DEVELOPMENT OF RECOMBINASE POLYMERASE AMPLIFICATION ASSAY FOR THE DETECTION OF *Mycobacterium tuberculosis*

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by

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LIST OF SYMBOLS

°C	Degree Celcius
µM	Micromolar
mM	Millimolar
µL	Microlitre
mL	Millilitre
rpm	Revolutions per minute
bp	Basepair
OD	Optical density
mV	Millivolt
ng	Nanogram
mg	Milligram
ng/µL	Nanogram per microlitre
pg	Picogram
nm	Nanometer
nt	Nucleotide
CFU/ml	Colony forming units per millilitre

LIST OF ABBREVIATIONS

3SR	Self-sustained sequence replication
AEC	Airway epithelial cells
AFB	Acid fast bacilli
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guerin
BSC	Biological safety cabinet
BTB	Bovine tuberculosis
CD	Communicable diseases
CD	Cluster of differentiation
CDS	Coding sequences
CFU	Colony forming units
CI	Confidence interval
CPA	Cross-priming amplification
DCs	Dendritic cells
DC-SIGN	DC specific intercellular adhesion molecule 3-grabbing non-integrin receptor
DDBJ	Data Bank of Japan
dsDNA	Double-stranded DNA
EMBL-EBI	European Bioinformatics Institute
E-MTD	Enhanced Mtb Direct Test
EtBr	Ethidium bromide
FISH	Fluorescence *in situ* hybridization
GEO	Gene Expression Omnibus
HDA	Helicase-dependent amplification
HGP	Human genome project
HIV	Human immunodeficiency virus
IGRA	Interferon Gamma
IL	Interleukin
IFN	Interferon
In	Integrons
INFORMM	Institute for Research in Molecular Medicine

IS	Insertion sequences
LAMP	Loop-mediated isothermal amplification
LPA	Line probe assay
LR-	Likelihood ratio negative
LR+	Likelihood ratio positive
LTBI	Latent TB infection
MDR	Multi-drug resistant
MGEs	Mobile genetic elements
MIRU	Mycobacterial interspersed repetitive units
MOH	Ministry of Health Malaysia
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NAA	Nucleic acid amplification
NAAT	Nucleic acid amplification test
NALC-NaOH	N-acetyl-L-cysteine sodium hydroxide method
NASBA	Nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
NCD	Non-communicable diseases
NK	Natural killer cells
NO	Nitric Oxide
NPV	Negative predictive values
NTM	Non tuberculous mycobacterium
OD	Optical density
ORFs	Open reading frames
PAS	Paraaminosalicylic acid
PCR	Polymerase chain reaction
PHLJB	Public Health Laboratory Johor Bahru
POCT	Point of care testing
POC	Point of care
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
PPV	Positive predictive values
PTB	Pulmonary tuberculosis
R&D	Research and development

RCA	Rolling circle amplification
RFLP	Restriction fragment length polymorphism
RD1	Region of difference 1
RFU	Relative fluorescence unit
RIF	Rifampin
RPA	Recombinase polymerase amplification
SDA	Strand displacement amplification
Se	Sensitivity
SNP	Single nucleotide polymorphisms
Sp	Specificity
SSBs	Single-strand binding proteins
STB	Smooth tubercle bacillus
TB	Tuberculosis
TE	Transposable elements
THF	Tetrahydrofuran
TNF	Tumour necrosis factor
Tn	Transposons
Tpases	Transposases
TST	Tuberculin skin test
WGS	Whole genome sequencing
WHA	World Health Assembly
WHO	World Health Organization
XDR	Extensively drug-resistant
ZN	Ziehl–neelsen

PEMBANGUNAN UJIAN AMPLIFIKASI RECOMBINASE POLIMERASE BAGI PENGESANAN *Mycobacterium tuberculosis*

ABSTRAK

Pertubuhan Kesihatan Sedunia (WHO) telah menggabungkan pelbagai strategi untuk mengukuhkan program kawalan tuberkulosis (TB). Kaedah diagnostik terkini dalam pengesanan TB yang menggunakan kaedah ujian asid nukleik (NA) memerlukan tahap kecanggihan makmal yang berbeza disebabkan oleh kemahiran teknikal yang rumit, memerlukan kepakaran serta mematuhi aspek biokeselamatan. Oleh itu, adalah agak mencabar untuk menjalankan ujian yang berasaskan NA di kawasan yang mempunyai sumber yang terhad. Dengan batasan sedemikian, terdapat keperluan untuk membangunkan kaedah baharu yang boleh digunakan di pusat kesihatan yang terletak di kawasan terpencil. Amplifikasi Recombinase Polymerase (RPA) merupakan kaedah amplifikasi NA yang memerlukan sumber haba yang optimal untuk diagnosis yang tepat terhadap penyakit tertentu dalam tempoh yang singkat. Kaedah ini berupaya untuk menjalankan amplifikasi NA spesifik pada suatu suhu yang rendah dan malar dengan menggunakan sampel yang minimum. Di dalam kajian ini, ujian pengesanan *Mycobacterium tuberculosis* (Mtb) yang pantas berasaskan ujian RPA yang menyasarkan gene B9 telah dibangunkan. Pengesanan Mtb DNA berasaskan RPA boleh dicapai dalam masa 20 minit pada suhu 39°C. Senstiviti analitikal bagi ujian ini adalah satu pg apabila diuji menggunakan DNA genomic MTB yang tulin. Prestasi klinikal RPA dievaluasi menggunakan 387 sampel kahak dengan kaedah kultur sebagai kaedah piawai. RPA dan mikroskopi dibandingkan dengan piawai daripada segi sensitiviti, spesifisiti, *positive predictive value*, *negative predictive value*, *false positive value* dan *false negative value*. Hasil kajian

menunjukkan sensitiviti, spesifisiti, *positive predictive value*, *negative predictive value*, *false positive value* dan *false negative value* RPA masing-masing adalah 97.2% (95% CI: 93.5, 99.0), 92.2% (95% CI: 87.6, 95.2), 91.7% (95% CI: 87.8, 95.6), 97.4% (95% CI: 95.2, 99.7), 7.8% (4.2, 11.4) dan 2.8% (95% CI:0.4, 5.1), manakala mikroskopi mempunyai sensitiviti, spesifisiti, *positive predictive value*, *negative predictive value*, *false positive value* dan *false negative value* 90.6% (95% CI: 85.4, 94.1), 88.8% (95% CI: 83.7, 92.5), 87.7% (95% CI: 83.0, 92.4), 91.5% (95% CI:87.6, 95.4), 11.2% (95% CI:6.9, 15.4) and 9.4% (95% CI:5.2, 13.6). Dapatan ini menunjukkan kaedah RPA lebih sensitif dan spesifik berbanding dengan kaedah mikroskopi. Ini mencadangkan bahawa kaedah ini mempunyai potensi untuk digunakan sebagai ujian *POC* bagi pengesanan TB.

DEVELOPMENT OF RECOMBINASE POLYMERASE AMPLIFICATION ASSAY FOR THE DETECTION OF *Mycobacterium tuberculosis*

ABSTRACT

The World Health Organization (WHO) has incorporated various strategies to strengthen tuberculosis (TB) control programmes. The current diagnostic tools for TB detection require different levels of laboratory sophistication due to technical complexities, expertise and biosafety requirements in nucleic acid (NA) based TB testing. Therefore, it is challenging to use NA-based assays in resource-constrained settings. With such limitations, there is a need to develop new approaches that can facilitate point-of-care (POC) diagnostics. Recombinase amplification assay (RPA) is an NA-based amplification platform that requires an optimal heat source for accurate diagnosis of a particular disease in a short time. The method is capable of amplifying specific NA at a single, low and constant temperature with minimal amount of sample preparation. In this study, a rapid assay for the detection of *Mycobacterium tuberculosis* (Mtb) based on the RPA targeting the B9 sequence was developed. The RPA-based detection of Mtb DNA was achieved within 20 minutes at 39°C. The analytical sensitivity of the test was one pg when tested using purified Mtb genomic DNA. The clinical performance of the RPA was evaluated using 387 sputum samples with the culture method as the gold standard. RPA and microscopy were compared to the gold standard in terms of sensitivity, specificity, positive predictive value, negative predictive value, false positive value and false negative value. The results showed that the sensitivity, specificity, positive predictive value, negative predictive value, false positive value and false negative value of RPA were 97.2% (95% CI: 93.5, 99.0),

92.2% (95% CI: 87.6, 95.2), 91.7% (95% CI: 87.8, 95.6), 97.4% (95% CI: 95.2, 99.7), 7.8% (4.2, 11.4), 2.8% (95% CI:0.4, 5.1), respectively, while microscopy had a sensitivity, specificity, positive predictive value, negative predictive value, false positive value and false negative value of 90.6% (95% CI: 85.4, 94.1), 88.8% (95% CI: 83.7, 92.5), 87.7% (95% CI: 83.0, 92.4), 91.5% (95% CI:87.6, 95.4), 11.2% (95% CI:6.9, 15.4) and 9.4% (95% CI:5.2, 13.6), respectively. RPA was found to be more sensitive and specific compared to microscopy, suggesting that the method has the potential to be used as a point-of-care (POC) TB diagnostic tool.

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease and is a major global public health problem, which predominantly affects low-and middle-income countries (Yates *et al*., 2016). *Mycobacterium tuberculosis* (Mtb) caused about 10.0 million new TB cases globally with about 1.5 million deaths, including 0.214 million deaths annually among HIV positive patients (World Health Organization, 2021).

TB is caused by Mtb and is transmitted when the person inhales droplets containing Mtb from an active pulmonary TB (PTB) patient. The bacterium crosses the upper respiratory tract and bronchioles to reach the alveolae. TB can also occur in other organs, such as the larynx, lymph nodes, pleura, brain, kidneys, bones and joints, which are known as extrapulmonary TB (Hopewell, 1994, Hopewell and Jasmer, 2005). The classical symptoms of active TB are chronic cough with mixed blood sputum, fever, nights sweats, and weight loss. Latent TB infection (LTBI) refers to a condition in which a person is infected with Mtb but the bacteria remain dormant and cause no symptoms). The elimination plan for TB nowadays becomes more challenging with the increasing level of multidrug-resistant (MDR) Mtb strains and co-infection of TB with human immunodeficiency (HIV). Although TB was considered to be under control in industrialized countries, its return in epidemic proportions shows that much is still needed to protect both patients and healthcare workers from its deadly consequences (Griffith and Kerr, 1996).

1.2 History of Tuberculosis

TB is an ancient disease, which is caused by a tubercle bacillus that has existed since 150,000 years ago (Hayman, 1984, Kapur *et al*., 1994). The earliest evidence of human TB was derived from a fossil bone and in abscess specimen from the lumbar spine from a mummy (Dubos, 1982). A study by (Gutierrez *et al*., 2005) concluded that Mtb has existed in East Africa around 3 million years ago and was believed to have caused infection among humans.

During epidemics in Europe and North America in the 18th and 19th centuries, about 800–1000 people died of TB per 100,000 population every year (Daniel, 2006). TB has been an endemic disease in high population areas, including villages and agriculture areas. For instance, the congestion in European cities and industrial revolution in Europe has contributed to the increase in TB cases, which was linked to the appalling socioeconomic conditions (overcrowding, poor nutrition, lack of hygiene and sanitation, a lack of medical care) during the earlier industrial revolution (Murray, 1989, Bates and Stead, 1993).

The chest physician, The'ophile Laennec, began work in the 19th century to understand the pathogenesis of TB. In 1865 Jean-Antoine Villemin demonstrated that TB is infectious and transmissible. Towards the end of the 19th century, two historic events occurred that had an incredible successive impact on the diagnosis and management of the disease. In 1882, the founder of tubercle the bacilli, Robert Koch, named the causative agent of TB as Mtb, and in 1895, Wilhelm Konrad Rontgen discovered X-ray (Murray, 2004, Cambau and Drancourt, 2014). Subsequently, paediatrician named Clemens Freiherrvon Pirquet developed the Tuberculin Skin Test (TST) in 1907. In 1908, Charles Mantoux introduced the use of a cannulated needle

and syringe to administer tuberculin intracutaneously, while Florence Seibert developed purified protein derivative (PPD) and used it to diagnose latent TB in asymptomatic children (Daniel, 2006).

In the late 19th century, the first sanatorium for TB patients was opened by Herman Brehmer (Warren, 2006). In these sanatoria, patients lived in an open area with fresh air, where they were provided with rich diet food and exercise regimen. Sanatoria treatment was supplemented with surgical treatment and pneumothorax therapy to reduce thoracic cavity volume and collapse tuberculous cavities, as well as to allow the patients to heal and prevent the spread of infection. However, this approach did not effectively cure the disease (Hurt, 2004).

In 1921, Albert Calmette and Camille Guerin developed the BCG vaccine, and the prevention program with BCG vaccination was widely employed following World War I (Grange *et al*., 1983). In the 1930s, William Well showed that TB is an air borne disease (Sepkowitz, 1996). The discovery and the used of streptomycin as anti-TB drug in 1944 indicated the beginning of the modern era of TB treatment and control, while isoniazid and para-aminosalicylic acid (PAS) was introduced in 1952 (Daniel, 2006). The emergence of MDR-TB was detected in the 1970s (Frieden *et al*., 1993) and TB cases continuously increased globally in 1980s. TB control program by WHO in 1993 has successfully decreased the number of TB cases. However, the first case of XDR-TB was reported in 2006 at the South Africa, (Singh *et al*., 2007) (Table1.1)

Table1.1 Timeline: The history of TB.

Year	Progress
3000BC	TB reported in ancient Egyptian mummies
19th century	The'ophile Laennec work on TB pathogenesis
1865	Jean Antoine Villlemin suggested that TB is contagious
1882	Robert Koch discovered the tubercle bacilli
1883	Tubercle bacilli named as *Mycobacterium tuberculosis*
1884	First Sanatorium in the US
1895	Wilhelm Konrad Rontgen discovered X-ray
1921	Albert Calmette & Camille Guerin-developed BCG Vaccine
1930's	William Wells showed that TB is air borne
1943	Streptomycin drug discovered to treat TB
1943–1952	Isoniazid & PAS drug introduced to treat TB
1970's	Emergence of MDR-TB
1980's	Unexpected rises in global TB cases
1993	The decline in TB cases-enhanced TB control efforts
2006	The emergence of XDR-TB (1st case was reported)

1.3 Global TB Burden

TB is contagious and air borne and has become a major global public health problem, which pre-dominantly affects low- and middle-income countries. TB kills five thousand people every day. Poverty, stigmatization, and discrimination are only a few of the social and economic impact. In 2019, TB was one of the top ten causes of mortality worldwide, and for the past five years, it has been the leading cause of death from a single infectious agent (World Health Organization, 2020). WHO reported that

last year, about 10 million people were infected with TB worldwide with 5.6 million men, 3.3 million women and 1.1 million children (World Health Organization, 2021)

Globally, TB incidence rate nearly reach the End TB strategy milestone: reduction by 20% between 2015 and 2020 (Figure 1.1). The disease's burden varies across countries, ranging from fewer to more than 500 new cases per 100,000 population per year, with a 9 percent reduction from 2015 to 2019 (World Health Organization, 2021).

TB occurs in every part of the world and can be found anywhere on the planet. The WHO South-East Asian Region had the highest number of new TB cases in 2020, accounting for 43% of all new cases, followed by the WHO African Region (25%), and the WHO Western Pacific (18%). According to WHO, the top 30 TB burden countries accounted for approximately 86% of new TB cases. Two-thirds of new TB cases were reported in eight Asian and African countries: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa (World Health Organization, 2021).

Multidrug-resistant TB (MDR-TB) remains a public health crisis and a threat to global health security. Globally in 2019, there were an estimated 206,230 new cases with multidrug- or rifampicin-resistant TB, 10% increase from 2018 (186,883 new cases) (World Health Organization, 2020). The global treatment success rate for MDR/RR TB patients was 59 percent. As a result, in 2020, WHO recommended a new shorter (9-11 month) and fully-oral regimen for patients with MDB-TB (World Health Organization, 2021).

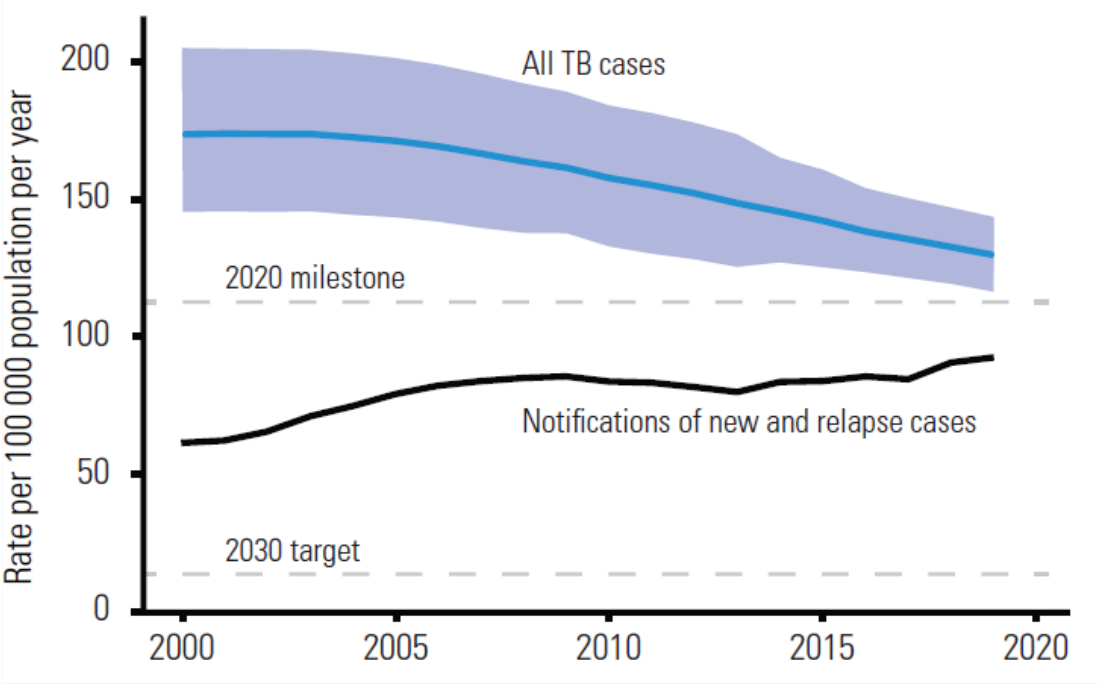

Figure 1.1 Global trend in the estimated TB incidence rate 2000-2019 (World Health Organization, 2020)

Statistics are not alarming, since TB is not a new epidemic, and has been in the world's population for thousands of years. Individuals who developed TB can be cured with a quick diagnosis and appropriate management (Padayatchi *et al*., 2019). However, to end the global TB epidemic, WHO aims to reduce the TB incidence by 80% and TB mortality by 90% by 2030. However, this goal will almost certainly be unachievable as the global decline in TB incidence is only 2% per year (World Health Organization, 2018). Thus, the Stop TB Partnership has revised the WHO Global Plan to End TB, 2018-2022 to reflect the current condition of the global community, as well as the lack of resources to establish more successful diagnosis, care and prevention interventions (Zumla *et al*., 2016). Therefore, WHO improvised the WHO`s End TB Strategy 2016-2035 from previous global strategies to respond to old and new challenges, as well as to create a framework addressing human rights and social justice concerns, as well as emphasizing public health issues, clinical fundamentals of TB

treatment and prevention (Figure 1.2) (Lönnroth and Raviglione, 2016, World Health Organization, 2017).

VISION	A WORLD FREE OF TB — zero deaths, disease and suffering due to TB			
GOAL	END THE GLOBAL TB EPIDEMIC			
INDICATORS	MILESTONES		TARGETS	
	2020	2025	SDG 2030[a]	END TB 2035
Percentage reduction in the absolute number of TB deaths (compared with 2015 baseline)	35%	75%	90%	95%
Percentage reduction in the TB incidence rate (compared with 2015 baseline)	20%	50%	80%	90%
Percentage of TB-affected households experiencing catastrophic costs due to TB (level in 2015 unknown)	0%	0%	0%	0%

PRINCIPLES

1. Government stewardship and accountability, with monitoring and evaluation
2. Strong coalition with civil society organizations and communities
3. Protection and promotion of human rights, ethics and equity
4. Adaptation of the strategy and targets at country level, with global collaboration

PILLARS AND COMPONENTS

1. **INTEGRATED, PATIENT-CENTRED CARE AND PREVENTION**
   - A. Early diagnosis of TB including universal drug–susceptibility testing, and systematic screening of contacts and high-risk groups
   - B. Treatment of all people with TB including drug-resistant TB, and patient support
   - C. Collaborative TB/HIV activities, and management of comorbidities
   - D. Preventive treatment of persons at high risk, and vaccination against TB
2. **BOLD POLICIES AND SUPPORTIVE SYSTEMS**
   - A. Political commitment with adequate resources for TB care and prevention
   - B. Engagement of communities, civil society organizations, and public and private care providers
   - C. Universal health coverage policy, and regulatory frameworks for case notification, vital registration, quality and rational use of medicines, and infection control
   - D. Social protection, poverty alleviation and actions on other determinants of TB
3. **INTENSIFIED RESEARCH AND INNOVATION**
   - A. Discovery, development and rapid uptake of new tools, interventions and strategies
   - B. Research to optimize implementation and impact, and promote innovations

[a] Targets linked to the Sustainable Development Goals (SDGs).

Figure 1.2 The WHO End TB Strategy (World Health Organization, 2017).

1.4 *Mycobacterium tuberculosis* (Mtb)

1.4.1 *Mycobacterium tuberculosis* Characteristics

Mtb is an acid fast, obligate aerob, non-spore forming and non-motile bacteria (Martínez *et al*., 1999). The bacteria form a straight or slightly curved rod about 3 X

0.3µm in size, either singly, in pairs, or in small clumps. The red colour acid fast bacilli (AFB) can be seen under a 100x objective microscope after being stained with Ziehl Neelsen staining (Figure 1.3). They are slow growers and *in-vitro* generation time is 14-15 hours. They require more than 7 days to form colonies when subcultured on Lowenstein-Jensen medium. Mtb forms off-white, rough colonies on Lowenstein-Jensen solid media (Pfyffer, 2015). The optimum growth temperature is 37°C, and growth does not occur below 25°C or above 40°C. While the optimum pH is between 6.4 and 7.0.

The *Mycobacterium* genus is divided into two major groups based on their growth rate. Slow-growing pathogens such as Mtb, *Mycobacterium bovis*, and *Mycobacterium leprae* (etiological agents of human tuberculosis (TB), bovine tuberculosis (BTB), and leprosy, respectively) belong to one group. The other group comprises of fast-growing bacteria such as *Mycobacterium smegmatis*, which are generally opportunistic or non-pathogenic mycobacteria (Forrellad *et al*., 2013). Mtb is a member of *Mycobacterium tuberculosis* complex (MTBC), which includes several closely related mycobacterial species (*M. africanum, M. canettii, M. bovis, M. microti, M. orygis, M. caprae, M. pinnipedii, M. suricattae* and *M. mungi*) that exhibit distinct phenotypic properties and host range (Brosch *et al*., 2002, Alexander *et al*., 2010). Although all MTBC members are obligate pathogens, not all species have been found to cause disease in human.

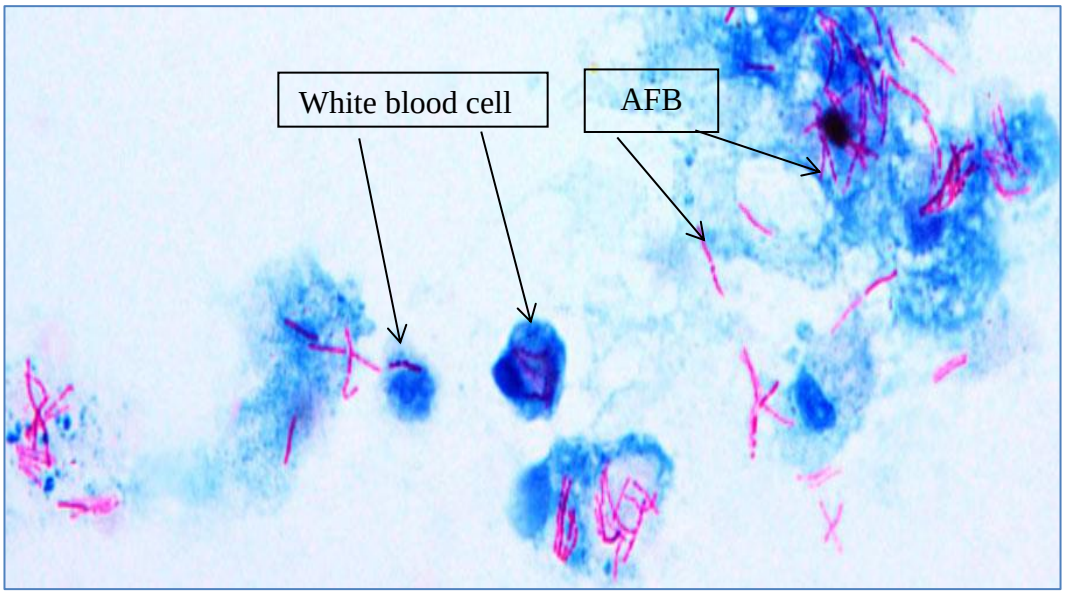

Figure 1.3 Acid Fast Bacilli (AFB) in sputum stained with Ziehl Neelsen staining (Bhonsle and Karpe, 2007).

The cellular envelope of the mycobacterium contains a major virulent component that is essential for the interaction of the host with *Mycobacterium* species. Mycobacteria are unique among prokaryotes due to their cell wall structure (Figure 1.4). The cell wall contains tightly packed mycolic acids and other structures, such as peptidoglycan, cord factor and wax-D. Mycolic acids are the major and specific lipid components of the mycobacterial cell envelope that are essential for *Mycobacterium* survival (Marrakchi *et al*., 2014). Cord factor triggers the formation of a distinctive serpentine cord; a molecule that is abundantly produced by virulent strains of Mtb. Cord factor can inhibit the migration of polymorphonuclear cells during the infection (Tu *et al*., 2003). Mtb cell wall also contains a lipid called Wax-D. The component is responsible for the impermeability to stains, dyes and also resistant to acidic and alkaline compounds (Samuel and William, 2006).

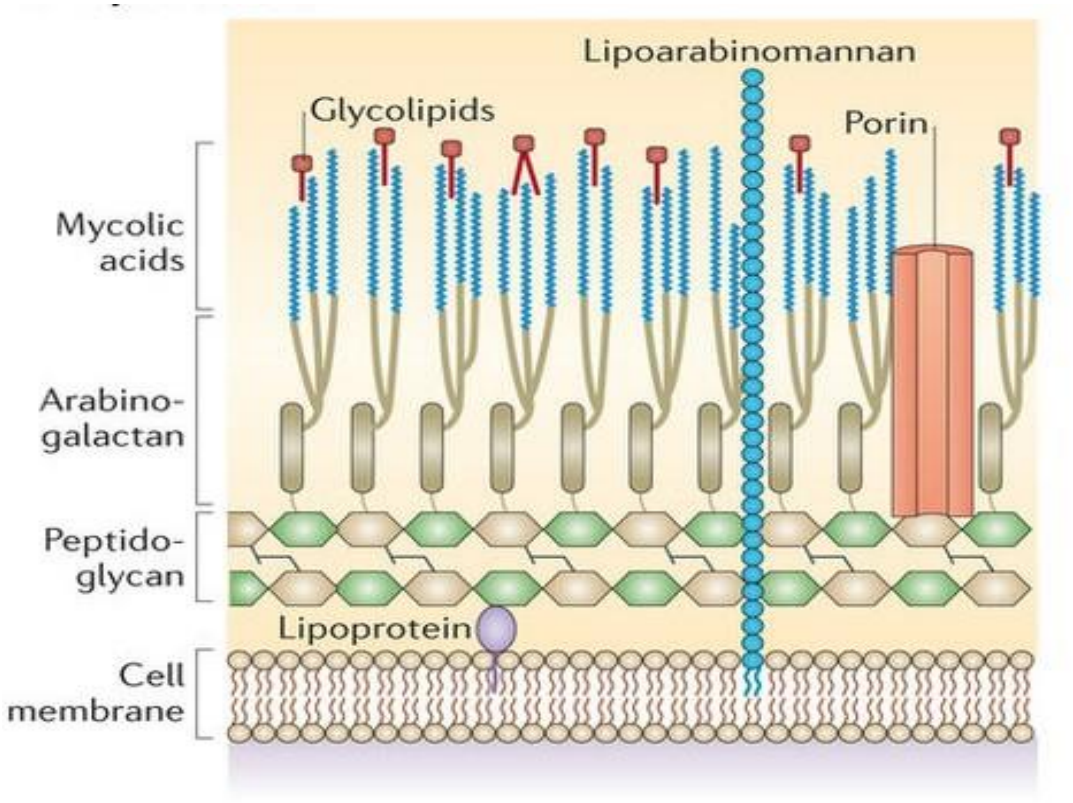

Figure 1.4 The structure of the Mtb cell wall (Adapted from Brennan, 2003).

1.4.2 TB Transmission

TB is an airborne disease caused by the bacterium Mtb (Pfyffer, 2015). Mtb will be detected as MTBC because of the close genetic relation between seven mycobacterial species (*M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti* and *M. mungi*). When an individual with active pulmonary or laryngeal TB coughs, sneezes, or sings, airborne droplet nuclei of 1–5 microns in diameter are generated. Depending on the environment, these tiny particles can persist in the air for several hours. Exposure to autopsy material, venereal transmission, and even percutaneous transmission (direct injury to the skin and mucous membranes through skin breaks) are other modes of transmission (Fleming and Hunt, 2006). The major

factor contributing to the high rate of transmission was suggested to be due to the long delay from the disease onset to diagnosis and commencing treatment (Uys *et al*., 2007).

Four factors contribute to the transmission of Mtb; 1) susceptibility of the exposed individual, 2) stage of TB infectiousness of the person (number of tubercle bacilli expels into the air), 3) environmental factors that affect the concentration of Mtb organism and 4) exposure (proximity, frequency and duration of exposure) (Desaleng and Koya, 2015, Alshammri, 2020). Several studies demonstrated that mycobacterial factors significantly contributed to the TB transmission: some Mtb strains can spread rapidly and form a larger cluster of cases than others (Van der Spuy *et al*., 2009, Verhagen *et al*., 2011). Furthermore, outbreaks can occur when the transmission of TB is not well controlled (Yuen *et al*., 2016). Individuals with pulmonary TB are more easily to transmit the disease than those with extrapulmonary TB (Behr *et al*., 1999, Neely *et al*., 2009).

At the onset of disease, the number of bacteria exhaled is low in individuals with active TB, but the number of bacteria will increase when the disease becomes progressively acute and significantly infectious (Uys *et al*., 2007). Subsequently, the persons with untreated TB of the respiratory tract are the source of transmission for new cases of TB infection (Comstock *et al*., 1974).

1.4.3 Immune Response Against TB

Immune response against TB is initiated upon invasion of the pathogen into the host, which may lead to latent infection, active tuberculosis, or pathogen clearance. Both the innate and adaptive immune responses play important roles in the protection against Mtb.

1.4.3(a) Innate Immunity

Innate immunity is the nonspecific first line response which is responsible to eliminate the pathogen from the target area (Hoffmann *et al*., 1999). Physical barriers, such as skin, and internal defenses, such as blood and blood derivatives, antimicrobial substances, and immune system cells that fight foreign cells in the body, are examples of these mechanism (de Martino *et al*., 2019).

The major innate immunity relies on airway epithelial cells (AEC), macrophages, neutrophils, dendritic cells (DCs), natural killer cells (NK), mast cells, and complement proteins, although the roles of mast cells and complement proteins in human Mtb infection are unknown (de Martino *et al*., 2019).

AEC act as a physical barrier and the first cells to come into contact with Mtb through pattern recognition receptors (PRRs) (Lee *et al*., 2009, Morales and Barrios, 2020). PPRs trigger the production of inflammatory cytokines, as well as the activation of mucosal-associated invariant T (MAIT) cells (Tissue-resident T cells), which stimulate IFN-γ and tumour necrosis factor (TNF), allowing Mtb to be detected (Scriba *et al*., 2017). Moreover, PRRs activation lead to the modification of the airway surface fluids composition, enhancing its antibacterial capacity (de Martino *et al*., 2019).

Macrophage acts as the first line of defense and is involved in the detection, phagocytosis and destruction of pathogen (Upadhyay *et al*., 2018). Macrophage eliminate Mtb by the production of Nitric Oxide (NO), cytokines, phagocytosis and autophagy of intracellular Mtb (Liu *et al*., 2017, de Martino *et al*., 2019). During the early stages of Mtb infection, alveolar macrophage cells recruit monocyte-derived macrophages to initiate phagocytosis. The macrophage PRRs (such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs))

recognize Mtb pathogen-associated molecular patterns (PAMPs) (such as glycolipids, lipoproteins, carbohydrates) and trigger production the inflammatory cytokines (IFN-γ, IL-12, IL-1β and macrophage inflammatory protein-1α) (Liu *et al*., 2017).

Granulocytes and neutrophils have been reported to be present in the BAL and sputum during active TB. These cells are involved in the defense against Mtb by secreting bactericidal enzymes and α-defensins to kill the pathogen (Dong *et al*., 2016). Peripheral blood neutrophils have also been reported to secrete a wide range of cytokines like IL-8, IL-1-β and IFN-γ in response to the Mtb infection (Hilda *et al*., 2020).

DCs are the principal APCs that link the innate and adaptive immunity. DCs exist in two forms, immature and mature. Immature DCs found in peripheral tissue are responsible for detecting pathogens or injured host cells. These cells, when triggered by pathogens developed into mature DCs (Abrahem *et al*., 2020). Mature DCs migrate into lymphoid organs and stimulate specific T cells response. DCs are also responsible for secreting cytokines (such as IL-12, IL-6) that induce CD4+ T cells to differentiate into Th1, Th2, Th17 and regulatory T cells. These T cells will then secrete effector cytokines such as IFN-γ and IL-4 and act as the immunological defense against Mtb (de Martino *et al*., 2019, Abrahem *et al*., 2020).

Natural killer (NK) cells play an important role in the initial defense against a wide variety of pathogens. During Mtb infection, NK cells stimulate macrophage to produce IFN-γ and IL-22 (Choreno Parra *et al*., 2017, de Martino *et al*., 2019). Both cytokines increase phagocytosis activity, which inhibits Mtb replication.

1.4.3(b) Adaptive Immunity

Besides the innate immune response, adaptive immunity also plays an important role against TB. The adaptive immune response depends on the immune system's prior contact with Mtb or Mtb antigens (immunogenic components). There are two types of adaptive immunity which are known as humoral and cellular immune response (Constant and Bottomly, 1997, Berger, 2000). During the Mtb infection, APC stimulate T- and B-cells and activate the mechanism of adaptive immunity (Khader *et al*., 2006).

CD4+ and CD8+ T cells were two important T cells subsets involved in the control of Mtb replication. Both T cell subsets can recognize Mtb antigen and produce cytokines (Beetz *et al*., 2008, Chan and Flynn, 2004). CD4+ T-helper cells can differentiate into various types of T-helper cells (Th1, Th2, Th17, Treg-cell) according to the type of cytokines produced by the cell (Joosten and Ottenhoff, 2008). Th1 cells produce IFNγ, TNFα, lymphotoxin and granulocyte-macrophage colony-stimulating factor which these cytokines promote the maturation and activation of macrophage and granulocytes. While Th2 cells produce B cell simulation factors (IL-4, IL-5, IL10, IL-13) which promote antibody production and suppress Th1 immune response. Th17 cells produce IL-17, IL17F, IL-21, IL-22 and stimulate recruitment of neutrophils and monocytes to the site of inflammation, and act as early host defense. In contrast, Treg-cells can inhibit T-cells proliferation and cytokine production (Joosten and Ottenhoff, 2008).Th1 cells and IFN-γ are crucial in immunity against mycobacterial infection (Ngai *et al*., 2007, Cooper, 2009). CD8+ lymphocytes stimulated additional IFN-γ production in response to the Mtb infection (Choreno Parra *et al*., 2017). This

mechanism is mediated by the cytokines IL-15 and IL-18, which are produced by infected macrophages (Choreno Parra *et al*., 2017).

The cytotoxic potential of CD8+ T cells to kill infected cells is facilitated by CD4+ T cells. Cytotoxic activity of CD8+ T cells involved at two mechanisms: apoptosis and intracellular mycobacterial cytotoxicity (via the release of the antimicrobial peptide, granulysin) (Stenger *et al*., 1997, Stenger *et al*., 1998, Sud *et al*., 2006). CD8+ T cells also secrete IFNγ and TNFα to activate macrophage to control Mtb infection (Lalvani *et al*., 1998, Marín *et al*., 2013).

Immune response against Mtb can also be initiated by B cells through several mechanisms. B-cells can act as APCs that interact with T cells which in turn trigger B-cell proliferation, somatic hypermutation, affinity maturation, class switching, and the formation of memory B cells and antibody-producing plasma cells (Lund and Randall, 2010). B-cells also produce a wide range of cytokines (IFNγ, TNFα, IL, lymphotoxin) when triggered through interaction with antigens, T cells or Toll-like receptor ligands lymphocytes (Chan *et al*., 2014). Antibodies produced by B-cells may trigger Mtb lysis or mediate phagocytosis process (Chan *et al*., 2014).

1.4.4 Latent TB

TB begins with inhalation of Mtb; before a period in which replication and spread of bacteria occurs, followed by immunologic containment of viable bacilli (Mack *et al*., 2009, Getahun *et al*., 2015b). Mtb can survive in the host for years without showing any symptoms or causing the disease and it is known as latent TB (TB).

LTBI is characterized by the presence of an immune response to a previous Mtb infection without showing any signs of active TB (Getahun *et al*., 2015a). This process results in asymptomatic latent tuberculosis infection, which is defined as a state of persistent bacterial viability, immune control, and no evidence of clinically manifested active TB (Mack *et al*., 2009). A person with latent TB infection is not infectious and do not spread Mtb to others (Khan *et al*., 2013). The TB infection indicated by a positive reaction to the tuberculin skin test (Mantoux test) or TB blood test.

One-third of the world's population has latent TB and this puts them at risk of active TB. Latent TB occurs when Mtb responds to stimuli that induce the activation of TB organism in a latent metabolic state (Parrish *et al*., 1998).The failure of immune-mediated clearance is due to the various strategies adopted by Mtb that interfere with the bactericidal mechanism of the infected cell and formation of a granuloma that can suppress the persistence of viable Mtb (Ulrichs *et al*., 2006, Cardona, 2007, Martínez *et al*., 2007, Ahmad, 2010). Previous studies showed that 5-10% individuals with LTBI develop active TB during their lifetime (Comstock *et al*., 1974, Park *et al*., 2020) and a higher percentage of reactivation, if the person is immunocompromised. Previous experimental data have shown that the constant endogenous reactivation of Mtb occurs in immunocompetent individuals (Cardona, 2009, Cardona, 2016).

LTBI is diagnosed by the response to *in-vivo* or *in-vitro* stimulation by Mtb antigens with the use of tuberculin skin test (Mantoux test) or interferon-γ release assays (IGRAs), respectively. The current diagnostic tests are not sufficient to measure the global latent TB infection rate, but a study that was conducted a decade ago

estimated that about one-third of the world's population (>2 billion people) were already infected with latent Mtb (Corbett *et al*., 2003).

TB treatment has been developed and expanded for the past 15 years. Human living conditions have improved worldwide with a declined in annual risk of infection in many countries. However, the global latent infection burden remains uncertain and needs to be reassessed (Getahun *et al*., 2015a). The main principle of testing and treatment for LTBI is to benefit individuals at risk. For the management of LTBI programs, National TB programmes and other stakeholders should give priority to risk groups, taking into consideration local TB epidemiology as well as the availability and efficient use of resources. For example, priority testing and treatment of LTBI should be given to high-risk groups, such as individuals living with HIV, adults and children who have contact with cases of pulmonary TB, as well as immigrants from countries with a high burden of TB (Malhotra, 2011, World Health Organization, 2012, World Health Organization, 2015a, Getahun *et al*., 2015a).

1.5 Risk Factors for Reactivation

Recent data show that the global disease burden reflects the demographic and lifestyle changes of the community, leading to an increase in non-communicable disease (NCD) and communicable disease (CD) infection rates. Most of the countries with high endemic TB infection face a large comorbidity burden from both NCD and CD. Population attributable to high TB risk include diabetes, malnutrition, smoking, excessive alcohol use and HIV infection. Integrated and efficient responses are needed, especially in low income and poor resource countries (Marais *et al*., 2013). There is a higher disease burden in developing countries due to the huge overlap between killer

infections, such as TB, HIV, malaria and severe viral infections with NCDs (Bates *et al*., 2015).

1.5.1 TB and HIV Co-infection

TB and HIV/AIDS are both chronic diseases and are regarded as global emergencies. TB is the most prevalent opportunistic disease in HIV-infected individual in a country with a low incidence of TB (Whalen *et al*., 1995, Corbett *et al*., 2003, Aichelburg *et al*., 2009). About 1.5 million TB deaths were reported globally including 0.208 million HIV positive patients (World Health Organization, 2020) Lawn *et al*.(2005) reported that the rate of progression from latent Mtb infection to active TB disease has increased being more aggressive in HIV-infected patients, even though they are receiving anti-retroviral treatment (Lawn *et al*., 2005). HIV influences the incidence, prevalence, clinical presentation, diagnostic yield and treatment options for patients with TB (Sharma *et al*., 2005). The difficulties in clinically diagnosing co-infected patients resulted in a high prevalence of HIV infection among TB patients. HIV–TB patients also encounter unique problems like drug-drug interactions, cumulative toxicity, lower plasma drug levels and the emergence of drug resistance during treatment despite adherence (Narendran and Swaminathan, 2016). TB may also be misdiagnosed as HIV due to the close resemble to TB. A diagnostic approach with a high-end diagnostic capability is required to avoid missing HIV-related TB, which is communicable to the general population (Gellete *et al*., 1997).

HIV infection is associated with immune activation and CD4+ cell depletion, which results in immune response defects (Brenchley *et al*., 2004). The use of antiretroviral therapy (ART) combinations is associated with the replenishment of

circulating CD4+ cells and an improvement in immune function, resulting in a reduction in the incidence of opportunistic infections (Group, 2006). The rapid increase in IFN-responses (Interferon responses) to region of difference 1 (RD1) antigens and purified protein derivative (PPD) suggests a significant contribution from ART-induced restoration of type 1 helper cell responses in the pathogenesis of ART-associated TB and a possible role for IGRA in the diagnosis and prediction of TB during early ART (Elliott *et al*., 2009).

Limited attention of infection control, inadequate investment in R&D and the HIV epidemic have caused TB to be a growing global threat (Mayer and Hamilton, 2010). In many countries, patient care and treatment facilities for HIV/AIDS and TB are overlapping, which creates opportunities for nosocomial infection and cross infection. This may be one of the reasons for high exogenous reinfection rates (Narayanan *et al*., 2010). In sub-Saharan Africa, correlation of TB mortality and HIV co-infection among adults, children and pregnant women showed the need for an integrated approach for the management and disease control (Marais *et al*., 2013). For several years, rapid and reliable tests, such as Xpert MTB/RIF are available, but implementation has been slow in the low- and middle-income countries. Further improvements in assay robustness, sensitivity and specificity are needed, especially for HIV co-infection, as current molecular tests are perform by low-skilled primary healthcare personnel in resource-limited, high-HIV burden setting (Rie *et al*., 2010, Scott *et al*., 2017).

1.5.2 TB in Non-Communicable Disease (NCD)

TB with Non-communicable diseases (NCDs) co-infection became a global crisis and require a global response (Beaglehole *et al*., 2011). The rise in several NCDs, such as diabetes mellitus, alcohol use disorders, and smoking-related disease, was responsible for a significant proportion of TB cases worldwide. As a result, it had an impact on TB epidemiology, TB case management, and TB treatment outcomes. NCD weakens the immune system and raises the chance of tuberculosis infection. Some NCDs can make detection more difficult, reduce the efficacy of TB drugs, deteriorate the patient's overall health, reduce treatment adherence, and increase the risk of treatment failure, recurrence, and mortality. These diseases and conditions, on the other hand, can be prevented and treated (Creswell *et al*., 2011).

Diabetes contributes substantially to TB incidence globally. Studies by Jeon *et al.* (2008) found that individuals with diabetes have a three-fold greater risk of developing active TB infection compared to individuals without diabetes (Cegielski *et al*., 2004, Lin *et al*., 2007, Jeon and Murray, 2008, Lönnroth *et al*., 2008). TB infection in diabetic individuals is often associated with poor glycaemic control. Reactionary hyperglycaemia is often associated with a TB infection since this reaction is antagonistic to insulin. It is triggered by increased pro-inflammatory conditions and release of regulatory stress hormones such as epinephrine, cortisol and glucagon (Kibirige *et al*., 2013). Moreover, anti-TB drugs, such as pyrazinamide may disrupt the glycaemic control while rifampicin may induce hyperglycaemia directly or indirectly via interactions with oral hypoglycaemic agents (Grant, 2013). The presence of diabetes probably impairs the immune responses needed to control bacterial infections (Dooley and Chaisson, 2009), thereby, increasing susceptibility to TB.

Co-morbid presentation of diabetes with TB can worsen treatment outcomes and exacerbate the severity of TB infection (Aikins *et al*., 2010). In the context of primary care and the general health system, TB programmes and clinicians must work more closely with those in general health services who provide NCD prevention and care (Creswell *et al*., 2011).

1.6 Multi-drug Resistant TB and Extensively Drug-resistant TB

Multi-drug resistant (MDR) TB is caused by Mtb that is resistant to isoniazid and rifampicin, whereas, extensively drug-resistant (XDR) TB is caused by Mtb that is resistant to rifampicin, plus any fluoroquinolone, plus at least one of the drugs bedaquiline and linezolid (Davies, 2004, Raviglione and Ditiu, 2013, World Health Organization, 2021). The types of drug resistance to Mtb may be summarized as follows: (1) Natural resistance: resulting from natural mutation, regardless of previous drug exposure, and proportional to the number of mutant bacilli; (2) Primary resistance: cases of resistance in individuals known not to have been previously exposed to anti-TB drugs; (3) Acquired resistance: cases of resistance in which patients were previously administered TB treatment, generally inadequately (Dalcolmo *et al*., 2007). Spontaneous mutation of the Mtb genome causes the alteration of enzymes / proteins that modify the antibiotics affinity for the target and make the bacteria resistant to drugs (Figure1.5) (Singh *et al*., 2020).

MDR-TB and XDR-TB continue to pose a major threat globally due to poor surveillance and testing facilities in developing countries (Sharma and Mohan, 2006, Migliori *et al*., 2010). According to the WHO, approximately 600,000 new cases were resistant to rifampicin (the most effective first-line drug), with 490,000 having MDR-TB. Almost half of these cases were from India, China and the Russian Federation. In

2016, an estimated 6.2% of people with MDR-TB had extensively drug-resistant TB (XDR-TB) (World Health Organization, 2018). XDR-TB is the most severe form of drug resistance, which has been reported worldwide.

The increase in the number of patients co-infected with Mtb and HIV, inefficient infection control procedures and facilities, laboratory delays in Mtb identification and susceptibility testing, uncontrolled drug use, and failure to recognize ongoing infectiousness in patients are all factors that contribute to MDR-TB and XDR-TB outbreaks and spread (Jacobs, 1996, Gandhi *et al*., 2010, Namouchi *et al*., 2010).

Drug-resistant TB poses a major threat to existing control programmes since treatment is less effective, more complex, toxic and involves expensive regimens. The treatment success rate is less than 40% in most of the patient population and the death rate is 50 to 80% (Liu *et al*., 2011, Falzon *et al*., 2013, Pietersen *et al*., 2014). Although MDR-TB presents a formidable challenge to TB control in several settings, the treatment for MDR-TB has proven to be achievable and effective in low-resource settings (Suárez *et al*., 2002). A more reliable estimate of MDR-TB magnitude, both globally and by country, is now needed for planning and expanding the programmatic management of drug-resistant TB within the context of a national TB control program, which is absent in most high-burden settings (Zignol *et al*., 2006, Gandhi *et al*., 2010).

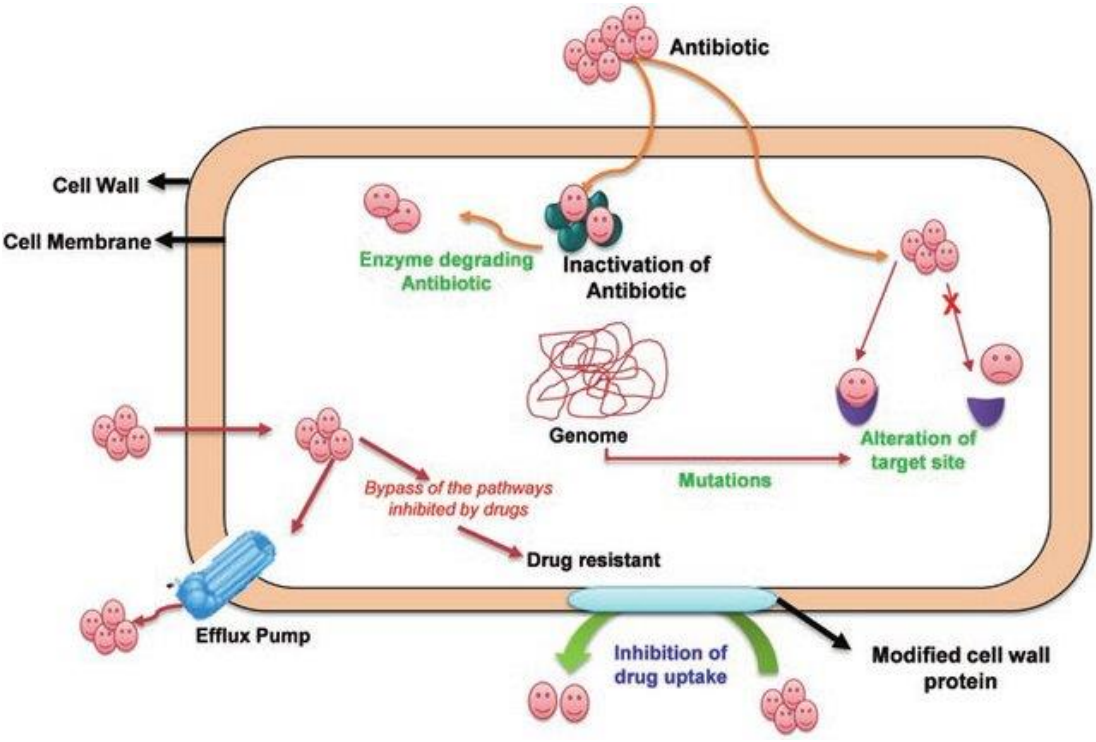

Figure1.5 Mechanism of Action of Drug-Resistant Mtb (Adapted from Singh *et al*., 2020).

1.7 TB Control Program

Various strategies have been carried out to strengthen the TB control program in terms of reducing mortality and morbidity rates together with TB reactivation. The rise in TB/HIV co-infection and the emergence of MDR-TB have hampered the existing TB control programme (Deribew *et al*., 2010). An early TB diagnosis will facilitate the initiation of appropriate treatment, especially for those who leave in the area with poor health facilities (Parsons *et al*., 2011). Misdiagnosis of TB in asymptomatic high-risk individuals at an early stage of infection may delay the receipt of treatment.

Control of infectious disease epidemics require active case detection, possible treatment, interference to transmissions and increased levels of immunity for susceptible individuals. If elimination is desired, the containment of infection

reservoirs is important (Rangaka *et al*., 2015). Tadolini *et al, (*2012) summarized the definitions of TB control and elimination as shown in Table 1.2.

Table 1.2 Definitions of TB control and elimination (Tadolini and Migliori, 2012)

	Definition
TB control	The strategy aimed to reduce the incidence of TB infection by focusing on early diagnosis and treatment of infectious cases of TB. Fewer and fewer people in the community will be newly exposed to the bacilli and will develop the disease.
Elimination	The point at which there is less than one infectious (sputum smear positive) case per 1,000,000 inhabitants in the general population each year, or when the prevalence of tuberculosis in the general population is less than 1% and continues to decline.

The 67th World Health Assembly (WHA) approved WHO's "Global strategy and targets for tuberculosis prevention, care, and control after 2015" on May 19, 2014 (World Health Organization, 2015b). The post-2015 global TB strategy (End TB Strategy) was developed in 2014. A committee was established in developing these guidelines; comprising the public and private sectors, including technical experts, financial and development partners, civil society and health activists and non-governmental organizations (Raviglione and Ditiu, 2013). The strategy has a vision of making the world free of TB with zero deaths, disease and suffering due to the disease. Prevention, care and control of TB have been presented not only scientifically but also in terms of ethical and policy concerns that need to be resolved in an appropriate manner (Uplekar *et al*., 2015).

The introduction of a new rapid diagnostic tool for tuberculosis (TB) and promising TB drugs, as well as the development of a new World Health Organization Strategy Post 2015, prompted a new discussion about how to control TB. The goal of