis (IPT)

(Telisinghe, Ruperez et al. 2021).

Testing rates are low with the traditional methods of TB detection as well, including sputum smear, sputum culture, tuberculin skin test, and interferon (IFN)-releasing test. To this day, autopsy results show that up to 45.8 percent of HIV-infected people in South Africa are not identified with TB before they die (Bates, Mudenda et al. 2015).

It's possible that current estimates of HIV-positive people with TB are significantly low. The diagnostic effectiveness of tuberculosis in HIV-positive persons might be improved by the use of rapid TB diagnostic techniques based on nonpreparatory material (Yang, Han et al. 2022).

The newest generation of Xpert MTB/RIF kits, Xpert Ultra, is more sensitive than standard kits. In multicenter studies comparing the clinical diagnostic accuracy of Xpert Ultra and Xpert, the sensitivities of Xpert Ultra and Xpert for patients with smear-negative and culture-positive sputum were 63% and 46% respectively. Xpert Ultra was more sensitive than Xpert at detecting RIF resistance in HIV-positive people with culture-positive sputum (90% vs 77% respectively) (Dorman, Schumacher et al. 2018).

The use of more accessible, more efficient, and less toxic drugs; early detection based on PCR technology of MDR-TB infection, as this sickness may be cured; and the creation of vaccinations are all potential strategies for reducing HIV-related tuberculosis infections. Using drugs like bedaquiline and delamanid, which may combat both sensitive and resistant strains (Tiberi, Carvalho et al. 2017) . There is a two- to fourfold increase in mortality for HIV-positive people receiving treatment for multidrug-resistant TB. HIV patients with MDR-TB may benefit from ART and high-quality anti-TB medicines (Bisson, Bastos et al. 2020).

Better treatment regimens are needed to stop the spread of drug-resistant TB in HIV-endemic areas (Khan, Yates et al. 2019). No QTc prolongation events of grades 3 or 4 were seen, and a 95% culture conversion rate at week 8 with the combination of Bedaquiline and Delamanid indicated a high early culture

conversion rate. Bedaquiline and delamanid have been shown to be effective treatments for people with multidrug-resistant TB and rifampin-resistant TB, respectively (including HIV-infected patients) (Dooley, Rosenkranz et al. 2021). Several clinical investigations have influenced new regulations and recommendations on a global scale. The effective and sustainable integration of TB and HIV care has made great strides in recent years, but there is still a need for high-quality evidence guiding practical implementation (Naidoo, Rampersad et al. 2019).

**2.4. Epidemiology**

In the past, MT favored young people and was referred to as the "thief of youth"; in most cases, it proved fatal, with a death rate in the pre-chemotherapy era of approximately 65 percent at 5 years, especially when it was associated with malnutrition, overcrowding, poor living and working conditions (Frith 2014). Tuberculosis is present everywhere in the world. Yet the majority of TB cases are found in countries with low or moderate per capita incomes. There is also an intolerable TB burden in a number of other countries throughout Asia, Africa, Eastern Europe, Latin America, and the Caribbean (Terracciano, Amadori et al. 2020).

TB remains a leading global cause of sickness and death despite advances in tuberculosis control and decreases in both new cases and mortality. In 2015, six nations accounted for 60 percent of the world's TB deaths: India, Indonesia, China, Nigeria, Pakistan, and South Africa (Pan, Zhang et al. 2020).

Among people living with HIV/AIDS, TB was the cause of mortality in 35 percent of all cases in 2015. One million cases of TB illness were reported in youngsters in 2015, making this demographic another at-risk category (W.H.O, 2017). According to WHO projections, by 2020, Europe and seven other nations will approach a tipping point when extreme loads become unsustainable (Kenya, Lesotho, Myanmar, Russia, South Africa, Tanzania, and Zimbabwe). Incidence and

mortality rates are rapidly decreasing in the African Region of the World Health Organization (Harding 2020). Because of these efforts, the annual incidence rate of tuberculosis is expected to have fallen by 1.5% globally since 2000 (WHO, 2017). Overall mortality rates due to tuberculosis have decreased considerably and steadily during the last several decades. According to the World Health Organization, the number of deaths caused by TB dropped by 22 percent between 2000 and 2015 (WHO, 2016).

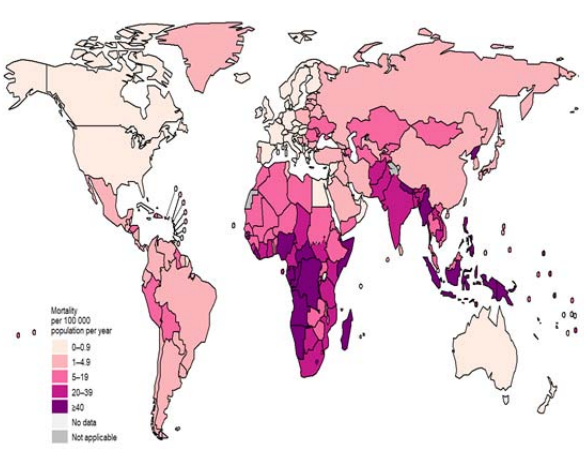


Figure 2.2. Estimated TB mortality rates in HIV-negative people, 2021.

Anti-tuberculosis chemotherapy has one of the highest costs-effectiveness ratios of any common treatment (McKee, Edwards et al. 2006).

The DOTS plan was developed by the World Health Organization (WHO) and the Stop Tuberculosis Partnership (STOP TB) (Bloom, Atun et al. 2017). More than 1.7 billion people have TB infection, according to estimates (Houben RM 2016). Since reaching a high in 2003, the number of new cases of TB throughout the globe has been steadily declining. In 2020, the World Health Organization predicts that 9.9 million people will get TB, with 1.5 million of them dying from the illness (Organization. 2021).

Possible contributing factors to this drop include increased food availability (better nutrition) and measures to reduce the spread of sickness. Initial records date back to 1901. Deaths from tuberculosis in the Ottoman Empire were 264 per 100,000 people, 30 percent higher in Istanbul than in England.

The infant mortality rate increased from 351 per 100,000 in 1918 to 268 per 100,000 in 1922 as a result of the First World War. After 1949, mortality and incidence rates in Turkey fell dramatically. There were an 88% drop-in incidence rates between 1949 and 1965, and a 75% drop-in fatality rates between 1949 and 1960. Out of a total 2010 population of 73,722,988, there were 15,183 TB patients in Turkey. The reported TB mortality rate in Turkey in 2011 was 0.72 per 100,000 (Çavuşoğlu 2014).

According to the World Health Organization, the prevalence of MDR-TB varies from zero in certain countries to sixty-five in others (Caminero 2003).

The true TB death rate ranges from as low as 5% in certain countries to as high as 20% in the African Region (Organization 2013). Nearly two-thirds of all cases of multidrug-resistant tuberculosis (MDRTB) are thought to be concentrated in China, India, and Russia at the present moment. Approximately 500,000 people were diagnosed with MDR-TB in 2018, but only about a third of them actually started treatment (Harding 2020).

Despite major advancements, tuberculosis (TB) remains a global public health emergency. An estimated 10 million people contracted TB in 2019, with 7.1 million of them people receiving a diagnosis they had it (Geneva 2020) .

The already sizable gap between the estimated and reported numbers worsened during the COVID-19 coronavirus disease pandemic. In contrast to other groups, this chasm has expanded (Maurer, Shubladze et al. 2021). In 2019, there were probably half a million newly diagnosed cases of MDR/RR-TB, but only 206,030 were recorded (Geneva 2020).

The highest rates of multidrug-resistant tuberculosis are still seen in several countries of the former Soviet Union, despite the belief that China, India, Russia,

and the nations of the former Soviet Union have the most MDR-TB patients. Overall, only 56% of patients were deemed cured by the therapy. By the end of the next several decades, when better diagnostics are in place, MDR-TB will be widely acknowledged as a major challenge to global TB control. Kazakhstan, Azerbaijan, Belarus, Moldova, Uzbekistan, Tajikistan, Ukraine, and Kyrgyzstan are among the countries in the former Soviet Union with the highest-burden of MDR-TB, along with several nations in Africa (including Angola, the Democratic Republic of the Congo, Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Somalia, and Zimbabwe) and several in Asia (Indonesia, Thailand, Pakistan, Philippines, and Bangladesh) (Organization 2000, Gandhi, Nunn et al. 2010, Singla, Singla et al. 2011, WHO 2011, Chen, Li et al. 2012, Loewenberg 2012, Taype, Agapito et al. 2012, Tessema, Beer et al. 2012, Zhao, Xu et al. 2012, Zignol, Gemert et al. 2012, Maharjan, Nakajima et al. 2018).

### 2.4.1. The Genome of *Mycobacterium tuberculosis*

This foresight relies on the high mutation rate of *M. tuberculosis*, which has been made feasible by the rapid development of molecular genetics tools (Gagneux, DeRiemer et al. 2006). Innate drug susceptibility in the *Mycobacterium TB* complex may differ across different strains of the bacterium (Köser, Feuerriegel et al. 2012).

The ability to do cross-species comparisons, such as when testing for pathogenicity, requires a system for categorizing species in relation to one another. kind of bacterium, virus, or other infectious agent that causes disease in humans or animals. The taxonomy of the *Mycobacterium TB* complex has grown significantly over the last 20 years due to widespread clinical isolation, advances in sequencing, and the delineation of constantly increasing loci (CRISPR and MIRU-VNTR).

Different online methods were developed to distinguish the isolates based on the known diversity in either CRISPRs (also called spoligotypes) or MIRU-VNTR profiles. The underlying taxonomies remain constant despite the fact that

they have different names and presentation levels. In 1882, Robert Koch initially isolated the organism now known as "*Mycobacterium tuberculosis*," and in 1883, Zopf introduced the label "Bacillus tuberculosis" to the scientific world (Lehmann and Neumann 1907).

In 1912, Calmette and Guerin led a series of experiments to isolate *M.bovis* from cows; today, it is used to create a vaccine; the researchers didn't use a different species name, likely because *M.bovis* infects humans and is known to induce very similar symptoms on *M. tuberculosis*, which was originally isolated from rodents and later elevated to species level as *M. tuberculosis var. Muris* in 1957 (Reed 1957). It was previously believed that mycobacterial diseases had spread from cattle to humans between 10,000 and 25,000 years ago, at which point the *Mycobacteria* would have adapted to the environment of their new host. TB developed thereafter. There was conclusive evidence that *M. bovis* is responsible for bovine tuberculosis (Haas and Haas 1996). More and more species have been given names that allude to their major animal hosts, even if these hosts are replacements, in accordance with this line of thought. Since 1970, many other mammalian hosts have been discovered for *Mycobacterium*, defying DNA similarity criteria: *Mycobacterium bovis* in 1970, *Mycobacterium caprae* in 1999, *Mycobacterium pinnipedii* in 2003, *Mycobacterium mungi* in 2010, *Mycobacterium orygis* in 2012, and *Mycobacterium surricatae* in 2013. Because of the similarities between the several species, the "*M. tuberculosis complex* (MTC)" name has been in use for over 30 years (Laszlo, Gill et al. 1983). Mutation rates are significant in *M tuberculosis* strains, which emerged anywhere from 250 to 1,000 years ago (Stead 1997).

Subtypes of MTC were triggered by CRISPR data (long "Direct Repeat" or "DR locus" and "Spoligotyping") and/or RFLP data detecting the IS6110 insertion sequence. MTC's taxonomy is based on DNA sequence data. Since 1999, scientists have used the CRISPR locus and its related taxonomy based on the presence or absence of particular spacers, leading to the creation of a worldwide

database first called SpolDB and now known as SITVIVEB (Sola, Devallois et al. 1999, Brudey, Driscoll et al. 2006, Demay, Liens et al. 2012). Names like LAM, CAS, S, X, and so on have been given to subordinate lineages that share a "signature," or the lack of a certain spacer, that is easily detectable by experts (Streicher, Victor et al. 2007). Around the year 2000, the TB taxonomy started showing signs of large deletions (Brosch, Gordon et al. 2002, Tsolaki, Hirsh et al. 2004), adding extra confusion to the situation. MIRU-VNTR is the name for it (Mycobacterial interspersed repeating units, variable number of tandem repeats) (Weniger, Krawczyk et al. 2010).

In genetic epidemiology, the 24-VNTR genotype is analogous because it has several sites with high discriminatory potential (Weniger, Krawczyk et al. 2010).

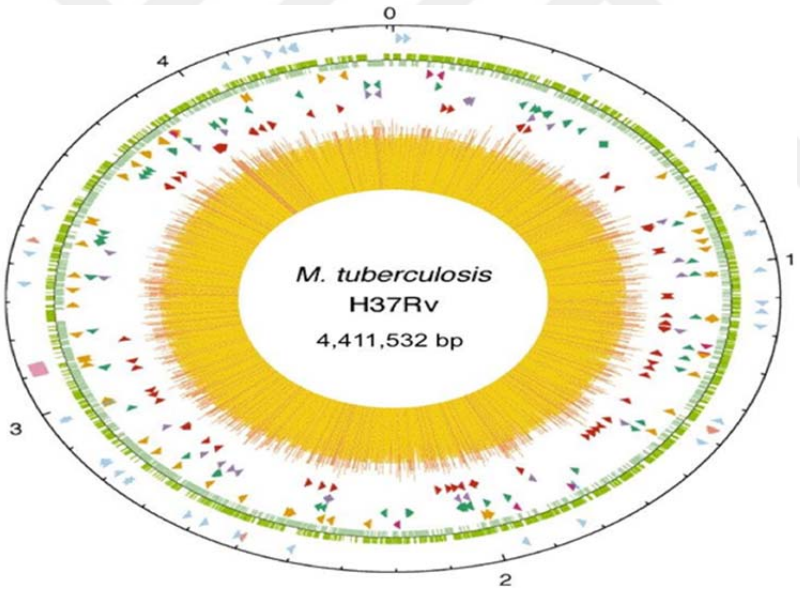


Figure 2.3. *M. tuberculosis* H37Rv Genome (Cole, Brosch et al. 1998).

Whether or whether spoligotyping is employed, 15-MIRU-VNTR sets have been developed due to the variability of loci for use in epidemiological research (Evans, Serafino Wani et al. 2011).

The next step in the investigation of MTC variation was the use of SNP analysis. High-throughput single-nucleotide polymorphism (SNP) typing or whole-genome sequencing (Karthik, Kumar et al. 2014). The slow growth, large GC genome content, and thick cell wall of the *Mycobacterium* genus set it apart from other bacterial groups (Maitra, Munshi et al. 2019). *M. tuberculosis*, *M. africanum, M. bovis, M. canetti, M. caprae, M. microti, M. mungi, M. orygis, P. pinnipedii*, and *M. suricattae* make up the *M. tuberculosis* complex, which is responsible for causing tuberculosis in humans and animals (Alexander, Laver et al. 2010, van Ingen, Rahim et al. 2012, Parsons, Drewe et al. 2013, Velayati and Farnia 2016).

As a result of their infection and growth in alveolar macrophages, these bacteria cause pulmonary TB (Parsons, Drewe et al. 2013), but they may also cause extrapulmonary illness if the bacteria spread beyond the lungs, such as to the central nervous system and/or lymph nodes (Field, Fisher et al. 2004, Yang, Kong et al. 2004). Non-tuberculosis mycobacteria, such as the *M. avium* complex (Horsburgh Jr, Gettings et al. 2001), *M. marinum*(Stamm and Brown 2004, Lai, Lee et al. 2005), and *M. ulcerans* are opportunistic pathogens that may infect immunocompromised persons (van der Werf, Stinear et al. 2003).

Fundamental to most studies is genetic analysis, which has allowed for the identification of 14 target locations (known as regions of difference or RD1–14). While these features are present in the M.TB H37Rv strain utilized in typical laboratory settings, they are absent in the vaccine-producing *M. Bovis var*. BCG, which helps pinpoint chromosomal regions containing genes involved in pathogenicity (Aranaz, Cousins et al. 2003).

There are six missing regions of the genome in the *M. tuberculosis* H37Rv strain. Deletions 1-5 from H37Rv and deletion 1 from *M. tuberculosis* are the names given to these regions (TbD1). On the other hand, all of the RD, RvD, and TbD1 genes can be found in the genome of *M. canettii*, the species presumed to be most closely related to the bacilli's progenitor. Strains of *M. africanum* found mostly in West Africa lack the RD9 region, whereas strains isolated in East Africa

contain both the RD9 and RD3 regions. *M. microti* does not have the regions RDmic, RD7, RD8, RD9, and RD10. When a pathogen is isolated from a single vole, it usually lacks data from the whole RD5 area. The most common strains of *M. bovis*, or "classical *M. bovis*," were found to be deficient in RD4, RD5, RD6, RD7, RD8, RD9, RD10, RD12, and RD13. Besides humans, these strains were found in bovines in Argentina, the Netherlands, the United Kingdom, and Spain. whereas *M. caprae* is closely related to *M. bovis*, its gyrB gene has many nucleotide changes that are absent in all other MTBC members (Aranaz, Cousins et al. 2003).

Multiple taxonomic isolates of the *M. tuberculosis* complex are now available and have been categorized accordingly. Extensive study and strong approaches applied to large datasets are needed to arrive at a consensus infra-specific MTC taxonomy that would guarantee agreement across relevant taxonomies. Species of *Mycobacteri*a that may be spread from animals to humans include: *Bovis*, is a bacterium that mostly infects ruminants including cows, sheep, goats, deer, like *M. tuberculosis* may cause infection in humans, apes, elephants, fish, swine, bovines, and even cervids like elk and moose. *M. avium* often infects pigs and poultry.

Cows, sheep, goats, deer/antelope, marsupials, monkeys, and horses are all examples of hosts. *Mycobacterium paratuberculosis* in wild ruminants of the bovine family. *M. intracellular* may cause infection in a wide variety of host species, including frogs, toads, turtles, birds, marsupials, monkeys, pigs, and cattle. Symptoms in fish, reptiles, amphibians, primates, swine, and cattle have been linked to *Mycobacterium chelonae*.

Illness caused by *M. fortuitum* has been linked to frogs, primates, and pigs. *M.leprae. murium*, often known as rat leprosy bacillus, is primarily a bacterium that causes disease in rats. The bacteria *Mycobacterium marinum*, which is responsible for lesions in fish and mollusks, is also the active ingredient in granuloma used by aquarists and fish breeders. Another pathogen that particularly

targets small rodents is *M. microti*, which causes disease in hedgehogs and field mice. Bovine, buffalo, and swine host the bacteria *Mycobacterium scrofulaceum*. Microbes similar to this one have been detected in both frogs and pigs (Brosch, Gordon et al. 2002).

Recently, however, a more worrisome scenario has evolved with the emergence of *M.tuberculosis* strains that have been shown to be resistant to all antibiotics that are now accessible for testing (Palomino and Martin 2014, Chakaya, Petersen et al. 2022). *Mycobacterium tuberculosis* complex (MTBC) bacteria are a family of closely related organisms that cause tuberculosis. One of the nine members, Mycobacterium tuberculosis (*M. tuberculosis*), remains the primary active ingredient of human TB, a leading cause of death and disability in many regions of the world. Although MTBC species diversity is low overall, there is considerable biological and behavioral variety across MTBC subgroups (Kanabalan, Lee et al. 2021).

### 2.4.2. Overview of the pathogenesis of human tuberculosis

People and tuberculosis germs, the relationship between *Mycobacteria* and their hosts is symbiotic. Changing during the next 50,000-70,000 years (Brites and Gagneux 2015).

The role of MTC in the development of human tuberculosis. Tuberculosis is a complex illness because of the reciprocal interaction between the host's immune system and the microorganisms that cause it. Latent tuberculosis infection (LTBI) or active tuberculosis is a result of a complex interaction between bacteria and humans. Because the host's genetic susceptibility and the infection's virulence are both important factors, the struggle against Mtb includes both innate and adaptive immune cell lines at different times. Mtb has been successful against humans for thousands of years because of its remarkable ability to avoid being killed by mechanisms present in the M8 from the onset of infection. When up against a microbe with sophisticated evasion mechanisms, the immune system has

just one chance to win: to respond rapidly and effectively the first time signs of infection arise (de Martino, Lodi et al. 2019). Inhalation of airborne infectious diseases is the initial stage in the pathophysiology of sickness. Core particle droplets (1–5 μ m in diameter) containing *Mycobacterium tuberculosis* from a person with active TB (Aurelie Cobat November 09 2009)

This infectious fluid subsequently makes its way to the terminal alveoli towards the very end of the lungs. The alveolar macrophages and other phagocytic immune cells consume them. Macrophages are the immune system's first line of defense, however if the infection has progressed too far, the intervention may be stopped immediately due to the clear strength ratio favoring the pathogen (Aurelie Cobat November 09 2009).

Pathogenic *Mycobacteria* are able to evade the microbicidal effects of macrophages because they have evolved resistant mechanisms. One of these is preventing apoptosis activation, while the other two are stopping the normal development of the phagosome and increasing resistance to host harmful substances (Forrellad, Klepp et al. 2013).

The buildup of Mtb in the lungs is the first stage in the initiation of many kinds of immunity, and the recruitment of cells, including macrophages, is an early phase in the response, an infection that may cause granulomas. These granulomas have the ability to spread and are very migratory. Means entering and leaving rapidly. The adaptive immune response, which follows the innate response, involves T cell infiltration of granulomas. As a consequence, a massive granuloma form around the Mtb site. When it comes down to the basics. If efforts are not taken to stop the spread of germs, infected granuloma sites become necrotic and caseous (Yousefi, Sankian et al. 2009).

After becoming liquid, the granuloma might be expelled via the respiratory system. The potential for further TB transmission now exists. Immunization and other tuberculosis preventative methods are crucial (Shakouri, Ghanei et al. 2015).

Macrophages, while keeping hosts; it also promoted the growth of invasive *Mycobacteria*. It also enables *M. tuberculosis* to enter the host's system during the early stages of infection and to stay in the latent phase of infection at all times. By crossing the alveolar barrier, pathogen-armed immune cells may be systemically disseminated (Teitelbaum, Schubert et al. 1999, Broxmeyer, Sosnowska et al. 2002). Toxic reactive oxygen and nitrogen species. The virulence of *M. tuberculosis* depends on the bacteria's ability to avoid being eaten by macrophages. Host cells destroy germs by a process called phagocytosis, in which they emit reactive oxygen species (ROS) and reactive nitrogen species (RNS). These ROI/RNI are able to react with a wide variety of substances, including nucleic acids, proteins, lipids, and carbohydrates. Like the great majority of intracellular infections, mycobacterial species have developed many resistance mechanisms to various treatments. However, these strategies seem to be unique to pathogenic mycobacterial species compared to other intracellular bacteria (Zahrt and Deretic 2002).

Many *Mycobacteria* manipulate the host macrophage such that lysosomal nitrous acid prevents the production of mature phagolysosomes. Because of this, intracellular *Mycobacteria* engage a variety of strategies to live and grow inside the host cells, including inhibiting the normal phagosome maturation process. As a result, the bacteria may continue to thrive in an internal environment that is not subject to acidification (Forrellad, Klepp et al. 2013).

Studies have indicated that *Mycobacterium. Tuberculosis* causes host cells to undergo apoptosis upon infection (Derrick and Morris 2007).

However, the individual MTBC strain affects the extent to which an infected cell undergoes apoptosis. Furthermore, the capacity of a bacterium to induce apoptosis is inversely linked to its pathogenicity (Keane, Remold et al. 2000).

Macrophages aid both the innate and adaptive arms of the immune system, while dendritic cells and CD4+ T cells work together to trigger adaptive immunity. *Mycobacterium.tuberculosis* infection elicits a response from the adaptive immune system (Gutierrez, Master et al. 2004). How *M. tuberculosis* infects unsuspecting cells. Cells' ability to go from the lungs to the lymph nodes may be hampered by Mtb's presence. Central caseous necrotic development lesions also include extracellular Mtb (Gholoobi A 2010). The immune system as a whole is failing to combat this invader. The host's sophisticated and thorough immune response to the detection of *Mycobacterium tuberculosis* may either result in a latent TB infection or the rapid eradication of the Mtb and the consequent abortive infection. Destroy the source of the illness entirely. The formation of granulomas, together with macrophages and CD4+ T cells, is widely seen as a cornerstone of the immune system's defense against Mtb. As a consequence of manipulation, the equilibrium may tip in favor of Mtb growth, leading to disease development, or against the host tissue, leading to damage from the immune system (de Martino, Lodi et al. 2019).

*M. tuberculosis* is an infectious bacterium that, depending on the host cell's immune response, may become permanently established inside the host cell. Inhibiting the presentation of antigens processed by the major histocompatibility complex; acting as an antagonist of the type 1 interferon receptor; and escaping the cell via the cytoplasm. This helps Mtb evade the host's immune system (Skjøt, Brock et al. 2002).

### 2.4.3. Overview of Mycobacterial essential virulence factors:

The degree to which an individual is able to contain or eliminate an infective inoculum is influenced by factors such as their preexisting immune system strength, genetic predisposition, and whether or not this is their first interaction with the organism. Alveolar macrophages have a hard time eliminating *M. tuberculosis* because of the bacterium's many pathogenicity features. The

elevated mycolic acid content in the bacterial outer capsule is a virulence factor because it hinders phagocytosis by alveolar macrophages. Other cell wall components, such as cord factor, may also directly injure alveolar macrophages.

Multiple reports suggest that *Mycobacterium tuberculosis* inhibits the formation of an effective phagolysosome, which in turn restricts or slows the clearance of the organisms (Adigun and Singh 2022).

Genes or cellular components of *Mycobacteria* that promote mycobacterial colonization, replication, and survival in their host are referred to as virulence factors. This is because several methods allow mycobacterial infections to colonize, replicate, and survive inside their host, contributing to their potential to cause illness. Whenever the development of the host organism is altered due to the elimination of an individual gene or cellular component, we call that element a virulence factor (Velayati and Farnia 2012). Clinic isolates with higher levels of medication resistance (i.e., MDR or XDR-TB) have been proven to be less virulent in studies (Smith, Saini et al. 2014).

*Mycobacterium tuberculosis* and other mycobacterial infections rely on a broad range of virulence factors, such as lipids and proteins on their surfaces and in their secretions, to infect and harm their hosts. Additionally, there is growing evidence that the virulence of pathogenic *Mycobacteria* is not the consequence of a single gene or cellular component, but rather the combined effect of multiple virulence factors. Mycobacterial surface lipids, Esx family proteins, and Pro-Glu (PE)/Pro-Pro-Glu (PPE) family proteins secreted by type VII secretion systems are all known to be harmful to the host (T7SS) (Velayati and Farnia 2012).

*Mycobacteria* have a capsule as their outermost layer, and unlike the other parts of the cell envelope, it was just recently found and defined. The capsule may include polysaccharides and a small amount of lipids. Mycobacterial adhesion and infection, solute transport (porins), and host cell survival are all mediated by proteins found in this matrix (Daffe and Etienne 1999).

*Mycobacteria* have been revealed to have about 500 different proteins in their cells. These proteins range from those present in the cell wall to lipoproteins (Mawuenyega, Forst et al. 2005, Wolfe, Mahaffey et al. 2010). A significant percentage (over 15%) of these putative cell wall proteins are engaged in lipid metabolism, and another significant percentage (over 5%) are involved in virulence and detoxification. Mycobacterial outer membranes have a bilayer rich in a kind of cell wall protein called outer membrane proteins (OMPs) (Hoffmann, Kohl et al. 2016)

These OMPs may be involved in the energetically demanding processes of nutrient intake, hydrophobic chemical absorption, and efflux. They aid in the attachment, invasion, and destruction of host structures by host cells. There are a few of these OMPs that are particularly important since they are the yet-to-be-discovered outer membrane component of the inner membrane transport mechanisms seen in MTBC (Song, Sandie et al. 2008).

The involvement of Erp (exported repetitive protein) in virulence has been well-documented, although its function remains a mystery. Berthet et al found that *M. tuberculosis* and *M. bovis* BGC erp mutants proliferated more slowly than the wild type in cultured macrophages and in BALB/c mice. Erp's importance to *M. tuberculosis* pathogenicity was shown by the fact that restoring erp to the mutants restored their ability to multiply (Al-Marzoqi and Shalan).

A protein complex known as fibronectin binding protein (Fbp) is essential to tuberculosis (TB) progression. Mycobacterial adhesion to mucosal surfaces and subsequent host cell invasion is facilitated by its affinity for FN (Wiker and Harboe 1992). OmpA is a porin from the outer membrane protein family, helping small hydrophilic molecules including arabinose, glucose, sucrose, and serine enter the cytoplasm (Senaratne, Mobasheri et al. 1998). Giris.666

The most noticeable surface protein is the adhesin HbhA (heparin-binding hemagglutinin). Because this protein binds sulphated glycoconjugates like heparin, *Mycobacteria* are only able to cling to epithelial and fibroblast cells and not

macrophage-like cells. allowing the bacteria to begin forming biofilms (Menozzi, Rouse et al. 1996). This includes both PhoT and PstA1. Inorganic phosphate transport proteins are encoded by the genes pstA1 and phoT (Braibant, Gilot et al. 2000).

Despite being very weakly attached to the cell membrane, the lipids and glycolipids of *Mycobacterium* may serve as diffusible components to modulate the host immune response or as signals to the pathogen in the early stages of infection. Because of their role in pathogenesis, the *M. tuberculosis* cell wall lipids that are abundant in many methyl-branched fatty acids might be targeted by innovative anti-mycobacterial therapeutics. Examples of lipids esterified with several methylbranched fatty acyl substituents seen in *M. tuberculosis* include sulfolipids (SL), di- and tri-acylated trehaloses (DAT and TAT), poly-acyltrehaloses (PAT), and phthiocerol dimycocerosates (PDM) (PDIM) (Cole, Brosch et al.).

The cell wall of *Mycobacteria* is unique due to its intricate lipid and protein composition. Mycolic acids, which are very long chain a-alkyl -hydroxy fatty acids, are joined to several strands of highly branched arabinofuran, which in turn are coupled to peptidoglycan, which forms the "core" of the structure. These lipids, which are oriented transversely to the plane of the membrane, provide a special barrier that contributes to the physiological and pathogenic properties of mycobacterial membranes. The PDIMs, TDM, SL, and PIMs are all sandwiched between the main body and the conclusion (Mougous, Petzold et al. 2004).

Lipomannan (LM) and lipoarabinomannan (LAM) are phosphatidylinositol mannosides that are also linked to the plasma membrane and extend outward to the cell wall (Daffe and Etienne 1999).

**2.4.3.1. Surface-Exposed Lipids and PE/PPE Family Proteins:**

Arabinogalactan is a component of the mycobacterial cell wall and is covalently linked to a large number of mycolic acids, which are long-chain (C60-C90) fatty acids. The mycolic acid layer forms the inner leaflet of the

mycobacterial outer membrane, whereas complex lipids, including glycolipids, form the outside leaflet (Chiaradia, Lefebvre et al. 2017). This feature of *Mycobacteria* is one reason why many medications fail to kill them (Velayati and Farnia 2012).

**2.4.3.2. Phthiocerol Dimycocerosates (PDIMs) and Phenolic Glycolipids (PGLs):**

Phthiocerol dimycocerosates is the chemical in question (PDIMs). The lipids PDIMs are unique to pathogenic *Mycobacteria* including *M. tuberculosis*, *M. bovis*, and *M. marinum* and include methyl-branched fatty acids (Velayati and Farnia 2012). In the early stages of infection, when *M. tuberculosis* bacilli first come into contact with host macrophages, PDIMs play a crucial role as virulence factors (Cole, Brosch et al.).

PDIMs have been shown to facilitate M. tb phagocytosis reliant on receptors , to contribute to the cell wall permeability barrier (Camacho, Constant et al. 2001), and to protect against reactive nitrogen intermediates in activated macrophages (Rousseau, Winter et al. 2004, Quigley, Hughitt et al. 2017).

Leprosy-causing *M. leprae's* phenolic glycolipid 1 (PGL-1) has been involved in the invasion of host phagocytic cells (Tabouret, Astarie-Dequeker et al. 2010).

**2.4.3.3. Sulfolipids (SLs):**

Family of sulfated acyl trehaloses found on the surface of *Mycobacteria*; detected by SLA two class-I and SLI-II tests (Goren 1970). It was via SLs' ability to inhibit phagosome-lysosome fusion and acidification in infected murine macrophages that SLs were first recognized as a virulence factor (Goren, D'Arcy Hart et al. 1976).

**2.4.3.4. PE/PPE Family Proteins:**

It is theorized that the PE/PPE proteins associated with the outer membrane of *Mycobacteria* have a role in communicating with the host's immune system (Delogu, Brennan et al. 2017). A technique for determining the virulence potential of certain PE/PPE protein, shows that PE11 is expressed in pathogenic *Mycobacteria* but not in *M. smegmatis.* Recent studies have shown an unexpected role for PE/PPE proteins: they act as small-molecule channels in the outer membrane, allowing nutrients to cross this normally impenetrable barrier into the cell (Singh, Rao et al. 2016).

Due to duplication and diversification, several distinct types of secretion systems have been identified, some of which have been linked to pathogenicity. Multiple gene clusters expressing proteins secreted by *Mycobacteria* through distinct pathways are thought to be crucial to the pathogen's ability to cause disease. There is emerging evidence that *Mycobacteria* have developed a specific secretion system to transport extracellular proteins over their hydrophobic and very impermeable cell wall (Forrellad, Klepp et al. 2013).

There are five T7SS in *M. tuberculosis*, also known as ESX, and they are quite similar to one another in terms of the genes they encode and the order in which they are located. These organisms host ESX-1 secretion-associated protein coding genes and ESX conserved component (Ecc) and (Esp) genes. Two of the four secretion systems encoded by the *M. tuberculosis* H37Rv genome are directly involved in the pathogenicity of the bacterium, which comes as a surprise to many (Forrellad, Klepp et al. 2013). The T7SS of MTBC secretes two key virulence-related proteins: an early secretory antigenic target (ESXA) of 6 kDa and a culture filtrate protein of 10 kDa (ESXB) (Pym, Brodin et al. 2003).

**2.4.3.5. Localization in the Inner Membrane: ESX Type VII Secretion Systems (T7SS):**

EsxA secretion system-1 (ESX-1) through ESX-5 are encoded by M. tb

and other pathogenic *Mycobacteria*. Although the roles of ESX-2 and ESX-4 are unclear, ESX-1, ESX-3, and ESX-5 are required for M. tb's complete pathogenicity. For ESX-1, ESX-3, and ESX-5, but not ESX-2 and ESX-4, protein transport across the inner membrane has been demonstrated, suggesting that this is a fundamental role of these ESX systems (Feltcher, Sullivan et al. 2010). Previous research has revealed that a fully functional ESX-5 system is necessary for M. tb to maintain cell wall integrity, secrete EsxN and PPE41, and exhibit complete pathogenicity (Bottai, Di Luca et al. 2012).

**2.4.3.6. Macrophage Antimicrobial Resistance Inhibiting Proteins:**

Acr kinship Mycobacteria have two members of the a-crystallin (Acr) family of molecular chaperones. Acr1, or 16-kDa a-crystallin (HspX), is a heat shock protein that is involved in the DosR regulon (a genetic program of *M. tuberculosis*). Circumstances that create TB control a wide range of dormancy-associated proteins by stifling aerobic respiration and thwarting bacillus reproduction (Hunter, Jagannath et al. 2007).

AhpC is an alkyl hydroperoxide reductase C, an enzyme that reduces organic peroxides, and is related to a family of bacterial and eukaryotic antioxidant proteins that directly reduce peroxides and peroxynitrites. Overexpressing AhpC in isoniazid-resistant *Mycobacterium TB* isolates devoid of catalase-peroxidase KatG data support a function for AhpC in *M. tubercu*losis peroxydative homeostasis (Silva, Senna et al. 2003). Despite lacking the catalase-peroxidase function of KatG, *M. tuberculosis* compensates by overexpressing AhpC (Sherman, Mdluli et al. 1999).

**2.4.3.7. SodC:**

Two genes, sodA and sodC, in *M. tuberculosis* code for proteins called superoxide dismutase (SOD). By splitting oxygen (O2) into molecular oxygen and hydrogen peroxide, sod enzymes eliminate ROS. Copper-containing SOD outer

membrane lipoprotein (SodC) is essential for *Mycobacterium tuberculosis* to protect itself from host-generated extracellular superoxide (D'orazio M and G 2001, Spagnolo, Törö et al. 2004).

**2.4.3.8. Mel2:**

The protein encoded by the mel2 gene is present in many different types of non-photosynthesizing pathogens and symbionts. Cirillo et al. have recently shown that Mel2 confers resistance to ROI in vitro in Mycobacterium TB (Cirillo, Subbian et al. 2009).

**2.4.3.9. KatG:**

KatG is the sole catalase-peroxidase enzyme in *Mycobacterium TB* capable of degrading hydrogen peroxide and organic peroxides. Almost all mutants that have evolved to be resistant to the anti-tuberculosis drug isoniazid (INH) have changes in a gene called katG, which causes INH to be activated and converted into numerous reactive species that inhibit a mycolic acid manufacturing enzyme (Timmins GS 2006;).

**2.5. Mycobacterial components that interfere with maturation of the phagosomal compartment:**

**2.5.1. Ndk:**

The enzyme encoded by the ndk gene has been demonstrated to bind and hydrolyze both ATP and GTP in vitro. Furthermore, it has been shown that *M. tuberculosis* autophosphorylates and secretes this protein into the culture medium, and that when coupled with ATP, pure Ndk promotes cytotoxicity in macrophages (Puneet Chopra 24 January 2003).

**2.5.2. PtpA:**

The host protein VPS33B, which regulates membrane fusion throughout endocytic trafficking, may be dephosphorylated by the small-molecule tyrosine phosphatase PtpA. The phosphatase activity of *M. tuberculosis* inhibited the maturation of phagosomes (Siobhán C. Cowley 2002).

Cell wall lipid synthesis proteins including PhoP254 and Ag85A contribute to MTBC's phagosome stopping activity (Muralidhar K. Katti 2008). One example is the PhoP/PhoR system, which regulates the synthesis of cell wall TDM and the SL, two lipids implicated in blocking phagosome/lysosome union (Kyle Rohde 10 September 2007 ).

**2.6. Inhibition of apoptosis:**

**2.6.1. NuoG:**

NuoG, one of the 14 subunits of the type I NADH dehydrogenase, NADH-1, was first discovered as an anti-apoptotic gene by Velmurugan et al. in *M. smegmatis* via a gain-of-function screening for anti-apoptotic *M. tuberculosis* genes. Later, an *M. tuberculosis* strain lacking nuoG was used to verify its function (Kamalakannan Velmurugan July 20, 2007).

**2.6.2. SecA2/SodA:**

The protein encoded by the secA2 gene is an ATPase that functions in the preprotein translocase. The protein superoxide dismutase A (SodA) and may be other proteins are released into the culture supernatant through a unique kind of secretion channel dubbed SecA2 (Braunstein, Espinosa et al. 2003, Gibbons, Wolschendorf et al. 2007).

**2.6.3 PknE:**

According to research by Jayakumar et al., deleting pknE from *M. tuberculosis* promotes nitric oxide-mediated apoptosis in human THP-1

macrophages and reduces production of pro-inflammatory cytokines, TNF-a and IL-6. Serine/threonine kinase E (PknE) is an enzyme that is encoded by the PknE gene (Jayakumar, Jacobs Jr et al. 2008).

**2.6.4 Proteases:**

The *M. tuberculosis* H37Rv genome encodes over a hundred proteases. *Mycobacterium.leprae*, *Mycobacterium.bovis*, *Mycobacterium.avium*, *M. paratuberculosis* and *Mycobacterium tuberculosis* share 38 of these proteins. However, information on the biochemistry of these enzymes in these species is scant (Forrellad, Klepp et al. 2013).

**2.6.5. Serine proteases:**

*Mycobacterium tuberculosis* H37Rv produces the five mycosins (mycosin-1 to -5) constantly. These mycosins belong to a family of highly conserved subtilisin-like serine proteases. The bacterial enzymes known as subtilases degrade proteins without particular sequence bias, facilitating the import of the resultant peptides (Gupta, Beg et al. 2002).

**2.6.6. Metalloproteases:**

There are three putative Zn-dependent metalloproteases in the *M. tuberculosis* genome. Rv1977, the third metalloprotease gene in *M. tuberculosis*, presumably functions as a Zn-dependent enzyme with chaperone function; however, this gene has been deleted in *M. bovis* and is unlikely to be involved in virulence (Forrellad, Klepp et al. 2013).

**2.6.7 Rip1:**

Rip1 is a major virulence determinant of *Mycobacterium TB* because of its role in regulating the composition of the cell membrane and *M. tuberculosis's* growth and persistence in vivo (Makinoshima and Glickman 2006).

### 2.6.8. Metal importers and exporters:

*Mycobacteria* clearly have a significant adaptive advantage thanks to the metal transporter proteins, which function as either importers or exporters, allowing them to survive in metal-limited environments like the phagosome or the host, or to tolerate environments with high metal concentrations that would otherwise be toxic and lead to bacterial death (Forrellad, Klepp et al. 2013).

### 2.6.9. Gene Expression Regulators:

*M. tuberculosis* encodes a large number of regulatory factors. These include a large number of orphan response regulators (RRs) and sensor kinases in addition to 11 complete two-component systems (TCSs) (SKs) (Forrellad, Klepp et al. 2013).

## 2.7. Proteins of Unknown Function:

### 2.7.1. The PE/PPE families:

About 10% of the *M. tuberculosis* H37Rv genome is made up of PE/PPE genes. Numerous genes like these may be identified in close proximity to the ESX operons. The Pro-Pro-Glu motif is represented by the PPE name, whereas the Pro-Glu located at positions 8 and 9 in the N-terminus gives birth to the PE name. The vast majority of these proteins are secreted or localized on the cell surface and they all act together to elicit a powerful immune response in the host. These proteins share a highly conserved N-terminus with many others, and their very varied C-termini are thought to be the source of antigenic and genetic variation (Brennan and Delogu 2002, Sampson 2011).

### 2.7.2 Other Virulence Proteins:

Unique enclaves (RD). Only the RD1 and RD2 portions of the MTBC genome have been demonstrated to be essential for full pathogenicity (Forrellad, Klepp et al. 2013).

**2.8. Morphological Characterization of *Mycobacterium tuberculosis*:**

Under a microscope, tubercle bacilli often take the form of either straight or slightly bent rods. Bacteria may range in size and shape from coccobacilli to elongated rods, depending on the culture's age and the conditions under which it was grown. In studies, bacilli have been measured between 0.2 and 0.6 micrometers in diameter and 1 to 10 micrometers in length (often 3-5 micrometers). Exceptional scientists, like Malassez and Vignal (1883), Nocard and Roux (1887), Metschnikoff (1888), Lubarsch (1899), Fischel (1893), and Vera and Rettberg (1893), tuberculosis bacilli's physical traits were emphasized for their potential. They showed that under stressful conditions, such as a lack of food or oxygen, *Mycobacterium* ballooned without developing vacuolar or globoid structures, giving the bacteria a bloated look. As time went on, it became clear that these initial results were built on contaminated data, and they were widely discredited(Velayati and Farnia 2012).

Modern microscopic methods, such as the transmission electron microscope (TEM), the scanning electron microscope (SEM), and the atomic force microscope (AFM), have led almost all scientists to conclude that the Koch bacillus does not always take on its signature rod shape. Additionally, when cultures develop, they have the potential to produce buds and branches in extensively drug-resistant strains (XDR-TB) (Velayati and Farnia 2012).

There are notable differences in the chemical compositions of the cell walls of *Mycobacteria* and those of other bacteria, which contribute to the unique construction of Mycobacterial cell walls (Velayati and Farnia 2012). The tubercle bacillus may switch between prototrophic and heterotrophic metabolisms. All of its building blocks, including carbon and nitrogen, can be synthesized by it, and it can also use carbon and energy from already-formed organic molecules (Hoffmann, Leis et al. 2008). It has been hypothesized that the tubercle bacillus's success as a pathogen is due to its extraordinary flexibility to environmental changes during the course of the infection.

Bacilli have a short lifespan that is often determined by variables such as nutrition quality and the surrounding environment (Velayati and Farnia 2012).

*Mycobacterium tuberculosis*, the causative agent of M.TB, is distinct from other bacterial illnesses in that its cell surface is covered with a significant number of complex lipids and lipoglycans. The function of these particular cell wall lipids is well-established. important function in disease; genes involved in their synthesis, degradation, and transport of virulence factors provide new avenues for therapeutic development (Converse, Mougous et al. 2003).

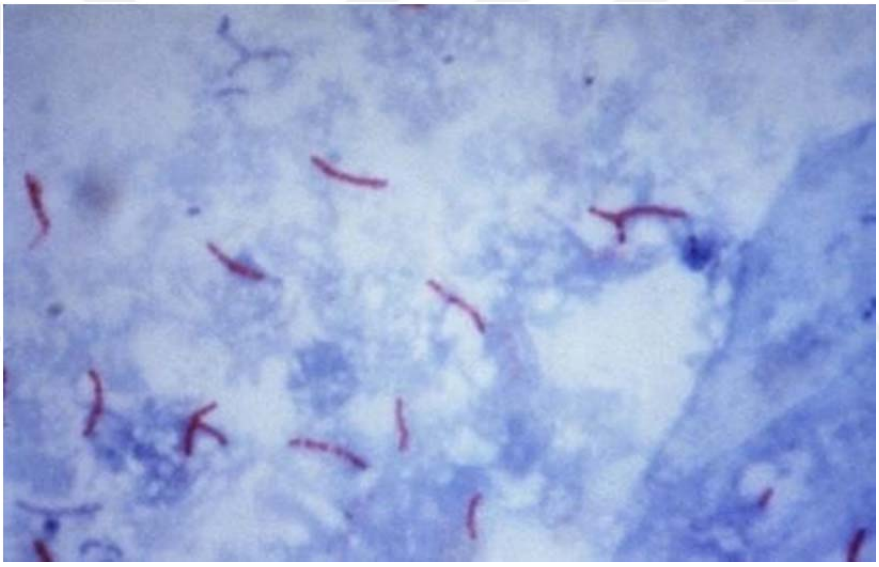

Figure 2.4. *M. tuberculosis-stained* Ziehl Neelsen from decontaminated sputum contained in oil immersion (x1000).

In order to stay alive and contagious, mycobacterial cells have a unique cell envelope structure and chemistry, including a peptidoglycan layer. Peptidoglycan biosynthesis, degradation, remodeling, and recycling are all relatively fresh areas of interest in the quest to find effective novel antimicrobials (Maitra, Munshi et al. 2019).

The outer capsule is comprised of proteins, glucan, and certain lipids. Cell walls are composed of an outside layer of mycomembrane (MM), an intermediate layer of arabinogalactan (AG), and an interior layer of peptidoglycan (PG). Phospholipids, trehalose mycolates, glycopeptidolipids, and lipoglycans make up outer leaflet, while long-chain mycolic acids compose the membrane's inner leaflet (MA). As with other lipids and the cytoplasmic membrane, the mycolic acid-arabinogactane-PG polymer generates a hydrophobic layer. Due to the permeability barrier established by the periplasmic gap, which separates the cell wall from the membrane lipid bilayer, antibiotics are unable to enter the cell (Nguyen 2016). Many antimicrobials fail to kill *Mycobacterium* TB because of the pathogen's peculiar cell wall structure, which has low permeability for many drugs and a large number of efflux pumps (Trias and Benz 1994, Brennan and Nikaido 1995). The unusual cell wall structure of *M. tuberculosis*, which has limited permeability for many medications and a high number of efflux pumps, may contribute to the pathogen's success and inherent traits, including its resistance to numerous antimicrobial treatments. *M. tuberculosis* has a highly impermeable envelope and the capacity to affect host defense mechanisms, which contribute to its long-term viability (Stanley and Cox 2013). *Mycobacterium tuberculosis*, the bacillus responsible for TB, is a massive, non-motile rod. To give you an idea of scale, a rod of this size would be between 2 and 4 micrometers in length and 0.2 and 0.5 micrometer in width.

They are considered obligatory aerobes since they can't undertake anaerobic metabolism and hence need oxygen to live. This bacterium is able to infect host cells and survive there without the host's knowledge or consent. The whole process of division usually takes between 15 and 20 hours. These bacteria are classified as acid-fast because of their capacity to produce acid-stable chemicals when exposed to the acrylmethane dye (Uhía, Galan et al. 2011).

*Mycobacterium* TB's thick cell wall is notoriously baffling to outsiders. It's brought on by bacteria with a very specific chemical composition (Jarlier and

Nikaido 1990). *M. tuberculosis* cells have a waxy covering and as a result of their high acidity, the mycolic acid bacteria prevalent in the environment serve as a fast acid. The GC content of the whole 4.4 MB genome is 67%. Of the 3,959 genes at issue, only 1,897 are really being utilized. Lipid metabolism genes are the most important parts of the bacterial genome (Bergey 1994).

Mycobacterial envelopes consist of a complex heteropolymer comprised of peptidoglycan and are located outside the cytoplasmic membrane. *Mycobacteria* are the only bacteria that can use the mycolic acids attached to the end of the arabinogalactane strand (Sutcliffe 1997). While fast-growing mycobacterial species like *M. smegmatis* have been shown to possess a major porin (MspA), slow-growing *Mycobacteria* like M. tb have been found to lack such a protein (Faller, Niederweis et al. 2004) .

*Mycobacteria* belong to the order *Actinobacteria*, which is itself a diverse and extensive collection of bacteria. The PG layer of the Mycobacterial cell wall is composed of mycolic acid (MA) and arabinogalactan (AG), but its structure and development are poorly understood, especially in the slow-growing intracellular pathogen *M. tuberculosis* (Petit, Adam et al. 1969, Mahapatra, Bhagat et al. 2008, Dhiman, Mahapatra et al. 2009). Mycobacterial species get their ability to withstand acids from the environment thanks to a lipid-rich outer membrane coating called peptidoglycan (Trivedi, Arora et al. 2004).

Peptides and glycan chains work together to form the peptidoglycon. N-acetylglucosamines (NAGs) linked to N-acetylmuramic acid make up the lengthy glycan strand in most cases (NAM). Peptides linked to the lactyl group of NAMs produced from various glycan chains form bridges between the chains. The L-alanyl-D-iso-glutaminyl-meso-diaminopimelic acid (DAP) from one strand of these peptide chains is usually linked to the terminal D-alanine residue of another strand of L-alanyl-D-iso-glutaminyl-meso-DAP-D-alanine (Velayati and Farnia 2012).

The heteropolysaccharide arabinogalactan, composed of arabinan and galactan, forms the second layer of macromolecules after the peptidoglycan. Mycolic acids, which have a very long chain length, are esterified to arabinogalactan. Because of their similarity in length but diversity in structural properties like cyclopropanations (cis or trans), keto, or methoxy groups, mycolic acids may be broken down into a number of different subfamilies.

In addition to serving as structural components, mycolic acids are esterified to glycerol and trehalose. Trehalose may include either one or two molecules of mycolic acid, generating trehalose dimycolates (TDM) and trehalose monomycolates (TMM), respectively. They both have complicated lipid and lypoglycan interactions with other components of the cell wall envelope (Barry III 2001).

**2.9. Diagnosis of tuberculosis infection:**

It is possible to identify the latent phase of tuberculosis infection. testing for tuberculosis by either a skin test or an interferon-gamma release assay (IGRA) Diagnosis by culture techniques, Molecular diagnostics, the cutting edge of a new technology for active tuberculosis (John Bernardo Oct 26, 2022.)

**2.9.1 Obtaining clinical samples:**

Although sputum may be created naturally (via coughing) or artificially, patients presenting sputum samples should be informed that nasopharyngeal discharge and saliva are not sputum. It is advised that at least 5-10 ml of high-quality sputum be collected to represent secretions from the lower respiratory tract, and there are proven ways for doing so (John Bernardo Oct 26, 2022.).

When *Mycobacterium tuberculosis* is isolated from bodily fluids (such as sputum, bronchoalveolar lavage fluid, or pleural fluid) or tissue (such as a biopsy, pleural, or lung biopsy), a definitive diagnosis of pulmonary TB may be obtained. Sputum acid-fast bacilli (AFB) and nucleic acid amplification (NAA) tests are

employed as complementary diagnostic tools; a positive NAA test plus an AFB smear is sufficient for a TB diagnosis. Before a chest X-ray is ordered for a patient suspected of having TB, a thorough evaluation of the patient's medical history and physical should be performed. If the display shows pulmonary or airway tuberculosis, it is indicated to send an AFB smear, three sputum samples for mycobacterial culture, and NAA test (collected by cough or induction at least eight hours apart, with at least one early morning sample). The interferon-gamma release assay (IGRA) or tuberculin skin test (TST) should also be conducted. These aids are used to detect TB infection that has been dormant for some time; Whether the result is positive or negative, neither confirms nor denies the presence of active tuberculosis (John Bernardo Oct 26, 2022.).

Traditional (phenotypic) drug susceptibility testing in which bacterial growth is measured in both a control medium and a medium containing drug is considered the gold standard for identifying drug-resistant tuberculosis. The planning and execution of a cultural event might take as much as a month. When the organism load is low, the time to a positive culture might be shortened in HIV-infected people. Before results from DST based on the explicit culture are available, drug-resistant TB molecular tests are useful for directing the first treatment selection (within hours to days) (John Bernardo Oct 26, 2022.).

Antibiotic resistance is only one example that can't be found in every lab. Sputum smears and cultures are examples of preliminary tests that certain community clinics and hospitals may do before sending the patient to a more specialist lab. Some procedures, such drug susceptibility testing, need extra orders beyond culture and identification, thus keeping an open channel of contact with the laboratory is essential. *Mycobacterium tuberculosis* complex-suspected culture isolates must be transmitted to a government-approved public health laboratory for confirmation. Authorities were also notified of promising culture findings so that they could keep tabs on things and deal with specific instances as needed (John Bernardo Oct 26, 2022.).

The microscopic examination of stained sputum smears for the presence of acid-fast bacilli is the fastest and cheapest approach for diagnosing tuberculosis (AFB). Direct smears from clinical specimens are best made with concentrated material (Lewinsohn, Leonard et al. 2017).

Three milliliters of sputum must be of adequate quality in order to proceed (Lewinsohn, Leonard et al. 2017).

Sputum smear microscopy is still considered the gold standard for examining persons with TB symptoms in regions with a high TB prevalence. Since the detection limit of sputum microscopy is just 5000 to 10,000 bacilli per milliliter, it is a rather insensitive test (LOD). Not only that, but sputum smear microscopy can't tell the difference between bacteria that are drug-sensitive and bacteria that are drug-resistant. The World Health Organization's TB programs have proposed using mWRDs to replace microscopy for detecting MTBC (Organization 2021).

So, while nucleic acid amplification (NAA) and culture are more sensitive than sputum AFB smears, normal light microscopy takes roughly 10,000 bacilli per mL to detect bacteria in an AFB smear (Hobby, Holman et al. 1973). AFB smear microscopy has a predicted sensitivity of 45–80 percent and a positive predictive value of 50–80 percent (Steingart, Ng et al. 2006, Behr, Kaufmann et al. 2021).

Microscopy continued to be the preferred method of isolation over the next decades because of the speed and accuracy with which diagnoses could be made. It was also cheap and simple to operate. Centrifugation, N-acetyl cysteine-sodium hydroxide, bleach, ammonium sulfate, and chitin are just some of the concentration methods that have been developed and employed in regions where the disease is common in order to boost sensitivity. Furthermore, immunofluorescence and Kinyoun staining were used instead of the conventional ZN staining (Singhal and Myneedu 2015).

More efficient and straightforward cold staining techniques have emerged in recent years. These include Gabbet's method and a modified two-reagent cold staining technique were utilized to diagnose TB (Gupta, Prasad et al. 2009).

Presently, positive smears are identified more reliably with the use of the fluorescent stain auramine. Changes to the fluorescent microscope's light source once every 200 to 300 hours previously limited its usefulness. The World Health Organization (WHO) now advises switching from traditional ZN microscopy for the detection of pulmonary TB even in resource-poor countries due to the novel usage of light-emitting diodes (LED), which endure for tens of thousands of hours (Hänscheid 2008, Chang, Page et al. 2016) . Anterior carbolfuchsin approaches (such as the Ziehl-Neelsen and Kinyoun methods with light microscopy) and the more rapid fluorochrome approach (using auramin-O or auramine-rhodamine dyes with fluorescent microscopy) are both widely employed for acid-fast staining (Peterson, Nakasone et al. 1999, Steingart, Henry et al. 2006, Marais, Brittle et al. 2008). Since the fluorochrome method may be up to 10 times more sensitive than carbolfuchsin methods, it is the preferred method (Shea, Davis et al. 2009, Lewinsohn, Leonard et al. 2016).

### 2.9.2. Mycobacterial culture:

#### 2.9.2.1. Conventional culture techniques:

The most sensitive conventional culture technique for detecting TB is sputum cultures, which have an 80% sensitivity and a 98% specificity. For purposes like taxonomy and drug testing, the capacity to grow organisms in a lab is vital (Morgan, Horstmeier et al. 1983, Ichiyama, Shimokata et al. 1993, Prevention May 26, 2016).

Popular types of standard culture medium include egg-based (Lowenstein-Jensen) media, agar-based (Middlebrook 7H10 or 7H11) media, and liquid culture media (Middlebrook 7H12 and other commercially available broth). Liquid medium growth typically takes between 1 and 3 weeks, but solid media growth takes much longer (three to eight weeks). Rapid growth is often seen on agar, however somewhat improved growth may be seen on egg-based medium. Colony morphology may be investigated on Agar medium, and mixed cultures can be easily distinguished (Morgan, Horstmeier et al. 1983).

After two or three days, monitor the growth using an automatic liquid system. For instance, MGIT 960 is one platform that offers continuous monitoring and rapid alerts if a new development is discovered. Every week or two, you should check on your solid media growth to make sure everything is going as planned. Once growth is confirmed, a sample is sent to a reference laboratory for further analysis, species identification, and drug susceptibility testing. High-pressure liquid chromatography (HPLC), biochemical methods, and mass spectrophotometry (matrix-assisted laser desorption/ionization-time of flight [MALDI-TOF]) are just a few of the methods that may be used to identify different species (Kent PT 1985., Shinnick and Good 1995, Lau, Drake et al. 2013, Hettick JM 2006).

Nowadays, the gold standard for this purpose is to culture TB bacteria on commercially available liquid media. Slow development in this field is hindered by factors such as culture, cost, infrastructure (biosafety level 3 [BSL-3] or TB containment lab), and time to results (1–3 weeks for a positive result and up to 6 weeks for a negative result). However, traditional microscopy and culture are still required to monitor a patient's response to treatment. To this day, culture is essential for the differential diagnosis of non-tuberculous *Mycobacteria* (NTM) infection and the identification of paucibacillary specimens from children with extrapulmonary TB and those with TB of the lymph nodes and skin (Organization 2021).

Worldwide, more and more people are becoming ill from bacteria that are resistant to several antibiotics, with a particular emphasis on identifying and treating drug-resistant TB (Zignol, Dean et al. 2016).

From a global perspective, based on information from 105 different regions and countries, the average ratio of *M. tuberculosis* strains among patients with MDR-TB was 20.1% (95% CI 15.5-25.0%). Despite improvements in diagnostic capacity, as measured by the number of rifampicin-resistant patients identified by countries in previous years, only 206 030 patients (44% of the projected number)

were informed globally in 2019, demonstrating that drug susceptibility testing coverage is severely insufficient. Eighty-six percent of the MDR/MDR-TB patients who were diagnosed received treatment ((WHO) 2020).

Early diagnosis is essential for effective treatment of TB, as is the detection of drug resistance as soon as possible. Get started right away on a powerful course of therapy. For this reason, it is critical that all TB patients have rapid access to accurate diagnostic tests and medication susceptibility testing (DST). In a perfect world, doctors would advise all TB patients on the best course of action. Before starting on anti-TB medication, a DST should be obtained. Let's get started with the treatment. However, postponing the start of treatment because of DST is not a smart idea. Improving laboratory capabilities (especially via DST) shouldn't slow down research or impair investigation. and enrolling those who have DR-TB in care initiatives (Maurer, Shubladze et al. 2021).

The World Health Organization's global strategy for the detection, diagnosis, and management of tuberculosis (TB). The End TB Strategy, which spans the years 2015-2035, places a premium on early diagnosis and universal access to drug-susceptibility testing (DST). All patients with bacteriologically confirmed TB, as defined by the World Health Organization, must have access to DST for at least RIF within 48 hours. Extra DSTs include the following: at the very least for fluoroquinolones (FQs) and second-line injectable medications for all TB patients with RIF resistance. According to the World Health Organization (WHO), rapid molecular diagnostic tests (for mwrd) should be available to everyone showing bacteriological indications or symptoms of tuberculosis. As of now, only RIF-resistant tuberculosis patients should get DST (of these patients, only around 61% were tested in 2018), but by the end of the year, all RR-TB patients should have received DST (Organization; 2020).

Specifically for pre-treatment assessments, the new World Health Organization guidelines strongly suggest using DST. Minor side effects are possible with drugs like FQs, INH, and RIF, but it is crucial to start them at the correct time (Organization 2021 ).

Drug-resistant tuberculosis should be suspected if the patient has a history of TB treatment, has advanced through TB clinics during treatment, has positive radiographic findings, lives in or has traveled to an area with a high prevalence of drug-resistant TB, or has been in close contact with a known or suspected carrier of the disease. The gold standard for diagnosing drug-resistant TB is the finding of *Mycobacterium tuberculosis* in sputum (or other clinical material) by a drug susceptibility test (DST) that is resistant to one or more antituberculosis drugs. Culture-based methods (providing phenotypic data) and molecular-analysis-based methods (revealing genotypic data) are both available for determining drug susceptibility (allowing genotypic information) (WHO. 2014. ).

A culture-based test is the gold standard for diagnosing drug-resistant TB; comparing the growth of the drug-resistant strain to that of a control strain on a control medium shows whether or not the strain is resistant to the medicine; the procedure may take at least a month. The rapid turnaround time (results may be achieved within hours or days) of a molecular test makes it useful for directing early treatment decisions before a full culture-based DST can be done (WHO. 2014. ).

The minimum inhibitory concentration (MIC) test includes culturing the organism at several drug concentrations to find the lowest medication concentration that inhibits the development of bacteria. For example, MIC testing might be used to decide whether a greater medication dosage would be helpful in cases of fluoroquinolone or injectable agent resistance (indicated by critical concentration). The sensitivity test for fluoroquinolones may be sped up if isoniazid resistance could be identified (Nahid, Mase et al. 2019)

If resistance to rifampin or other first-line medicines is found, testing for second-line drugs such as amikacin, streptomycin, fluoroquinolones, cycloserine, para-aminosalicylic acid, ethionamide, rifabutin, bedaquiline, linezolid, and clofazimine should be expedited. Molecular testing for drug resistance should be considered here as well (Nahid, Mase et al. 2019).

Rapid culture approaches that use liquid rather than solid medium include the Mycobacteria Growth Indicator Tube (MGIT) and the Microscopic Observation of Drug Susceptibility (MODS) test. *Mycobacteria* growth in the presence and absence of various antituberculosis medications is tested using liquid culture in MGIT. This might be an issue if, during cultivation, drug-resistant bacteria are discovered to be present. Unlike conventional solid culture methods, MGIT yields visible results in only a few days (Iseman and Heifets 2006, Minion, Leung et al. 2010).

The two main types of molecular analysis are the probe-based (non-sequence) tests and the array-based tests. Drug-resistance genes may be uncovered by using nucleic acid amplification (NAA) assays, often known as probe-based testing due to the use of a nucleic acid probe to evaluate a specific nucleic acid sequence. Molecular methods may be used to identify the DNA of *Mycobacterium tuberculosis complex* as well as the most common mutations that result in resistance to antituberculosis medications. Both array-based tests and probe-based (non-sequence) tests are common types of molecular analysis. The presence or lack of a mutation in such tests is not accompanied by any sequence information, in contrast to probe-based assays, which may determine the presence or absence of a mutation. Evidence that not all mutations in a given gene region cause medication resistance. However, information regarding the nature of a mutation may be gleaned via array-based tests, leading to more accurate predictions of drug resistance. For this reason, results from probe-based tests that imply resistance should be verified using an array-based test or culture (John Bernardo Oct 26, 2022.).

Both the *Mycobacterium tuberculosis* (MTB) Direct amplification test and the Xpert MTB / RIF test are validated by the Food and Drug Administration (FDA) of the United States. NAA is more sensitive than smear but less sensitive than culture, with results being positive at concentrations as low as 1–10 organisms/mL (Catanzaro, Perry et al. 2000, Lim, Mukhopadhyay et al. 2003, Cheng, Yew et al. 2005, Conaty, Claxton et al. 2005, Wiener, Della-Latta et al. 2005).

In the right clinical and epidemiological situations, a positive smear in conjunction with a positive nuclear antigen absorbance (NAA) is considered sufficient for the diagnosis of TB (Havlir and Barnes 1999, Catanzaro, Perry et al. 2000, Campos, Quartin et al. 2008). A lack of a positive result does not rule out the presence of active TB drug resistance. When choosing NAA test, it is important to consider the patient's clinical history (including any prior drug susceptibility testing and any prior treatment regimens for the patient and source case, if applicable), the patient's proximity to the laboratory performing the test, and the nature of the potential drug resistance. A false-positive result on NAA test may linger after curative treatment has been given since the test may identify nucleic acid from both living and nonliving species (John Bernardo Oct 26, 2022.). Because of this, NAA should not be used beyond the first diagnostic phase and instead only during the treatment phase. Not only will this help people avoid looking for people they already know or taking medicine they don't need, but it will (Campos, Quartin et al. 2008, Marks, Cronin et al. 2013).

Assays (WRDs); It is defined by the use of molecular diagnostic tests or biomarkers; It is the first line of defense at the WHO against misdiagnosis of tuberculosis. The World Health Organization recommends rapid molecular tests like Xpert MTB/RIF and Xpert Ultra as first diagnostic tests for diagnosing tuberculosis and rifampicin resistance in those exhibiting symptoms of the disease. Mutations in the 81-bp region (codons 426 to 452) of the rpoB gene, commonly known as the rifampicin resistance determining region, are detected by the Xpert

MTB/RIF assay, a genetic marker test for *Mycobacterium tuberculosis* (RRDR) (Boehme, Nabeta et al. 2010, Steingart, Schiller et al. 2014).

Rapid testing for rifampicin resistance, a marker for multidrug-resistant tuberculosis (MDR-TB), is now possible thanks to Xpert MTB/RIF. In locations with a high prevalence of HIV or multidrug-resistant TB, the test was originally advised to be used as a first-line diagnostic to replace microscopy and to identify a variety of extrapulmonary illnesses. While the testing method is sophisticated and expensive, it is surprisingly simple to utilize. Price reductions are available to government institutions in 145 poor and middle-income countries where tuberculosis is prevalent (Walzl, McNerney et al. 2018).

The signs and symptoms of pulmonary tuberculosis in adults justify a switch from smear microscopy , culture and phenotypic to DST Xpert MTB / RIF as the first line of diagnostic testing to determine TB and RIF resistance (Organization 2021).

There is no need to do microscopy or culture and phenotypic DST on sputum from children exhibiting symptoms of pulmonary tuberculosis in order to determine whether or not they are RIF resistant; instead, they should undergo an Xpert MTB / RIF test. Instead of smear microscopy or culture, Xpert MTB/RIF in cerebrospinal fluid (CSF) should be conducted initially in adults and children with symptoms of tuberculous meningitis. Compared to Xpert, Xpert Ultra seems to be more sensitive in detecting MTB in smear-negative culture-positive specimens, pediatric specimens, extrapulmonary specimens (particularly CSF fluid), and specimens from HIV-infected individuals (Organization 2021).

Resistance to rifampin and isoniazid may be detected using a molecular line-probe test called MTBDRplus (rpoB gene for rifampin resistance; katG and inhA genes for isoniazid resistance). U.S. Food and Drug Administration has not cleared this test for usage. MTBDR molecular line-probe test may be used to detect resistance to fluoroquinolones and injectable drugs (second-line antituberculosis agents); the gyrA gene is responsible for resistance to fluoroquinolones, while the

rrs gene is responsible for resistance to injectable treatments (Theron, Peter et al. 2014, Theron, Peter et al. 2016).

All national TB programs (NTPs) should develop SOPs, appropriate QA processes, adherence to biosafety requirements for all testing, and sufficient Human Resources to guarantee reliable and timely TB laboratory test findings. Guidelines for the diagnosis and treatment of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB); rapid tests. Additionally, there is a need to upgrade drug-resistant tuberculosis testing infrastructure. As part of MDR-TB treatment plans, only drugs with validated DST methods should be used, hence phenotypic DST is necessary (including, but not limited to, bedaquiline [BDQ], linezolid [LZD], clofazimine [CFZ], and delamanid [DLM]). Governments need better ways to monitor whether or not DR-TB patients are able to effectively convert their cultures while receiving therapy (Organization 2021).

Table 2.1. TB diagnostic tools (approved by WHO).

| Method | Intended use | Main strengths | Main weakness | Method |
|---|---|---|---|---|
| Sputum smear Microscopy for acid fast bacilli | Rapid, point ofcare test for TB case detection | Minimal infrastructure | Low sensitivity | Sputum smear Microscopy for acid fast bacilli |
| In vivo solid culture | TB case detection | Good sensitivity | Slow growth time | In vivo solid culture |
| Culture inliquid media | TB case detection and as a prerequisite for drug—susceptibilitytesting | High sensitivity | High contaminationrate | Culture inliquid media |
| Chest radiology | TB case detection (pulmonary TB) | Indicative ofTB | Low specificity, low sensitivity, trained interpreter needed | Chest radiology |
| Tuberculin skin test(Mantoux) | Detection of *M. tuberculosis* infection | Practical | Sensitivity decreases with immunocompromise, cross reaction with BCG vaccine | Tuberculin skin test(Mantoux) |
| Interferongama releaseassay | Detection of *M. tuberculosis* infection | Highly specific for*M. tuberculosis* | Requires moderate training andequipment | Interferongama releaseassay |
| Line probe assays | TB case Detection anddrug susceptibility testing | Short reporting time | Potential for cross contamination, requires extensivetraining | Line probe assays |

**2.10. Phenotypic and genotypic DST:**

Recently developed drugs and proposed treatment regimens for tuberculosis have led to revisions in the DR-TB criteria. MDR/RR-TB is tuberculosis caused by *M. tuberculosis* strains that are immune to all fluoroquinolones. Current criteria for the diagnosis of XDR-TB need the presence of at least one additional Group A drug, such as BDQ or LZD, and any FQ (Organization 2021).

Total drug-resistance tuberculosis (TDR-TB) is an isolate of Mycobacterium tuberculosis that is insensitive to every treatment option tried in the patient's immediate area It is now essential to test for resistance to BDQ and LZD, with particular emphasis on BDQ resistance, since the current definition of XDR-TB calls for DST findings for FQs, BDQ, and LZD (Velayati, Masjedi et al. 2009, Udwadia, Amale et al. 2013).

Phenotypic DST of BDQ and LZD in culture may be performed using Mycobacterial Growth Indicator Tubes (MGITs) or Middlebrook 7H11 media. The HIV Reagent Program of the United States National Institutes of Health (NIH) provides free pure drug material for BDQ phenotypic DST (Kibirige, Manak et al. 2022). Delamanid (delamanid: NR-51636) powder is available for purchase from the manufacturer, Otsuka. Orders may be initiated with the company through the ATCC website. Other pure medicinal substances for laboratory DST and associated catalogs are also available via the Global Drug Facility (Organization 2021).

Although phenotypic DST remains the gold standard, it is laborious to set up and requires trained professionals to interpret the data. Cutting-edge, next-generation environmental technology has the potential to minimize treatment durations and improve patient outcomes. The following recommended target goods have each been given a profile by the World Health Organization (Organization 2021). New direct methods for detecting DR-TB in sputum have been developed, using NGS-based targeted amplification. The Deeplex-MycTB test, has entered commercial distribution and provides estimates of resistance to 15 short-acting

anti-TB drugs with a response time of less than 48 hours (Kayomo, Mbula et al. 2020).

**2.11. Mycobacterial mechanisms of drug resistance:**

Tuberculosis antibiotics are so powerful that a single dosage may wipe out 99 percent of bacteria. Minimal metabolic activity in persisters renders antibiotic-targeted cellular proteins unneeded. This is in agreement with transcriptome studies demonstrating decreased production of the target protein for these medicines in Mtb strains that are unable to reproduce (Nguyen 2016).

Despite the fact that most strains of Mtb already have resistance to antibiotics, it is typical for Mtb to acquire drug resistance. Mutations, chromosomal alterations, cellular envelope permeability, drug efflux, drug degradation, and modifications to target replacement and target mimics are just some of the intrinsic resistance and combinations of acquired drug resistance mechanisms that make most Mtb cells resistant to the antibiotic class (Singh, Dwivedi et al. 2020).

Several mechanisms contribute to the development of drug resistance in Mtb. These include: compensatory evolution; epistasis; clonal interference; cellular membrane permeability; efflux pumps; drug degradation and modification; target mimicry; phenotypic drug tolerance (Zurita, Espinel et al. 2019, Singh, Dwivedi et al. 2020).

The inability to effectively treat TB is due in part to intrinsic (high levels of naturally existing antibiotic resistance) and extrinsic (newly acquired mutations) antibiotic resistance. To provide just one example, Mtb have a built-in resistance to the -lactam family of antibiotics because they are genetically designed to manufacture the -lactamase enzyme (Pinto and Menzies 2011, Chang, Yew et al. 2013, Klopper, Warren et al. 2013, Nguyen 2016).

Patient non-adherence during long-term TB treatment is largely responsible for the rapid development of multidrug- and extensively drug-resistant Mtb. The astonishing diversity of antibiotic resistance mechanisms in Mtb has been the

subject of much research in recent years, thanks to advancements in genetics and biology. However, further insight into the molecular basis of antibiotic resistance in this bacterial species is required in spite of significant progress.

The development of new anti-TB drugs and the therapeutic use of existing antibiotics are both impeded by these processes of resistance and tolerance. The best hope for the future effectiveness of TB therapy lies on a deeper understanding of the specific causes of antibiotic resistance in Mtb (Singh, Dwivedi et al. 2020).

Mtb's intricate molecular mechanisms are the product of evolutionary adaptations to the cytotoxicity of antibiotics and other toxic compounds, as well as the evolution of innate drug resistance (Nguyen 2016).

### 2.11.1. Impermeability of cell envelope:

The Mtb cell envelope is exceedingly thick, highly hydrophobic, and prevents diffusion of even hydrophobic compounds like antibiotics like rifamycins, macrolides, fluoroquinolones, and tetracyclines, leading to virulence and intrinsic drug resistance (Nasiruddin, Neyaz et al. 2017).

Scientists have determined the importance of cell envelope lipids in imparting intrinsic drug resistance in *Mycobacteria* by studying mutants deficient in cell envelope lipids, which exhibit increased susceptibility to antibiotics (Nguyen 2016).

Drug sensitivity in combination therapy is hypothesized to be enhanced by Mtb's viscosity, which is determined by the sequence and structure of mycolic acids as well as the presence of a functional group. Therefore, a Mtb cell may become susceptible to various drug types after being treated with ethambutol, which enhances permeability. Damage to the cell wall caused by missing or dysfunctional Mtb enzymes and proteins may increase its sensitivity to treatment (Nasiruddin, Neyaz et al. 2017).

Trehalose dimycolate (TDM) is generated in the Mtb cell as part of the antigen 85A (Ag85A) protein complex, which is also responsible for the synthesis of other proteins necessary for the integrity of the cell wall. When the Ag85 gene is disabled, *Mycobacterium* TB becomes more susceptible to first-line and other broad-spectrum antibiotics (Nasiri, Haeili et al. 2017).

**2.11.2. Drug efflux:**

Pharmaceuticals are expelled into bacteria through active transport caused by drug efflux pump proteins (Nasiruddin, Neyaz et al. 2017).

There are five superfamilies that may be used to categorize the numerous kinds of drug efflux pumps observed in *Mycobacteria*: First, there's the ATP-binding cassette (ABC) family, then there's the major facilitator family, the small multidrug resistance (SMR) family, and finally the resistance superfamily. Superfamily RND (responsible for nodulation and cell division) and Family MATE (multidrug and toxic compound extrusion) (multidrug and toxic compound extrusion) (Bloom, Graham et al. 2013).

Efflux pumps have an important role in isoniazid and RR even in the absence of mutations. mutations in 15 distinct gene regions have been related to resistance to isoniazid. FurA-katG, fabG1-inhA, and ahpC-oxyR are the most common double-stranded DNA combinations. Even mutations have been reported. Isoniazid resistance was shown in vitro, and it was linked to the putative RND transporter mmpL (Narang, Giri et al. 2017).

Most efflux pumps were upregulated in response to isoniazid stress in multidrug-resistant clinical isolates carrying wild-type katG, inhA, and oxyR-ahpC associated with isoniazid resistance. The discovery of new anti-tuberculosis drugs targeting drug efflux pumps is a promising avenue for the control and ultimate reversal of multidrug resistance (Meehan, Goig et al. 2019).

### 2.11.3. Degradation and alteration of drugs

*M. tuberculosis* has evolved several enzymes that modify or breakdown various classes of antibiotics, including aminoglycosides, B -lactams, and macrolides. The effectiveness of B-lactam medications against Mtb is minimal, at best. The low affinity of PBPs for -lactams and the impenetrability of cell walls are further factors in their ineffectiveness. Antibiotic resistance may be avoided by chemical modifications that make antibiotics useless, such as acetylation and methylation, which prevent the antibiotic from inhibiting the target protein's ability to bind to it (Nguyen 2016).

### 2.11.4. Changing and mimicking targets

The capacity to alter, and hence avoid binding to, the target antibiotic drug target, contributes to Mtb's innate drug resistance to important antibiotics. Antibiotics including lincosamides, macrolides, and streptomycin have a reduced effect on Mtb due to target changes (Nguyen 2016).

Inhibiting mycobacterial growth, these drugs target the bacterial 50S ribosomal RNA subunit by binding reversibly to a specific region within the peptidyl-tRNA. It has been discovered that Mtb have a natural resistance to the antimicrobial drugs lincosamides and macrolides. The erm gene in *M. tuberculosis* codes for an enzyme called erythromycin resistance methylase (erm). To prevent macrolides from binding, this enzyme methylates a specific area of 23S ribosomal RNA. Resistance to capreomycin, neomycin, and vancomycin is encoded by the tly gene, which also encodes rRNA methyltransferase and methyltransferase loss activities (streptomycin resistance is caused by the methylation of 16S rRNA, another gene) (Nguyen 2016).

A protein called RNA polymerase binding protein (RBP) inhibits rifampicin from binding to RNA polymerase. Mtb adopts the unique approach of molecular mimicry of drug targets to negate the efficiency of fluoroquinolones. *Mycobacterium* fluoroquinolone resistance protein A (mfpA) binds to DNA gyrase

and blocks the binding of fluoroquinolones to DNA gyrase by imitating the shape, size, and surface of the target site in the form of B DNA. Therefore, Mtb, which are resistant to several drugs, adapt by mimicking DNA gyrase, an antibacterial target of fluoroquinolones (Zhao, Wang et al. 2013, Nguyen 2016).

### 2.11.5. Phenotypic drug tolerance

Long-term, rigorous treatment for tuberculosis is necessary. Due to the fact that certain *Mycobacterium tuberculosis* cells are immune to medicines and may continue to grow even while on therapy, TB medication must be administered for a longer period of time. Epigenetic alterations in drug-tolerant cells, often known as "persisters," which are metabolically or physiologically inert. It is believed that the capacity to expand in the face of adversity, such as the presence of antibiotics, is crucial for the survival of cells at the expense of subpopulations, and that this ability is an intrinsic property of bacterial populations. The results of several investigations suggest that (Nguyen 2016).

Adapts to life in the presence of antibiotics by adopting a dormant, or non-proliferative, condition in which metabolic activity is very low or nonexistent and survival is ensured. Dormancy is characterized by a reversible slowing of metabolism, a suspension of physiological processes, a slow growth rate, difficulties in growing on solid media, a lack of multiplication, a loss of acid fastness, resistance to antibiotics, and triglyceride accumulation in intracellular lipid bodies (Lipworth, Hammond et al. 2016).

In addition to Mtb's inherent and acquired drug resistance, the transition from the metabolically active, proliferating form to the dormant, non-replicating form causes phenotypic drug tolerance and operates as an intractable component in TB therapy (Singh, Dwivedi et al. 2020).

Due to host defenses and pharmaceutical treatment, latent TB is notoriously hard to eradicate. Phenotypic tolerance of Mtb cells consists only of lower metabolic or physiological activity, in contrast to direct genetic or

chromosomal harboring innate and acquired drug resistance mutations (Nguyen 2016).

### 2.12. New Regimens and Emerging Resistance

As a result of their intricate physiology, morphology, and virulence mechanisms, mycobacterial pathogens provide a formidable obstacle to the development of effective antimycobacterial therapies (Velayati and Farnia 2012). The new, all-oral regimens for rifampin-resistant TB that have been approved by the World Health Organization revolve on bedaquiline (Organization 2019).

Multidrug-resistant TB is diagnosed when a patient does not respond to at least two of the most common drugs used to treat the illness. Extended drug-resistance tuberculosis is diagnosed when MDR TB additionally develops resistance to any fluoroquinolone and any of the three second-line injectables (amikacin, capreomycin, or kanamycin) (XDR TB) (Mbuagbaw, Guglielmetti et al. 2019).

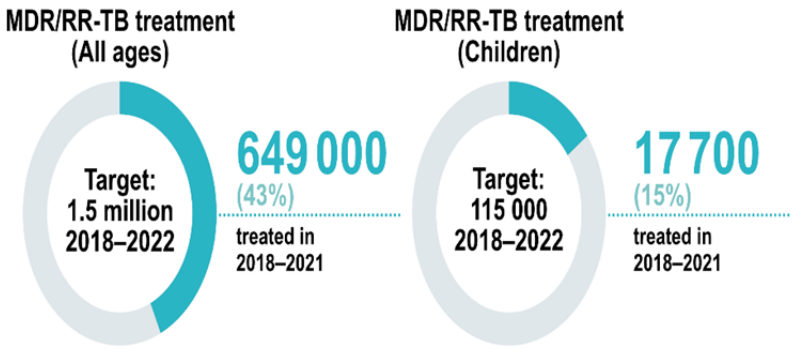


Figure 2.5. Global progress in the number of people treated for MDR/RR-TB between 2018 and 2021, compared with cumulative targets set for 2018–2022 at the UN high-level meeting on TB.

Treatment-resistant tuberculosis (MDR-TB) has proven challenging because of its chronic nature, lack of effective therapies, and poor patient tolerance, as a result, more chemotherapeutic interventions are required. Multidrug-resistant tuberculosis (MDR-TB) dramatically reduces the success rate of tuberculosis therapy because of late drug-resistance diagnosis, lower efficacy and increased toxicity of second-line TB drugs, and longer treatment regimens (World Health Organization, 2019b; Pontali et al., 2019).

Treatment for multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis requires drugs that are less effective, more toxic, and more costly, and which must be administered for a longer duration of time. After a 40-year hiatus, the development of novel therapies is once again of interest due to the emergence of multidrug-resistant and extensively drug-resistant TB. Treatment results might be vastly improved with the availability of drugs that attack both drug-sensitive and drug-resistant strains (Goel 2014).

Pharmaceutical companies have shown little motivation for creating new anti-tuberculous drugs, and the research process itself is time-consuming and expensive. To add insult to injury, TB is more prevalent in countries with weak economies , in addition research in this area has also accelerated in the last decade, after the establishment of the global plan to eradicate tuberculosis (Organization 2013).

Problems in curing multidrug-resistant and extensively drug-resistant tuberculosis (TB) with second-line medications and a high risk of recurrence are among the difficulties in treating this form of the illness. This is especially true in developing countries like India, where the rate of multidrug-resistant tuberculosis (MDR-TB) cases has been increasing. India is the third country to report zero response to the spread of drug-resistant TB (Rowland 2012).

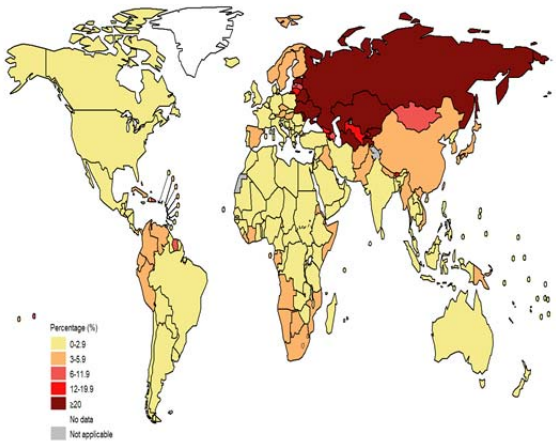


Figure 2.6. Percentage of new TB cases with MDR/RR-TB, 2021.

Newer antimicrobial drugs are more effective against multidrug-resistant tuberculosis notwithstanding the presence of cross resistance between older and newer medications. A key issue that might compromise its usefulness is, however, cross resistance to CFZ and BDQ. Combating TB with a BDQ (Hartkoorn et al., 2014; Ismail et al.,2018).

There must be conclusive evidence linking specific mutations to the development of MDR-TB or to the intricate interplay between drug resistance and fitness. That way, drug-resistant TB might be assessed more accurately, and its future course could be anticipated with more certainty. Such data is urgently needed to help in the creation of new diagnostic tools and therapies in light of the increasing prevalence of drug-resistant variants throughout the globe (Njire, Tan et al. 2016).

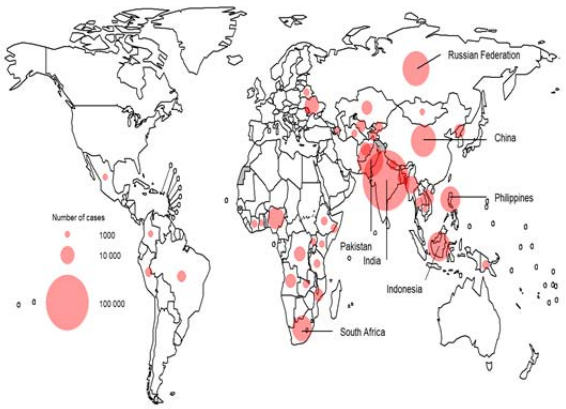


Figure 2.7. The proportion of TB cases with MDR/RR-TB varies considerably among regions and countries.

**2.13. Bedaquiline:**

TMC207, R207910, compound were earlier names for bedaquiline (BDQ), a revolutionary last-ditch anti-tuberculosis medication. It belongs to the family of drugs known as diarylquinolines (Lohrasbi, Talebi et al. 2018).

first recognized it in 2005. Using a whole-cell assay, they investigated a panel of compounds in an effort to discover prototypes, and then examined their capacity to inhibit cell divisions in *Mycobacterium smegmatis* (Andries, Villellas et al. 2014, Diacon, Pym et al. 2014).

No new medicine class for treating MDR-TB or extensively drug-resistant TB has been approved in the United States since rifampicin in 1971 (XDR-TB) (Nguyen, Cao et al. 2016).

Janssen Pharmaceuticals developed bedaquiline (BDQ; previously known as TMC207 or R207910) in 2005. In 2013, the World Health Organization (WHO) approved BDQ, a diarylquinoline, for the treatment of multidrug-resistant tuberculosis (Zumla, Gillespie et al. 2014).

BDQ, sold under the brand Sirturo1, is the first innovative anti-TB therapy approved for use in Europe and the United States since treatment for multidrug-

resistant tuberculosis (MDR-TB) was originally authorized by the FDA in both countries (Organization 2013, Villellas, Coeck et al. 2017).

New antitubercular drug bedaquiline was approved for usage in 2012. There are now a lot of anti-tuberculosis medications in phase II and III clinical trials. Bedaquiline is the first drug of its type, and it shows promise in helping to treat TB more effectively (Goel 2014).

In 2018, the WHO revised its guidelines for treating drug-resistant tuberculosis (TB) in light of an evidence-based policy. The new guidelines advocate include bedaquiline into the conventional treatment regimen for rifampin-resistant TB (Organization 2019).

In 2018, the World Health Organization reclassified bedaquiline as a member of category A medication that, according to the results of a meta-analysis of patient data, should be given to all patients with multidrug-resistant or rifampicin-resistant tuberculosis in order to enhance treatment success and minimize mortality (Ndjeka, Schnippel et al. 2018).

BDQ may have an influence on the efficacy of ongoing treatment for LTBI. One of the greatest ways to prevent contracting drug-resistant tuberculosis is via long-term, intensive interaction with a patient who already has it. There is currently no accepted treatment protocol for LTBI, making it especially challenging to care for patients with multidrug-resistant tuberculosis (MDR/XDR-TB). Evidence from a mouse model shows that BDQ has potent sterilizing effect against latent (non-replicating) tubercle bacilli, indicating that it might be utilized to treat DRLTBI in as little as three to four months (Zhang, Li et al. 2011, Mirsaeidi 2013).

The diarylquinoline bedaquiline has alcohol and amine groups on its side chains. The anti-mycobacterial effects of the molecule are due to the presence of these two side chains. It has a quinolinic core heterocyclic nucleus (Matteelli, Carvalho et al. 2010).

Both a hydrophobic region containing N(CH3)2 (a critical role in interacting to the ATP synthase) and an H2-bonding acceptor/donor (contributing to stability) that facilitates hydrogen bonding. The anti-tuberculosis activity of BDQ may be attributed to its diarylquinoline ring, N, N-dimethyl amino-terminated side chain, hydroxyl group, and naphthalene moiety (Palomino and Martin 2013, Chahine, Karaoui et al. 2014, He, Preiss et al. 2017).

Bedaquiline fumarate is created by combining bedaquiline and fumaric acid in a 1:1 ratio (Gaurrand, Desjardins et al. 2006). There are a number of documented inactive ingredients in BDQ including croscarmellose sodium, lactose monohydrate, polysorbate 20, microcrystalline cellulose, hypromellose 2910, colloidal silicon dioxide, maize starch, magnesium stearate, and purified water (Kunin and Ellis 2000, Savini, Chiasserini et al. 2002, Lohrasbi, Talebi et al. 2018).

The "multicentric trial of TMC 207 in adult patients with newly confirmed smear-positive lung TB caused by MDRTB strains" evaluated its stage 2 efficacy. 47 people participated in the first 8-week phase. Each patient was given either a placebo and a background regimen (BR) of second-line drugs, or bedaquiline at a dose of 400 milligrams (mg) once daily for two weeks, followed by 200 milligrams (mg) three times weekly for six weeks. Sputum conversion occurred more quickly in the bedaquiline group than in the placebo group, as measured by the Cox regression analysis (hazard ratio -11.8, 95% CI: 23-61.3, P = 0.003). In contrast, just 9% of patients in the placebo group had their sputum converted whereas 48% of patients in the bedaquiline group did (Diacon, Pym et al. 2009). After 8 weeks of treatment with just BR, patients were re-examined at 24 weeks; by then, the bedaquiline-treated group had a substantially greater incidence of sputum conversion (hazard ratio -2.253:95% CI -1. CI 1.084.71; P= 0.031). There were 161 people who made it to Phase 2, with 79 receiving bedaquiline (400 mg daily for 2 weeks, followed by 200 mg three times weekly) and BR and 81 receiving placebo and BR. At the conclusion of 24 weeks, 81% of individuals who took bedaquiline and 58% of those who received a placebo had changed sputum (P = 0.008) (Diacon, Donald et al. 2012).

**2.13.1. Mechanism of action:**

Although BDQ has a structural similarity with the fluoroquinolones, it acts differently and does not block DNA gyrase. ATP is the target of BDQ, the only anti-TB medicine licensed by the FDA that works by blocking the proton pumping mechanism (Hards, Robson et al. 2015).

Bedaquiline has been shown to have potent and targeted antimycobacterial action. This compound blocks the proton pump of mycobacterial adenosine triphosphate (ATP) synthase. The production of ATP is slowed by bedaquiline. It interacts with the oligomeric and proteolipid component c of mycobacterial ATP synthase. Therefore, it results in the death of bacteria (Matteelli, Carvalho et al. 2010, Lakshmanan and Xavier 2013).

It has been shown that bedaquiline binds to mycobacterial ATP synthase with an affinity that is over 20,000 times higher than its binding affinity to human mitochondrial ATP synthase. Because of this, *Mycobacterium* are targeted specifically, whereas host cells are only little affected (Haagsma, Abdillahi-Ibrahim et al. 2009).

Both the hydrophilic F1 complex and the membrane-bound F0 complex are required for ATP synthase enzyme function. Bedaquiline inhibits ATP generation by binding to the hydrophobic C subunit of the F0 complex and preventing the complex from rotating. Since ATP is essential for the survival of all living creatures, it is effective against both those that are actively reproducing and those that are not (Koul, Vranckx et al. 2008).

**2.13.2. Drug resistance:**

Approximately one in every $10^8$organisms have a mutation that confers resistance to bedaquiline. There are two potential mutational routes for bedaquiline. The expression of the efflux pump and mutation of the atpE gene are two of these methods. Mutations in the atpE gene, namely at locations 63 and 66, modify bedaquiline's affinity for the c subunits of the ATP synthase enzyme (Petrella,

Cambau et al. 2006, Matteelli, Carvalho et al. 2010).

*Mycobacterium.tuberculosis* also acquires bedaquiline resistance because to a mutation in the gene Rv0678. The term "heteroresistance" is used to describe situations whereby an isolate contains both antibiotic-resistant and antibiotic-susceptible bacterial populations (Chesov, Chesov et al. 2022). Bedaquiline resistance occurs because of an upregulation of an efflux pump that prevents the uptake of the antimalarial medication. Rv0678 mutations activate efflux pump expression (Andries, Villellas et al. 2014).

**2.13.3. Absorption:**

Bedaquiline is well absorbed in humans after both single and repeated oral dosing. It has a linear pharmacokinetic profile, which means that its peak plasma concentration is attained 4–6 hours after injection. Bedaquiline's bactericidal properties heighten as its concentration rises (van Heeswijk, Dannemann et al. 2014).

To treat pulmonary tuberculosis caused by MDR *M. tuberculosis* in adults ( 18 years) as part of combination therapy is the sole approved use (Field 2015). Bedaquiline has been linked to a number of gastrointestinal, musculoskeletal, and central nervous system adverse medication events, including nausea, vomiting, diarrhea, abdominal pain, limb pain, arthralgia, back pain, headache, and dizziness. In addition to these, bedaquiline has been associated with a host of additional undesirable side effects, including rash, pruritus, acne, hemoptysis, pleuritic discomfort, pharyngolaryngeal pain, deafness, hyperuricemia, QT interval prolongation, and increased transaminases (Diacon, Pym et al. 2009, Matteelli, Carvalho et al. 2010, Chahine, Karaoui et al. 2014). QTc prolongation caused by delamanid and bedaquiline was not synergistic. In addition, no patients had a QTc event of grade 3 or 4, and the maximal QTc rise observed was comparable between bedaquiline and delamanid and bedaquiline alone. These findings encourage physicians about the safety of using bedaquiline and delamanid together to treat

patients with MDR-TB or rifampicin-resistant TB (Dooley, Rosenkranz et al. 2021).

### 2.13.4. Cross-reactivity between medications:

#### 2.13.4.1. Antitubercular Drugs:

As the ratio of bedaquiline to rifampicin drops from 1:1 to 0.5:1, the bedaquiline is rendered completely ineffective. Therefore, bedaquiline is not recommended for persons using a strong CYP3A4 inducer. Ethambutol, kanamycin, isoniazid, pyrazinamide, ofloxacin, and cycloserine are all viable alternatives for treating TB, and they all have little potential for drug interactions (Field 2015).

In combination therapy when bedaquiline and ketoconazole are used. When used with additional drugs such ketoconazole, fluoroquinolones, macrolide, and clofazimine, bedaquiline's QT prolongation was either additive or synergistic (Organization 2017).

African programmatic data, assessed by the World Health Organization, indicated that shorter regimens incorporating bedaquiline result in a 13% greater success rate compared to shorter regimens including injectables (Vatsyayan, Pattery et al. 2022).

Treatment regimens using the medications bedaquiline, pretomanide, and linezolid have been suggested by the World Health Organization (WHO) (BPaL). MDR-TB patients who also exhibit resistance to fluoroquinolones are a good candidate for this treatment, according to the NTEP. If the patient's culture is still positive after 16 weeks of therapy, the total length of time in treatment might be increased from 26 to 39 weeks. Nearly 90% of patients with XDR (89%), MDR with FQ resistance (92%), and treatment intolerance/non-responders (92%), all had positive outcomes after receiving BPaL (Vatsyayan, Pattery et al. 2022).

Both bedaquiline and a placebo were added to the standard treatment plan in a randomized controlled trial including 160 participants. Cox regression analysis showed a significant reduction in the median culture conversion time from 125 days to 83 days in the medication group (95% confidence interval, p0.001) (Diacon, Pym et al. 2014).

The efficacy of the bedaquiline-containing regimen in treating multidrug-resistant TB was the subject of a meta-analysis that analyzed data from 25 observational studies and 4 experimental trials in 2022. The success rate of treating patients with bedaquiline was reported to be 74.4% in observational studies and 86.1% in experimental trials (Hatami, Sotgiu et al. 2022).

According to a cross-sectional cohort study, a high cure rate and avoiding development of bedaquiline resistance can only be achieved with the use of a practicable background regimen in conjunction with a bedaquiline-containing regimen for DRTB. This study also confirmed the need of regular and accurate drug-resistance monitoring, particularly for novel bedaquiline regimens (Gao, Gao et al. 2021).

A cross-sectional analysis of 2023 people and a longitudinal study of 695 patients both found that bedaquiline resistance was associated with worse treatment outcomes. The importance of quick bedaquiline resistance testing cannot be overstated (Ismail, Omar et al. 2022). For patients with a history of bedaquiline exposure or those who continue to show culture positivity after two months of treatment. Research conducted on 177 Chinese patients with MDR-TB or XDR-TB found that adding bedaquiline to the standard treatment regimen was associated with a significant increase in the rate of culture conversion. Culture conversion rates for MDR-TB and XDR-TB were 84.6% and 86.6%, respectively, with a 95% confidence range (Gao, Gao et al. 2021).

In 2014, the first case of MDR-TB using a BDQ-resistant isolate was reported. After 24 months of treatment and recurrence, a Tibetan patient was found to have isolates resistant to both BDQ and CFZ (Rv0678 mutation) (Hoffmann,

Kohl et al. 2016). Therefore, it is recommended that patients who have previously received an anti-TB regimen comprising CFZ have their susceptibility to BDQ validated prior to commencing a therapy with BDQ (Riccardi, Giacomelli et al. 2020).

In 2019, it was reported that a 50-year-old XDR-TB patient has acquired a mild form of BDQ resistance. MICs were up to 8-fold higher than in the baseline sample, as measured by the colorimetric resazurin microtitre assay (REMA). Using Mycobacterium Growth Indicator Tube (MGIT) assay, it has seen that the original isolate responded well to BDQ (Polsfuss, Hofmann-Thiel et al. 2019).

**2.13.4.2. Synergism of bedaquiline with pyrazinamide:**

As a powerful antibiotic, pyrazinamide (PZA) blocks the bacterial proton motive force and ribosomal translation. Because BDQ is an ATP synthase inhibitor and both drugs have the potential to slow down bioenergetic processes, they may operate together to deplete cellular energy supplies (Lu, Lill et al. 2015). PZA has a synergistic function with BDQ in the indirect inhibition of ATP synthase (Ibrahim, Andries et al. 2007), which may be due to its interference with the proton motive force and membrane integrity. Therefore, combining BDQ and PZA has the potential to hasten the removal of germs and shorten the length of therapy for patients (Nguyen, Cao et al. 2016).

**2.13.5. Mechanisms of resistance to bedaquiline:**

**2.13.5.1. Mutations Associated with BDQ Susceptibility:**

One of the recognized targets of BDQ is the transmembrane component of the ATP synthase complex that is encoded by the Mtb gene atpE. Based on mutational and computational molecular modeling results, AtpE was shown to be a BDQ target, and the amino acid Glu-61 in its native state was found to be the BDQ binding site (Segala, Sougakoff et al. 2012).

Bacteria resistant to BDQ were found to have mutations in seven different genes. In addition to the therapeutic target atpE, the off-target gene Rv0678 also displayed a significant incidence of mutations. In the atpE gene, four non-synonymous SNPs were detected often, whereas five were discovered in the Rv0678 gene. Mtb clinical strains with a high MIC to BDQ have been shown to have other mutations at this location (Asp28Asn and Asp28Pro) by PCR-Sanger sequencing (Huitric, Verhasselt et al. 2010, Zimenkov, Nosova et al. 2017).

Three single-nucleotide variations (SNVs) were found in atpE at amino acid position. This latter observation bolsters the potential diagnostic use of atpE mutations impacting residue 28 of the enzyme in the context of BDQ resistance. Other mutations in pepQ were associated with varying degrees of BDQ susceptibility (MICs of 0.12 mg/L, using APM) (Almeida, Ioerger et al. 2016). Andries et al. previously revealed one pathway, which is linked to mutations in the atpE gene, which produces a transmembrane protein of F1/F0-ATP synthase (Andries, Villellas et al. 2014).

Sequencing the atpE gene in BDQ-exposed *M. tuberculosis* isolates revealed six distinct amino acid substitutions and mutations in the subunit c forming the C ring in the ATP synthase. These changes may cause a 10-133-fold rise in BDQ's MIC (Andries, Villellas et al. 2014).

Secondly, Rv0678 mutations may alter the expression of the MmpS5-MmpL5 efflux pump. Mutations leading to a significant 2- to 8- fold rise in the BDQ MIC and a 2- to 4-fold increase in the CFZ MIC have been found in isolates following in vitro exposure to BDQ or clofazimine (CFZ) and in some of the isolates of patients treated with the BDQ (Nguyen, Cao et al. 2016).

Some researchers believe that Rv0678, also known as mmpR, inhibits the Mmps2-MmpL2, MmpS4-MmpL4, and MmpS5-MmpL5 transporter/transport systems (Radhakrishnan, Kumar et al. 2014).

MmpS-MmpL proteins transfer lipids, virulence factors, and certain drugs across the membrane of *Mycobacterium tuberculosis* cells. In particular, MmpS5-

MmpL5 contributes to the export of BDQ, clofazimine, and azoles like econazole (Milano, Pasca et al. 2009).

Increased BDQ and clofazimine efflux is caused by Rv0678 mutations because these mutations inactivate the MmpS5-MmpL5 repressor. Resistance-associated variants (RAVs) in clinical isolates identified to date are almost exclusively caused by Rv0678 mutations which can raise *Mycobacterium tuberculosis* minimum inhibitory concentrations (MICs) for bedaquiline and clofazimine (Hartkoorn, Uplekar et al. 2014).

The clinical significance of key Rv0678 variants is uncertain. Occurrence of Rv0678 mutations documented during treatment, but the incidence is unknown (Hartkoorn, Uplekar et al. 2014).

Bedaquiline exhibits little cross-resistance with first- and second-line anti-TB medicines currently in use (Andries, Verhasselt et al. 2005, Fox and Menzies 2013).

It is hypothesized that the low rate of primary resistance in clinical observations is due to CFZ's ability to target many locations in tubercle bacilli (Mirnejad, Asadi et al. 2018).

As an alternative, people who have mutations in the Rv0678 gene, which codes for a transcriptional regulator that inhibits the production of the efflux pump mmpS5-mmpL5, have a lower risk of developing CFZ (Lange, Chesov et al. 2019).

Researchers found that Rv0678 mutations were present in 97% of CFZ-resistant mutants created in the lab (Zhang et al., 2015).

Interesting results were observed by Villellas et al., who discovered that a sizeable percentage of MDR-TB isolates (23/347; 6.6%) contained Rv0678 resistance-associated mutations (RAVs). Only seven patients (a rather low number) had previous exposure to BDQ or CFZ. The prevalence of Rv0678 RAVs in MDR-TB strains was 9-fold higher than in non-MDR-TB isolates (0.7%) (Villellas, Coeck et al. 2017).

Andries et al. also reported a high incidence of mutations (6.6%) and discovered a major role for Rv0678 in increasing MICs of BDQ (Andries, Villellas et al. 2014) , The Rv0678 mutation was found in five MDR-TB isolates, and it remained even after the drug's use was drastically reduced for more than a year (Bloemberg, Keller et al. 2015).

New evidence from a case study of an MDR-TB patient treated with BDQ for 6 months suggests that a mutation in position 2 (GTG GCG) in Rv0678 is responsible for the patient's high degree of resistance (Bloemberg, Keller et al. 2015). BDQ or CFZ resistance in *M. tuberculosis* isolates has been linked to mutations in the protein synthesis gene pepQ. Cross-resistance between CFZ and BDQ has been related to mutations in pepQ.

PepQ mutations caused a moderate 4-fold increase in BDQ and CFZ MICs. Because of these adjustments, the antibiotic is not as effective in vivo, yet resistance is not completely achieved (Almeida, Ioerger et al. 2016).

Bedaquiline (BDQ) is a promising drug, although it may have limited efficacy in patients who have already been exposed to is that (CFZ) due to mutations in the gene Rv0678 (Hartkoorn et al., 2014; Nguyen et al., 2018).

Although atpE mutations are uncommon in the clinic, some authors have hypothesized that this might be due to the lower fitness cost associated with some atpE mutations (Zimenkov, Nosova et al. 2017).

Additionally, certain mmpL5 Mtb mutants and the pepQ mutant have shown impaired fitness (Lamichhane, Tyagi et al. 2005, Almeida, Ioerger et al. 2016). Thousands upon thousands of patients throughout the globe have gotten BDQ since it was first made available as a treatment for MDR-TB very recently. Resistance to BDQ arose rapidly, raising concerns about the drug's potential involvement in future anti-TB regimens. Mutations in the intergenic region between the efflux pump genes Rv0678 (MmpL5) and Rv0677c (efflux pump MmpS5) and the atpE (BDQ target) gene confer resistance to BDQ in *M. tuberculosis* isolates (Nguyen, Anthony et al. 2018, Ghajavand, Kargarpour Kamakoli et al. 2019).

**2.14. Delmanid:**

Delamanid was authorized by the European Medicines Agency (EMA) for use in treating tuberculosis in April 2014, making it just the second medicine from a novel drug class to achieve such approval in over forty years (after bedaquiline). Similarly, the Japanese regulatory agency green-lighted Delamanid in July 2014. For those with multidrug-resistant tuberculosis (MDR-TB) who have few options for therapy that are both effective and well tolerated, delamanid (marketed as Deltyba) is a promising new alternative (Organization 2014).

Delamanid (DLM) is a dihydro-nitroimidazooxazole derivative (formerly known as OPC-67683). It was created by Otsuka Pharmaceutical in 2003. DLM suppresses production of mycolic acids in cell walls (Gler, Skripconoka et al. 2012). In 2014, the World Health Organization (WHO) approved DLM for the treatment of people with MDR-TB due to its minimal interaction with antiretroviral therapy (Organization 2014).

Delamanid has been shown to be safe, effective, and rapidly increase culture conversion, treatment result, and dramatically decrease mortality in patients with multidrug-resistant tuberculosis. Resistance to at least isoniazid and rifampin allows for the diagnosis of Multidrug-Resistant Tuberculosis (MDR-TB), which may be treated with delamanid (D'Ambrosio, Centis et al. 2012, Blair and Scott 2015, Wells, Gupta et al. 2015).

The nitroimidazole substances of delamanid seem to be lethal to TB bacteria because they prevent the production of mycolic acids, a crucial component of the bacterium's cell walls (Masjedi, Tabarsi et al. 2010).

There is evidence that the nitric oxide produced during metabolism of nitroimidazoles is toxic to tuberculosis bacteria and contributes to the medications' lethal effects (Haver, Chua et al. 2015).

Delamanid (formerly known as OPC-67683) is a nitroimidazole that is a nitro-dihydro-imidazooxazole derivative. in April 2014 received the light from EMA (European Medicines Agency). Otsuka Pharmaceutical Development and

Commercialization (Osaka, Tokyo, Japan) created and manufactured it (Sotgiu, Pontali et al. 2015).

The World Health Organization (WHO) endorses its use in the treatment of multidrug-resistant tuberculosis. After being reviewed in 2016, the Dlm interim policy now covers youth between the ages of 6 and 17.

The World Health Organization now recognizes delamanid as an essential medicine. It was released in June of 2017 for kids and December of 2015 for adults (Organization 2017).

World Health Organization, a hastened external Expert evaluation of Trial 213 data in early December 2017 to assess the findings' implications for 2014 and 2016 interim policy recommendations (Moja and Organization 2018). Trial 213's findings were limited, but they suggest that it may have a protective function in avoiding Dlm and subsequent drug resistance. Consequently, the terms under which Dlm is administered to certain individuals remain unchanged. Guidelines, necessary medicine lists, and access to purchase Dlm vary by country (Moja and Organization 2018).

The MICs for DEL have been reported in the literature to be between 0.006 to 0.024 mg/L, making it an effective inhibitor of both drug-sensitive and -resistant TB, Low doses of the chemical are shown to have significant therapeutic efficacy in vivo (Matsumoto, Hashizume et al. 2006).

Delamanid and placebo participants had similar rates of significant adverse effects throughout the course of the two-month experiment, with the exception of QT prolongation. Compared to twice-daily dosing of 200 milligrams, the 100 milligram dose of delamanid seems to have less adverse effects (Gler, Skripconoka et al. 2012). QT prolongation, the most concerning adverse effect of delamanid, is also produced by other MDR-TB medications.

Delamanid is a pro-drug that is metabolically activated by the enzyme Rv3547, which is an F420-dependent mycobacterial nitroreductase (ddn). The enzymes fgd1, fbiA, fbiB, and fbiC are responsible for the synthesis and

reactivation of co-factor F420. In vitro delamanid resistance has been linked to polymorphisms in any of these five genes (EMA 2013).

Delamanid drug susceptibility testing (DST) capabilities need to be improved since the rate of spontaneous mutations imparting delamanid resistance is predicted to be between $6.4\times10^{26}$ and $4.2\times10^{25}$.

Several lines of evidence indicate that F420 is crucial to *M. tuberculosis* survival that F420 is required by at least 28 different enzymes (Greening, Ahmed et al. 2016), F420 plays important roles in hypoxic survival, protection against oxidative and nitrosative damage, and evasion of the host immune system (Haagsma, Abdillahi-Ibrahim et al. 2009).

Ddn's remarkable degree of conservation across almost all Mycobacterial species (with the exception of *Mycobacterium leprae*) indicates that its physiological function is subject to intense evolutionary selection, ddn has been proposed as a menaquinone reductase (Haslbeck and Vierling 2015).

Compared to pretomanid, delamanid is more effective against MDR- and XDR-TB strains. In a follow-up study, Upton and co-workers found similar results, demonstrating that delamanid is at least eight times more effective against MTB than pretomanid. Although delamanid is more effective, evidence shows that it has the same frequency of resistance as pretomanid and a similar plasma concentration (Upton, Cho et al. 2015).

Gain-of-function mutations in the F420 biosynthesis pathway or the F420-dependent glucose 6-phosphate dehydrogenase (FGD) that catalyzes F420 reduction to F420H2, may lead to acquired resistance to pretomanid and delamanid. Resistance may also be conferred by genetic alterations that eliminate ddn activity, according to in vitro research While these modifications may render *M. tuberculosis* harder to cure in those who are already infected, there is no evidence that they may be passed on to healthy hosts (Manjunatha, Boshoff et al. 2006, Haver, Chua et al. 2015, Schena, Nedialkova et al. 2016).

A Potential anonymous substitution in fbiC providing DMD resistance in *M. tuberculosis* isolates adds to the body of data on mutations in the ddn and fgd1 genes (Feuerriegel, Köser et al. 2011).

Coenzyme FbiC is a component of the biosynthetic route leading to F420. The hydroxybenzyl group of 4-hydroxyphenylpyruvate may be transferred to pyrimidinedione with its help. According to recent research by Haver et al., fbiC may have a role in nitroimidazole resistance since it is responsible for the greatest amount of SNP diversity among self-generated fbiC. Mutant strains resistant to PA-824 (Haver, Chua et al. 2015).

DEL has no cross-resistance with other first-line TB drugs. This further indicates that resistance to DEL monotherapy would emerge quickly, calling for in vitro combination testing (Chandramohan, Padmanaban et al. 2019).

### 2.14.1. Mutations Associated with DLM Susceptibility:

The majority of DLM-resistant genes have roles in transforming the prodrug into the active drug. In particular, most of them have been discovered in the deazaflavin (F420)- dependent mycobacterial nitroreductase ddn (Rv3547) (Yu, Gao et al. 2019).

*Mycobacterium leprae*, like many other mycobacterial species, is resistant to DLM and pretomanid because it lacks the ddn gene (Manjunatha, Lahiri et al. 2006). Similarly, fgd1 (Rv0407) and the glucose-6-phosphate dehydrogenase it encodes, fbiA (Rv3261), fbiB (Rv3262), and fbiC (Rv1173), are required for the full conversion of DLM to its active form (Matsumoto, Hashizume et al. 2006).

Considering that F420 is crucial to Mtb physiology and in vivo growth, it is not surprising that mutations in this gene result in a fitness loss for ddn mutants. During hypoxia development and in defense against the host immune system's redox reactions, F420 has been shown to play a crucial role. Loss of native enzyme function might reduce the fitness of the bacterium with the ddn mutation. Furthermore, particular ddn mutants did not exhibit reduced development relative to wild type strains in a mouse model study (Lee, Harold et al. 2020).

**2.15. Delamanid and bedaquiline:**

Many compounds in the TB therapeutic research pipeline are now being tested in humans (Hartkoorn, Uplekar et al. 2014).

The mission of the Global Alliance for the Development of Anti-Tuberculosis Drugs (TB Alliance) is to find effective treatments for tuberculosis, using of strong molecular combinations to delay the emergence of TB strains resistant to standard treatment (Ginsberg 22 Aug 2011).

The novel mechanisms of action of already approved medications have shown encouraging outcomes in clinical studies. Proven efficacious against both drug-sensitive and drug-resistant TB line (BDQ) [FDA-approved]. European Medicines Agency (EMA)-approved delamanid (DEL). Two promising new drugs, bedaquiline (BDQ) and delamanid (DMD), have emerged in the fight against drug-resistant tuberculosis.

The FDA and EMA have given conditional approval to two medications for the treatment of MDR-TB: bedaquiline (BDQ) and delamanid (DLM). These medications have been classified as WHO Group 5 due to a lack of information on their long-term effectiveness and safety (Bonnet, Bastard et al. 2016).

The combination of BDQ with delamanid (DLM) has been viewed with concern due to the possibility of additive cardiac damage (Pontali, Sotgiu et al. 2018). Recent studies have shown that these medications are effective for MDR/XDR-TB patients, especially when a regimen including four active antibiotics is not possible (Pontali, Sotgiu et al. 2018).

After reviewing the available PK (pharmacokinetic) data, the WHO has only recently authorized delamanid for use in children older than 6 years. Those two medications could also have bacterial inhibitory actions against various NTM species, based on the similarities in genotype and phenotype within the genus *Mycobacterium*, however there is currently little evidence to support this hypothesis (Yu, Gao et al. 2019).

DLM/BDQ and CFZ were used together to effectively treat the first case of XDR-TB in 2016. Since the patient did not improve while using the second-best medicine, combo treatment was started. After eight doses of BDQ with DLM administration, the recommended regimen was halted due to ongoing concerns regarding QTc prolongation. The patient was closely monitored with ECGs performed every two weeks (Tadolini, Lingtsang et al. 2016).

A median of 119 days after starting BDQ and/or DLM therapy, 39 (70.9%) of 55 patients with initially positive sputum cultures had achieved sputum culture conversion. Six percent of patients had their treatment discontinued due to extended QTc (Kim, Kim et al. 2018).

Research published in 2017 by Achar J et al. describes the successful use of BDQ to treat MDR-TB in 27 children and adolescents less than 18 years old. Patients responded well to therapy, and no ones were removed from the study owing to unwanted side effects (Achar, Hewison et al. 2017).

In the beginning, the radioactive Bactec 460TB was used to record bedaquiline AST. The minimum inhibitory concentration (MIC) for delamanid against *Mycobacterium tuberculosis* isolates has been found to range from 0.006 mg/liter to 0.05 mg/liter (Matsumoto, Hashizume et al. 2006). There have been medications like bedaquiline and delamanid for 10 years, but no standardized methods exist for automated in vitro AST of these drugs. Drug resistance may be detected using either phenotypic or molecular testing (DR). Provisional threshold concentration values for BDQ and DLM to indicate phenotypic resistance are currently specified by the World Health Organization or the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (Matsumoto, Hashizume et al. 2006).

Although BDQ or DLM resistance thresholds have been identified, they vary widely depending on the culture system (Organization 2018).

Based on the available evidence, it seems that mutations in the drug target atpE, which encodes for the component c of the ATP synthase complex, are

responsible for BDQ resistance. In addition, BDQ resistance is associated with off-target mutations in Rv0678, pepQ (Rv2535c), and Rv1979c(Villellas, Coeck et al. 2017). As with fgd1 (Rv0407), ddn (Rv3547), fbiA (Rv3261), fbiB (Rv3262), and fbiC (Rv1173), off-target mutations in these genes were linked to DLM (Nieto Ramirez, Quintero Vargas et al. 2020).

Resistance to BDQ and DLM using WGS was first documented a year after these drugs were approved for the treatment of MDR-TB patients (Hartkoorn, Uplekar et al. 2014, Bloemberg, Keller et al. 2015).

BDQ resistance alleles in the Rv0678, atpE, and pepQ genes have been identified using PCR-Sanger sequencing, and these variations have been shown to be frequent in BDQ resistance in the studies that have been compared (discovered by WGS) (Huitric, Verhasselt et al. 2010, Torrea, Coeck et al. 2015, Villellas, Coeck et al. 2017), including the ones that employed "old school" sequencing methods. DLM RAM have been uncovered by a number of different studies, much like PCR-Sanger sequencing (Feuerriegel, Köser et al. 2011, Haver, Chua et al. 2015).

It has been demonstrate that the same four genes (ddn, fgd1, fbiA, and fbiC) implicated in DLM resistance are also molecular markers of pretomanid resistance (Dookie, Rambaran et al. 2018).

During the review, that reviewed 18 publications and carefully analyzed their findings. Mutations in pepQ were connected to intermediate resistance to BDQ, whereas changes in Rv0678 conferred complete resistance. Phenotypically resistant instances were discovered to have mutations in the genes ddn, fgd1, fbiA, and fbiC, but all known alterations in fbiB were detected solely in DLM-susceptible strains. It was also possible to identify heteroresistance to both medications by WGS analysis (Nieto Ramirez, Quintero Vargas et al. 2020).

Evidence from the longitudinal studies included in that article suggests that an increase in MIC values is a good predictor of the development of drug-resistant microorganisms. In these cases, whole genome sequencing (WGS) would be

crucial for locating and verifying new variations that explain medication resistance.

Future efforts to define standard protocols for BDQ and DLM resistance detection, provide standard (or reference) antibiotic compounds and drug-resistant reference Mtb strains, and provide external quality assurance should involve a partnership between the World Health Organization and the European Committee for Antimicrobial Susceptibility Testing (EUCAST). This has the potential to help bring together the CC and CB standards necessary for DLM (Nieto Ramirez, Quintero Vargas et al. 2020).

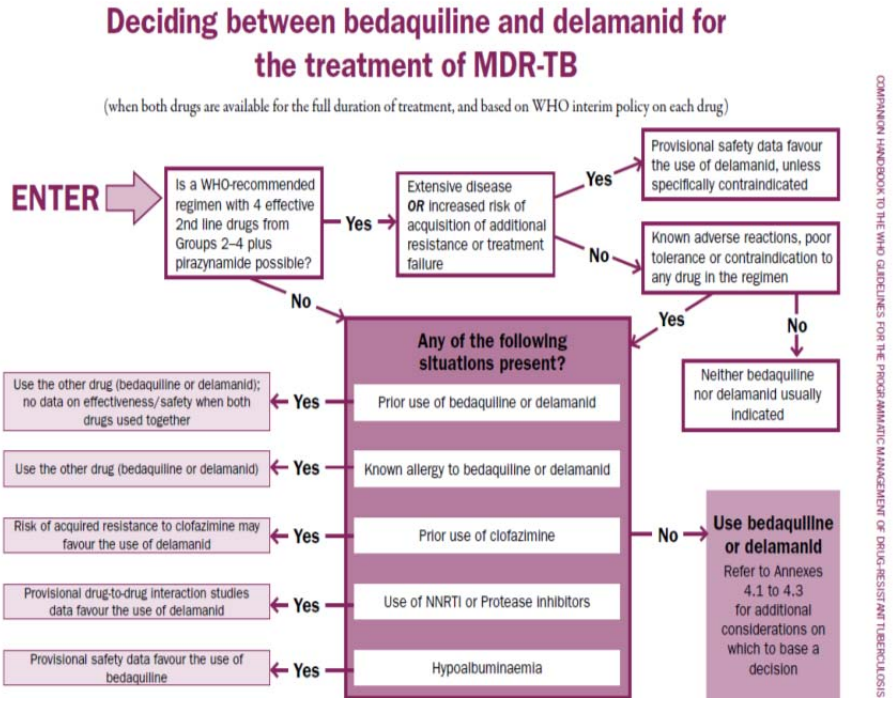


Figure 2.8. Deciding between Bedaquiline and Delamanid For the treatment of MDR.

**2.16. Linezolid:**

The protein synthesis inhibitor LZD belongs to the oxazolidinone family of anti-TB drugs. Previously assigned to group 5 (the very last group) for the treatment of MDR-TB by WHO in 2014, LZD is now in group A, the recommended medication category for the treatment of both MDR-TB and RR-TB. Because of this, LZD is an essential component in the management of TB that has developed resistance to many drugs (Organization 2018).

Evidence that linezolid inhibits protein synthesis came from its association with domain V of the 23S rRNA (Shinabarger 1999). Evidence for a novel method of action is provided by the fact that oxazolidinones do not cause cross-resistance to other antibiotics. Chloramphenicol, macrolides, and lincosamides all bind in this domain (Lin, Murray et al. 1997, Zurenko, Gibson et al. 2001, Wolter, Smith et al. 2005). As well as showing bacteriostatic effectiveness against *Mycobacterium* TB in vitro and in animal models, the antibiotic linezolid was granted FDA approval in 2000 for the treatment of different Gram-positive infections (Lee, Lee et al. 2012, Zhang, Pang et al. 2014)

Linezolid's use to treat drug-resistant tuberculosis has been validated by safety and effectiveness trials (Ramírez-Lapausa, Pareja et al. 2016).

Linezolid binds the ribosome at the peptidyl-transferase center, and resistant strains from in vitro and clinical studies include mutations in the genes for the 23S rRNA and the L3 ribosomal protein (Hillemann, Rüsch-Gerdes et al. 2008, Zhang, Pang et al. 2014).

Due to its strong antibacterial characteristics (MIC of 0.125 to 0.5 mg/liter) against *Mycobacterium tuberculosis*, linezolid has been used by several TB clinics for the treatment of resistant forms of the illness (Brickner, Hutchinson et al. 1996, Alcalá, Ruiz-Serrano et al. 2003) .

Linezolid's treatment-limiting adverse effects, such as anemia, necessitated the development of lower dose approaches (Gerson, Kaplan et al. 2002). The problem is that the negative effects still exist even when the dose is reduced. To

identify a new, more successful therapy for MDR- and XDR-TB, researchers have launched phase I/II studies of PNU-100480, a structural counterpart of linezolid. In contrast to linezolid, which contains an oxygen atom in its ring structure, this one has a sulfur atom (Alffenaar, Laan et al. 2011).

The 8-month success rate for a clofazimine-containing regimen in Bangladesh was 84% , whereas the 6-month success rate for a bedaquiline-containing regimen was 73% (levofloxacin, LZD, and clofazimine as an optimized background regimen) (Chiang, Van Deun et al. 2016). In addition, MDR-TB and extensively drug-resistant tuberculosis may soon have a shorter, all-oral, and more cost-effective treatment option thanks to the Nix-TB study (NCT02333799), the first clinical trial to evaluate the innovative regimen BPaL, which comprises of three medicines (bedaquiline, PA-824, and LZD) (XDR-TB). Ninety percent of the 98 patients who took part in the Nix-TB clinical study were deemed cured 6 months after completing treatment, according to an intention-to-treat analysis (Nimmo, Naidoo et al. 2020).

Linezolid (LZD) is a widely available antibacterial drug that is seeing widespread use. Multidrug-resistant tuberculosis patients use it. Treatment with LZD has been demonstrated to be effective for patients with MDR and XDR pulmonary TB in a number of clinical studies (Villar, Sotgiu et al. 2011, Koh, Kang et al. 2012). PNU-100480's use in first-line regimens has been proven to increase TB activity. PNU-100480's superiority over INH in boosting rifampin's sterilizing activity suggests that treatment durations might be shortened. It is unclear why certain isolates are susceptible to PNU-100480 yet resistant to INH (Williams, Brickner et al. 2009).

When it comes to treating multidrug-resistant tuberculosis, LZD has moved from group 5 (the very end of the list) to group A since 2014. (The first) drugs belonging to this group are used to treat both drug-resistant and drug-sensitive forms of TB. Since tuberculosis has developed resistant to several medications, LZD is crucial for its treatment (Organization 2018).

The Bactec MGIT 960 method was created by Rusch-Gerdes et al. to determine a person's level of vulnerability to LZD. Multiple research institutions have confirmed the method's accuracy (Rüsch-Gerdes, Pfyffer et al. 2006).

With the rising use of LZD in clinical anti-TB treatment, there have been reports of MTB clinical isolates showing resistance to the drug (LES, LEUR et al. , Beckert, Hillemann et al. 2012).

Isolates with resistance to bedaquiline and LZD or bedaquiline with delamanid have recently been donated by patients in treatment investigations (an equivalent of PA-824) (Bloemberg, Keller et al. 2015, Zimenkov, Nosova et al. 2017).

Sander and coworkers found mutations of *Mycobacterium smegmatis* that were resistant to linezolid, and the strains belonged to either class I or class II, showing that the two classes of bacteria used different resistance mechanisms. In class I mutants, the MIC for 23S rRNA shifted by 64 g/ml. The MICs for class II mutants ranged from 4-8 g/ml, and they grew properly and preserved wild-type peptidyl transferase activity. Sander and coworkers postulated a non-ribosomal resistance mechanism for class II mutants (Sander, Belova et al. 2002).

From 2003 to 2005, the German *Mycobacteria* National Laboratory examined 210 strains of *Mycobacteria* for resistance to a 1.0 g/ml minimum inhibitory concentration due to the appearance of M. TB strains resistant to the first-line antituberculosis medication (LZD) in clinical samples. A total of 19 scientists used Bactec 460 or Bactec 960 in their studies. The LZD method uses a 1.0 g/ml threshold concentration (Richter, Rüsch-Gerdes et al. 2007).

Recently, at the Aga Khan University Hospital (AKUH) in Karachi, Pakistan, LZD was tested against XDR and Pre-XDR isolates. The results showed that 94% of the isolates could be killed by LZD at a minimum inhibitory dosage of 0.5 g/ml. The Aga Khan University Hospital (AKUH) is a major tertiary care treatment and diagnostic institution in the southern region of Pakistan. These two studies highlight the therapeutic potential of LZD for the treatment of drug-

resistant TB (Ahmed, Jabeen et al. 2013).

Most cases of linezolid resistance may be traced back to either a nucleotide sequence change (rrl) at position 2061 or a point mutation (rplC) at T460C. Linezolid resistance has been related to the T460C mutation in rplC and the rrl mutation at nucleotide position 2061 (Hillemann, Rüsch-Gerdes et al. 2008, Beckert, Hillemann et al. 2012).

Mutations that confer resistance to linezolid have been the subject of a great deal of previous research. Hillemann et al. (2008) observed nucleotide alterations at rrl at positions 2016 and 2576 in *M. tuberculosis* mutants that were resistant to the antibiotic linezolid. The T460C mutation in rplC was found by Beckert et al. to be associated with higher linezolid MIC values (>2 mg/L) in vitro in a subset of mutants and clinical isolates (Beckert, Hillemann et al. 2012, Zhang, Chen et al. 2016).

# 3. MATERIAL AND METHODS

## 3.1. Materials

### 3.1.1. Ethics Approval of Research

The research used only regularly received isolates from the Tropical Diseases Research and Application Center in Adana, Turkey, with approval from the Ethical Review Committee of Cukurova University/College of Medicine.

### 3.1.2. Selection of *M. tuberculosis* complex clinical isolates

This descriptive cross-sectional laboratory research. This study included a total of 41 MDR-TB and 30 susceptible strains isolated from clinical samples of tuberculosis suspects sent between January 2021and October 2022 to the Cukurova University Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory from eight different cities in the Cukurova Region of Turkey. Adoption of nonprobability convenience sampling. All of the isolates were verified to be *M. tuberculosis* complex (MTBC) using the Bactec MGIT 960 TB system, the gold standard, and sufficient isolation profiles were done. Duplicate specimens were removed from our investigation. All isolates were independently tested for resistance to the first-line medicines (streptomycin, isoniazid, rifampin, and ethambutol), pyrazinamide, 30 isolates were sensitive to all first line drugs (fully drug-susceptible M. tuberculosis strains) and 41 isolates were MDR-TB that were resistant to at least INH and RIF. There were no *M. tuberculosis* complex (MTBC) investigations that weren't carried out in a Biosafety Level 3 lab by qualified and experienced researchers. *M. tuberculosis* reference strain H37Rv (ATCC 27294) was utilized as a control for each batch of DST in the phenotypic and genotypic susceptibility detection procedures employed in this work.

### 3.1.3. Materials and Kits Used in Experiments:

Bactec MGIT Tube 100, BD, Bactec MGIT OADC Supplement, BD, Bactec MGIT PANTA, BD, Streptomycin (STR), Isoniazid (INH), Rifampicin (RIF), Ethambutol (ETM) were purchased from Becton Dickonson and Each solution was made fresh on the day of the experiment. exoSAP (Applied Biosystems™), Sephadex (Sigma-Aldrich), BigDye Terminator v3.1 Cycle sequencing kitUSA,10µl Filtered Pipette Tip, Biopointe Scientific, USA, 200µl Filtered Pipette Tip, Biopointe Scientific, USA.

### 3.1.4. Devices Used in Experiments

Bactec MGIT 960 Liquid Culture System, BD, 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 and an ABI Prism 33l0 Genetic DNA analyzer.

## 3.2. Methods

### 3.2.1. Phenotypic Drug Susceptibility Testing Method:

For quantitative drug susceptibility testing, we used a semi-automated MGIT 960 system and EpiCenter software with a TB eXiST module to facilitate adoption in ordinary labs. For growth detection, the MGIT 960 platform is a semi-automated device that employs a fluorescence-quenching-based oxygen sensor. This technique is commonly used in ordinary labs for *M. tuberculosis* drug susceptibility testing (DST).

To execute the DST, the EpiCenter System TB/eXIST software was used to introduce new medications to the MGIT 960 device and the dilution of bacteria from the culture, creation of drug solutions, preparation of the tubes to be cultured, and cultivation processes. For first-line and PZA, phenotypic DST findings were obtained between 4 and 13 days and 21 days, respectively. The Anti Mycobacterial

Susceptibility Testing of *M. tuberculosis* strains commonly conducts DST against first-line medications (streptomycin, isoniazid, rifampin, and ethambutol) and pyrazinamide DST, employ pyrazinamide (PZA) DST supplement; MGIT 960-based antimicrobial testing was used to examine strains (Becton, Dickinson and Company) Inoculum was prepared in accordance with the manufacturer's guidelines. The tubes contained Isoniazid (0.1 g/ml), Rifampin (1.0 g/ml), Ethambutol (5.0 g/ml), Streptomycin (1.0 g/ml) (Springer, Lucke et al. 2009), and For each batch of DST. In this investigation, the *M. tuberculosis* reference strain H37Rv (ATCC 27292) was employed as a control for each batch of drug susceptibility testing (DST).


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

### 3.2.2. Equipment and Materials for first-line DST:

The BACTEC MGIT 960 SIRE kit (catalog number 245123, BD catalog number GDF 106028) consists of one vial of lyophilized streptomycin, isoniazid,

rifampin, and ethambutol plus eight vials of the SIRE supplement. Rifampicin 322.0 grams, streptomycin 322.0 grams, isoniazid 33.2 grams, and ethambutol 1660.0 grams. To obtain the required stock concentration, each medicine from the BACTEC MGIT SIRE kit was reconstituted with 4ml of sterile distilled water (Table 1). Each medicine in the BACTEC MGIT PZA kit was reconstituted with 2.5 ml of sterile, distilled water to obtain the required stock concentration (Table 1). Complete only after the growth supplement has been added to the MGIT medium. Becton Dickson SIRE supplement was available, the BBL OADC growth supplement (catalog number: 245116) was not used substituted in the same amount (800µl). For pyrazinamide DST, we used pyrazinamide (PZA) DST supplement.

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

| **Drug Lyophilized** | **Volume Added** | **Concentration of reconstituted agent µg/mL** | **Volume added to each MGIT tube** | **Final concentration in MGIT tubes** |
|---|---|---|---|---|
| Isoniazid | 4ml | 8.3 | 100 | 0.1 µg/ml |
| Rifampicin | 4ml | 83 | 100 | 1.0 µg/ml |
| Ethambutol | 4ml | 415 | 100 | 5.0 µg/ml |
| Pyrazinamide | 2.5ml | 8000 | 100 | 100 µg/ml |

### 3.2.3 Preparation of Drug Solutions:

#### 3.2.3.1. Anti-TB drugs:

Susceptibility tests were carried out with three anti-tuberculosis drugs (BDQ, DEL, and LZD). Because bedaquiline and delamanid were unavailable as pure medications, we decided to create AST in tablet form. In theory, the prescription information may reveal the drugs' ingredients and dosages. Janssen

Pharmaceutica provided the bedaquiline SIRTURO 100 mg tablets (Beerse, Belgium). Colloidal anhydrous silica, croscarmellose sodium, hypromellose, lactose monohydrate, magnesium stearate, maize starch, microcrystalline cellulose, polysorbate, and polysorbate were all included in the formulation of each tablet, with the active component totaling 100 mg(Janssen Products. 2012. Highlights of prescribing information Sirturo (bedaquiline) tablets. Janssen Pharmaceutical Research and Development Keller et al. 4354 aac.asm.org Volume July 2015). Otsuka Pharmaceutical (Tokyo, Japan) Deltyba supplied 50 mg Delamanid film-coated tablets, According to the manufacturer's summary of product characteristics, one tablet of Delamanid contained 50 mg of the active ingredient, as well as hypromellose phthalate, povidone, cellulose, microcrystalline, sodium starch glycolate (type A), carmellose calcium, colloidal hydrated silica, magnesium stearate, lactose monohydrate, hypromellose, macrogol 8000, titanium dioxide, talc and yellow iron oxide (E172) (GmbH 2012), The 600 mg linezolid pills were purchased from kocak Pharmaceutical (Turkey). One tablet of linezolid contained 600 mg of the active ingredient and, according to the manufacturer's summary of product characteristics, Maize starch (corn derived), Microcrystalline cellulose (E460), Hydroxypropylcellulose (E463), Sodium starch glycollate type A, Magnesium stearate (E571), and Opadry, white, YS-1-18202-A(e) containing: Hypromellose (E464), Titanium dioxide (E (E903). following the protocol of Keller and colleagues (Keller, Hömke et al. 2015) with slight modifications.

After the tablet was powdered, the powder was dissolved in 100% pure dimethyl sulfoxide (DMSO, Merck, USA), and small aliquots of the stock solution were kept at -70°C. Included in the determination of drug concentrations were the WHO critical value, the EUCAST MIC value, and the values from the research' guiding papers. Twofold serial dilutions in DMSO were used to produce test concentrations. After thawing, stock solutions were employed in tests conducted on the same day. The stabilities of all stock solutions were evaluated simultaneously. For AST, MGIT tubes containing 0.8 ml of oleic acid-albumin-dextrose-catalase

(OADC) supplement (Becton Dickinson) were inoculated with 0.1 ml of the antibiotic in DMSO solution and 0.5 ml of the test strain suspension (2.4% ultimate DMSO concentration).

In order to prepare the drug-free growth control tube, the organism suspension was diluted 1:100 with sterile saline solution, and then 0.5 ml was injected into the tube containing 2.4% (vol/vol) DMSO. MGIT subcultures were used to produce the bacterial suspensions. Introduction of a New Medicine MGIT 960 Equipment Prior to the trial, bedaquiline, delamanid, and linezolid medication concentrations were reported to the MGIT 960 device in accordance with the recommendations and publications. The company's technical staff input the medication concentrations we want to test into the TB/eXIST system. Installing a computer, barcode reader, and barcode printer linked to the MGIT 960 system allowed for the operation of the software system. The MGIT960 equipment was used to incubate drug-containing MGIT960 tubes at 37°C for up to 28 days.

As the MGIT system is automated, the equipment constantly measures growth unit (GU) levels at 60-minute intervals for up to 28 days using fluorescent sensors. Between days 4 and 28, when the control attained a growth unit value of 400, the instrument recognized the DST set as "complete." When the GU value of the drug-containing tube was 100 and the GU value of the growth control tube was 400, bacteria were considered resistant. The interpretation of the results was as follows: When the growth unit (GU) value for the drug-free control tube was more than 400, the strain was classified as resistant (R) if the GU value for the drug-containing tube was less than 100. If the GU value for the drug-containing tube at this time point was less than 100, the strain was classified as sensitive (S). The minimal inhibitory concentration (MIC) of each strain was defined as the lowest drug concentration classified as sensitive by the preceding criterion. According to EUCAST (the European Committee for Antimicrobial Susceptibility Testing), the epidemiological cutoff (ECOFF) value is the minimum inhibitory concentration (MIC) value that identifies the top limit for the wild-type population (Ängeby K

2012).

The applied approach was one of the researchers' procedures, which is also included in the WHO Critical Drug Concentrations Guidelines (Keller, Hömke et al. 2015, Organization 2018). Following were the calculations for preparing various drug concentrations:

**3.2.3.2. Concentrations and solutions required for anti-TB agent preparation for use with the BACTEC MGIT 960 system**

**3.2.3.2.(1). Stock solution and solvent**

To create and sterilize anti-TB agent solutions, we prepared the necessary dilutions so that when 0.1 ml of anti-TB agent solution is added to 7 ml of MGIT medium, the appropriate test concentration is reached depending on the volume of the medium in milliliters.

**Total Volume** = MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + drug (0.1 ml) + inoculum (0.5) = 8.4 ml

**Calculation of dilution factor** =

Total volume / drug volume = 8.4 / 0.1 = 84

**3.2.3.2.(1). According to the dilution factor, several bedaquiline concentrations from 0.25 to 6.4 g/ml were prepared:**

One tablet of bedaquiline contains 100 milligrams of bedaquiline fumarate. This corresponds to 100 mg of bedaquiline was dissolved in 2.5 ml of sterile DMSO, dissolve 12 mg of BDQ fumarate salts (equal to 10 mg of BDQ base).

10 mg 2.5 ml

100 mg X

X= 25 ml sterile DMSO

In 25 ml of sterile DMSO, 100 mg of BDQ fumarate salts (equal to 100 mg of BDQ base) were dissolved. (4000 µg /ml Solution A).

1 mL solution A was diluted to final volume of 5 mL with DMSO (800 µg/mL Solution B). 1.05 mL of solution B was added to 8.95 mL of DMSO. (84 µg/ml Working solution)

**To prepare:**

**0.25 µg/ml drug concentration:**

1 µg/ml 0.1ml

0.25 µg/ml X

X= 0.025 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.325 ml

**0.4 µg/ml drug concentration**:

0.04 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.34 ml

**0.65 µg/ml drug concentration**:

0.065 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.365 ml

**0.8 µg/ml drug concentration:**

0.08 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) =

8.38 ml

**1.6 µg/m l drug concentration:**

0.16 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.46 ml

**2 µg/ml drug concentration:**

0.2 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) 8.5 ml

**4 µg/ml drug concentration:**

0.4 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.7 ml

**6.4 µg/ml drug concentration:**

0.64 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.94 ml

**3.2.3.2.(1).b. The preparation of various Delamanid concentrations ranging from 0.005 to 0.32 µg/ml was based on the following dilution factor:**

One delamanid tablet contains 50 mg of delamanid fumarate. This corresponds to 50 mg of delamanid. Each film-coated tablet contains fifty milligrams of delamanid.

Dissolve 10 mg DLM in 2.5 ml sterile DMSO. Using sterile water, dilute to a final level of 10 ml (1,000 µg /ml Solution A)

10 mg 2.5 ml

50 mg X

X= 12.5 ml sterile DMSO

50 mg DEL fumarate salts (equivalent to 50 mg DEL base) were dissolved in

12.5 ml sterile DMSO. (1,000 µg/ml Solution A)

1 mL of solution A was diluted with 9 mL of SDW to get 100 µg /mL Solution

B.

0.5 mL of solution B was added to 9.5 mL of SDW (5 µg/mL Working solution)

**To prepare:**

**0.01 µg/ml drug concentration:**

0.06 µg/ml 0.1ml

0.01 µg/ml X

X= 0.0166 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.3166 ml

**0.02 µg/ml drug concentration:**

X= 0.0333 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.633 ml

**0.04µg/ml drug concentration:**

X= 0.0666 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.0966 ml

**0.08 µg/ml drug concentration:**

X= 0.1333 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (6.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.0633 ml

**0.16 µg/ml drug concentration:**

X=0 .266 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.566 ml

**0.32 µg/ml drug concentration:**

X= 0.533 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.833 ml\

**1.6 µg/ml drug concentration:**

X= 2.666 ml from drug 5 µg/mL (Working solution) stock was added to MGIT Medium (5.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.63 ml

**3.2.3.2.(1).c. The preparation of various Linezolid concentrations ranging from 0.25 to 4 g/mL was estimated using the following dilution factor:**

One Linezolid tablet contains 600 mg of Linezolid fumarate. This corresponds to 600 mg of Linezolid is included in each film-coated Linezolid 600 mg tablet.

Dissolve 10 mg LZD in 10 ml SDW (1000 µg /mL Solution A).

10 mg 10 ml

600 mg X

X= 600 ml sterile DMSO

In 600 ml of sterile DMSO, 600 mg LZD fumarate salts (equal to 600 mg LZD

base) were dissolved (1,000 µg /ml Solution A).

4 mL of Solution A was diluted to a final volume of 5 mL with SDW (800 µg/mL Solution B).

1.05 mL of solution B was added to 8.95 mL of SDW (84 µg/mL Working solution).

**To prepare**

**0.25 µg/ml drug concentration:**

1 µg/ml 0.1ml

0.25 µg/ml X

X= 0.025 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.325 ml

**0.4 µg/ ml drug concentration:**

0.04 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.34 ml

**0.65 µg/ ml drug concentration:**

0.065 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) =

8.365 ml

**0.8 µg/ ml drug concentration:**

0.08 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.38 ml

**1.6 µg/ ml drug concentration:**

0.16 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.46 ml

**2 µg/m ml drug concentration:**

0.2 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.5 ml

**4 µg/ ml drug concentration:**

0.4 ml from drug 84 µg/ml (Working solution) stock was added to MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + inoculum (0.5) = 8.7 ml

### 3.3. Determination of resistance profiles via genotypic approaches:

The genomes of *M. tuberculosis* include mutations in the gene areas responsible for bedaquiline, delamanid, and linezolid resistance, using the Sanger DNA sequencing approach, strains of 71 *M. tuberculosis* complex MTB isolates including the fully drug-susceptible strains (n = 30) and MDR strains (n = 41) were

studied. In order to do this, DNA isolation, PCR, visualization of the produced amplicons on agarose gel, and Sanger sequencing were conducted, and the results were evaluated.

### 3.3.1. Isolation of genomic DNA

*M. tuberculosis* complex strains' genomic DNA was extracted using the Mickle mechanical extraction technique and kept at –20°C until use

### 3.3.2. DNA extraction by Mickle Mechanical methods and sequence analysis

1. The study's samples were placed in a microcentrifuge tube containing 0.5 milliliters.
2. The bacteria were killed by incubating them at 80 degrees Celsius for 30 minutes.
3. Spin at 15000xG for 15 minutes and discard the supernatant.
4. Spin at 15000xG for 15 minutes and discard the supernatant.
5. Spin at 15000xG for 15 minutes and discard the supernatant.
6. 0.25 milliliters of 1xTE buffer were added.
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.

### 3.3.3. Bedaquiline, delamanid, and linezolid resistance gene DNA sequencing

For bedaquiline, each isolate's atpE and Rv0678 genes were sequenced; for delamanid, each isolate's fbiA and ddn genes were sequenced; and for linezolid, each isolate's rrl and rplC genes were sequenced. Table 2 displays the primers used to amplify and sequence the genes for resistance to bedaquiline, Delamanid, and

linezolid. Table 3 displays PCR mix required for a single reaction in PCR Standard conditions for PCR amplification were as follows: initial denaturation at 95 degrees Celsius for 5 minutes; 35 cycles of denaturation at 95 degrees Celsius for 30-60 seconds; annealing at 53.8 degrees Celsius (fbiA), 54 degrees Celsius (ddn), 59.0 degrees Celsius (rrl and rplC), or 58 degrees Celsius (Rv0678 and atpE) for 30- 60 seconds; extension at 72 degrees Celsius for 30-60 seconds and a final extension at 72°C for 10 minutes. Amplicons generated by polymerase chain reaction were examined after being placed onto an agarose gel. Sequencing of amplicons was done right away or they were frozen at -20 degrees Celsius for subsequent analysis. The PCR products' DNA sequences were determined using a Sanger sequencing kit, a BigDye Terminator cycle sequencing kit, and an ABI Prism 33l0 Genetic DNA analyzer. Basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST) was used to examine the findings. We also did an alignment of the sequences to the H37Rv *Mycobacterium tuberculosis* genome

Table 3.2. The primer sequences used for PCR.

| Antimicrobial agent | Primer | Sequence (5′ to 3′) | Amplicon size |
|---|---|---|---|
| Bedaquiline | atpE_F | GGACCCCACTATCGCTGCCGGCG | 244 |
| | atpE_R | TTACTTGACGGGTGTAGCGAA | |
| | Rv0678_F | TGAAGTTCACGCCGGTCTGG | 724 |
| | Rv0678_R | CAGCGAACCGGAGAGGACGACTGA | |
| Delamanid | fbiA_F | GGCCAGTTTGCTGCCAATTC | 727 |
| | fbiA_R | TGGAATCCACCCCGATAACC | |
| | ddn_F | CCGCTCAGCGACTTCTTTATC | 299 |
| | ddn_R | GCGGTAAGGTCCAGCACTT | |
| Linezolid | rrl_F | TACCAAGGCGTACGAGAT | 576 |
| | rrl_R | GGGTTCGAGGTTAGATGCCC | |
| | 2rrl_F | GGGCATCTAACCTCGAACCC | 503 |
| | 2rrl_R | GTGCACCAGAGGTTCGTCC | |

Table 3.3. PCR mix required for a single reaction in PCR.

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

**3.3.3.1. Investigation of Resistance to Bedaquiline:**

Bedaquiline resistance has been linked to mutations in *Mycobacterium tuberculosis* atpE (Rv1305) and Rv0678 genes, thus our study looked for these mutations. The findings of DNA isolation, polymerase chain reaction (PCR), gel

electrophoresis of amplicons, and Sanger sequencing were evaluated for this aim.

**Amplification of atpE (Rv1305) gene:**

The PCR procedure yielded a 244-bp-long product.

atpE gene amplification and sequence primers:

| |
|---|
| 1ATGGACCCCACTATCGCTGCCGGCGCCCTCATCGGCGGTGGACTGATCATGGCCGGTGGC |
| 61GCCATCGGCGCCGGTATCGGTGACGGTGTCGCCGGTAACGCGCTTATCTCCGGTGTCGCC |
| 121CGGCAACCCGAGGCGCAAGGGCGGCTGTTCACACCGTTCTTCATCACCGTCGGTTTGGTT |
| 181 GAGGCGGCATACTTCATCAACCTGGCGTTTATGGCGCTGTTCGTCTTCGCTACACCCGTC |
| 241 AAGTAA |

**Amplification of Rv0678 gene:**

| |
|---|
| 1GTGAGCGTCAACGACGGGGTCGATCAGATGGGCGCCGAGCCCGACATCATGGAATTCGTC |
| 61GAACAGATGGGCGGCTATTTCGAGTCCAGGAGTTTGACTCGGTTGGCGGGTCGATTGTTG |
| 121GGCTGGCTGCTGGTGTGTGATCCCGAGCGGCAGTCCTCGGAGGAACTGGCGACGGCGCTG |
| 181GCGGCCAGCAGCGGGGGGATCAGCACCAATGCCCGGATGCTGATCCAATTTGGGTTCATT |
| 241GAGCGGCTCGCGGTCGCCGGGGATCGGCGCACCTATTTCCGGTTGCGGCCCAACGCTTTC |
| 301GCGGCTGGCGAGCGTGAACGCATCCGGGCAATGGCCGAACTGCAGGACCTGGCTGACGTG |
| 361 GGGCTGAGGGCGCTGGGCGACGCCCCGCCGCAGCGAAGCCGACGGCTGCGGGAGATGCGG |
| 421GATCTGTTGGCATATATGGAGAACGTCGTCTCCGACGCCCTGGGGCGATACAGCCAGCGA |
| 481 ACCGGAGAGGACGACTGA |

Genes atpE (Rv1305) and Rv0678 have been amplified as follows:

95 °C - 5 min - pre-denaturation

95 °C – 30-60 sec – denaturation
58 °C – 1min annealing
72 °C - 30 sec - extension
} 35-40 cycles

72 °C - 10 min Final extension /

4 °C ∞

In 2% Agarose Gel electrophoresis, products of atpE and Rv0678 were found to be 244 bp and 724bp, respectively. Sequencing of amplicons was done right away or they were frozen at -20 degrees Centigrade for subsequent analysis.

**3.3.3.2. The Sanger DNA Sequencing Analysis of atpE (Rv1305) and Rv0678:**

Because of automation in sequence analysis technologies, DNA can now be studied rapidly. The reactions in these techniques (dye-terminator sequencing) were performed in a single tube containing four ddNTPs, all of which were tagged with dyes that radiate at distinct wavelengths, allowing for laser readout.

The 246-bp atpE (Rv1305) and 560-bp Rv0678 gene regions of the M. tuberculosis were sequenced using the Sanger DNA sequencing technique. Yellow highlights indicate genomic areas where primers were successfully implemented (Table 3). Sanger DNA sequence analysis of PCR results was performed using a BigDye Terminator Cycle Sequencing kit and an ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST) was used for the analysis of the data. The genome of *Mycobacterium tuberculosis* H37Rv was used for this evaluation (GenBank accession no. AL123456.3)

**3.3.4. Purification of PCR Products:**

After PCR products were obtained, they were purified using the "ExoSAP-IT" kit according to the manufacturer's instructions by adding 2µl of enzyme to 5 µl of PCR product, incubating at 37°C for 30 minutes, and then maintaining the mixture at 85°C for 15 minutes.

**3.3.5. Cycle Sequence PCR Amplification**

Using BigDye® Terminator v3.1 CycleSequencing Kit (AppliedBiosystems) with dNTP and ddNTP-containing reaction mixture and forward primer, purified PCR products were amplified using a single chain amplification.

Each sample was treated with the following mixture:

Ready Reaxion Mix (RR-100) 5x 1µl

Sequencing Buffer 5x 2µl

atp E –F/ Rv0678 F 2µl

dH2O 3µl

Pure PCR product 2µl

Samples were amplified in a thermal cycler according to the cycle sequence as follows:

96˚C -10 seconds
50˚C -5 seconds } 25 cycles
60˚C -4 minutes
4˚C ∞

**3.3.6. Cleaning and loading the sequencer with purified PCR products derived from a cycle sequence:**

Before being loaded into the sequencing machine, 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 1gr of sephadex chemical.

1. To begin, 1.5 ml collecting tubes were set up with empty spin columns.
2. Seven hundred microliters of sephadex mixture were placed into each column.
3. The next step was a 2-minute centrifugation run at 4500 rpm. No damage was done to the sephadex column created by emptying the liquid in the collecting tube before the amplicons were added.

4. The pure cycle sequencing product was collected by centrifuging the sample for 3 minutes at 4500 rpm, and then was put into an ABI Prism 3130 sequence instrument for analysis according to the manufacturer's instructions.
5. Sequencing Analysis 5.1 (Applied Biosystems) was used to interpret the result.

### 3.3.7. Investigation resistance to delamanid Antibiotics:

Resistance to Delamanid was traced back to changes in *Mycobacterium tuberculosis's* fbiA and ddn genes. This was achieved by using the same apparatus, equipment, and software as for bedaquiline to conduct DNA isolation, PCR, visualization of the generated amplicons on an agarose gel, and Sanger sequencing

### 3.3.7.1. Amplification of fbiA and ddn genes

Resistance

fbiA: product with a length of 727 bp was obtained by PCR process.

fbiA gene amplification and sequence primers:

| |
|---|
| 1GTGAAGGTCACCGTTCTGGCCGGTGGAGTCGGCGGCGCCCGCTTCCTGCTCGGGGTCCAG |
| 61CAGCTGCTCGGCCTGGGCCAGTTTGCTGCCAATTCTGCCCACTCGGACGCCGACCACCAA |
| 121CTGAGCGCTGTCGTCAACGTCGGCGACGACGCCTGGATCCACGGGCTGCGTGTCTGCCCG |
| 181GATCTGGACACCTGCATGTATACCCTGGGCGGCGGGGTGGACCCCCAGCGCGGCTGGGGC |
| 241CAGCGTGACGAAACTTGGCACGCCATGCAGGAACTGGTGCGCTATGGCGTGCAGCCCGAC |
| 301TGGTTCGAGCTCGGGGACCGCGATCTGGCCACCCATCTGGTGCGCACCCAGATGCTGAG |
| 361GCCGGCTACCCCCTGTCACAGATCACCGAGGCCCTATGCGATCGCTGGCAACCGGGCGCC |
| 421CGCTTGCTGCCTGCCACCGACGACCGTTGCGAAACCCATGTAGTGATCACCGACCCGTC |
| 481GACGAAAGCCGCAAGGCGATCCATTTTCAGGAGTGGTGGGTGCGCTACCGTGCCCAGGTG |
| 541CCGACGCACAGCTTTGCTTTTGTCGGCGCTGAAAAGTCCAGCGCTGCAACCGAAGCGATC |
| 601GCCGCCCTGGCCGACGCCGACATCATCATGCTGGCGCCGTCTAATCCGGTGGTCAGCATC |
| 661GGCGCCATCCTGGCCGTCCCCGGGATTCGCGCGGCGTTGCGGGAAGCAACCGCACCGATC |
| 721GTCGGCTACTCGCCGATCATCGGCGAAAAGCCGTTGCGCGGCATGGCCGATACGTGCCTT |
| 781TCGGTTATCGGGGTGGATTCCACCGCGGCCGCTGTGGGCCGGCACTACGGCGCGCGGTGC |
| 841GCCACCGGGATACTGGACTGCTGGCTGGTGCACGACGGCGACCACGCTGAGATTGACGG |

ddn: product with a length of 846 bp was obtained by PCR process.

ddn gene amplification and sequence primers:

| |
|---|
| 1ATGCCGAAATCACCGCCGCGGTTTCTGAATTCGCCGCTCAGCGACTTCTTTATCAAGTGG |
| 61ATGTCACGGATTAATACCTGGATGTACCGCCGCAACGACGGGGAGGGTCTGGGCGGCACC |
| 121TTCCAGAAGATTCCGGTCGCGCTGCTGACCACCACCGGCCGCAAGACCGGCCAGCCGCGG |
| 181 GTCAACCCGCTCTACTTCCTGCGCGACGGTGGGCGGGTCATTGTCGCGGCCTCCAAGGGC |
| 241GGCGCGGAGAAGAACCCGATGTGGTACCTCAACCTCAAGGCCAACCCCAAGGTTCAGGTA |
| 301CAGATCAAAAAGGAAGTGCTGGACCTTACCGCGCGGGACGCGACCGACGAGGAGCGCGCC |
| 361GAATATTGGCCACAGTTGGTCACGATGTACCCAAGTTATCAGGACTACCAGTCCTGGACC |
| 421 GACCGCACGATCCCGATCGTGGTTTGCGAACCCTGA |

The amplification steps for fbiA gene have been completed as follows:

95 °C - 5 min - pre-denaturation

95 °C – 30-60 sec – denaturation
53.8 °C – 30-60 sec annealing } 35-40 cycles

72 °C - 30 sec - extension

72 °C - 10 min Final extension / 4 °C ∞

The amplification steps for ddn gene have been completed as follows:

95 °C - 5 min - pre-denaturation

95 °C – 30-60 sec – denaturation
54 °C – 30-60 sec annealing } 35-40 cycles

72 °C – 1min- extension

72 °C - 10 min Final extension / 4 °C ∞

The presence of 727 bp and 299 bp products for fbiA & ddn genes respectively, were

detected in 2% Agarose Gel electrophoresis. Amplicons were immediately sequenced or

stored at -20ºC for later study.

### 3.3.8. Examining the Prevalence of Linezolid Resistance:

The existence of linezolid-resistance-causing mutations in *Mycobacterium TB* was examined by testing for mutations in the rrl and rplC genes. To do this, we employed the same apparatus, equipment, and software for bedaquiline to do DNA isolation, PCR, visualization of the generated amplicons on agarose gel, and Sanger sequencing**.**

#### 3.3.8.1. Amplification of rrl , 2rrl and rplC genes

rrl: product with a length of 576 bp and 503 bp was obtained by PCR process.

rrl gene amplification and sequence primers:

| |
|---|
| TTGTAAGTGTCTAAGGGCGCATGGTGGATGCCTTGGCATCGAGAGCCGATGAAGGACGTGGGAGGCTGCG |
| ATATGCCTCGGGGAGCTGTCAACCGAGCGTGGATCCGAGGATTTCCGAATGGGGAAACCCAGCACGAGTG |
| ATGTCGTGCTACCCGCATCTGAATATATAGGGTGCGGGAGGGAACGCGGGGAAGTGAAACATCTCAGTAC |
| CCGTAGGAGGAGAAAACAATTGTGATTCCGCAAGTAGTGGCGAGCGAACGCGGAACAGGCTAAACCGCAC |
| GCATGGGTAACCGGGTAGGGGTTGTGTGTGCGGGGTTGTGGGAGGATATGTCTCAGCGCTACCCGGCTGA |
| GAGGCAGTCAGAAAGTGTCGTGGTTAGCGGAAGTGGCCTGGGATGGTCTGCCGTAGACGGTGAGAGCCCG |
| GTACGCGAAAACCCGGCACCTGCCTAGTATCAATTCCCGAGTAGCAGCGGGCCCGTGGAATCCGCTGTGA |
| ATCCGCCGGGACCACCCGGTAAGCCTAAATACTCCTCGATGACCGATAGCGGATTAGTACCGTGAGGGAA |
| TGGTGAAAAGTACCCCGGGAGGGGAGTGAAAGAGTACCTGAAACCGTGTGCCTACAATCCGTCAGAGCCT |
| CCTTTTCCTCTCCGGAGGAGGGTGGTGATGGCGTGCCTTTTGAAGAATGAGCCTGCGAGTCAGGGACATG |
| TCGCAAGGTTAACCCGTGTGGGGTAGCCGCAGCGAAAGCGAGTCTGAATAGGGCGACCCACACGCGCATA |
| CGCGCGTGTGAATAGTGGCGTGTTCTGGACCCGAAGCGGAGTGATCTACCCATGGCCAGGGTGAAGCGCG |
| GGTAAGACCGCGTGGAGGCCCGAACCCACTTAGGTTGAAGACTGAGGGGATGAGCTGTGGGTAGGGGTGA |
| AAGGCCAATCAAACTCCGTGATAGCTGGTTCTCCCCGAAATGCATTTAGGTGCAGCGTTGCGTGGTTCAC |

rplC: product with a length of 589 bp was obtained by PCR process.

rplC gene amplification and sequence primers:

| |
|---|
| 1ATGGCACGAAAGGGCATTCTCGGTACCAAGCTGGGTATGACGCAGGTATTCGACGAAAGC |
| 61AACAGAGTAGTACCGGTGACCGTGGTCAAGGCCGGGCCCAACGTGGTAACCCGCATCCGC |
| 121ACGCCCGAACGCGACGGTTATAGCGCCGTGCAGCTGGCCTATGGCGAGATCAGCCCACGC |
| 181AAGGTCAACAAGCCGCTGACAGGTCAGTACACCGCCGCCGGCGTCAACCCACGCCGATAC |
| 241CTGGCGGAGCTGCGGCTGGACGACTCGGATGCCGCGACCGAGTACCAGGTTGGGCAAGAG |
| 301 TTGACCGCGGAGATCTTCGCCGATGGCAGCTACGTCGATGTGACGGGTACCTCCAAGGGC |
| 361 AAAGGTTTCGCCGGCACCATGAAGCGGCACGGCTTCCGCGGTCAGGGCGCCAGTCACGGT |
| 421 GCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGATGTGCCACGCCGGCGCGGGTG |
| 481 TTCAAGGGCACCCGGATGGCCGGGCGGATGGGCAATGACCGGGTGACCGTTCTTAACCTT |
| 541TTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGCGGTTCCTGGC |
| 601 CGCACCGGTGGACTGGTCATGGTCCGCAGTGCGATCAAACGAGGTGAGAAGTGA |

The amplification steps for rrl and rplC genes have been completed as follows:

95 °C - 5 min - pre-denaturation

95 °C – 30-60 sec – denaturation
59 °C – 30-60 sec annealing } 35-40 cycles

72 °C - 30 sec - extension

72 °C - 10 min Final extension / 4 °C ∞

The presence of 576 bp, 503 bp and 589 bp length products for rrl and rplC genes respectively, were detected in 2% Agarose Gel electrophoresis. Amplicons were immediately sequenced or stored at -20ºC for later study.

### 3.3.9. Analyzing fbiA, ddn, rrl, and rplC by Sanger DNA sequencing.

Using the same apparatus, equipment, procedure, and software as for bedaquiline, we looked for mutations in the fbiA, ddn, rrl, and rplC gene areas based on the H37Rv reference strain genome.

Figure 3.2. (ABI PRISM$^{tm}$ 310 Genetic Analayzer) instrument, department of Microbiology/ Medicine Faculity, Cukurova University.

# 4. RESULT AND DISCUSSION

## 4.1. Susceptibility Testing

A total of 75 strains isolated from clinical samples of tuberculosis suspects sent to the Cukurova University Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory from eight different cities in the Cukurova Region of Southern Turkey.

Duplicate specimens were removed from our investigation, whereas 4 strains were excluded from the study due to subculture failure. All of the isolates were verified to be *M. tuberculosis* complex (MTBC) using the Bactec MGIT 960 TB system, the gold standard, and sufficient isolation profiles were done. All samples were chosen after susceptibility test. All isolates were independently tested for resistance to the first-line medicines (streptomycin, isoniazid, rifampin, and ethambutol), pyrazinamide, 30 isolates were sensitive to all first line drugs (fully drug-susceptible *M. tuberculosis* strains) and 41 isolates were MDR-TB that were resistant to at least INH and RIF (see Figure 4.2). 26.8(11/41) of MDR were pyrazinamide sensitive strains and 73.2% (30/41) were pyrazinamide resistant strains.

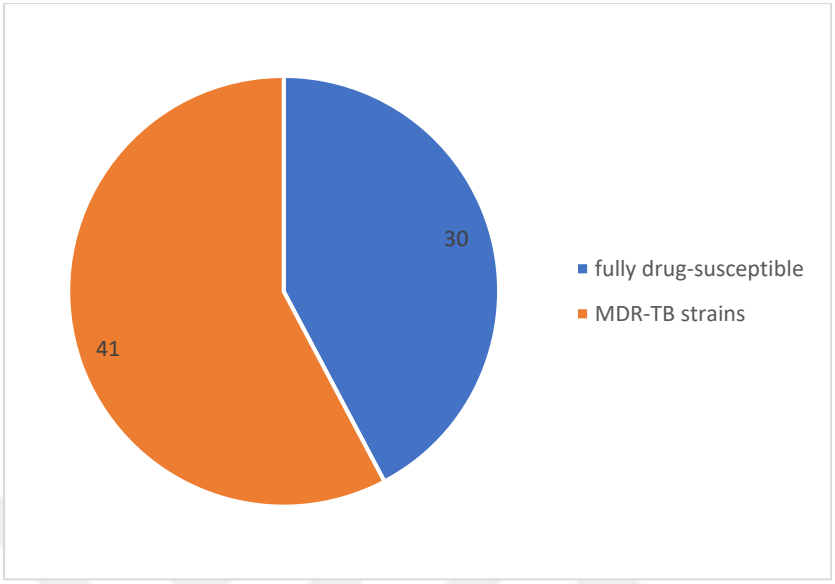


Figure 4.1. Distribution of MTB isolates.

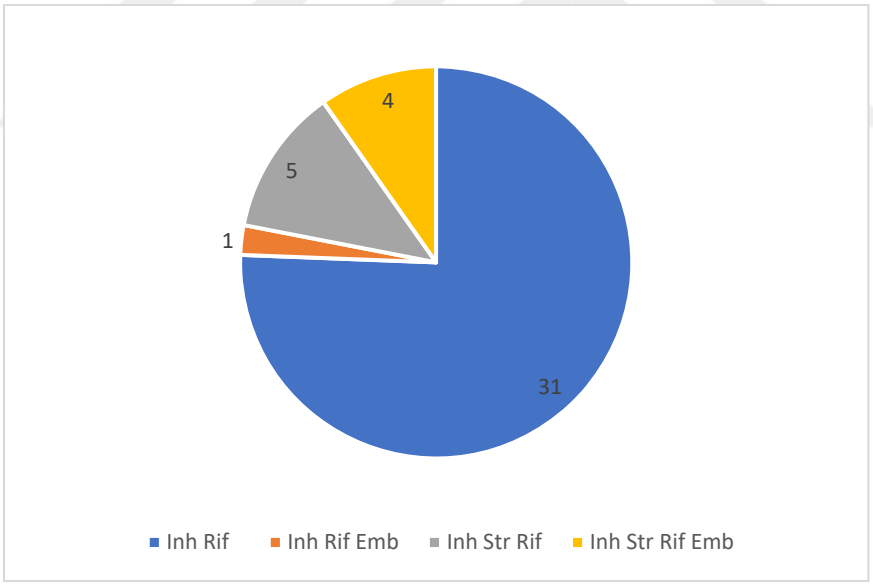


Figure 4.2. Drug susceptibility testing (DST) profiles of MDR-TB strains.

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

| Stains | Susceptibility Profiles | No. of isolates | Percentage (%) of resistant strains |
|---|---|---|---|
| **MDR-TB strains** | Inh Rif | 31 | 75.61% |
| | Inh Rif Emb | 1 | 2.44% |
| | Inh Str Rif | 5 | 12.19% |
| | Inh Str Rif Emb | 4 | 9.76% |
| | Total | 41 | 100% |

All MDR-TB and fully drug-susceptible *M. tuberculosis* strains (n 71; 41 MDR, and 30 fully drug-susceptible) from bedaquiline, delamanid and linezolid treatment-naive patients were independently tested for resistance to three anti-TB medications (BDQ, DEL and LZD), according to our approach.

We used a semiautomated MGIT 960 system and EpiCenter software equipped with a TB eXiST module for quantitative drug susceptibility testing. We chose 6 to 10 concentrations for AST (see Table 4.2).

Semiautomated MGIT 960 system and EpiCenter software with a TB eXiST module for quantitative drug susceptibility testing were used to analyze the MICs of BDQ, DEL, and LZD, and the results were in accordance with previously published MICs.

In this experiment, we employed BDQ, DEL, and LZD at concentrations ranging from 0.25 to 6.4 mg/mL, 0.01-3.2 mg/mL, and 0.0125 to 16 mg/mL.

With the BACTECTM 960 reader showing 400 growth units for the drug-free control, the MIC of three anti-TB medicines (BDQ, DEL, and LZD) in MGIT was calculated as the lowest drug concentration that maintained growth at100 growth units.

The susceptibility results of *M. tuberculosis* strains to bedaquiline, delamanid and linezolid studied in the MGIT 960 system with the TB/eXIST software, the bacterial growth amount in the growth control tube reached the threshold value of 400 on the 8th day. Growth in the tube containing >1 µg/ml, > 0.2 µg/ml and >1 µg/ml bedaquiline, delamanid and linezolid respectively, occurred on the 4th day and the strain exceeded the 100 GU threshold. Therefore, this strain is resistant to bedaquiline, delamanid and linezolid respectively.

The overall drug MIC distribution is shown in Table 4.2**.** For bedaquiline, the fully drug-susceptible strains had drug MIC values between 0.2 and 0.8 mg/ liter. the MIC arithmetic mean for fully drug-susceptible *M. tuberculosis* strains was 0.27 mg/liter and the median was 0.25 mg/liter. *M. tuberculosis* H37Rv had a drug MIC of 0.4 mg/liter. In an analysis of 41 MDR isolates, The MDR strains had drug MIC values between 0.2 and 6.4 mg/ liter, the arithmetic mean of the drug MIC was 0. 62 mg/liter and the median of the MIC was 0.5 mg/liter.

For delamanid, the fully drug-susceptible strains had drug MIC values between 0.005 and 0.08 mg/ liter. The MIC arithmetic mean for fully drug-susceptible *M. tuberculosis* strains was 0.015 mg/liter; the median was 0.01 mg/liter. *M. tuberculosis* H37Rv had a drug MIC of 0.01 mg/liter. In an analysis of 41 MDR isolates, The MDR strains had drug MIC values between 0.02 and0.32 mg/ liter, the arithmetic mean of the drug MIC was 0.06 mg/liter and the median of the MIC was 0.08 mg/liter.

For linezolid, the fully drug-susceptible strains had drug MIC values between 0.125 and 0.5 mg/ liter. The MIC arithmetic mean for fully drug-susceptible *M. tuberculosis* strains was 0.2 mg/liter; the median was 0.25 mg/liter. *M. tuberculosis* H37Rv had a drug MIC of 0.5 mg/liter. In an analysis of 41 MDR isolates, the MDR strains had drug MIC values between 0.125 and 2 mg/ liter. The arithmetic mean of the drug MIC was 0.569 mg/liter and the median of the MIC was 0.5 mg/liter.

For delamanid of the 71 strains (see Figure 4.1), 69 (97.2%) were

susceptible and 2 (2.8%) were resistant to delamanid, when setting 0.2 mg/L as a critical concentration to distinguish susceptible and resistant isolates. All delamanid resistant isolates were MDR and all fully drug-susceptible strains were susceptible to delamanid (see Table 4.3).

For bedaquiline (see Figure 4.3), 65 (91.5%) clinical strains were susceptible, and 6 (8.45%) were resistant, based on WHO breakpoint 1mg/L. All bedaquiline resistant were MDR and 83.3% (5/6) were İNH+RİF resistant MDR-TB strains, and 16.7% (1/6) were Inh, Rif and Emb resistant MDR-TB strains. According to EUCAST the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint of 0.25 µg/ml 21 (29.6%) clinical strains were susceptible, and 50 (70.4%) were resistant to bedaquiline, 78% (39/50) were MDR and 22% (11/50) were fully drug-susceptible strains (see Table 4.3).

For linezolid (see Figure 4.5), 66 (92.96%) clinical strains were susceptible, and 5 (7.04 %) were resistant, based on WHO and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint of 1mg/L. All linezolid resistant, were İNH+RİFresistant MDR-TB strains (see Table 4.5).

According to the bedaquiline, delamanid, and linezolid MIC values of fully drug-susceptible and MDR strains determined by WHO and EUCAST, the resistance rates of the strains are given in tables 4.3, 4.4, and 4.5 respectively.

Table 4.2. Susceptibility of bedaquiline, delamanid and Linezolid strains distributions.

| Strain (n) | Bedaquline (µg/ml) | | | | | | Delamanid (µg/ml) | | | | | | Linezolid (µg/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.2 | 0.4 | 0.5-0.8 | 1.6 | 2-4 | 6.4 | ≤0.01 | 0.02 | 0.04 | 0.08 | 0.16 | 0.32 | ≤0.125 | 0.25-.04 | 0.5 | 1 | 1.6 | 2 |
| **Susceptible** | 22 | 49 | 66 | 66 | 70 | 71 | 17 | 40 | 49 | 59 | 69 | 71 | 19 | 37 | 55 | 66 | 67 | 71 |
| **Resistant** | 49 | 22 | 5 | 5 | 1 | 0 | 54 | 31 | 22 | 12 | 2 | 0 | 52 | 34 | 16 | 5 | 4 | 0 |

Table 4.3. Bedaquiline resistance rates of *M. tuberculosis* strains according to WHO and EUCAST guidelines.

| *M.tuberculosis* strains | 1µg/ml according to WHO | | | | MIC 0.25 µg/ml according to EUCAST | | | |
|---|---|---|---|---|---|---|---|---|
| | Resistant | | Senstive (n) | | Resistant | | Senstive | |
| | N | % | n | % | n | % | N | % |
| Fully drug-susceptible strains | 0 | 0% | 30 | 42.3% | 11 | 15.5% | 19 | 26.8% |
| MDR-TB strains (İNH+RİFresistant) | 5 | 7.0% | 26 | 36.6% | 30 | 42.3% | 1 | 1.4% |
| MDR-TB strains (Inh Rif Emb resistant) | 1 | 1.41% | 0 | 0% | 1 | 1.41% | 0 | 0% |
| MDR-TB strains (Inh Str Rif resistant) | 0 | 0.0% | 5 | 7.0% | 4 | 5.6% | 1 | 1.41% |
| MDR-TB strains (Inh Str Rif Emb resistant) | 0 | 0% | 4 | 5.6% | 4 | 5.6% | 0 | 0% |
| Total | 6 | 8.5% | 65 | 91.5% | 50 | 70.4% | 21 | 29.6% |

Table 4.4. Delamanid resistance rates of *M. tuberculosis* strains when setting 0.2 mg/L as a critical concentration to distinguish susceptible and resistant isolates.

| ***M.tuberculosis* strains** | Setting 0.2 mg/L as a critical concentration | | | |
|---|---|---|---|---|
| | **Resistant** | | **Sensitive** | |
| | **n** | **%** | **n** | **%** |
| Fully drug-susceptible strains | **0** | **0%** | **30** | **4%** |
| MDR-TB strains (İNH+RİFresistant) | **1** | **1.41%** | **30** | **42.3%** |
| MDR-TB strains (Inh Rif Emb resistant) | **0** | **0%** | **1** | **1.41%** |
| MDR-TB strains (Inh Str Rif resistant) | **1** | **1.41%** | **4** | **5.6%** |
| MDR-TB strains (Inh Str Rif Emb resistant) | **0** | **0%** | **4** | **5.6%** |
| Total | **2** | **2.8%** | **69** | **97.2%** |

Table 4.5. Linezolid resistance rates of *M. tuberculosis* strains according to WHO and EUCAST guidelines.

| ***M.tuberculosis* strains** | **1 µg/ml according to WHO and EUCAST** | | | |
|---|---|---|---|---|
| | **Resistant** | | **Sensitive** | |
| | **n** | **%** | **n** | **%** |
| fully drug-susceptible strains | **0** | **0.0%** | **30** | **42,3%** |
| MDR-TB strains (İNH+RİFresistant) | **5** | **7.0%** | **26** | **36.6%** |
| MDR-TB strains (Inh Rif Emb resistant) | **0** | **0.0%** | **1** | **1.41%** |
| MDR-TB strains (Inh Str Rif resistant) | **0** | **0.0%** | **5** | **7.0%** |
| MDR-TB strains (Inh Str Rif Emb resistant) | **0** | **0%** | **4** | **5.6%** |
| Total | **5** | **7.0%** | **66** | **93%** |

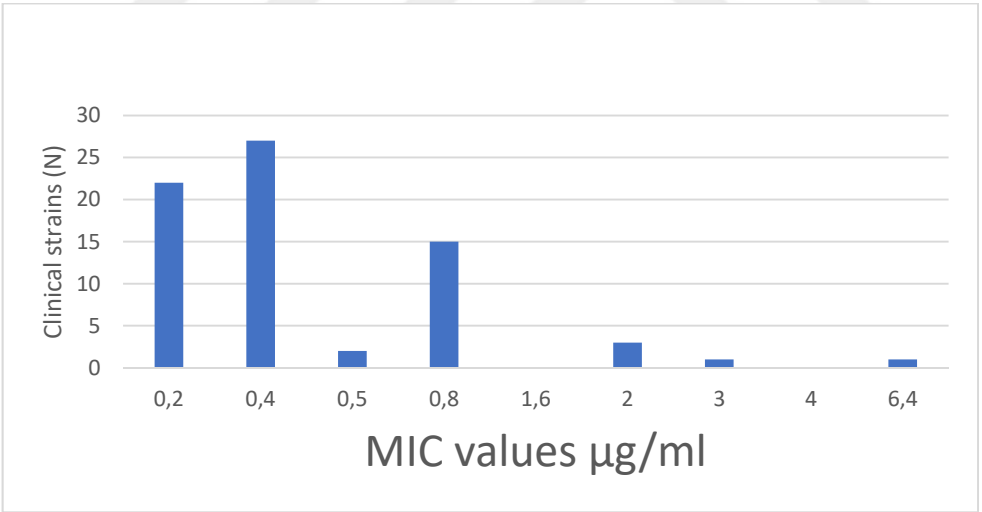


Figure 4.3. MGIT MIC distribution of Bedaquiline 71 clinical strains.

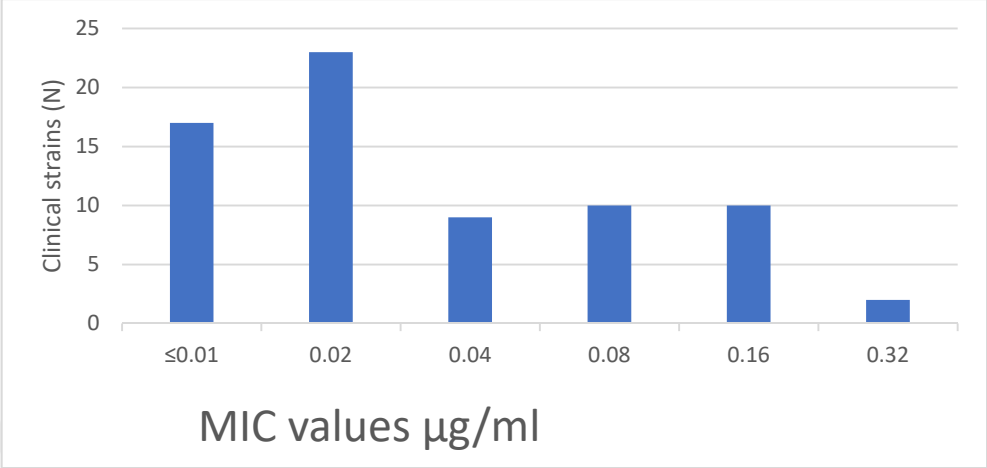


Figure 4.4. MGIT MIC distribution of Delamanid 71 clinical strains.

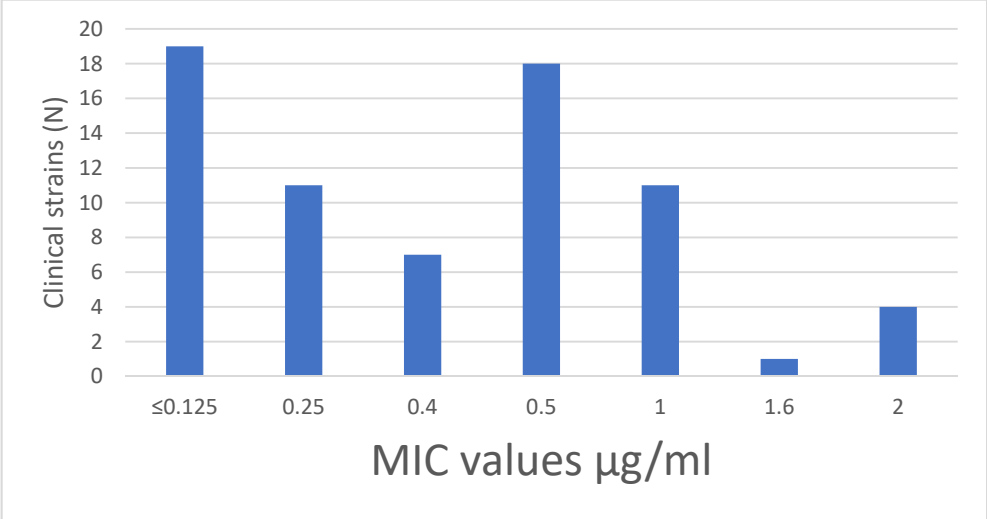


Figure 4.5. MGIT MIC distribution of Linezolid 71 clinical strains.

**4.2. Sanger Sequencing Results:**

First, to demonstrate a correlation between the amount of phenotypic drug resistance and the existence of resistance-related mutations, we first screened for mutations in genes linked with drug resistance to the antibiotic under study. The resultant DNA sequences were evaluated using the basic local alignment search tool (http://www.ncbi.nih.gov/BLAST) and the Finch tv program to determine the presence of mutations.

**4.3. Gene mutations conferring resistance to bedaquiline:**

Mutations possibly related to resistance to BDQ, atpE and Rv0678 were screened by Sanger Sequencing. The atpE and Rv0678 genes were sequenced in 71 MTB isolates including the fully drug-susceptible strains (n = 30) and MDR strains (n = 41). A total of 9 out of 71 (12.7 %) MDR-TB isolates carried a mutation located in the atpE gene.

No mutations within the atpE gene were observed in these fully drug-susceptible isolates. Interestingly, all these Nine bedaquiline-mutant isolates were clinical isolates from patients who were bedaquiline naive, exhibited BDQ MICs values >0.25µg/ml compared with the majority of fully drug-susceptible isolates with MICs lower than 0.25µg/ml, 55.6% (5/9) were phenotypically resistant to bedaquiline, and 44.4 (4/9) were phenotypically sensitive to bedaquiline (see Figure 4.7). 21 (33.9%) of the 62 wild-type isolates had a MGIT MIC of 0.25 µg/mL or less and 41 (66.1 %) of 62 isolates had an MIC of 1 µg/mL or less (see Figure 4.6).

Further sequence analysis revealed that all mutant strains harbored nucleotide substitution in the atpE gene contained one or more atpE variants (see Table 4.6). Overall, 8 variants were observed the 244 base-pair-long coding regions. No insertions or deletions were observed.

No mutation was observed with RV0678 gene in either fully drug-susceptible isolates (n = 30) or MDR isolates (n = 41) isolates.

Table 4.6. Mutations identified in bedaquiline resistance-related the atpE gene.

| Gene | Nucleotide substitution | Mutation type | Strains (N) | MIC (mg/L) |
|---|---|---|---|---|
| atpE | G73A | SNV | 5 | 0.5-2 |
| | G70C and C72G | SNV | 1 | 0.8 |
| | C60G | SNV | 3 | 0.5-3 |
| | G61C | SNV | 2 | 2-3 |
| | A64C | SNV | 1 | 0.5 |
| | C66G | SNV | 1 | 2 |
| | C228G | SNV | 1 | 6.4 |

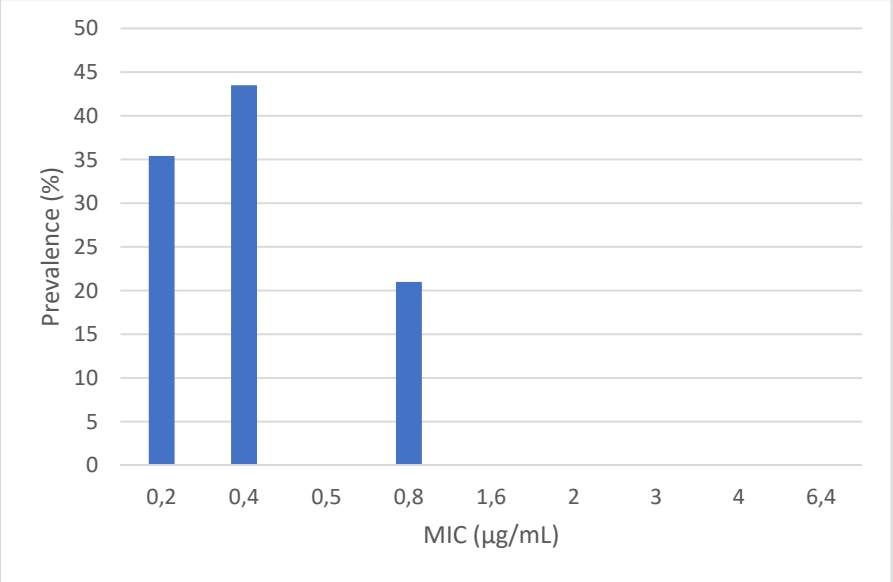


Figure 4.6. MGIT MIC distribution of bedaquiline wild-type samples (62 isolates).

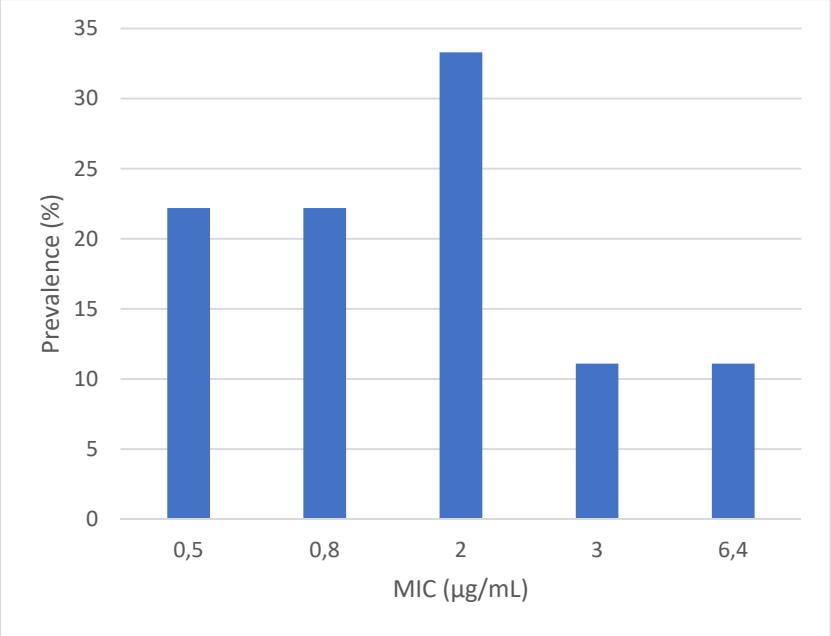


Figure 4.7. MGIT MIC distribution of isolates with one or more atpE variants and wild-type Rv0678 (9 isolates).

**4.4. Mutations conferring DMD resistance:**

We sought to identify mutations in delamanid resistance-related genes, we analyzed the nucleotide sequences of 71 MTB isolates 71 MTB isolates including the fully drug-susceptible strains (n = 30) and MDR strains (n = 41), their fbiA (Rv3261) and ddn (Rv3547) genes had sequenced by Sanger Sequencing.

The results are summarized in Table 4.7. We found one mutation in ddn gene. A total of 1 out of 71 (1.4%) fully drug-susceptible TB isolates carried a mutation located in the ddn gene, was phenotypically susceptible to delamanid and exhibited DEL MICs values 0.16 µg/ml.

Mutant strain harbored nucleotide substitution in the ddn gene contained one ddn variants (see Table 4.4). No mutations within the fbiA gene were observed in MDR and fully drug-susceptible isolates. 68 (97.1%) of the 70 wild-type isolates had a MGIT MIC of 0.2 µg/mL or less and 2 (2.9%) of 70 isolates had an MIC of 0.32µg/mL (see Figure 4.8).

Table 4.7. Mutations identified in Delamanid resistance-related ddn.

| Delamanid | Gene | Nucleotide substitution | Mutation type | Strains (N) | MIC (mg/L) |
|---|---|---|---|---|---|
| susceptible | ddn | **G242A(**Gly81Asp) | SNV | **1** | 0.16 |

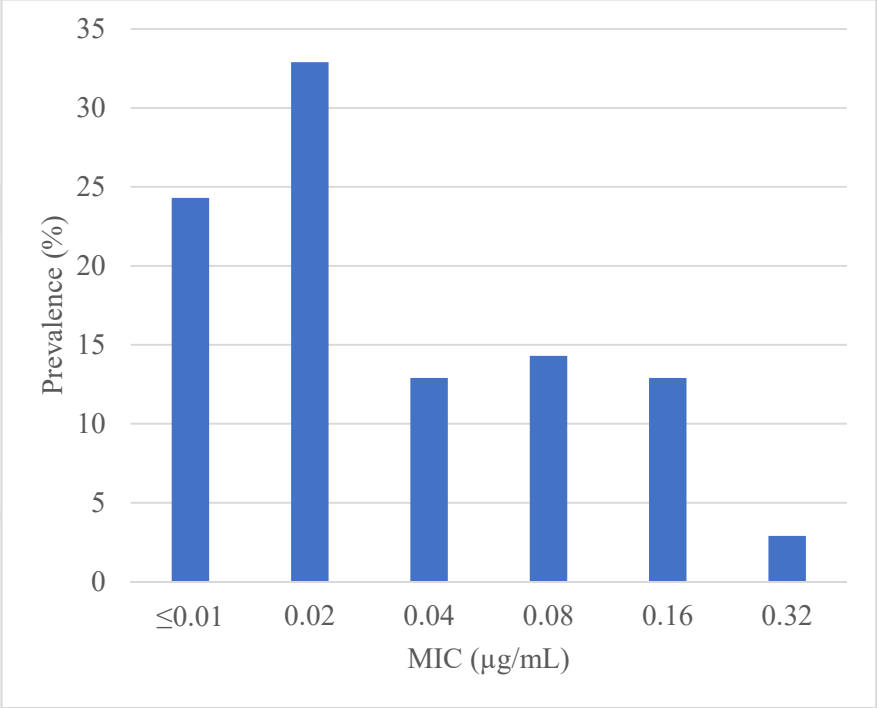


Figure 4.8. MGIT MIC distribution of Delamanid wild-type samples (70 isolates).

**4.5. Mutations conferring LZD resistance:**

To investigate the mutations associated with LZD resistance, we examined, rplC, and rrl among 71 MTB isolates including the fully drug-susceptible strains (n = 30) and MDR strains (n = 41). No mutations within two genes were observed in MDR and Fully drug-susceptible isolates. 66 (93%) of the71wild-type isolates had a MGIT MIC of 1 µg/mL or less and 5 (7.0%) of 71 isolates had an MIC of 1.6- 2 µg/mL (see Figure 4.9).

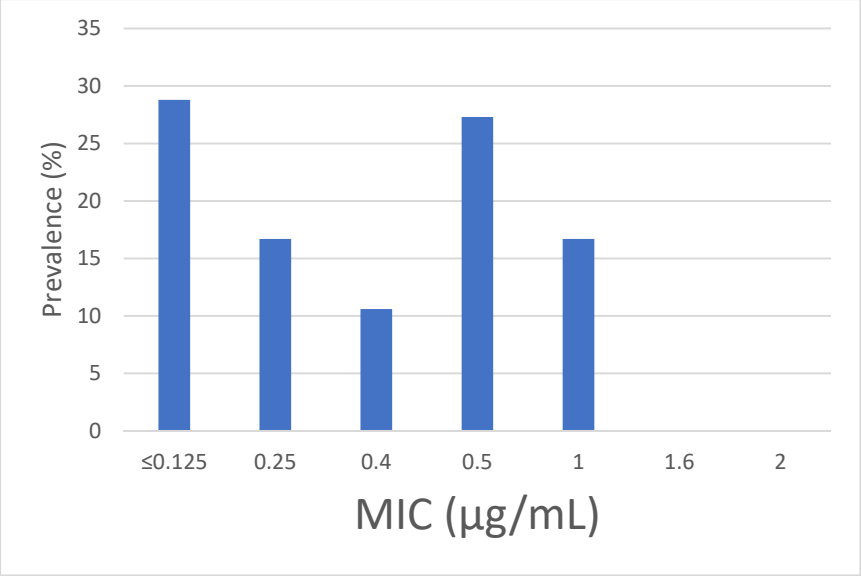


Figure 4.9. MGIT MIC distribution of Linezolid wild-type samples (66 isolates)

The development of BDQ and DLM has increased the number of therapy options available to patients with pulmonary RR-, MDR-, and XDR-TB, including children with MDR-TB (Organization 2016, Achar, Hewison et al. 2017).

Identifying BDQ and DLM resistance is crucial for treating DR-TB patients and stopping the spread of the disease. Despite growing evidence for the efficacy of established and standardized processes for MICs and the placing of breakpoints for BDQ and DLM, there is space for improvement (Organization 2018, Rancoita, Cugnata et al. 2018, Meehan, Goig et al. 2019, Kaniga, Aono et al. 2020). However, there is a clear lack of sufficient information on genetic variants associated with resistance (Köser, Maurer et al. 2019).

On top of that, phenotypic techniques are too time-consuming to provide a prompt evaluation of susceptibility status at the time therapy is commenced. Correctly classifying genetic variants according to their association with drug resistance is necessary to give interpretable molecular DST for BDQ and DLM and to steer the development of treatment regimens (Cabibbe, Walker et al. 2018,

Andres, Merker et al. 2020).

The correlation analysis between mutations connected to resistant phenotypes is supported by a meta-analysis showing no connections between BDQ or DLM resistance and strain lineage or drug resistance profiles of MTBc isolates. According to DST profiles, the great majority (68.7%) of BDQ- and DLM-resistant strains were from newly diagnosed TB patients globally. Additionally, only 6% were MDR-TB (Schena, Nedialkova et al. 2016, Villellas, Coeck et al. 2017).

More than 50 countries are now making use of BDQ. Given the limited treatment options for those with MDR/XDR-TB, the release of this innovative class of antimycobacterial medication has been met with great excitement. This study is the first to examine phenotypic and genotypic BDQ susceptibility among treatment-naive *M. tuberculosis* isolates in this area.

There is still a great deal of confusion around the genetics of bedaquiline resistance. Improper diagnostics for tracking drug resistance after drug introduction might lead to an increase in untreatable patients. For bedaquiline to be used effectively in the future, we need quick molecular biology procedures that are based on accurate phenotypic drug susceptibility testing.

The purpose of this research was, therefore, to examine the in vitro susceptibility to bedaquiline of MTB isolates from South Turkey and to identify gene changes leading to resistance to bedaquiline. Disagreements were found between the acquired results and those of other research, which may be due to variations in experimental circumstances, susceptibility profiles of the isolates, sample size, statistical method, and other aspects.

A total of 71 strains that remained viable and were free of contamination after selection and passage isolated from clinical samples of TB patients from January 2021 to October 2022 sent to the Cukurova University Tropical Disease Research and Application Center, Ministry of Health, Public Health Institution, Adana Regional Tuberculosis Laboratory from eight different cities in the Cukurova Region of South Turkey.

The World Health Organization has recommended for the use of successive minimum inhibitory concentration (MIC) tests to detect BDQ resistance in the absence of particular medication susceptibility testing.

To meet the guidelines set out by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the following MIC cutoffs apply to BDQ: resistant > 0.25 mg/ L; susceptible 0.25 mg/ L (Cholo, Mothiba et al. 2016, Dookie, Rambaran et al. 2018). It is crucial to develop standardized and reproducible methodologies for in vitro DST concurrently with the introduction of new antimicrobials in order to halt the fast development of drug-resistant bacteria. DST in liquid medium yields results in,10 days on average, which is 3 weeks faster than the agar-based proportion technique, allowing for earlier discovery of drug resistance, earlier adjustment of treatment, and earlier cessation of transmission of drug-resistant strains of TB. In light of these factors, it has been created standard methods for bedaquiline, delamanid and Linezolid DST within the MGIT (Campanerut, Ghiraldi et al. 2011).

To investigate the in vitro susceptibility to bedaquiline among MTB isolates, A total of 71 including culture isolates of the fully drug-susceptible strains (n = 30) and MDR strains (n = 41), phenotypic DST was performed using semiautomated MGIT 960 system and EpiCenter software equipped with a TB eXiST module for quantitative drug susceptibility testing, which was considered the reference method for bedaquiline DST, and bedaquiline resistance genes were sequenced for 71 MTB isolates.

In this study, MIC values for bedaquiline against *M. tuberculosis* H37Rv reference strain were 0.4 mg/liter, indicating that all batch test results passed quality control testing, based on WHO breakpoint 1mg/L for bedaquiline 65 (91.5%) clinical strains were susceptible, and 6 (8.45%) were resistant to bedaquiline, all resistant strains were MDR, 6 out of the 41 MDR-TB strains (14.6%) were resistant to bedaquiline; this rate of bedaquiline resistance is higher than that reported by Hu et al. (6.6%) (Hu et al., 2019). However, using the

EUCAST preliminary criteria breakpoint of 0.25 µg/ml to testing for MGIT would result in 70.4% (50/71) of isolates being falsely resistant, necessitating method-specific criteria. The WT MIC in MGIT960 ranged from ≤0.25 to 0.8 mg/L.

Our results are comparable to those of Torrea et al and Ismail, Omar et al, who established 1 mg/L. as the ECV for MGIT in a small number of MDR-TB isolates (Torrea, Coeck et al. 2015, Ismail, Omar et al. 2018), whereas Keller et al calculated the value to be 1.6 g/ml using crushed tablets (Keller, Hömke et al. 2015). When looking at the WT MIC distributions, MGIT960 had WT MIC values that were four times greater than BACTEC 460 (Andries, Verhasselt et al. 2005). This might be because of the fluorescent component embedded in silicone at the bottoms of MGIT tubes or the polymers used in their construction (Torrea, Coeck et al. 2015).

Cases of clinical failure of BDQ-based therapy regimens have been documented from all around the globe in recent years (Keller, Hömke et al. 2015), mutations in the Rv0678 gene encoding an efflux pump pathway were found to account for resistance in the vast majority of cases (Hartkoorn, Uplekar et al. 2014, Somoskovi, Bruderer et al. 2015). The study conducted by Villellas et al. in Belgium found that a high percentage of MDR-TB patients who had never been exposed to CFZ or BDQ had mutations in the Rv0678 gene (Villellas, Coeck et al. 2017). In contrast, in the current analysis, we found no mutations in the Rv0678 gene among the completely drug-susceptible (n = 30) or MDR (n = 41) populations. Finding no mutations in the Rv0678 gene, our results are consistent with those of the research by Singh, Soneja et al. and Ghajavand, Kargarpour Kamakoli et al (Ghajavand, Kargarpour Kamakoli et al. 2019, Singh, Soneja et al. 2020).

Although yang, Pang et al. stated that a mutation in the RV0678 gene is a main factor leading to bedaquiline resistance, we could not find any such mutation in our investigation (Yang, Pang et al. 2020).

Prior CFZ exposure enhances BDQ resistance linked with Rv0678 and pepQ gene mutations, as shown by several experimental and clinical trials (Hartkoorn, Uplekar et al. 2014, Almeida, Ioerger et al. 2016).

Despite the fact that all BDQ-resistant strains were also resistant to CFZ, only a third of CFZ-resistant bacteria were also resistant to BDQ, as noted by Tiberi et al. Potentially significant implications for BDQ resistance as a "surrogate marker" for CFZ resistance (Tiberi, Cabibbe et al. 2018).

As a result of this, we did not do CFZ DST in the present inquiry to confirm the same thing. Mutations in the intergenic region mmpS5-Rv0678, alterations in cell wall permeability, and the expression of genes that regulate the two-component system have all been proposed as potential resistance mechanisms (Zhou, Yang et al. 2015, Villellas, Coeck et al. 2017).

Considering that not a single bedaquiline-resistant isolate has the requisite Rv0678 mutation. Further experimental investigation into the mechanism(s) through which these isolates may be resistant to bedaquiline is urgently needed.

For new drugs like bedaquiline, understanding both target and nontarget processes for lowering medication resistance is essential. This information might be useful for both drug susceptibility testing (DST) and a drug susceptibility monitoring program.

In the present study mutations associated with atpE gene linked with BDQ drug target site were 9 out of 71 (12.7 %) MDR-TB isolates carried a mutation located in the atpE gene. No mutations within the atpE gene were observed in these fully drug-susceptible isolates, suggests a role of prior exposure to TB drugs. Interestingly, all these Nine bedaquiline-mutant isolates were clinical isolates from patients who were bedaquiline naive**,** exhibited BDQ MICs values > 0.25µg/ml compared with the majority of fully drug-susceptible isolates with MICs lower than 0.25µg/ml. 55.6% (5/9) were phenotypically resistant to bedaquiline 44.4% (4/9) were phenotypically sensitive to bedaquiline.

The high prevalence of atpE RAVs in baseline isolates is especially striking given that none of these MDRTB patients had ever been treated with bedaquiline, based on the medical history of these people, it's feasible that some of them contracted an infection caused by bacteria resistant to the antibiotic bedaquiline. Since bedaquiline has just been given the green light, it's safe to assume that TB patients haven't acquired a resistance to the drug. In contrast, bedaquiline resistance may develop spontaneously, even in the absence of prior exposure to the drug (Villellas, Coeck et al. 2017, Jian, ZHANG et al. 2019). Therefore, investigating MTB isolates' level of bedaquiline resistance is important.

Further sequence analysis revealed that all mutant strains harbored nucleotide substitution in the atpE gene contained one or more atpE variants.

Overall, 8 variants were observed the 244 base-pair-long coding regions. No insertions or deletions were observed.

The fact that mutations in the atpE gene conferring bedaquiline resistance have been reported by other researchers. Our findings regarding atpE mutations were distinct from those found by others (Segala, Sougakoff et al. 2012, Andries, Villellas et al. 2014).

An overwhelming majority of the bedaquiline-resistant strains had the same mutation, 73G→A was previously reported by Ismail, Rivière et al. 2021, as associated with bedaquiline resistance in *M. tuberculosis* clinical strains (Ismail, Rivière et al. 2021).

In sequencing, we have found types of mutation in atpE gene at position G70C, C72G, C60G, G61C, A64C, C66G, C228G, which have not been reported in previous studies. The clinical significance of this new mutations needs to be determined.

atpE mutation were not only identified in isolates with high baseline MICs: 44.4% of the isolates (4/9) were phenotypically sensitive to bedaquiline, had similar Resistance-associated variants (RAVs), not resulting in bedaquiline MICs exceeding the bedaquiline breakpoint, suggesting a role of additional genes in

determining the bedaquiline MIC.

In total, RAVs in atpE were observed in 55.6% (5/9) of the MDR-TB isolates and they were associated with bedaquiline MICs exceeding the breakpoint in only 5 out of 9 cases (5 out of 41).

It is alarming that 8.5% of patients without prior exposure to bedaquiline or clofazimine had high MICs (MIC > ECOFF) to bedaquiline. More surveillance studies are needed to better understand the cause of these high MICs and how they affect treatment results.

In a set of in vitro-selected isolates, the frequency of target-based (atpE) and nontarget-based (Rv0678) RAVs for bedaquiline was comparable, but the latter are more likely to emerge first, as efflux-based mutations generally result in lower levels of resistance (2 to 16-fold higher MICs) than target-based mutations (16 to 1000-fold higher MICs) (Huitric, Verhasselt et al. 2010).

This raises the possibility that non-target-based resistance will serve as a stepping stone to higher-level, target-based resistance in patients whose treatment regimen is failing.

Because bedaquiline resistance is possible among TB patients in Turkey, DST should be performed when the drug is included into the treatment regimen. Mutations in atpE conferring resistance to bedaquiline were detected in 44.4% (4 of 9) of isolates that were phenotypically sensitive to bedaquiline, leading us to hypothesize that exposure to anti-TB agents other than clofazimine may have played a role in inducing the occurrence of these mutations. Naturally, this requires more experimental testing.

This study identifies novel atpE gene variants associated with BDQ in MDR-TB isolates. Despite the low frequency of atpE and gene alterations in this investigation, as well as the absence of mutation in the Rv0678 gene, a larger sample size with a wider range of strains is needed to assess the pattern of resistance to this innovative therapy.

An overview of the in vitro sensitivity of the MTB isolates in this investigation to bedaquiline. It is unclear if this resistance is the consequence of treatment with other anti-TB drugs that produce bedaquiline resistance through atpE mutations, or whether it is the product of spontaneous genetic diversity. This study was the first to examine the molecular characteristics and in vitro susceptibility of MTB isolates to bedaquiline in South Turkey.

Against MTB isolates from southern Turkey, the lab data show that bedaquiline has strong antibacterial effect. As an added note, there was no correlation between treatment history and resistance to bedaquiline. Molecular investigation of resistance-associated genes revealed that alterations to the atpE gene are responsible for bedaquiline resistance in MTB isolates from southern Turkey. Since no bedaquiline-resistant isolates tested positive for Rv0678 mutations, it is clear that identifying hotspots of currently resistance-associated genes is not sufficient to understand the processes by which certain bacteria become resistant to this drug (atpE, Rv0678, and pepQ). As a result, measuring bedaquiline susceptibility at this time is best done using phenotypic DST.

To investigate the MIC distributions of delamanid among MTB isolates, we performed phenotypic DST using a semiautomated MGIT 960 system and EpiCenter software equipped with a TB eXiST module for quantitative drug susceptibility testing and sequencing of drug resistance-related genes for 71 clinical *M. tuberculosis* strains, including culture isolates of the fully drug-susceptible strains (n = 30) and MDR strains (n = 41) using 0.2 mg/L MIC as a cutoff, we found that the vast majority of sensitive strains had a MIC between 0.005 and 0.16 mg/L.

When compared to previous estimates (Keller, Hömke et al. 2015, Schena, Nedialkova et al. 2016, Yang, Kim et al. 2018), this MIC distribution across sensitive strains is different, unlike previous studies and showed different MIC distribution profiles.

The majority of the resistant bacteria in our study had MICs of 0.32 mg/L for delamanid. Our findings are similar to the study by Keller et al reported (Keller, Hömke et al. 2015) that MICs for delamanid >0.32 mg/L were found in three strains, and a different investigation by Yang, Kim et al (Yang, Kim et al. 2018), that reported the majority of delamanid-resistant bacteria were found to have MICs between 0.4 and 0.8 mg/L, but other investigations revealed MICs of >8 mg/L (Schena, Nedialkova et al. 2016)

For delamanid, a genetic analysis of 71 MTB isolates (n 71; 41 MDR, and 30 fully drug-susceptible), from patients with no previous exposure to DLM, we analyzed the nucleotide sequences of their fbiA (Rv3261) and ddn (Rv3547) genes by Sanger Sequencing. One non-silent mutation (Gly81Asp) in ddn was found in delamanid-susceptible strains. Mutant strain harbored nucleotide substitution in the ddn gene and contained one ddn variants.

The positions of nucleotide substitutions were different from those reported by Schena et al (Schena, Nedialkova et al. 2016). Haver et al. (Haver, Chua et al. 2015) the Gly81Asp mutation (MIC=1 mg/L), which is related with drug activation, is particularly noteworthy; a total of 19 mutations were identified, including stop mutations, at different locations in ddn.. This was the single mutation detected in our study. The mutant strain was phenotypically sensitive to delamanid, with MICs of 0.16 g/ml. Our results matched those of Yang, Kim, et al. (Yang, Kim et al. 2018) who also found the Gly81Asp mutation (MIC 1.6 mg/L) and demonstrated a significant level of resistance. However, in our work, we found that the same mutation was present in delamanid-susceptible strains (MIC 0.16 g/ml), therefore the role it plays in drug resistance is unclear.

Since we employed both multidrug-resistant and totally drug-susceptible isolates that were likely never exposed to delamanid, we conclude that this is not the product of acquired resistance

Among the two (2.8%) delamanid-resistant isolates, no ddn mutations were detected. No mutations in the fbiA gene were identified in either sensitive or resistant strains to delamanid.

Although several mutations in fbiA and ddn have been reported in resistant strains, our results were dissimilar from those mutations. Schena et al. (Schena, Nedialkova et al. 2016) the premature stop codon resulting from four different nonsense mutations in fbiA and ddn was discovered.

The Asp49Thr mutation in fbiA was found in a resistant strain in another investigation (Bloemberg, Keller et al. 2015).

The choice to add DLM to the treatment regimen of MDR TB patients must be based on individualized assessments of drug susceptibility information in order to detect the earliest emergence of drug resistance and prevent the spread of resistant isolates (Stinson, Kurepina et al. 2016).

In our investigation, Bdq, Dlmand Lzd showed good in vitro activity against MDRTB more than 85 % of the MDR-TB strains were susceptible, which was comparable with other country's studies (Wang, Jiang et al. 2021).

Our findings support the use of these novel medicines in patients who have experienced treatment resistance. The change in sensitivity to these new medications after widespread usage can only be monitored with access to baseline data on susceptibilities prior to this time. Although resistant genes had been found for each of these innovative medications, the lack of mutation, silent mutation, or very low mutation rates in these genes suggested that the major drug resistance mechanisms for these therapies remain unexplained and need more research.

The minimum inhibitory concentrations (MICs) we found for *M. tuberculosis* H37Rv in our investigation were much higher than those found in the literature (bedaquiline MIC, 0.4 mg/liter; delamanid MIC,.01 mg/liter) (Andries, Verhasselt et al. 2005, Rustomjee and Zumla 2015).

This most likely represents a fundamental methodological difference. The pharmacokinetics and pharmacodynamics (PK/PD) results demonstrate that 99.9% of bedaquiline and delamanid are bound to proteins in the plasma (Rouan, Lounis et al. 2012, Rustomjee and Zumla 2015).

Bedaquiline's minimum inhibitory concentration (MIC) is shown to rise in the presence of 5% bovine serum albumin (Rouan, Lounis et al. 2012). Albumin was often not present in the previous research that examined drug susceptibility Adding OADC (this research) or BD's MGIT growth supplement increased the albumin concentration in the MGIT 960 tube to around 4% (wt/vol), comparable to the physiological plasma protein concentration (Upton, Cho et al. 2015).

Based on a critical concentration of 1 mg/L for linezolid, the MIC for the most susceptible strains was between 0.125 and 0.5 mg/L, while the MIC for the most resistant strains was between 1.6 and 2 g/mL. Two genes (rplC and rrl) in 71 MTB isolates (n=71, 30 fully drug-susceptible strains, 41 MDR strains) had no mutations.

Several previous studies have shown linezolid-resistance-conferring mutations; however, our results vary from all these. Hillemann et al. (Hillemann, Rüsch-Gerdes et al. 2008) linezolid-resistant *Mycobacterium* TB mutants selected in vitro include nucleotide substitutions at positions 2016 and 2576 in rrl, according to their research. Beckert et al. (Beckert, Hillemann et al. 2012) in vitro-selected mutants and clinical isolates both had a T460C mutation in rplC, and this mutation was linked to high linezolid MIC values (>2 mg/L) (Zhang, Chen et al. 2016).

Our findings were similar to those found in a study that was conducted by Yang, Kim, and colleagues (Yang, Kim et al. 2018). The MICs for the most sensitive strains were found to be between 0.25 and 0.5 mg/L, and whereas nucleotide alterations in rrl and rplC were present in the sensitive strains, they were not in the resistant strain.

Non-ribosomal mutation mechanisms may be responsible for LNZ resistance in *M. tuberculosis*, as the development of resistant isolates without mutations suggests. Consequently, the low association between the LNZ-resistant phenotype and target gene mutations conferring LNZ resistance demonstrates that molecular detection of LNZ resistance by scanning these target genes is not suitable for target design for molecular drug resistance detection in MDR-TB strains circulating in Turkey under current conditions.

This research concludes that the frequency of LNZ-resistant isolates among MDR-TB isolates circulating in Turkey is 12.1%. The inadequate association between mutant genotypes and an MDR phenotype demonstrates the urgent need to discover a previously unknown mechanism for LNZ resistance.

Before initiating treatment with these new drugs, it is crucial to do phenotypic DST, including the measurement of MICs and perhaps sequencing, to assess the baseline resistance levels. In addition, both delamanid and bedaquiline must be subjected to frequent and repeated quality-controlled DSTs prior to treatment.

Although there were certain limits to our research, such as inadequate sample size is one of the study's limitations, the need to analyze a larger sample set comprised of different strains, we consider that the AST findings we obtained are reliable. which enable a discrimination to be made between populations of wild types and those of resistant types.

Our results demonstrate that standard clinical laboratories may use MGIT 960s equipped with TB eXiST to perform AST for bedaquiline, delamanid, and linezolid, as also shown by previous studies (Keller, Hömke et al. 2015).

Both multidrug-resistant and totally drug-susceptible isolates that were likely never exposed to these three drugs were employed in this study. Since no evidence of acquired resistance in the form of high MIC values was found, no causal link between the identified mutations and resistance can be established.

The findings of this study demonstrated that the drug-sensitive *M. Tuberculosis* H37Rv strain grown in the lab and MDR-TB isolates were effectively killed by BDQ, DEL, and LZD, even at extremely low drug doses.

We did not assess clinical isolates having acquired resistance to the three medicines, which is a limitation of our study.

Furthermore, we were able to calculate the MICs for three anti-TB drugs in MDR and fully drug-susceptible bacteria and identify a few resistant strains. To establish the genetic basis and identify the underlying mechanisms of resistance to these anti-TB treatments, more research is required.

# 5. CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions

Based on the results of our investigation, in which we showed that the drug-sensitive H37Rv strain generated in the lab and MDR-TB isolates were successfully killed by BDQ, DEL, and LZD, even at very low drug dosages, we think that the AST results we got are trustworthy. which allow for a differentiation between wild type populations and resistant type populations. Our findings, along with those of other research, reveal that MGIT 960s equipped with TB eXiST may be used by routine clinical labs to conduct AST for bedaquiline, delamanid, and linezolid (Keller, Hömke et al. 2015).

In our investigation, Bdq, Dlm and Lzd showed good in vitro activity against MDRTB more than 85% of the MDR-TB strains were susceptible, which was comparable with other country's studies (Wang, Jiang et al. 2021).

Our findings support the use of these novel medicines in patients who have experienced treatment resistance. Phenotypic DST, including the measurement of MICs and perhaps sequencing, must be performed before to starting therapy with these new medications in order to determine the baseline resistance levels. More so, prior to therapy, delamanid and bedaquiline need to be exposed to frequent and recurrent DSTs under quality control.

## 5.2. Recommendations

Patients with multidrug- and extensively-resistant tuberculosis (MDR/XDR TB) have a lower chance of recovering from their illness after receiving therapy. A three of the antituberculosis drugs we tested are effective in treating patients with both multidrug- and extensively-resistant strains of the disease. If we are serious about stopping the abuse of antimicrobial drugs, we must keep doing phenotypic drug susceptibility testing on them.

More study is needed to confirm the genetic basis of drug resistance to anti-TB drugs and to determine the underlying mechanisms of this phenomenon.

Our findings support the use of these novel medicines in patients who have experienced treatment resistance. The change in sensitivity to these new medications after widespread usage can only be monitored with access to baseline data on susceptibilities prior to this time.

Although resistant genes had been found for each of these innovative medications, the lack of mutation, silent mutation, or very low mutation rates in these genes suggested that the major drug resistance mechanisms for these therapies remain unexplained and need more research.

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**CURRICULUM VITAE**

Huda Ali MohamedAli Ahmed Sudanese in Nationality, finished my Primary and Middle School in Khartoum in 2003 and finished my High school in Khartoum city 2006, Attended to Medical Microbiology department/ College of Medical laboratory Science in Al -Neelain University/Sudan and five years to graduate in 2011 as a Microbiologist.

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