CUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

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COMPARISON OF GENE EXPRESSION PROFILES BETWEEN HIGH AND LOW DOSE ISONIAZID RESISTANCE IN MULTIDRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

DEPARTMENT OF BIOTECHNOLOGY

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ABSTRACT

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COMPARISON OF GENE EXPRESSION PROFILES BETWEEN HIGH AND LOW DOSE ISONIAZID RESISTANCE IN MULTIDRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS

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Tuberculosis (TB) is one of the oldest serious infection disease in developing countries caused by Mycobacterium tuberculosis complex (MTBC). The aim of the current study is to see the effect of low (0.1 μg/ml) and high (0.4 μg/ml) doses of INH respectively on MDR and sensitive *M. tuberculosis* complex strains by investigating the expression levels on 18 efflux pump genes. *polA* was considered as a housekeeping gene to see the effectiveness of the drug in the two different doses in comparison with Pan-sensitive isolates. In addition to that to investigate the relation between the efflux pump genes expression levels and the mutations on four genes *katG, rpoB, inhA and oxyR-ahpC* on *M. tuberculosis* complex isolates. It was found that eight of the efflux pump genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB* and *Rv2994* were overexpressed in 100% of the isolates when comparing the three groups with the sensitive isolates, however there were no significant differences among these three groups, we also found that 100% of the isolates which have mutations on the genes *rpoB, katG* and *inhA* were overexpressed.

Key words: Tuberculosis, efflux pump, Isoniazid, Susceptibility, Isoniazid resistance

ÖZ

DOKTORA TEZİ

ÇOKLU İLAÇ DİRENÇLİ MYCOBACTERİUM TUBERCULOSİS COMPLEX SUŞLARINDA YÜKSEK VE DÜŞÜK DOZ İZONİAZİD DİRENCİ ARASINDAKİ GEN İFADE PROFİLLERİNİN KARŞILAŞTIRILMASI

Hassan Thamer Shatub SHATUB

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BİYOTEKNOLOJİ ANABİLİM DALI

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Tüberküloz (TB), *Mycobacterium tuberculosis* kompleksinin (MTBC) neden olduğu gelişmekte olan ülkelerdeki en eski ciddi enfeksiyon hastalıklarından biridir. Bu çalışmanın amacı, sırasıyla düşük (0,1 µg / ml) ve yüksek (0,4 µg / ml) INH dozlarının MDR ve duyarlı *M. tuberculosis* kompleks suşları üzerindeki etkisini 18 eflux pompası geni üzerindeki ekspresyon seviyelerini araştırarak görmektir. *polA*, Pan-sensitive izolatlara kıyasla ilacın iki farklı dozdaki etkinliğini görmek için bir Housekeeping geni olarak kabul edildi. Buna ek olarak, *M. tuberculosis* kompleks izolatlarında eflux pompası gen ekspresyon seviyeleri ile *katG, rpoB, inhA* ve *oxyR-ahpC* olmak üzere dört gen üzerindeki mutasyonlar arasındaki ilişkiyi araştırmak. Üç grup hassas izolatlarla karşılaştırılırken izolatların % 100'ünde efflux pompası genlerinden *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB* ve *Rv2994* genlerinden sekizinin aşırı eksprese edildiği, ancak anlamlı olmadığı bulundu. Bu üç grup arasındaki farklılıklar, *rpoB, katG* ve *inhA* genleri üzerinde mutasyonlara sahip izolatların% 100'ünün aşırı eksprese edildiğini de bulduk.

Anahtar Kelimeler: Tüberküloz, Eflüks pompa, Isoniazid, Duyarlılık, Isoniazid direnci

EXPANDED ABSTRACT

Tuberculosis (TB) disease occurs in every part of the world and large numbers of cases from all over the world has been noted. About one third of the population is infected with TB, and every year, millions of people die. The disease which is caused by *Mycobacterium tuberculosis* complex (MTB), which usually attacks the lungs but can infect other organs as well. The emergence of multidrug resistance (MDRTB) and extensive drug resistance (XDRTB) strains in the treatment of this disease, which remains a critical but largely unresolved problem, is a major problem in the current situation. MDR-TB refers to TB bacteria resistant to first-line antibiotics such as isoniazid and rifampicin, whereas XDR-TB refers to TB bacteria resistant to fluoroquinolone (FQ) in addition to isoniazid and rifampicin and to at least one of the second-line medicines such as amikacin, kanamycin or capreomycininin (Francis *et al.*,2014).

The growth of bacterial resistance has various mechanisms involved. For example, complex cell walls are an effective way to prevent a drug from reaching its target and being absorbed by the cell. Bacteria do this by altering their membranes' penetrability or reducing the number of channels available for drugs to flow through (Brennan *et al.*,1995). For many antibiotics, another mechanism involves drug-modifying & inactivating enzymes that work by sticking to their target and preventing them from interacting within the cell with other molecules. Some types of bacteria, for instance, produce enzymes called betalactamases that degrade penicillin (Lomovskaya *et al.*,2001). Some bacteria react by modifying the target's structure (or even replacing it completely with another molecule) so that the antibiotic can no longer recognize or bind to it (Lambert *et al.*,2005). By getting a copy of a gene encoding an altered protein, even from those of a different species, spontaneous mutations in the bacterial genome can also acquire resistance. FQ target, DNA gyrase required as a DNA supercoiling enzyme with subunits and substitution results in mutation in these subunits (Ramaswamy *et al.*,2004).

According to the World Health Organization (WHO), there were approximately 10.0 million new cases of TB reported and 1.3 million HIV-negative deaths (range, 1.2-1.4 million) among HIV-negative people, and there were an additional 300 000 TB deaths (range, 266 000-335 000) among complicated TB co-infection with HIV-positive people worldwide in 2017.

The best estimates for 2017 were that 90% of cases were adults (aged about 15 years), 64% were male, 9% were people living with HIV. There are different mechanisms involved in the development of resistant bacteria, including complex cell wall is an effective way to keep a drug from reaching its goal and preventing it from being taken up by the bacterial resistance (Munita and Arias,2016). Some bacteria react by modifying the target's structure (or even replacing it completely with another molecule) so that the antibiotic can no longer recognize or bind to it (Lambert *et al.*,2005). By obtaining a copy of a gene encoding an altered protein even from those of a different species FQ target, DNA gyrase that is required as a DNA supercoiling enzyme with subunits and substitution in these subunits results in mutation is a major concern in the control of the global TB epidemic. Spontaneous mutations in the bacterial genome can also acquire resistance. Due to a lack of extrachromosomal DNA or plasmids, *M. tuberculosis* does not acquire drug resistance through horizontal gene transfer. Most resistance is due to high- or low-level resistance mutations in drug-targeting genes; however, these mutations do not account for resistance in all strains, indicating that there are other mechanisms such as drug-inactivating enzyme production, inactive derivative formation, target alteration, drug entry inhibition in the cell, and efflux pump extrusion (Hameed et al, 2018).

Membrane proteins that are capable of actively transporting a wide range of substrates, including drugs, from the cytoplasm to the outside of the cell are bacterial efflux pumps. They are involved in physiological processes, such as division of the cell wall, pH homeostasis maintenance, and intracellular metabolite secretion. A low-level-resistant phenotype is provided by up regulation of efflux

pump genes, and it has been suggested that bacteria have higher drug resistance levels under these circumstances. The inhibition of efflux pumps, which would also restore the efficacy of antimicrobials that are subject to efflux, would be a method to stop this chain of events. A few efflux pumps have been defined in *M. tuberculosis*, but their contribution to clinical drug resistance remains to be fully clarified. Of the small multidrug resistance (SMR) family of transporters, Mmr (Rv3065) is one of these pumps (Rodrigues *et al.*,2012).

The aim of the current study was to evaluate the expression in clinical isolates of multidrug-resistant (MDR) *M. tuberculosis* of 18 putative efflux pump genes in considering of *polA* as a house-keeping gene. These genes have been annotated in the as likely/hypothetical drug efflux genes. These 18 genes were examined to determine the expression in MDR *M. tuberculosis* Clinical isolates after the growth were occurred on Control, high 0.1 μg/ml INH and low 0.4 μg/ml INH tubes as a sign to be resistance in high INH dose in *M. tuberculosis* Clinical isolates.

The research will further enhance our understanding of the mechanism of active efflux in the resistance of *M. tuberculosis* to INH and RIF and is the first study of the expressional differences between clinical isolates of MDR in two different doses (low and high) INH in comparing to efflux pump expression levels in isolates of pan-sensitive. Moreover, profiles of Resistance of *M. tuberculosis* Susceptibility tests were determined by using the BACTEC MGIT 960 system, and target-gene mutations such as, *katG* and *inhA* regulator sequences and *oxyR-ahpC* genes for INH *rpoB* for the RIF (reported to carry major INH and RIF resistance related mutations) were analyzed for resistance in *M. tuberculosis*.

Statistic analysis were done by SAS 9.1 software at p 0.05 to recognize that there were insignificant differences among the studied groups (Control, high and low doses INH) when comparing with pan-sensitive isolates and for all the efflux pump genes except *emrB* which was showing significant differences when p 0.05 and in comparing between control group and the other two groups according to

sensitive isolates as there were significant decrease in the two o.1 μg/ml and o.4 μg/ml INH when p 0.05.

As a result of genotypic resistance tests, mutations are often (n=24/28) detected in codon 531 in rpoB gene for rifampicin resistance, also a mutation in 526 were detected in one isolate, while for *katG* gene the mutations were often (n=25/28) found on codon 315 for isoniazid resistance, the mutation C15T were found on the gene *inhA* (n=13/28), and no mutation were found on *oxyR-ahpC* gene. In the current study we found that eight of the efflux pump genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB* and *Rv2994* were overexpressed in 100% of the isolates when comparing the three groups with the sensitive isolates, however there was no significant differences among these groups. Although we did not find a significant difference among the INH resistant groups, but we still agree that the efflux pump gene expression plays an important role to become resistant-TB.

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CONTENTS **PAGE**

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

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
qPCR	: Real-time quantitative PCR
RIF	: Rifampin/Rifampicin
RR-TB	: Rifampicin-resistant tuberculosis
SNP	: Single nucleotide polymorphism
TB	: Tuberculosis
TE	: Tris/EDTA
µg	: Micrograms
µl	: Microlitres

WHO	: World Health Organization
XDR TB	: Extensively drug-resistant tuberculosis
ZN	: Zielh Neelsen

1. INTRODUCTION

1.1. Global Tuberculosis History and Current Status

Historically, TB is an elderly disease known to present an enormous global health problem at the moment. Historically, through several studies of human remains, human traces have been infected with a historic pathogen called *Mycobacterium tuberculosis*, which is really the main potential cause of TB, for over hundreds of years. The disease also was identified from Hippocrates through from the 1800s as Phthisis and Consumption, the white death and the great white plague during the 19th century (Hershkovitz et al, 2015).

Pulmonary TB is one of most common bacterial infections, which can spread easily by coughing, sneezing or spitting and pushing or dispersing TB germs into the air (Smith,2003). In order for one person to become infected, it is only appropriate to inhale a few or some of these germs. Around 1/4 of the population worldwide is contaminated with TB. There is a lifetime chance of 5-15% TB for people infected with TB bacillus. However, for people living with HIV, people with compromised immune systems, for instance (Mohajan, 2015).

A few parameters can clarify this epidemiological problem, such as social and health situations, the correlation with HIV/AIDS (1.2 million of patients are co-infected by MTB/HIV) (Nerlich and Losch, 2009), the movement of populations and the presence of multidrug-resistant variants (Hiroshi, 2009). Tuberculosis occurs for both genders and in all age ranges and can affect almost all of the body's organs (Chaisson *et al.*,2003).

Since the first drug with anti-mycobacterial activity was found, dramatically changing took a place in the history of TB. The introduction of Streptomycin in 1943 for the therapy of TB resulted in a reduction in TB-related death rates. Shortly afterward, particularly in 1948, the very first antibiotic for the therapy of TB was administered. However, antibiotic resistance evolved largely

due to the use of streptomycin as monotherapy shortly after the first antibiotic was used in 1943. (WHO TB report 2020)

TB continues to be among the most destructive infectious diseases with HIV and malaria, and according to World Health Organization, as well as TB is the world's highest and ranks as the world's second largest cause of infectious disease deaths coming after Human Immunodeficiency Virus (HIV) or is also listed as one of the top ten causes of death and also the major cause of mortality. With the development of many other medications with anti-TB activity worldwide and much more recently, multidrug therapy has become important for disease control by Enabling the healing of patients and disrupting the transmission chain. The observation of Robert Koch led the way for the creation of the skin tests of Pirquet and Mantoux tuberculin, the BCG vaccine of Albert Calmette and Camille Guérin, streptomycin of Selman Waksman and other anti-tuberculous drugs.

The ratio of TB incidence is decreasing in the global, still not quick enough to achieve the first milestone of the End TB Strategy; that is, a reduction of 20 percent between 2000 and 2020 (Figure 1.1). Between 2015 and 2019, the overall decline worldwide was 9 percent (from 142 to 130 new cases per 100,000 population), such as a 2.3 percent reduction during 2018 and 2019.

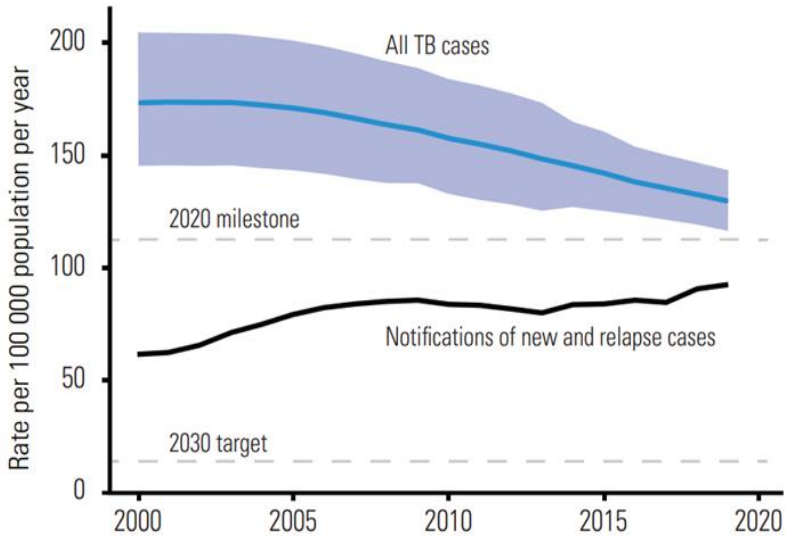

Figure 1.1. Global estimated TB incidence rate (WHO report 2020)

Moreover, the WHO European Area has come nearly to the 2020 target, declining by 19 percent from 2015 to 2019, while the Africa has made strong strides, achieving a reduction of 16 percent in just a few years. The decreases in other regions worldwide were 3.5%, 8.7%, and 6.1%, respectively. In the WHO Area of the Americas, incidence is growing in Brazil.

78 countries in the world are expected to hit the 2020 mark. This includes seven regions that have reported the highest rates of people with tuberculosis (TB) along with three others that are in progress to achieve it (Lesotho, Myanmar and Zimbabwe). In 2019, 54 countries were at low risk of tuberculosis infection, mainly in the WHO area of the Americas and European area, with a few countries in the Eastern Mediterranean and Western Pacific areas (Figure 1.2).

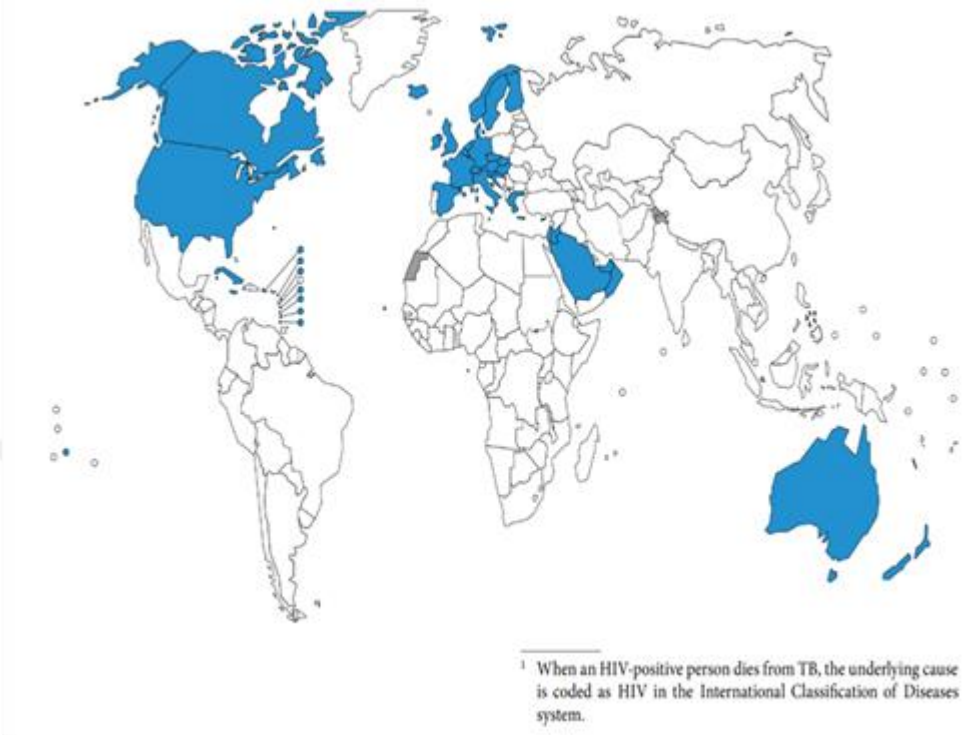

Figure 1.2. Countries TB incidence rate of less than 10 per 100 000 population (WHO 2020 report)

These countries are very well positioned to meet the goal of tuberculosis eradication. In 2019, a staggering 10.0 million people became ill with tuberculosis. In total, 56% were men, 32% were women and 12% were children (aged <15); overall, 8% of people with TB were living with HIV. 30 highest Incidence burden countries account for 87% of the total global cases; and eight countries account for around 70 % of the total global cases (Figure 1.3.)

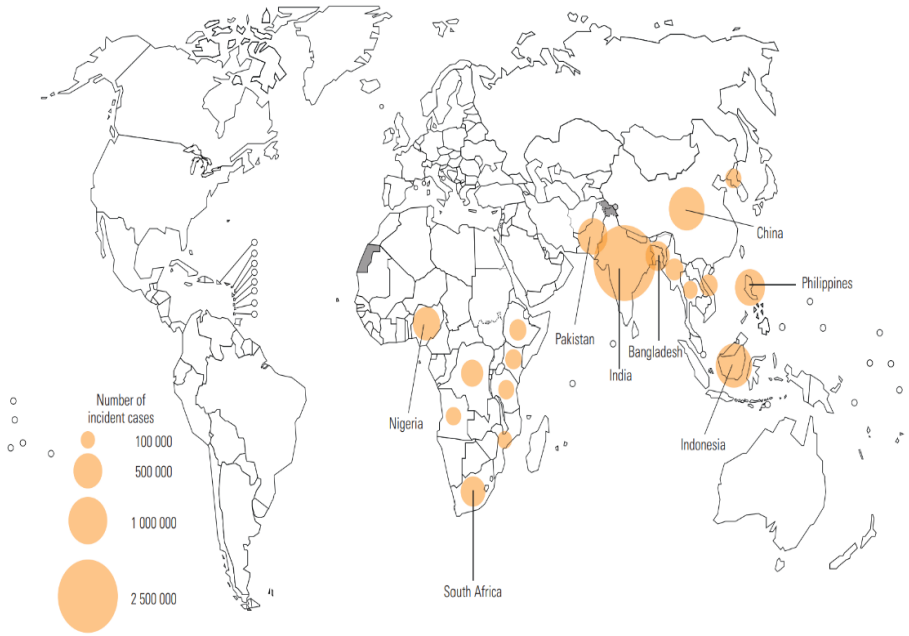

Figure 1.3. Eight countries that ranked first to eight in terms of number of TB cases and accounted for two-thirds of global cases in 2019. (WHO report 2020)

TB is the world's leading killer of infectious diseases and one of the 10 leading causes of death. In 2019, 1,4 million people were killed, including 208 000 people with HIV. Though tuberculosis is difficult to treat, it takes 6 months of a combination of medications which will increase the chances of drug-resistance. It is known as the drug-resistant TB, generally referred to as Multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). the recent emergence of multi drug resistant (MDR) tuberculosis is a serious problem which potentially threaten therapeutic control of tuberculosis. Contrary to global TB rates, the emergence of MDR and XDR-TB infections continues to be a major concern. is one of the greatest problems for global tuberculosis prevention efforts.

There are many mechanisms that lead to the creation of bacterial resistance, including an excess of cell wall surfaces through which a drug will not be able to get into a cell. Bacteria modify their cell membrane towards improving drug permeability (Pal et al.,2014). And additional mechanisms of drug-related resistance, e.g. extruder efflux pumps (EPs) and drug modifiers and targets for inactivating or altering various enzymes, have also been identified, for example, in order to keep their aims and prevent them from interacting with other molecules in cells. Some bacteria generate certain enzymes that kill the drug penicillin (Lomovskaya *et al.*,2001). Some bacteria react by modifying the structure of the antibiotic so that the antibiotic cannot recognize or bind to them (Lambert *et al.*,2005).

1.2. Objective

1. The aim of the current study is to see the effect of low (0.1 µg/ml) and high (0.4 µg/ml) doses of INH on MDR and sensitive *M. tuberculosis* complex strains by investigating the expression levels on MDR isolates for 18 efflux pump genes and comparing them with pan-sensitive isolates, *polA* was considered as a housekeeping gene to see the effectiveness of the drug in the two different doses in comparison with Pan-sensitive isolates.
2. To investigate the mutations on four genes *katG, rpoB, inhA* and *oxyR-ahpC* on *M. tuberculosis* complex isolates.
3. Investigating the relation between the present of mutations and expression levels o efflux pump genes.

2. LITERATURE REVIEW

2.1. General characteristics

M. tuberculosis was described in 1882 by Robert Koch as non-moving bacilli, aerobic, dry with rough colonies and without pigments and form a cord-like structure in liquid medium Figure 2.1. (A), When stained with a Ziehl-Neelsen stain, the mycolic acids rapidly discolor and retain them in the bacillus. Under the microscope, the bacillus surface is bright blue, while the bacillus is seen as a bright red bar. Figure 2.1. (B). (Naveen et al.,2012; Adler et al.,2001; Portevin *et al.*,2011, Hershberg *et al.*,2008).

The genus is split into two broad taxonomic groups on the basis of the growth rates of individual species. The first category that takes over a week to establish colonies, known as slow growers, is slow-growing species such as the well-known pathogens MTB, *M. bovis*, and *M. leprae*. Fast growers or non-pathogenic/opportunistic bacteria, are the other categories that deliver colonies within seven days, such as *M. smegmatis* (Forrellad *et al.*,2013).

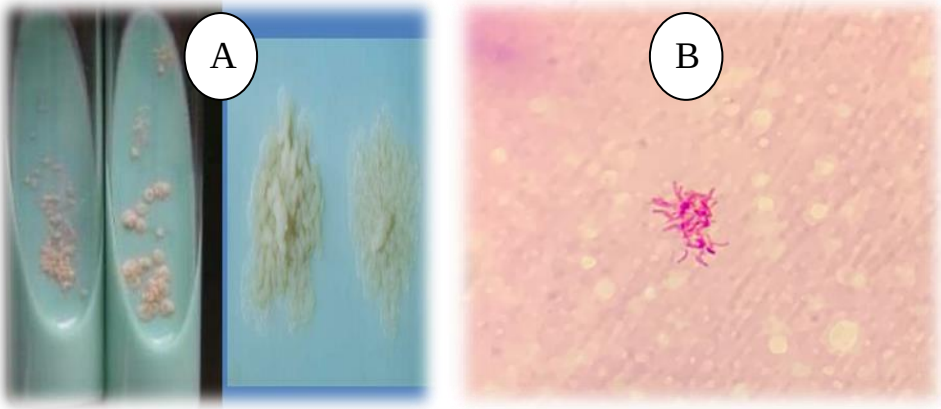

Figure 2.1. (A) Visualization of *M. tuberculosis* colonies on Lowenstein-Jensen medium. (B) M. tuberculosis visualization using the Ziehl–Neelsen stain

Under electron microscope, bacterium is about 0.2- 0.5 µm in width and 2 – 4 µm in length (Figure 2.2). (Zuber et al. 2008, Reed et al. 2004, Rocha-Ramirez et al. 2008; Gengenbacher *et al.*,2012)

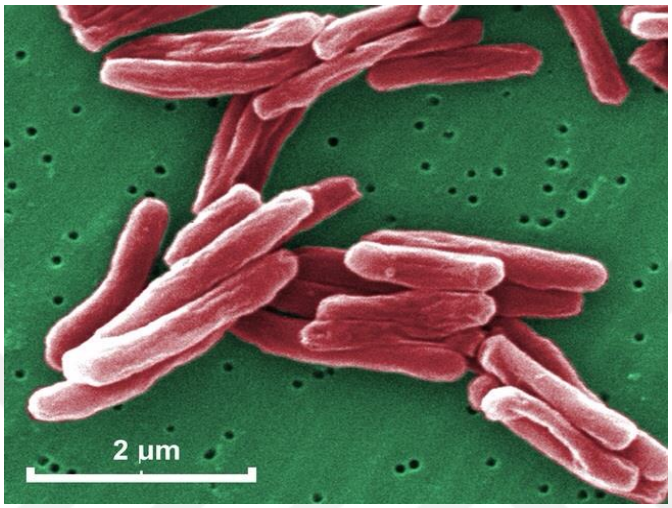

Figure 2.2. Mycobacterium tuberculosis scanning electron micrograph (https://images.app.goo.gl/opB8V6Pzop2sCdKL7)

Although the potential biosynthetic pathways of cell wall components make them suitable for new drugs for the treatment of TB, the organism apparently does not generate any toxins; although there are no typical virulence factors such as recently developed pathogenicity islands; it has a massive repertoire of structural and physiological features that helps in its survival within its host, including the ability to detoxify oxygen radicals. MTBC contains *Mycobacterium tuberculosis sensu stricto* (MTB), *Mycobacterium mungi*, *Mycobacterium bovis*. *Mycobacterium suricattae*, *Mycobacterial caprae*, *Mycobacterium microti*. However, with a high degree of DNA similitude (>99.95%), these animals are genetically monomorphic, with the exception of *M. Canettii*, they have different host ranges. The major causative agents of human tuberculosis *are Mycobacterium tuberculosis stricto* and *Mycobacterium africanum. Mycobacterium microti* affects

Voles, *Mycobacterium orygis* pathogen of Antelope (Wells 1937; Wells 1946; Wayne et al. 1986; Frota et al. 2004) (van Ingen et al. 2012) (Aranaz *et al.*,1999).

M. Pinnipedii, a seal and sea lion pathogen, *Mycobacterium mungi nangoose* (A *et al.*,2003) The widest variety of host affecting animals and people reveals *Mycobacterium Bovis* (Garnier *et al.*,2003). While *Mycobacterium bovis* is sometimes human isolated and affected by less than 1% of all human cases of TB, it lacks the capacity to maintain or sustain the period of infection in human populations. This may be because of the three mutations shown to be essential regulators of virulence factors, including several major lipids and protein, in the two-component PhoP/PhoR (PhoPR) regulatory system. These mutations lower PhoP regulon expression, which leads to less spreading capability among humans (Berg and Smith, 2014; Smith *et al.*,2006). The most phenotype-distinguished component of the complex is *Mycobacterium canettii*, also considered to be a member of the *Mycobacterium tuberculosis* complex(MTB). *Mycobacterium canettii* and other so-called Smooth TB bacilli (STBs) are distinguished by the presence of lipooligosaccharides in the cell wall and the smooth glossy white colony (Gutierrez *et al.*,2005). STBs seem strong evidence of continuous horizontal gene exchange, but without records of transmission among humankind. These involve STBs in the mycobacterial populations suggested as causes of MTBC (Gutierrez *et al.*,2005).

2.1.1. Taxonomy

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 first scientific taxonomy of mycobacteria began in 1896, when the first genus Lehmann and Neumann (Jagielski *et al.*,2016), consisting of genus and MTB, was established in 1896. *Mycobacterium Leprae* seems to be the only genus that is taxonomically placed in the *Mycobacteriaceae*,

belonging to the class of *Actinomycetales* and *Actinobacteria*, (Saviola & Bishai., 2006). The full Mycobacterium taxonomic lineage is shown in Table 2.1. According to the most current edition of the List of prokaryotic names standing in the nomenclature database (http://www.bacterio.net.,2017), it now accommodates a total of 197 featured species. All of these organisms were classified into three major categories: MTBC, M. Non-tuberculous mycobacteria and leprosy (Jagielski *et al.*,2016).

Table 2.1. Full Mycobacterium taxonomic lineage (http://www.bacterio.net.,2

Kingdom	*Bacteria*
Phylum	*Actinobacteria*
Class	*Actinobacteria*
Subclass	*Actinobacteridae*
Order	*Actinomycetales*
Suborder	*Corynebacterianeae*
Family	*Mycobacteriaceae*

Mycobacterium is the only genus in the family Mycobacteriaceae that is taxonomically placed, belonging to the order Actinomycetales and the class of Actinobacteria. The structure of the mycobacterial cell wall is unique among procaryotes and is related to the pathogenesis of MTB (Smith, 2003; Dubnau *et al.*,2000; Glickman *et al.*,2000). The complexity of the cell wall reflects the richness of high molecular weight lipids (Brennan *et al.*,2003; Christensen *et al.*,1999; Daffé *et al.*,1998). Unusual impermeable properties of the MTB cell wall are believed to support bacilli in stressful osmotic shock conditions (Hett & Rubin, 2008) and the polymers, covalently connected with peptidoglycan and trehalose dimycolate, provide a thick layer of antibiotic resistance to MTB and the host protection mechanism (Takayama *et al.*,2005).

An external layer and an internal layer covering the plasma membrane are contained in the mycobacterial cell wall (Figure 2.3). Both proteins and lipids make up the external compartment. The inner compartment contains covalently

connected peptidoglycan (PG), arabinogalactan (AG) and mycolic acid (MA) to structure an insoluble complex known as the essential center of the MTB cell wall (Brennan *et al.*,2003).

Arabinogalactan (AG) is essential for the integrity of the cell wall and a major factor of bacterial type maintenance protecting bacilli from osmotic turgor pressure is to anchor the impermeable MA layer to the PG layer, murein or PG, external to the bacterial cell membrane. The peptidoglycan structure is peculiar to bacteria and therefore is a good target for therapies (Hett & Rubin 2008). Lipoarabinomannan, a main lipoglycan engaged in mycobacterium virulence (Chan *et al.*,1991) and in altering the host response during infection, is another significant component of the cell wall (Cox *et al.*,1999).

The Cord Factor is a glycolipid formed most abundantly in MTB virulent strains, which is responsible for Animal granulomas identical to those typical of TB infection are caused (Hunter *et al.*,2006b); Increasing production of cytokines (Ryll *et al.*,2001); Inhibiting the movement of phagocytose bacteria in macrophages to acid compartments (Indrigo *et al.*,2003.); Affecting the morphology of colonies of mycobacteria (Hunter *et al.*,2006b).

The cell wall is a key to mycobacteria's survival and areas of great importance are a more complete understanding of biosynthetic pathways and gene activities and the production of antibiotics to prevent the formation of the cell wall (Joe *et al.*,2007). Mycolic acids, powerful hydrophobic molecules which form a lipid shell around the organism and effect cell surface permeability characteristics, have been shown to be essential for MTB survival (Draper *et al.*,2005). *M. tuberculosis* does have a specialized complex of cell walls consisting of four main components: mycosides, arabinogalactan, mycolic acids, and peptidoglycan. Mycolic acids, which in general are the key lipids of the cell wall of mycobacteria, are major components of the external permeability barrier and liable for this community of microorganisms 'acid-fastness' (Kaur *et al*., 2009). In addition, fat acids are associated with components of carbohydrates that form a special envelope

that prevents phagolysosome fusion (Nguyen and Thompson 2006). Like a lot of other bacteria, *M. tuberculosis* does not produce spores but is capable of being dormant, a non-replicative state marked by low metabolic rate and extended persistence (Gengenbacher *et al.*,2012).

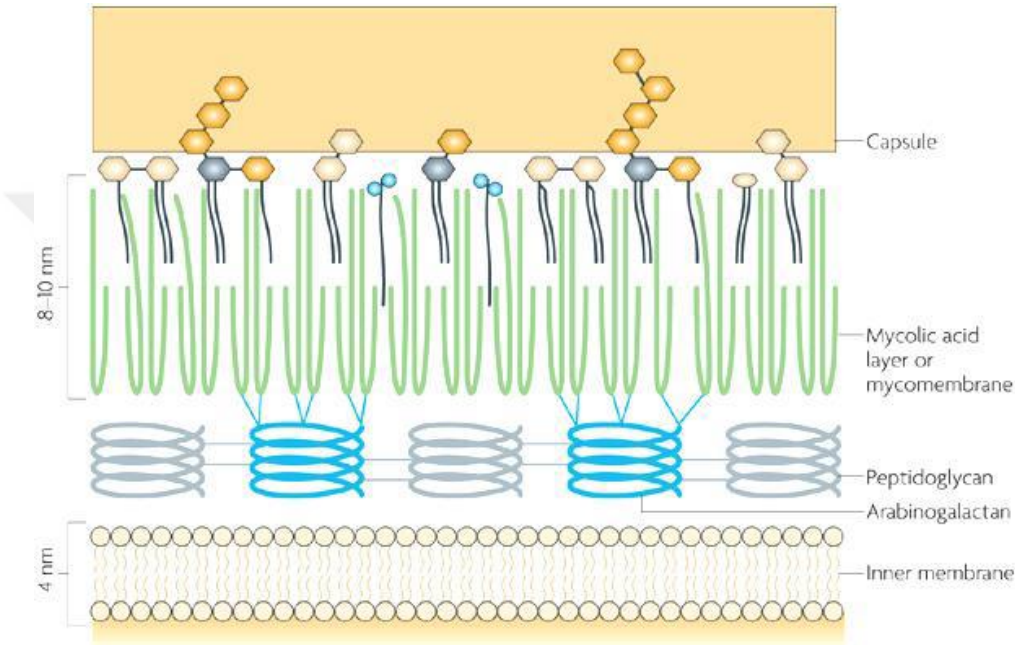

Figure 2.3. Mycobacterial cell wall (Schematic representation of the cell envelope of Mycobacterium tuberculosis. | Nature Reviews Microbiology)

2.1.2. Morphology

Mycobacteria are usually rod-shaped bacteria, non-spore forming, acid-fast bacteria. The size of bacillus has been recorded as 1-10 micrometres long, normally 3-5 micrometres long. The Variable morphology can be observed when bred on solid media (Belisle & Brennan 1989).

Recorded multivariate morphological variation in *M. tuberculosis* is graded in two forms, active and latent. Multi-branched growth exemplifies

exponential stages of growth (Figure 2A). Those that are seen constantly under stressful or heavily prejudiced circumstances (Velayati *et al.*,2009, 2011; Farnia *et al.*,2010).

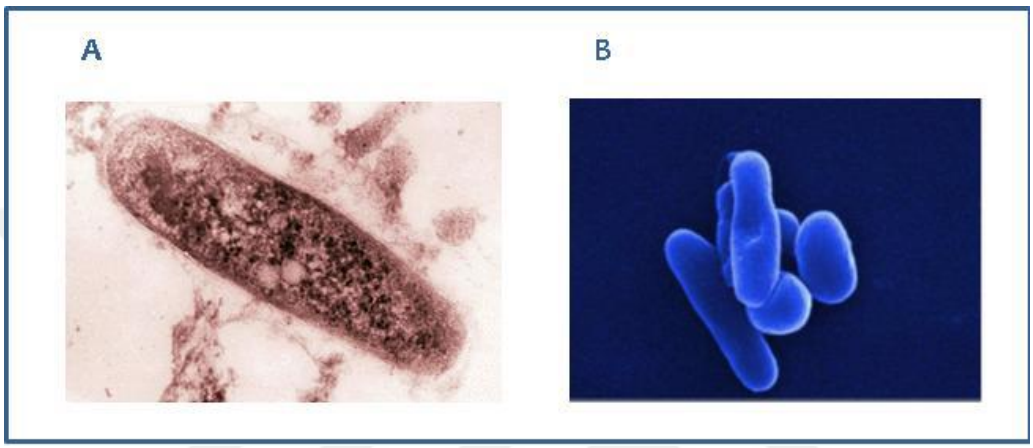

Figure 2.4. morphological variances among TB
(A) Thin section transmission electron micrograph of MTB.
(A) Scanning electron microscope reveals odd shapes at the rising process of MTB (Velayati & Farnia, 2012).

Furthermore, Mycobacterium tuberculosis is obligate aerobe, and hence is present in the well-aerated upper lobes of the lungs in patient with TB. The bacterium is an obligate intracellular parasite, normally of macrophages, and has a generation time of 15-20 hours, which is extremely slow compared to other bacteria.

2.1.3. Cell Wall

The structure of the mycobacterial cell wall is unique among procaryotes and is linked to the pathogenesis of MTB (Smith, 2003; Barry *et al.*,1998; Dubnau *et al.*,2000; Glickman *et al.*,2000). The complexity of the cell wall is represented by the richness in high molecular weight lipids (Brennan *et al.*,2003; Christensen *et al.*,1999; Daffé *et al.*,1998).

Uncommon impermeable properties of the MTB cell wall are considered to be beneficial to bacilli in stressful osmotic shock conditions (Hett & Rubin, 2008) and the polymers, covalently associated with peptidoglycan and trehalose dimycolate, provide a thick layer of MTB antibiotic resistance and host defense mechanisms (Takayama *et al.*,2005). An internal layer and an external layer surrounding the plasma membrane are found in the mycobacterial cell wall (Figure 2.3). Both proteins and lipids make up the external compartment. The inner compartment consists of covalently linked arabinogalactan (AG), peptidoglycan (PG) and mycolic acids (MA) to structure an insoluble complex known as the basic core of the Mtb cell wall (Brennan *et al.*,2003).

Arabinogalactan (AG) is important for the integrity of the cell wall and a major factor of bacterial form maintenance protecting bacilli from osmotic turgor pressure is to anchor the impermeable MA layer to the PG layer, murein or PG, external to the bacterial cell membrane. The peptidoglycan structure is specific to bacteria and is therefore a good target for therapeutics. (In 2008, Hett & Rubin).

Lipoarabinomannan, a major lipoglycan involved in mycobacterium virulence (Chan *et al.*,1991) and in modifying the host response during infection, is another significant component of the cell wall (Cox *et al.*,1999). It has been shown that mycolic acids, the powerful hydrophobic molecules which always make up a lipid shell it around organism and affect permeability properties on the cell surface, are critical for MTB survival (Draper *et al.*,2005). The membrane features, however, do not correspond to Gram-positive ones. Indeed, as expected for Gram-positive bacteria, the bacteria do not retain the crystal violet dye (Nguyen and Thompson 2006), sometimes resulting in the appearance of the 'ghost' after washing with alcohol or acetone. A specialized cell wall complex, consisting of four major components, mycosides, mycolic acids, arabinogalactan and peptidoglycan.

Mycolic acids, the main lipids of the mycobacterial cell wall in general, were also major components of the external permeability barrier and are responsible for the acid-fastness of this microorganism group (Kaur et al.,2009).

In addition, fatty acids are associated with components of carbohydrates that form a unique envelope that inhibits phagolysosome fusion (Nguyen and Thompson 2006 48). *M. tuberculosis* does not form spores like many other bacteria, but has the ability to become dormant, a non-replicating status affected by poor metabolic rate and continuous persistence (Gengenbacher *et al.*,2012).

2.1.4. The Genome of Mycobacterium tuberculosis

The paradigm reference strain H37Rv became the first MTB strain fully sequenced in 1998 (Cole et al. 1998). The circular genome of MTB H37Rv consists of 4,411,532 bp and shows a high content of 65% G+C. Over 4000 protein-coding and 50 stable RNA genes are included, using about 91% of its potential coding capacity.

It displays a gene density that is comparable to other prokaryotes, at one gene per kilobase. On the forward and reverse strands, genes are distributed evenly, and 59 percent of the transcription is at the same polarity as the replication fork.

More than half of the MTB coding sequences have arisen from events of gene duplication or domainshuffling (Tekaia et al. 1999). Several characteristics of the biochemistry, physiology, genetics and immunology of MTB were highlighted by genome sequence analysis.

As the most widely used strain in TB research, continued efforts have been made to retain a precise and up-to-date genome annotation of MTB H37Rv that used a combination of bioinformatics, data gathering, comparative genomics, scientific literature and manual curation (Cole et al. 1998; Camus et al. 2002; Lew et al. 2011). The TubercuList database (Lew et al. 2011) (http://tuberculist.epfl.ch) has provided access to the gene-based annotation for MTB H37Rv for more than 10 years. There are 4095 annotated features in the current release (R25-April

2012), including 4022 protein genes and 73 RNAs (tRNAs, ribosomal RNAs, small RNAs, stable RNAs).

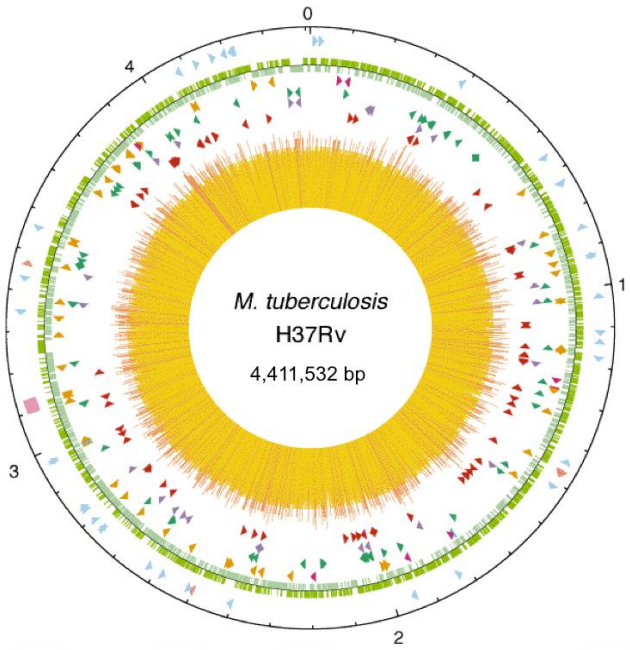

Figure 2.5. *M. tuberculosis* H37Rv Genome (Cole et al. 1998).

The position of stable RNA genes (tRNAs are blue and others are pink) and the direct-repeat region (pink cube) are indicated by the first ring from the outside; the second ring reveals the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring shows repeated DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the repetitive DNA (insertion sequences, orange) (dark red). With <65 percent G+C in yellow and >65 %G+C in red, the histogram (center) represents the G+C content (Cole et al. 1998).

The genes of *M. tuberculosis* have been widely categorized into 11 functional categories (Table 2.2).

Table 2.2. The *M. tuberculosis* genes classified accourding to functional categories

Category	Function	Number of features	% of total
0	Virulence, detoxification, and adaptation	238	5.8
1	Lipid metabolism	272	6.6
2	Information pathways	242	5.9
3	Cell wall and cell processes	773	18.9
4	Stable RNAs	73	1.8
5	Insertion seqs and phages	147	3.6
6	PE/PPE proteins	168	4.1
7	Intermediary metabolism and respiration	936	22.9
8	Unknown	16	0.4
9	Regulatory proteins	198	4.8
10	Conserved hypotheticals	1032	25.2

2.2. Efflux pumps in Mycobacteria:

The efflux pumps are present in almost all bacteria and related proteins can be found on plasmids or chromosomes. This has five families: Resistance-Nodulation-Division (RND), Major Facilitator Superfamily (MFS), ATP (Adenosine Triphosphate) Binding Cassette (ABC), Small Multidrug Resistance (SMR), and Multidrug and Toxic Compound Extrusion (MATE). The RND superfamily is only present in Gram- negative bacteria, however, pathways of four other families: MFS, ABC, SMR and MATE are universally found in both Gram-negative and Gram- positive bacteria. There are several movement channels for efflux pumps in the single compartment membrane (Sun *et al.*,2014).

Several mycobacterial drug efflux pumps have recently been discovered and described (Louw et al., 2009; Viveiros et al., 2003). About 2.5% of the *M. tuberculosis* genome is composed of genes encoding ABC transporters, and at least 37 complete and incomplete ABC transporters have been identified (Braibant et al, 2000). Only a few of these transporters have been identified, and they appear to play a role in drug resistance. *M. tuberculosis* contains the drrABC operon, which is thought to be involved in doxorubicin resistance (Choudhuri et al., 2002). The *M. smegmatis drrA* and *drrB* genes confer resistance to a wide range of antibiotics, including tetracycline (TET), erythromycin (EMB), norfloxacin (SM), and chloramphenicol. Efflux pump inhibitors like reserpine or verapamil are used to reverse the resistant phenotype (Choudhuri et al., 2002). According to studies, the main function of *M. tuberculosis drr* proteins is to transport lipids to the cell's periphery, and *drrC*, in particular, appears to be involved in the transport of phthiocerol dimycocerosates (Camacho et al., 2001). When produced from a multicopy plasmid, the *M. tuberculosis Rv2686c-Rv2687c-Rv2688c* operon encodes an ABC transporter responsible for fluoroquinolone efflux. This operon increases the MIC of ciprofloxacin and norfloxacin by 8 and 2 folds, respectively, when overexpressed in *M. smegmatis*. In the presence of reserpine, carbonyl cyanide mchlorophenylhydrazone (CCCP), and verapamil, resistance is reduced (Pasca et al., 2004).

The *LfrA* protein of Mycobacterium smegmatis was the first efflux pump described in the genus Mycobacterium, and it may be responsible for low-level resistance to fluoroquinolones, acridine, and quaternary ammonium compounds (Liu et al., 1996; Sander et al., 2000; Takiff et al., 1996). When overexpressed in *M. smegmatis*, *lfrA* appeared to play a key role in ciprofloxacin resistance. In the absence of overexpression, however, *lfrA* has no effect on fluoroquinolone susceptibility. In fact, *lfrA* disruption reduced the MICs of ethidium bromide (EtBr) and acriflavine by 8-fold and 2-fold, respectively, for ciprofloxacin, doxorubicin, and rhodamine (Li et al., 2004). If EtBr is a better substrate for *lfrA*, the difference

in results between EtBr and ciprofloxacin can be explained (Li et al., 2004). In a clinical strain of *M. tuberculosis*, *Rv1258c* expression was increased in the presence of R and ofloxacin (Siddiqi et al., 2004). Furthermore, the MIC of TET in an *M. smegmatis* strain was reduced by carbonyl cyanide m-chlorophenyl hydrazone (CCCP), reserpine, and chlorpromazine (Ramon-Garcia et al., 2006). CCCP and reserpine inhibit the efflux activity of *tap* protein from *M. fortuitum*, which is consistent with the reduction of TET's MIC in the presence of these compounds. Furthermore, the MIC of TET in an *M. smegmatis* strain was reduced by CCCP, reserpine, and chlorpromazine (Ramon-Garcia et al., 2006). *Rv1634* has been proposed as a new fluoroquinolone efflux transporter in *Mycobacterium tuberculosis* (De Rossi et al., 2002). When this gene was overexpressed in *M. smegmatis*, it was discovered that it reduced susceptibility to a variety of fluoroquinolones. Furthermore, *Rv1634* appears to be involved in the efflux of norfloxacin and ciprofloxacin (De Rossi et al., 2002). The *P55* efflux pump has been linked to low-level drug resistance to a variety of drugs, including TET, aminoglycosides, and R. (Ramon- Garcia et al., 2009; Silva et al., 2001). *P55* is required for normal bacterial growth on both solid and liquid media, according to a recent study: I it provides resistance to several drugs (including rifampin); (ii) it is part of the oxidative stress response; and (iii) it is required for normal bacterial growth on both solid and liquid media (Ramon-Garcia et al., 2009).

The protein that is thought to be involved in efflux The transporter motifs found in *efpA* of *Mycobacterium tuberculosis* are similar to those found in the efflux-mediated antiseptic resistance gene *qacA* of *S. aureus*, including those related to proton antiporter function and those specific to drug transporters. It was discovered that when INH was present, the expression of *efpA* increased, implying that the protein encoded by this gene transports molecules involved in mycolic acid synthesis (Wilson et al., 1999). Although deletion of the *efpA* homologue in *M. smegmatis* increased susceptibility to EtBr, gentamicin, and fluoroquinolones by 2-fold and to acriflavine by 8-fold, it reduced susceptibility to rifamycins and

chloramphenicol by 4-fold and reduced susceptibility to INH and erythromycin by 2-fold (Li et al., 2004). Furthermore, this *efpA* deleted mutant grew slower than the wild-type strain, suggesting that its increased susceptibility is due to impaired growth (Li et al.,2004). As a result, since the deletion of *efpA* increases susceptibility to EtBr, EtBr could be a substrate of this gene (Li et al., 2004). The genome of *M. tuberculosis* contains several genes that code for RND superfamily putative transport proteins. *MmpL* (mycobacterial membrane proteins, large) is the name given to these proteins, which are thought to be involved in the transport of fatty acids (Tekaia et al, 1999). *MmpL7* protein was found to export phthiocerol dimycocerosate (PDIM), a lipid component of the outer membrane in *M. tuberculosis* (Camacho et al., 2001). The *fadD28* gene, which encodes an Acyl-CoA synthase, is located upstream of the *mmpL7* gene and is likely involved in the release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. Dimycocerosate (DIM) is produced by a strain with an insertion in the *mmpL7* gene and is retained in the cytoplasm or cytoplasmic membrane. *MmpL7* protein production increased the MIC of INH 32-fold in *M. smegmatis*. This phenotype is reversed when *fadD28* and *mmpL7* are both expressed at the same time, implying that DIM and INH compete for the same *MmpL7* transporter (Pasca et al., 2005).

2.3. Treatment

One of the health priorities of the Sustainable Development Goals is the reduction of the TB epidemic by 2030. Tuberculosis (TB) is one of the world's top 10 causes of death (Figure 2.6.) Since the invention of p-aminosalicylic acid (PAS) and streptomycin (STR) in the 1940s, TB has been well treated with successful chemotherapy since the discovery of p-aminosalicylic acid (PAS) and streptomycin (STR) in the 1940s, 54 million lives have been saved by TB diagnosis and treatment between 2000 and 2017. At first thought, It might seem paradoxical, that the bacteriological aspects the importance of tuberculosis should be increased as

more efficient means of treating the disease become available. With lobar, this was not the case, when sulfonamides, and later penicillin, were available, pneumonia or infection with 8-streptococcus. Failure to acknowledge that this is the case in tuberculosis has resulted in a prolonged lag in providing the additional facilities and staff that necessarily entails the increased need for bacteriological diagnosis and treatment monitoring (Middlebrook and Chon, 1958).

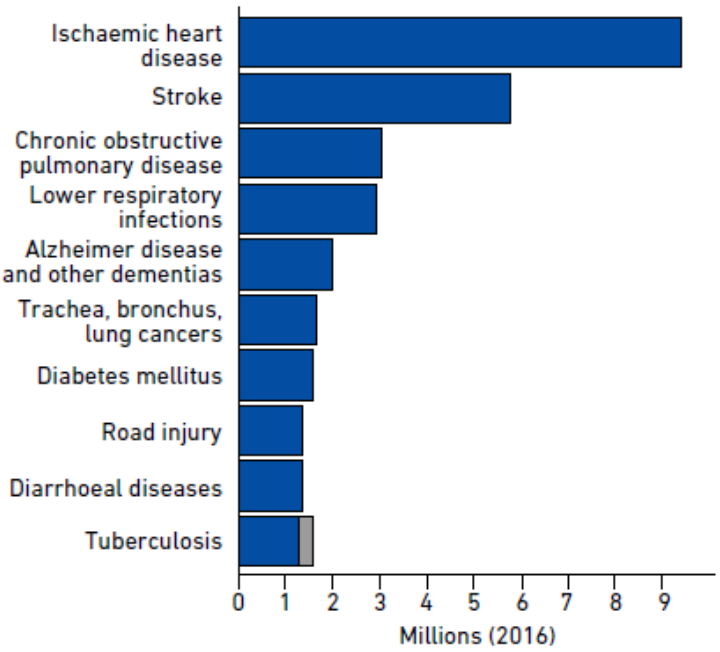

Figure 2.6. Leading worldwide causes of death. TB among HIV-positive people are shown in grey.

TB therapy is used not only to cure the disease, but also to cut off transmission and avoid recurrence (most recurrences occur within 6-12 months of treatment completion) (Böttger and Springer, 2008). The Specifically Witnessed Therapy Short Course (DOTS), a procedure for the diagnosis and treatment of tuberculosis, was implemented by the WHO in 1994 in which patients are required to take every dose of anti-TB medicine before the end of treatment (WHO, 2001).

Sputum samples are taken on a monthly basis before 2 consecutive tests are negative. However, due to the rise of Multidrug Resistant Tuberculosis (MDRTB) and the development of Extensivley Drug Resistant Tuberculosis, the effectiveness of DOTS faces new challenges (XDRTB).

This led to a new policy called DOTS-plus, a systematic DOTS-based administration program aimed at preventing multidrug resistant tuberculosis (MDRTB) from evolving and spreading further (WHO, 2006; WHO/IUATLD, 2008). Rifampcin (RIF), isoniazid (INH), streptomycin (STR) pyrazinamide (PZA) and ethambutol (EMB) are the first-line drugs used, and fluoroquinolones, aminoglycosides such as kanamycin and amicacin, cyclic peptides such as Streptomycin can be used in the primary phase of therapy as an interchangeable treatment for EMB. Only if the organism is proven to be sensitive to the medication or the patient is from a culture in which Streptomycin tolerance is unlikely is STR advised to be exchangeable with EMB (Bigi et al, 2003; WHO, 2001). For many factors, TB has been treated with combination therapy for more than fifty years. Unlike most of the other infectious disease where a single medication regimen is being used for treatment, TB has been treated for over fifty years using combination therapy for several reasons:

1) Decreasing the chances of drug resistance acquisition.

2) Combined drug modes of action help to efficiently clear bacteria: rifampicin prevents the synthesis of RNA and has a sterilizing effect (McClure and Cech, 1978). (PZA) is very effective in killing bacteria living in acidic conditions and found within macrophages or in sites of acute inflammation, although it is weakly bactericidal (Zhang *et al.*,2013). EMB inhibits the polymerization steps of the synthesis of arabinogalactan (Mikusova *et al.*,1995). By inhibiting mycolic acid synthesis, INH is a pro-drug and bactericidal against replicating bacteria (Zhang *et al.*,1992), fluoroquinolones act on Dna synthesis (Drlica *et al.*,2009), para-aminosalicylic avoid the formation folic acid (Rengarajan *et al.*,2004), whereas

ethionamide (ETD) also inhibits the synthesis of fatty acids required for mycolic acid synthesis (Banerjee *et al.*,1994).

The standard therapy for new TB patients (defined as patients who have not received previous anti-TB therapy or who have received prior anti-TB therapy with less than one month) consisted of an intense phase of two months with daily INH/RIF/EMB/PZA followed by a continuous phase of 4 months with daily INH/RIF. The most effective drugs for the treatment of TB are INH/RIF: INH is responsible for the initial killing of approximately 95% of species on the first days of treatment, augmented by RIF and PZA for the remainder of the intensive period, for the continuation phase RIF is the main active agent against persistents from the intensive phase (World Health Organization, 1994). Globally, previously treated patients are five times more likely to have TB caused by MDR strains and should therefore be treated according to the findings of the drug susceptibility test (DST). However, in the absence of DST findings, patients typically follow an 8-month drug regimen consisting of 2 months of INH/RIF/EMB/PZA (intensive phase), 1 month of INH/RIF/EMB/PZA (intensive phase) and 5 months of INH/RIF/EMB/PZA (continuation phase).

Table 2.3. Anti-TB drugs and their mechanism of action (Muller et al., 2013)

Drug (year of discovery	Year of discovery	Effect on bacterial cell	Mechanism of action	Targets
First line drugs				
Streptomycin	1944	Bactericidal	Inhibition of protein synthesis	Ribosomal S12 protein and 16SrRNA
Isoniazid	1952	bacteriocidal against replicating tubercle bacilli	Inhibition of cell wall mycolic acid synthesis and other multiple effects on DNA, Lipids, carbohydrates and NAD metabolism	Multiple targets including acyl carrier protein reductase (InhA)
Pyrazinamide	1952	Bacteriostatic/ bacteriocidal against slow replicating bacilli in acidic lesions	Disruption of membrane transport and energy depletion	Membrane energy metabolism
Ethambutol	1961	Bacteriostatic	Inhibition of polymerization of cell wall arabinogalactan	Arabinosyl transferase
Rifampicin	1966	A semi derivative of Rifamycin. Bacteriocidal activity against tubercle bacilli	Inhibition of RNA synthesis	RNA polymerase β subunit
Second line drugs				
ρ-aminosalicylic acid (PAS)	1946	Bacteriostatic	Inhibition of folic acid and iron metabolism synthesis	ρ-aminosalicylic acid (PAS)
Cycloserine	1952	Bacteriostatic	Blocks enzyme of cell wall biosynthesis	D-alanine racemase
Ethionamide	1956	Bacteriostatic	Inhibition of mycolic acid synthesis	Acyl carrier protein synthesis (InhA)
Kanamycin	1957	Bacteriocidal	Inhibition of protein synthesis	16S rRNA
Capreomycin	1960	Bacteriocidal	Inhibition of protein synthesis	30s ribosomal subunit
Quinolones	1963	Bacteriocidal	Inhibition of DNA synthesis	DNA gyrase

2.4. Drug resistance

When drug resistant mutants are selected as a result of ineffective treatment, this is known as acquired resistance, and when a patient is infected with a resistant *M. tuberculosis* strain, this is known as primary resistance (Figure 2.7). The long generation time of *M. tuberculosis*, its low metabolic activity, and its capacity for dormancy all contribute to the selection of drug resistant mutants (Wayne, 1994). Furthermore, because *M. tuberculosis* can be found inside pulmonary cavities, where antibiotic access is difficult, compartmentalization of the infection increases the likelihood of monotherapy exposure (Elliott et al, 1995). This effect is amplified in the presence of insufficient anti-TB drug dosage, which can occur as a result of a physician's insufficient prescription or the patient's non-compliance. To avoid drug resistance, it is necessary to understand the mechanisms by which *M. tuberculosis* develops resistance. Several studies have described the mechanisms of action of most anti-TB drugs used in clinical practice over the years. MDR-TB is a type of tuberculosis in 2017, there is still a public health crisis and a threat to health security. According to the WHO, there were 558 000 new cases of rifampicin resistance, and MDR-TB had the most effective first-line drug – which was used in 82% of cases. The burden of MDR-TB falls primarily on three countries: India, China, and the United States. Together, the Russian Federation and China account for nearly half of all global cases. In 2017, approximately 8.5% of MDR-TB cases had drug-resistant TB in general (XDR-TB). However, 55% of MDR-TB patients are currently being treated successfully around the world. WHO and in the year 2016 was issued the employ of a short, standardized regimen for MDR-TB patients who do not have strains that are resistant to the second-line TB drugs. This regimen is much less expensive than the standard MDR-TB treatment, which can take up to two years, and lasts 9–12 months. Patients with XDR-TB or second-line anti-TB drug resistance, on the other hand, must be put on longer MDR-TB regimens, to which one of the new drugs (bedquiline or delamanid) may be added.

In the year 2017, Globally, 3.5% (95% confidence interval [CI]: 2.5–4.7%t) of new cases had MDR/RR-TB, while 18% (95% CI: 6.3–34%) of previously treated cases had MDR/RR-TB Figures 2.8. and 2.9. show the proportions of new and previously treated TB cases with MDR/RR-TB at the country level in 2017, an estimated 558 000 (range, 483 000– 639 000) incident cases of MDR/RR-TB were reported, but the proportion of cases with MDR-TB was estimated to be 82% (460 000 out of 560 000) The countries with the highest number of MDR/RR-TB cases (47% of the global total) were India, the Russian Federation, and China (Figure. 2.11.). MDR/RR-TB claimed the lives of about 230 000 people in 2017 (range: 140 000–310 000), which is similar to the best estimate for 2016 published in the 2017 edition of the WHO global TB report (WHO, 2017).

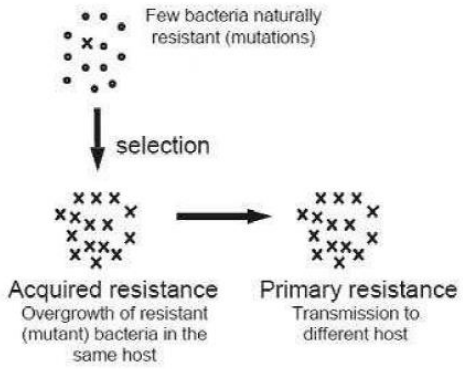

Figure 2.7. Development of resistance in TB

As a result of the collection of resistant mutants, acquired tolerance grows as a consequence of inadequate treatment and non-compliance. The primary resistance happens by transmitting the antibiotic resistance to the new host (adapted from Johnson R. et al, 2006).

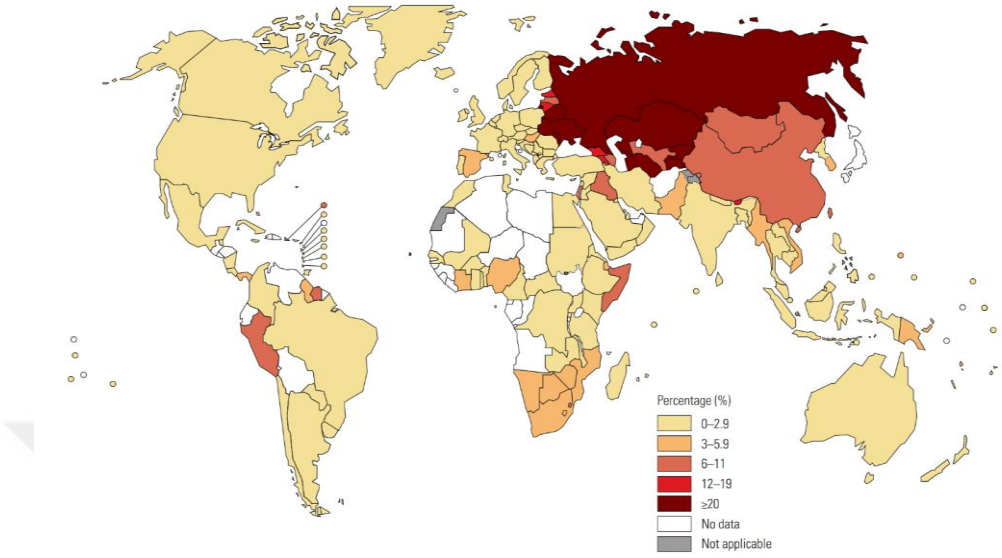

Figure 2.8. New MDR/RR-TB cases (WHO,2020)

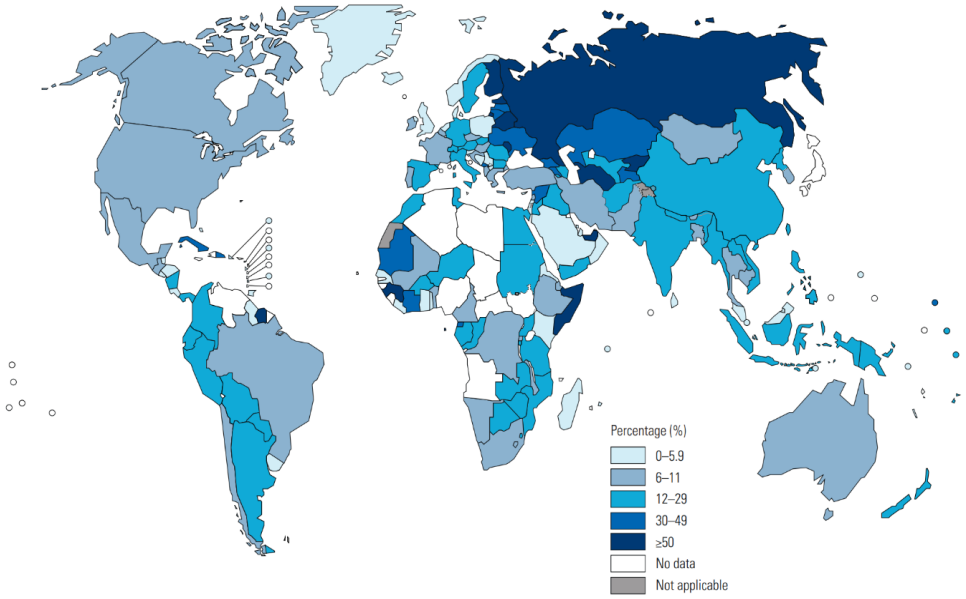

Figure 2.9. Previously treated MDR/RR-TB patients percentege

The statistics are based on the most recent year for which data has been published, which varies between countries. The data will cover the 2005-2019 period. High percentages in Belize, Guam and Sao Tomé and Principe of previously treated RR-TB TB cases apply to only a small number of recorded cases (range: 1-8 reported previously treated cases of TB).

Figure 2.10. Estimated incidence of MDR/RR-TBa in 2019, for countries with at least 1000 incident cases.

2.4.1. Resistance intrinsic

Classically, *M. tuberculosis* intrinsic resistance was due to the peculiar structures of its mycolic acid like cell wall that gave the bacteria a low permeability for the many compounds such as antibiotics and other chemotherapeutic agents (Jarlier and Nikaido., 1994: Niederweiss M. *et al*, 2010). The role of the efflux mechanism has recently been recognized as an important factor in the natural resistance of antibiotic mycobacteria, including fluoroquinolones, tetracyclines and aminoglycosides. (De Rossi *et al*.,2006) And in Mycobacterium smegmatis, it was domenstrate that the MIC of the b-lactam antibiotics Ampicillin and Cefaloridine

was increased for mutants missing the main porin *MspA*. Deletion of the *mspA* gene also increased the MIC of Rifampicin, Erythromycin and Vancomycin by 2 to 10 times. (Stephan *et al.*, 2004.).

Latest research at *M. tuberculosis* has shown that *Rv1698* can contribute to intrinsic resistance to hydrophilic components with the same function as *MspA*. (Siroy *et al.*,2008). And both *Rv1973* and *Rv1698* have been identified as mycobacterial outer membrane proteins (OMPs) with a possible role in the intrinsic resistance to several antibiotics in bioinformatics research (Song *et al.*,2008)

The intrinsic resistance to antibiotics in mycobacteria is not only due to permeability barriers or *B-lactamases*. However, antibiotic resistance can also be due to physiological adaptations within the host (Nguyen and Pieters., 2008). Finally, the whiB7 in *M. tuberculosis* is an MDR determinant that deletion produces a phenotype susceptible to multidrugs, indicating its position as an ancestral determinant of MDR. Therefore, intrinsic resistance is significant because it restricts the number of medications available for treatment and facilitates the development of high-level drug resistance strains. As a result, drugs that inhibit these mechanisms will allow for future new use against M. Many of the antibiotics currently active but not previously used in the treatment of TB are tuberculosis (Lomovskaya and Bostian., 2006: Nguyen and Thompson., 2006).

2.4.2. Acquired drug resistance

In general and unlike other bacteria, the acquired drug resistance is mediated by the horizontal transfer of mobile genetic components, such as transposons or plasmids and integrins, developed drug resistance in *M. tuberculosis*, primarily due to spontaneous mutations in chromosomal genes, generating resistant strain selection during suboptimal drug treatment (Kochi *et al.*, 1993) In Prokaryotes, spontaneous mutations occur at a really low rate of 0.0033 per replication (Safi *et al.*,2008). The per-basepair mutation rate is inversely

proportional to genome size. In previous research, the mutation rate has shown that it may vary depending on the nature of the drug range, but this appears at a rate with about 1029 mutations per cell division for the majority of the key anti-TB drugs. This is the key reason why anti-TB drugs are used as a blend, since 102188 is the possibility of a mutant comprising two resistance mutations (Gillespie 2007). Mutations in the *rpoB* gene encoding the *β-RNA* polymerase subunit lead to high-level resistance to rifampicin, and Jin and Gross have identified three clusters of *rpoB* gene site mutations in rifampicin-resistant Escherichia coli strains. Nucleotide sequences of the cluster I and II regions of the *rpoB* gene have recently been investigated by several researchers, and mutations from rifampicin-resistant *Mycobacterium tuberculosis* strains have been shown. In addition, several distinct regions in the *rpoA* and *rpoC* genes are potentially important regions in *rpoB*'s compensatory evolution (Tawgozy and Qarasnji 2020).

Table 2.4. Anti-TB drugs and their mechanism of drug resistance (Muller *et al*., 2012)

Drugs	Genetic region involved in resistance formation	Natural function of gene	Role in resistance formation when mutated
	First Line drugs		
Isoniazid	ahpC	Alkyl hyperperoxide reductase	Compensatory mutations
	fabG	3-Oxoacyl-thioester reductases	Unknown
	fadE24	Involved in fatty acid b-oxidation n	Unknown
	inhA	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of InhA	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	katG	Catalase/peroxidase	Elimination of pro-drug conversion
Rifampicin	rpoA	α-Subunit of RNA polymerase	Compensatory mutations
	rpoB	β-Subunit of RNA polymerase	Alteration of drug target
	rpoC	β-Subunit of RNA polymerase	Compensatory mutations
Pyrazinamide	pncA	Nicotinamidase	Elimination of pro-drug conversion
Streptomycin	gidB	7-Methylguanosine methyltransferase	Alteration of drug target
	rpsL	S12 ribosomal protein	Alteration of drug target
	rrsb	16S rRNA	Alteration of drug target

Table 2.4. continued

Ethambutol	embA	Arabinosyl transferase	Alteration of drug target
	embB	Arabinosyl transferase	Alteration of drug target
	embC	Arabinosyl transferase	Alteration of drug target
	embR	Regulator of embCAB operon expression	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	rmlD	dTDP-4-dehydrorhamnose reductase	Unknown
	Second line drugs		
Fluoroquilones	gyrAb	DNA gyrase	Alteration of drug target
	gyrBb	DNA gyrase	Alteration of drug target
Injectables (Kanamycin/amikacin)	rrsb t	16S rRNA	Alteration of drug target
	rrs	16S rRNA	Compensatory mutations
Capreomycin/viomycin	tlyA	rRNA methyltransferase	Alteration of drug target
	rrsb t	16S rRNA	Alteration of drug target
Ethionamide	inhA	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of inhA	Overexpression of drug target
Para-amino salicylic acid	thyA	Thymidylate synthase A	Elimination of pro-drug conversion
PA-824 and OPC-67683	Rv3547	Hypothetical 16.4 kDa	Alteration of drug target
TMC207	atpE	ATP synthase	Alteration of drug target

2.4.2.1. First-line oral anti-TB drugs

In Turkey first line drugs used in the treatment of tuberculosis are Streptomycin (STR), Isoniazid (INH), Rifampicin (RIF), and Ethambutol (EMB), respectively. WHO envisages treatment options with pyrazinamide (PZA) added to the four major drugs (Rattan *et al.,* 1998; Ramaswamy and Musser 1998).

2.4.2.1.1. Isoniazid

Isoniazid, which is a bactericidal drug in synthetic structure and effective on proliferating bacilli, is a prodrug and is activated by the enzyme catalase-peroxidase. Mutations in the *katG* gene, which encodes the catalase-peroxidase enzyme, are responsible for 60-70% of high MIC resistance against isoniazid. The most common *Ser 315 Thr* mutation (40%). It has been shown that *Arg 463 Leu* mutation, which is frequently detected in the *KatG* gene, is a polymorphism and is also found in isolates susceptible to isoniazid in China, Russia and some Asian countries (Somoskovi et al., 2001). Mutations in the *inhA* gene (<10%) encoding the *enoyl-ACP* (acyl carrier protein) reductase enzyme (<10%) and the *kasA* gene encoding the beta ketoacyl ACP synthase enzyme, which is important in fatty acid elongation, were also held responsible for isoniazid resistance. However, the relation of this gene to isoniazid resistance is doubtful since isolates with mutations in the *kasA* gene also have mutations in the *katG* or *inhA* genes. Mutations (~ 10%) in the *ahpC* gene encoding the alkyl-hydroperoxide reductase enzyme, which is responsible for the cellular response to oxidative stress, were also thought to be responsible for resistance, but it was observed that mutations in the *katG* gene could accompany this mutation (Rattan *et al.* 1998; Ramaswamy and Musser 1998; Somoskovi *et al.,* 2001).

2.4.2.1.2. Rifampicin (RIF)

Rifampicin is a semi-synthetic (semisynthetic) bactericidal drug produced from *R. Mediterranei* bacteria. This drug acts by binding to the beta subunit of

DNA-dependent RNA polymerase and inhibiting RNA synthesis. Mutations in the 81 bp region (27 codons, 507-533) of the *rpoB* gene encoding the beta subunit of RNA polymerase were shown to be responsible for rifampicin resistance in 96% of MTBK strains resistant to approximately 500 mg rifampicin. The most common mutations responsible for high MIC resistance are 526 and 531 at their codons (65-86%). Rifampicin resistance with low MIC values 511, 516, 518 and 522. It has been associated with mutations in codons The most common Ser 531 Leu (42%) and His 526 Tyr (23%) mutations were determined. The resistance of *Mycobacterium tuberculosis* to rifampicin is mainly mediated by *rpoB* gene mutations. Secondary mutations in *rpoA* or *rpoC* genes relieve the effects of *rpoB* mutations (Tawgozy and Qarasnji 2020; Ramaswamy and Musser 1998; Somoskovi *et al.,* 2001).

2.4.2.1.3. Streptomycin (STR)

Streptomycin is a bactericidal aminoglycoside derived from *S. griseus* bacteria. Streptomycin binds to the bacterial ribosome and inhibits protein synthesis. Mutations in the *rpsL* gene encoding the ribosomal protein S12 (60%) and the mutations in the *rrs* gene encoding the 16S rRNA (<10%) are responsible for Streptomycin resistance. The most common is the *Lys* 43 *Arg* mutation in the rpsL gene, and less frequently, the *Lys* 43 *Thr* mutation is also seen. *Lys* 88 *Arg* or *Lys* 88 *Gln* mutations have also been identified in this gene. Mutations in this gene have been found to be responsible for high MIC Streptomycin resistance. Other mutations responsible for streptomycin resistance are found in the 530 rings and 915 regions of the *rrs* gene. Nucleotide changes of C 491 T, C 512 T, C 516 T, A 513 C, A 513 T were seen in the 530 ring. In the 915 region, C 903 A, C 903 G, A 904 G nucleotide changes are common. It is thought that low-value MIC resistance against streptomycin may be due to the decrease in the entry of the drug into the cell as a result of changes in the cell membrane (Rattan *et al.,* 1998; Ramaswamy and Musser 1998).

2.4.2.1.4. Ethambutol (EMB)

Ethambutol is a synthetic, bacteriostatic drug. Ethambutol disrupts cell wall synthesis of MTBK by interacting with arabinogalactan and arabinosyl transferase enzyme involved in lipoarabinomannan synthesis. Mutations in the *emb* ABC gene encoding the arabinyl transferase enzyme are responsible for 70% ethambutol resistance. In particular, the most common mutations are due to 60% change in the *Met* amino acid in the 306th codon of the *embB* gene. High MIC values of ethambutol resistance were associated with *Met* 306 *Leu*, *Met* 306 *Val* mutations rather than *Met* 306 *Ile* mutation. (Rattan *et al.*, 1998; Ramaswamy and Musser 1998) .

2.4.2.1.5. Pyrazinamide (PZA)

PZA is the structural analog of nicotinamide. PZA, whose importance as an anti-tuberculosis agent was recognized in 1952 and was effective in the creation of short-term treatment protocols in the 1980s, is effective on semi-dormant tuberculosis bacilli showing low metabolic activity in macrophages under acidic conditions. Although PZA and INH are nicotinamide derivatives, their mechanism of action is different. However, it creates strong synergy with INH and RIF. PZA is a prodrug and the sensitivity of mycobacteria to PZA depends on the presence of the specific Pyrazinaminidase (Pzaze). PZA is thought to be effective in the acidic environment of phagolysosomes by converting to pyrazinoic acid, its active form, with Pzaze produced by tuberculosis bacillus. As a matter of fact, other mycobacteria that do not have this enzyme activity, such as *M. bovis*, show intrinsic resistance to PZA. It becomes active at pH 5.5 in macrophage phagolysosomes. While MIC is 1650 μg/ml at pH 5.5, it is 100 μg / ml at pH 6.0, it is not effective at neutral pH. Apart from pH, the effectiveness of pyrazinamide is determined by the ecrease in the number of bacteria, iron, efflux, energy inhibitors and oxygen. Resistance to PZA develops easily, but cross resistance development is not in responsible with other drugs. The decrease in nicotinamidase activity is blamed for the development of resistance. It is known that there are various types of mutations in the *pncA* gene encoding the pyrazinamidase enzyme in 72-94% of

the strains resistant to PZA. Mutations that show a lot of variability and are distributed throughout the gene is a condition that is not seen in resistance genes other than *pncA*. In contrast, the mutations (in the three regions of the *pncA* gene between codons 3-17, 61-85 and 132-142) show a certain clustering (Sanic *et al.,* 1999).

2.4.2.2. Second line drugs used in TB treatment

Second-line drugs are used in the treatment of MTBC, especially in cases where rifampicin and isoniazid resistance are combined. In addition, second-line drugs are used in the treatment of MOTT (Mycobacterium Other Than Tuberculosis) (Somoskovi *et al.,* 2001).

2.4.2.2.1. Para-amino Salicylic Acid (PAS)

It is a very narrow spectrum drug that is effective only against *M. tuberculosis*. PAS is the structural analog of paraaminobenzoic acid and can have a bacteriostatic effect on *M. tuberculosis* by competitively blocking the conversion of para-aminobenzoic acid to folic acid. It is much weaker than STR and INH. The development of resistance in mycobacteria against this drug will be more late and more difficult than against STR and RIF. When used together, it delays the development of resistance to these drugs and isoniazid. It is currently used in the combined treatment of tuberculosis in children under 2 years of age. Since it is difficult or impossible to detect the onset of the visual toxic effect of EMB, it is necessary to use PAS instead. (Somoskovi *et al.,* 2001)

2.4.2.2.2. Ethionamide

Ethionamide, used only orally, is a second-line agent, used in combination with other drugs when primary drugs are ineffective or contraindicated. Ethionamide is produced from isonicotinic acid such as isoniazid and has bactericidal action. Although the actual mechanism of action is unknown, it is thought to act by inhibiting mycolic acid synthesis in the mycobacterial cell wall as in INH. (Somoskovi *et al.,* 2001)

2.4.2.2.3. Protionamid

It is an INH derivative such as ethionamide and its pharmacological properties are similar to ethionamide. The effect and side effects are the same as ethionamide, and both drugs can be effective on INH-resistant mycobacteria. Cross resistance is seen between ethionamide and protionamide (Somoskovi *et al.*, 2001)

2.4.2.2.4. Cycloserine

Cycloserine is a broad spectrum antibiotic produced by Streptococcus orchidaceus. It is an analog of *D-alanine*, and competitively inhibits the action of *alanine* racemase and D-alanyl-D-alanine synthase. These enzymes inhibit the precursors of the cell wall, and their inhibition leads to regression of cell growth and cell death, possibly by lysis. When used for tuberculosis treatment, it should be given with other effective drugs (Somoskovi *et al.*, 2001) .

2.4.2.2.5. Thioacetazone

It is widely used together with isoniazid in developing countries because it is very cheap. Information on its in vitro efficacy and pharmacokinetics is limited. The drug concentrates in the adrenal glands and reaches bacteriostatic concentrations in the lung. Thioacetazone has pronounced side effects and significant toxicity. This drug is similar in structure to ethionamide and has a similar side effect profile including nausea, vomiting, abdominal discomfort and anorexia. The most important side effects are lethal erythema multiforme and toxic epidermal necrosis (Somoskovi *et al.*, 2001).

2.4.2.2.6. Kanamycin

Kanamycin has similar properties in many respects with the aminoglycoside amikacin and capreomycin. These drugs, which are used only in the treatment of disease caused by resistant microorganisms or non-tuberculosis mycobacteria (TDM) and should be administered parenterally, have similar pharmacokinetics and toxicity. Two drugs from this group should not be combined and should not be combined with Streptomycin, as they are potentially ototoxic and

nephrotoxic. Small groups of patients with tuberculosis were treated with 1 g kanamycin daily. Its common toxic effects are neuromuscular paralysis, respiratory depression, agranulocytosis, anaphylaxis and nephrotoxicity (Somoskovi *et al.*, 2001).

2.4.2.2.7. Amikacin

an aminoglycoside, is very active against many mycobacteria species. It may also be an important drug in the treatment of TDM infections (Somoskovi *et al.*, 2001).

2.4.2.2.8. Capreomycin

Capreomycin, an antimycobacterial cyclic peptide formed by *Streptococcus capreolus*, is effective in both in vitro and experimental tuberculosis. When given alone, bacterial resistance develops against capreomycin; these microorganisms show cross resistance with 50 kanamycin and neomycin. Capreomycin is used in combination with other antituberculosis drugs when bactericidal agents are not tolerated or in the presence of resistant microorganisms (Somoskovi *et al.*, 2001).

2.4.2.2.9. Viomycin

It is effective against many resistant tuberculosis strains. Cross-resistance to capreomycin and viomycin often occurs (Somoskovi *et al.*, 2001).

2.4.2.2.10. Fluoroquinolones

Fluoroquinolones that inhibit DNA gyrase show bactericidal effects. DNA gyrase inhibition leads to deficiency in DNA replication and protein synthesis. Its interaction with other antituberculosis drugs is important. RNA and protein synthesis inhibitors such as RIF can reduce the bactericidal effect of quinolones. Moxifloxacin, a broad spectrum fluoroquinolone, has been found to exhibit similar in vitro activity to rifampicin. There is complete cross-resistance between ofloxacin and ciprofloxacin, and cross-resistance between ciprofloxacin and moxifloxacin

and gatifloxacin in vitro has also been demonstrated. It is thought to be 2 times more effective than ofloxacin, the isomer of levofloxacin. Rarely, allergic reactions, diarrhea, photosensitivity, elevated liver function tests and peripheral neuropathy may be seen. Dose adjustment of ciprofloxacin, ofloxacin and levofloxacin is required in renal failure, this requirement is not available for moxifloxacin (Somoskovi *et al.*, 2001).

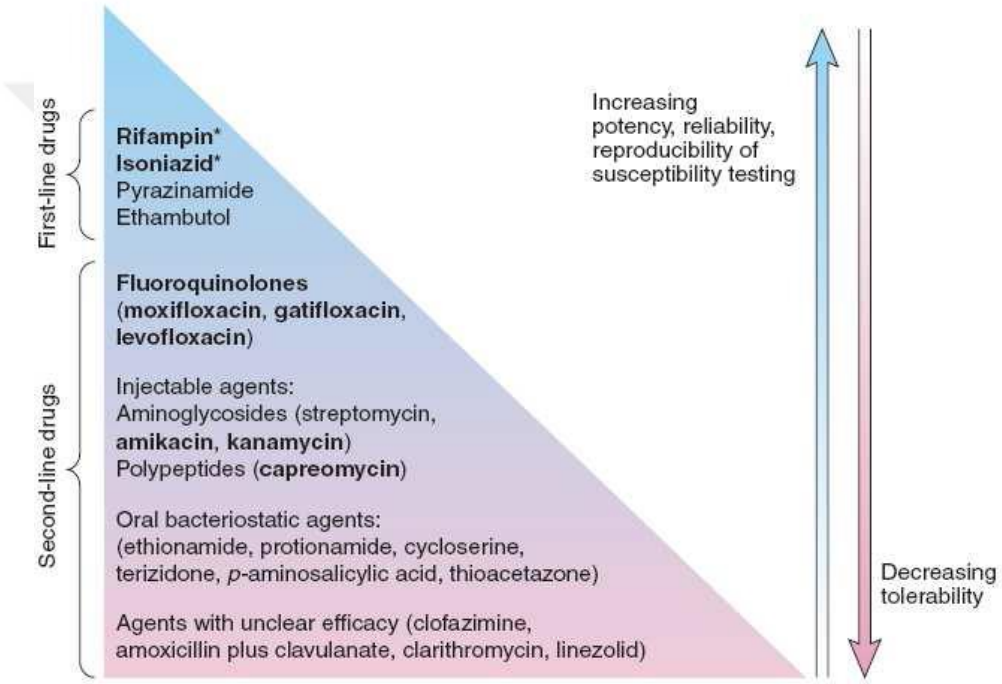

Figure 2.11. Effectiveness of first-line and second-line anti-TB drugs (Dorman S.E. *et al.*, 2004).

2.5. Tuberculosis Diagnosis

This is the main approach used in this study in identifying tuberculosis. Pulmonary tuberculosis is diagnosed from a chest x-ray that shows lung disease, whereas extra-pulmonary tuberculosis is diagnosed through the process of biopsy or fine needle aspiration. The most effective methods that can be used to diagnose TB are microscopy, commercial molecular marker tests, and culture.

2.5.1. Microscopy with Direct Smear

It is most common diagnostic technique applied to active pulmonary tuberculosis in developing countries. The preservation of after alcohol-acid decolourisation of the red carbol fuschion dye relies on the light microscopy smear analysis after Ziehl Neelsen staining (Forrellad *et al.*,2013). Sputum smear microscopy is relatively easy and cheap to do. Unfortunately, its low sensitivity (~50%) combined with its inherent dependency on the development of the sampling sputum limits its use in certain vulnerable groups, such as children and people with HIV (Shingadia and Novelli, 2003; Getahun *et al.*,2007).

There have been attempts to develop more rapid procedures by using fluorescent dyes to do automated sampling and staining instead of the traditional Ziehl Neelsen procedure Figure 2.12. these protocols are 10% more sensitive than light microscopy, are less time-consuming but high prices (World Health Organization, 2011). Despite these disadvantages Sputum smear microscopy is good for speed and requires no specialised technology, making it ideal for endemic areas of Africa with scarce resources.

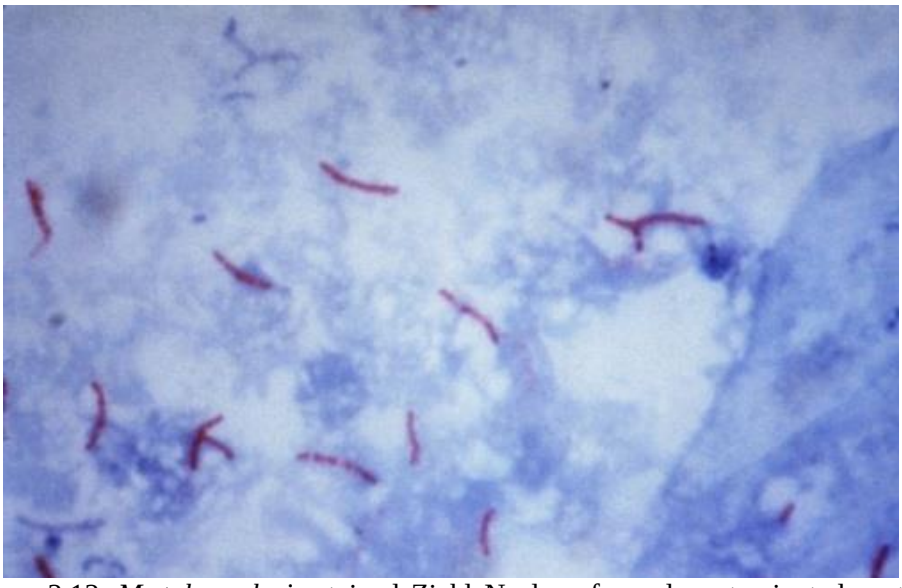

Figure 2.12. *M. tuberculosis* stained Ziehl Neelsen from decontaminated sputum contained in oil immersion (x1000)

2.5.2. Radiology of the Chest

Chest X-rays are also used as an additional technique for microscopy diagnosis in the diagnosis of TB. Anomalies seen with chest x-rays in the upper lungs are usually testamentary to tube but usually not definite (such as infiltration or cavities). Chest X-rays are also used to rule out lung tuberculin in people who have a positive tuberculin reaction and no symptoms of disease (Kumar *et al.*,2007).

2.5.3. Culturing of Mycobacterium tuberculosis in vitro

Causative isolation is a clear proof of the disease and known as the TB diagnostic gold standard. In addition, bacterial isolates can be acquired which can be used for in-depth research. Although this technique is highly sensitive and requires only a few sustainable baccili to start growth (Allen and Mitchison 1992), the slow growth rate of MTBC (3-4 weeks) and requirement that a clear decontamination solution be used not only as a prize-in-person fast test for the diagnosis of active TB prevents this technique from being used (Palomino *et al.*,1998).

2.5.4. Alternative diagnostic instruments for MTBC detection

Several molecular diagnoses based on the amplification of specific markers have been produced as complementary instruments for traditional microbiological diagnosis of TB. Most of these tests also benefit from TB diagnosis and simultaneous detection of drug resistance. The Xpert MTB/RIF (Kurtatova *et al.*,2012), and the HAIN life science line testing are currently in use in several endemic countries. Xpert MTB/RIF based on PCR real time chain reaction amplification in the *rpoB* gene detects sputum-to-rifampicin resistance directly in less than 2 hours, regardless of smoothness. Moreover, as all reagents are integrated, this method does not require any additional reagents, which decreases costs. In contrast, the GenoType MTBDR plus line test detects resistance of both

the isoniazide and rifampicin in less than 2 hours from pulmonary patient specimens (Miotto *et al.*,2008; Barnard *et al.*,2008; Bazira *et al.*,2010).

In addition to molecular assays, there are currently several immunological assays for the diagnosis of latent TB. The Mantoux test is a major worldwide test of tuberculin skin that replaces several tests including the Tine test (Mendel, 1908). This test consists of a delayed measurement of the hyperensitive reaction following intradermal tuberculin injection. The Mantoux test, irrespective of its simplification and utility, is limited by poor specific characteristics, particularly among vaccinated individuals who are Bacille Calmette-Guerin (BCG).

In recent times, the commercial antigens-specific testing of Interferon-μ (interferon-gamma) releases from T-lymphocytes has been developed as an alternative to the Mantoux test, as well as commercial immune tests (Quantiferon-gold in tube (Cellestis, Australia) and enzymes-linked immune spot (T-Spot TB (Oxford, Immunotec, UK) (Ferrara *et al.*,2006). Both tests are based on the ability of MTBC antigen to boost interferon gamma (ESAT-6) host production and culture filtrated protein 10 10 (CFP-10). The tests are unique for MTBC infection because these antigens are not also present in any of the BCG vaccine variants or tubercular mycobacteria (Al-Hajoj SA, 2009). (Pai *et al*. 2004). Unfortunately, latent TB cannot be distinguished from active MTB infections in these tests (Rangaka *et al.*,2011). In addition, the need for specialized resources and training to restrict their adoption in developed countries is part of these approaches.

Table 2.5. TB diagnostic tools (approved by WHO)

Method	Intended use	Main strengths	Main weakness	Method
Sputum smear Microscopy for acid fast bacilli	Rapid, point of care 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 in liquid media	TB case detection and as a prerequisite for drug – susceptibility testing	High sensitivity	High contamination rate	Culture in liquid media
Chest radiology	TB case detection (pulmonary TB)	Indicative of TB	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)
Interferon gama release assay	Detection of *M. tuberculosis* infection	Highly specific for *M. tuberculosis*	Requires moderate training and equipment	Interferon gama release assay
Line probe assays	TB case detection and drug susceptibility testing	Short reporting time	Potential for cross contamination, requires extensive training	Line probe assays

2.6. TB pathology

TB is a compulsory aerobic disease pathogen with a passion for areas rich in oxygen (Raja, 2004). Therefore in the well aerated upper lung lobes, the classic tube bacillus is always found. An individual with a tuberculosis active disease must inhale airborne goutlets, but active disease must result in the bacteria being transmitted (Gagneux, 2012). Driple nuclei should be small enough for physical barriers to the lower respiratory tract, a longer exposure time between uninfected and infected persons and low ventilation levels to avoid the upper respiratory tract and nasopharynx. The bacteria loaded in golets therefore need to be high and the

droplet nuclei small enough (1 to 2 mm or less). The bronchial trees enter the lungs after the bacterium is inhaled and are swallowed by alveolar macrophages in the lungs (Kang *et al.*,2011). When bacillos enter the human lung, different results and host protection mechanisms are encountered. The survival in the bacillus lungs thus depends upon the host system's ability to withstand abolition (van Crevel *et al.*,2002).

The bacilli can spread from the lung to the lymph nodes by the lymph system. 5-10% of the infections are progressive for reasons we don't know; the initial immune answer to bacteria is extremely efficient and complex in the lungs (Kang *et al.*,2011). TB infection means the immune system is controlled by baccili in the body. TB infection (TB infection). However, if the removal of an invasive pathogen does not succeed in additional immune cells such as lymphoacytes and dendritic cells, they recruit the invasive pathogen from nearby blood vessels to an infectious focal point. The Immune Reaction (Ernst, 2012). Hosts immune cells attract granuloma, also known as the giant wall, to the infection site to prevent the spread of bacteria into neighboring cells (Russell *et al.*,2010). As the granuloma changes its composition: originally unorganized cells, it is more organic in the center with peripheral lymphocytes and macrophages (Ulrichs and Kaufmann 2006).

In that organization, the complex interaction between innate and cell-mediated immune cells following infection is reflected. The elimination of MTBC infection depends in large part on the success of theB & T lymphocyte and the interaction between the infected macrophages. In the beginning antibodies and B cells were deemed unimportant for the immune defense of MTBC and are now helping to increase immune response to MTBC by modulating various immune components in the host with T-cells (Achkar *et al.*,2014). However, the host developed certain bacilli despite its strong immune protection, that developed efficient solutions to avoid killing the immune response and to enter a dormancy state. The 'containment' stage is also referred to as a latent TB asymptomatic characteristic. The infected person is not infectious at this stage, because the

infection is not able to spread to other people. Granuloma dormant bacilli remain medium through a complex blending of inflammatory cells with mediated cells for decades. Nevertheless the center of granuloma undergoes necrosis if the balance is scaled to favor bacilli, as in the case of systemic immune abolition in the event of co-infection with HIV. The patient is released live and infectious into the alveoli. Spew in the airways to cause productive toxins that spread infectious bacteria to the air. Included or unable to control or eliminated from the bacial lymphocytes, the series of immune response caused by exposure to bacilli for a pathogen, such as MTB, clearly defines infection. The clinical course of the infection and its consequences depend therefore largely on the interaction between various bacterial factors and the host (Russell *et al.*,2009).

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Ethics Approval of Research

The study only used isolates which were routinely obtained from the Tropical Diseases Research and Application Center in Adana / Turkey, which was approved by the Ethical Review Committee in Cukurova University/College of Medicine.

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

Isolates of mycobacteria in MGIT tubes were examined under a microscope to distinguish M. tuberculosis from non-tuberculosis mycobacteria. The presence of cords on Ziehl-Neelsen staining and the formation of turbidity clumps in the medium without appearance led to the identification of these isolates as M. tuberculosis. Reference strain (H37Rv), collected from the Tropical Diseases Research and Application Center in Adana, Turkey.

3.1.3. Reagents and chemicals

MGIT tubes, Streptomycin (STR), Isoniazid (INH), Rifampicin (RIF), Ethambutol (ETM) were purchased from Becton Dickonson and All solutions were prepared on the day of the experiment.

exoSAP (Applied Biosystems™), Sephadex (Sigma-Aldrich), BigDye Terminator v3.1 Cycle sequencing kit, NucleoZOL was purchased from MACHERY-NAGEL (Düren, Germany), cDNA high capacity kit (applied Biosystems).

3.2. Methods

3.2.1. Collection of Bacterial strains

M. tuberculosis clinical isolates were selected from the storage collection of the Tropical Disease Research and Application Center, Cukurova University, the isolates were sputum specimens obtained from patients and cultured on BACTEC-MGIT 960 (figure 3.1), A total of 56 clinical isolates *M. tuberculosis* along with *M. tuberculosis* reference strain H37RV were included in this study. Isolates were selected and divided into two groups the first group includes 28 MDR-TB isolates, which are resistant to INH, and 28 sensitive-TB isolates which are sensitive to the Antibiotics were used.

3.2.2. Testing for drug susceptibility BACTEC MGIT 960 system

All clinical isolates have been tested for susceptibility to antibiotics. MGIT 960 Based Antimicrobial Testing (Becton, Dickinson and Company) has analyzed strains. the concentrations were used are INH (0.1 μg/ml) as a low dose while INH (0.4 μg/ml) was considered as high dose Figure 3.2. Resistance is expressed as the proportion of colonies that grow on the substances' critical concentrations. The interpretation will be based on the customary resistance criteria.

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

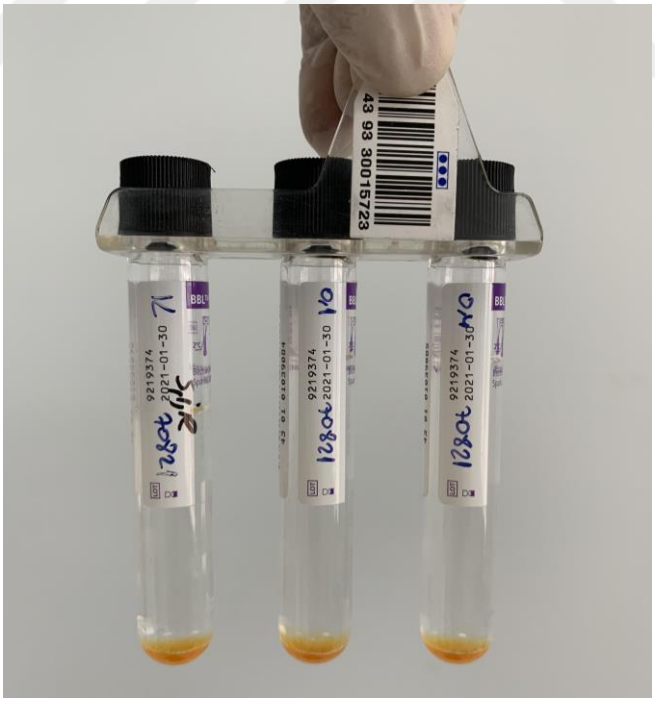

Figure 3.2. MGIT tubes containing isolates under low and high doses INH

3.2.3. RealTime PCR

3.2.3.1. Total RNA Isolation

Total bacterial RNA was isolated using NucleoZOL reagent according to the manufacturer's instructions as follows:

1- 0.2 ml liquid sample was placed in sterile (1.5 ml) micro centrifuge tubes Then 0.5 ml of nucleoZOL™ Reagent was added and homogenized by pipetting.
2- 0.2 ml of water was added and mixed vigorously for 15s then incubated 5 min at room temperature, centrifuge at 12,000 g for 15 min to precipitate contaminates.
3- 0.5 ml of the supernatant was transferred to RNase-free micro centrifuge tubes, 0.5 ml of 100% isopropanol was added to the transferred supernatant and then samples were incubated for 10 min at room temperature, centrifugation at 12,000 × g for 10 min to precipitate total RNA and discard the supernatant.
4- Pellet was washed with 0.5 ml 75% ethanol samples were centrifuged at 8,000 g for 3 min to wash total RNA and supernatant was discarded, washing step was repeated, followed by decant the supernatant and air dry the pellet for 2–5 min, RNA pellet was dissolved in 20µl of RNase-Free H2O by vortexing at room temperature for 3 min and finally samples were stored at –70°C.
5- The ratio of A260nm/A280nm of the RNA was measured according to the value range of 1.8-2.1 using a NanoDrop™ Lite Spectrophotometer.

Figure 3.3. NanoDrop ND-1000 device for DNA and RNA measurements.

3.2.3.2. cDNA synthesis:

RNA was reverse transcribed according to the manufacturer's recommendations High Capacity RNA to cDNA kit (Applied Biosystems). The additions are described in Table 3.1.

Table 3.1. RNA to cDNA kit component and volume (µL) per sample were used

Component	volume
2X RT buffer	10 µL
20X RT Enzyme mix	1.0 µL
RNA sample	9.0 µL
Total per reaction	20.0 µL

Subsequently, tubes were immediately placed in a thermal cycler and the program was as follows:

37°C 60 min

95°C 5 min

4°C infinity

The cDNA stored at −20°C till it was used.

3.2.3.3. Quantification of gene expression using RealTime quantitative PCR (qPCR)

The primers of the 21 genes are described in Table 3.6. The assay was performed using a SYBR green qPCR kit (Promega Company, UK) in Rotor Gene 3000 Rotor 36 x 0,2 ml. Real-Time PCR Cycler.

PCR mix required for a single well in Real-Time PCR.

Syber green master mix	12.5 µl
Forward primer.	0.5 µl
Reverse primer.	0.5 µl
Water	9.0 µl
cDNA	2.5 µl
Total	25 µl

The thermal cycling conditions were as follows:

95°C for 15 min,

then 40 cycles of:

denaturation at 95°C for 15-30 sec,

annealing at 55-65°C for 60 sec.

and the last step consisted of a melting curve analysis (65–95°C).

first choosing *polA* as housekeeping and get the ct values for all samples to count the Δct values for the primers were used (Table 3.2).

Table 3.2. Primers used in this study to quantify gene expression of efflux

No.	Gene	Primer sequence (5′→3′)	Amplicon size (bp)
1	*efpA*	CGCCCTACGGGAAACCAACAAAGA	226
		GCGGAACAAGTGGAACGGCACGAC	
2	*emrB*	ACCGCACAGAACATCCGCTCATAG	148
		GATTGGTGCAACACTTGCTGGAGG	
3	*Rv0849*	GTCGTTCGCAACCGTCCGTTTCTG	94
		CCTGCATGGGCAGAGCCAGATAGA	
4	*Rv1250*	GCAGCCTTGGATTTGGGCGGTGAT	133
		GGACAAGCTGAAGTTCCGGTCGTT	
5	*tap (Rv1258c)*	CGTCTGGAACCTGCGGGTATTGCG	118
		CGGTTGCTGGTGGTCGGTGAAGTA	
6	*Rv1634*	TCGATACCTACGTGCCGCTGTTCG	157
		GCTGCCACGACATGCCCGATAACT	
7	*Rv2994*	ATGCGTCCCGTCCGCCTGAT	147
		GGTGGCTTCTAGCCCGTTGTCC	
8	*Rv1877*	TGTGCTGCGTCCTGTCCTTCGT	235
		AGGAACGCAACCGCCATCAG	
9	*Stp*	TCCGATGATGGATCTGACCCTG	217
		GCCAACCAGGTGCCCAACA	
10	*jefA (Rv2459)*	CGTCGCCCTGATCGCATACA	213
		CAGGACATCACCACGAAGTAGACG	
11	*Rv2265*	CGGTTGTCCTCGGTAATCCT	112
		AACCCGAACGTGCCAAAC	
12	*Rv3239c*	GCCGATTCCTGGCACTTTT	146
		ATGTGGATGGCGGTGTGTT	
13	*mmpl13a*	GACGACCTGCTGGTGATGGAGTTG	242
		CGACTGACGATGAGCAGCGTGTAG	
14	*mmpl13b*	ATGTTCGGCCTCGGCCTGACTTTA	182
		GAACGTCTCCTCGAAACCGGCTCT	
15	*drrA*	TAGACATCGCGTGCGGATTGGT	147
		GCGTGGTCAACAACGTGGCAAT	
16	*drrB*	TCGCCAGCAACTTAGGGCAATACA	233
		TCCGATGACGTAGCCGCAAACTAG	
17	*Mmr*	TAGTGGGTTATGGCATCGCTTTCG	167
		GACGCCAACCACCTTCATCACAGA	
18	*polA*	GTCGTGGTTGGACCTTGGAGGG	181
		GCGTCCGTATCGTCGTCATCG	

3.2.4. DNA Sequencing

3.2.4.1. Genomic DNA Extraction & Amplification

Genomic DNA was extracted from cultures grown on BACTEC-MGIT 960, bacteria were killed for 30 minutes by heating at 95 ° C And DNA was extracted by mickle method, the extracted DNA was immediately analyzed or stored at -20 ° C, PCR amplification assays for genes *rpoB, katG, inhA* and *oxyR-ahpC* were performed. Primers have been chosen from published literature or designed for each of the genes. Specific annealing temperatures and primer sequences used for amplification are included in Table 3.3.

Table 3.3. Primers used fro amplifying and sequencing

Gene	**Primer**	**Sequence (5′→3′)**	**Amplicon size (bp)**
rpoB	F	ACCGACGACATCGACCACTT	450
	R	GTACGGCGTTTCGATGAACC	
katG	F	AATCGATGGGCTTCAAGACG	500
	R	CTCGTAGCCGTACAGGATCTCG	
inhA	F	CCTCGCTGCCCAGAAAGGGA	248
	R	ATCCCCCGGTTTCCTCCGGT	
oxyR-ahpC	F	GAGACCGGCTTCCGACCACC	293
	R	GCTGGTAGGCGGGGAATTGAT	

Each 25-µL PCR mixture contained 12.5 µL of HotStarTaq master mix, 0.25 µL forward primer, 0.25 µL reverse primer, 6 µL of distilled H_2O, 1 µL of Dimethyl sulfoxide (DMSO) and 5 µL of genomic DNA, the reaction was performed in applied Biosystems thermal cycler (MJ Research, Inc.)(Figure 3.3A) for *rpoB, katG, inhA and oxyR-ahpC* genes under the following conditions, Amplification was carried out for 30 cycles (an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 30 s), annealing temperature for three genes (*katG,, rpoB* and *inhA*: 60 °C) for 30 s and elongation at 72 °C for 30 s , with

a final elongation step at 72 °C for 5 min, and the *oxyR-ahpC* gene Amplification was carried out for 35 cycles (an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 45 s, annealing temperature 60 °C for 45 s and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 7 min, The amplification of the gene was detected by 2% agarose gel electrophoresis with ethidium bromide staining and visualized under UV light

3.2.4.2. Agarose gel electrophoresis

In genomic research, agarose electrophoresis is a significant technique. Based on its size and charge, it separates biological molecules. The influence of the negatively charged DNA molecules migrates under the constant current towards the positive charge, and the separation also depends on the DNA mass and charge. The molecules of DNA are forced to move through the pores of the 2% agarose gel (geneticeducation.co.in).

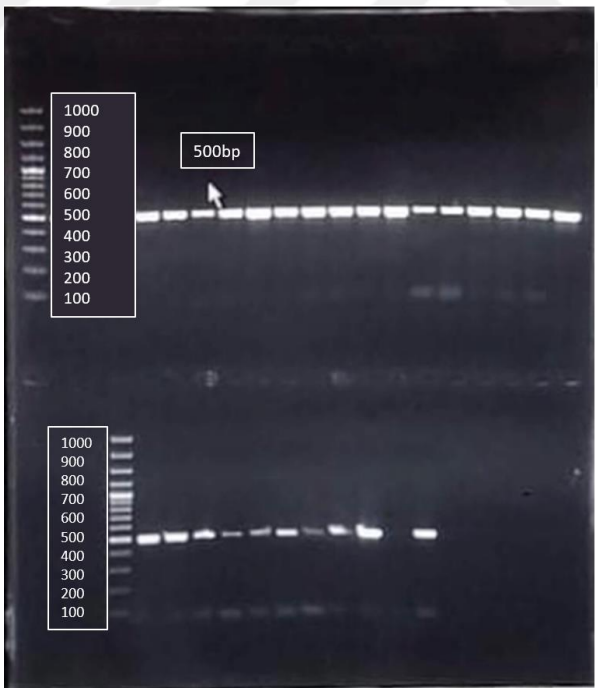

Figure 3.4. Gel Image using *katG* primer on 2% Agaaose gel.

3.2.4.3. Clean Up the PCR Products

1. 2 µl of ExoSAP-IT™ was added to each 5 µl amplified reaction to a new PCR tube.

3. Set the reaction volume to 7 µl and program the thermal cycler as shown in table (3.4)

Table 3.4. Program the thermal cycler conditions for Clean Up the PCR Products

Stage	Description	Temp.	Time
1	Enzyme Incubation	37° C	30 min
2	Enzyme Inactivation	80° C	15 min
3	Hold	4° C	Indefinite Hold

Note: cover heat set 50 °C.

3.2.4.4. Cycle Sequence Reaction

Preparing the samples Reaction for Cycle Sequencing using BigDye applied bysystems kit as follows:

Table 3.5. Reaction mixture for the perform DNA sequnencing reactions

Component	**Volume µL per specimen**
BigDye Terminator Mix Red Reaction	1.0
Sequencing Buffer	2.0
Sequencing Forward Primer	2.0
Distilled Water	3.0
Sample	2.0
Total	10.0

Program the thermal cycler conditions for perform Cycle sequencing stages as following:

96°C	1 min	
96°C	10 sec	25 cycles
50°C	5 sec	
60°C	4 min	
4 oC	∞	

3.2.4.5. Purification of Extension Products

1. 700 µL Sephadex mix was added into a 2 ml tube.
   Note: preparing Sephadex, 1 gram Sephadex to 14 mL ultra-pure water, vortex and shake it gently minimum 30 minutes.
2. The tube was centrifuged for 2 minutes at 4500 rpm and collecting tube with flow-through was discarded.
3. The column was placed back or into the same collecting tube and whole Cycle Sequence product was added to the center of column.
4. The tube was centrifuged for 3 minutes at 4500 rpm and the sample went throw the column to the bottom of the collecting tube and ready to be added to the (ABI PRISM^{tm} 310 Genetic Analayzer) instrument Figure 3.5.

Figure 3.5. (ABI PRISM^{tm} 310 Genetic Analayzer) instrument, department of Microbiology/ Medicine Faculity, Cukurova University.

3.2.5. Data analysis

All data was collected and listed and arranged by Microsoft Excel, then were analyzed by SAS software versio 9.1. the SAS 9.1 software The ANOVA Procedure t Test Least Significant Difference (LSD), the mean values were compared with the Least Significant Difference (LSD) at p 0.05.

4. RESULT AND DISCUSSION

4.1. Susceptibility Testing

All samples were chosen after suseptibility test, 28 isolates were resistant to at least the first-line drug isoniazid (INH) and 28 isolates were sensitive to all frist line drugs. susceptibility test against INH, RIF,EMB, and STR, The concentrations of INH, RIF, EMB, and STR were 0.1 μg/ml, 1.0 μg/ml, 5.0 μg/ml, and 1.0 μg/ml, respectively (Adami *et al.*,2017). Clinical isolates of *M. tuberculosis* that have shown resistance to at least two drugs, including INH and RIF, are considered to be MDR-TB (Nathanson *et al.*,2010).

28 samples were chosen to grow under stress of two different stress concentrations of INH which were 0.1 μg/ml and 0.4 μg/ml in addition to control tube all togather were placed on BACTEC MGIT 960 system and the tubes were collected after a period of 14 days and then samples were ready for RNA extraction, (table 4.1.)

Table 4.1. Drug Susceptibility Profiles

Stains	Susceptibility Profiles (0.1 INH R)	No. of isolates	INH(0.4) resistant
M. tuberculosis complex	Inh^{r}	2	2
	$Inh^{r,}$ Rif^{r}	10	10
	$Inh^{r,}$ $Rif^{r,}$ Emb^{r}	8	8
	$Inh^{r,}$ $Str^{r,}$ Rif^{r}	2	2
	$Inh^{r,}$ $Str^{r,}$ $Rif^{r,}$ Emb^{r}	6	6
	Total	28	28

4.2. Effect of high and low doses of INH on Efflux Pump Gene Expression

The ratio of A260nm/A280nm of the RNA was measured according to the value range of 1.8-2.1 using a NanoDrop™ Lite Spectrophotometer. cDNA was synthesid and 18 genes were analysed using Rotor gene 3000, the values of threshold cycle (ct) were collected (Figure 4.1.a and Figure 4.1.b) and using the ct values $2^{-\Delta\Delta CT}$ were calculated to see the diffences on expression levels between the INH low, high doses comparing with sensitive isolates, furthermore to compare the low and high dose groups according to control group.

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		20	Unknown	22.75			
10		21	Unknown	28.05			
11		22	Unknown	26.77			
12		23	Unknown	23.47			
13		24	Unknown	25.09			
14		25	Unknown	21.54			
15		26	Unknown	20.20			
16		27	Unknown	30.45			
17		28	Unknown	26.76			
18		29	Unknown	29.82			
19		30	Unknown	23.86			
20		31	Unknown	22.63			
21		32	Unknown	25.34			
22		33	Unknown	20.25			
23		34	Unknown	19.06			
24		35	Unknown	19.18			

Figure 4.1.a Ct values obtained from Rotor gene Software

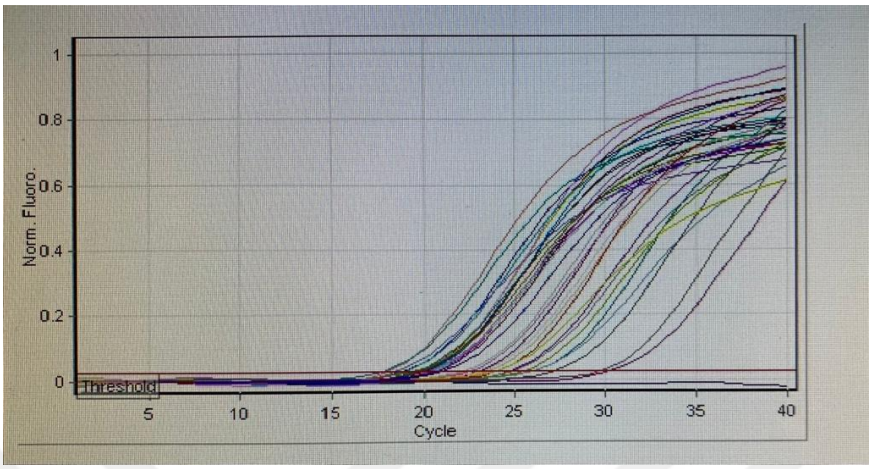

Figure 4.2.b Ct values obtained from Rotor gene Software

18 genes including House-keeping gene *polA* were examined to recognize the effect caused by INH drug stress, it was noticed that all of the 28 *Mycobacterium tuberculosis* complex resistant isolates were over-expressed in eight genes in the control group which were *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239, emrB and Rv2994, Rv1877* was noticed to be overexpressed in 24 isolates and the up- regulation showed in only two isolate, however 22 isolates were overexpressed in 3 genes *drrB, tap* and *Rv2265* and up-regulated in 2, 2 and 4 isolates respectively. Meanwhile *drrA, mmrb* and *Rv1250* showed overexpression in only two of the 28 isolates while not even one isolate was over expressed in jefA (Table 4.2).

Table 4.2. Expression levels of efflux pump genes in control group INH resistant and MDR-TB clinical isolates

stress condition	Gene	No. of MDR isolates with different fold change ($2^{-\Delta\Delta CT}$) value, n = 28									
		0-		1-		2-		3-		>4-	
		Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%
Isoniazid Control	*Rv0849*	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv1634	0	0%	0	0%	0	0%	0	0%	28	100%
	mmpl 13a	0	0%	0	0%	0	0%	0	0%	28	100%
	mmpl 13b	0	0%	0	0%	0	0%	0	0%	28	100%
	stp	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv3239c	0	0%	0	0%	0	0%	0	0%	28	100%
	emrB	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv2994	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv1877	2	7%	2	7%	0	0%	0	0%	24	86%
	drrB	4	14%	2	7%	0	0%	0	0%	22	79%
	Rv2265	2	7%	4	14%	0	0%	0	0%	22	79%
	tap	4	14%	0	0%	2	7%	0	0%	22	79%
	efpA	0	0%	8	29%	2	7%	2	7%	16	57%
	drrA	20	71%	4	14%	2	7%	0	0%	2	7%
	mmrB	24	86%	2	7%	0	0%	0	0%	2	7%
	Rv1250	26	93%	0	0%	0	0%	0	0%	2	7%
	jefA	28	100%	0	0%	0	0%	0	0%	0	0%

The second group (0.1ug/ml) and among the 18 genes, four genes (*mmpl 13a*, *mmpl 13b*, *Rv2265* and *emrB*) showed overexpression in all 28 isolates, 24 isolates showed overexpression in four genes *Rv0849*, *Rv1634*, *stp* and *Rv329c* while at least two of the rest four isolates were up regulated. In *Rv1877* and *Rv2994* 22 isolates showed to be overexpressed out of 28 while only four isolate was up regulated in both. The genes *drrB* and *efpA* showed overexpression in 20 of the isolates while six isolates were up regulated in both. And 18 isolates were overexpressed in tap while six isolates were up regulated. While most of the genes showed overexpression in most of the isolates *drrA*, *jefA*, *mmrB* and *Rv1250* genes did not show any overexpression in any of the isolates and showed an upregulatation in only 2, 4 and 4 isolates in the genes *drrA*, *mmrB* and *Rv1250* respectively, (Table 4.3.).

Table 4.3. Expression levels of efflux pump genes in 0.1 µg/ml INH group resistant and MDR-TB clinical isolates

stress condition	Gene	No. of MDR isolates with different fold change ($2^{-\Delta\Delta CT}$) value, n = 28									
		0-		1-		2-		3-		>4-	
		Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%
Isoniazid 0.1 µg/ml	*Rv0849*	0	0%	2	7%	0	0%	2	7%	24	86%
	Rv1634	2	7%	2	7%	0	0%	0	0%	24	86%
	mmpl 13a	0	0%	0	0%	0	0%	0	0%	28	100%
	mmpl 13b	0	0%	0	0%	0	0%	0	0%	28	100%
	stp	2	7%	0	0%	2	7%	0	0%	24	86%
	Rv3239c	2	7%	2	7%	0	0%	0	0%	24	86%
	emrB	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv2994	2	7%	0	0%	2	7%	2	7%	22	79%
	Rv1877	2	7%	2	7%	0	0%	2	7%	22	79%
	drrB	2	7%	4	14%	0	0%	2	7%	20	71%
	Rv2265	0	0%	0	0%	0	0%	0	0%	28	100%
	tap	4	14%	4	14%	2	7%	0	0%	18	64%
	efpA	2	7%	4	14%	0	0%	2	7%	20	71%
	drrA	26	93%	0	0%	2	7%	0	0%	0	0%
	mmrB	24	86%	2	7%	2	7%	0	0%	0	0%
	Rv1250	24	86%	4	14%	0	0%	0	0%	0	0%
	jefA	28	100%	0	0%	0	0%	0	0%	0	0%

The third group showed growth under the stress of 0.4 µg/ml INH drug, the overexpression was occurred in 13 of the genes furthermore 8 genes (*Rv0849, mmpl 13a, mmpl 13b, stp, Rv3239c, Rv1877* and *Rv2994*) were showing the overexpression in all of the 28 isolates while *Rv2265* and *emrB* showed overexpression in 26 of 28 isolates and up regulation in two isolates for *Rv2265*. The overexpression in *drrB* and *tap* showed in 24 isolates and at least two isolates were up regulated, *efpA* showed overexpression in 18 isolates and up-regulation in 10 isolates. Meanwhile *drrA* showed only two overexpressed isolate and four others were up-regulated, however *jefA* did not show any overexpression. *mmrb* showed that only four isolates were up-regulated and *Rv1250* showed up-regulation in two isolates with not even one overexpression, (Table 4.4.).

Table 4.4. Variance expression levels of efflux pump genes in 0.4 µg/ml INH group resistant and MDR-TB clinical isolates

stress condition	Gene	No. of MDR isolates with different fold change ($2^{-\Delta\Delta CT}$) value, n = 28									
		0-		1-		2-		3-		>4-	
		Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%
Isoniazid 0.4 µg/ml	*Rv0849*	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv1634	0	0%	0	0%	0	0%	0	0%	28	100%
	mmpl 13a	0	0%	0	0%	0	0%	0	0%	28	100%
	mmpl 13b	0	0%	0	0%	0	0%	0	0%	28	100%
	stp	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv3239c	0	0%	0	0%	0	0%	0	0%	28	100%
	emrB	2	7%	0	0%	0	0%	0	0%	26	93%
	Rv2994	0	0%	0	0%	0	0%	0	0%	28	100%
	Rv1877	0	0%	0	0%	0	0%	0	0%	28	100%
	drrB	2	7%	0	0%	0	0%	2	7%	24	86%
	Rv2265	0	0%	2	7%	0	0%	0	0%	26	93%
	tap	0	0%	0	0%	2	7%	2	7%	24	86%
	efpA	0	0%	4	14%	4	14%	2	7%	18	64%
	drrA	22	79%	4	14%	0	0%	0	0%	2	7%
	mmrB	24	86%	4	14%	0	0%	0	0%	0	0%
	Rv1250	26	93%	2	7%	0	0%	0	0%	0	0%
	jefA	28	100%	0	0%	0	0%	0	0%	0	0%

Using the SAS 9.1 software The ANOVA Procedure t Test Least Significant Difference (LSD) it was recognized that there were no significant differences among the three groups in comparing with the sensitive isolates fro the same efflux pump genes for all the genes except *emrB* which showed significant

differences (LSD 14.394, control group mean 28.502, 0.1 μg/ml INH mean 17.631 and 0.4 μg/ml INH mean 13.412), while all the other genes did not show any significant diferrence when comparing the three groups with the sensitive isolates, *Rv1877* (LSD 18.906, control group mean 23.429, 0.1 μg/ml INH mean 28.308 and 0.4 μg/ml INH mean 38.278), *Rv3239c* (LSD 103.55, control group mean 109.83, 0.1 μg/ml INH mean 121.18 and 0.4 μg/ml INH mean 115.53), *Rv2994* (LSD 69.387, control group mean 88.84, 0.1 μg/ml INH mean 77.81 and 0.4 μg/ml INH mean 80.53), *Rv2265* (LSD 53.994, control group mean 72.52, 0.1 μg/ml INH mean 85.78 and 0.4 μg/ml INH mean 33.75), *tap* (LSD 25.965, control group mean 18.08, 0.1 μg/ml INH mean 26.20 and 0.4 μg/ml INH mean 23.85), *drrA* (LSD 0.4706, control group mean 0.7138, 0.1 μg/ml INH mean 0.5908 and 0.4 μg/ml INH mean 0.6285), *drrB* (LSD 57.302, control group mean 48.57, 0.1 μg/ml INH mean 34.34 and 0.4 μg/ml INH mean 41.95), *efpA* (LSD 5.3859, control group mean 4.593, 0.1 μg/ml INH mean 7.864 and 0.4 μg/ml INH mean 9.284), *jefA* (*Rv2459*) (LSD 0.1439, control group mean 0.25000, 0.1 μg/ml INH mean 0.25929 and 0.4 μg/ml INH mean 0.17143), *mmrB* (LSD 0.5736, control group mean 0.7529, 0.1 μg/ml INH mean 0.5900 and 0.4 μg/ml INH mean 0.5693), *Rv0849* (LSD 22.725, control group mean 28.97, 0.1 μg/ml INH mean 42.16 and 0.4 μg/ml INH mean 40.23), *Rv1250* (LSD 0.6426, control group mean 0.6393, 0.1 μg/ml INH mean 0.4871 and 0.4 μg/ml INH mean 0.4357), *RV1634* (LSD 51.24, control group mean 54.89, 0.1 μg/ml INH mean 71.27 and 0.4 μg/ml INH mean 51.46), *mmpl13a* (LSD 53.622, control group mean 74.60, 0.1 μg/ml INH mean 127.62 and 0.4 μg/ml INH mean 104.64), *mmpl13b* (LSD 188.54, control group mean 177.09, 0.1 μg/ml INH mean 257.41 and 0.4 μg/ml INH mean 222.86), *stp* (LSD 51.152, control group mean 74.17, 0.1 μg/ml INH mean 76.53 and 0.4 μg/ml INH mean 61.20) Figure 4.3. and Figure 4.4.

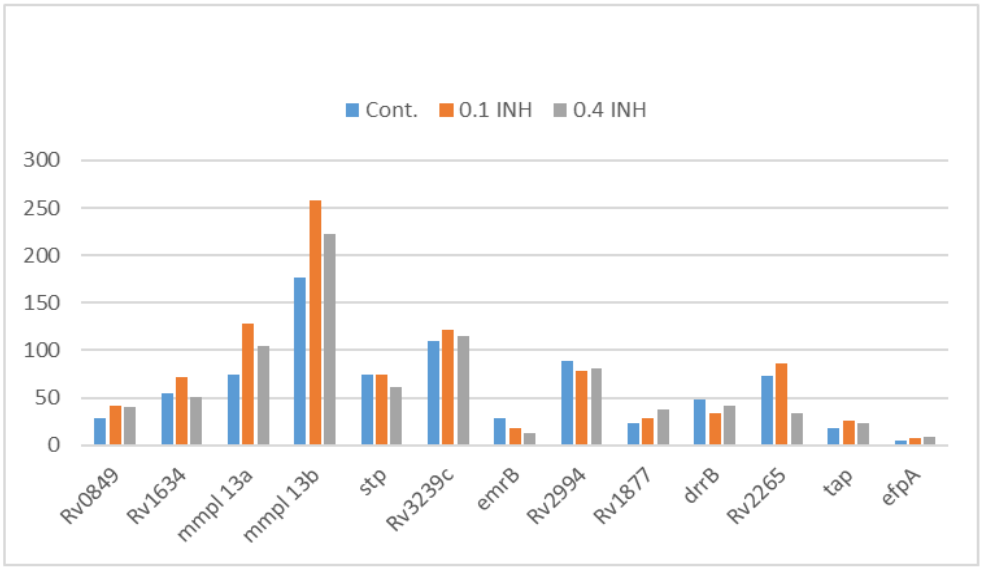

Figure 4.3 Comparison efflux pump genes expression levels between sensitive isolates and resistant isolates (Control, 0.1 µg/ml INH 0.4 µg/ml INH groups) for the genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB, Rv2994, Rv1877, drrB, Rv2265, tap and efpA.*

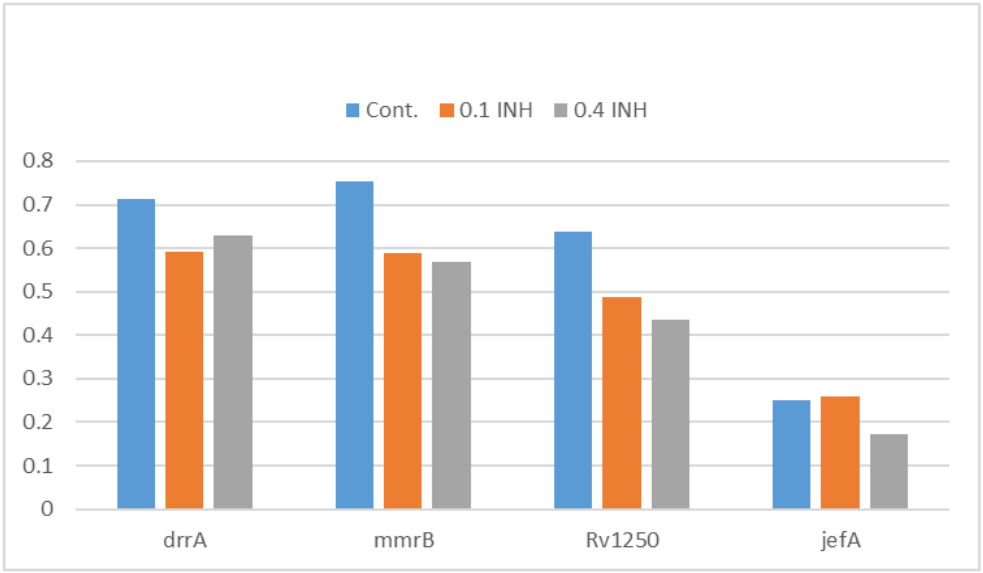

Figure 4.4 Comparison efflux pump genes expression levels between sensitive isolates and resistant isolates (Control, 0.1 µg/ml INH 0.4 µg/ml INH groups) for the genes *drrA, mmrB, Rv1250 and jefA.*

Sult as its listed in Table 4.5. describing the previously fine detailed results with an abbreviation for the three groups when fold changing values ($2^{-\Delta\Delta CT}$) are betwwn 0-1, the most overexpressed genes show 0 or almost 0 isolates were listed while the other genes showed all or almost all the isolates were included. On the other hand, same comparision is listed in Table 4.5. describing the previously detailes results with an abbreviation for the three groups when fold changing values ($2^{-\Delta\Delta CT}$) are $\geq$ 4, the most overexpressed genes show that all or almost all the isolates were included when fold changing values $\geq$ 4 meanwhile the other genes showed 0 or almost 0 isolates were overexpressed.

Table 4.5. Expression levels of efflux pump genes in MDR-TB clinical isolates with 0- to 1 fold change

Gene	No. and percentage of MDR isolates with different fold change ($2^{-\Delta\Delta CT}$) value, n = 28					
	Isoniazid Cont.		Isoniazid 0.1		Isoniazid 0.4	
	Freq.	%	Freq.	%	Freq.	%
Rv0849	0	0%	0	0%	0	0%
Rv1634	0	0%	2	7%	0	0%
mmpl 13a	0	0%	0	0%	0	0%
mmpl 13b	0	0%	0	0%	0	0%
stp	0	0%	2	7%	0	0%
Rv3239c	0	0%	2	7%	0	0%
emrB	0	0%	0	0%	2	7%
Rv2994	0	0%	2	7%	0	0%
Rv1877	2	7%	2	7%	0	0%
drrB	4	14%	2	7%	2	7%
Rv2265	2	7%	0	0%	0	0%
tap	4	14%	4	14%	0	0%
efpA	0	0%	2	7%	0	0%
drrA	20	71%	26	93%	22	79%
mmrB	24	86%	24	86%	24	86%
Rv1250	26	93%	24	86%	26	93%
jefA	28	100%	28	100%	28	100%

Table 4.6. Expression levels of efflux pump genes in MDR-TB clinical isolates with >4- fold change

Gene	No. and persentage of MDR isolates with different fold change ($2^{-\Delta\Delta CT}$) value, n = 28					
	Isoniazid Cont.		Isoniazid 0.1		Isoniazid 0.4	
	Freq.	%	Freq.	%	Freq.	%
Rv0849	28	100%	24	86%	28	100%
Rv1634	28	100%	24	86%	28	100%
mmpl 13a	28	100%	28	100%	28	100%
mmpl 13b	28	100%	28	100%	28	100%
stp	28	100%	24	86%	28	100%
Rv3239c	28	100%	24	86%	28	100%
emrB	28	100%	28	100%	26	93%
Rv2994	28	100%	22	79%	28	100%
Rv1877	24	86%	22	79%	28	100%
drrB	22	79%	20	71%	24	86%
Rv2265	22	79%	28	100%	26	93%
tap	22	79%	18	64%	24	86%
efpA	16	57%	20	71%	18	64%
drrA	2	7%	0	0%	2	7%
mmrB	2	7%	0	0%	0	0%
Rv1250	2	7%	0	0%	0	0%
jefA	0	0%	0	0%	0	0%

The expression levels were determined once again to see the effect of the low and high doses of INH drug on efflux pump genes expression levels but this time in compring to the resistant control group (Table 4.7. and Table 4. 8.) to investigate the differences btween the groups were under stress and their control group, also to investigate the diferrence between the two different doses groups (Table 4. 9.) using the SAS 9.1 software The ANOVA Procedure t Test Least Significant Difference (LSD) and it was recognized that there were no significant differences among the three groups for all the genes, *emrB* (LSD 0.7566, control group mean 1.0679, 0.1 µg/ml INH 1.1214 and 0.4 µg/ml INH 0.8421), *mmpl13b* (LSD 0.9627, control group mean 1.5870, 0.1 µg/ml INH 1.5710 and 0.4 µg/ml INH 1.3280), *mmpl13a* (LSD 0.7582, control group mean 1.7909, 0.1 µg/ml INH 1.4064 and 0.4 µg/ml INH 1.2236), *Rv2265* (LSD 1.1062, control group mean 1.0573, 0.1 µg/ml INH 1.2618 and 0.4 µg/ml INH 1.1364), *efpA* (LSD 1.821, control group mean 2.2408, 0.1 µg/ml INH 2.2123 and 0.4 µg/ml INH 2.1177), *jefA* (Rv2459) (LSD 0. 8626, control group mean 1.3654, 0.1 µg/ml INH 1.0377 and 0.4 µg/ml INH 1.2138), *mmrB* (LSD 0.9037, control group mean 1.1615, 0.1 µg/ml INH 1.0723 and 0.4 µg/ml INH 1.6992), *Rv1634* (LSD 1.388, control group mean 1.6269, 0.1 µg/ml INH 1.6408 and 0.4 µg/ml INH 1.4385), *stp* (LSD 0.8973, control group mean 1.3215, 0.1 µg/ml INH 1.2877 and 0.4 µg/ml INH 1.1777), *Rv3239c* (LSD 2.02809, control group mean 1.6162, 0.1 µg/ml INH 1.6969 and 0.4 µg/ml INH 1.6969), *drrA* (LSD 0.7731, control group mean 1.0661, 0.1 µg/ml INH 1.0748 and 0.4 µg/ml INH 1.4090), *drrB* (LSD 1.5428, control group mean 1.8167, 0.1 µg/ml INH 1.8000 and 0.4 µg/ml INH 1.4317), *Rv0849* (LSD 0.9042, control group mean 1.2967, 0.1 µg/ml INH 1.4267 and 0.4 µg/ml INH 1.5517), *Rv1250* (LSD 3.2099, control group mean 1.385, 0.1 µg/ml INH 2.489 and 0.4 µg/ml INH 2.738), *Rv1877* (LSD 2.149, control group mean 2.145, 0.1 µg/ml INH 2.103 and 0.4 µg/ml INH 1.754), *Rv2994* (LSD 0.9756, control group mean 1.1442, 0.1 µg/ml INH 1.5067 and 0.4 µg/ml INH 1.6542), *tap* (LSD 4.532, control group mean 2.912, 0.1 µg/ml INH 2.780 and 0.4 µg/ml INH 3.214) Figure 4.5.

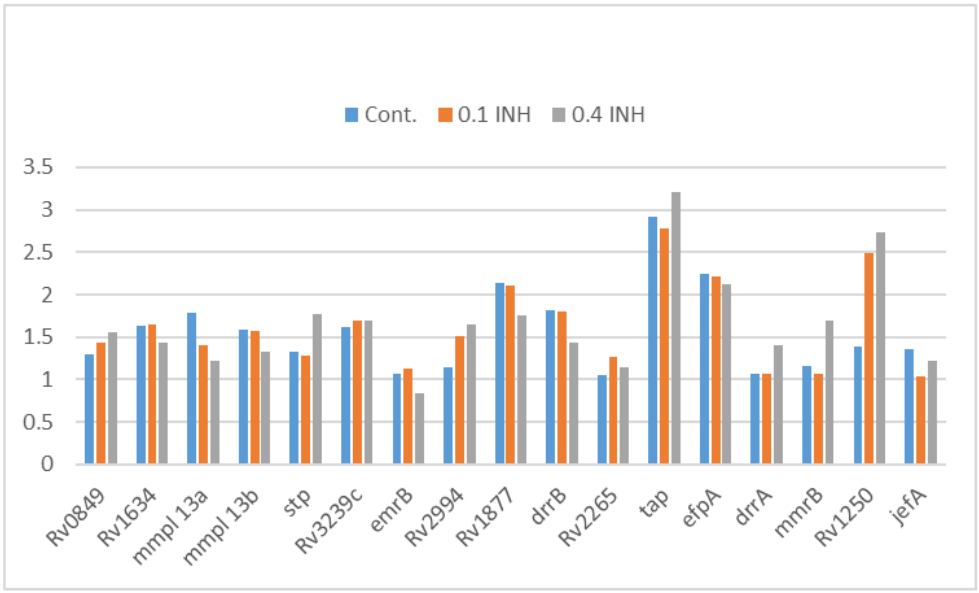

Figure 4.5 Comparison efflux pump genes expression levels among resistant isolates (Control, 0.1 µg/ml INH 0.4 µg/ml INH groups) for all the genes were used in the study considering *polA* as housekeeping gene.

Table 4.7. Comparison of 0.1ug/ml resistant MDR-TB Expression levels with control (ΔΔCT = ΔCT 0.1 – ΔCT control)

Gene	No. and percentage of MDR isolates with different fold change ($2^{-\Delta\Delta Ct}$ value, n = 28									
	0-		1-		2-		3-		>4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	12	43%	10	36%	0	0%	4	14%	2	7%
Rv1634	12	43%	8	29%	2	7%	0	0%	6	21%
mmpl 13a	4	14%	8	29%	12	43%	0	0%	4	14%
mmpl 13b	8	29%	8	29%	2	7%	0	0%	10	36%
stp	14	50%	6	21%	4	14%	0	0%	4	14%
Rv3239c	14	50%	8	29%	2	7%	0	0%	4	14%
emrB	16	57%	10	36%	0	0%	2	7%	0	0%
Rv2994	16	57%	8	29%	0	0%	0	0%	4	14%
Rv1877	12	43%	3	21%	4	14%	2	7%	4	14%
drrB	14	50%	2	14%	0	0%	0	0%	10	36%
Rv2265	12	43%	8	29%	2	7%	0	0%	6	21%
tap	14	50%	2	7%	4	14%	4	14%	4	14%
efpA	3	21%	12	43%	2	7%	6	21%	2	7%
drrA	20	71%	4	14%	4	14%	0	0%	0	0%
mmrB	12	43%	14	50%	0	0%	2	7%	0	0%
Rv1250	12	43%	6	21%	6	21%	0	0%	4	14%
jefA	12	43%	8	29%	4	14%	2	7%	2	7%

Table 4.8. Comparison of 0.4ug/ml resistant MDR-TB Expression levels with control (ΔΔCT = ΔCT 0.4 – ΔCT control)

Gene	No. and percentage of MDR isolates with different fold change ($2^{(-\Delta\Delta Ct)}$ value, n = 28									
	0-		1-		2-		3-		>4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	12	43%	8	29%	2	7%	0	0%	6	21%
Rv1634	14	50%	4	14%	4	14%	2	7%	4	14%
mmpl 13a	6	21%	16	57%	0	0%	0	0%	6	21%
mmpl 13b	6	21%	8	29%	8	29%	4	14%	2	7%
stp	12	43%	10	36%	2	7%	4	14%	0	0%
Rv3239c	10	36%	12	43%	0	0%	2	7%	4	14%
emrB	16	57%	6	21%	2	7%	4	14%	0	0%
Rv2994	8	29%	10	36%	6	21%	4	14%	0	0%
Rv1877	8	29%	6	21%	8	29%	0	0%	6	21%
drrB	12	43%	4	14%	4	14%	2	7%	6	21%
Rv2265	14	50%	0	0%	4	14%	4	14%	6	21%
tap	18	64%	0	0%	0	0%	2	7%	8	29%
efpA	10	36%	2	7%	8	29%	2	7%	6	21%
drrA	16	57%	6	21%	6	21%	0	0%	0	0%
mmrB	12	43%	14	50%	2	7%	0	0%	0	0%
Rv1250	12	43%	10	36%	0	0%	2	7%	4	14%
jefA	18	64%	6	21%	4	14%	0	0%	0	0%

Table 4.9. Comparison of 0.4 ug/ml resistant with 0.1 ug/ml resistant TB Expression levels (ΔΔCT = ΔCT 0.4 – ΔCT 0.1)

Gene	No. of MDR isolates with different fold change ($2^{(-\Delta\Delta Ct)}$) value, n = 28									
	0-		1-		2-		3-		>4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	12	43%	8	29%	4	14%	0	0%	4	14%
Rv1634	18	64%	6	21%	0	0%	0	0%	4	14%
mmpl 13a	20	71%	4	14%	2	7%	2	7%	0	0%
mmpl 13b	14	50%	12	43%	0	0%	2	7%	0	0%
stp	18	64%	4	14%	2	7%	2	7%	2	7%
Rv3239c	10	36%	10	36%	2	7%	2	7%	4	14%
emrB	22	79%	2	7%	4	14%	0	0%	0	0%
Rv2994	12	43%	4	14%	4	14%	4	14%	4	14%
Rv1877	8	29%	10	36%	2	7%	2	7%	6	21%
drrB	8	29%	10	36%	4	14%	2	7%	4	14%
Rv2265	16	57%	8	29%	0	0%	0	0%	4	14%
tap	14	50%	2	7%	2	7%	0	0%	10	36%
efpA	14	50%	8	29%	0	0%	4	14%	2	7%
drrA	20	71%	4	14%	4	14%	0	0%	0	0%
mmrB	12	43%	6	21%	2	7%	2	7%	6	21%
Rv1250	10	36%	8	29%	0	0%	2	7%	8	29%
jefA	16	57%	10	36%	0	0%	0	0%	2	7%

4.3. Correlation between the Mutations Associated with Antibiotic Resistance and Efflux pump genes

First, in order to establish a relationship between the level of phenotypic drug resistance and the presence of resistance-associated mutations, we checked for mutations in genes associated with drug resistance to the antibiotic studied. All pan-susceptible strains showed no mutation in any of the genes evaluated. Resistance to isoniazid was associated with mutations in the katG and inhA genes (Cambau *et al.*,2015; Domínguez *et al.*,2016).

The resulting DNA sequences were analyzed using the basic local alignment search tool (http://www.ncbi.nih.gov/BLAST) and using of the software Finch tv to indicate the present of the mutations, mutations were found as described in, the most comon mutation was determind is 315 AGC/ACC on *katG* gene (n=25/28) the amino acid variation Ser 315 Thr, then the 531 TCG/TTG on *rpoB* as it was determined (n=24/28) and the amino acid variation is Ser 531 Leu, following by the mutation C15T on *inhA* which occured only in 13 samples (n=13/28)n finally the least determined mutation was 526 CAC/GAC on the gene *rpoB*, while oxyR-ahpC did not show any mutation, (Table 4.10.)

Table 4.10. The mutations on different genes and isolates of the current study (n=28)

Gene	Codon	Nucleotide Variation	Amino Acid Variation	Isolates No.	
				n	%
rpoB	531	TCG/TTG	Ser 531 Leu	24	92.3%
	526	CAC/GAC	His 526 Asp	2	7.7%
katG	315	AGC/ACC	Ser 315 Thr	25	89.3%
inhA	-15	C/T	C15T	13	46.4%
oxyR-ahpC	-	-	-	0	0%

The relationship between the mutaions and the present of the efflux pumps overexepression

It was recognized that 100% of the isolates (n=25) in which the mutation *katG* 315 AGC-ACC occurred these isolates were showing overexpression in all the isolates and for the genes *Rv0849, Rv1634, mmpl 13a*, *mmpl 13b*, *stp*, *Rv3239c*, *emrB, Rv2994* and *Rv1877*. While most of the isolates (n=23/25) showed overexpression in the genes *Rv2265* and *tap* in the present of the mutation, 20 and 17 out of 25 isolates were showed to be overexpressed in *efpA* and *drrB* in the present of the mutation, (Table 4.11.)

Table 4.11. The relationship between *katG* 315 AGC-ACC mutation and efflux pump genes

Genes	***katG*, 315 AGC-ACC**									
	0-		1-		2-		3-		4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	0	0%	0	0%	0	0%	0	0%	25	100%
Rv1634	0	0%	0	0%	0	0%	0	0%	25	100%
mmpl 13a	0	0%	0	0%	0	0%	0	0%	25	100%
mmpl 13b	0	0%	0	0%	0	0%	0	0%	25	100%
stp	0	0%	0	0%	0	0%	0	0%	25	100%
Rv3239c	0	0%	0	0%	0	0%	0	0%	25	100%
emrB	0	0%	0	0%	0	0%	0	0%	25	100%
Rv2994	0	0%	0	0%	0	0%	0	0%	25	100%
Rv1877	0	0%	0	0%	0	0%	0	0%	25	100%
drrB	5	20%	3	12%	0	0%	0	0%	17	68%
Rv2265	2	8%	0	0%	0	0%	0	0%	23	92%
tap	2	8%	0	0%	0	0%	0	0%	23	92%
efpA	0	0%	5	20%	0	0%	0	0%	20	80%
drrA	14	56%	5	20%	3	12%	0	0%	3	12%
mmrB	19	76%	3	12%	0	0%	0	0%	3	12%
Rv1250	25	100%	0	0%	0	0%	0	0%	0	0%
jefA	25	100%	0	0%	0	0%	0	0%	0	0%

Meanwhile the mutation *rpoB* 531 TCG-TTG presented in 24 isolates out of the 28 isolates, in the present of this mutation it was shown that 100% of these isolates were overexpressed for the genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239, emrB* and *Rv2994* and 91.6 % of the isolates were overexpressed in *Rv1877,* while 79.2% of the isolates for both *drrB* and *Rv2265* were overexpressed, (Table 4.12.)

Table 4.12. The relationship between *rpoB* 531 TCG-TTG mutation and efflux pump genes

Genes	***rpoB*, 531 TCG-TTG**									
	0-		1-		2-		3-		4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	0	0%	0	0%	0	0%	0	0%	24	100%
Rv1634	0	0%	0	0%	0	0%	0	0%	24	100%
mmpl 13a	0	0%	0	0%	0	0%	0	0%	24	100%
mmpl 13b	0	0%	0	0%	0	0%	0	0%	24	100%
stp	0	0%	0	0%	0	0%	0	0%	24	100%
Rv3239c	0	0%	0	0%	0	0%	0	0%	24	100%
emrB	0	0%	0	0%	0	0%	0	0%	24	100%
Rv2994	0	0%	0	0%	0	0%	0	0%	24	100%
Rv1877	2	8.3%	0	0%	0	0%	0	0%	22	91.6%
drrB	2	8.3%	2	8.3%	0	0%	0	0%	19	79.2%
Rv2265	2	8.3%	2	8.3%	0	0%	0	0%	19	79.2%
tap	2	8.3%	0	0%	2	8.3%	0	0%	19	79.2%
efpA	0	0%	7	29.2%	0	0%	2	8.3%	15	62.5%
drrA	15	62.5%	5	20.8%	2	8.3%	0	0%	2	8.3%
mmrB	19	79.2%	2	8.3%	0	0%	0	0%	2	8.3%
Rv1250	22	91.6%	0	0%	0	0%	0	0%	2	8.3%
jefA	24	100%	0	0%	0	0%	0	0%	0	0%

It was obsereved that *inhA*, C-T, C15T mutation was present in 13 isolates out of 28 isolates and the relationship btween the present of this mutation and the overexpression on the different genes was determind and it was showed that all of the 13 isolates were overexpressed on the genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, Rv1877* and *tap,* while *Rv2265* and *efpA* showed overexpression in only 10/13 isolates when the mutation C15T was present, and drrB showed to be overexpressed in 9 isolates in cmparing of the present of the C15T mutation, (Table 4. 13.).

Table 4.13. The relationship between *inhA*, C-T, C15T mutation and efflux pump genes

Genes	***inhA*, C-T, C15T**									
	0-		1-		2-		3-		>4-	
	n	%	n	%	n	%	n	%	n	%
Rv0849	0	0%	0	0%	0	0%	0	0%	13	100%
Rv1634	0	0%	0	0%	0	0%	0	0%	13	100%
mmpl 13a	0	0%	0	0%	0	0%	0	0%	13	100%
mmpl 13b	0	0%	0	0%	0	0%	0	0%	13	100%
stp	0	0%	0	0%	0	0%	0	0%	13	100%
Rv3239c	0	0%	0	0%	0	0%	0	0%	13	100%
emrB	0	0%	0	0%	0	0%	0	0%	13	100%
Rv2994	0	0%	0	0%	0	0%	0	0%	13	100%
Rv1877	0	0%	0	0%	0	0%	0	0%	13	100%
drrB	2	15.4%	2	15.4%	0	0%	0	0%	9	69.2%
Rv2265	3	23.1%	0	0%	0	0%	0	0%	10	76.9%
tap	0	0%	0	0%	0	0%	0	0%	13	100%
efpA	0	0%	3	23.1%	0	0%	0	0%	10	76.9%
drrA	6	46.2%	4	30.7%	3	23.1%	0	0%	0	0%
mmrB	9	69.2%	2	15.4%	0	0%	0	0%	2	15.4%
Rv1250	13	100%	0	0%	0	0%	0	0%	0	0%
jefA	13	100%	0	0%	0	0%	0	0%	0	0%

When comparing the obtained results with other studies it was found that there are some differences in the findings and that might be back to the differences between experiments conditions, differences of isolates susceptibilty profiles, number of the samples, the used statistic system and many other reasons.

Rodrigues *et,al;* (2012) made a study showed that the genes *efpA, mmpL7, mmr, p55* and *the Tap-like gene Rv1258c* were the most overexpressed genes when in the current study it was shown that *efpA* (the only gene included in both studies) was not one of the eight most overexpressed genes however this gene showed overexpression in most of the isolates. While Li *et, al;* (2015) in determination of efflux pump genes overexpression levels it was found that the genes *drrA, drrB, efpA, jefA (Rv2459), mmr, Rv0849, Rv1634, Rv1250* were showing the highest overexpression levels, the listed difference might be related to the number of the isolates were used in the mentioned study which were only 9 isolates (Table 4.14)

Narang *et, al;* (2020) showed that the genes *Rv1634, Rv0849, efpA, p55, Rv1273c, Rv0194, Rv1250, Rv3823c, Rv0507 and jefA* showed the heighst overexpression and that is agreed with our study only in the genes *Rv1634* and *Rv0849* while it does not agree with the rest of the genes, meanwhile the study showed a relationship between the efflux pumps and the mutation *katG* 315 AGC–ACC which is corresponding with the results of our study as listed in table (4.14.) Also *Kanji et, al;* (2016) showed that the mRNA expression levels of efflux genes *Rv2688c* (p = 0037), *Rv1634* (p = 0.0042), *drrA* (p = 0.0078) and *drrB* (p = 0.0003) were upregulated in XDR-TB strains as compared with DS MTB strains.

Kardan-Yamchi et, al; (2019) in their study showed that *drrA, stp and drrB* were found to be the most commonly overexpressed. Whilst no elevation was observed in the expression of mmr, *Rv1250, Rv1634 and Rv1258c* which is again did not agree with our current study as we noticed that *Rv1634* was one of the genes that showed highest overexpression levels. Also Almatar (2018) obtained in his study different results in comparing with the current study as highest

overexpressed genes were *efpA* and *drrA* and the reason behind that might be the using of the pomegranate juice effect against bacterial efflux pump activities.

Albayati (2019) and his study and between mostly the same genes there were differences in the determind results and that might be return to the effect of the INH concentrations were used in our current study, meanwhile the mutations were the agreed part between both studies as *katG G315C, inhA C15T, rpoB C526T* were alos deterimind in both studies (Table 4.14.).

Balgouthi (2019) also determind the same mutations *katG G315C, inhA C15T and rpoB C526T* which were also determined on our current study.

Table 4.14 Other studies compared to the current study

Researcher and the year	The research	Which genes were included	The most overexpressed genes	Concentrations were used	Common mutations	Number of resistance isolates
Rodrigues et,al; 2012	Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex	*16S rRNA, Rv2942 (mmpL7), Rv1258c, Rv1410c, efpA and mmr*	*efpA, Rv2942 (mmpL7), mmr, p55, the Tap-like gene Rv1258c.*	the MGIT tube containing INH (0.1 mg/L)	-----	
Li et,al; 2015	Efflux Pump Gene Expression in Multidrug Resistant *Mycobacterium tuberculosis* Clinical Isolate	*efpA, emrB, Rv0849, Rv1250, tap, Rv1634 Rv2994, Rv1877, stp, jefA, Rv2265, Rv2456c, Rv3239c, mmpL13a, mmpL13b pstB, drrA, drrB, mmr, polA and P55*	*drrA, drrB, efpA, jefA (Rv2459), mmr, Rv0849, Rv1634, Rv1250*	using Lowenstein-Jensen slants with the following: INH, 0.2 µg/mL	-----	9
Narang et, al ; 2020	Contribution of Putative Efflux Pump Genes to Isoniazid Resistance in Clinical Isolates of *Mycobacterium tuberculosis*	*Rv1272c, Rv1456c, Rv1457c, Rv1686c, Rv1687c, Rv0842, Rv0849, Rv0876c, Rv2265, Rv2456c, jefA, rrs, Rv1634, Rv1250, Rv1877, Rv0676c, Rv3823c, Rv0507, efpA, p55, pstB, sigA*	*efpA, Rv1273c, Rv1456c, Rv0842, Rv1457c, jefA, Rv0849*	LJ medium 0.2 µg/mL	*Mutations on katG 315 AGC – ACC But no mutations on inhA*	6
Kanji et, al; 2016	Increased expression of efflux pump genes in extensively drug-resistant isolates of Mycobacterium tuberculosis	*Rv0194, Rv2688c, Rv1634, drrA, and drrB*	*Rv2688c (p = 0.0037), Rv1634 (p = 0.0042), drrA (p = 0.0078) and drrB (p = 0.0003) were upregulated*	----	---	10
Kardan-Yamchi et, al; 2019	Expression analysis of 10 efflux pump genes in multidrug-resistant and extensively drug-resistant Mycobacterium tuberculosis clinical isolates	*efpA, mmr, stp, drrA, drrB, mmpL7, Rv1250, Rv1634, Rv2994 and Rv1258c*	*drrA, stp and drrB*	-----	*rpoB, katG and inhA*	31

Table 4.14. continued

Manaf ALMATAR 2018	COMBINATION OF ANTI-TUBERCULOSIS DRUGS WITH POMEGRANATE JUICE AGAINST MULTIDRUGRESISTANT TB AND EXAMINATION OF THE INFLUENCE OF THIS MIX ON RESISTANT AND EFFLUX PUMP GENES	*efpA, Rv1250, Rv161634, Rv2459, drrA, drrB, polA*	*drrA, drrB, efpA, Rv2459, Rv1634, and Rv1250 were overexpressed under INH/RIF plus FPJ stress*	the MGIT tube containing INH (0.1 ug/mL)	*katG, inhA rpoB, oxyR-ahpC*	9
Aymen ALBAYATI 2019	EFFLUX PUMP GENE EXPRESSION AND EFFLUX PROTEINS IN MULTIDRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS COMPLEX	*Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239, emrB, Rv2994, Rv1877, drrB, tap, Rv2265, efpA, drrA,mmrB,Rv1250,jefA and p55*	*drrA, drrB, efpA, jefA, mmr, Rv0849, Rv1634, and Rv1250 were overexpressed under INH or RIF stress.*	the MGIT tube containing INH (0.1 ug/mL)	*katG G315C inhA C15T rpoB C526T*	40
Khaoula BALGOUTH 2019l	MOLECULAR GENETIC DIFFERENCES OF HIGH AND LOW DOSE ISONIAZID RESISTANCE IN MULTI DRUG MYCOBACTERIUM TUBERCULOSIS	---	--------	the MGIT tube containing INH (0.1 ug/mL and 0.4ug/mL)	*katG G315C (inhA C15T C122T C126T) furAlG, fabGI-pro*	91
Our study	Comparison Of Gene Expression Profiles Between High And Low Dose Isoniazid Resistance In Multi Drug Resistant Mycobacterium tuberculosis	*Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239, emrB, Rv2994, Rv1877, drrB, tap, Rv2265, efpA, drrA,mmrB,Rv1250, polA and jefA*	*Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239, emrB and Rv2994*	the MGIT tube containing INH (0.1 ug/mL and 0.4ug/mL)	*katG G315C inhA C15T rpoB C526T*	28

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

In the current study we found that eight of the efflux pump genes *Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB and Rv2994* were overexpressed in 100% of the isolates when comparing the three groups with the sensitive isolates, however there was no significant differences among these groups. In relation between mutations and efflux expression levelsit was found that specific genes (*Rv0849, Rv1634, mmpl 13a, mmpl 13b, stp, Rv3239c, emrB and Rv2994)* were overexpressed.

Although we did not find a significant difference in over among the INH resistant groups, but we still agree that the efflux pump gene expression plays an important role to become resistant-TB.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusıons

Efflux pumps may play a significant role in the resistance acquired by INH in *M. tuberculosis* complex isolates, particularly in strains with *katG* and *inhA* mutations that are associated with resistance to INH.

In the current study we found that eight of the efflux pump genes *Rv0849, Rv1634, mmpl 13a , mmpl 13b, stp, Rv3239c, emrB* and *Rv2994* were overexpressed in 100% of the isolates when comparing the three groups with the sensitive isolates, however there was no significant differences among these groups.

Although we did not find a significant difference among the INH resistant groups, but we still agree that the efflux pump gene expression plays an important role to become resistant-TB.

5.2. Recommendatıons

Determining same efflux pump genes expression levels on same antibiotic but different doses (if there will be growth on all tubes and if there will not be growth on all isolates then a table showes the numbers and percentage of these isolates will be required), for example 0.2 μg/ml INH as low dose and 0.8 μg/ml INH as a high dose. Moreover, determining the expression levels of different genes is also an other suggestion.

investigating the relationship between efflux pump genes and mutation on different genes while putting *M. tuberculosis* under high and low doses stress for different antibiotic for example RIF, STR and EMB and the mutations related with these antibiotics.

We also would suggest more investigations on expression levels on the genes that were found to be overexpressed (*Rv0849, Rv1634, mmpl 13a , mmpl 13b, stp, Rv3239c, emrB* and *Rv2994*).

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