CUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Aymen Azeez Khalid AL-BAYATI

EFFLUX PUMP GENE EXPRESSION AND EFFLUX PROTEINS IN MULTIDRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS COMPLEX

DEPARTMENT OF BIOTECHNOLOGY

ADANA-2019

ÇUKUROVA UNIVERSITY
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DEPARTMENT OF BIOTECHNOLOGY

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This study supported by Çukurova University Scientific Research Projects Unit.
Project No: FDK-2018-11361

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ABSTRACT

PhD THESIS

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Aymen Azeez Khalid AL-BAYATI

ÇUKUROVA UNIVERSITY I
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DEPARTMENT OF BIOTECHNOLOGY

Supervisor : Prof. Dr. Fatih KÖKSAL
Year: 2019, Pages:130
Jury : Prof. Dr. Fatih KÖKSAL
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Isoniazid (INH) and rifampicin (RIF) are the most effective drugs in the treatment of tuberculosis. Known of molecular resistance mechanisms for these important tuberculosis drugs is essential to quick diagnoses of multidrug-resistant (MDR) tuberculosis and extensive drug-resistant tuberculosis.

Numerous studies show efflux pump as an important mechanism for antibiotic resistance and using of efflux inhibitors with antituberculosis drugs during treatment, enhanced success.

40 clinical MDR Mycobacterium tuberculosis isolates and 40 clinical pan-sensitive isolates were included to evaluate the expression of 20 putative drug efflux pump (EP) genes and sequence mutations in rpoB (RIF), katG (INH), the inhA promoter (INH), and oxyR-ahpC (INH). Nine and three MDR isolates were induced to over- express efflux pump genes by INH and RIF, respectively.

MDR isolates that carried the wild-type katG, inhA, and oxyR-ahpC efflux pump genes were most overexpressed under INH stress in a than in those that carried mutations. drrA, drrB, efpA, jefA, mmr, Rv0849, Rv1634, and Rv1250 were overexpressed under INH or RIF stress.

Without drug inducement, efpA, Rv0849, Rv1250, Rv1634, Rv2994, stp, Rv2459, pstB, drrA, and drrB expression levels were significantly higher ($P < 0.05$) in 40 MDR isolates than in 40 pan- sensitive isolates.

In conclusion, EP may play an important role in INH acquired resistance in MDR tuberculosis especially wild type katG and INH genes. Understanding of expression levels for EP genes between MDR and sensitive tuberculosis some may be helpful to diagnose and treat resistant tuberculosis.

Key words: Tuberculosis, efflux pump, MDR, Isoniazid, Treatment.

ÖZ

DOKTORA TEZİ

ÇOKLU ILAÇ DİRENÇLİ MYCOBACTERIUM TUBERCULOSIS KOMPLEKS SUŞLARINDA EFLÜKS POMPASINI OLUŞTURAN GENLERIN EKSPRESYONLARI VE EFLÜKS PROTEİNLERİ

Aymen Azeez Khalid AL-BAYATI

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BİYOTEKNOLOJİ ANABİLİM DALI

Danışman : Prof. Dr. Fatih KÖKSAL
Yıl: 2019, Sayfa:130
Jüri : Prof. Dr. Fatih KÖKSAL
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İzoniyazid (INH) ve rifampisin (RIF) tüberküloz tedavisinde kullanlan en etkili anti tüberküloz ilaçlardır. Bu önemli tüberküloz ilaçları için moleküler direnç mekanizmalarının bilinmesi, çoklu ilaç dirençli (MDR) tüberküloz ve yaygın ilaç dirençli tüberkülozun hızlı teşhisi için çok önemlidir.

Çok sayıda çalışma, dışa atım (eflüks) pompalarının antibiyotik direnci ile ilişkili olduğun ve antitüberküloz ilaçlarla birlikte dışa atım inhibitörlerinin kullanımının başarıyı arttığını göstermektedir.

Çalışmamıza 20 dışa atım pompa (EP) geninin ekspresyonunu ölçmek ve dirence sebep olan rpoB (RIF), katG (INH), inhA promotör (INH) ve oksiR-ahpC (INH) genlerindeki mutasyonlarını değerlendirmek için 40 MDR Mycobacterium tuberculosis izolatı ve 40 klinik duyarlı izolat dahil edildi.

katG, inhA ve oksiR-ahpC mutasyonu olmayan MDR izolatlarında, mutasyonları taşıyanlara kıyasla eflüks pompa genlerinde daha fazla ekspresyon tespit edilmiştir. drrA, drrB, efpA, jefA, mmr, Rv0849, Rv1634 ve Rv1250 INH veya RIF stresi altında aşırı ekspresyon gözlemlenmiştir.

İlaç etkisi olmadan, MDR izolatlarında duyarlı olanlara göre; efpA, Rv0849, Rv1250, Rv1634, Rv2994, stp, Rv2459, pstB, drrA, ve drrB ekspresyon düzeyleri dikkate değer şekilde daha yüksekti. ($P < 0.05$)

Sonuç olarak, eflüks pompa mekanizması özellikle katG ve inhA genlerinde mutasyonu olmayan INH direncinde oldukça önemlinde önemli rol oynayabileceği düşülmektedir. MDR ve hassas suşlar arasındaki EP genleri ekspresyon seviyelerinin anlaşılması, dirençli tüberküloz tanı ve tedavisinde faydalı olacaktır.

Anahtar Kelimeler: tüberküloz, eflüks pompa, çoklu ilaç dirençli, tedavi

EXPANDED ABSTRACT

Tuberculosis (TB) disease occurs in every part of the world and large numbers of cases have been noted from all over the world. About one third of the population has TB infection and millions of people are dying every year. The disease is caused by *Mycobacterium tuberculosis* (MTB) which usually attacks lungs but can infect other organs also. In the current situation, the major problem in treating this disease is emergence of Multidrug resistance (MDRTB) and Extensive drug resistance (XDRTB) strains, which remains a critical but largely unresolved issue. MDR-TB refers to the TB bacteria which are resistant to first line drugs such as isoniazid and rifampicin, while XDR-TB refers to the TB bacteria which are resistant to fluoroquinolone (FQ) in addition to isoniazid and rifampicin and to at least one of the second line drugs like amikacin, kanamycin or capreomycin (Francis et al., 2014). There are various mechanisms which are involved in development of bacterial resistance. For instance, complex cell wall is an effective way to keep a drug from reaching its target and to prevent it from being taken up by the cell.

Bacteria do this by changing the penetrability of their membranes or by lessening the number of channels available for drugs to diffuse through (Brennan et al., 1995). Another mechanism includes drug modifying & inactivating enzymes for many antibiotics which work by sticking to their target and preventing them from interacting with other molecules inside the cell. For example, some kinds of bacteria produce enzymes called as betalactamases that degrade penicillin (Lomovskaya et al., 2001). Some bacteria respond by changing the structure of the target (or even replacing it with another molecule altogether) so that the antibiotic can no longer recognize or bind to it (Lambert et al., 2005). Spontaneous mutations in bacterial genome can also acquire resistance by getting a copy of a gene encoding an altered protein even from those of a different species. FQ target, DNA gyrase which is required as DNA supercoiling enzyme having subunits and

substitution in these subunits results in mutation (Ramaswamy et al., 2003). According to the World Health Organization (WHO), there were about 10.0 million new cases of TB reported and 1.3 million deaths caused (range, 1.2–1.4 million) among HIV-negative people, and there were an additional 300 000 deaths from TB (range, 266 000–335 000) among complicated by co-infection with HIV-positive people by TB globally in 2017 Estimates of absolute and occurs in every part of the world and all age groups, the largest number of new TB cases occurred in the South-East Asia and Western Pacific regions, with 62% of new cases, followed by the African region, with 25% of new cases . and about Age groups overall the best estimates for 2017 were that 90% of cases were adults (aged ≥15 years), 64% were male, 9% were people living with HIV There are various mechanisms which are involved in development of bacterial resistance , includ complex cell wall is an effective way to keep a drug from reaching its target and to prevent it from being taken up by the cell (Munita and Arias,2016). Bacteria do this by changing the penetrability of their membranes or by lessening the number of channels available for drugs to diffuse through (Pal et al.,2014). Another resistance mechanisms associated with drug transport, such as efflux pumps by extrusion have also been reported, (Ball et al., 1980), and also drug modifying & target alteration or inactivating enzymes for many antibiotics which work by sticking to their target and preventing them from interacting with other molecules inside the cell. For example, some kinds of bacteria produce enzymes called as betalactamases that degrade penicillin (Lomovskaya et al., 2001). Some bacteria respond by changing the structure of the target (or even replacing it with another molecule altogether) so that the antibiotic can no longer recognize or bind to it (Lambert et al., 2005). Spontaneous mutations in bacterial genome can also acquire resistance by getting a copy of a gene encoding an altered protein even from those of a different species FQ target, DNA gyrase which is required as DNA supercoiling enzyme having subunits and substitution in these subunits results in mutation is a major concern in the control of the global TB epidemic.

M. tuberculosis does not acquire drug resistance through horizontal gene transfer because of lack of extrachromosomal DNA or plasmids. Most resistance is due to mutations in drug-targeting genes contributing to high- or lowlevel resistance; however, these mutations do not account for resistance in all strains, indicating that other mechanisms such as production of drug-inactivating enzymes, formation of inactive derivatives, target alteration, inhibition of drug entry into the cell, and extrusion by efflux pumps are present (Adnan Hameed et al, 2018).

Bacterial efflux pumps are membrane proteins that are capable of actively transporting a broad range of substrates, including drugs, from the cytoplasm to the exterior of the cell. They are involved in physiological processes, such as cell wall division, maintenance of the pH homeostasis, and secretion of intracellular metabolites. Increased expression of efflux pump genes confers a low-level-resistant phenotype, and it has been suggested that under these conditions, bacteria have greater higher levels of drug resistance. A strategy to prevent this chain of events would be the inhibition of efflux pumps, which, in addition, would restore the effectiveness of antimicrobials that are subject to efflux. In *M. tuberculosis*, several efflux pumps have been described, but their contribution to clinical drug resistance remains to be completely clarified. One of these pumps is Mmr (Rv3065) of the small multidrug resistance (SMR) family of transporters. the aim of present study was to to evaluate the expression of 20 putative efflux pump genes in multidrugresistant (MDR) *M. tuberculosis* clinical isolates. These genes were annotated as probable/ hypothetical drug efflux genes in the *M. tuberculosis* genome and are available at the website of the Wellcome Trust Sanger Institute (www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/functional_classes/III.A.6.shtml). The expression of these 20 genes in MDR *M. tuberculosis* clinical isolates was examined previously, as was their contribution to RIF resistance in *M. tuberculosis* clinical isolates. The study will further increase our understanding of the mechanism of active efflux in the INH and RIF resistance of *M. tuberculosis* and is the first investigation of the expressional differences between MDR clinical

isolates and pan-sensitive clinical isolates. Resistance profiles of *M. tuberculosis* were determined by susceptibility test using BACTEC MGIT 960 system and the sequences target-genes mutations as rpoB for RIF, katG and inhA regulator sequence, and oxyR-ahpC genes for INH (reported to carry major mutations associated with RIF and INH resistance) were analyzed for the resistance in *M. tuberculosis.*

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to the following people, Prof. Dr. Fatih KÖKSAL my supervisor, for his invaluable and untiring efforts and for all his support and encouragement over the years.

Tropical Diseases Research and Application Center- for providing excellent material and logistical conditions helping me in these first steps of scientific demand and, consequently, in the development and conclusion of this research work. In particular Dr. Begum KAYAR who made my access simpler to the research facilities and laboratory and gave an opportunity to become part of their team as an intern.

My Colleagues and friends at the Department of Medical Microbiology, past and present, for their incredible assistance and support. In particular Hassan SHATUB, I would also like to thank Ms. Suna GOKMEN, Secretary of the Department for my guidance and assistance in administrative matters *My Parents and my family*, I am truly blessed to have your love and support. Thank you for every sacrifice, for celebrating my joys and easing my failures. You are the pillars of strength in life, without you, none of this would be possible.

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LIST OF SYMBOLS AND ABBREVIATONS

AIDS	: Acquired Immune Deficient Syndrome
BCG	: Bacille Calmette-Guerin
bp	: Base pair
°C	: Degree Celcius
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic 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. History and Current Situation of Global Tuberculosis

Pulmonary TB is usually the most common infectious site and can spread to the disease by coughing, sneezing or spitting when pushing or dispersing TB germs into the air (Smith,2003). In order for an individual to become infected, only a few or some of these germs must be inhaled. About a quarter of the world's population is infected with TB. People infected with TB bacteria have a lifetime risk of 5-15% TB. However, for people living with HIV, for example, people with weakened immune systems (Mohajan, 2015).

Historically, TB is an an old disease considered as currently presents an immense international health challenge. historically through some studies of human skeletons They found traces since over tens of thousands of years human has been infected with an ancient pathogen named *Mycobacterium tuberculosis* which is the main causative agent of TB, the disease was also known as phthisis and Consumption from Hippocrates through to the 18th century, the white death and the great white plague during the 19th century, and other names which evoked the despair and horror of the disease such as the robber of youth ,was discovered by Robert Koch in 1882 (Hershkovitz et al, 2015). Several parameters can explain this epidemiological situation, such as the social and health conditions, the association with HIV/AIDS (1.2 million of patients are co-infected by MTB/HIV) (Nerlich and Losch, 2009), the movement of populations and the existence of multidrug-resistant strains (Hiroshi, 2009). Tuberculosis occurs in both sexes, in all age groups and can affect virtually all organs of the body (Shafi et al., 2008; Chaisson et al., 2008).

The history of tuberculosis (TB) changed significantly after the discovered of the first drugs with anti-mycobacterial activity. The introduction of Streptomycin in 1943 to treat TB resulted in a decrease in death rates associated with TB disease" Shortly thereafter, especially in 1948, the first antibiotic was

given for the treatment of TB. However, shortly after the first antibiotic was introduced in 1943, drug resistance developed mainly due to the use of streptomycin as monotherapy.

TB continues to be one of the most devastating human infectious diseases with HIV and malaria, and also still TB as a most worldwide and it's ranking as the second highest cause of death from an infectious disease in worldwide after the human immunodeficiency virus (HIV) or is also classified as one of the top 10 causes of death, and the leading cause from a single infectious agent (above HIV/AIDS) According to the World Health Organization (WHO) and remains a threat with sub-Saharan Africa having the highest prevalence of TB in the world It is a major public health problem worldwide and millions of people continue to fall sick with the disease each year. in worldwide and more recently with the discovery of several other drugs with anti-TB activity, multidrug therapy became fundamental for the control of the disease by promoting the cure of the patients and interrupting the chain of transmission. Robert Koch's discovery paved the way for the development of the Pirquet and Mantoux tuberculin skin tests, Albert Calmette and Camille Guérin's BCG vaccine, Selman Waksman's streptomycin and other anti-tuberculous drugs.

Tuberculosis is still a major public health problem today according to the World Health Organization (WHO), there were about 10.0 million new cases of TB reported and 1.3 million deaths caused (range, 1.2–1.4 million) among HIV-negative people, and there were an additional 300 000 deaths from TB (range, 266 000–335 000) among complicated by co-infection with HIV-positive people by TB globally in 2017.

Estimates of absolute numbers are shown in Table 1.1 and occurs in all part of the world and all age groups, the highest number of new TB cases infected in the South-East Asia and Western Pacific regions, and 62% of new cases, followed by the African region, with 25% of new cases and about age groups overall the best estimates for 2017 were that 90% of cases were adults (aged ≥15 years), 64% were male, 9% were people living with HIV were recorded in 2017 Widely varying incidence rates are demonstrated in figure 1.1.

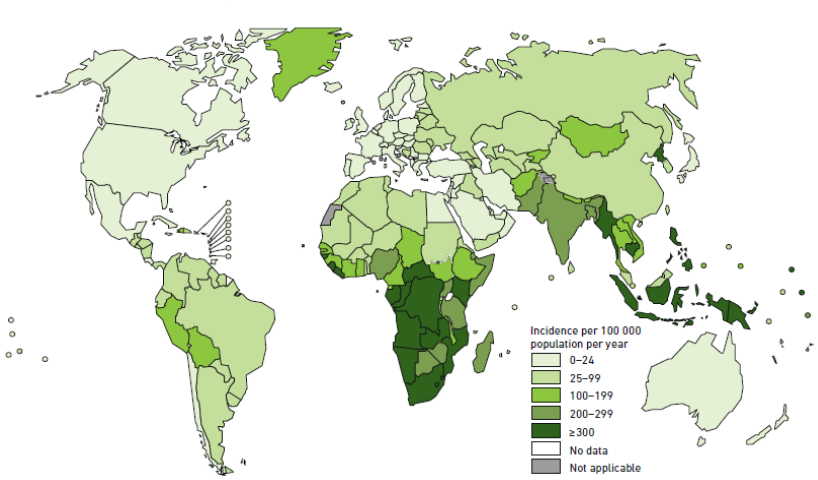


Figure 1.1. Estimated TB incidence rates, **2017** (WHO, **2018**)

In 2017, there were close to 10.0 million cases of Tuberculosis worldwide. (range, 9.0–11.1 million). Estimates of absolute numbers and rates per capita are shown in **Table 1.1**.

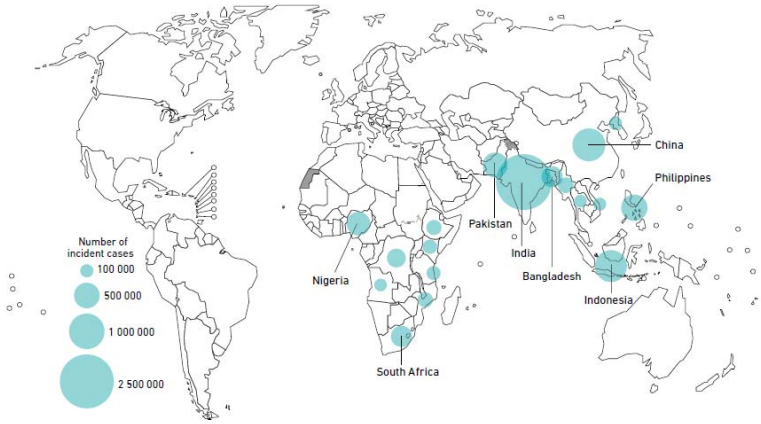


Figure 1.2**.** Estimated TB incidence rates, 2017

Table 1.1. Estimated epidemiological burden of TB in 2017 for WHO regions and globally. Rates per 100 000 population.

WHO regions	HIV-NEGATIVE TB MORTALITY		HIV-POSITIVE TB MORTALITYa		TOTAL TB INCIDENCE		HIV PREVALENCE IN INCIDENT TB (%)	
	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL
Africa	39	33–46	24	21–27	237	211–263	27	23–31
The Americas	1.8	1.7–1.9	0.60	0.53–0.67	28	26–30	11	9.6–12
Eastern Mediterranean	13	11–15	0.43	0.26–0.66	113	90–139	1.3	0.73–2.0
Europe	2.6	2.5–2.7	0.54	0.41–0.69	30	26–34	12	9.1–16
South-East Asia	32	30–35	1.4	1.1–1.8	226	179–277	3.5	2.4–4.7
Western Pacific	4.9	4.5–5.3	0.26	0.20–0.34	94	78–112	1.8	1.2–2.4
Global	17	16–18	4.0	3.5–4.5	133	120–148	9.2	7.9–11

The infection is difficult to treat, requiring six months of a combination of medications, which increases the chances of drug-resistance in the bacteria Eliminate TB remains a challenge, due More recently, new forms of antibiotic resistance have emerged. Clinical forms of Multidrug-resistant TB (MDR-TB), refers to the TB bacteria which are resistant to rifampicin(RIF) and isoniazid(INH) the first-line antitubercular (anti-TB) drugs, and extensively drug-resistant TB (XDR-TB), which caused by strains of *M. tuberculosis* are resistant to fluoroquinolone (FQ)., in addition to being MDR, and to at least one of the three injectable drugs like kanamycin, capreomycin and amikacin, again threaten adequate control of the disease (3,4). Despite the undeniable progresses that has

been made in global TB control, with a decrease of TB prevalence and mortality rate since 1990, the emergence of MDR and XDR-TB remains a critical but largely unresolved issue. is one of the main challenges threatening the success of global TB control program.

There are various mechanisms which are comprised in development of bacterial resistance, includes complex cell wall is an efficient way to keep a drug from reaching its target and to prevent it from being taken up by the cell (Munita and Arias,2016). Bacteria do this by changing the penetrability of their membranes or by lessening the number of channels available for drugs to diffuse through (Pal et al.,2014). and another resistance mechanisms associated with drug transport, for example efflux pumps (EP) by extrusion have also been described, (Ball et al., 1980), and drug modifying and target alteration or inactivating enzymes for numerous antibiotics which work by sticking to their target and preventing them from interacting with other molecules inside the cell. Some types of bacteria produce enzymes known as betalactamases that degrade penicillin (Lomovskaya et al., 2001). Some bacteria respond by changing the structure of the target (or even replacing it with another molecule altogether) so that the antibiotic can no longer recognize or bind to it (Lambert et al., 2005).

Objective: This study aimed to evaluate the expressional differences of 20 putative efflux pump genes between MDR clinical isolates and pan-sensitive clinical isolates.

- These genes were annotated as probable/hypothetical drug efflux genes in the *M. tuberculosis* genome and are available at the website of the Wellcome Trust Sanger Institute (www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/functional_classes/III.A.6.shtml).

- The expression of these 20 genes in MDR *M. tuberculosis* clinical isolates was investigated previously, as was their contribution to RIF resistance in *M. tuberculosis* clinical isolates.
- The study will further increase our understanding of the mechanism of active efflux in the RIF and INH resistance of *M. tuberculosis* and is the first examination of the expressional variances between MDR clinical isolates and pan-sensitive clinical isolates.

2. LITERATURE REVIEW

2.1. General characteristics

M. tuberculosis was described in 1882 by Robert Koch. They are non-moving bacilli, aerobic, dry colonies with colonies rough 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. (WHO., 201520; Tortoli et al.,1999; Somoskovi et al.,1999; Naveen et al.,2012; Adler et al.,2005 49-52 (Figure 5) (Comas et al., 2011, Smith et al., 2006; Brosch et al., 2002; Gagneux et al., 2008; Garnier et al., 2003; Frota et al., 2004; Cousins et al., 2003). Under electron microscope, the bacteria are about 2 – 4 μm in length and 0.2- 0.5 μm in width (Figure 2.2). ((Zuber et al. 2008, Reed et al. 2004, Rocha-Ramirez et al. 2008) Gengenbacher et al., 2012 48)

The genus is divided into two wide taxonomic groups depend on the growth rates of individual species. The first group which takes more than a week known as slow growers to form colonies includes slow-growing species such as the well-known pathogens *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae*. The other group that give colonies within seven days such as *Mycobacterium smegmatis*, those are termed fast growers or non-pathogenic/ opportunistic bacteria (Ethiological agents of human tuberculosis (TB), bovine tuberculosis (BTB) and leprosy respectively) (Forrellad et al., 2013).

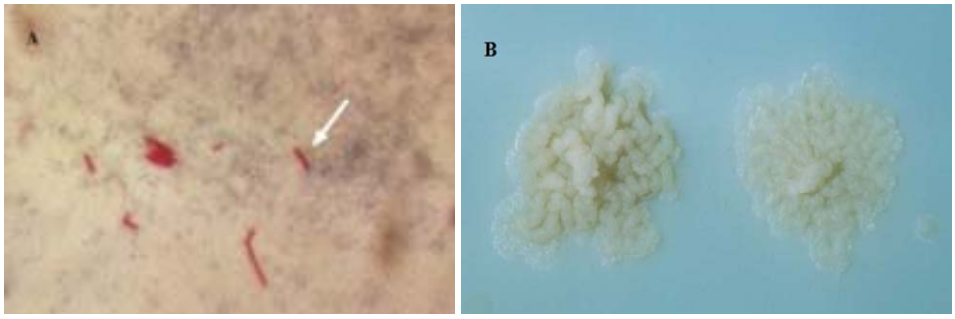


Figure 2.1. (A) *M. tuberculosis visualization* using the Ziehl–Neelsen stain (B)Visualization of *M. tuberculosis* colonies on Lowenstein-Jensen medium(Source from http://textbookofbacteriology.net/tuberculosis.html)

Under electron microscope, the bacteria are about 2 – 4 μm in length and 0.2- 0.5 μm in width (Figure 2.2) (Orduz and Ribón.,2015). The cell wall is the most distinguishing characteristic of all Mycobacterium species, it is thicker than cell walls in the other bacteria and also it is so essential for growing and surviving intracellularly (Bhamidi, 2009). Lipids (mycolic acids, cord factor and wax-D) are the main content in the mycobacterial cell wall with more than 60% lipids are covalently linked to arabinogalactan and attached to peptidoglycan. In addition, mycomembrane and cell wall contain differnt free lipids, like phthiocerol dimycocerosates, phenolic glycolipids, dimycolyltrehalose or cord factor, phosphatidylinositol mannosides and sulpholipids are intercalated with the mycolic acids (Abdallah et al., 2007). The benefits of lipids high concentration in the cell wall to the bacteria are linked to the impermeability to dyes and normal stains used for the common bacteria identification, also, increasing resistance to some antibiotics which are high potent, attack by lysozymes also killing by alkaline and acidic compounds (Biberstein and Hirsch, 1999).

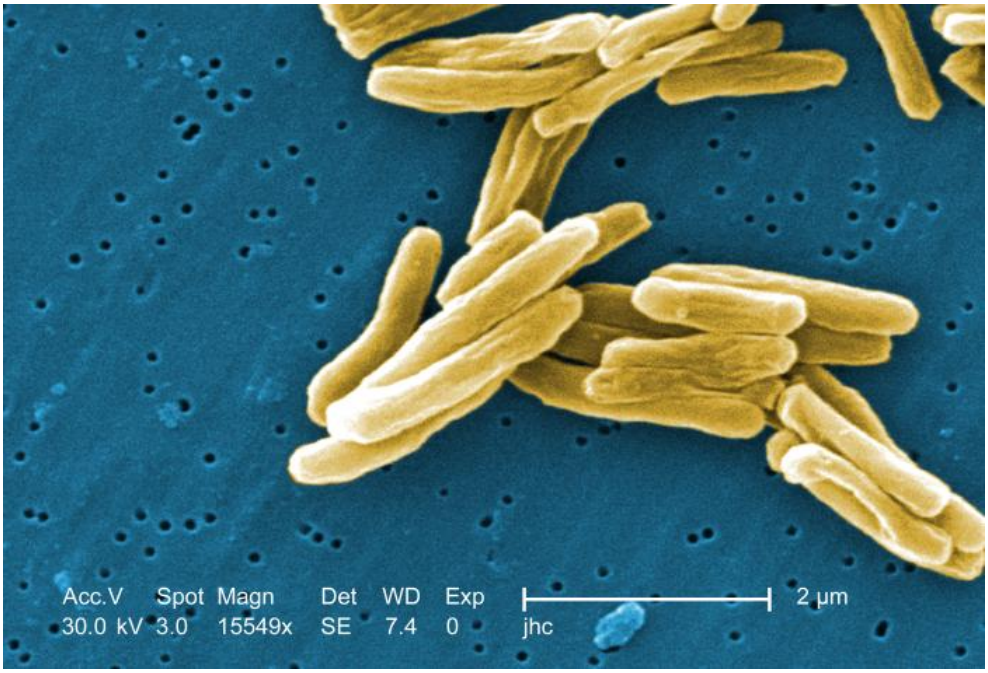


Figure 2.2. *Mycobacterium tuberculosis* scanning electron micrograph (Source from http://phil.cdc.gov/phil/details.asp?pid=9997)

The actual biosynthetic pathways of cell wall components make them targets for new drugs for treating TB. Although the organism apparently does not produce any toxins, has no classical virulence factors such as recently acquired pathogenicity islands; it possesses a huge repertoire of structural and physiological properties that aids in its survival within its host, including ability to detoxify oxygen radicals.

MTBC includes *Mycobacterium tuberculosis* sensu stricto (MTB), *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium suricattae, Mycobacterium microti, Mycobacterium mungi, Mycobacterium pinnipedii and Mycobacterium orygis*. Despite the fact that these species show up genetically monomorphic with a high level of DNA sequence similarity (>99.95%), with exception of *M. canettii*, they have varying host ranges: *Mycobacterium africanum* and *Mycobacterium tuberculosis* sensu stricto are the

foremost causative agents of tuberculosis in humans. *Mycobacterium microti* affects voles, (Wells 1937; Wells, 1946; Wayne et al., 1986; Frota et al., 2004), *Mycobacterium orygis* pathogen of antelope (van Ingen et al., 2012), *Mycobacterium caprae* a pathogen of sheep and goats (Aranaz et al., 1999). *Mycobacterium mungi* nangoose pathogen, *M. pinnipedii* a pathogen of seals and sea lions (Cousins et al., 2003). *Mycobacterium bovis* displays the widest spectrum of host affecting animals and humans (Garnier et al., 2003). However, although *Mycobacterium bovis* sometimes isolated from human, affecting less than 1% of all human TB cases, it lacks the ability to keep an infection cycle in human population or transmit in a sustainable way. This could be due to three mutations in the two-component regulation system PhoP/PhoR (phoPR) previously shown to be important regulator of virulence factors including several important lipids and proteins (ESAT-6). These mutations decrease the expression of the PhoP regulon leads to reduced ability to spread between humans (Berg and Smith, 2014; Smith et al., 2006). *Mycobacterium canettii* often considered a member of *Mycobacterium tuberculosis* complex (MTBC), It is the most phenotypically distinguished member of the complex. *Mycobacterium canettii* and the other so-called “Smooth TB bacilli (STBs)” are characterized by smooth glossy white colony due to the presence of lipooligosaccharides in the cell wall (Gutierrez et al., 2005). The STBs appearance clear evidence of continuous horizontal gene exchange (Gutierrez et al., 2005), but with no record of transmission between human (van Soolingen et al., 1997; Koeck et al., 2010; Fabre et al., 2010). These places the STBs among the population of mycobacteria proposed as the originators of MTBC (Gutierrez et al., 2005).

2.1.1. Taxonomy

Mycobacterium is thought to have existed a hundred and fifty years ago. The first known representative of this group was discovered in 1875 by Hansen under the name Bacillus leprae, and therefore the first scientific taxonomy of mycobacteria started in 1896, when the first genus Lehmann and Neumann

(Jagielski et al., 2016), comprising genus and MTB, was established in 1896. Leprae (Saviola & Bishai., 2006) Mycobacterium is the only genus that is taxonomically located in the Mycobacteriaceae family, belonging to the Actinomycetales order and Actinobacteria class. Complete taxonomic lineage for Mycobacterium is shown in Table 2.1. It is now accommodates a total of 197 featured species, according to the most recent version of the List of prokaryotic names with standing in nomenclature database (http://www.bacterio.net.,2017). All of these species divided into three main groups, the *M. leprae*, MTBC and non-tuberculous mycobacteria (Jagielski et al., 2016).

Table 2.1.Complete taxonomic lineage of Mycobacterium (Orduz and Ribón., 2015). Mycobacterium is the only genus that is taxonomically located in the Mycobacteriaceae family, belonging to the Actinomycetales order and Actinobacteria class.

Table 2.1. Complete taxonomic lineage of Mycobacterium

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

2.1.2. Cell wall

The mycobacterial cell wall structure is unique between procaryotes, is linked with the pathogenicity of MTB (Smith, 2003; Barry et al., 1998; Dubnau et al., 2000; Glickman et al., 2000). The richness in high molecular weight lipids represents the complexity of the cell wall (Brennan et al., 2003; Christensen et al., 1999; Daffé et al., 1998). Unusual impermeable properties of MTB cell wall are thought to be advantageous for the bacilli in stressful conditions of osmotic shock (Hett & Rubin, 2008) and the polymers, covalently linked with peptidoglycan and

trehalose dimycolate, provide a thick layer involved in MTB resistance to antibiotics and the host defense mechanisms (Takayama et al., 2005).

The mycobacterial cell wall contains of an internal layer and an External layer that surround the plasma membrane (Figure 2.3). The External compartment consists of both proteins and lipids. The internal compartment contains of, arabinogalactan (AG), peptidoglycan (PG) and mycolic acids (MA) covalently linked together to structure an insoluble complex referred to as the basic core of the Mtb cell wall (Brennan et al., 2003).

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

There is another important component of the cell wall is lipoarabinomannan, a major lipoglycan involved in virulence of mycobacterium (Chan et al., 1991) and in modifying the host response during the infection (Cox et al., 1999).

Cord Factor, a glycolipid most abundantly produced in virulent strains of MTB, is responsible for:

i) Inducing animal granulomas similar to those characteristic of TB infection (Hunter et al., 2006b);
ii) Increasing cytokine production (Ryll et al., 2001);
iii) Inhibiting the transfer of phagocytosed bacteria to acidic compartments in macrophages (Indrigo et al., 2003.);
iv) Influencing the morphology of mycobacterial colonies (Hunter et al., 2006).

The cell wall is a key to the survival of mycobacteria and a more complete understanding of the biosynthetic pathways and gene functions and the development of antibiotics to prevent formation of the cell wall are areas of great interest (Joe et al., 2007).

Mycolic acids, the strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface, has been shown to be critical for the survival of MTB (Draper et al., 2005).

Nevertheless, the membrane characteristics do not correspond to Gram-positive ones. Indeed, the bacteria do not retain the crystal violet dye as expected for Gram-positive bacteria (Nguyen,2016), sometimes resulting in “ghost” appearance after washing with alcohol or acetone. *M. tuberculosis* has a specialized cell wall complex, which consists of four major components, mycoside, mycolic acids, arabinogalactan and peptidoglycan. Mycolic acids, the major lipids of the cell wall of mycobacteria in general, are major components of the outer permeability barrier and are responsible for the "acid-fastness" of this group of microorganisms (Kaur et al.,2009 53).

Furthermore, the fatty acids are linked with carbohydrate components that form an unique envelope which inhibits phagolysosome fusion (Nguyen,2016 48). Like many other bacteria, *M. tuberculosis* does not form spores but has the capacity to become dormant-a non-replicating state characterized by low metabolic activity and prolonged persistence (Gengenbacher et al., 2012 48).

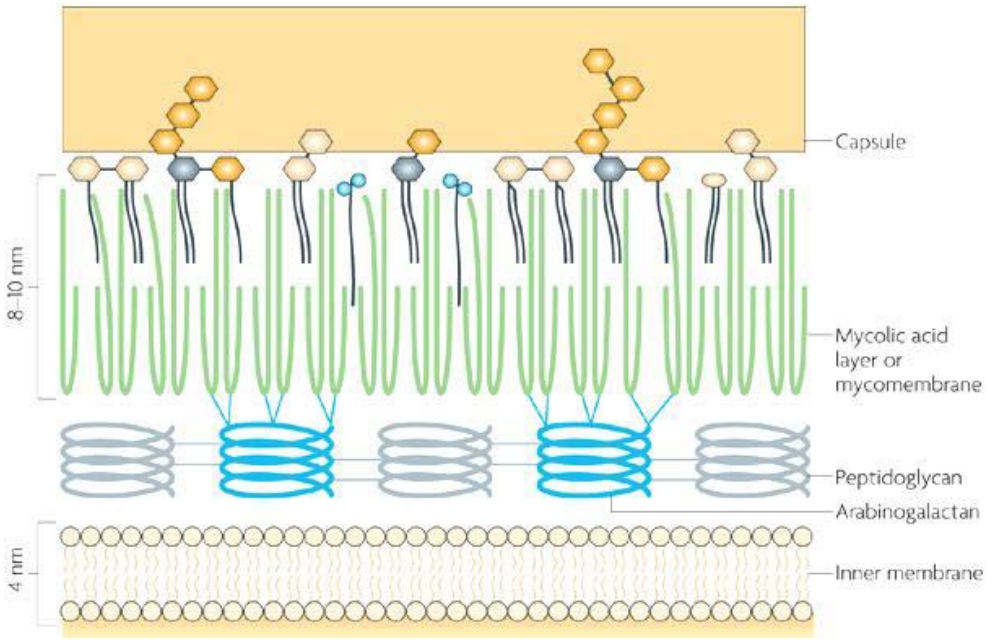


Figure 2.3. Schematic of the bacterial cell wall (Abdallah et al., 2007).

2.1.3. The Cord Factor

In general, *M. tuberculosis* bacilli produce rough textured colonies on solid media and are arranged in serpentine cords on microscopic smears. The recognition of these two characteristics allows an experienced microbiologist to separately identify *M. tuberculosis* from other mycobacteria in cultured specimens (Rodrigues., 2010). These characteristics have been attributed to the glycolipid trehalose 6, 6'- dimycolate, also known as cord factor, which is present in the mycobacterial cell-wall (Hunter N. et al, 2006). It is responsible for: (i) inducing animal granulomas similar to those characteristic of TB infection (Hunter N. et al, 2006b); (ii) increasing cytokine production ((Rodrigues., 2010; Ryll R. et al, 2001); (iii) inhibiting the transfer of phagocytosed bacteria to acidic compartments in macrophages (Indrigo J. et al, 2003.); and (iv) influencing the morphology of mycobacterial colonies (Hunter N. et al, 2006). In liposomes, trehalose dimycolate inhibits vesicle fusion, which could explain its role in preventing the

phagosomelysosome fusion in vivo (Spargo B.J. et al, 1991). However, the role that trehalose dimycolate may play outside the host environment remains to be completely clarified. (Rodrigues., 2010).

2.1.4. Morphology

Mycobacteria are typically rod-shaped, non–spore forming, aerobic bacteria, classified as acid-fast bacilli. The dimensions of the bacilli have been reported to be 1-10 μm in length (usually 3-5 μm), and 0.2-0.6 μm width. Variable morphology can be observed when grown on solid media and some species exist as shorter cocci-bacilli or curved rods on artificial media (Belisle & Brennan 1989).

The reported morphological variation in *M. tuberculosis* are classified in two categories; i) those which are frequently seen at exponential phase of growth that is rod, V, Y-shape, branched or buds (Figure. 2.4.A), and ii) those that are seen occasionally under stress or environmental conditions which are round, oval, ultra-virus, spore like, and cell wall defiant or L-forms (Figure. 2.4.B) (Velayati et al., 2009, 2011; Farnia et al., 2010).

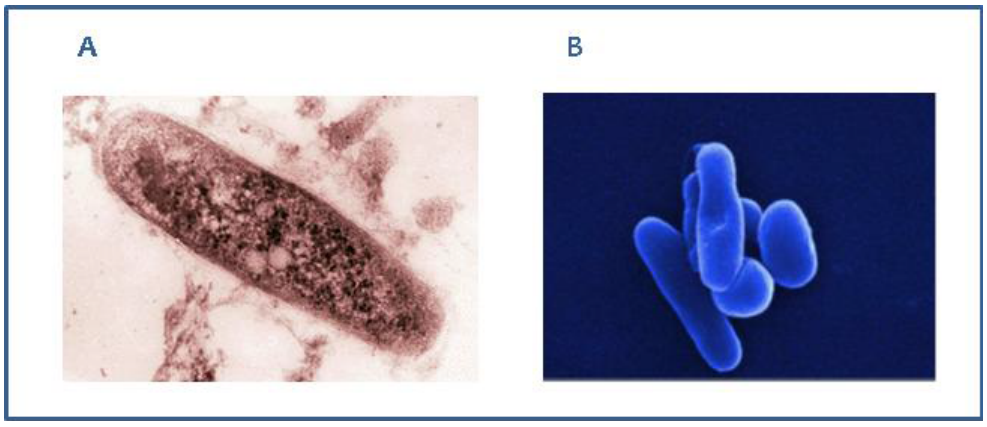


Figure 2.4. Morphological variations in *M. tuberculosis*. (A). Thin section transmission electron micrograph of MTB (extracted from www.wadsworth.org/databank/mycotubr.htm). (B). Scanning electron microscope shows shape variation in MTB at exponential phase of growth (Velayati & Farnia, 2012).

Mycobacterium tuberculosis is an obligate aerobe. For this reason MTB complexes are always found in the well-aerated upper lobes of the lungs in patient with TB. The bacterium is a facultative intracellular parasite, usually of macrophages, and has generation time of 15-20 hours, which is extremely slow compared to other bacteria with division times measured in minutes.

2.1.5. The Mycobacterium tuberculosis genome

In 1998, the paradigm reference strain H37Rv became the first fully sequenced MTB strain (S. Cole et al. 1998). The circular MTB H37Rv genome consists of 4,411,532 bp and displays a high G+C content of 65% (Figure 2.5). It contains over 4000 protein-coding and 50 stable RNA genes, using around 91% of its potential coding capacity. It displays a gene density at one gene per kilobase, which is comparable to other prokaryotes. Genes are evenly distributed on the forward and reverse strands, and 59% of the transcription is in the same polarity as the replication fork. More than half of the coding sequences in MTB have arisen from gene duplication or domainshuffling events (Tekaia et al. 1999). Analysis of the genome sequence highlighted several characteristics of the biochemistry, physiology, genetics and immunology of MTB. Being the most widely used strain in TB research, continued efforts have been carried out (Cole et al 1998; Camus et al. 2002b; Lew et al. 2011) to maintain an accurate and up-to-date genome annotation of MTB H37Rv using a combination of bioinformatics, data mining, comparative genomics, scientific literature and manual curation. For over 10 years, the TubercuList database (Lew *et al*. 2011) (http://tuberculist.epfl.ch) has been providing access to gene-based annotation for MTB H37Rv. The current release (R25 – April 2012) contains 4095 annotated features, which includes 4022 protein genes and 73 RNAs (tRNAs, ribosomal RNAs, small RNAs, stable RNAs).

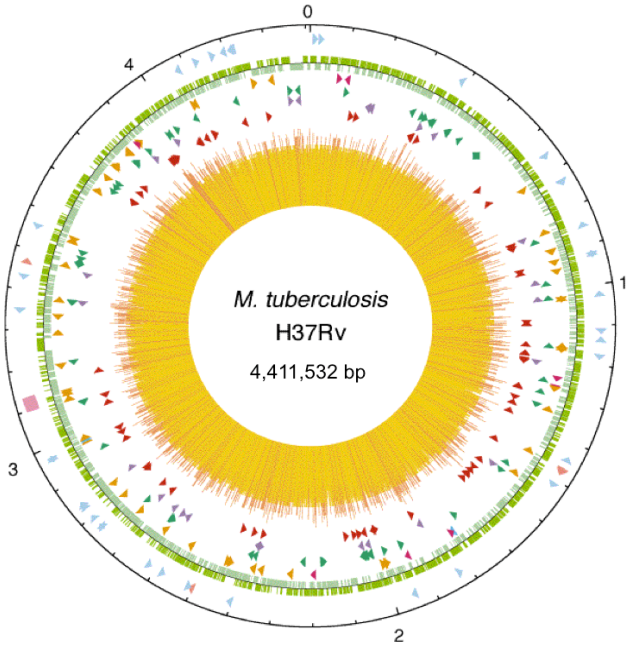


Figure 2.5. Circular map of the chromosome of *M. tuberculosis* H37Rv (S. Cole et al. 1998).

The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red (Cole et al. 1998).

The *M. tuberculosis* genes have been broadly classified into eleven 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

2.1.6. Virulence determinants

The virulence of *Mycobacterium tuberculosis* is extraordinarily complex. Although the organism apparently does not produce any toxins, it possesses a huge repertoire of structural and physiological properties that have been recognized for their contribution to mycobacterial virulence and to pathology of tuberculosis. Subsequently, virulent *M. tuberculosis* species have developed strategies to avoid or modulate the immune response in their favour.

Among a great number of virulence factor of *M. tuberculosis* (Table 2.3), exported proteins are one of the most important factors responsible for the virulence (Rajni & Meena, 2011). Culture filtrate proteins, found in the culture medium in which *M. tuberculosis* grows, are actively studied by many researchers,

since a great deal of them is recognized by the sera of TB patients (Smith, 2003). The virulent components can be assembled in different clusters: i) Cell envelope proteins, including cell wall proteins, lipoproteins and secretion systems; ii) Proteins inhibiting antimicrobial effectors of the macrophage; iii) Gene expression regulators, including two component systems, sigma factors and other transcriptional regulators; iv) Enzymes involved in general cellular metabolism; and v) Other proteins, including the ones of unknown function (Forrellad *et al*., 2013; Smith, 2003).

M. tuberculosis has developed highly specialized mechanism to proliferate in the host during infection, such as: i) Slow generation time, in consequence to which the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them; or ii) Lipid and fatty acid metabolism, including catabolism of cholesterol. It has been also shown that *M. tuberculosis* produces pili during human infection, which could be involved in initial colonization of the host (Alteri *et al*., 2007).

Table 2.3. Virulence factors of *Mycobacterium tuberculosis*.

Virulence factor	**Category**	**Role**	**Virulence characterization**	**Reference**
Antigen 85 complex	Exported protein	Fibronectin-binding	Possible impact in walling off bacteria from the immune system and facilitation of tubercle formation	Belisle *et al.*, 1997
HbhA	Adhesin	Heparin-binding hemagglutin	Involvement in binding *Mtb* to epithelial cells	Delogu & Brennan, 1999
ESX-1	Secretion-associated proteins	Virulence proteins delivery during infection	Avoidance of excessive virulence by maintaining ESX-1 activity that leads to long term survival of *Mtb*	Raghavan *et al.*, 2008
WhiB3 protein	Putative transcription regulator	Implication in sensing oxygen tension and redox state	Adaptation of mycobacteria to changes in oxygen tension	Banaiee *et al.*, 2006
Acr1 (hspX)	α-crystalline protein homolog	Dormancy-associated protein	Inhibition of antimicrobial effectors of the macrophage	Yuan *et al.*, 2008
19-kDa protein	Lipoprotein antigen	Blockage of IFN-γ signaling through a TLR-2 dependent mechanism	Inhibition of MHC-II antigen processing and presentation in macrophages	Tobian *et al.*, 2003
Sigma factors	Gene expression regulators	Help in regulation of expression of specific genes during stress or morphological development	Adaptation to the changing environment within the host	Browning *et al.*, 2004
STPKs	Serine-threonine protein kinases	Regulation of cell shape; macrophages modulation	Regulation of host-pathogen interactions and developmental changes through signal transduction using reversible phosphorylation of proteins	Av-Gay ; Leonard *et al.*, 1998

2.1.7. Growth rate and growth phase

One of the important factors in how *M. tuberculosis* has become a formidable pathogen is the inherent slow growth rate. Doubling times of 16 hours are achievable in optimal laboratory conditions but in reality doubling times vary depending on the site and stage of infection, often being much slower (Beste et al., 2009). Since virtually all antibiotics preferentially kill rapidly replicating bacteria, a slow growth rate poses a problem for treatment. When the cell wall itself contains the primary target for drug action a reduction in the relative abundance of the target material may reduce the overall susceptibility of the cells. Altering the porin availability of the cell wall through reduced growth rate can also slow down the transport of hydrophilic agents (Brown et al., 1990). Slowing down replication is an effective way to induce drug tolerance against many of the antibiotics that target cell division and cell wall synthesis.

Tuberculosis has two distinct phases, an acute phase in which bacteria are actively growing and a persistent phase in which the bacteria are very slow growing or in a non-replicating state. *M. tuberculosis* can remain in this dormant state for many years leading to latent disease. The mechanism behind the ability of *M. tuberculosis* to remain in a host with a competent immune system for decades had puzzled researchers for many years, the observation in mouse models that the bacteria causing infection were replicating very slowly or not replicating at all supported the hypothesis that it is persistent non-replicating organisms that cause latent tuberculosis disease (Rees and Hart, 1961). In this state, the organism is refractory to immune clearance but primed for reactivation; treatments are largely inactive against this population of non-dividing bacteria allowing the disease to become latent. After reactivation this population becomes infectious once again. It has been shown that switching from fast to slow growth is a carefully controlled process in mycobacteria involving a unique set of genes and not simply acceleration or deceleration of the same cellular processes (Beste *et al.*, 2009)

Growth phase can also affect the efficacy of antibiotic in similar ways to the growth rate. Bacteria that are exponentially growing and actively dividing are generally the most susceptible phase to antibiotics with cell wall and replication based targets. As populations enter stationary and then death phase the proportion of the population that is rapidly dividing or dividing at all decreases and therefore the antibiotic susceptibility decreases. In vitro experiments have demonstrated that the number of persister cells in a population is directly proportional to the number of stationary phase cells inoculated into the culture (Balaban et al., 2004), giving some indication as to the importance of the growth phase of an organism when studying antibiotic effects. Stationary phase cells of *M. tuberculosis* have been shown to reduce the sterilising activity of isoniazid nearly 1000 fold (Wallis et al., 1999) and whilst rifampicin tends to be more active than isoniazid against this population the rates of killing are slowed (Paramasivan et al., 2005).

2.2. The *M. tuberculosis* Complex

Mycobacterial species that cause TB in humans and other mammalian hosts are collectively known as tubercle bacilli and are grouped within the *Mycobacterium tuberculosis* complex (MTBC). In humans, TB is primarily caused by *M. tuberculosis* and *M. africanum* (sub-Saharan Africa). In addition, several animal-adapted members of MTBC have been identified. These include, *M. bovis* (cattle) and its attenuated derivative *M. bovis* BCG (vaccine strain), *M. microti* (voles and other small rodents), *M. pinnipedii* (seals and sea lions), and *M. caprae* (goats and sheep) (Schaaf & Zumla 2009: Jagielski *et al*., 2016).

The genomic revolution in the last decade has facilitated the application of a variety of powerful tools, such as large-scale transcriptomics, proteomics, comparative genomics, and structural genomics studies in the field of TB research that have led to great advances in our understanding of the biology and pathogenesis of the tubercle bacilli. (Pfyffer, 2015)

2.2.1. Mycobacterium tuberculosis

Robert Koch is considered the first to describe *M. tuberculosis* in 1882, About the origin of the MBTC strains, *Mycobacterium tuberculosis* is the main agent of TB in humans (Koch ,1932: Orduz and Ribón., 2015). In the past there was a presumption that *M. tuberculosis* had evolved from *M. bovis* by specific adaptation of an animal infectins to the human host (Stead et al,1995: Brisse et al, 2006: Smith et al,2006). In addition, genomic analysis has appear that *M. bovis* carries a smaller genome, that means it is evolutionary younger according to some studies (Brosch et al, 2002).

M. tuberculosis can be identified by phenotypically using some analysis such as resistance to thiophene-2-carboxylic acid hydrazide (TCH) , production of niacin, nitrate reductase and sensitivity to pyrazinamidase (Hoffner et al,1993 Niemann *et al*,1999) in addition to that genotypically by Spacer Oligonucleotide Typing (Spoligotyping), *Mycobacterium tuberculosis* has been classified into different phylogenetic lineages (Brudey *et al*, 2006).

2.2.2. Mycobacterium bovis and Mycobacterium bovis BCG

Mycobacterium bovis causes a disease called bovine tuberculosis is the major zoonotic disease caused by mycobacteria, infecting cattle, also certain free or captive wildlife species and other domesticated animals. The disease is spreads to human, typically through ingestion of contaminated meat or unpasteurized milk, causing extrapulmonary tuberculosis, but can also be transmitted by inhalation of aerosols causing pulmonary TB (Ayele *et al*, 2004, Cosivi *et al*, 1998).

TB caused by *M. bovis* and TB caused by *M. tuberculosis* cannot be distinguished clinically, radiographically, or pathologically in individual patients (Grange et al, 2001). Thus, the identification of these causative agents can only be through mycobacterial culture and subsequent use of biochemical or molecular methods (Grange, 2001). However, containment facilities to identify the causative agent of TB are largely absent in low income countries (Müller et al, 2013).

In high income countries, zoonotic TB accounted for a relevant proportion of the TB cases until the introduction of regular milk pasteurization programs (Müller *et al*, 2013). In our days, bovine TB is well controlled or eliminated, and zoonotic TB cases are rarely seen; however, reservoirs in wildlife can make complete eradication challenging (Michel et al, 2010, Torgerson PR and Torgerson DJ, 2010).

In Africa, the situation is somehow more critical, as bovine TB is an economical and public health threat in low income countries (Ayele et al, 2004: Stefan Niemann., 2014). In most African countries, effective control of bovine TB is largely absent, including regular milk pasteurization and slaughterhouse meat inspection (Ayele et al, 2004, Cosivi et al, 1998). Additionally, the presence of multiple risk factors such as human behaviour and HIV infection (Cosivi et al, 1998: Ayele et al, 2004: Michel et al., 2010) makes the situation even worse. The reported median proportion of bovine TB in Africa is 2.8% (range 0%–37.7%) of human TB cases (Ayele et al, 2004). Control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations (Cosivi et al, 1998: Ayele et al, 2004: Lari et al., 2011).

There is also a non-virulent strain of *M. bovis* called Bacillus Calmette Guerin (BCG), which has its origin from a virulent *M. bovis* strain (Calmette, 1928). Calmette and Guerin performed 230 in vitro passages of *M. bovis* until the organism lost its virulence. While this strain has been used worldwide as a live attenuated vaccine to immunize people against TB, with highly variable efficacy (Andersen and Doherty, 2005), it may cause disease in humans. In many high TB incident countries, the BCG vaccination is mandatory and free of charge, given on the first three days after birth, showing protection in children against more serious forms of TB (Colditz et al, 2005, Mittal et al, 1996: Singh et al., 2015), although in adults, protection varies from 0 to 80% (Fine, 1995).

2.2.3. Mycobacterium africanum

M. africanum was first described in 1968 in a Senegalese patient (Castets et al, 1968: Mostowy et al.,2004), after that it was found almost exclusively in West Africa. The prevalence of *M. africanum* varies from 5,3% in the Ivory Coast (Bonard et al, 2000), 47,1% Guinea-Bissau (Groenheit et al, 2011) and 67,7% in Uganda (Niemann et al, 2002). *M. africanum* is phenotypically heterogeneous, with characteristics common to both *M. tuberculosis* and *M. bovis*. Based on their geographic origin and biochemical characteristics, two subgroups of *M. africanum* have been described, in western Africa (subtype I) and eastern Africa (subtype II) (Collins et al, 1982: de Jong et al., 2010).

2.2.4. Mycobacterium canettii

Mycobacterium canettii, a novel rare variant of MTC with a smooth colony morphology was first isolated from a Somali-born patient in 1969 by Canetti (van Soolingen et al., 1997). Daffe (Daffe et al., 1991) demonstrated that this particular strain differed from the commonly rough strains by having large amounts of lipooligosaccharides. The smooth and glossy colonies produced are highly exceptional for this species. This smooth phenotype is however unstable and can switch to a rough colony morphology (van Soolingen et al., 1997: Niemann et al., 2016: Fabre et al., 2010).

2.2.5. Mycobacterium microti

Mycobacterium microti typically causes disease in voles, wood mice and shrews, although it was also detected in a limited number of other mammalian species. The causative agent was named *M. tuberculosis subsp. muris*, and later this species was designated *M. microti* and classified as a member of the MTC . It was first reported in humans in 1998 in immunocompromised patients (van Soolingen et al., 1998), although human to human transmission of *M. microti* infection seems to be rare (Emmanuel et al., 2007). *M. microti* differs from other MTC strains in its

S-shaped cell morphology, extremely slow growth in vitro, and distinct host-specific pathogenicity for laboratory animals. Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis, M. africanum*, or M. *bovis* but *M. microti* strains display characteristic IS6110 banding patterns and spoligotypes, distinct from types previously observed in other MTC strains (van Soolingen et al., 1998).

2.2.6. Mycobacterium pinnipedii

In 1993, it was reported for the first time that isolates from seals captured on the coast of Argentina had a characteristic IS6110 RFLP pattern (Cousins et al.,1993). This seal bacillus was later designated *M. pinnipedii* and appeared to have a unique position in the *M. tuberculosis* complex ((Cousins et al.,1993)). Later on, reports have described *M. pinnipedii* infections in various marine mammals (Forshaw and Phelps,1991: Hunter et al., 1998: Thompson et al.,1993).

Transmission of *M. pinnipedii* to humans has been reported in individuals who are in close contact with marine mammals (Thompson et al., 1993; Kiers et al., 2008).

M. pinnipedii isolates present a distinct spoligotype pattern when compared to other members of the MTC (Cousins et al., 2003). Later designated *M. pinnipedii* and appeared to have a unique position in the MTC (Cousins et al., 2003).

2.2.7. Mycobacterium caprae

Mycobacterium caprae was first isolated from goats in Spain (Aranaz et al., 1999), but has since been found in other animals, such as cattle (Prodinger et al., 2002; Erler et al., 2004; Boniotti et al., 2009), pigs (Pavlik et al., 2002), red deer (Pavlik et al., 2002), and wild boars (Erler et al., 2004). Its isolation from humans has also been described (Erler et al., 2004, Kubica et al., 2003); often, a contact with livestock has been suggested as a likely means of transmission

(Prodinger et al., 2002). To our knowledge, this pathogen has never been isolated outside continental Europe, except from a European patient in Australia (Sintchenko et al., 2006) and a cow in Algeria (Sahraoui et al., 2009). For a long time this species was considered as *M. bovis*, because the biochemical test results were similar to *M. bovis* and *M. bovis* BCG. By spoligotyping, *M. caprae* species form a homogeneous cluster easily recognizable by the absence of spacers 1,3-16, 30-33 and 39-43. The lack of spacers 39-43 has also been described in *M. bovis* and *M. microti* (Aranaz et al., 1999: Aranaz et al., 2003: Prodinger et al., 2014).

2.2.8. Novel variants of the *M. tuberculosis* complex

The novel *M. mungi*, was identified as the causative agent of TB in banded mongooses (Mungos mungo), in Botswana *M. mungi* was characterizes as highly virulent, causing high numbers of deaths in a short period of time (2–3 months from clinical presentation to death), apparently through environmental transmission (nonrespiratory route) (Alexander et al.,1960).

The Dassie bacillus, the causative agent of TB in the dassie (Procavia capensis), is considered an infrequent variant of the *M. tuberculosis* complex characterized as being similar to *M. microti*, based on morphology and growth requirements (Smith., 1960), although, they differ in growth preferences and bacillary morphology under microscopy. In terms of pathogenicity, the Dassie bacillus was reported to have a very low level of virulence in rabbits and guinea pigs (Cousins et al., 1994). Genome comparison of nine regions of difference (RD) shows that five are shared with *M. microti* (RDs 3, 7, 8, 9, and 10). Although the Dassie bacillus does not share the other documented deletions in *M. microti* (RD1mic, RD5mic, MID1, MID2, and MID3) (Mostowy et al., 2004).

The *M. orygis* or Oryx bacilli was identified to be the causative agent of TB in oryxes and gazelles (van Soolingen et al., 1994), deer, antelope and waterbucks (Smith et al., 2006), although their exact host range remains uncertain. Combined findings based on Single nucleotide polymorphisms (SNP), RD,

spoligotyping and 24-locus Mycobacterial Interspersed Repetitive Units–Variable-Number Tandem Repeat (MIRU-VNTR) analysis, placed *M. orygis* at a distinct phylogenetic position between the Dassie bacillus and *M. microti*. It was proposed by Ingen and colleagues that *M. orygis* was attributed a subspecies status (van Ingen et al., 2012).

M. suricattae, closely related to the Dassie bacillus was first isolated in meerkats from South Africa, it was proposed as a novel member of the *M. tuberculosis* complex, the deletion of the direct-repeat region spacers, i.e. no amplification of any spacer by spoligotyping, distinguishes this strain from all other *M. tuberculosis* complex members (Parsons et al., 2013).

2.3. The global diversity of human-associated MTBC

The phylogenetic structure of human-associated MTBC has been extensively studied using LSPs in 875 globally representative strains from 80 different countries (Gagneux et al., 2006). This study found that human-associated MTBC consists of six major lineages which show biogeographic specificities in that the individual lineages are associated with particular geographic locations (Figure 2.6). Lineages 1, 5 and 6, which are referred to as the “ancient” lineages are predominant in Africa (Lineage 5 and 6) and around the Indian Ocean (Lineage 1), while the “modern lineages are more widespread but still strongly associated with particular geographic settings; Lineage 4 in Europe, the Americas and Africa, Lineage 2 in East Asia, and Lineage 3 in South-Asia. The same authors used another approach using maximum parsimony analysis of 89 genes in a global collection of 108 human and animal strains (Hershberg et al., 2008). This analysis yielded a single comprehensive phylogenetic tree (Figure 2.7), which showed analogy to ancient and modern lineages defined based on the presence/absence of TbD1. Analysis of genetic distances revealed that human MTBC strains are genetically diverse as represented by different phylogenetic lineages. Lineages from Africa and animal hosts are represented mostly in ancient lineage while others are represented in modern lineages

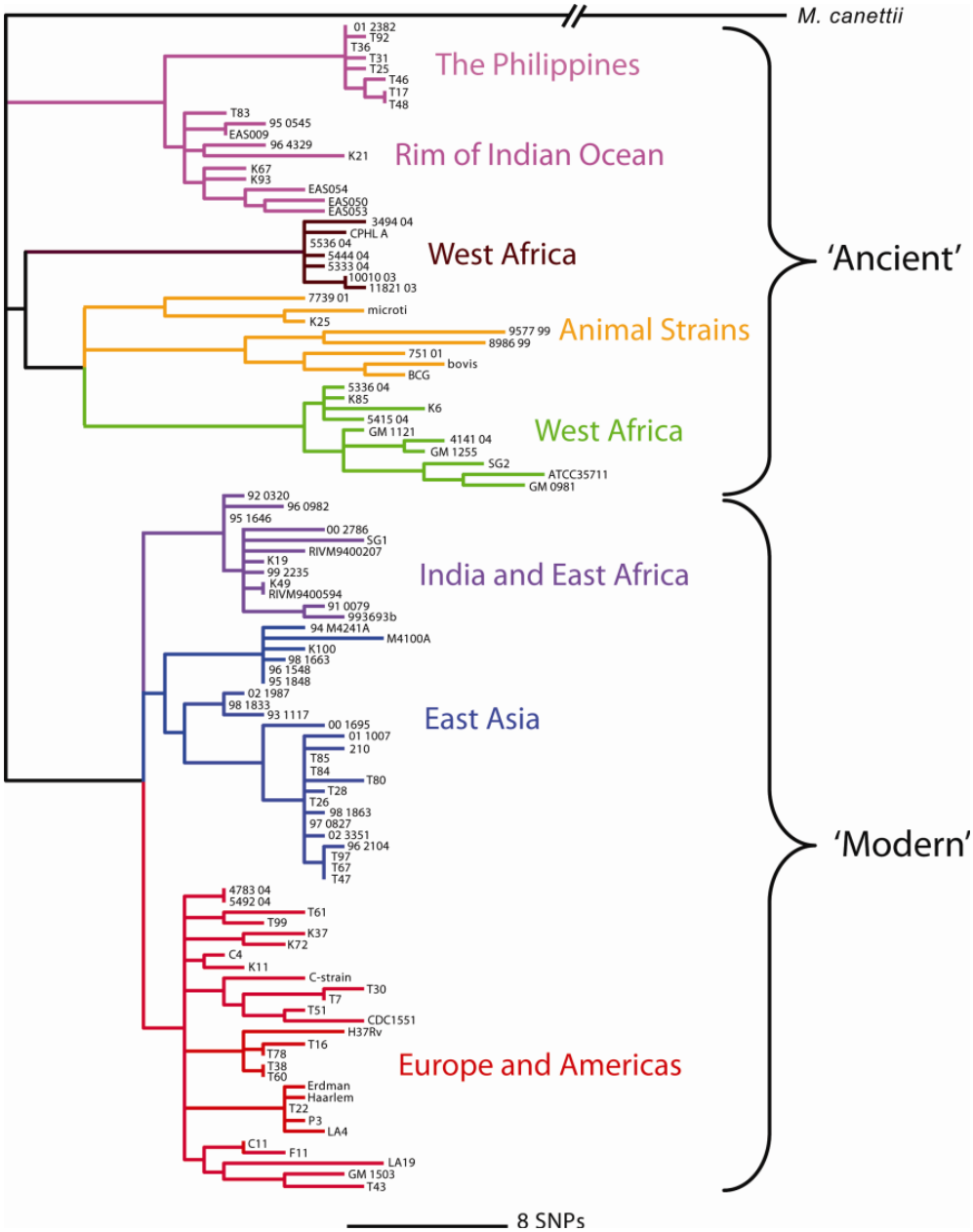


Figure 2.6. Phylogeny of *M. tuberculosis* showing six major lineages (Source: Hershberg et al, 2006)

More recently, the global phylogeny of human-associated MTBC was defined into six major lineages based on whole genome sequences (Coscolla and

Gagneux, 2010). This lineage classification corresponds to genotypes as detected and defined by other techniques.

Although the explanation on genetic diversity of global MTBC continues using different genes and molecular biological tools, it is equally important to understand whether this variability has biological consequences in terms of host immune recognition, pathogenesis, and the outcome of infection and disease in clinical settings (Portevin et al., 2011, Brites and Gagneux, 2012).

2.4. Diagnosis of Tuberculosis

Mainly, TB is diagnosed by direct bacteriological identification of MTBC bacteria in a clinical sample taken from a suspected TB patient. Pulmonary TB, the most common form of TB is diagnosed from sputum collected from a patient with an abnormal chest x-ray while for the more aggressive less common form, extra-pulmonary TB, a biopsy or fine needle aspirates from the infection site such as enlarged lymph nodes is collected and examined using histology or the microscopy (Table 2.3). At present, methods with proven clinical utility for the diagnosis of active TB include microscopy, commercial kits to detect molecular markers, and culture.

2.4.1. Direct Smear Microscopy

This procedure is the most widely and routinely used diagnostic tool for active pulmonary TB in most developing countries. Smear examination by light microscopy after Ziehl Neelsen staining relies on the retention of the red carbol fuschion dye afteralcohol-acid decolourisation (European Centre for Disease Prevention and Control, 2011; _TB_control.pdf; Forrellad et al., 2013).

Sputum smear microscopy is fast and relatively cheap. Unfortunately, its low sensitivity (~50% in average; requires a concentration of 104 bacilli per millilitre for positive smear test) combined with its intrinsic reliance on sputum production limits its use in some vulnerable groups such as children and HIV-positive patients who often produce little sputum with low bacillary load (Shingadia and Novelli, 2003; Getahun et al., 2007).

A faster procedure based on fluorescent dyes with shorter reporting time such as auramine-rhodamine staining procedure is gradually replacing basic fuchsin Ziehl Neelsen staining procedures as an alternative staining procedure. These procedures are 10% more sensitive than light microscopy and are less time consuming, however they come with a high cost of fluorescent microscopes (World Health Organization, 2011).

Notwithstanding these drawbacks, sputum smear microscopy is good for its rapidity and does not require sophisticated equipment, making it suitable for endemic regions in Africa where resources are scarce.

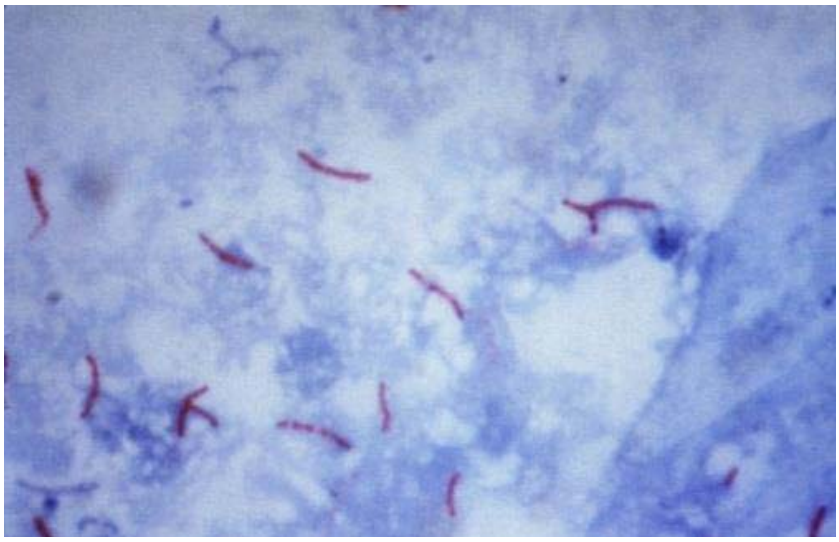


Figure 2.7. Ziehl Neelsen stained smear of M. tuberculosis from decontaminated sputum observed under oil immersion (x1000) (http://en.wikipedia.org)

2.4.2. Chest radiology

Chest X-ray is often used as a complementary tool to smear microscopy in diagnosing TB. Generally, abnormalities seen in the upper lungs (infiltration or cavities) on chest radiographs are often suggestive of but not necessarily definite for TB. Chest X-ray is often used to rule-out the possibility of pulmonary TB in a

person with positive reaction to the tuberculin skin test and no symptoms of the disease (Kumar et al., 2007).

2.4.3. In vitro culturing of *Mycobacterium tuberculosis*

Isolation of the causative agent provides definite evidence of the disease and is considered the gold standard for diagnosing TB. In addition, it offers the opportunity for obtaining bacterial isolates that can be used for in-depth studies. Although this technique is highly sensitive and needs only a few viable baccili to initiate growth (Allen et al., 1992), the slow growth rate of MTBC (3-4 weeks) and the requirement of specific decontamination solution in addition to a costly biosafety level 3 laboratory prevent its usage as a first hand rapid test for the diagnosis of active TB (Palomino et al., 1998).



Figure 2.8. Macroscopic mycobacterial culture on Lowenstein Jensen media (TB reference lab, Noguchi Memorial Institute for Medical Research, Legon)

2.4.4. Alternative diagnostic tools for the identification of MTBC

Several molecular diagnostic tests based on DNA amplification of specific markers have been developed as complementary tools to conventional microbiological diagnosis of TB. Most of these assays have the added advantage of

simultaneously diagnosing TB and detecting drug resistance. Two of such assays; Xpert MTB/RIF (Kurbatova et al., 2012) and line probe assays from HAIN life science are currently in use in many endemic countries. Xpert MTB/RIF based on real-time polymerase chain reaction PCR amplification of rpoB gene detects resistance to rifampicin directly from sputum, regardless of the smear status in less than 2 hours. Furthermore, this method requires no additional reagents since all reagents are in-built, minimizing the cost. The line probe assay GenoType MTBDR plus on the other hand detects resistance to both isoniazid and rifampicin from pulmonary patient specimen in less than 2 hours (Miotto et al., 2008; Barnard et al., 2008; Bazira et al., 2010).

Apart from molecular assays, there are several immunological based assays currently in use for diagnosing latent TB. One of the major tuberculin skin tests used around the world, largely replacing multiple-puncture tests such as the Tine test is the Mantoux test (Mendel, 1908). This assay is based on the measurement of delayed hypersensitive reaction, following intradermal injection of tuberculin. Regardless of its simplicity and usefulness, the Mantoux test is limited by poor specificity especially among Bacille Calmette-Guerin (BCG)–vaccinated individuals and high levels of cross-reactivity with atypical mycobacteria.

In recent times, commercial antigen specific assays measuring Interferon-γ (interferon-gamma) release from T lymphocytes by enzyme linked immunoassay (Quantiferon Gold in Tube (Cellestis, Australia) and enzyme linked immune spot (T-spot TB (Oxford, Immunotec, UK) have been developed as alternative to the Mantoux test (Ferrara et al., 2006). Both tests are are based on the ability of MTBC antigens for early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) to stimulate host production of interferon-gamma. Because these antigens are not present in non-tuberculous mycobacteria or in any of the BCG vaccine variants (Al-Hajoj SA, 2009), these tests are specific for MTBC infection (Pai et al., 2004). Unfortunately, these tests cannot distinguish active MTB infections from latent TB (Rangaka et al., 2011). Additional, these methods require the need for sophisticated instruments and training which limits their implementation in developing countries.

Table 2.4. Gives A Summary of the Recommended TB Diagnostic Tool (Dorman, 2010)

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

2.5. Pathogenesis of TB

TB is an obligatory aerobic pathogen with a penchant for areas rich in oxygen supply (Raja, 2004). For this reason, always the classical TB bacillus in well aerated upper lobes of the lungs is found. To occur an infection, one has to inhale airborne droplet from a person with active disease containing live tubercle bacilli generated, however to be able to transmit the bacteria needs to cause active disease (Gagneux, 2012). The establishment of an infection is based on several factors: high bacteria load in the droplets, the droplet nuclei must be small enough in size (1 to 2 mm or less) to avoid exclusion from the lower respiratory tract by the physical barriers of the upper respiratory tract and nasopharynx, the longer duration of exposure between uninfected and infected persons and poor degree of ventilation and. After inhalation the bacterium, travels down the bronchial tree into the lungs where they are engulfed by alveolar macrophages of the lungs (Kang et al., 2011). Upon entry into the human lung, the bacilli undergo a series of encounters with different outcomes and different host defense mechanisms. In the lungs hence the survival of bacilli depends on its ability to resist elimination by the host immune system (van Crevel et al., 2002).

The bacilli from the lungs can spread to the lymph nodes via lymphatic system. Against the presence of the bacteria in the lung, the initial immune response is quite efficient and very complex; only 5-10% of these infections in fact will lead to progressive disease for reasons unknown (Kang et al., 2011). TB 'infection' means the baccili are in the body but are kept under control by the host immune system. The initial immune response however in the event where is not effective in clearing the invading pathogen, additional immune cells such as lymphocytes and dendritic cells and are recruited from neighbouring blood vessels to the focal site of infection where they engulf the invading pathogen (Ernst, 2012). The attraction of host immune cells to the site of infection initiates the formation of granuloma also known as the giant wall which functions as a barrier for preventing the spread of bacteria to neighbouring cells (Russell et al., 2010). As it matures the

granuloma makes-up changes: initially made up of disorganized cell but becomes more organized with lymphocytes at the periphery and macrophages in the centre (Ulrichs et al., 2006).

The complex successful interaction this organization reflects between the innate and cell mediated immune cells following infection. Elimination of MTBC infection in other words, mainly depends on the success of the interaction between B and T lymphocytes and infected macrophages. Initially thought to play no role in immune defense against MTBC, antibodies and B cells are now believed to contribute to an enhanced immune response against MTBC and with T-cells together modulate various immunological components in the infected host (Achkar et al., 2014). However, by the host despite the strong immune defense put up, some bacilli which have evolved effective strategies to evade the immune response escape killing and enter a state of dormancy, and persist in a low replicating phase by avoiding elimination by the immune system. Otherwise this asymptomatic stage known as the 'containment' phase is a hallmark of latent TB. At this stage Infected individuals are not infectious as they cannot spread the infection to other people. In the granuloma the dormant bacilli remain for decades mediated by the complex interplay between inflammatory cells and cell mediated. In the event, nonetheless where this balance is tilted in favour of the bacilli like in the event of systemic immune suppression as occurs in HIV co-infection, the center of the granuloma undergoes necrosis and active disease develops and eventually becomes caseous. Into the alveoli live baccili are released and the patient becomes infectious. Infectious bacilli viable, spew into the airways resulting in productive cough spreading the infectious bacteria into the air (Russell et al., 2009). With MTB, the final outcome of infection largely depends on the balance between (i) killing or outgrowth of MTB and (ii) the extent of tissue fibrosis, necrosis, and regeneration.

For a pathogen like MTB, the series of immune responses triggered by exposure to the bacilli clearly defines the course of infection: be it containment, or inability to control the bacilli, or be it total eliminationt. The clinical course of

infection thus and its consequences depends largely on the interplay between several bacterial factors and host.

2.6. Treatment

Tuberculosis (TB) is one of the top 10 causes of death worldwide. Between 2000 and 2017, according to the WHO report in 2017, 54 million lives were saved through TB diagnosis and treatment, ending the TB epidemic by 2030 is one of the health goals of the Sustainable Development Goals. In the absence of resistance to medication or deugs, Since the discovery of p-aminosalicylic acid (PAS) and streptomycin (STR) in the 1940s (Table 2.5), TB has been successfully treated with effective chemotherapy, Then the discovery of isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) followed in the 1950s and 1960s.

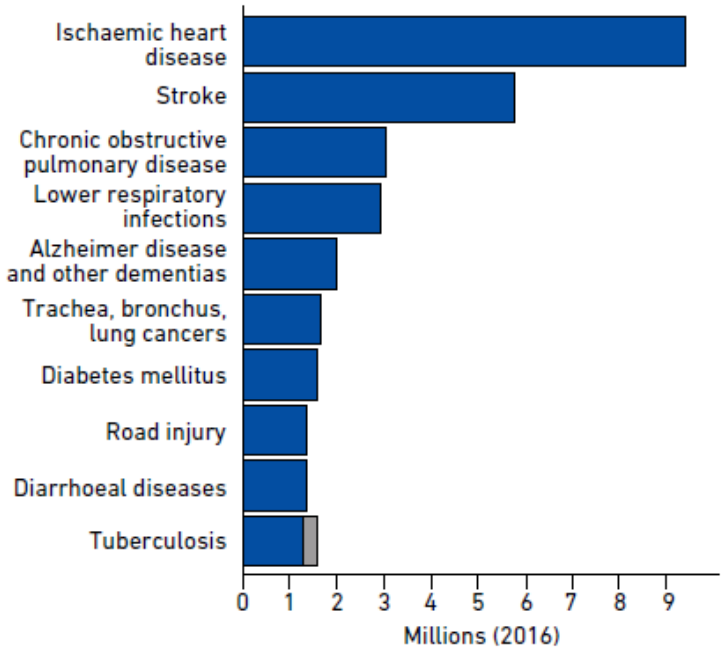


Figure 2.9. Top causes of death worldwide in 2016.a,b Deaths from TB among HIV-positive people are shown in grey.

Treatment of TB is used not just to cure the disease nevertheless also to cut off the transmission and to prevent relapse (most relapses happen within 6-12 months after the finish of treatment) (Böttger and Springer, 2008). The WHO In 1994 introduced the Directly Observed therapy Short Course (DOTS), a method for the detection and treatment of tuberculosis, in which patients are observed to take each dose of anti-TB medication, till the end of Treatment (WHO, 2001). Monthly sputum samples are then taken until 2 consecutive specimens are negative. However, the efficacy of DOTS is facing new challenges Because of the increase of Multidrug Resistant Tuberculosis (MDRTB) and the emergence of Extensivley drug Resistant Tuberculosis XDRTB. This lead to a new strategy so-called DOTS-plus, a comprehensive Administration initiative based on the DOTS strategy with the aim of preventing more development and spread of multidrug resistant tuberculosis (MDRTB) (WHO, 2006; WHO/ IUATLD, 2008). Treatment can be divided into first-line and second-line drugs (Table 2.5 lists the current first and second line drugs available for TB treatment): the first-line drugs used are Rifampcin (RIF), Isoniazid (INH), pyrazinamide (PZA), streptomycin (STR) and ethambutol (EMB) and the second-line drugs comprise fluoroquinolones, aminoglycosides for example amikacin and kanamycin, cyclic peptides such as ethionamide ,capreomycin, r-amino salicylic acid and D-cycloserine , every treatment regimen for pulmonary TB it causes by susceptible organisms has an primary 2 months intensive phase with RIF, INH, EMB and PZA,then followed by a continuation phase with RIF and INH for (4 -7) months. Streptomycin can be used as an interchangeable drug with EMB in the primary phase of therapy. Nevertheless, STR is only recommended to be exchangeable with EMB if the organism is known to be susceptible to the drug or the patient is from a society in which Streptomycin resistance is improbable (Blumberg H.M. et al, 2003; WHO, 2001).

Unlike most other bacterial diseases where single drug regimen is used for treatment, TB has been treated for over fifty years using combination therapy for

several reasons: 1) Reducing the chances of acquiring drug resistance 2) The combined modes of action of the drugs aid in effectively clearing the bacteria: rifampicin inhibits RNA synthesis and has a sterilizing effect (McClure and Cech, 1978). Pyrazinamide (PZA) although weakly bactericidal, is very effective against bacteria located in acidic environments found inside macrophages, or in areas of acute inflammation (Zhang et al., 2003). EMB inhibits the polymerization step of arabinogalactan synthesis (Mikusova et al., 1995). INH is a pro-drug and bactericidal against replicating bacteria by inhibiting mycolic acid synthesis (Zhang et al., 1992), para-aminosalicylic acid inhibits folic acid (Rengarajan et al., 2004), fluoroquinolones act on DNA replication (Drlica et al., 2008) whiles Ethionamide (ETD) also a prodrug, inhibits fatty acid synthesis required for mycolic acid synthesis (Banerjee et al., 1994).

The standard therapy for new TB patients (defined as patients with no previous anti-TB treatment or with previous anti-TB treatment for less than 1 month) consists of two months intensive phase with daily INH/RIF/PZA/EMB, followed by a 4 months continuous phase of daily INH/RIF. INH/RIF are the most important drugs for TB treatment: INH is responsible for the initial killing of about 95% organisms during the first days of treatment, complemented by RIF and PZA during the remaining intensive phase, whilst for the continuation phase RIF is the main active drug against persisters from the intensive phase (World Health Organization, 1994). Previously treated patient are globally 5 times more likely to present with TB caused by multidrug-resistant (MDR) strains, and therefore, should be treated according to drug susceptibility test (DST) results. However, in the absence of DST results, patients are normally placed on a 8 months drug regimen comprising two months of INH/RIF/PZA/EMB (intensive phase), one month of INH/RIF/PZA/EMB (intensive phase) and five months of INH/RIF/EMB (continuation phase).

Table 2.5. 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

2.7. Drug resistance

Clinical drug resistance in TB is classified as acquired resistance, when drug resistant mutants are selected as a result of ineffective treatment, or as primary resistance, when a patient is infected with a resistant *M. tuberculosis* strain (Figure 2.10). The selection of drug resistant mutants has to do with the long generation time of *M. tuberculosis*, its low metabolic activity and capacity for dormancy (Wayne, 1994). Additionally, the compartmentalization of the infection increases the probability of exposure to monotherapy, since *M. tuberculosis* may be located inside pulmonary cavities, with difficult antibiotic access (Elliott et al, 1995). This effect is enhanced in the presence of an inadequate dosage of anti-TB drugs, due to inadequate prescription by the physician or non-adherence by the patient. It is necessary to understand the mechanisms by which *M. tuberculosis* becomes resistant, in order to avoid drug resistance. Several studies over the years have described the mechanisms of action of most of the anti-TB agents used in clinical practice.

MDR-TB In 2017 remains a health security threat and a public health crisis. WHO respect that there were 558 000 new cases with resistance to rifampicin and MDR-TB had the most effective first-line drug – of which 82%. The MDR-TB on 3 countries burden largely falls– India. Russian Federation and China which jointly calculate for nearly half of the global cases. In 2017about 8.5% of MDR-TB cases had overally drug-resistant TB (XDR-TB).

Worldwide, however 55% of MDR-TB patients are currently successfully treated. WHO In 2016 approved the employ of a short, standardised regimen for MDR-TB patients who do not have strains that are resistant to second-line TB drugs. This regimen is much less expensive than the conventional treatment for MDR-TB, which can take up to 2 years and this regimen takes 9–12 months. Patients cannot use this regimen with XDR-TB or resistance to second-line anti-TB drugs, however, and need to be put on longer MDR-TB regimens to which 1 of the new drugs (bedquiline and delamanid) may be added.

In 2017 Globally, an estimated 3.5% (95% confidence interval [CI]: 2.5–4.7%) of new cases and 18% (95% CI: 6.3–34%) had MDR/RR-TB of previously treated cases. The proportions of new and previously treated TB cases with MDR/RR-TB at the country level are shown in Figure 2.11 and Figure. 2.12. There were an estimated 558 000 (range, 483 000– 639 000) incident cases of MDR/RR-TB in 2017, however, the proportion of cases estimated to have MDR-TB was 82% (460 000 out of 560 000) the countries such as India, the Russian Federation and China were the largest numbers of MDR/RR-TB cases (47% of the global total) (Figure. 2.13). In 2017 there were about 230 000 (range, 140 000–310 000) deaths from MDR/RR-TB, similar to the best estimate for 2016 that was published in the 2017 edition of the WHO global TB report (WHO, 2017).

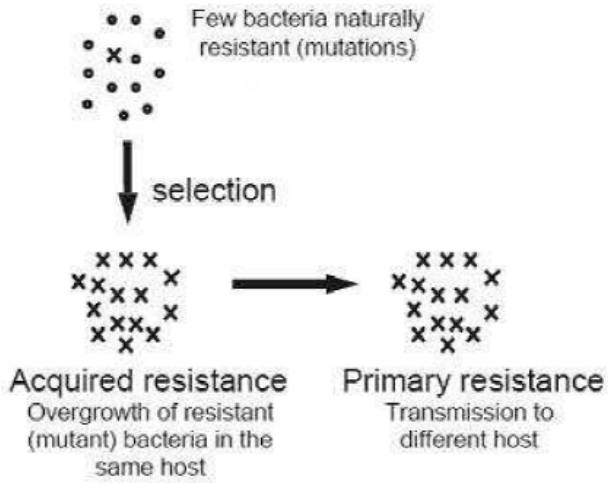


Figure 2.10. Development of resistance in TB.

Acquired resistance develops due to the selection of resistant mutants as a consequence of ineffective treatment and non-compliance. Primary resistance occurs through the transmission of the resistant bacteria to a new host (adapted from Johnson R. et al, 2006).

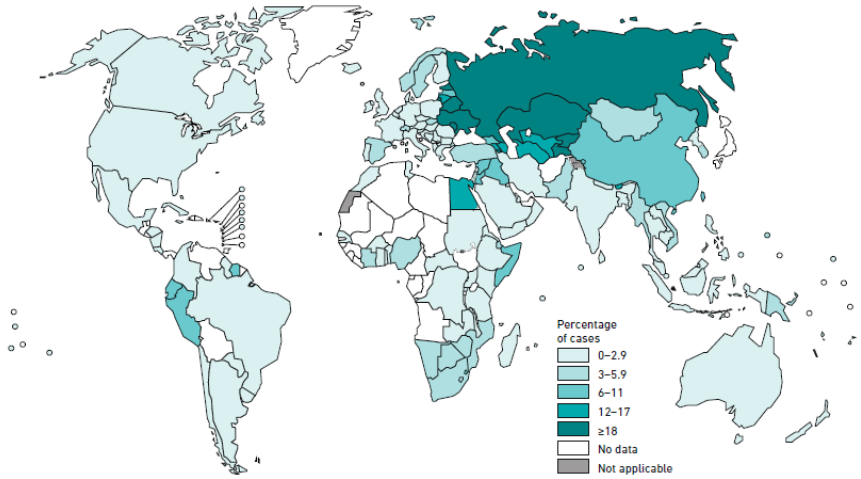


Figure 2.11. Percentage of new TB cases with MDR/RR-Tb (WHO,2018)

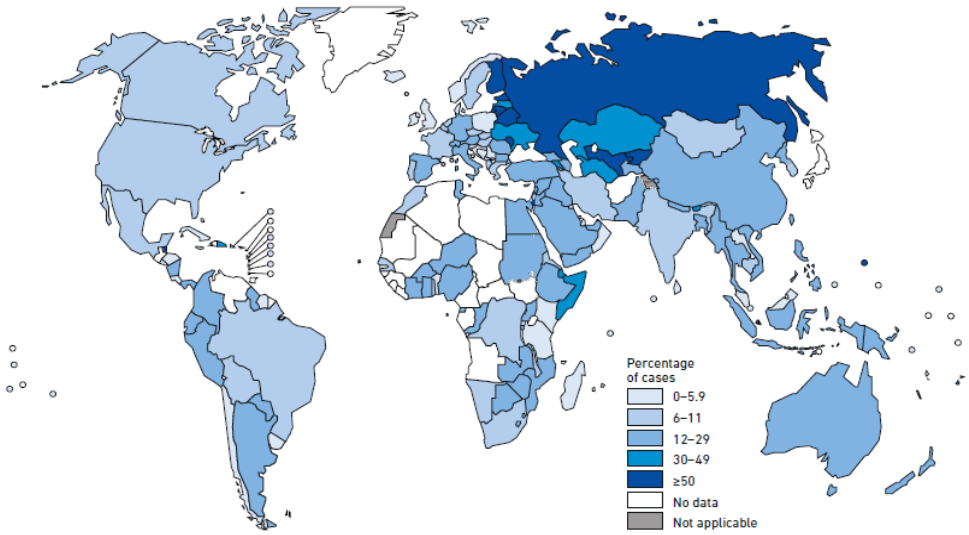


Figure 2.12. Percentage of previously treated TB cases with MDR/RR-TBa

A figures are based on the most recent year for which data have been reported, which varies among countries. Data cover the period 2005–2018. The high percentages of previously treated TB cases with RR-TB in Belize, Guam and Sao Tomé and Principe refer to only a small number of notified cases (range: 1–8 notified previously treated TB cases).

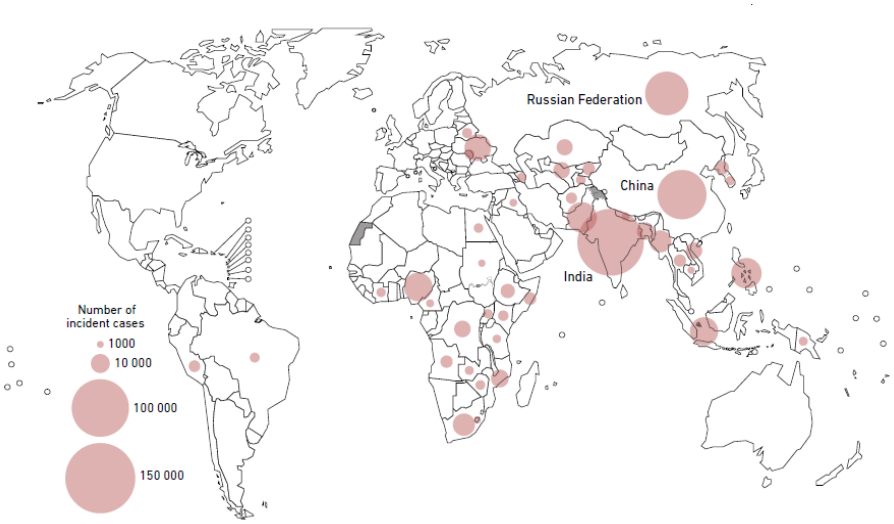


Figure 2.13. Estimated incidence of MDR/RR-TB in 2017, for countries with at least 1000 incide

2.7.1. Intrinsic resistance

Intrinsic drug resistance of *M. tuberculosis* has classically been due to the unusual structure of its mycolic acid-including cell wall that confer the bacteria a low permeability for many compounds like antibiotics and other chemotherapeutic agents (Jarlier and Nikaido., 1991: Niederweiss M. et al, 2010). More recently, the role of efflux mechanisms has too been recognized as an important factor in the natural resistance of mycobacteria against antibiotics such as tetracycline, fluoroquinolones and aminoglycosides, among others.(Table 2.6) (De Rossi et al.,2006) In *Mycobacterium smegmatis*, it has been domenstrate that mutants lacking the major porin MspA had an increased MIC of the b-lactam antibiotics cefaloridine and ampicillin. Deletion of the mspA gene as well increased the MIC of erythromycin, rifampicin and vancomycin by 2- to 10-fold. (Stephan et al.,2004)

Recent studies in *M. tuberculosis* have shown that Rv1698 can have the same function as MspA in contributing to intrinsic resistance to hydrophilic components (Siroy et al., 2008). Moreover, bioinformatics analysis has identified

both Rv1698 and Rv1973 as presence mycobacterial outer membrane proteins (OMPs) with a probable role in the intrinsic resistance to several antibiotics (Song et al., 2008)

But not only permeability barriers or b-lactamases are responsible for the intrinsic resistance to antibiotics in mycobacteria. Also Physiological adaptations revolving within the host can be responsible for antibiotic tolerance (Nguyen and Pieters., 2008) Eventually, whiB7 in *M. tuberculosis*, is an MDR determinant whose deletion generates a multidrug-susceptible phenotype, suggesting its role as an ancestral MDR determinant. Intrinsic resistance is thus important since it limits the number of drugs available for treatment and favours the emergence of strains with a high level of drug resistance. Consequently, drugs inhibiting these mechanisms would enable potential new use against *M. tuberculosis* of many antibiotics that are already available but have not been used before in the treatment of TB (Lomovskaya and Bostian., 2006: Nguyen and Thompson., 2006).

2.7.2. Acquired drug resistance

Acquired drug resistance generally unlike the situation in other bacteria is mediated through horizontal transfer by mobile genetic elements, such as transposons or integrons and plasmids, acquired drug resistance in *M. tuberculosis*, is caused mainly by spontaneous mutations in chromosomal genes, producing the selection of resistant strains during sub-optimal drug therapy (Kochi et al., 1993), Although in *M. tuberculosis* no single pleiotropic mutation has been found to cause an MDR phenotype, a possible complex association between classical mutations associated with resistance to one drug could be related to initial steps in the resistance to other drugs. (Safi et al., 2008) Spontaneous mutations in prokaryotes, occur at a low rate of 0.0033 per replication. The mutation rate per bp is inversely proportional to the genome size. The rate of mutation in previous studies have shown that depends on the nature of the drug selection, but for most of the main anti-TB drugs, this occurs at a rate of 1029 mutations per cell division. This is the

main reason why anti-TB drugs are given as a combination, as the risk of a mutant containing two resistance mutations is 10218 (Gillespie., 2007).

Table 2.6. 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

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

2.7.2.1. First-line oral anti-TB drugs

Any drug used in the anti-TB regiment is assumed to have an effective sterilizing activity that is capable of shortening the duration of treatment. Currently, a four-drug regiment is used consisting of INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). Resistance to first line anti-TB drugs has been related to mutations in at least 10 genes; inhA, ahpC, katG, kasA and ndh for INH resistance; pncA for PZA resistance, rpoB for RIF resistance, embB for EMB resistance, and rpsL and rrs for STR resistance.

2.7.2.1.(1). Isoniazid

A first-line pro-drug is Isoniazid (isonicotinic acid hydrazide) that is requiring activation by the catalase/peroxidase enzyme encoded by katG (Zhang et al., 1992). The active species (isonicotinic acyl radical or anion) reacts with nicotinamide adenine dinucleotide (H), forming INH76 NAD adduct, which inhibition of cell wall mycolic acid synthesis by inhibits InhA. INH is active only against growing MTB, but not against non-growing bacilli (Zhang et al., 1992). INH tolerance on the other hand, in non-growing organisms may be caused by mycobacterial DNA-binding protein 1 (MDP1), a histone-like protein, which down regulates katG transcription and could lead to tolerance to INH (Niki et al., 2012).

Resistance to INH is a complex process. Mutations in several genes, including inhA, ahpC, katG, kasA and ndh, all have been with INH resistance associated. The major mechanism of INH resistance is by mutations in katG (Zhang et al., 1992: Zhang and Yew., 2009: Hazbon et al., 2006). The most common mutation in INH resistant strains is by The katG S315 mutation is, accounting for 50–95% of INH resistant clinical isolates (Zhang et al.,1992: Zhang and Telenti., 2000: Colangeli et al., 2005). Mutations in inhA or its promoter region are usually associated with low level resistance (minimum inhibitory concentrations [MICs] of 0.2–1 μg/ml) and are less frequent than katG mutations, in contrast to katG mutations, which usually cause high86 level resistance, (Zhang

and Yew., 2009: Zhang and Telenti., 2000: Fu and Shinnick., 2007). Mutations in inhA 88 is confer cross-resistance to the structurally related drug ethionamide (ETH) not only cause INH resistance (Banerjee et al., 1994). Other genes including kasA, ahpC, ndh, and the ahpC-oxyR intragenic region have been associated with resistance to INH, but their impact on resistance among clinical isolates remains unclear (Campbell et al., 2011: Guo et al., 2006: Shekar et al., 2014).

2.7.2.1.(2). Rifampicin

In 1972 RIF was first introduced as an anti-TB drug and has excellent sterilizing activity (Rattan et al., 1998; Ramaswamy and Musser, 1998). The action of RIF has allowed a shortening of routine TB treatment from 1 year to 6 months in combination with PZA. RIF in combination with INH forms the backbone of short-course chemotherapy. It is interesting to note that mono resistance to RIF is quite rare but mono resistance to INH is common. It has thus been proposed that resistance to RIF can be used as a surrogate marker for MDR-TB as nearly 90% of RIF resistant strains are also INH resistant (Somoskovi et al., 2001: Zhang and Yew 2009). RIF interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits (α, β, β' and σ) encoded by rpoA, rpoB, rpoC and rpoD genes respectively. RIF binds to the β-subunit hindering transcription and thereby killing the organism. Extensive studies on the rpoB gene in RIF resistant isolates of *M. tuberculosis* identified a variety of mutations and short deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletion and 38 multiple nucleotide changes have been reported (Herrera et al., 2003). In a 51bp core region more than 95% of all missense mutations are located (Rifampicin resistance determining region) of the rpoB gene between codons 507–533 with the most common changes in codons Ser531Leu, His526Tyr and Asp516Val. In more than 70% of RIF resistant isolates these changes occur (Rattan et al., 1998; Ramaswamy and Musser, 1998; Herrera et al.,

2003). Furthermore, the minimal inhibitory concentration (MIC) appeared, with mutations in codon 526

2.7.2.1.(3). Ethambutol

Like PZA, EMB interferes with the biosynthesis of cell wall arabinogalactan. Arabinosyl transferase, encoded by embB, an enzyme involved in the synthesis of arabinogalactan, is the target of EMB in MTB Mutations in embCAB operon, in particular embB, and occasionally embC, are responsible for resistance to EMB. embB codon 306 mutation is most frequent in clinical isolates 110 resistant to EMB, accounting for as much as 68% of resistant strains. While mutations at EmbB306 leading to certain amino acid changes caused EMB resistance, other amino acid substitutions had little effect on EMB resistance. However, about 35% of EMB resistant strains (MIC of 10 g/L) do not have embB mutations, suggesting other mechanisms of resistance. Mutations in ubiA, encoding the decaprenylphosphoryl-5- phosphoribose (Dppr) synthase involved in cell-wall synthesis, have recently been found to cause higher-level EMB resistance in conjunction with embB mutations

2.7.2.1.(4). Streptomycin

STR, an aminocyclitol glycoside, is an alternative first line anti-TB drug recommended by the WHO (Cooksey et al., 1996). STR is therefore used in the retreatment of TB cases together with the four drug regimen that includes INH, RIF, PZA and EMB (Brzostek et al., 2004). The effect of STR has been demonstrated to take place at the ribosomal level (Telenti et al., 1993). STR interacts with the 16S rRNA and S12 ribosomal protein (rrs and rpsL) (Escalante et al., 1998; Finken et al., 1993; Sreevatsan et al., 1996; Abbadi et al., 2001), inducing ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis. Although STR is a recommended anti-TB drug, is it less effective against M. tuberculosis than INH and RIF. Point mutations in STR

resistant isolates have been reported in rrs and rpsL genes in 65–67% of STR resistant isolates (Ramaswamy and Musser, 1998). In the rrs gene a C-T transition at positions 491, 512 and 516, and a A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl–tRNA binding site and is involved in the decoding process (Carter et al., 2000). The C-T transition at codon 491 is not responsible for resistance to STR as it occurs in both STR resistant and susceptible isolates but is strongly associated with the global spread of *M. tuberculosis* with a Western Cape F11 genotype (van Rie et al., 2001; Victor et al., 2001). Other mutations in the 915 loop [903 (C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance (Carter et al., 2000). Mutations in the rpsL gene at codon 43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAGAGG/ CAG) (Lys-Arg/Gln) are associated with STR resistance. MIC analysis of STR resistant isolates indicate that amino acid replacements in the rpsL genes correlate with a high level of resistance, whereas mutations in the rrs gene correlate with an intermediate level of resistance (Cooksey et al., 1996; Meier et al., 1996). In addition, it has been suggested that low levels of STR resistance are also associated with altered cell permeability or rare mutations which lie outside of the rrs and rpsL genes.

2.7.2.1.(5). Pyrazinamide

Pyrazinamide is a paradoxical drug with unique sterilizing activity in anti-TB treatment (Zhang *et al*., 2013: Zignol *et a*l., 2016). In striking contrast to common antibiotics that kill or inhibit growing bacteria, PZA inhibits non-growing bacteria and has activity against growing bacteria only at acidic pH. One key characteristic of PZA is its ability to inhibit semidormant bacilli residing in acidic environments (Zhang et al., 2003). PZA is a pro-drug that has to be converted to its active form, pyrazinoic acid (POA), for activity by the pyrazinamidase/nicotinamidase enzyme encoded by the pncA gene of MTB (Scorpio and Zhang, 1996). Pyrazinamide has multiple effects on MTB, including

interference with membrane energy production, and inhibition of rpsA (ribosomal protein S1), which is involved in translation, and PanD, which is involved in pantothenate and co-enzyme A synthesis (Zhang et al., 2013: Zhang et al., 2003, Shi et al.,2011: Shi et al.,2014). Mutations in the pncA gene are the main mechanism of PZA resistance in MTB Scorpio and Zhang, 1996: Scorpio et al., 1997). Most PZA-resistant MTB strains (72–99%, average 85%) have mutations in pncA, but some do not (Scorpio et al., 1997: Cheng et al., 2000: Sreevatsan et al.,1997: Hirano et al., 1998: Morlock et al., 2000). Those do not have mutations in the drug target RpsA Feuerriegel et al., 2013:, Finken et al., 1993). RpsA target mutations are usually associated with low-level PZA resistance (MICs of 200– 300 μg/ml PZA). More recently, panD was found to be involved in PZA resistance (Zhang et al., 2013). panD mutations were identified in naturally PZA-resistant Mycobacterium canettii strains and some PZA-resistant MDR-TB strains. M. canettii, a member of the MTB complex, is intrinsically resistant to PZA, and has mutations in both rpsA48 and panD (Finken et al., 1993).

2.7.2.2. Second line drugs used in TB treatment

When the recommended short-course treatment with first-line drugs fails due the emergence of MDRTB, management of TB in these cases is based on the use of second-line drugs (WHO, 2001). However, these drugs are more toxic and less effective than first-line drugs and treatment is more prolonged and expensive (Figure 2.14). There are six classes of second-line drugs used for the treatment of TB: aminoglycosides, such as amikacin and kanamycin; cyclic peptides, such as capreomycin; fluoroquinolones, such as ciprofloxacin, levofloxacin and moxifloxacin; thioamides, like ethionamide; D-cycloserine; and p-aminosalicylic acid. The increased use of these drugs has contributed to the emergence of XDRTB strains and, therefore, it is important to understand the mechanisms by which *M. tuberculosis* becomes resistant to these drugs.

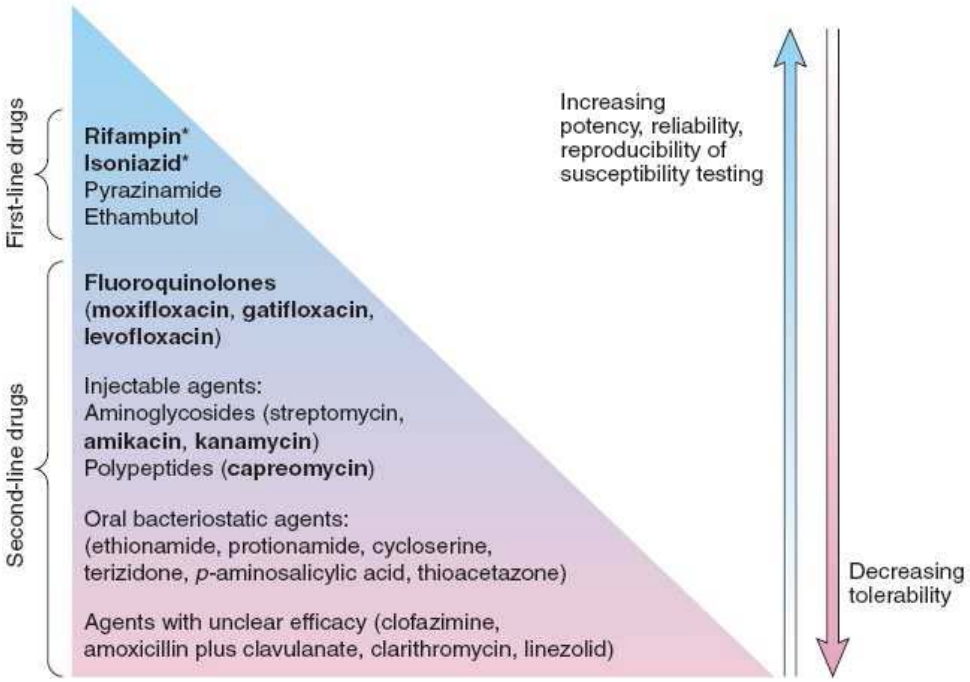


Figure 2.14. Effectiveness and tolerability relation of first- and second-line drugs used in TB treatment (Dorman S.E. et al, 2004).

2.8. Efflux Pumps

Bacterial efflux pumps are identified for their association with antimicrobial resistance. Nevertheless, the existence of efflux pumps in bacteria is previous to the development of antibiotics. Therefore, their natural physiological role is not related with the use of antibiotics, but instead consists in the extrusion of noxious agents from the cell, allowing the bacteria to survive in a hostile environment (Piddock L.J., 2006; Poole K., 2007). An example of a natural function of efflux pumps is the secretion of intracellular metabolites and protection against bile salts and fatty acids in enteric bateria as a response to their natural environment. Antimicrobial resistance due to an increased efflux activity can be caused by the genetic overexpression of the efflux pump, or by aminoacid substitutions in the protein itself that can render the pump more efficient. Both mechanisms cause the reduction of the intracellular concentration of the

antimicrobial and, consequently, the organism becomes less susceptible to that agent. Efflux pumps may be substrate-specific or transport a broad range of structurally dissimilar compounds (including antibiotics of multiple classes), the latter of which may be associated with multiple drug resistance. Genes coding for efflux pumps can be found on the bacterial chromosome or on transmissible elements such as plasmids (Piddock L.J., 2006; Poole K., 2007). The following sections will address the characteristics of efflux pumps, their role on drug resistance and strategies to prevent efflux-mediated multidrug resistance.

2.8.1. Classes and Organization of Efflux Pump Systems

Efflux pump systems can be organized into five different families according to their energetic and structural characteristics (Figure 2.15): the ATP-binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family; and the resistance nodulation division (RND) family. Efflux pumps that are included in the ABC superfamily are considered primary transporters because they hydrolyze ATP as a source of energy, whereas the other families of efflux pumps use the proton (or sodium in the case of MATE family) gradient as an energy source and are thus called secondary transporters (Lomovskaya O. et al, 2007; Marquez B., 2005; Piddock L.J, 2006; Poole K., 2007).

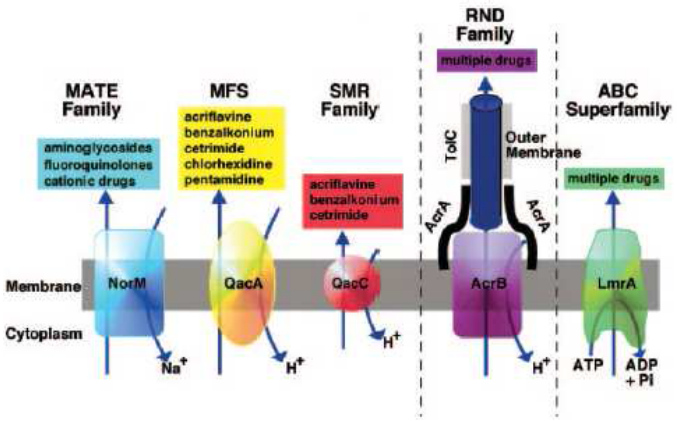


Figure 2.15. Schematic representation of the different classes of efflux pump systems (Piddock L.J., 2006).

1-ATP-binding cassette (ABC) superfamily ABC transporters are involved in different transport functions such as the efflux of toxins, metabolites and drugs. These systems consist of two cytoplasmic domains that bind ATP and two hydrophobic transmembrane domains (Davidson A.L. et al, 2008;

Higgins C.F., 2001). The nucleotide binding domains are highly homologous and possess the Walker A and B motifs, common to all ATP-binding proteins, and a Signature Motif specific to ABC transporters (Davidson A.L. et al, 2008; Kerr I.A., 2002). One of the most studied ABC transporters is the mammalian P-glycoprotein (Pgp) that, when overexpressed, confers resistance to compounds used in cancer chemotherapy (Lage H., 2003). Other ABC transporters have been described in Grampositive and Gram-negative bacteria (Davidson A.L. et al, 2008; Lubelski J. et al, 2007). An example is LmrA, a well studied ABC transporter from Lactococcus lactis that confers multidrug resistance and presents structural and functional homologies with the human P-gp (Poelarends G.P. et al, 2002; Lubelski J. et al, 2007). Genome sequencing revealed putative LmrA homologues in other bacteria such as E. coli, Bacillus subtilis, Helycobacter pylori, Haemophillus influenzae, Mycoplasma genitalium and Staphylococcus aureus (Davidson A.L. et al, 2008; Lubelski J. et al, 2007).

2- Major facilitator superfamily (MFS)

MFS is a large superfamily of transporters involved in symport, uniport or antiport of various substrates. Most proteins are 400-600 aminoacid residues in size and possess either 12 or 14 putative transmembrane domains (Fluman N. et al, 2009; Law C.J. et al, 2008; Saidijam M. et al, 2006). An example of an MFS drug efflux protein in Gramnegative bateria is the MdfA from E. coli, which can extrude compounds like chloramphenicol and various cationic compounds (Bibi E. et al, 2001). MdfA is a typical MFS 12 transmembrane helix protein with a large and complex multidrug recognition pocket. In addition to its function as a multidrug transporter, other studies revealed that MdfA plays a physiological role in alkaline pH homeostasis, possibly through its K+/proton antiporter activity (Lewinson O. et al, 2004). EmrD is another MFS drug/proton antiporter from E. coli and was first identified as an efflux pump for uncouplers of oxidative phosphorylation, which can rapidly inhibit bacterial growth by depleting the proton gradient (Naroditskaya V. et al, 1993). It was later discovered that EmrD could also transport detergents such as benzalkonium and sodium dodecylsulfate (Nishino K. et al, 2001). EmrD is a close homologue to other MFS transporters, including MdfA from E. coli, NorA from S. aureus, LmrP from L. lactis and Bmr from B. subtilis (Paulsen I.T. et al, 1996).

3- Multidrug and toxic compound extrusion (MATE) family Efflux pumps of the MATE family have been described for various bacteria, including Vibrio parahaemolyticus (NorM), Vibrio cholerae (VcrM; VcmA), *Bacteroides thetaiotaomicron (BexA), Haemophilus influenzae (HmrM), Pseudomonas aeruginosa (PmpM), Clostridium difficile (CdeA),* and *S. aureus (MepA)* (McAleese F. et al, 2005; Omote H. et al, 2006; Otsuka M. et al, 2005). Most MATE members consist of 400–550 residues with 12 transmembrane helices. Although no apparent consensus sequence is conserved in all MATE proteins, all proteins share 40% sequence similarity. Two energy sources have been identified

for MATE transporters: the proton motive force and the sodium ion gradient. Some MATE transporters, such as NorM of *V. parahaemolyticus*, have been shown to be energetically coupled to the sodium ion gradient force across the plasma membrane. This force is established by either a primary Na+ pump or a Na+-proton antiporter coupled with respiration. However, in the case of AbeM (*Acinetobacter baumannii*) and PmpM (*P. aeruginosa*) activity is coupled to the proton motive force across the plasma membrane (Omote H. et al, 2006). It was demonstrated that these transporters confer resistance against cationic drugs such as ethidium bromide (EtBr), tetraphenylphosphonium (TPP), berberine, acriflavine and norfloxacin (Omote H. et al, 2006). In particular, MepA from *S. aureus* has been associated with resistance to tigecycline, an antibiotic that shows antimicrobial activity against methicillin-resistant S. aureus (MRSA) (McAleese F. et al, 2005).

4- Small multidrug resistance (SMR) family

The smallest secondary transporters belong to the SMR family. These proteins are typically around 110 aminoacid residues in length with 4 predicted transmembrane helices. SMR transporters can confer resistance to several compounds, such as methyl viologen, TPP, benzalkonium, cetyltrimethylammonium bromide, cetylpyridinium chloride, EtBr, acriflavine, proflavin, crystal violet, pyronine Y and safranin O (Bay D.C. et al, 2008). The most characterized members of this family are the plasmid encoded Smr from *S. aureus*, which confers resistance to EtBr and other quartenary compounds, and chromosomal encoded EmrE from *E. coli* that is now considered the structural archetype of all SMR proteins (Paulsen I.T. et al, 1996b; Schuldiner S., 2007; Tate C.G., 2006). EmrE confers resistance to monovalent cations, such as ethidium, proflavine, pyronin Y and safranin O, as well as to erythromycin, sulfadiazine, TPP and TET.

5- Resistance nodulation division (RND) family

RND transporters have been mostly studied in Gram-negative bacteria, because they are involved in intrinsic antibiotic resistance in these microorganisms. However, in Gram-positive bacteria their function is still mostly unknown. These transporters present a broad range of substrates and can extrude positive, negative or neutral charged molecules, hydrophobic and hydrophilic compounds (Piddock L.J., 2006; Poole K., 2007). Most RND transporters are composed of a polypeptide chain with 700- 1300 aminoacid residues and are predicted to span the membrane

12 times with two large periplasmic domains located between transmembrane helices 1 and 2 and between 7 and 8 (Seeger M.A. et al, 2008). Examples of the RND superfamily include AcrB and AcrF from E. coli, MexB from P. aeruginosa and MtrD *from Neisseria gonorrhoeae*. Two other putative *E. coli* proteins, AcrD and YhiV, may also be RND multidrug efflux proteins (Piddock L.J., 2006; Poole K., 2007). These transporters combine with membrane fusion proteins and outer membrane proteins (also called outer membrane factor) to form a tripartite efflux pump system. The most studied example of such structure is the AcrAB-TolC system of *E. coli* (Figure 2.16). This tripartite efflux system comprises the following components: a RND transporter protein in the inner membrane (AcrB), a periplasmic membrane fusion protein (AcrA) and an outer membrane protein channel (TolC) (Piddock L.J., 2006; Poole K., 2007). AcrB binds the substrate within either the phospholipid bilayer of the inner membrane of the bacterial cell-wall or the cytoplasm and transports them to the exterior of the cell using TolC. AcrA mediates the cooperation between AcrB and TolC (Piddock L.J., 2006; Seeger M.A. et al, 2008). The AcrAB-TolC system transports a wide range of antibiotics such as b-lactams (e.g. oxacillin), macrolides (e.g. erythromycin), fluoroquinolones (e.g. ciprofloxacin) and tetracyclines, RIF, novobiocin, fusidic acid and nalidixic acid, and also other compounds such as EtBr, acriflavine, bile salts and shortchain fatty acids (Piddock L.J., 2006; Poole K., 2007).

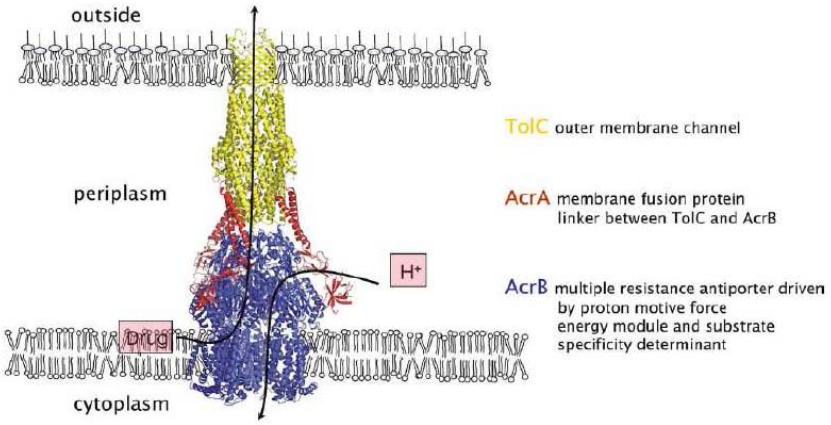


Figure 2.16. Schematic representation of AcrAB–TolC of E. coli.

AcrB is responsible for substrate recognition and energy transduction. Drugs are extruded in exchange with protons. TolC forms a pore in the outer membrane, which is extended by a long periplasmic channel. AcrA mediates the interaction between AcrB and TolC (Seeger M.A. et al, 2008).

2.8.2. Efflux Pumps in Mycobacteria

Some Mycobacterial drug efflux pumps have been identified and characterized to the present-day (Louw G.E. et al, 2009; Viveiros M. et al, 2003).

1- Genes encoding ABC transporters include nearly about 2.5% of *the M. tuberculosis genome* and at least (37) complete and incomplete ABC transporters have been identified (Braibant M. et al, 2000). Nevertheless, only a few of these transporters have been characterized and shown to be involved in drug resistance. *M. tuberculosis* contains a putative doxorubicin-resistance operon, drrABC (Choudhuri B.S. et al, 2002). The DrrAB genes expressed in *M. smegmatis* confer resistance to a broad range of antibiotics, including TET, erythromycin, ethambutol, norfloxacin, STR and chloramphenicol. The resistant phenotype is reversed by treatment with reserpine or verapamil, compounds known to inhibit efflux (Choudhuri B.S. et al, 2002). Studies have suggested that the main role of

the Drr proteins of *M. tuberculosis* may be the export of lipids to the exterior of the cell and, in particular, DrrC seems to be involved in the transport of phthiocerol dimycocerosates (Camacho L.R. et al, 2001). The *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon encodes an ABC transporter responsible for fluoroquinolone efflux when produced from a multicopy plasmid. When overexpressed in *M. smegmatis*, this operon increases 8-fold the MIC of ciprofloxacin and 2-fold the MIC of norfloxacin. The level of resistance decreases in the presence of reserpine, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil (Pasca M.R. et al, 2004).

2-The plasmid-encoded TET efflux pump Tet(V) was isolated from *M. smegmatis* and, when overexpressed, increases the MIC of TET from 2- to 4-fold. The distribution of the tet(V) gene among the genus Mycobacterium has been investigated by polymerase chain reaction (PCR). However, *M. smegmatis* and *M. fortuitum* were the only species tested that revealed a tet(V) gene (De Rossi E. et al, 1998).

3- The LfrA protein of *M. smegmatis* was the first efflux pump to be described in the genus Mycobacterium and may be responsible for low-level resistance to fluoroquinolones, acridine and quaternary ammonium compounds (Liu J. et al, 1996; Sander P. et al, 2000; Takiff H.E. et al, 1996). When overexpressed, LfrA seems to play an important role in the resistance to ciprofloxacin in *M. smegmatis*. However, in the absence of overexpression, LfrA is thought to have no effect on the susceptibility to fluoroquinolones. In fact, disruption of the lfrA gene decreased 8-fold the MIC of EtBr and acriflavine and decreased only 2-fold the MICs of ciprofloxacin, doxorubicin and rhodamine (Li X.Z. et al, 2004). This discrepancy of results between EtBr and ciprofloxacin can be explained if EtBr is a better substrate for LfrA (Li X.Z. et al, 2004). Other explanation resides in the fact that efflux pumps other than LfrA may extrude ciprofloxacin in M. smegmatis. The region upstream from the lfrA gene revealed the presence of an open reading frame encoding a putative polypeptide of 195

amino acids, LfrR, homologous to several transcriptional regulators of the TetR family (Li X.Z. et al, 2004). The lfrR and lfrA genes are organized into an operon probably controlled by LfrR. It has been demonstrated that deletion of the lfrR gene enhances lfrA expression, increasing the resistance to ciprofloxacin, norfloxacin, EtBr and acriflavine (Li X.Z. et al, 2004). No known homologue of the lfrA gene as been described in the M. tuberculosis genome (De Rossi E. et al, 2002).

4-The *M. fortuitum* Tap efflux pump and its *M. tuberculosis* homologue Rv1258c confer resistance to TET and aminoglycosides, including STR (Ainsa J. et al, 1998). When cloned on a plasmid, Tap increased the resistance of M. smegmatis mc2155 to gentamicin, STR and TET (Ainsa J.A. et al, 1998). In the case of a clinical strain of *M. tuberculosis*, the expression of rv1258c increased in the presence of RIF and ofloxacin (Siddiqi N. et al, 2004). Furthermore, TET accumulation experiments showed that the efflux activity of Tap from *M. fortuitum* is inhibited by CCCP and reserpine, a result consistent with the decrease of the MIC of this antibiotic in the presence of these compounds. In addition, CCCP, reserpine and also chlorpromazine reduced the MIC of TET in a *M. smegmatis* strain expressing the Tap protein (Ramon-Garcia S. et al, 2006).

5-Rv1634 is a MFS efflux pump that has been suggested as a new fluoroquinolone efflux transporter in *M. tuberculosis* (De Rossi E. et al, 2002). It was demonstrated that this pump decreased susceptibility to various fluoroquinolones when overexpressed in *M. smegmatis*. Furhermore, accumulation assays suggest that Rv1634 is also involved in norfloxacin and ciprofloxacin efflux (De Rossi E. et al, 2002).

6- The P55 efflux pump from *M. bovis* and *M. tuberculosis* has been associated with low-level resistance to several drugs including TET, aminoglycosides and RIF (Ramon- Garcia S. et al, 2009; Silva P.E. et al, 2001). Rv1410c, the gene for P55 in *M. tuberculosis*, forms an operon with Rv1411c, encoding the lipoprotein LprG. It is thought that both genes support in vivo growth

of *M. tuberculosis* and studies performed in *M. smegmatis* have shown that this operon is required for survival in the presence of EtBr and for normal cell surface composition (Bigi F. et al, 2004; Farrow M.F. et al, 2008). A recent study has demonstrated that P55 plays a role in at least three important processes: (i) it extrudes and provides resistance to several drugs (including rifampicin); (ii) it is part of the oxidative stress response; and (iii) it is needed to maintain normal growth characteristics on solid and in liquid media (Ramon-Garcia S. et al, 2009).

7-The *M. tuberculosis* putative efflux protein EfpA presents the transporter motifs characteristic of QacA of *S. aureus*, including those associated with proton antiporter function and those specific to drug transporters. It was shown that expression of efpA increases in the presence of INH, which could suggest that the protein encoded by this gene transports molecules involved in mycolic acid synthesis (Wilson M. et al, 1999). The deletion of the efpA homologue in *M. smegmatis* resulted in a 2-fold increased susceptibility to EtBr, gentamicin and fluoroquinolones and an 8-fold increased susceptibility to acriflavine. However, it also resulted in a 4-fold decreased susceptibility to rifamycins and chloramphenicol and a 2-fold decreased susceptibility to INH and erythromycin (Li X.Z. et al, 2004). Moreover, this efpA deleted mutant grew more slowly than the wild-type strain, which could mean that its higher susceptibility may be because of impaired growth (Li X.Z. et al, 2004). Thus, the role of EfpA in drug resistance remains unclear. However, since the deletion of this gene increases susceptibility to EtBr, it is pssible that EtBr is a substrate of this pump (Li X.Z. et al, 2004).

8-The genome of *M. tuberculosis* contains several genes that code for putative transport proteins of the RND superfamily. These proteins have been designated MmpL (mycobacterial membrane proteins, large) and are thought to be involved in the transport of fatty acids (Tekaia F. et al, 1999). In M. tuberculosis, MmpL7 exports phthiocerol dimycocerosate (PDIM), a lipid component of the outer membrane (Camacho L.R. et al, 2001). Upstream from the mmpL7 gene is the fadD28 gene, which encodes an acyl-CoA synthase probably involved in the

release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. A strain with an insertion in mmpL7 produces a dimycocerosate (DIM) molecule that is retained in the cytoplasm or the cytoplasmic membrane. The production of MmpL7 in *M. smegmatis* promotes a 32- fold increase of the MIC of INH. However, this phenotype is reversed if fadD28 and mmpL7 are expressed simultaneously, which suggests that DIM and INH compete for the same MmpL7 transporter (Pasca M.R. et al, 2005).

9- Mmr is the only protein of the SMR family that has been described in *M. tuberculosis* (De Rossi E. et al, 1998b). The chromosomal gene mmr, when inserted into a multicopy plasmid, decreases the susceptibility of *M. smegmatis* to TPP, EtBr, erythromycin, acriflavine, safranin O and pyronin Y. Accumulation assays have shown that Mmr extrudes TPP, in a process dependent of the proton motive force. The presence of mmr-like genes in other Mycobacterium species (M.simiae, M. gordonae, M. marinum and M. bovis) has been demonstrated by Southern hybridization (De Rossi E. et al, 1998b).

10- The operon Rv0341-Rv0342-Rv0343 was demonstrated to be induced by treatment with INH (Alland D. et al, 2000). The three genes that form this operon were designated as iniB, iniA and iniC (for isoniazid inducible gene) in the order that they appeared in the operon. From the three genes the most studied is iniA and it has been shown that it may be involved in the development of tolerance to INH and EMB (Colangeli R. et al, 2005). In fact, deletion of iniA from M. tuberculosis increased the susceptibility to INH, whereas the overexpression of this gene in M. bovis allowed the survival of the organism for a longer period of time in the presence of INH and EMB and also resulted in resistance to EtBr. The exposure of the iniA overexpressing M. bovis BCG strain to reserpine reversed both tolerance to INH and resistance to EtBr. The fact that IniA forms multimeric structures containing a central pore suggests that this protein could be a pump component. By this manner, IniA may function through an efflux pump like mechanism, although it does not seem to directly transport INH from the bacterial cell (Colangeli R. et al, 2005).

Table 2.7. Putative mycobacterial efflux pumps and genes that might be associated with drug reistance in mycobacteria

Efflux pump	Microorganism	Possible substrates	Family	Ref.
PstB	*M. smegmatis* *M. tuberculosis*	INH, RIF, EMB, CIP	ABC	Bhatt K. *et al*, 2000
Rv2686c-Rv2687c-Rv2688c	*M. tuberculosis*	CIP	ABC	Pasca M.R. *et al*, 2004
Rv1747	*M. tuberculosis*	INH	ABC	Braibant M. *et al*, 2000
DrrA-DrrB-DrrC	*M. tuberculosis*	TET, STR, EMB	ABC	Choudhuri B.S. *et al*, 2002
Tet(V)	*M. smegmatis*	TET	MFS	De Rossi E. *et al*, 1998a
LfrA	*M. smegmatis*	Fluoroquinolones, EtBr, acriflavine	MFS	Takiff H.E. *et al*, 1996
Rv1258c (Tap homologue)	*M. tuberculosis*	INH, RIF, EMB, OFL	MFS	Siddiqi N. *et al*, 2004
Rv1877	*M. tuberculosis*	TET, KAN, ERY	MFS	Li X.Z. *et al*, 2004
Rv1634	*M. tuberculosis*	Fluoroquinolones	MFS	De Rossi E. *et al*, 2002
EfpA	*M. tuberculosis*	Possibly INH	MFS	Doran J.L. *et al*, 1997
P55	*M. tuberculosis* *M. bovis*	Aminoglycosides, TET, RIF	MFS	Silva P.E. *et al*, 2001
Tap	*M. fortuitum*	Aminoglycosides, TET	MFS	Ainsa J.A. *et al*, 1998
MmpL7	*M. tuberculosis*	INH	RND	Pasca M.R. *et al*, 2005
Mmr	*M. tuberculosis*	TPP, EtBr, ERY, acriflavine	SMR	De Rossi E. *et al*, 1998b
IniA-IniB-IniC	*M. tuberculosis*	INH	Membrane protein	Colangeli, R. *et al*, 2005

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Ethics approval of research

This study has been performed in accordance with the Declaration of Helsinki. Since the study used only isolates that were routinely collected from Tropical Disease Research and Application Center in Adana /Turkey, The Ethical Review Committee at the cukurova university /college of medicine approved the study procedures.

3.1.2. Selection clinical isolates of M. tuberculosis complex

Mycobacterial isolates in MGIT tubes have been examined under microscope in order to distinguish *M. tuberculosis* from non-*tuberculous mycobacteria*. The presence of cording on Ziehl-Neelsen staining and formation of clumps with nonappearance of turbidity in the medium led to identify these isolates as *M. tuberculosis*. Reference strain (H37Rv) collected from National Tuberculosis Reference and Research Laboratory in Ankara, Turkey.

3.1.3. Reagents and chemicals

MGIT tubes and Rifampicin (RIF), and Isoniazid (INH) ,were purchased from Becton Dickonson and All solutions were prepared on the day of the experiment. NucleoZOL will purchased from MACHERY-NAGEL (Düren, Germany)., Turkey.

3.2. Methods

3.2.1. Bacterial strains and drug susceptibility test using BACTEC MGIT 960 system

M. tuberculosis clinical isolates were selected from the storage collection of the Tropical Disease Research and Application Center,university of cukurova The isolates were from sputum specimens obtained from patients and cultured on BACTEC-MGIT 960 (figure 3.1) , A total of 80 clinical isolates *M. tuberculosis* along with *M. tuberculosis* reference strain H37RV were included in the present study. were selected and divided into two groups the first group includes Forty MDR-TB isolates, which are resistant to INH and RIF, have been selected and Forty sensitive-TB isolates which are sensitive to Antibiotics used . The isolate profiles of drug susceptibility were evaluated by the proportional method using BACTEC MGIT 960 system with the following: RIF 1.0 µg/mL and INH 0.1 µg/mL.

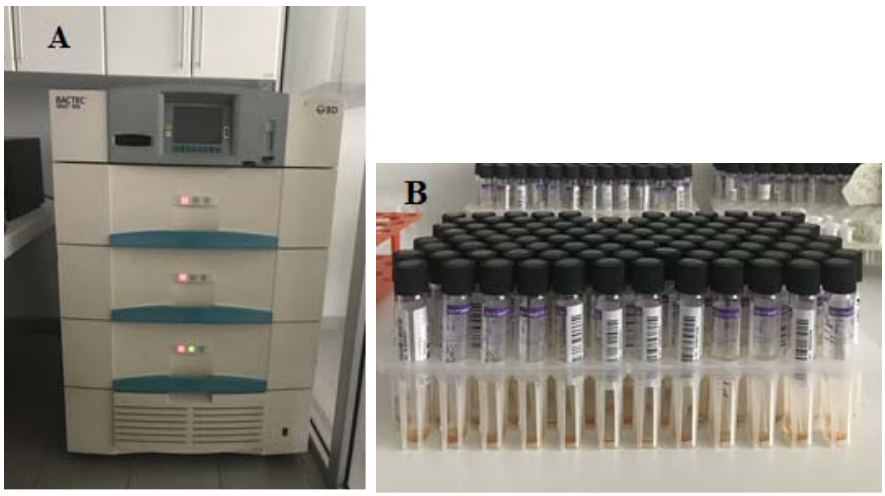


Figure 3.1. (A) Displayed BACTEC MGIT 960 system, (B) MGIT Tubes

3.2.2. Genomic DNA Extraction & Amplification

Genomic DNA was extracted from cultures grown on BACTEC-MGIT 960 were harvested and killed by heating at 80°C for 30 min. using the mickle method (Figure 4.2A), as described previously and the extracted DNA was analyzed immediately or was stored at −20°C , PCR amplification assays were carried out for the *rpoB, katG, inhA and oxyR-ahpC* genes. Primers for each of the genes were selected from published literature or designed. Table 3.1 contains specific annealing temperatures and primer sequences used for amplification.

Each 25-µL PCR mixture contained 12.5 µL of HotStarTaq master mix , primers 0.25 µL of the forward, and 0.25 µL of the reverse ; 6 µL of double-distilled H2O,1 µL of Dimethyl sulfoxide (DMSO) ; and 5 µL of genomic DNA,the reaction was performed in applied biosystem thermal cycler (MJ Research, Inc.)(Figure 3.2B) 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.



Figure 3.2. (A) Mickle device. (B) Applied Biosystems Thermal cycler PCR

Table 3.1. Primers used to amplify and sequence

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	

3.3. DNA Sequencing and mutation detection

The sequences of genes including rpoB for RIF, katG and inhA regulator sequence, and oxyR-ahpC genes for INH (reported to carry major mutations associated with RIF and INH resistance) were analyzed by PCR, positive isolates and DNA sequencing It amplified a product was designed to identify the presence or absence of the gene. The sequences of the specific primers and the sizes of the amplicons are presented in (Table 3.1). Partial PCR products were characterized by DNA sequencing using the forward primers on an Applied Biosystems 3130xl Genetic Analyzer. The resulting DNA sequences were analyzed using the basic local alignment search tool (http://www.ncbi.nih.gov/BLAST), and the specific mutations in protein sequences of the individual isolates were identified.

The PCR products were used as templates for targeted DNA sequencing. Sequencing of both strands of the PCR product was performed on an ABI373 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Mutations were determined by comparing with *M. tuberculosis* H37Rv sequence of katG, rpoB, oxyR-ahpc, and inhA genes where a PCR product was detected, it was purified using the SeqFinder Sequencing System MEFV Kit Protocol (Talstrasse, Altendorf, Switzerland) following manufacturer‘s instructions. A cycle sequencing reaction was set up using the purified PCR product as below:

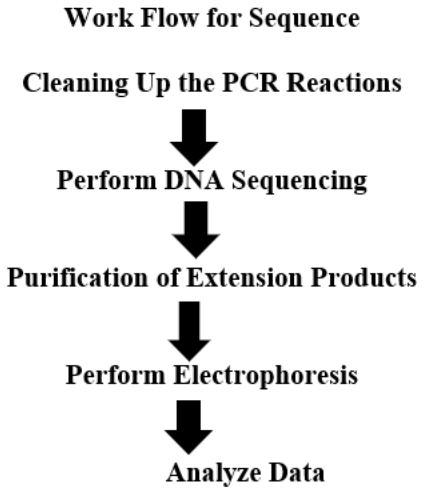


Figure 3.3. An overview of DNA sequencing procedures

To Clean Up the PCR Products :

1. 2 µl of GML ExoSAP was added to each 5 µl amplified reaction and the plate with MicroAmp Clear Adhesive Cover Film was sealed.
2. Vortex the plate briefly to mix the contents and Centrifuge the plate at 1600 g for 30 seconds.
3. Set the reaction volume to 7 µl and program the thermal cycler as shown in table (3.2)

Table 3.2. Program the thermal cycler conditions for Clean Up the PCR Products

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

To Perform DNA Sequencing

Preparing the Sequencing Reactions for Perform DNA Sequencing as follows:

Table 3.3. Reaction mixture for the perform DNA sequnencing reactions

Component	Volume µL per specimen
BigDye Terminator Mix	1.0 µL
Sequencing Buffer	2.0 µL
Sequencing Forward Primer	2.0 µL
Distilled Water	3.0 µL
Total	8.0 µL

Program the thermal cycler conditions for perform DNA sequencing stage as follows:

96°C	1 min	
96°C	10 sec	40 cycles
50°C	5 sec	
60°C	4 min	
4 oC	∞	

To Purification of Extension Products

1. 700 µL Sephadex mix was added (1 g Sephadex + 14 mL distilled water) into a 2 ml tube.
2. Note: Before using Sephadex mix please vortex and shake it gently minimum 30 minutes.
3. The tube was centrifuged for 2 minutes at 4500 rpm and collecting tube with flow-through was discarded.
4. The column was placed into a new 2 ml collecting tube or into the same back and whole PCR product was added to the center of column.
5. The tube was centrifuged for 2 minutes at 4500 rpm and PCR product was at the bottom of the tube and ready to load on instrument.



Figure 3.4. Applied Biosystems 3130xl Genetic Analyzer

3.3.1. Total RNA isolation and cDNA synthesis

All 80 *M. tuberculosis* strains were sub-cultured in MGIT tubes with ADC supplement, and collected for RNA extraction,Total bacterial RNA was isolated using NucleoZOL reagent according to the manufacturer's instructions as follow : 0.2 ml liquid sample was placed in sterile (1.5 ml) microcentrifuge tubes Then added 0.5 ml of nucleoZOL™ Reagent and homogenized by pipeting , 0.2 ml of water was added with 0.5 ml of nucleoZOL™ Reagent again to the lysate were Mixed vigorously for 15 s and then incubated 5 min at room temperature followed by centrifugation at 12,000 × g for 15 min to precipitate contaminates , 0.5 ml of the supernatant was transferred to RNase-free microcentrifuge tubes, 0.5 ml of 100 % isopropanol was added to the transferred supernatant and then samples were incubated for 10 min at room temperature, followed by centrifugation at 12,000 × g for 10 min to precipitate total RNA and discard the supernatant, pellet was washed with

0.5 ml 75% ethanol samples were centrifuged at 8,000 × g for 3 min to wash total RNA and discard supernatant .repeat washing, 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. 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 3.4). RNA was reverse transcribed according to the manufacturer's recommendations (Thermo Fisher Scientific Company, USA). 10 µL of 2X RT master mix (Table 3.2) was pipetted into individual tube. Afterward, the 2X RT master mix was placed on ice and mix gently. Next, 15 µL of RNA sample was added to each tube and mixed gently by pipetting up and down two times



Figure 3.5. NanoDrop ND-1000 device for DNA and RNA measurements.

Table 3.4. Reaction mixture for the reverse transcription reactions (cDNA)

Component	volume
10X RT buffer	3.0 µL
10X RT Random Primers	3.0 µL
25X dNTP Mix (100 mM)	1.2 µL
Nuclease-free H2O	6.3 µL
MultiScribe™ Reverse Transcriptase	1.5 µL
RNA sample	15.0 µL
Total per reaction	30.0 µL

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

25°C 10 min

37°C 120 min

85°C 5 min.

85°C ∞

The cDNA stored at −20°C.

To synthesize single-stranded cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kits:

3.3.2. Quantification of gene expression using real-time quantitative PCR (qPCR)

The primers of the 21 genes are described in Table 3.6. The assay was performed using a Syber green qPCR kit (Promega Company, UK) in Applied Biosystems 7300 Real-Time PCR System (Figure 3.6). Briefly, each 0.2 mL tube contained 12.5 µL 2× Syber green master mix, 0.25 µL each primer, 150 ng cDNA, and 9.5 µL RNase-free water(Table 3.5). The thermal cycling conditions were as

follows: 95°C for 10 min, then 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 1min, and the last step consisted of a melting curve analysis (65–95°C). The fold change in the genes expression in eighty (10) MDR-TB isolates was calculated by 2-ΔΔCT method of Livak and Schmittgen (Livak and Schmittgen, 2001). When the expression of genes compared with nonexposed control genes displaying levels more than 1 were found to be increased; equal to or above four were found to be overexpressed (Livak and Schmittgen, 2001; DeMarco et al., 2007; Rodrigues et al., 2012; Bustin et al., 2010). The expression of genes of (10) MDR-TB isolates without drug inducement was calculated by 2-ΔCT method. The delta Ct values were derived by subtracting the CT value of housekeeping gene (*polA*) from the CT value obtained for each gene. The values of ΔΔCT were derived by subtracting the value of ΔCT obtained for each gene without drug stress from the value of ΔCT of genes induced by INH/RIF. polA which is expressed at a stable level in the isolates and can used as an internal invariant control, is described as a housekeeping gene

Table 3.5. PCR mix required for a single well in Real-Time PCR.

Syber green master mix	12.5 µl
Forward primer.	0.25 µl
Reverse primer.	0.25 µl
Water	9.5 µl
cDNA	2.5 µl
Total	25 µl



Figure 3.6. Applied Biosystems 7300 Real Time PCR System

Table 3.6. Primers used in this study to quantify gene expression of efflux pump

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	*P55(Rv1410c)*	ATCCCGACGGCAAACACGTACTGC	205
		ACATCAACCAGCGTCACCATCAGC	

7	*Rv1634*	TCGATACCTACGTGCCGCTGTTCG	157
		GCTGCCACGACATGCCCGATAACT	
8	*Rv2994*	ATGCGTCCCGTCCGCCTGAT	147
		GGTGGCTTCTAGCCCGTTGTCC	
9	*Rv1877*	TGTGCTGCGTCCTGTCCTTCGT	235
		AGGAACGCAACCGCCATCAG	
10	*Stp*	TCCGATGATGGATCTGACCCTG	217
		GCCAACCAGGTGCCCAACA	
11	*jefA (Rv2459)*	CGTCGCCCTGATCGCATACA	213
		CAGGACATCACCACGAAGTAGACG	
12	*Rv2265*	CGGTTGTCCTCGGTAATCCT	112
		AACCCGAACGTGCCAAAC	
13	*Rv2456c*	CAGCGAACCCCACCAAA	140
		GCACAATCGAGACGAAGGAA	
14	*Rv3239c*	GCCGATTCCTGGCACTTTT	146
		ATGTGGATGGCGGTGTGTT	
15	*mmpL13a*	GACGACCTGCTGGTGATGGAGTTG	242
		CGACTGACGATGAGCAGCGTGTAG	
16	*mmpL13b*	ATGTTCGGCCTCGGCCTGACTTTA	182
		GAACGTCTCCTCGAAACCGGCTCT	
17	*pstB*	CTGGACCCGACTACCACCGAGAA	95
		GCCTGGGCAAGGTTATGGGTC	
18	*drrA*	TAGACATCGCGTGCGGATTGGT	147
		GCGTGGTCAACAACGTGGCAAT	
19	*drrB*	TCGCCAGCAACTTAGGGCAATACA	233
		TCCGATGACGTAGCCGCAAACTAG	
20	*Mmr*	TAGTGGGTTATGGCATCGCTTTCG	167
		GACGCCAACCACCTTCATCACAGA	
21	*polA*	GTCGTGGTTGGACCTTGGAGGG	181
		GCGTCCGTATCGTCGTCATCG	

3.3.3. Data analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) will be used to perform Mann-Whitney test, and the difference will be considered to be statistically significant when $P < 0.05$.

4. RESULT AND DISCUSSION

4.1. Selection strains and Susceptibility testing

For the study, 80 clinical isolates were used (Table 4.1). Of these, 40 isolates were pan-susceptible, 40 isolates were resistant to the first-line drugs, i.e. isoniazid (INH) and rifampin (RIF). The concentrations of INH were 0.1 μg/ml and RIF were 1.0 μg/ml (Adami et al., 2017). *M. tuberculosis* clinical isolates, which showed resistant to both drugs, including INH and RIF, are considered as MDR-TB (Nathanson et al., 2010; Organization, 2010). Whereas, XDR-TB is caused with additional resistance to a fluoroquinolone such as ciprofloxacin and one second-line injectable agent including amikacin or kanamycin (Kolyva and Karakousis, 2012).

Table 4.1. Characteristics of sample

Mycobacterial Strains	Number of TB isolates	Notes
Sensitive TB	40 sample	Sensitive to INH and RIF
MDR-TB	40 sample	Resistance to INH and RIF

4.2. DNA Amplification

A total of 89 bacterial strains for both strains (sensitive and resistant starins) were isolated from the tuberculosis center as that mentioned in material and method section, Moreover all strains were subjected to the molecular identification procedure by means of PCR amplification of inhA ,katG, oxyR-ahpC and rpoB gene and show in table 4.2. all strains , For this purpose genus specific primers were used in PCR ,all isolated were amplified were used previous genes as described to confiremed the mycobacterium tuberculosis were identified using specific primers sets that mentioned earlier in Table 3.1 (Materials and Methods section).

Table 4.2. PCR result were used for genes to detect gene mutation

Sample No	rpoB	KatG	inhA	OxyR-ahpC
S*[1] 1	Positive	Positive	Positive	Positive
S2	Positive	Positive	Positive	Positive
S3	Positive	Positive	Positive	Positive
S4	Positive	Positive	Positive	Positive
S5	Positive	Positive	Positive	Positive
S6	Positive	Positive	Positive	Positive
S7	Positive	Positive	Positive	Positive
S8	Positive	Positive	Positive	Positive
S9	Positive	Positive	Positive	Positive
S10	Positive	Positive	Positive	Positive
S11	Positive		Positive	Positive
S12	Positive	Positive	Positive	Positive
S13	Positive	Positive	Positive	Positive
S14				
S15	Positive	Positive	Positive	Positive
S16	Positive	Positive	Positive	Positive
S17	Positive	Positive	Positive	Positive
S18	Positive	Positive	Positive	Positive
S19	Positive	Positive	Positive	Positive
S20	Positive	Positive	Positive	Positive
S21	Positive	Positive	Positive	Positive
S22	Positive	Positive	Positive	Positive
S23	Positive		Positive	
S24	Positive	Positive	Positive	Positive
S25	Positive		Positive	
S26	Positive	Positive	Positive	Positive
S27	Positive	Positive	Positive	
S28	Positive	Positive	Positive	Positive
S29	Positive	Positive	Positive	Positive
S30	Positive	Positive	Positive	Positive
S31	Positive	Positive	Positive	Positive

S32	Positive	Positive	Positive	
S33	Positive		Positive	
S34	Positive	Positive	Positive	Positive
S35	Positive		Positive	Positive
S36	Positive	Positive	Positive	Positive
S37	Positive	Positive	Positive	
S38	Positive	Positive	Positive	Positive
S39	Positive	Positive	Positive	
S40			Positive	
S41	Positive	Positive	Positive	Positive
S42	Positive		Positive	
S43	Positive	Positive	Positive	Positive
S44	Positive	Positive	Positive	
S45			Positive	
MDR[2*] 46	Positive	Positive	Positive	Positive
MDR 47			Positive	
MDR 48	Positive	Positive	Positive	
MDR 49	Positive	Positive	Positive	Positive
MDR 50	Positive		Positive	
MDR 51	Positive	Positive	Positive	Positive
MDR 52			Positive	Positive
MDR 53			Positive	
MDR 54	Positive		Positive	
MDR 55	Positive		Positive	
MDR 56	Positive	Positive	Positive	Positive
MDR 57	Positive	Positive	Positive	Positive
MDR 58	Positive	Positive	Positive	Positive
MDR 59	Positive	Positive	Positive	Positive
MDR 60	Positive	Positive	Positive	Positive
MDR 61	Positive	Positive	Positive	Positive
MDR 62	Positive	Positive	Positive	Positive
MDR 63	Positive	Positive	Positive	Positive

MDR 64	Positive	Positive	Positive	Positive
MDR 65	Positive	Positive	Positive	Positive
MDR 66	Positive	Positive	Positive	
MDR 67	Positive	Positive	Positive	Positive
MDR 68	Positive	Positive	Positive	Positive
MDR 69	Positive	Positive	Positive	
MDR 70	Positive	Positive	Positive	
MDR 71		Positive	Positive	
MDR 72	Positive	Positive	Positive	Positive
MDR 73			Positive	
MDR 74	Positive	Positive	Positive	
MDR 75	Positive	Positive	Positive	Positive
MDR 76	Positive	Positive	Positive	
MDR 77	Positive	Positive	Positive	Positive
MDR 78	Positive	Positive	Positive	Positive
MDR 79	Positive	Positive	Positive	
MDR 80	Positive	Positive	Positive	
MDR 81	Positive	Positive	Positive	
MDR 82	Positive	Positive	Positive	
MDR 83			Positive	
MDR 84	Positive	Positive	Positive	Positive
MDR 85	Positive	Positive	Positive	
MDR 86	Positive	Positive	Positive	
MDR 87	Positive	Positive	Positive	
MDR 88	Positive		Positive	Positive
MDR 89	Positive	Positive	Positive	

S[1]* Sensitive Strains
MDR[2]* Multidrug resistant Strains

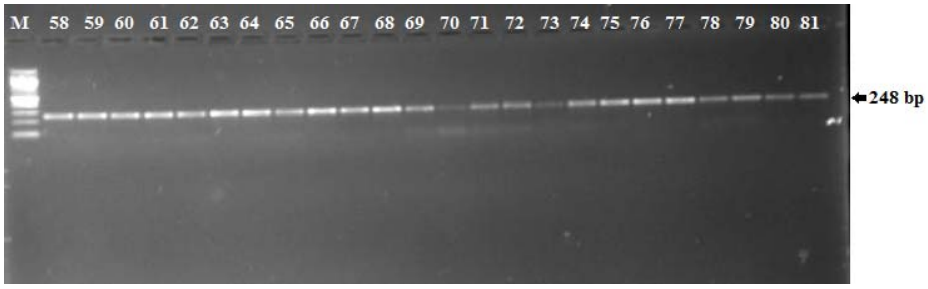


Figure 4.1. PCR products of amplified inhA gene MDR-TB isolated Lanes: M, 100 bp DNA marker

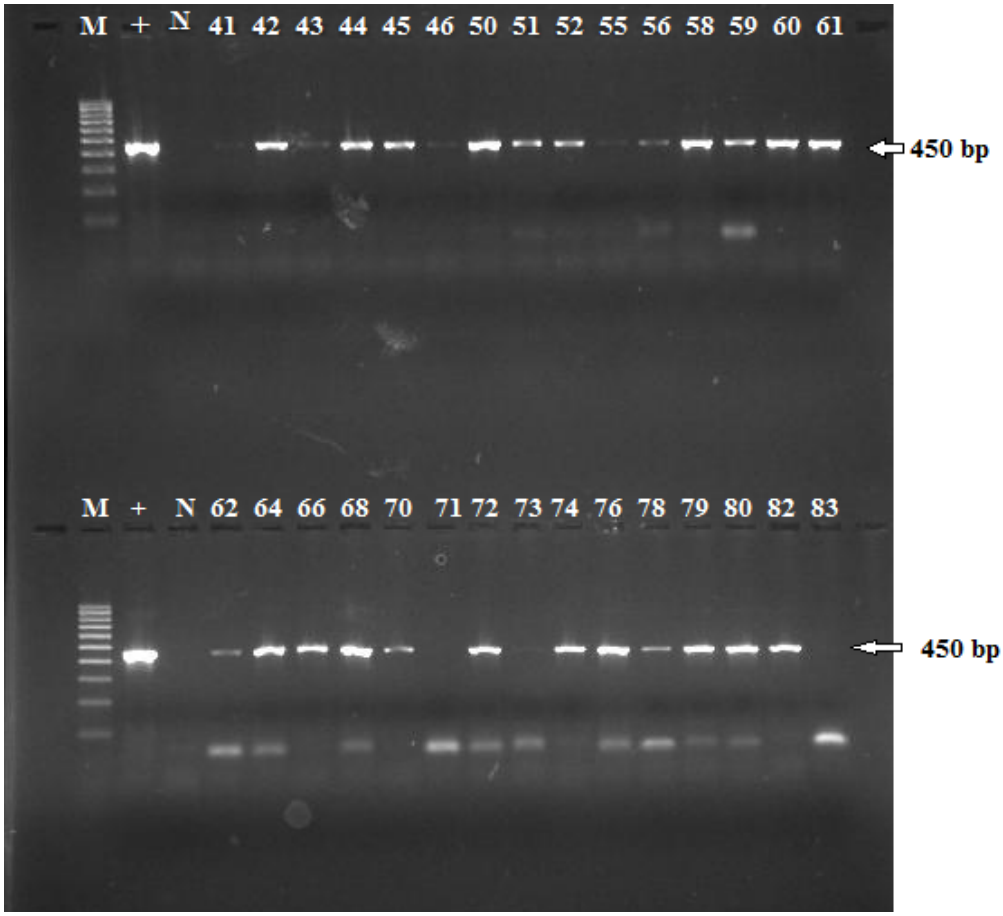


Figure 4.2. PCR products of amplified rpoB gene Pan sensitive and MDR-TB isolated Lanes: M, 100 bp DNA marker

4.2.1. Correlation between the Phenotype and Mutations Associated with Antibiotic Resistance

First, in order to establish a relationship between the level of phenotypic drug resistance and the presence of resistance-associated mutations, we looked for mutations in genes associated with drug resistance to the antibiotics 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 according to published data (Cambau et al., 2015; Domínguez et al., 2016). Among the MDR strains, 28 harbored mutations in katG, of which 27 had the S315T mutation and one has a rare mutation at codon 735. The remaining 12 strains harbored double mutations in inhA. The double mutations C-15T/S94A were detected in strains MtbPT7 and MtbPT9 and the pair C-15T/I194T was detected in MtbPT10 and MtbPT11.

As for rifampicin resistance, all MDR strains and two rifampicin monoresistant strains showed S531L mutation in rpoB. It is the most common mutation in rifampicin resistant clinical isolates.

According to the most widely accepted dogma on the mechanisms of drug resistance in *M. tuberculosis*, the only cause of resistance levels recognized in these strains is expected to be derived from the reduced affinity of the mutated genes against the respective antibiotic only. (Zhang and Yew, 2009; Domínguez et al., 2016).

4.2.2. Effect of Antibiotics on Efflux Pump Gene Expression

To further confirm these findings, we analyzed the transcriptional profile of the possible pumps described in the literature that the *M. tuberculosis* strains were associated with antibiotic resistance phenotypes. (Viveiros et al., 2012; Black et al., 2014; Silva da et al., 2016). RT-qPCR was done for efflux genes previously shown to be overexpressed in response to antibiotic exposure.

The cDNA concentrations of the transcripts were calculated by comparing the 2-ΔΔCT values ofthe tested cDNA samples with those of the external controls.

The results showed that significant changes in expression levels occurred in all strains after exposure to antibiotics. MDR strains were exposed to rifampicin or isoniazid and similar models were observed(Table 4.3). Three to six genes were overexpressed after exposure to isoniazid or rifampicin. P55 showed the highest level of expression in both strains and for both antibiotics. In general, EfpA was overexpressed in all strains, independent of the antibiotic exposure. For the remaining flow pumps analyzed, we were unable to find any correlation antibiotic to overexpression of a particular efflux pumps. We observed a general pattern of gene expression upon exposure to antibiotics.

Drug resistance in *M. tuberculosis* has long been associated with the development of mutations in genes encoding drug targets, but it has recently been recognized that waste flow pump activity plays an important role in the development of drug-resistant phenotypes in *M. tuberculosis*. Several recent studies have demonstrated the importance of overexpression of overflow pump genes in clinical strains of MDR and XDR *M. tuberculosis*. However, most of these studies are based on a simple assessment and evaluation of the expression levels of the *M. tuberculosis* discharge pump genes, and few have identified the effect of discharge inhibitors on the MICs of antituberculosis drugs and their over-expressed flow systems. Therefore, further studies are needed to investigate the contribution of overactivity of overflow pumps to resistance levels in *M. tuberculosis* strains. (Kanji et al., 2016; Machado et al., 2017; Oh et al., 2017).

Next, we analyzed the expression levels of putative efflux pumps upon exposure to the antibiotics in the H37Rv susceptible strain, and in the monoresistant, MDR *M. tuberculosis* clinical isolates. The efflux pump genes mmr, mmpL7, Rv1258c, p55, and efpA were shown to be overexpressed in presence of antibiotics demonstrating the contribution of these efflux pumps to the resistance phenotype of the strains studied, with the exception of the H37Rv strain. We have noticed a general overexpression of almost all efflux genes studied upon exposure to the antibiotics in the drug resistant strains independently on the genotype of the strains.

Table 4.3. The differences in drug efflux pump gene expression between MDR isolates and pan-sensitive isolates

No.of strain	susceptibility	*Rv3239c*	*mmpL13a*	*Rv2265*	*mmpL13b*	*Rv1877*	*mmr*	*Rv2456c*	*stp*	*tap(Rv1258c)*	*emrB*	*Rv1634*	*Rv0849*	*Rv2994*	*jefA(Rv2459)*	*pstB*	*P55(Rv1410c)*	*Rv1250*	*drrB*	*efpA*	*drrA*
1	MDR	0,188	0,301	0,047	0,265	0,509	0,598	0,210	4,955	1,375	0,794	0,537	3,401	1,549	33,063	6,353	2,338	31,194	3,809	6,492	13,501
2	MDR	0,242	0,697	0,294	0,661	0,465	0,365	0,402	0,910	1,568	1,594	1,075	1,585	2,115	2,371	2,305	2,006	4,004	3,627	3,757	6,493
3	MDR	0,097	0,105	0,014	0,165	0,297	0,338	0,145	6,151	0,383	0,720	0,381	1,133	0,931	29,890	5,235	1,683	10,060	2,696	4,902	8,071
4	MDR	0,247	0,698	0,246	0,437	0,871	0,345	0,868	1,434	1,198	1,762	1,812	1,584	3,076	5,291	5,897	8,933	5,322	9,075	12,932	16,932
5	MDR	0,093	0,099	0,041	0,191	0,105	0,102	0,143	1,191	0,459	0,092	0,377	0,751	1,079	5,601	2,211	1,540	9,350	5,171	7,069	9,455
6	MDR	0,061	0,102	0,039	0,122	0,185	0,173	0,165	2,440	0,309	0,373	0,535	0,426	0,853	4,579	2,511	1,761	4,976	2,967	6,329	5,632
7	MDR	0,061	0,070	0,059	0,160	0,169	0,283	0,138	1,220	0,725	0,585	0,413	3,976	0,508	8,744	1,945	1,554	7,736	2,310	5,582	3,753
8	MDR	0,176	0,202	0,164	0,204	0,341	0,375	0,373	1,297	1,147	1,161	1,516	1,743	1,447	5,371	3,287	3,593	5,039	4,491	9,196	11,894
9	MDR	0,174	0,271	0,298	0,384	0,477	0,567	0,657	1,295	1,299	1,309	1,880	2,066	2,324	4,270	6,073	7,424	9,943	10,488	16,155	65,983
10	pan-sensitive	0,146	0,306	0,356	0,377	0,466	0,225	0,666	0,478	1,318	1,071	0,375	1,503	0,348	0,739	1,387	1,155	3,850	1,092	1,989	4,766
11	pan-sensitive	0,167	0,460	0,617	0,147	0,567	0,275	0,723	0,720	2,171	1,162	0,397	1,034	0,470	1,051	1,082	1,616	3,287	1,269	2,505	5,015
12	pan-sensitive	0,134	0,672	0,417	0,133	1,082	0,270	0,717	1,280	0,667	1,172	0,269	0,421	0,547	1,327	0,563	0,717	0,895	1,078	0,522	1,561
13	pan-sensitive	0,178	0,732	0,476	0,234	0,527	0,194	0,629	0,766	0,672	0,799	0,342	0,424	0,365	1,475	0,750	0,243	0,886	1,138	1,145	3,034
14	pan-sensitive	0,111	0,252	0,867	0,205	0,758	0,518	0,325	0,674	1,291	1,007	0,336	0,242	0,611	0,997	1,364	1,496	9,821	2,495	10,801	10,221
15	pan-sensitive	0,141	0,643	0,305	0,176	0,482	0,186	0,519	0,335	0,790	0,809	0,216	0,307	0,468	0,694	0,674	0,687	0,869	0,546	0,570	1,494
16	pan-sensitive	0,156	0,754	0,251	0,113	0,655	0,244	0,451	0,688	0,561	0,925	0,232	0,371	0,444	0,888	0,335	0,801	0,534	0,883	0,495	1,493
17	pan-sensitive	0,207	0,473	0,201	0,159	0,478	0,186	0,423	0,601	0,618	0,881	3,205	0,277	0,625	0,836	0,528	0,501	0,581	0,579	1,633	1,964
18	pan-sensitive	0,090	0,228	0,271	0,120	0,474	0,182	0,301	0,336	0,732	0,671	0,530	0,216	0,491	0,975	0,594	1,226	2,818	1,495	2,637	5,919
19	pan-sensitive	0,060	0,563	0,135	0,224	0,709	0,330	0,207	0,802	1,590	0,988	0,384	0,374	0,373	0,791	0,623	1,234	3,035	1,495	1,075	3,999
20	pan-sensitive	0,063	0,306	0,867	0,113	0,474	0,244	0,325	0,688	0,276	0,667	0,232	0,277	0,491	0,888	0,674	1,226	9,942	3,150	2,214	3,501
H37RV	pan-sensitive	0,591	0,520	0,040	0,426	0,344	0,312	0,579	0,132	0,276	0,559	0,723	0,506	1,043	0,840	1,467	3,293	9,942	3,150	2,214	3,501

These results indicated that *M. tuberculosis* efflux pumps are promiscuous in their activity as we cannot associate the extrusion of drugs to a specific gene. Moreover, the RT-qPCR data combined with the real-time fluorometry results showed that the drug resistant clinical strains are more prompt to respond, via an efflux-mediated response, to the antibiotics, whereas the susceptible H37Rv strain shows a less prompt detoxifying response to the drugs to which it was exposed (Machado et all, 2017). Altogether, the results obtained demonstrate that *M. tuberculosis* clinical strains are primed to efflux noxious(figure 4.3) and (figure 4.4) and (figure 4.5) and (figure 4.6) compounds and showed that efflux pump induction is involved in the antibiotic resistance pattern of the strains in study, reinforcing the validity of the hypotheses that efflux contributes to the resistance level of these strains, contributing also to their MDR phenotype.

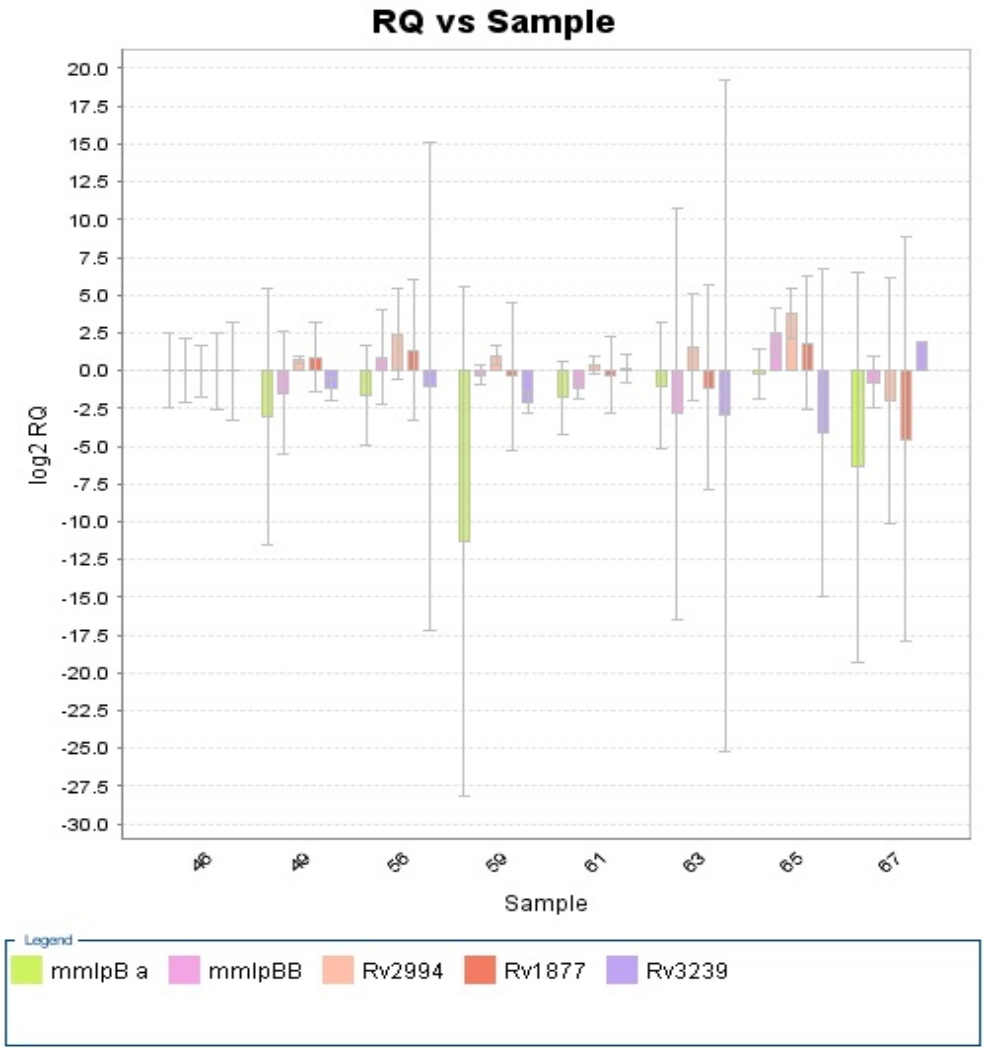


Figure 4.3. result of quantative Real time PCR for MDR strains (46,49,56,59,61,63,65,67) caluculated by log 2RQ

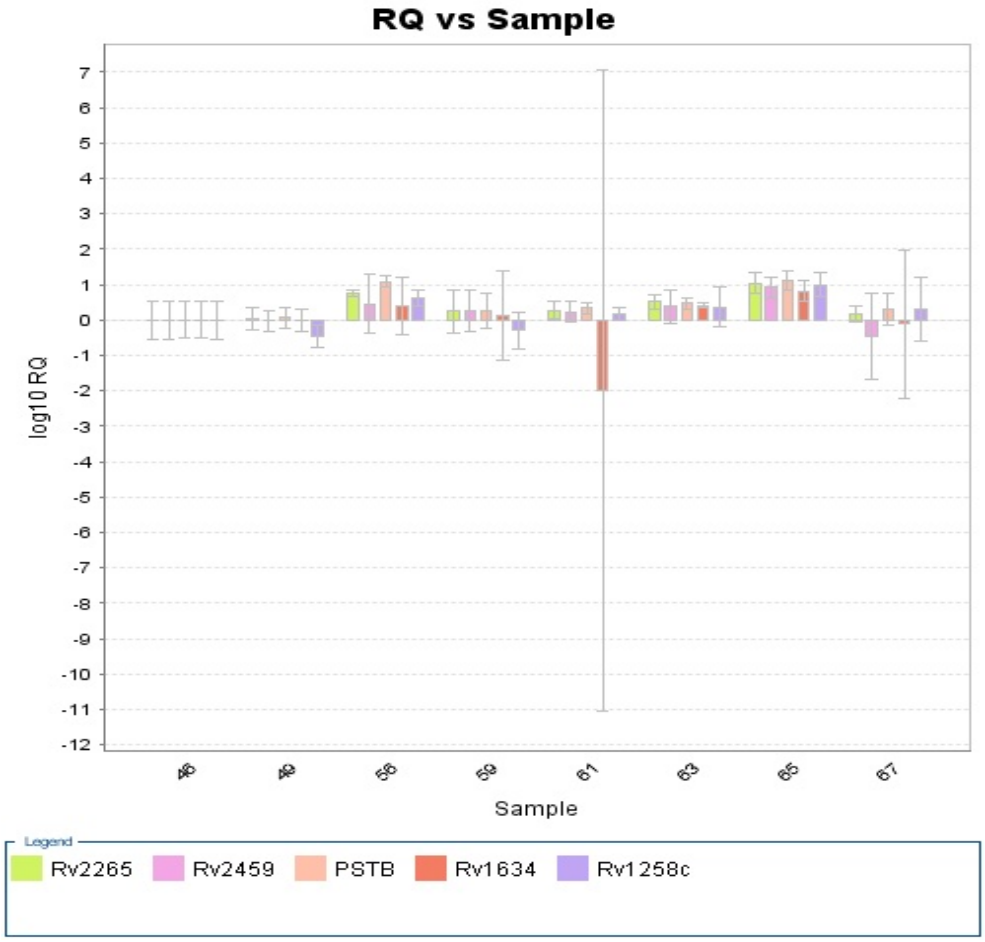


Figure 4.4. result of quantative Real time PCR for MDR strains (46,49,56,59,61,63,65,67) for 5 efflux pump genes caluculated by log 2RQ

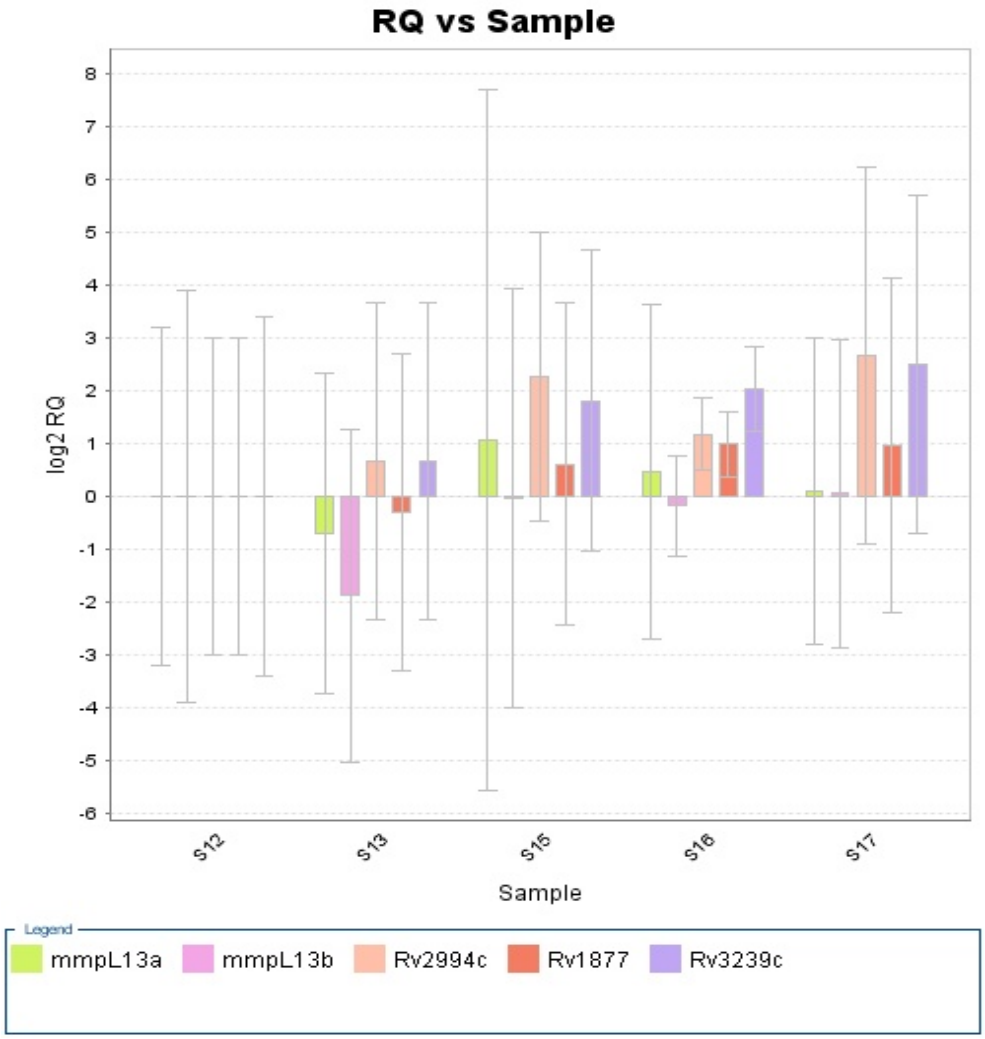


Figure 4.5. result of quantative Real time PCR for Sensitive strains (S12,S13,S15,S16,S17) for diffrent genes and housekeeping caluculated by log 2RQ

Gupta et al. showed that the induced efpA expression was 4-and 4.5-fold greater under INH stress, respectively, which was consistent withthe resultsin ourstudy. jefA (Rv2459) has been reported to respond to INHstress, and Gupta et al. found tha tthe increased transcription ofjefA (Rv2459) leads to increased resistance to EMB and INH in *M.tuberculosis*. Mmr has been reported to mediate

the efflux of different chemical classes and antibiotics. Gupta et al found thatmmr had two-fold higher expression in two isolates under the INH-stressed condition and three-fold higher expression in three isolates under the levofloxacin-stressed condition. Rv1634 is a member ofthe majorfacilitator superfamily in *M. tuberculosis,* similar to many antibiotic resistance (efflux) proteins, including DTDP-glucose dehydratase fromStreptomyces violaceoruber. Rv1634 has been reported to decrease susceptibility to various fluoroquinolone swhen overexpressed in *M. smegmatis* and involve in norfloxacin and ciprofloxacin efflux. Louwet al. reported that Rv1634was up regulated in strains with a Beijing genotype after exposure to RIF for 24h. Balganesh et al. reported that the efflux pump encoded by Rv0849 mediates the efflux of RIF Rv1250 isaprobable drugtransport integral membrane protein having 579 amino acid (aa) and highl ysimilar to the tetracenomycin Cp rotein from *Streptomyces glaucescen* (32.9% identity in517 aaoverlap). Rv1250is alsosimilartoRv3239C from *M. tuberculosis* (31.9% identity in 423aa overlap). No further information on this protein ha sbeen reported. Together, these results indicate thatmore efflux pumps may respond toINH than RIF in *M. tuberculosis*. A possible explanation for this may be that INH exerts its bactericidal activity byattacking the cellwall mycolic acidafter being converted to arangeof oxygenated andorganic toxic radicals b ybifunctional bacterial enzyme catalase-peroxidase (KatG), whereas RIF acts by arresting DNA-directed RNA synthesis inthe cytoplasm of *M. tuberculosis.*

In conclusion, this study allowed us to show that the main mechanisms associated with drug resistance in *M. tuberculosis* correlates mutations in target genes with increased efflux and that compounds that inhibit efflux activity can significantly reduce the phenotypic level of such resistance. The level of drug resistance in *M. tuberculosis* is a combination between the presence of a mutation in the drug target genes and a general stress response to the presence of noxious compounds that regulates the intracellular level of a drug. The data obtained in the presented study corroborated our previous findings (Machado et al., 2017) now

tested on a larger and diverse panel of *M. tuberculosis* clinical strains. The demonstration that the efflux activity modulates the levels of antibiotic resistance by complementing the resistance due to target-gene mutations, is a very relevant finding in the context of the ongoing discussion on the ability and clinical reliability of sole molecular based detection of the target-gene mutations as the future routine DST for *M. tuberculosis* (Böttger, 2011; Domínguez et al., 2016; Pankhurst et al., 2016; Schön et al., 2016). The use of efflux inhibitors as adjuvants of the antituberculosis therapy may be a promise for the development of new and shorter therapeutic strategies as they may potentiate the activity of the current antituberculosis drugs shortening the recommended 6 month treatment for cure, they can increase the activity of drugs that are no longer used due to the emergence of resistance, and they may also be used to protect the activity and usefulness of the new antituberculosis drugs from the development of drug resistance.

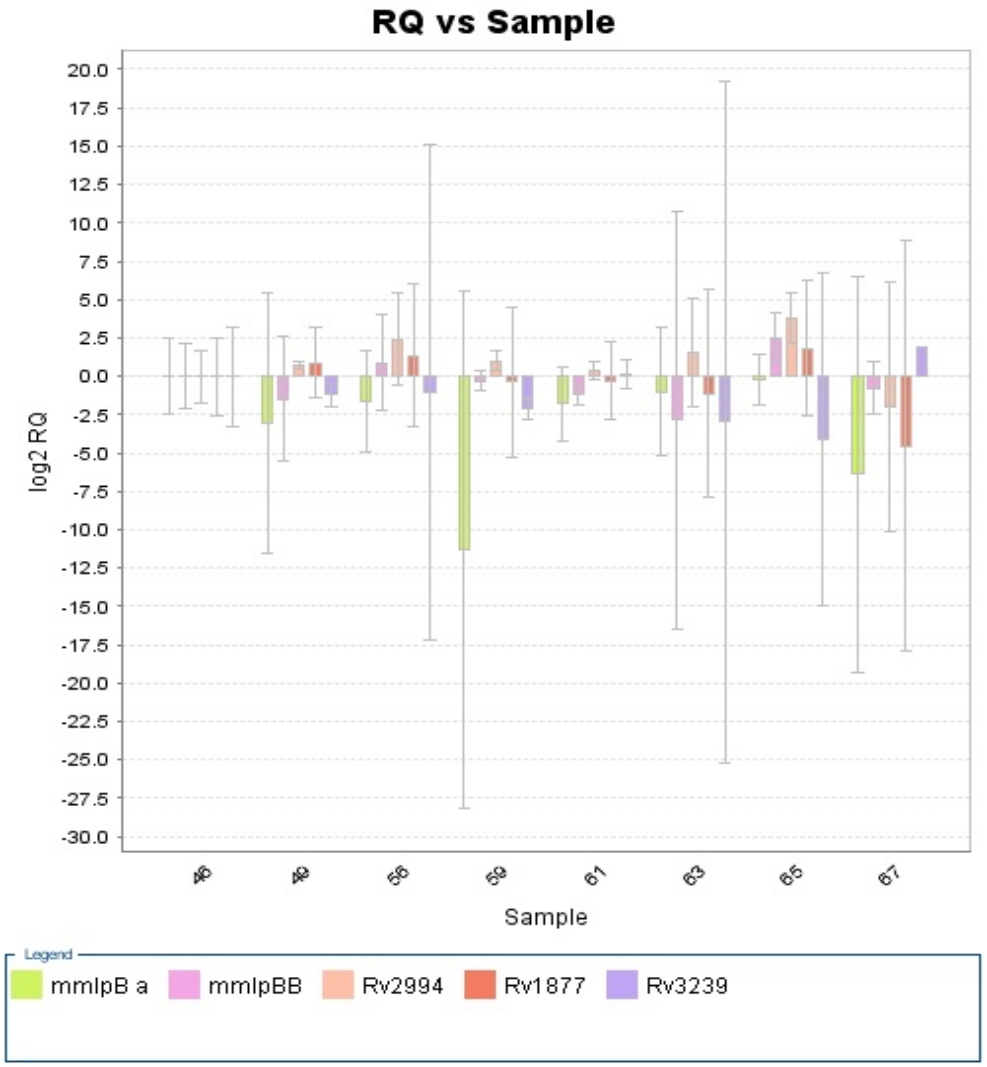


Figure 4.6. result of quantative Real time PCR for MDR strains (46,49,56,59,61,63,65,67) for 5 gene with housekeeping caluculated by log 2RQ

5. CONCLUSION

Efflux pumps may play an important role in INH acquired resistance in MDR M. tuberculosis, especially in strains without mutations in katG, inhA, and oxyR-ahpC, which are associated with INH resistance.

The basal expressional differences of some drug efflux pump genes between MDR and pan-sensitive isolates may be helpful to diagnose and treat resistant tuberculosis. However, we acknowledge that we were unable to demonstrate a direct relationship between the resistance level of INH and/or RIF and the activation of specific genes.

Further efforts are required to elucidate the actual roles of drug efflux pumps in the drug resistance of *M. tuberculosis.*

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