IN SILICO SCREENING FOR THE IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL MUTATIONS THAT CONFER TO ANTIBIOTIC RESISTANCE IN MALAYSIAN MDR-TB ISOLATES

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg/µl	Microgram per microliter
µg/ml	Microgram per milliliter
µg	Microgram
µl	Microliter
µm	Micrometer
ATP	Adenosine triphosphate
BC	Before Christ
BCG	Bacille Calmette-Guérin
bp	Base pair
DR	Drug resistance
DNA	Deoxyribonucleic acid
dNTP	Dideoxynucleotide triphosphate
DST	Drug susceptibility test
EDTA	Ethylenediaminetetraacetic acid
ETB	Ethambutol

Hcl	Hydrochloric acid
HIV	Human immunodeficiency virus
i.e.	id est (that is)
INH	Isoniazid
Kcl	Potassium Chloride
L	Liter
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
MDR	Multidrug resistance
MDR-TB	Multidrug resistance tuberculosis
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
ng	Nanogram

$(NH_4)_2 SO_4$	Ammonium sulfate
NGS	Next generation sequencing
PG	Peptidoglycan
PCR	Polymerase chain reaction
PZA	Pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic acid
RTP	Room temperature
rpm	Revolutions per minute
s	Second
STM	Streptomycin
TAE	Tris-Acetate-EDTA
TB	Tuberculosis
XDR-TB	Extensively drug resistance tuberculosis
U	Unit
µL/mL	Microgram per milliliter
V	Volt
WHO	World Health Organization

WGS	Whole genome sequencing

SARINGAN *IN SILICO* UNTUK MENGENALPASTI DAN PENCIRIAN MUTASI BERPOTENSI YANG TERLIBAT DALAM KERINTANGAN ANTBIOTIK MDR-TB DI MALAYSIA

ABSTRAK

Kemunculan tuberkulosis rintangan multidrug (MDR-TB) disebabkan oleh penyesuaian Mycobacterium tuberculosis untuk bertahan dengan kehadiran antibiotik, yang juga disumbangkan oleh mutasi dalam gen yang berkaitan dengan MDR. Kajian terdahulu menunjukkan bahawa kejatuhan ekspresi gen FhaA membawa kepada pengumpulan prekursor peptidoglycan (PG) pada septum dan kutub bacillary, yang menunjukkan kemungkinan kecacatan dalam biosintesis PG. Akibatnya, dinding sel menjadi tidak telap kepada antibiotik, menyebabkan MDR. Dalam kajian ini, analisis bioinformatik telah dilakukan pada pengasingan MDR-TB daripada 24 sampel klinikal untuk mencari mutasi baru yang menyumbang kepada rintangan antibiotik. Kami mendapati potensi penghapusan nukleotida yang mengodkan 6 asid amino dalam 13 sampel, dalam gen FhaA (RV0020c). Analisis struktur kami yang seterusnya menunjukkan bahawa pemadaman berada pada kedudukan lokus 243-248, terletak berhampiran terminal N protein FhaA. Kami mengandaikan bahawa pemadaman berpotensi menyebabkan kehilangan pertalian mengikatnya kepada MviN, mengakibatkan perencatan dan penyumbatan pempolimeran peptidoglikan, menyebabkan MDR dalam MTB. Selain itu, sejumlah dua belas sampel memperoleh 6 penghapusan bes asid amino dalam gen hbha. Beberapa kajian menunjukkan bahawa glikoprotein ini mengandungi beberapa ulangan kaya lisin pada hujung terminal ekor

karboksil dan mendorong pengagregatan auto bakteria. Protein HBHA mungkin merupakan faktor yang mendorong apoptosis dalam MTB disebabkan oleh perubahan struktur di hujung terminal-C strukturnya. Glikokonjugasi sulfat seperti heparin telah dikurangkan kerana ketiadaan protein HBHA. Pengurangan ini boleh menjejaskan protein HBHA yang boleh menghalang autophagy dalam makrofaj. Oleh itu, perencatan autophagy boleh memudahkan apoptosis dalam makrofaj menyebabkan ia menggalakkan jangkitan MTB dan mungkin membawa kepada kekurangan dalam penyebaran ekstrapulmonari. Pada masa akan datang, pengesahan eksperimen diperlukan untuk membuktikan persatuan mutasi ini dengan MDR.

IN SILICO SCREENING FOR THE IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL MUTATIONS THAT CONFER TO ANTIBIOTIC RESISTANCE IN MALAYSIAN MDR-TB ISOLATES

ABSTRACT

The emergence of multidrug resistance tuberculosis (MDR-TB) is caused by the adaptation of *Mycobacterium tuberculosis* to survive in the presence of antibiotic, which is also contributed by mutations in the MDR-associated genes. Previous studies showed that the knockdown of *fhaA* gene expression led to peptidoglycan (PG) precursors the accumulating at the bacillary septum and poles, this indicates a probable defect in PG biosynthesis. As a result, the cell wall becomes impermeable to antibiotic, causing MDR. In this study, bioinformatics analyses were performed on MDR-TB isolates from 24 clinical samples to search for novel mutations that contribute to antibiotic resistance. We found out a potential deletion of nucleotides encoding 6 amino acids in 13 samples, in *fhaA* gene (RV0020c). Subsequent structural analysis shows that the deletion is located at the locus position 243-248, located near the N-terminal of the FHAA protein. Since FHAA protein has a function by binding to MviN protein, it regulates cell growth and peptidoglycan synthesis, we postulated that the deletion will potentially cause the loss of its binding affinity to MviN, resulting in the inhibition and blockage of the peptidoglycan polymerization, causing MDR in MTB. Similarly, another 6 amino acid deletion was also detected for *hbhA* gene (Rv0475) in 12/14 drug resistant samples. HBHA protein has a function of inducing aggregation in mycobacterial. Several studies shows that this glycoprotein contains several lysine rich repeats at the terminal end of

the carboxyl tail and induces bacterial auto aggregation. HBHA protein may be a possible apoptosis-inducing factor in MTB due to the change of structure in the C-terminal end of its structure. The sulfated glycoconjugates such as heparin was reduced due to the absence of HBHA protein. This reduction may affect HBHA protein which could inhibit autophagy in macrophage. Thus, autophagy inhibition may facilitate apoptosis in macrophage causing it to promote MTB infection and might lead to the deficiency in extrapulmonary dissemination. In conclusion, we detected deletions in both *fhaA* and *hbhA* genes which is associated with antibiotic inhibition and promote MTB infection respectively. Future experimental validation is necessitated to substantiate the association of these mutations with MDR.

CHAPTER 1 INTRODUCTION

1.1 Introduction

An ancient pathogen, *Mycobacterium tuberculosis* (MTB) is one of the most successful pathogen that infects and causes tuberculosis (TB). This infectious disease is one of the top ten death causing disease with an estimation of 10 million people infected globally (WHO, 2021). Malaysia, a middle-class country in Southeast Asia with an approximate population of 32 million, the estimated TB rate are 92 per 100,000 population in 2019 (Tan et al., 2020). TB can be spread via coughing or sneezing through the aerosol droplets produced by the infected individuals (Churchyard et al., 2017). MTB primarily affects the lungs causing pulmonary TB. However, TB infection can spread to other parts of the body and cause TB lymphadenitis, pleural TB, ocular TB, skeletal TB, and gastrointestinal TB which is known to be extrapulmonary TB (Chai et al., 2018). In a contagious stage, the symptoms such as cough, fever, night sweats and weight loss will be observed on the infected individuals (Pai et al., 2016).

MTB infection evolves from the containment of the TB cells in the host, where the bacteria are isolated within the granulomas and this is known as latent TB infection. During the TB latent infection, the host response to the persistent infection by establishing an organized structure of immune cells. Thus, shaping the lifestyle of TB cells to reside in granulomas and utilizing the effector protein in the host immune system (Chai et al., 2018). MTB pathogenicity is mainly based on the reprogramming of

the host macrophages by preventing the elimination of the bacilli from the host after primary infection. This is followed by the formation of granulomas, resulting in the pathogen survival in equilibrium alongside with the host defense. Finally, the formation of the dormant state that is characterized by the slow control of bacterial central metabolism and replication leading to the MTB resistant to the host defense and therapy (Miggiano et al., 2020).

Since the year 1995 cases of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) have been reported among TB patients in Malaysia. MDR-TB is defined as a resistance of MTB to the two of the strongest first line anti-TB drugs which are isoniazid (INH) and rifampicin (RIF) (WHO, 2021). Whereas, XDR-TB is defined as resistant to RIF and INH, in addition to any of the fluoroquinolone, along with at least one of the drugs, bedaquiline and linezolid (WHO, 2021). MDR-TB and XDR-TB has become a huge challenge to the public health and TB control programme. Thus, detecting drug resistance in the early stage is important in the emergence and wide spread of MDR-TB along with XDR-TB strains to ensure the functional management of TB patients (Miggiano et al., 2020).

Three main factors have been shown to be responsible for the establishment of the drug resistance which are poor adherence of TB patients, mistreating of treatments and the expensive costs of treatment. According to Pai et al. (2016), although antibiotic resistance of MTB develops through genetic mutations, the development of resistance

through the acquisition of new DNA is yet to be reported. Genetic mutations in MTB genome plays a major role in drug resistance of MTB. The most common form of genetic variation in MTB is point mutations that involves in a single nucleotide polymorphisms (SNPs) and single nucleotide insertions or deletions (Stucki, 2013).

Early detection and effective treatment are vital in order to curb the emergence and spread of MDR-TB (Iketleng et al., 2018). The advancement of biocomputational tools rapidly and high-throughput system have been applied for the diagnostic purpose of infectious diseases. The identification of SNP mutations in bacterial genome has aided by next-generation sequencing (NGS). For surveillance of bacterial pathogens, this approach has been proposed to be included in the "priority list" by WHO (Cabibbe et al., 2018). Therefore, NGS has great potential for the diagnosis of MDR-TB in diverse clinical reference MTB laboratory settings worldwide (WHO, 2018a). It is a powerful tool to obtain an accurate detection of all clinically relevant mutations in MDR-TB.

1.2 Research objectives

To gain a greater insight into mutations associated with antibiotic resistance in MDR-TB, genome wide analysis by using NGS on MDR-TB strains that have been isolated in Malaysia was conducted. This study was carried out to detect potential novel mutations that are associated with antibiotic resistance. Further analysis was done to validate and to obtain a better understanding in the potential mutations involved. Therefore, the main

objective of this study is to detect a potential mutation in MDR-TB associated with antibiotic resistance by using biocomputational approach.

The specific objectives of this study are

i. To experimental validate the detected mutations by using biocomputational approach and PCR validation
ii. To highlight the potential mechanism between mutations and drug resistance in MDR-TB.

CHAPTER 2 LITERATURE REVIEW

2.1 Tuberculosis

TB is still one of the important causes of mortality. This contagious disease is caused by the genus, *Mycobacterium* that has existed for more than 150 million years ago (Barberis et al., 2017). TB is ranked as the 13th leading cause of death and the 2nd leading infectious killer disease after COVID-19 (Figure 2.1). Until 2020, WHO WHO (2021) has reported that nearly 10 million people worldwide have been infected by TB with a total of 1.3 million of death cases which also includes 214,000 people who are infected with HIV.

In 1882, Dr Robert Koch has discovered MTB as the causative agent of TB. According to the ancient history, TB has been given many names such as “Phtisis” in the Ancient Greece, while in the 12th century, TB was known as “King’s evil” in England. In the mid-19th century, the term ‘tuberculosis’ was suggested by Johann Lukas Schönlein, a German naturalist and professor of medicine at universities of Würzburg (Barberis et al., 2017). Other *Mycobacterium tuberculosis* complex (MTBC) which includes *Mycobacterium bovis* and *Mycobacterium africanum* are more rarely to cause TB infection.

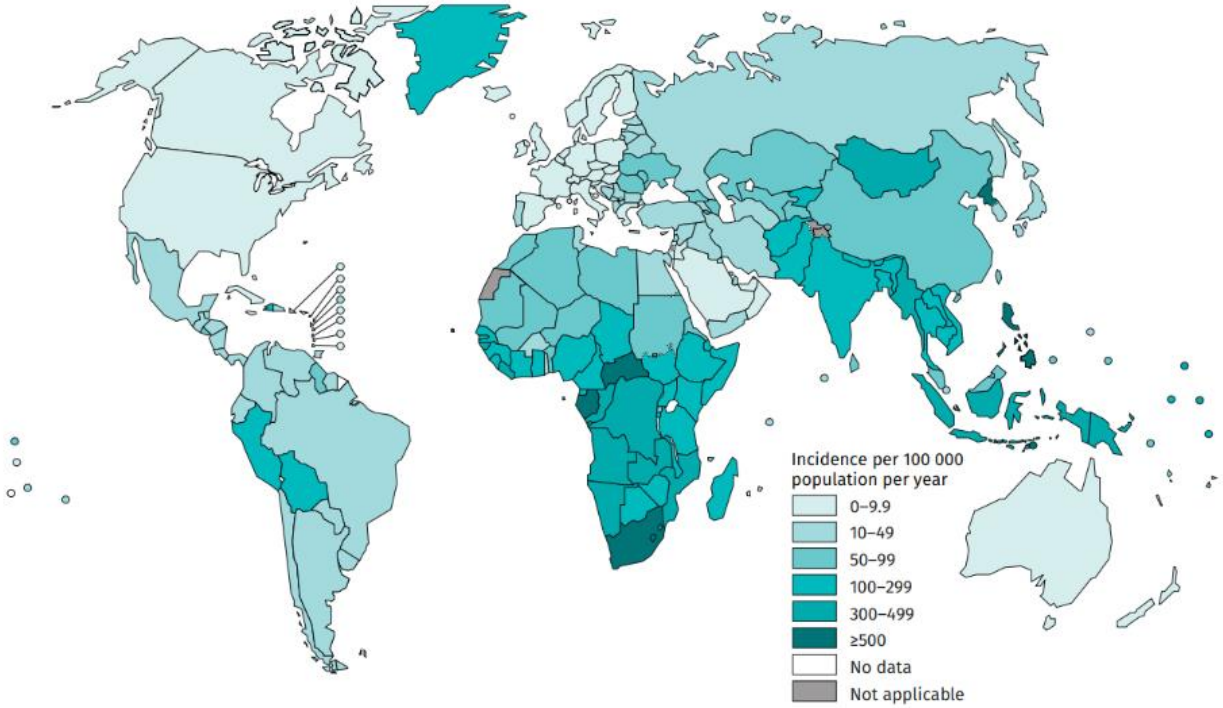

Figure 2.1 Schematic representation of the global TB incidents rate in 2020. Taken from WHO (2021).

2.2 *Mycobacterium tuberculosis* (MTB)

MTB is a non-motile, non-spore forming, rod-shaped microorganism with the size of 0.2-0.6 μm wide and 1.0-10 μm long length. The MTB colonies grows as rough, dry, and unpigmented colonies. MTB are classified as aerobic organism that requires oxygen to grow. MTB is a slow growing bacillus in nature and it has a multiplication time of 16–22 hours (Rustad et al., 2013).

The unusual, waxy coating on the MTB cell surface, that is known as mycolic acid, causes the cell impervious to Gram staining. Thus, Ziehl-Neelsen staining, or acid-fast staining are used to stain MTB. The cell wall plays an important role for the mycobacteria physiology. These includes the protection of the bacterial cells from hostile environments, resistance to drugs, transportation of solutes, proteins, and adhesion of receptors (Chiaradia et al., 2017).

The cell wall of MTB comprises of four very thick layers which includes the inner most layer that is composed of peptidoglycan, followed by lipoarabinomannan as shown in figure 2.2. It also consists of up to 60% of lipids. The exceptionally long fatty acids at the outermost layer which are mycolic acid and arabinogalactan are covalently link to the cell wall. Mycobacteria species are resistant to most of the broad-spectrum antibiotics due to the presence of these lipids (Chiaradia et al., 2017). MTB tend to manipulate its physiology to withstand and adapt to the hostile environment condition in the host (Rustad et al., 2013).

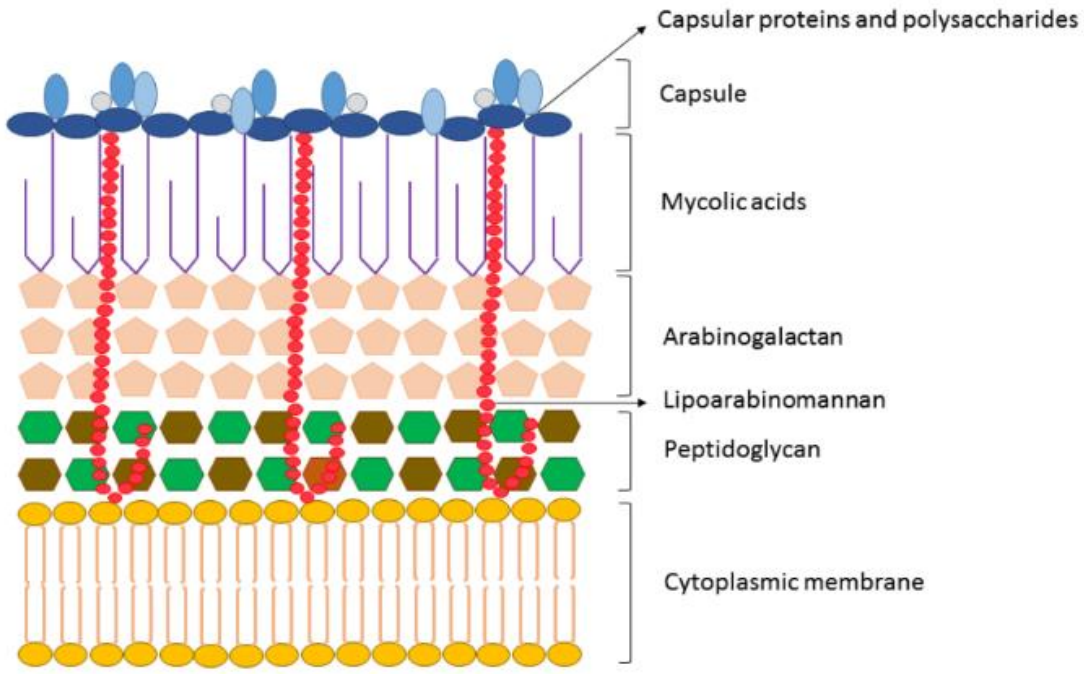

Figure 2.2: Schematic diagram of the structure arrangement of the MTB cell wall. Taken from (Squeglia et al., 2018).

2.3 TB in Malaysia

Malaysia, a middle-class country in Southeast Asia with an estimated population of 32 million people, the estimated TB rate are 92 per 100,000 population in 2019 (Tan et al., 2020). Regarding TB incidence, Malaysia is ranked 76th worldwide and has been classified as a medium-to-low level of endemic country for TB. Sociodemographic factors such as age, gender, race, residency, nationality, and country of origin contributes to the cases in TB (Mohidem et al., 2021). Even though in 2018, TB cases has been reported to decrease 1.26% from 26,168 cases to 25,837 cases due to the Direct Observed Treatment and Short Course Chemotherapy (DOTS) programme. However, this reduction is insignificant as it indicates that the transmission of TB is still active in Malaysia.

2.4 Transmission and Pathogenicity

When individuals with pulmonary TB cough and sneeze, infectious aerosol particles or droplet nuclei containing MTB is generated. Even though, the mucous lining of the human nasal and respiratory tract surface is the first line of defense preventing MTB from entering the alveoli, susceptible individual have a high potential of developing TB when the infectious MTB particles are inhaled (Mohidem et al, 2021). A primary pulmonary TB affects mainly the bronchus and lungs. However, MTB may also cause extrapulmonary TB that affects other parts or the body. The pathogenicity of MTB is mostly based on the capability of the bacteria to infect and reprogram host macrophage after primary infection, thus averting the process of elimination (Miggiano et al., 2020).

During the early stage of TB infection, the host immune system inhibition of the MTB proliferation inside the alveolar macrophage lead to the formation of granuloma in the lungs (Mohidem et al., 2021). The granulomas formation is associated with the slowing down of the MTB central metabolism and replication which characterized the dormant state that causes the MTB to be resistant to host defenses and therapy (Miggiano et al., 2020). For an individual with TB, the infectiousness and duration of infection depend on the host and MTB factors. An individual with smear-positive pulmonary TB is highly infectious. However, individual with smear-negative TB cases have also been shown to transmit TB (Churchyard et al., 2017).

Poor adherence of TB patients, mistreatment, and high treatment cost in some developing countries have been associated to the establishment of DR-TB. MTB has

been shown to gradually becoming resistant to all existing drugs. In Malaysia, Direct Observed Treatment and Short Course Chemotherapy (DOTS) programme by using isoniazid, rifampicin, ethambutol, and pyrazinamide have been applied to rapidly reduce the infectiousness.

2.5 Mechanism of drug resistance

2.5.1 Acquired drug resistance

The failure in the treatment of TB remains a major challenge. Thus, poses an immense threat to the public health. One of the factor that contributes to the failure is the development of antibiotic resistant of TB isolate (Zeng et al., 2018). In general, antibiotic resistance in bacteria is usually acquired either through mutations or horizontal gene transfer through plasmid, transposon elements or phages (Nguyen, 2017). In the MTB population, antibiotic resistance becomes a predominant trait because the survival advantage give rise to the mutant strains under selective pressure. Several factors that caused antibiotic resistance are associated with the poor treatment adherence, prolong period of treatment, economic wastage, psychological problems, and consequently treatment failure (Mulu et al., 2015). The continuous exposure to antibiotics along with the individual's non-adherence to the antibiotic treatment has impacted the evolution of MTB to become resistant (Palomino and Martin, 2014). In addition, sublethal exposure to antibiotics have been suggested to mediate radical-induced mutagenesis (Kohanski et al., 2010).

The resistant in MTB is not a single homogeneous biological unit (Pym and Cole, 2007). The genetic mutation in MTB strains is generally characterized by single-nucleotide polymorphisms (SNPs) followed by insertion and deletion (Indels) (Godfroid et al., 2020). There are three major mechanisms that underlie antibiotic resistance in MTB.

Antibiotic resistances have been shown to cause cell wall impermeability, slow metabolic mechanism, and overactivation of numerous efflux pump (R. Singh et al., 2020).

i) Impermeable cell wall.

The cell wall of the MTB is comprised of three major components which are mycolic acid, Wax-D and cord factor (Figure 2.2). The arabinogalactan is a hydrophilic layer that results in the impermeability of the cell wall towards hydrophobic chemicals. Moreover, the hydrophobic mycolic acid impedes the entrance of hydrophilic molecules. Since antibiotics used in the MTB is a hydrophilic this causes the cell wall to be impermeable to antibiotics. The impermeability of the MTB cell wall results in the accumulation of antibiotics at the surrounding of the cell. The antibiotics are slowly detoxified by different cellular components or enzyme released by MTB such as β-lactamases that effectively degrades β-lactams antibiotics.

ii) Slow metabolic mechanism

Carbapenems could overcome common barriers of penicillin against MTB, making it potentially an attractive anti-tuberculosis agent (Jaganath et al., 2016). The reduction in metabolic processes of MTB is due to acetyl CoA that is an essential component in tricarboxylic acid cycle involved in the triacylglycerol synthesis. MTB extensively produced triacylglycerol under stress conditions such as acidic pH, oxygen deprivation, and iron deficiency. Carbapenems are used to treat MDR-TB and XDR-TB when it is

difficult to identify the recommended four active drugs to be included in the regimen (Sotgiu et al. 2016). However, carbapenem is an unstable antibiotic due to the short 3.8-hour half-life that loses their activity at a faster rate compared to the very slow growth rate of MTB (Lechtig-Wasserman *et al.,* 2021) . The slow growth rate of MTB also plays an important role in drug resistance. MTB is a slow growing bacillus in nature and it has a multiplication time of 16–22 hours (Rustad et al., 2013). This will effects any antibiotics that are used for the treatment of TB such as carbapenem.

iii) The presence of numerous efflux pump

MTB have been shown to posses numerous efflux pumps. Efflux pump are protein channels that play a major role in normal metabolism and physiology of MTB such as cell signaling and transporting of molecules (toxin, waste, and nutrient) across cell well. Multidrug efflux pump serve as a passage for antibiotics to move through the inner and outer membrane of the cell (Pym and Cole, 2007). The drug efflux pump in MTB has been found to consist of a regulatory protein system, which has a function of regulating the efflux protein pump expression. One of the key mechanisms of resistance in MTB is the efflux pump. These efflux pumps function as a pump to pump out solutes out of the cell. By removing toxic substances, including antimicrobial agents, this allows the MTB to regulate their internal environment metabolites and quorum sensing signal molecules (Soto, 2013).

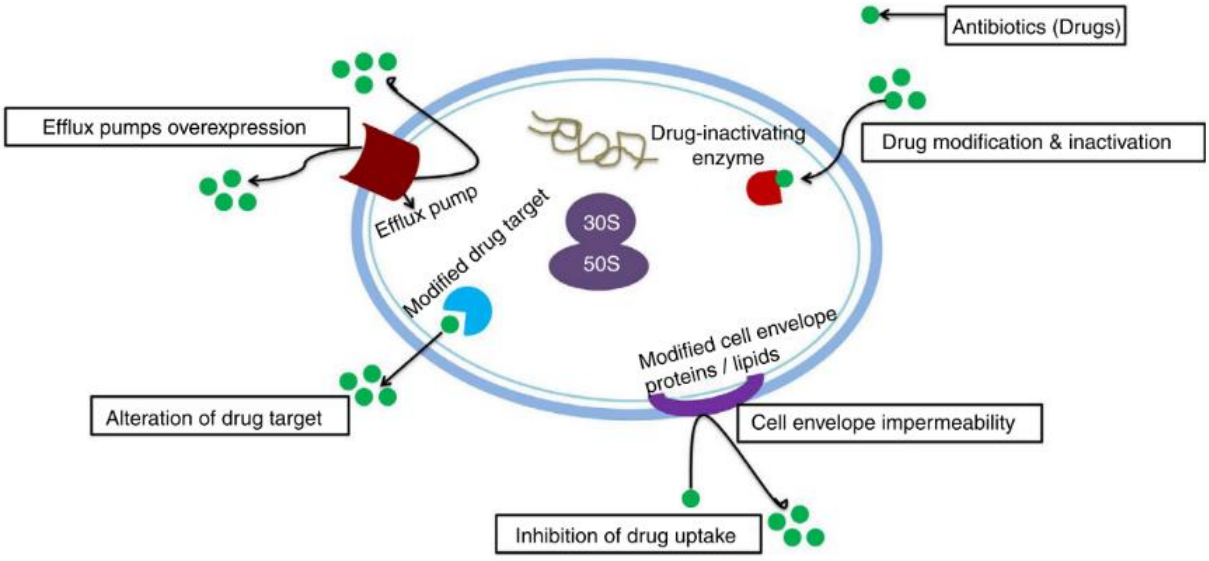

Figure 2.3 Schematic diagram showing the drug resistance mechanism in MTB (R. Singh et al., 2020)

2.5.2 Molecular mechanism of drug resistance

There are four types of MTB drugs used for the early treatment of TB are isoniazid, rifampicin, ethambutol and pyrazinamide (WHO, 2009). MTB has been shown to acquire antibiotic resistance from spontaneous mutations in various chromosomal genes. These mutations were found to alter the interaction between antibiotics and their specified target. In the 1970s, a fundamental lipophilic annamycin chemotherapeutic agent, rifampicin (RIF) is introduced into the multidrug treatment of tuberculosis. RIF plays a role in inhibiting the growth of MTB. Besides that, RIF function by binding to the β-subunit of RNA polymerase and it also inhibits the elongation of messenger RNA (mRNA). The resistance of MTB to RIF is due to the mutations in *rpoB* gene that reduce the affinity of the drug towards RNA polymerase.

Isoniazid (INZ) is also another antibiotic used in the treatment of TB. INZ is usually in the inactive form and converted into a pharmacologically active form in the body of the individual. The conversion into the active form happens when the KatG peroxidase, which is encoded by the *KatG* gene exerts its activity. Once it is activated, it has a strong bacteriostatic effect on metabolically and physiologically active MTB. The list of antibiotic used in treating TB, mechanism of actions, and gens involved in resistance is shown in table 2.1.

Drug (year of discovery)	MIC µg/ml	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency %
Isoniazid (1952)	0.02–0.2	*kat*G *inh*A	Catalase-peroxidase Enoyl ACP reductase	Pro-drug conversion Drug target	Inhibition of mycolic acid biosynthesis and other multiple effects	50–95 8–43
Rifampicin (1966)	0.05–1	*rpo*B	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis	95
Pyrazinamide (1952)	16–50 (pH 5.5)	*pnc*A	Nicotinamidase/pyrazinamidase	Pro-drug conversion	Depletion of membrane energy	72–97
Ethambutol (1961)	1–5	*emb*B	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	47–65
Streptomycin (1944)	2–8	*rps*L *rrs* *gid*B	S12 ribosomal protein 16S rRNA rRNA methyltransferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis	52–59 8–21 ?
Amikacin/kanamycin (1957)	2–4	*rrs*	16S rRNA 16S rRNA	Drug target	Inhibition of protein synthesis	76
Capreomycin (1960)		*tly*A	2′-O-methyltransferase			
Quinolones (1963)	0.5–2.5	*gyr*A *gyr*B	DNA gyrase subunit A DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	75–94
Ethionamide (1956)	2.5–10	*eta*A/*eth*A *inh*A	Flavin monooxygenase	Prodrug conversion Drug target	Inhibition of mycolic acid synthesis	37 56
PAS (1946)	1–8	*thy*A	Thymidylate synthase	Drug activation?	Inhibition of folic acid and iron metabolism?	36

MIC = minimum inhibitory concentration; ACP = acyl carrier protein; PAS = para-aminosalicylic acid.

Table 2.1 List of antibiotics, gene functions, role and mechanism.

Taken from (Wade and Zhang, 2009)

2.6 Next generation sequencing of MTB

Next generation sequencing (NGS) is defined as a high throughput method to sequence large scale of DNA or RNA that aids in the field of genome exploration (Behjati and Tarpey, 2013). NGS technologies are revolutionizing clinical microbiology practice by enabling massive parallel sequencing (Cabibbe et al., 2018). They allow nonprecedential resolution for genotyping, determination of known sequence variants involved in antibiotic resistance, and outbreak investigation. The application of NGS involves in the assembly of genome that is achieved with the aid of a reference genome or *de novo* assembly, which map the small fragment of genome sequence without a reference. This approach has been proposed by WHO for surveillance of bacterial pathogens in the "priority list" (Cabibbe, 2018).

NGS is a very useful method with a great potential for the rapid diagnosis of DR-TB in diverse clinical reference laboratory worldwide (WHO, 2018b). Furthermore, the development of comprehensive analysis and collection of whole genome sequencing (WGS) data of MTB have provide us an opportunity to understand TB infections at a molecular level (Bradley et al., 2015). The first MTB strain to undergo WGS was H37Rv. The genome of H37Rv is 4,411,532 bp in size with 65.6% GC rich content (Cole et al., 1998). MTB has a high GC content compared to other bacteria such as *Escherichia coli* that has only 50% GC content. The rate of polymorphism and recombination of MTB genome is expected to be low due to its high GC content. Comparative genome analysis and identification of the genetic variants in MTB that

confer antibiotic resistance can be conducted in order to explain the impact of genetic difference.

2.7 Comparative genome analysis of MTB

Early identification of drug resistance in TB can be used as the strategy to mitigate the spread of MDR-TB (Palomino and Martin, 2014). To diagnose of MDR-TB, drug susceptibility test (DST) is required. There are two methods for the diagnosis of MDR-TB which are the phenotypic or culture-based method and the genotypic or molecular method (Seung et al., 2015). Collection of sputum from TB patients followed by a test for the growth of MTB in the presence of anti-TB drugs is known as the culture-based method. The culture-based method has a disadvantageous since it takes weeks to months in order to obtain results due to the slow replication rate of MTB. Moreover, the method also requires high cost, high-level biosafety infrastructure face problems such as poor reproducibility and some uncertainties around the proposed critical concentrations for some drugs (Cabibbe et al., 2018). This makes it difficult and mostly unavailable in resource-limited setting.

Molecular methods such as polymerase chain reaction (PCR) technique has revolutionized the diagnosis of MDR-TB in detecting genetic mutation that are known to confer resistance to anti-TB drugs (Seung et al., 2015). The detection of resistance is based on the knowledge of genetic mechanisms that causes drug resistance (Rosales-Klintz et al., 2012). Molecular methods allow faster diagnosis of MTB drug resistance

compared to the culture-based DST method. The transmission events and the rapid identification of drug-resistant TB aides to achieve TB elimination, (Cabibbe et al., 2018).

2.8 Drug resistance in MTB

The emergence of MDR remains a major challenge in the treatment of TB that poses immense threat to the public health. The development of extensively drug-resistant TB (XDR-TB) is due to the mismanagement and failure of MDR-TB treatment. According to WHO, DR-TB can be classified into five types which are

i. Monoresistance which defines as resistance to only one of the first-line anti TB drug.
ii. Rifampicin resistance (RR) which is only resistance to RIF antibiotic
iii. Poly-DR which is resistance to more than one first-line anti-TB drug besides isoniazid and rifampicin.
iv. Multidrug resistance (MDR) is defined as resistance to at least both isoniazid and rifampicin, which is two of the strongest first line anti-TB drug.
v. Extensive drug resistance (XDR) refers to resistance to any fluroquinolone and at least one of the three second -line injectable drugs

The effective drug treatment was first developed in 1940s. The recommended drug used for treating TB are INF, RIF, PZA and ETB for 6 months as shown in table 2.2 (Miggiano et al., 2020). The regimes to treat MDR-TB are PZA, EMB, RFB and one of the second line injectable drugs (kanamycin, capreomycin and amikacin)

The biological phenomenon of drug resistant that has been observed in MTB ever since the very first discovery streptomycin (SM), the first anti-TB drug, in 1944. With the increase emergence of MDR-TB and XDR-TB in the era of HIV, it is a major threat to effective control of TB. MTB has been shown to possess a very high frequency of spontaneous chromosomal mutations at the 10^{-6} to 10^{-8} of mycobacterial replications, thus contribute to the rapid development of genetic resistance to anti-TB drugs (Wade and Zhang, 2009). It also contributed to the sequential accumulation of mutations in difference genes that involved in anti-TB drugs leading to MDR-TB phenotype.

The use of drug combination for the chemotherapy of TB was the solution to prevent the development of MTB resistance with the discovery of new drugs such as thioacetazone and para-aminosalicylic acid in 1948 and isoniazid in 1952 (Seung et al., 2015). Initially, the treatment using the combination of drug treatment regime required 18 months. Since after 1957, the discovery of the most powerfully sterilizing anti-TB drug, rifampicin paves a shorter and more effective rifampicin-containing treatment regime for TB known as short-course chemotherapy. According to WHO, the recommended treatment is a six month regimen of four of the first line drugs which are

isoniazid, rifampicin, ethambutol, and pyrazinamide for patients with drug susceptible TB (Figure 2.11) (WHO, 2021)

Table 2.2 Drug Regimen for Culture-Positive Pulmonary TB

caused by Drug-Susceptible Organisms Intensive. Taken from (Johnson et al., 2006).

Intensive Phase			Continuation Phase		
Regimen	Drugs	Doses	Regimen	Drugs	Doses
1	INH, RIF PZA, EMB	7 d/wk for 56 doses (8wk) or 5 d/wk for 40 doses (8wk)	1	INH/RIF	7 d/wk for 126 doses (18 wk) or 5d/wk for 90 doses (18 wk)
			1	INH/RIF	2d/wk for 36 doses (18wk)
			1	INH/ RPT	1 wk for 18 doses (18wk)
2	INH, RIF PZA, EMB	7 d/wk for 14 doses (2wks), then 2 d/wk for 12 doses (6wks) or 5 d/wk for 10 doses (2wk), then 2 d/wk for 12 doses (6 wk)	2	INH/RIF	2d/wk for 36 doses
			2	INH/ RPT	1 wk for 18 doses (18wk)
3	INH, RIF PZA, EMB	3d/wk for 24 doses (8wk)	3	INH/RIF	3 wk for 54 doses (18wk)
4	INH, RIF, EMB	7 d/wk for 56 doses (8wk) or 5 d/wk for 40 doses (8 wk)	4	INH/RIF	7 d/wk for 217 doses (31 wk) or 2d/wk for 62 doses (31 wk)

INH- Isoniazid, RPT- Rifapentine, RIF- Rifampicin, PZA- Pyrazinamide, EMB- Ethambutol

Table 2.3 Shows the recommended doses of first line anti-tuberculosis drugs for adult

. *I*t is recommended to reduce the dose to 10 mg/kg per day *for p*atients aged over 60 years *as they* may not be able to tolerate more than 500–750 mg daily.

Drug	Recommended dose			
	Daily		3 times per week	
	Dose and range (mg/kg body weight)	Maximum (mg)	Dose and range (mg/kg body weight)	Daily maximum (mg)
Isoniazid	5 (4–6)	300	10 (8–12)	900
Rifampicin	10 (8–12)	600	10 (8–12)	600
Pyrazinamide	25 (20–30)	–	35 (30–40)	–
Ethambutol	15 (15–20)	–	30 (25–35)	–
Streptomycin[a]	15 (12–18)		15 (12–18)	1000