DEVELOPMENT OF *Mycobacterium tuberculosis* 16 kDa PROTEIN DETECTION USING IMMUNOCHROMATOGRAPHY TEST

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By

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LIST OF SYMBOLS AND ABBREVIATIONS

List of Symbol

Symbol	Definition	Symbol	Definition
<	Less than	˚C	Degree Celcius
>	More than	λ	Lamda
≈	Approximate	μ	Micro
%	Percentage	γ	Gamma
®	Registered	δ	Beta
™	Trademark	α	Alpha

List of Abbreviation

Abbreviation	Definition
AD	Anno Domini
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BC	Before century
BCE	Before common era
BCG	Bacille Calmette-Guèrin
bp	Base pair
BSA	Bovine serum albumin
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit
CPG	Clinical Practice Guidelines
CSF	Cerebral spinal fluid
CT	Chest computed tomography
ddH_2O	Double distilled water
dH_2O	Distilled water
DOTS	Directly observed treatment short course
DNA	Deoxyribonucleic acid
DST	Drug sensitivity testing
ECL	Enhanced Luminol-based Chemiluminescent
ELISA	Enzyme Linked Immunosorbent Assay
et al.	And others
FDA	Food Drug Administration
g	Relative centrifugal force
HIV	Human immunodeficiency virus
HRP	Horseradish peroxsidase

Abbreviation	Definition
hr	Hour
HSP	Heat shock proteins
IC control	Immunochromatographic control
ICT	Immunochromatographic test
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
kDa	Kilodalton
LAM	Lipoarabinomannan
LJ	Löwenstein–Jensen
LOD	Limit of detection
LPS	Lipopolysaccharide
LTBI	Latent TB infection
M	Molar
mAb	Monoclonal antibody
MDR-TB	Multi drug resistant TB
MHC	Major Histocompability Complex
min	Minute
MMWR	Morbidity and Mortality Weekly Report
MODS	Microscopic Observation Drug Susceptibility
MOH	Ministry of Health
Mtb	*Mycobacterium tuberculosis*
MTBC	Mtb Complex
n	Sample size
NC	Nitrocellulose
NAAT	Nucleic Acid Amplification Technique
NF-kB	Nuclear factor kB
NK	Nature killer cell
NTM	Non-tuberculous mycobacteria
NTP	National TB Control Programme
OD	Optical density
OD_{525}	Optical density at 525 nm
OADC	Oleic acid/ albumin/ dextrose/ catalase enrichment
pAb	Polyclonal antibody
PAGE	Polyacrylamide Gel Electrophoresis
PHE	Porcine hemagglutinating encephalomyelitis
PB	Phosphate buffer
PBS	Phosphate buffer saline
PBS-T_{20}	Phosphate buffer saline-Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

Abbreviation	Definition
POC	Point of care
PPD	Purified protein derivative
PVP	Polyvinylpyrrolidone
Q_{10}	Reaction rate factor
RH	Relative humidity
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real time-polymerase chain reaction
s	second
SDA	Strand displacement amplification
SDS	Sodium dodecyl sulphate
spp.	species
TB	Tuberculosis
TBM	Tuberculous meningitis
TLA	Thin layer agar
TLRs	Toll-like receptor
TMA	Transcription mediated amplification
TNF-α	Tumor necrosis factor-alpha
TST	Tuberculin skin test
TU	Tuberculin unit
U	Unit
UNITAID	International facility for the purchase of diagnostics and medicines for diagnosis and treatment of HIV/AIDS, Malaria and TB
UV	Ultraviolet
WHO	World Health Organization

PEMBANGUNAN ASAI PENGESANAN PROTEIN 16 kDa DARIPADA *Mycobacterium tuberculosis* MENGGUNAKAN UJIAN IMUNOKROMATOGRAFI

ABSTRAK

Mycobacterium tuberculosis (Mtb) adalah patogen yang boleh menyebabkan penyakit tuberculosis (TB). Penyakit TB yang berjangkit ini masih kekal menjadi masalah kesihatan yang utama di seluruh dunia. Penyakit ini ditular melalui pernafasan atau titisan udara yang mengandungi Mtb yang terhasil daripada pesakit TB. Pengesanan awal penyakit ini adalah penting bagi mengelakkan pemindahan penyakit tersebut kepada orang lain yang berada rapat dengan pesakit. Sehingga hari ini, belum ada lagi ujian diagnostik yang mudah, cepat, sensitif dan spesifik untuk penyakit TB. Kajian-kajian terdahulu telah menunjukkan antigen Mtb 16 kDa mempunyai potensi dalam pendiagnosian TB. Dalam kajian ini, kaedah asai imunokromatografi aliran melintang (ICT) dipstik terhadap antigen 16 kDa telah dibangunkan dan dinilai sebagai kaedah yang cepat dan berpotensi untuk mendiagnos jangkitan TB. Antibodi monoklon anti-16 kDa telah dioptimumkan sebagai antibodi penambat dan antibodi pengesan pada pelantar ICT. Parameter untuk garisan kawalan, bahan penebat, larutan penimbal ujian, pengalas konjugat dan pengalas sampel telah dioptimumkan sebelum prototaip dipstik TB-antigen ICT dilengkapkan. Kajian sensitiviti terhadap prototaip dipstik TB-antigen ICT menunjukkan dipstik tersebut mampu mengesan antigen 16 kDa yang tulen serendah 125 ng. Walau bagaimanapun, kajian spesifisiti prototaip tidak dapat dimuktamadkan kerana kaedah yang digunakan untuk penyediaan sampel memerlukan kajian lanjut. Hasil daripada analisis sensitiviti, (teknik ICT) berpotensi untuk aplikasi klinikal yang pelbagai.

DEVELOPMENT OF *Mycobacterium tuberculosis* 16 kDa PROTEIN DETECTION USING IMMUNOCHROMATOGRAPHY TEST

ABSTRACT

Mycobacterium tuberculosis (Mtb) is a pathogenic bacterium that can cause tuberculosis (TB). This contagious disease remains a severe health problem in the world. The disease is transmitted via inhalation of airborne droplets carrying Mtb from TB patients. Early detection of infection is vital to prevent transmission of the disease to people in close contact with the patients. To date, there is no simple, rapid, sensitive and specific diagnostic test for TB. Previous studies showed the potential of Mtb 16 kDa antigen in TB diagnosis. In this study, an immunochromatographic (ICT) lateral flow dipstick assay against 16 kDa antigen was developed and assessed as a potential rapid method to diagnose TB. A 16 kDa monoclonal antibody was optimized as the capturing and detection antibody on the ICT platform. The parameters of control line, blocking reagent, running buffer, conjugate and sample pad were also optimized before a complete prototype of TB-antigen ICT dipstick was developed. Sensitivity studies on TB-antigen ICT prototype dipstick showed that the dipstick was capable to detect purified 16 kDa antigen as low as 125 ng. However, the specificity of the assay was inconclusive probably due to the method used for sample preparation, which needs further study. The analytical sensitivity suggests its potential usefulness in different clinical applications.

CHAPTER 1

LITERATURE REVIEW

1.1 Background

Tuberculosis (TB) is still a leading cause of mortality from infectious disease and remains a major global health problem worldwide. Even though the Bacillus Calmette–Guérin (BCG) vaccine and anti-TB drugs have been used for the past 90 and 50 years respectively, the mortality rate caused by TB is still considerably high. The Stop TB Partnership revealed in its Global Plan To Stop TB 2011-2015 that one major challenge is to rapidly and effectively diagnose TB in an early stage of infection (WHO, 2010a). The development of rapid diagnostic tests is very crucial to initiate treatment and control of the disease. Previous studies conducted by Trilling *et al.* (2011), Siddiqui *et. al* (2011), Kaushik *et. al* (2012) and Srivastava *et al.* (2013) indicated that the 16 kDa protein has a potential role in TB diagnosis. In this study, the 16 kDa antigen was evaluated as a candidate marker for development of an immunodiagnostic test for the diagnosis of TB. Lateral flow immunochromatographic test (ICT) has been evaluated for the diagnosis of TB since it utilise a simple, rapid, inexpensive, sensitive and specific platform as previously reported by Said *et. al* (2011).

1.2 History of Tuberculosis (TB)

TB is an ancient illness. TB has been discovered in humans since antiquity. The archeological evidence of TB disease has been recognized in the fragments of the spinal columns of Egyptian mummies from 2400 BCE (Madkour, 2004). The history of TB is documented in ancient Babylonian, and Chinese writings. The term “phthisis” appear in Greek literature around 460 BCE. Hippocrates in his writing

identified and described phthisis as the most widespread disease that caused death (Palomino *et al.*, 2007). Hippocrates defined phthisis as a chronic disease accompanied by productive cough, sweating, and fever. Later, the Greek physicians Aristotle (384-322 BC), and Galen (131-200 AD) stated TB as a transmissible disease reported (Kanai, 1990).

In 1720, the English physician Benjamin Marten in his publication "A New Theory of Consumptions", hypothesized that TB could be caused by "minute living creatures" as cited by Doetsch (1978). A further significant advance was observed in 1865 when a French military physician, Jean-Antoine Villemin (1827-1892) indicated that TB is caused by a specific microorganism (cited in Daniel, 2006). *Mycobacterium tuberculosis* (Mtb) was finally identified by Robert Koch in 1882 with a staining technique which gave birth to TB diagnostics (Koch, 1882, cited in Blevin and Bronze, 2010). This early bacteriologist's work was first presented in Berlin on 24th March 1882 (Koch, 1882, cited in Daniel 2005, cited in Palomino, 2007, WHO, 2009). Koch had created a guideline to determine the causative agents of several specific infectious diseases. This guideline was named as "Koch's Postulates". According to this guideline, bacterium suspected as a causative factor of a disease must exist in the infected tissue (such as blood) of an infected person or animal (Tortora *et al.*, 2010). When the infected tissue was inoculated onto solid media, it must produce pure colonies and that must be infectious to experimental animals. Then, the bacteria must be retrieved as pure colonies from the second infected animal tissues (Palomino *et al.*, 2007; Blevins and Bronze, 2010; Tortora *et al.*, 2010). Lehmann and Newman then classified the microorganism as *Mycobacterium* genus that belongs to the Mycobacteriaceae family in 1896 (Grange, 1980; Grange, 1985).

1.3 Global incidence of TB

1.3.1 Epidemiology of TB

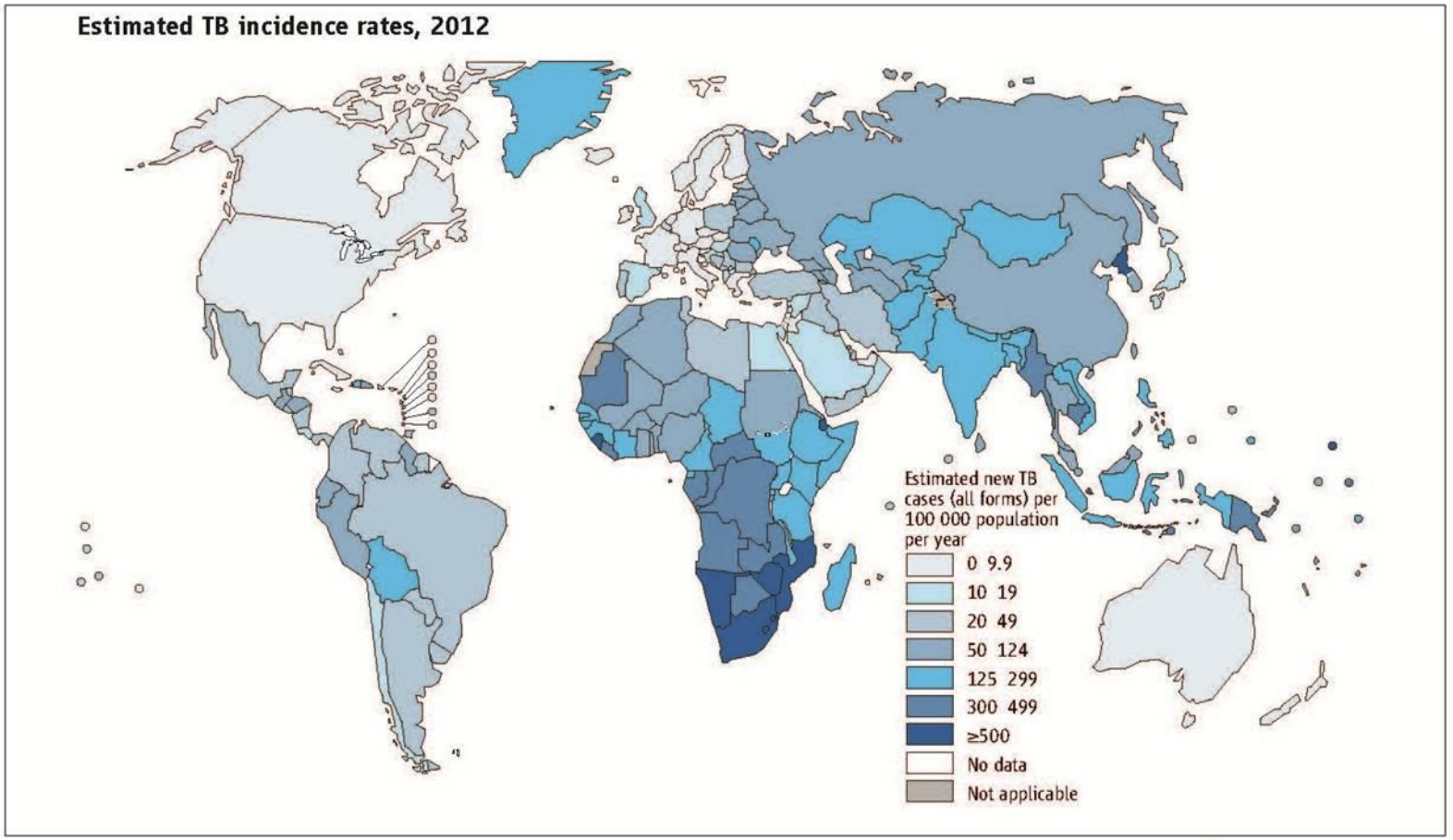
World Health Organization (WHO) declared TB as a global emergency in 1993 with the need to implement immediate control measures (WHO, 1994). According to WHO (2012), TB remains a major global health problem and is responsible for millions of incident cases each year. TB was reported as the second leading cause of death from an infectious disease worldwide, after acquired immune deficiency syndrome (AIDS) which is caused by human immunodeficiency virus (HIV) (WHO, 2012).

According to Lange and Mori (2010), TB epidemics can be classified into high prevalence and low prevalence areas based on the extent of TB incidence. The low prevalence areas are composed of countries that experienced serious TB epidemics after the 18th century but the incidence rate have gradually and successfully reduced to 100 cases per 100 000 population or less. They are industrialized countries. On the other hand, the high prevalence areas are countries that have suffered TB epidemics after the 20th century with an incidence rate of more than 100 cases per 100 000 population. These high prevalence areas mostly comprise developing countries.

Global Tuberculosis Report 2013 reported that in 2012, there were an estimated 8.6 million people who developed TB and 1.3 million deaths due to TB including 320 000 who were HIV-positive. South –East Asia documented the highest cases worldwide in 2012 (29%) followed by African (27%) and Western Pacific regions (19%). The report also shows that the most populous countries of Asia such as India and China have the largest number of incidence with 26% and 12% of the total global cases respectively (WHO, 2013).

Globally, there are numerous challenges, such as multi drug resistant TB (MDR-TB), poor diagnosis of TB, TB infection among immigrants from high-prevalence areas, and co-infection with HIV (Frieden *et al.*, 2003; Lange and Mori, 2010). Currently, it is estimated that about 1.1 million (13%) of the 8.6 million new TB patients in 2012 were HIV infected and about 75% of these incident cases were from Africa (WHO, 2013). WHO (2013) also reported that 450 000 people developed MDR-TB and there were an estimated 170 000 deaths from MDR-TB in 2012 with Eastern Europe and central Asia reporting the highest cases. Some countries have more than 20% of new TB cases with MDR-TB and more than 50% of those previously treated for TB have MDR-TB.

However, the large number of TB incident cases started to decline around 2003 as a consequence of the global effort in TB control by the implementation of the Directly Observed Therapy Short-course (DOTS) strategy for TB care since 1990s (Lange and Mori, 2010; WHO, 2012; WHO, 2013).



Source : Global Tuberculosis Report 2013. (WHO, 2013)

World Health Organization

Figure 1.1 Estimated number of new TB cases (all forms) 2012 worldwide (Adapted from WHO Tuberculosis Report, 2013).

1.3.2 TB in Malaysia

In the early 1940s and 1950s, TB was the leading cause of death in Malaysia (Iyawoo, 2004). However, since the National TB Control Programme (NTP) was launched in 1961, morbidity and mortality rates decreased rapidly. TB cases were successfully reduced in Malaysia, and Malaysia was categorized as a country with intermediate burden of TB (Mohamed Naim, 2004). Nevertheless, since 1995, new cases of TB increased and in 1998, TB was ranked 11th among the top causes of death in Malaysia (Iyawoo, 2004; Mohamed Naim, 2004). The high prevalence of TB during 2000-2001 is probably due to the migration of foreign workers into Malaysia who originated from high prevalence TB countries. Approximately 10% of TB cases reported in Malaysia involved the immigrant population, 90% of whom are from the Philippines and Indonesia. These two countries have been classified as high burden countries of TB by WHO (Iyawoo, 2004). According to Atif *et. al.* (2012), TB incidence rates were around 82-85 cases of 100 000 population in 2007-2011. It was also reported that there were increases of new cases from 15000 cases (2002) up to 16665 (2006) and 20000 cases in 2011 (Rafiza *et al.*, 2011; Atif *et al.*, 2012; Ministry of Health, 2012a).

Ministry of Health Malaysia (MOH) stated that there was a steady increase in TB cases from 2007 to 2011. In 2012, 22710 TB cases were recorded with 1520 deaths with a notification rate of 72 per 100,000 cases in 2011 and 78.7 per 100 000 cases in 2012. The highest incidence was reported in Sabah with 4426 TB cases followed by Selangor (3560 cases) and Sarawak (2430). MOH also reported that foreigners contributed to the sudden surge of TB cases in Malaysia especially from higher TB burden countries (Benedict, 2014).

	2007		2008		2009		2010		2011		2012	
STATE	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS
PERLIS	112	13	129	12	119	18	121	18	141	18	185	13
KEDAH	812	63	843	61	903	78	1000	101	1084	85	1174	100
PENANG	880	98	943	127	1039	150	1062	148	1126	128	1245	117
PERAK	1210	181	1160	152	1250	179	1389	151	1309	159	1554	190
SELANGOR	2484	84	2374	87	2342	100	2829	111	3242	216	3560	108
WPKL/ PUTRAJAYA	1253	22	1387	47	1429	57	1455	33	1907	83	1906	64
N. SEMBILAN	395	35	506	44	408	41	426	35	449	35	480	16
MALACCA	349	58	337	48	339	54	395	32	511	48	546	55
JOHOR	1591	188	1700	189	1855	180	2058	134	2038	151	2046	182
PAHANG	723	116	779	109	838	130	806	107	788	132	890	112
TERENGGANU	661	107	730	125	762	125	664	108	667	81	733	114
KELANTAN	1265	289	1354	295	1324	205	1333	232	1448	232	1436	140
LABUAN	45	3	67	3	66	2	80	3	106	1	99	1
SABAH	3433	164	3376	140	3515	179	3728	233	3794	188	4426	245
SERAWAK	1705	83	1821	93	1913	84	1991	111	2056	87	2430	63
TOTAL	**16918**	**1504**	**17506**	**1532**	**18102**	**1582**	**19337**	**1557**	**20666**	**1644**	**22710**	**1520**

Figure 1.2 TB incidence according to states in Malaysia, 2007-2012.

(The table was taken from Benedict (2014) [Online] Available from: http://www.themalaymailonline.com/malaysia/article/tb-kills-more-annually-than-dengue-says-ministry#sthash.QZltWk92.dpuf)

1.4 *Mycobacterium tuberculosis* (Mtb)

1.4.1 Causative Agent

Mtb and *M. bovis* species of Mtb complex (MTBC) infect humans and a wide variety of animal species (Kubica *et al.*, 2006; Cleaveland *et al.*, 2007). These microbial species are actually closely related to each other at the genomic and antigenic levels. *M. bovis* causes TB mainly in the bovine species. However, *M. bovis* can also infect humans especially through drinking infected milk (Collins *et al.*, 1997; Ayele *et al.*, 2004; Kubica *et al.*, 2006; Tortora *et al.*, 2010). Mtb is identified as the etiologic agent of TB in humans and it is reported as one of the most devastating human pathogens in the world (Dietrich and Doherty, 2009; Gengenbacher and Kaufmann, 2012).

1.4.2 Features and Life Cycle

Mtb is a bacterium belonging to the family of Mycobacteria (Mycobacteriaceae) (Brighenti and Lerm, 2012), a non-motile organism, that is slender or slightly curved rod shape bacterium distantly related to Actinomycytes. Many nonpathogenic mycobacteria are components of the normal flora of humans (Todar, 2012; Sharma *et al.*, 2013).

Mtb is 2-4 μm in length and 0.2-0.5 μm in breadth (Todar, 2012; Sharma *et al.*, 2013). Mtb has a lipid containing cell wall which is responsible for acid-fastness, namely when stained with carbol fuchsin dye and is resistant to decolorization by acid-alcohol. This characteristic is used in Ziehl-Neelsen staining. The lipid containing cell wall is also associated with the resistance of Mtb to environmental stresses such as drying and chemical antimicrobials (Tortora *et al.*, 2010). Figure 1.3 shows the image of Mtb bacillus under scanning electron micrograph.

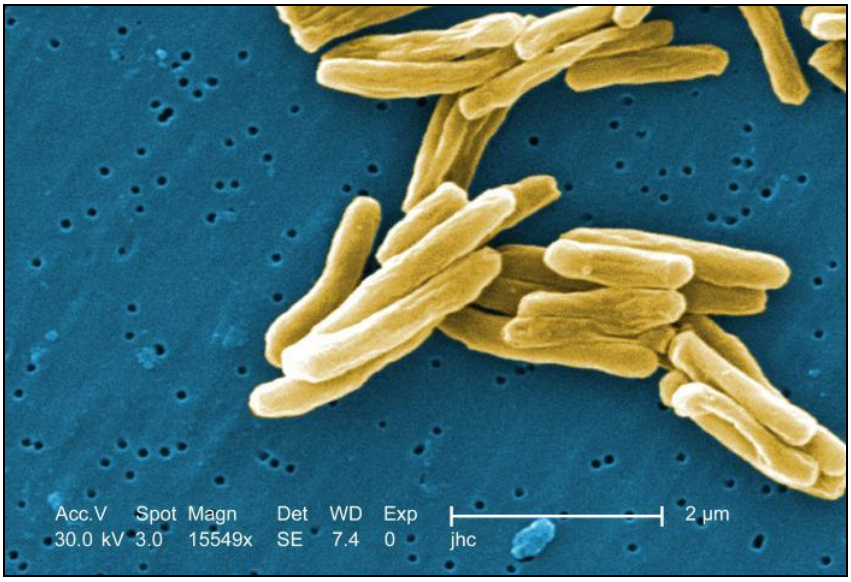


Figure 1.3 Scanning electron micrograph of the Mtb bacillus.

(The picture was adapted from Todar (2012) [Online]. Available from: http://textbookofbacteriology.net/tuberculosis.html).

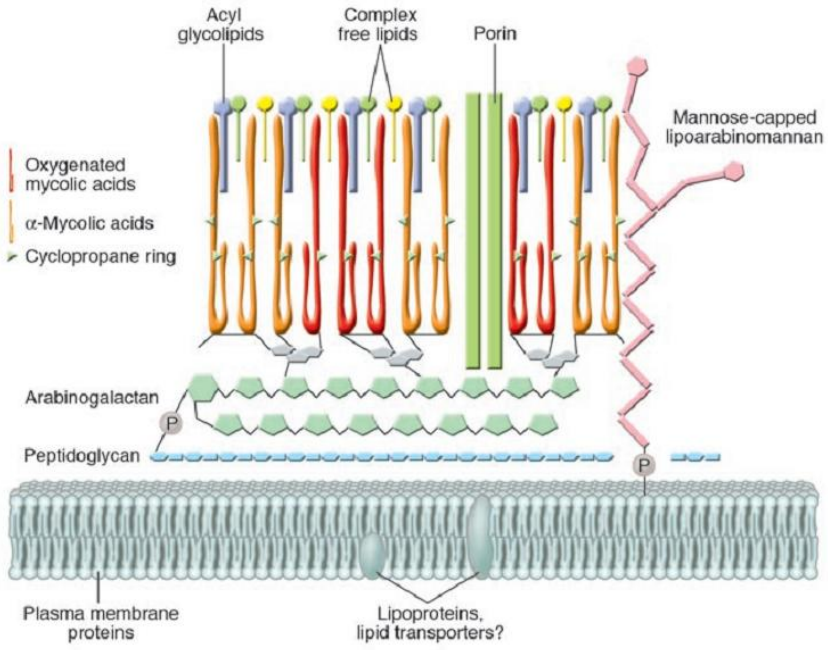


Figure 1.4 Composition of Mtb cell wall (Adapted from Riley, 2006).

The Mtb cell wall structure of Mtb consists of peptidoglycan, peptide side-chains, glycolipid and mycolic acids (Figure 1.4). The peptidoglycan forms the first layer or innermost layer of the Mtb cell wall, which is linked to the arabinogalactan layer and also to mycolic acids. Mycolic acids are strong hydrophobic molecules that form a special lipid barrier or shell around the organism and affect permeability properties at the cell surface (Brennan, 2003; Riley, 2006; Todar, 2012).

Besides mycolic acids, other molecules such as lipoarabinomannan (LAM), lipomannan and 19 kDa lipoprotein are also associated with the cell wall of Mtb. These molecules are able to stimulate host immune responses (Riley, 2006).

Mtb is an obligate aerobe. For this reason, in the classic case of TB, Mtb complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite of macrophages with a slow growth (15-20 hours or longer generation time), a physiological characteristic that may contribute to its virulence (Tortora *et al.*, 2010; Gengenbacher and Kaufmann, 2012; Todar, 2012; Sharma *et al.*, 2013).

1.4.3 Genetics of Mtb

The genome is defined as the whole set of genes within a cell (Ratledge and Stanford, 1982). The entire genome of Mtb H37Rv strain was published by Cole *et al.* (1998) with the complete deoxyribonucleic acid (DNA) sequence and annotation (Figure 1.5). The genetics of Mtb was subsequently studied extensively by Smith (2003).

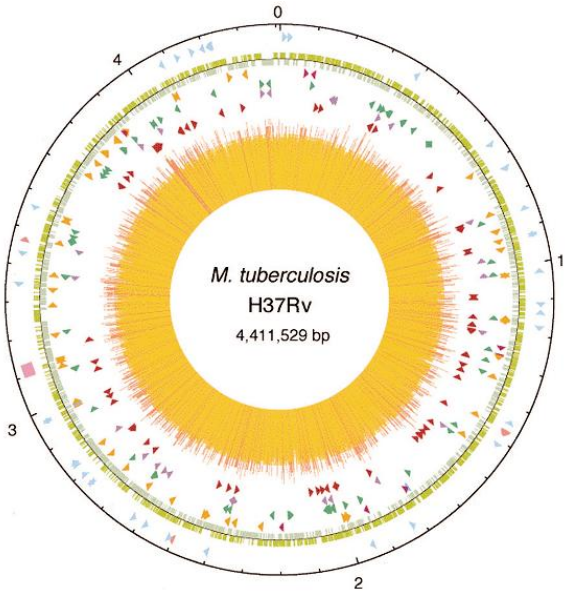


Figure 1.5 Circular map of the chromosome of Mtb H37Rv.

(The picture was adapted from Cole *et al.* (1998), Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685), 537-44.)

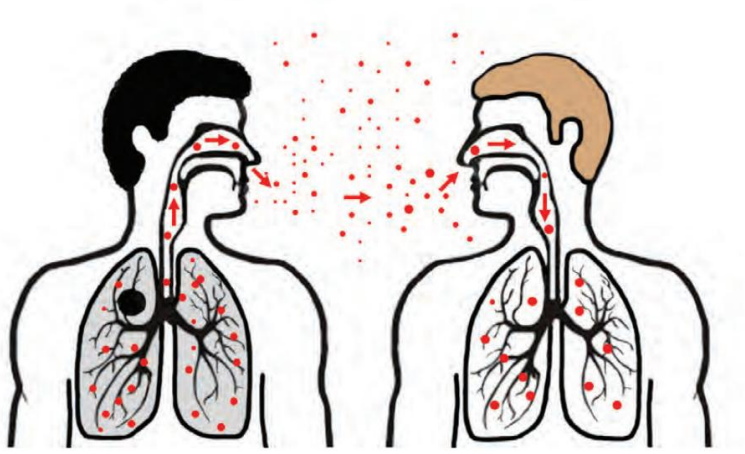


Figure 1.6 TB is spread from person to person through the air. The dots in the air represent droplet nuclei containing germs (Mtb).

(The picture was adapted from CDC (2012) [Online]. Available from : http://www.cdc.gov/TB/TOPIC/basics/default.htm)

According to Smith (2003), the size of a Mtb H37Rv genome is 4.4 X 10^6 bp which consists of approximately 4000 genes. The genome of Mtb has several unique characteristics compared to other bacterium. Mtb has more than 200 genes involved in the metabolism of fatty acids. These various fatty acid enzymes genes are probably associated with the ability of this organism to grow within macrophages where fatty acid is the major carbon source. Mtb genome also contains PE and PPE genes which encode for PE (Pro-Glu) and PPE (Pro-Pro-Glu) proteins located in 2 conserved locations in the N-terminal region of these protein families. These proteins may be involved in virulence and antigenicity of mycobacteria.

1.4.4 Transmission of TB

TB disease is spread by small airborne droplets (particles 1 to 5 μm in diameter) called droplet nuclei. A person with active TB can generate or produce TB germs (Mtb) in the lungs or throat. Those germs are expelled into the air via aerosol by coughing, sneezing, talking or singing (Figure 1.6). The germs (in droplet nuclei) can remain airborne for minutes to hours after expectoration (CDC, 2000; Knechel, 2009; Ahmad, 2011). The number of bacilli in the droplets, the virulence of the bacilli, exposure of the bacilli to UV light, degree of ventilation, and occasions for aerosolization all influence transmission (CDC, 2000). Only people who subsequently breathe these germs into their lungs may become infected (CDC, 2012; Colin, 2014). Those who breathe in tuberculosis germs usually have had very close contact with someone who has the disease (CDC, 2011). Introduction of Mtb into the lungs leads to infection of the respiratory system. However, Mtb can also spread and infect other organs and cause extrapulmonary TB (Knechel, 2009).

1.4.5 Pathophysiology of TB

Studies showed that TB is spread through small or large water droplets containing a single or a few viable bacilli during coughing, sneezing, talking or singing from active TB patients (Palomino *et al.*, 2007). Everybody within the surrounding area can be exposed to the droplets. TB is most commonly acquired when an individual is exposed and inhales droplets containing the viable bacilli (Mtb) (Tortora *et al.*, 2010). After inhalation of tubercle bacilli, the bacilli will enter the alveolar spaces in the lungs which will then be ingested by alveolar macrophages and dendritic cells (van Crevel *et al.*, 2002).

Alveolar macrophages act as the first line of defence against Mtb which can destroy tubercle bacilli. However the destruction of tubercle bacilli depends not only on the microbicidal activity of the alveolar macrophages because the virulence of the tubercle bacilli has an important role in this process too (van Crevel *et al.*, 2002). If the bacteria survive from the destructive machinery of alveolar macrophages, they will multiply slowly (dividing approximately 25 to 32 hr) within the alveolar macrophages which cause the weakening and disruption of the macrophages (CDC, 2000). The disruption of infected alveolar macrophages will attract blood monocytes and other inflammatory cells to the lungs. In the lungs, the blood monocytes will develop into macrophages and readily take in the tubercle bacilli. However the blood-derived macrophages will be unable to destroy the tubercle bacilli (van Crevel *et al.*, 2002). The ingested tubercle bacilli will multiply within the blood-derived macrophages and produce mycobacterial foci in the lungs (Kaufmann, 2004).

While the infected alveolar macrophages remain in the lung tissues, some of the infected dendritic cells will migrate to the draining lymph nodes. In the draining lymph nodes, T cells will be activated and after migration, T cells recognize the

mycobacterial foci in the lung (Kaufmann, 2001). The activated T cells together with macrophages will form a surrounding layer at the infection foci site. Then the surrounding layer will become a lump (granuloma) or walled-off lesion called tubercle. After several weeks, the interior part of the tubercle will become caseous (cheese-like appearance) (Gonzalez-Juarrero *et al.*, 2001; Tortora *et al.*, 2010; Ahmad, 2011). This caseous lesion will inhibit the highly aerobic Mtb from receiving air supply. Accordingly, the growth of Mtb will be restricted and TB infection becomes stationary or dormant until months or even years (van Crevel *et al.*, 2002). This condition is also known as latent TB infection (LTBI).

However, a few patients (with primary infection) may develop active TB disease which will progress rapidly into active pulmonary, extrapulmonary or disseminated disease. Persons susceptible to such a rapid course include those who have compromised immune systems (such as HIV/AIDS patients), children and the elderly (McKinley Health Center, 2008; CDC, 2012). TB disease which develops soon (after infection), seen as initial infection (primary infection) without previous natural contact with Mtb is called primary TB (Palomino *et al.*, 2007). Symptoms of primary TB are generally mild and include low-grade fever (Poulsen, 1950; Poulsen, 1957). However, most of persons with primary pulmonary TB remain asymptomatic (Hoffmann and Churchyard, 2009; Knechel, 2009).

Sometimes, the caseous lesion may become larger and more liquid. This process is called liquefaction. The liquefied lesion will become air filled and provide an aerobic condition within the lesion (Tortora *et al.*, 2010). This aerobic condition favours the growth of tubercle bacilli within the liquefied lesion. At this time point, Mtb will rise from dormancy and multiply extensively within and/or outside of the infected macrophages (Figure 1.7).

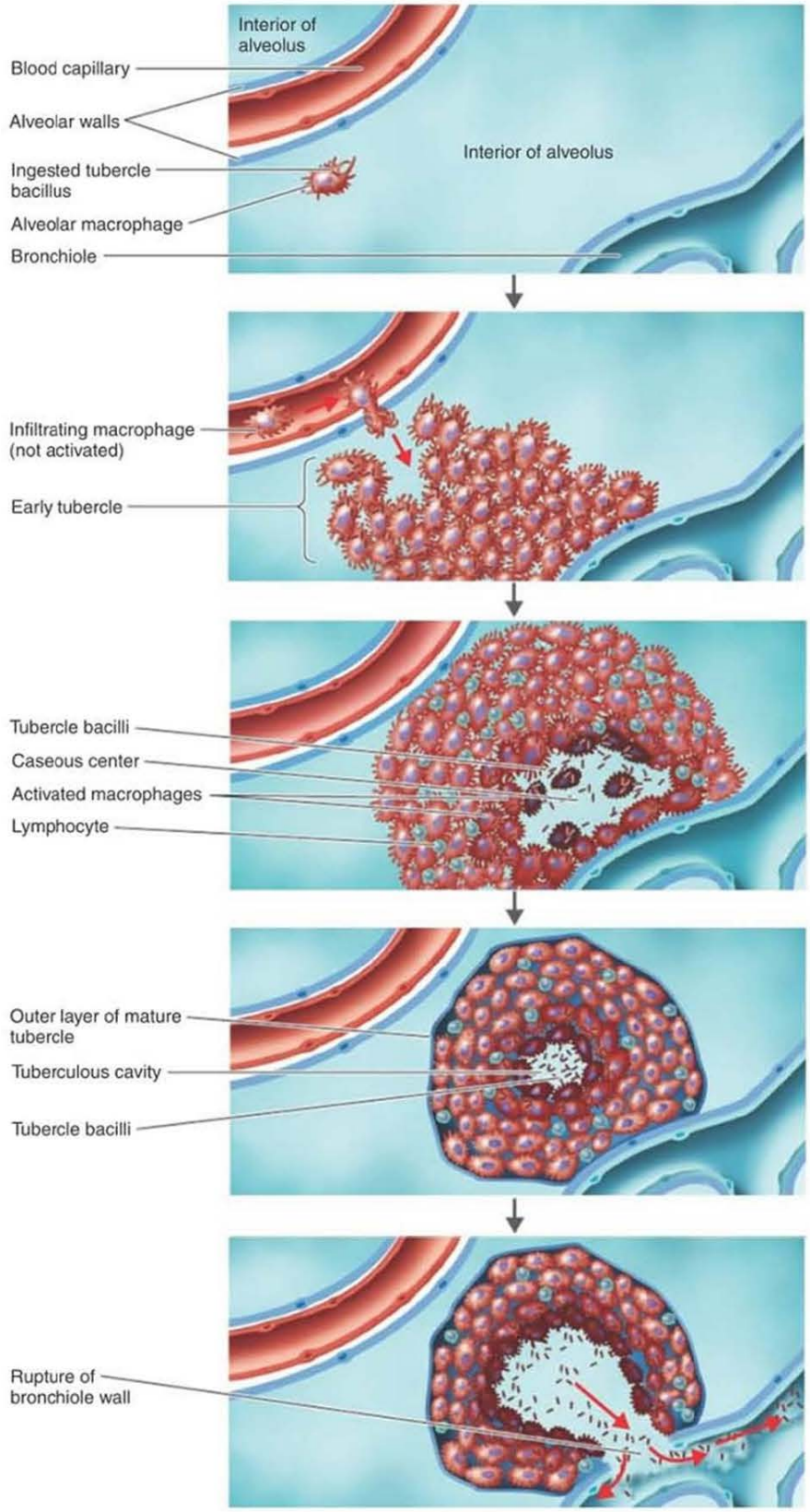


Figure 1.7 The pathogenesis of tuberculosis. This figure represents the progression of the disease when the defenses of the body fail. The picture was adopted from Tortora et al. (2010).

The liquefied lesion may rupture into the bronchioles spreading tubercle bacilli to other parts of the lung and body systems (van Crevel *et al.*, 2002; Tortora *et al.*, 2010). The progression of TB after a period of LTBI is called secondary TB. Secondary TB is also known as post-primary or reactivation TB. Common symptoms of secondary TB include cough, fever, fatigue, night sweats, anorexia, weight loss, and malaise (Hoffmann and Churchyard, 2009; Knechel, 2009; Sia and Wieland, 2011; Delong, 2013).

1.5 Immunity against TB

1.5.1 General View of Immune System in Human

Our environment contains various pathogens such as viruses, bacteria, protozoa, fungi and parasites. These pathogens are able to infect and cause diseases in humans. In normal individuals, most infections are short-lived and cause little permanent damage (Roitt *et al.*, 2001; Roitt *et al.*, 2006). The human immune system recognizes foreign agents and develop an immune response against these agents in order to eliminate them. There are two types of immune responses known as innate and adaptive immune response (Abbas and Lichtman, 2004; Abbas *et al.*, 2010). The innate immune response mediates an early immune reaction against microbes, follows by adaptive immune response (Abbas *et al.*, 2010). This early response involves several components of defence, including cytokine production, phagocytosis by phagocytes (macrophages and neutrophils), complement system and also physical and chemical barriers (Abbas *et al.*, 2010). In contrast, adaptive immune responses involves specific recognition systems mediated by T and B lymphocytes. Adaptive immune response is able to produce long lasting immunity against a specific pathogen upon activation. Thus, adaptive immune response

provides specificity and memory against previously encountered pathogens (Roitt *et al.*, 2006).

1.5.2 Innate Immune Responses against TB

Mtb is an air-borne microorganism, which infects primary lung tissues. The inhalation of Mtb into the alveolar space will activates the alveolar resident macrophages, the cell type involved in the phagocytosis (Smith, 2003; Palomino *et al.*, 2007; Kleinnijenhuis *et al.*, 2011). The phagocytosis of Mtb by resident macrophages is mediated by various receptors expressed on their surface (Palomino *et al.*, 2007; Dheda *et al.*, 2010).

Recognition of Mtb antigens by the immune system is an important step to achieve an effective protective response against these pathogens. Mtb antigens such as LAM, lipoproteins and Mtb DNA are recognized by a set of receptors called as Toll-like receptors (TLRs) (Smith, 2003; Roitt *et al.*, 2006). TLRs are found on the surface of macrophages and dendritic cells (Roitt *et al.*, 2006; Palomino *et al.*, 2007; Kleinnijenhuis *et al.*, 2011). TLRs play a central role in immune recognition of Mtb. The interaction of TLRs with Mtb activates nuclear factor kB of signalling pathways (NF-kB), causing secretion of pro-inflammatory cytokines (such as TNF-α, IL-6, IL-12, IL-18 and IFN-γ), chemokines. This will promote phagocytosis and killing of the Mtb, and antigen presentation by macrophages (Dheda *et al.*, 2010; Kleinnijenhuis *et al.*, 2011). The processed Mtb antigen is further eliminated by T and B cells of adaptive immune response following antigen-presentation by activated macrophages (Roitt *et al.*, 2006).

1.5.3 Adaptive Immunity against Mtb

Adaptive immune response against Mtb begins with three processes of antigen presentation, co-stimulation and cytokine production (van Crevel *et al.*, 2002). Mycobacterial antigen presentation by antigen presenting cell (APC) to T-cells involves three distinct mechanisms. The first mechanism involves extracellular antigen presentation by APC to $CD4^+$ T-cells via major histocompatibility complex (MHC) class II molecules. The second mechanism involves intracellular antigen presentation by APC to $CD8^+$ T-cells via MHC class I molecules. The third mechanism involves the presentation of mycobacterial antigens through Type 1 CD1 (-a, -b, and -c) molecules on the APC to CD1 restricted T cells. CD1 molecules is a non-polymorphic MHC class I molecule expressed on the surface of APC which is able to recognize mycobacterial lipoprotein (van Crevel *et al.*, 2002).

T-cell activation requires cytokines produced by activated macrophages and dendritic cells. Both cells are capable of producing Type 1 cytokines IL-12, IL-18 and IL-23 (Oppmann *et al.*, 2000). T-cell activation may be decreased or delayed if the Type 1 cytokine production is reduced (van Crevel *et al.*, 2002).

Cytokines including IL-12, IL-23, IL-7, IL-15 and TNF-α are released from the infected macrophages and dendritic cells. The effector cytokine, IFN-γ is later secreted by these T-cells together with TNF-α, which in turn activates macrophages that kill intracellular mycobacteria by reactive oxygen and nitrogen immediates (Dheda *et al.*, 2010).

1.6 Control of Mtb Infection

WHO has recommended the DOTS to control Mtb infection in mid-1990s. DOTS strategy includes political commitment, initial diagnosis by sputum-smear

microscopy, short-course treatment, continuous drug supply and systematic monitoring for every patient that started on treatment (Frieden and Driver, 2003). To date, DOTS remains as core elements of the Stop TB strategy (WHO, 2013).

According to the Global TB Control Report 2013, a cumulative total of 56 million TB patients were successfully treated in DOTS programmes between 1995 and 2012. Thus, up to 22 million lives were saved including women and children (WHO, 2013). In Malaysia, Clinical Practise Guidelines (CPG) was introduced by the MOH to assist clinicians and other healthcare providers in making evidence-based decisions on appropriate management and treatment of TB (Ministry of Health, 2012b).

1.6.1 Therapy and Anti-TB Drug Treatment

Anti-TB drug treatment was started in 1944 after the discovery of streptomycin and para aminosalicylic acid (Shatz *et al.*, 1944; Lehmann, 1964; Iseman, 2002; cited in Palomino, 2007). Currently, more than 10 anti-TB drugs are available for the treatment of TB. The most frequently used are Isoniazid, Kanamycin, Pyrazinamide, Ethambutol and Rifampicin. According to Onyebujoh *et al.* (2005), there are 2 main objectives of anti-TB treatment, to kill Mtb living extracellularly in lung cavities and to achieve complete sterilization and elimination of Mtb intracelullarly. Generally, the mechanisms of action for these anti-TB drugs are by killing or inhibiting the growth of Mtb. The recommended treatment consists of 6 months regimens with 2 phases. TB patients are treated with Isoniazid, Rifampicin, Pyrazinamide and either Ethambutol or Streptomycin for the first 2 months intensive phase treatment follow by continuation phase using Isoniazid and Rifampicin for the remaining 4 months (Iyawoo, 2004).

1.6.2 BCG Vaccination

To date, the almost one-century old BCG vaccine remains as the only available vaccine against TB and is widely administered worldwide (Luca and Mihaescu, 2013). Calmette and Guerin developed the BCG vaccine which consists of a live attenuated strain derived from *M. bovis* in 1908-1920 (at Pasteur Institute in Lille). The BCG was then administrated to humans during the 1920s and showed high protective efficacy against TB infection (Sakula, 1983).

1.7 TB Diagnosis

1.7.1 Conventional Methods

1.7.1.1 Microscopy

Direct sputum smear microscopy is the main conventional technique used in most diagnostic laboratories for decades (WHO, 2010c; UNITAID, 2012). The method of choice is Ziehl-Neelsen staining but fluorescent acid-fast staining has also been used (Toman, 2004b). The Ziehl-Neelsen method still remains the mainstay of point of care (POC) diagnosis in most TB endemic countries (Dheda *et al.*, 2013).

According to Laszlo (1999), this method is rapid and has great value especially in the detection of active infectious cases. However, it has limited sensitivity, requiring at least 10^5 bacilli per millilitre of specimen for positive detection (Toman, 2004a). Moreover it cannot distinguish Mtb from other non-tuberculous mycobacteria (NTM) (Hobby *et al.*, 1973; WHO, 2010b). In addition, this technique is also ineffective for the diagnosis of extra-pulmonary TB and requires well-trained and motivated technicians (WHO, 2006; UNITAID, 2012).

1.7.1.2 Culture

Active TB infection is defined when the organism is isolated from specimens using microbiological culture. The culture method remains as the diagnostic "gold standard" for both diagnosis and drug sensitivity testing (DST) of TB (Laszlo, 1999; Guillerm *et al.*, 2006; WHO, 2011a). This technique can identify the Mtb organism in over 80% of TB cases with a specificity of over 98% (WHO, 2006). The sensitivity of culture is much higher than microscopy, requiring just 10 to 100 bacilli per millilitre of specimen (Laszlo, 1999). The most commonly used solid media in the laboratory are egg-based media (Lowenstein Jensen (LJ), Ogawa) and the agar-based growth medium (Middlebrook 7H10, 7H11) (Guillerm *et al.*, 2006; Palomino *et al.*, 2007). However, this conventional method takes 2 to 6 weeks to obtain culture results and requires highly trained personnel (WHO, 2006). New rapid commercial techniques based on liquid and broth growth systems such as BACTEC 460TB, BACTEC MGIT 960, MB/BacT, SeptiChek-AFB, ESP Culture II System and many others were introduced in order to improve the detection time as well as to increase recovery. Many laboratories reported an average time for detection of 1 to 4 weeks using these systems (Rom and Garay, 2004; WHO, 2006; Anochie *et al.*, 2012). Although the broth or liquid based growth systems are much better than conventional culture in terms of sensitivity and time of detection, it still has limitations because they require much higher capital and operating cost (Guillerm *et al.*, 2006; WHO, 2006). Furthermore, the only well-established rapid method for detecting mycobacteria in clinical specimens is the BACTEC 460TB system. However this method uses a radioactive component, thus introduces concerns related with waste disposal (Laszlo, 1999).

Recently, new rapid non-commercial microcolony culture based methods of Microscopic Observation Drug Susceptibility (MODS) and Thin Layer Agar (TLA) have been reported (Guillerm *et al.*, 2006). Both methods appear to be rapid, inexpensive and accurate tools for active TB detection. However, a systematic review on both methods reported that they did not find sufficient evidence on the feasibility and costs of implementation, nor on the impact of these methods on patient outcomes (Leung *et al.*, 2012). Other than that, both methods require well-trained personnel and appropriate containment facilities.

1.7.1.3 Biochemical tests

A battery of biochemical tests are used for the identification of mycobacteria (Rom and Garay, 2004). According to Babady and Wengenack (2012), there are several well-standardized techniques of conventional biochemical tests. These include niacin accumulation, nitrate reduction, detection of pyrazinamidase, activity inhibition of thiophene-2-carboxylic acid hydrazide (T_2H), iron uptake, urease production, catalase test, Tween 80 hydrolysis, tellurite reduction, and many others. Although these conventional biochemical assays are relatively inexpensive and simple to perform, they still have limitations especially due to the fact that the test can only be performed after primary culture are successful (which already requires incubation periods of up to 4 weeks) resulting in major delays in identification.

1.7.1.4 Radioghraphy

Chest radiography is fast, convenient and used widely for the detection of pulmonary TB. This method is sensitive in the diagnosis of pulmonary TB but of low specificity, especially in HIV infected patients (WHO, 2009). The conventional chest X-ray is the most common used radioghraphic method for screening, diagnosis, and treatment response follow-up in pulmonary TB cases. The more sensitive method to use is

chest computed tomography (CT), especially the high resolution CT, as it is able to detect early parenchymal lesions or enlargement of mediastinal lymph nodes and in determination of disease activity in TB (Lange and Mori, 2010). This technique could be useful and may be cost-effective for the management of patients. However the main drawback is its nonspecificity, resulting in over diagnosis when used alone. Furthermore, the whole capital and operational cost are considered highly expensive (WHO, 2006; Lange and Mori, 2010).

1.7.2 Nucleic Acid Amplification Test (NAAT)

The NAAT for the diagnosis of TB or to detect drug resistance is a sensitive method that can produce a much faster result than conventional culture methods (Dheda *et al.*, 2013). The most common amplification techniques widely used are polymerase chain reaction (PCR), strand displacement amplification (SDA) and transcription mediated amplification (TMA) (WHO, 2006). PCR was originally described by Mullis and Faloona in 1987. PCR is a nucleic acid based technique that uses oligonucleotide primers to amplify specific DNA target sequences. DNA is amplified by repeated cycles of DNA denaturation, annealing of primer to the DNA and primer extension (Guatelli *et al.*, 1989). Because of specificity of the designed primers, PCR has become a powerful means to detect small numbers of bacterial DNA present in clinical specimens. In comparison to PCR, SDA is an isothermal amplification of DNA sequence. It is based on the ability of a *Hincll* restriction enzyme to nick a hemi-modified recognition site and the ability of a polymerase to displace a downstream DNA strand during replication (Down *et al.*, 1996; Roth *et al.*, 1997). TMA is also an isothermal process that can amplify DNA and Ribonucleic acid (RNA) targets to a billion fold in less than 1 hour's time. The method employs 2 enzymes to perform the reaction which consists of RNA polymerase and reverse

transcriptase. The entire reaction is performed in a water bath at the same temperature. Thus, this technique also does not require a thermal cycler to perform the test (Roka Bioscience, 2013).

According to Dheda *et al.* (2013), there are several commercially available NAAT techniques for TB diagnosis from a few companies. These includes (i) PCR based NAAT; Amplicor MTB, Cobas Amplicor, and LightCycler Mycobacterium Detection kits (Roche, USA); Genotype MTBDR*plus* and GenotypeMTBDR*sl* (Hain Lifescience, Germany), INNO-LiPA Rif TB line probe assays (Innogenetics, Belgium); and Xpert MTB/RIF nested real-time PCR assay (Cepheid), (ii) SDA based; BD-ProbeTec-ET (Becton Dickinson, USA), (iii) TMA based; Amplified *M. tuberculosis* Direct (AMTD) (Gen-probe, USA). The Amplicor MTB test (Roche, USA) and AMTD test (Gen-probe, USA) techniques have been approved by the United States Food and Drug Administration (FDA) for use in Mtb detection procedure (Babady and Wengenack, 2012; UNITAID, 2012; Delong, 2013; Sharma *et al.*, 2013). In general, the application of NAAT has created some very sensitive and specific diagnostic tools for TB. The principal advantages of NAAT is that a result can be obtained in only a few hours and the test process can be adapted for high- or low-throughput screening depending upon demand. However, these techniques require well-trained personnel to perform the test. In addition, due the cost of the instrument and high operational cost, this technique is not feasible to be used in many low income setting areas (WHO, 2006).

1.7.3 Immunological Tests

1.7.3.1 Tuberculin Skin Test (TST)

TST is the method used for screening suspected TB patients since 1930s (MMWR, 1982; Rossman and MacGregor, 1995; CDC, 2003). Several methods were