STUDIES ON THE ACTIVITY OF ISONIAZID DERIVATIVES AND THEIR COMBINATIONS WITH OTHER ANTI-TB DRUGS ON *MYCOBACTERIUM TUBERCULOSIS*

by

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Thesis submitted in fulfillment of the

requirements for the degree

of Master of Science

April 2012

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude to Assoc. Prof. Dr. Pazilah Ibrahim and Prof. Dr. Amirin Sadikun, whom I am fortunate to have them as my supervisors, for their admirable guidance, encouragement and motivation for making this thesis a reality. I would also like to thank Madam Suriyati Mohamad for her valuable guidance during the laboratory work.

I am also grateful to my lab mates; Naveen, Popi, Adilah, Nabihah, Ang, and Anis for creating sweet memories to cherish and making life more amusing as well as meaningful in TB Lab. I offer my good luck and best wishes for their future undertakings.

Not forgetting, truthful gratitude to my family members specially my parents for their encouragement and tolerance with me all these years. No words can ever be expressed for my gratefulness and love for you, mom and dad.

I also would like to express my warmest appreciation to Veterinary Research Institute (Bukit Tengah, Pinang), Electron Microscope Unit (School of Biological Sciences, USM) and Multimedia Unit (School of Pharmaceutical Sciences, USM) for their generous help in the microscopy studies. Last but not least, this work would not have been possible without the funding provided by Funding Research Grant Scheme (FRGS) (203/PFARMASI/671157) and USM Fellowship Scheme.

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LIST OF ABBREVIATIONS

µg/mL	:	Micro gram per milliliter
CFU/mL	:	Colony forming unit per milliliter
CO_2	:	Carbon dioxide
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EMB	:	Ethambutol
ETL	:	Electron transparent layer
FAS	:	Fatty acid synthesis
FIC	:	Fractional inhibitory concentration
g/mol	:	Grams per mole
INH-NAD	:	Isoniazid-nicotinamide adenine dinucleotide
INH-C6	:	1-isonicotinoyl-2-hexanoyl hydrazine
INH-C7	:	1-isonicotinoyl-2-heptanoyl hydrazine
INH-C8	:	1-isonicotinoyl-2-octanoyl hydrazine
INH-C9	:	1-isonicotinoyl-2-nonanoyl hydrazine
INH-C10	:	1-isonicotinoyl-2-decanoyl hydrazine
INH-C11	:	1-isonicotinoyl-2-undecanoyl hydrazine
INH-C12	:	1-isonicotinoyl-2-dodecanoyl hydrazine
INH-C13	:	1-isonicotinoyl-2-tridecanoyl hydrazine
INH-C14	:	1-isonicotinoyl-2-tetradecanoyl hydrazine
INH-C15	:	1-isonicotinoyl-2-pentadecanoyl hydrazine
INH-C16	:	1-isonicotinoyl-2-hexadecanoyl hydrazine
INH-C17	:	1-isonicotinoyl-2-heptadecanoyl hydrazine
INH-C18	:	1-isonicotinoyl-2-octadecanoyl hydrazine
INH	:	Isoniazid
mRNA	:	Messenger ribonucleic acid
MIC	:	Minimum inhibitory concentration
NADP	:	Nicotinamide adenine dinucleotide phosphate
O_2	:	Oxygen
PBS	:	Phosphate buffer saline
PM	:	Plasma membrane
PTL	:	Peptidoglycan layer
PZA	:	Pyrazinamide
RIF	:	Rifampicin
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal ribonucleic acid
STR	:	Streptomycin
TEMA	:	Tetrazolium microplate assay
°C	:	Degree celcius

Kajian tentang aktiviti terbitan isoniazid dan gabungannya dengan drug anti-tibi yang lain ke atas *Mycobacterium tuberculosis*

ABSTRAK

Sel membran yang kurang telap terhadap drug anti-tibi yand berciri hidrofilik terutamanya isoniazid (INH), merupakan salah satu faktor penting yang menyumbang kepada ketahanan intrinsik dalam *Mycobacterium tuberculosis*. Banyak kajian menghipotesiskan bahawa aktiviti anti-mikobakteria bagi INH dapat ditingkatkan dengan menjadikannya sebatian hidrofobik/lipofilik. Berasaskan hipotesis ini 13 terbitan yang berbeza hidrofobisitinya disintesis melalui penambahan rantai asil hidrofobik kepada INH. Kajian ini bertujuan untuk mengkaji aktiviti anti-tibi terbitan-terbitan tersebut dan saling tindak mereka dengan drug-drug anti-tibi yang lain seperti INH, streptomisin (STR), rifampisin (RIF), dan etambutol (EMB). Kajian ini juga bertujuan mengkaji aktiviti terbitan INH yang berpotensi terhadap kehidupan *M. tuberculosis* H37Rv dan perubahan morphologi sel pada fasa kitaran hidup yang berlainan. Morfologi sel *M. tuberculosis* H37Rv ATCC 25618 semasa kitaran pertumbuhan didapati lebih baik dikaji menggunakan medium agar kerana peringkat pertumbuhan dalam medium ini lebih jelas lagi berbanding dengan medium cecair. Aktiviti anti-tibi terbitan-terbitan ini telah dikaji dengan menentukan kepekatan perencatan minimum (MIC) terhadap *M. tuberculosis* H37Rv menggunakan "tetrazolium microplate assay" (TEMA). Antara terbitan yang diuji, 1-isonikotinoil-2-heksadekanoil hidrazina (INH-C16), 1-isonikotinoil-2-heptadekanoil hidrazina (INH-C17) dan 1-isonikotinoil-2-oktadekanoil hidrazina (INH-C18) mempunyai nilai MIC dua kali ganda lebih rendah daripada INH. Justeru itu, saling tindak terbitan-terbitan tersebut

dengan drug anti-tibi yang lain telah dikaji menggunakan kaedah "fixed-ratio isobologram". INH-C16 menunjukkan saling tindak yang sangat baik dengan STR dan RIF. Berdasarkan penemuan ini, INH-C16 telah dipilih untuk kajian selanjutnya ke atas sel. Keputusan yang diperolehi menunjukkan bahawa *M. tuberculosis* sangat sensitif terhadap INH-C16 pada fasa permulaan, logaritma dan kematian dalam kitaran hidupnya. Pemerhatian di bawah mikroskop cahaya menunjukkan bahawa sel-sel yang dirawat dengan INH-C16 cenderung untuk membentuk kelompok dan hilang sifat ketahanan asid pada fasa permulaan, logaritma dan pegun. Sel-sel yang dirawat kemudian dilihat di bawah mikroskop elektron penskanan dan transmisi. INH-C16 menyebabkan dinding sel pecah lalu menyebabkan kehilangan integriti sel justeru menyebabkan sel-sel lisis dan mati. Pemerhatian ini mencadangkan bahawa mekanisma tindakan INH-C16 mungkin melalui perencatan sintesis asid mikolik. Maka, ia boleh disimpulkan bahawa INH-C16 mempunyai potensi untuk penyelidikan selanjutnya sebagai drug anti-tibi.

Studies on the activity of isoniazid derivatives and their combinations with other anti-TB drugs on *Mycobacterium tuberculosis*

ABSTRACT

The low permeability of the cell envelope against hydrophilic anti-tuberculosis (TB) drugs especially isoniazid (INH), is one of the crucial factors that contribute to the intrinsic resistance in *Mycobacterium tuberculosis*. Previous studies have floated the idea that the anti-mycobacterial activity of INH can be enhanced by augmenting the hydrophilic INH into a hydrophobic/lipophilic compound. Therefore, a series of 13 derivatives with different hydrophobicity were synthesized by adding different hydrophobic acyl chain to the parent compound, INH. The current study was aimed to investigate the anti-TB activity of these derivatives and their interactions with the first-line anti-TB drugs such as INH, streptomycin (STR), rifampicin (RIF) and ethambutol (EMB). The study was also aimed to elucidate the activity of potential hydrophobic INH derivative on the viability of *M. tuberculosis* H37Rv at the cellular level at different phases of the growth cycle. The cellular morphology of *M. tuberculosis* H37Rv ATCC 25618 during the growth cycle was best studied using solid medium as the growth stages were well defined compared to the liquid medium. The anti-TB activity of these derivatives was carried out by determining the minimum inhibitory concentration (MIC) against *M. tuberculosis* H37Rv using a tetrazolium microplate assay (TEMA). Among the derivatives tested, 1-isonicotinoyl-2-hexadecanoyl hydrazine (INH-C16), 1-isonicotinoyl-2-heptadecanoyl hydrazine (INH-C17) and 1-isonicotinoyl-2-octadecanoyl hydrazine (INH-C18) were shown to have two-fold lower MIC value than INH. Their interactions with first-line anti-TB drugs were then

performed using a fixed-ratio isobologram method. INH-C16 showed most promising interaction with STR and RIF. Based on these findings, INH-C16 was selected for further studies on the cells. The results obtained indicated that *M. tuberculosis* H37Rv was most susceptible to INH-C16 at the lag, log and death phase of the growth cycle. Observation under light microscope indicated that INH-C16 treated cells tend to form clumps and lost their acid-fastness properties at lag, log and stationary phase. The treated cells were further viewed under scanning and transmission electron microscope. INH-C16 caused the cell wall to rupture that made the cells to lose their cellular integrity and ultimately led to cell lysis and death. These observations suggest that the action mechanism of INH-C16 could be via the inhibition of mycolic acid synthesis. Hence, it could be concluded that INH-C16 has the potential to be a drug lead worthy of further investigations.

SECTION 1

INTRODUCTION

1.1 PROBLEM STATEMENT

Mycobacterium tuberculosis is a resilient human pathogen which causes tuberculosis (TB). According to the World Health Organization (WHO), there were 8.8 million TB cases in 2010 globally (WHO, 2011b). During the same year, a total of 1.45 million people died of the disease, of which, 0.35 million were human immuno-deficiency virus (HIV) positive patients. Moreover, the emergence of multidrug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) render the control of TB even more difficult. In 2008, around 390 000 - 510 000 MDR-TB incidence cases were reported worldwide with 150 000 deaths (WHO, 2010c). In Malaysia, it was reported that the incidence rate of TB was around 63.95 cases per 100 000 population and the mortality rate was 5.49 death per 100 000 population in 2008 (Ministry of Health Malaysia, 2009). Apparently, TB had caused more death than any other communicable infectious diseases such as dengue, hand, food, and mouth diseases etc. This suggests that urgent attention should be given to circumvent this harrowing phenomenon.

The modern, standard short-course therapy for TB recommended by WHO is based on a combination of at least three first-line anti-TB drugs regimen that relies on direct observation of patient compliance to ensure effective treatment (WHO, 2003). TB patients receive combination therapy instead of mono therapy because the latter can also delay the emergence of drug resistant *M. tuberculosis* strains and reduce the resistant effects to existing drugs (Cynamon, 2001). While TB is curable, MDR-TB may be fatal and the management of MDR-TB is more complicated,

costlier, and needs highly skilled medical personnel (da Silva and Aínsa, 2007). MDR-TB also demands treatment with second-line drugs that are often less effective, more toxic and expensive. Hence, the urgent need to develop new drugs which are more effective, less toxic and cheaper than the present anti-TB agents cannot be overemphasized.

Among the first-line anti-TB agents, isoniazid (INH) is the most prominent drug which is exquisitely potent with a minimum inhibitory concentration (MIC) ranging from 0.02 to 0.2 µg/mL (Makarov *et al.*, 2009). However, in the last decade, the number of INH resistant *M. tuberculosis* strains isolated from TB patients had been increasing at an alarming rate (WHO, 2010b). One of the intrinsic factors contributing to INH resistance in *M. tuberculosis* is the underlying architecture of the bacterial cell envelope (Rastogi *et al.*, 1981, Rastogi *et al.*, 1986, Rastogi and Goh, 1990, Jarlier and Nikaido, 1994, Brennan and Nikaido, 1995). Unlike other bacteria, the cell wall of *M. tuberculosis* is double-layered, comprising of an inner electron-dense layer of peptidoglycan and an outer electron-transparent layer containing mycolyl arabinogalactan complex covalently bound to the peptidoglycan (Takade *et al.*, 2003, Dahl, 2004). The outer layer of *M. tuberculosis* functions as an exclusion barrier towards hydrophilic drugs, such as INH (Rastogi and Goh, 1990, Brennan, 2003). As a hydrophilic compound, INH molecules diffuse into the bacteria via the protein channel known as porin (Liu *et al.*, 1999). This penetration via mycobacterial porin had been shown to be very slow because it is inefficient in allowing the permeation of solutes and also exists in low concentration (Faller *et al.*, 2004). Thus, the cell wall structure and INH penetration through the lipid domain provide opportunities for rational anti-TB drug design strategies focused on drug lipophilicity.

Previous studies have emphasized that chemical modifications of INH by increasing its lipophilic property resulted in enhanced activity of INH against *M. tuberculosis* (Rastogi and Goh, 1990, Bardou *et al.*, 1996, Billington *et al.*, 1998, De Logu *et al.*, 2002). A study by Mohamad *et al.* (2004) also reported that a lipophilic INH derivative, 1-isonicotinyl-2-nonanoyl hydrazine (INH-C9) had a lower MIC value compared to INH. Furthermore, this observation was also supported by molecular modelling study that INH derivatives with longer acyl chain have greater binding affinity to InhA enzyme which plays a crucial function in mycolic acid biosynthesis in *M. tuberculosis* cell wall (Qamar, 2004). On the basis of these antecedents, the major objectives of the current study is to further prove and validate the hypothesis that the anti-TB activity of INH could be enhanced by augmenting it into a hydrophobic compound. In addition, the interaction between the potential derivatives and common first-line anti-TB drugs was also studied. Finally, the effects of the potential lipophilic derivative on *M. tuberculosis* H37Rv cells at the cellular level were also observed.

1.2 GENUS *MYCOBACTERIUM*

Genus *Mycobacterium* is classified as an ancient genus because it is assumed that this genus was originated more than 150 million years ago (John, 1984). Theoretically, the name "myco" which means fungus was given by their special characteristic to exhibit filamentous growth in liquid medium (Tortora *et al.*, 1989).

The taxonomy lineage of genus *Mycobacterium* is shown in Figure 1.1. The prominent member of this genus is *Mycobacterium tuberculosis* which is an obligate human pathogen (Saviola and Bishai, 2006). Its variants or subtypes *Mycobacterium africanum* and *Mycobacterium canetti* are also pathogenic to human. *Mycobacterium*

bovis and *Mycobacterium microti* cause TB in animals and can be transmitted to humans (Barrera, 2007). *Mycobacterium caprae* and *Mycobacterium pinnipedii* were isolated and identified from TB-infected goats and seals (Barrera, 2007).

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	***M. tuberculosis***
	M. africanum
	M. microti
	M. canetti
	M. caprae
	M. bovis
	M. pinnipedii

Figure 1.1 Taxonomy lineages of TB agents (Source : Barrera, 2007)

Bacteria from this genus are Gram-positive, aerobic, non-motile, non-sporulate, and long, slender, straight, or curved rod-shaped with a slight tendency to be filamentous or branching (Tortora *et al.*, 1989, Saviola and Bishai,

2006, Barrera, 2007, Talaro, 2008). These microorganisms also show a unique staining characteristic known as an acid-fastness property (Madigan and Martinko, 2006). This characteristic is due to high lipid content in their cell wall, probably the highest among all the bacteria. This lipid is known as mycolic acid (Saviola and Bishai, 2006, Talaro, 2008). The mycolic acid content imparts to cell wall hydrophobicity which renders immunological properties, resistance against wide range of antimicrobial agents and to injuries, and also contributes to the slow growth rate in some of mycobacterial species (Barrera, 2007).

Generally, this genus can also be further divided into fast growers and slow growers. The mycobacteria are categorized as fast growers if they are able to form visible colonies on selective media in less than 7 days, whereas slow growers take more than 7 days (Saviola and Bishai, 2006). Table 1.1 shows the list of fast growers and slow growers of the genus *Mycobacterium*.

Table 1.1 List of fast growers and slow growers of the genus *Mycobacterium*

Fast growing *Mycobacterium*	Slow growing *Mycobacterium*
M. fortuitom	*M. triviaic*
M. farginogenes	*M. simae*
M. senegalense	*M. genavense*
M. chelorne	*M. interjectum*
M. penegrum	*M. intermedium*
M. neaurum	*M. terrar*
M. diernhoferi	*M. hibernine*
M. abscessus	*M. nonchromogenicum*
M. chitae	*M. cookii*
M. fullax	*M. xenopi*
M. aurum	*M. celatum*
M. vaccae	*M. gordonae*
M. confluentis	*M. asiaticam*
M. madagascariense	***M. tuberculosis* complex**
M. flavescens	*M. marinum*
M. smegmatis	*M. leprae*
M. thermoresistible	*M. scrofmlaceni*
M. phlei	*M. gratrif*
	M. kansasii
	M. szugni
	M. malmacuse
	M. intracellulars
	M. paratuberculosis
	M. avium

(Source : Saviola and Bishai, 2006)

Mycobacteria can also be grouped based on their ability to form yellow, orange, or pink carotenoid pigments (Madigan and Martinko, 2006, Talaro, 2008). The pigmentation can be photochromogenesis (forming pigment only when cultured in the light, such as *M. kansasii* and *M. marinum*), scotochromogenesis (forming pigment when cultured in the dark for example *M. gordonae*), or nonpigmented (such as *M. tuberculosis* and *M. bovis*) (Madigan and Martinko, 2006).

Occasionally, the members of this genus are clustered into complexes based on genetic similarities, similar disease syndrome and identical 16S ribosomal ribonucleic acid (RNA) gene sequence (Gillespie, 2006), such as *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex. *M. tuberculosis* complex comprises of human pathogenic microorganisms such as *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. caprae*, and *M. pinnipedii* (Saviola and Bishai, 2006, Barrera, 2007). The members of this complex are also known as "tubercle bacilli" (Collins *et al.*, 1984). Meanwhile, *M. avium* complex is composed of *M. avium* and *M. intracellulare* which can cause opportunistic and disseminated infection in AIDS patients (Inderlied *et al.*, 1993).

Thus, it can be concluded that the genus *Mycobacterium* is medically pivotal because this genus consists of a number of human pathogenic species of bacteria that can cause severe diseases in human which may lead to fatality.

1.3 *MYCOBACTERIUM TUBERCULOSIS*

1.3.1 Classification

M. tuberculosis is a well-studied and medically most important member of the *M. tuberculosis* complex (Collins *et al.*, 1984). It is a nonpigmented, obligate human pathogen and very seldom identified in other mammals (Madigan and

Martinko, 2006, Saviola and Bishai, 2006). It does not have a significant environmental reservoir and it is only transmitted from person to person (Saviola and Bishai, 2006).

The genomic studies of *M. tuberculosis* H37Rv revealed that it consisted of 4 411 529 base pairs with very high guanine plus cytosine (G + C) content and 4 000 genes (Cole *et al.,* 1998). The study also highlighted that large portion of the genes were coded for; (1) production of enzymes involved in lipogenesis and lipolysis which makes the *M. tuberculosis* differ radically from other bacteria and, (2) glycine-rich proteins with a repetitive structure that functions as a source of antigenic variation of *M. tuberculosis* H37Rv (Cole *et al.,* 1998).

1.3.2 Microscopic morphology

Microscopically, the tubercle bacilli are observed as straight or slightly curved rods. The bacilli are 1 - 10 µm in length (usually 3 - 5 µm) and 0.2 - 0.6 µm in width (Barrera, 2007). The bacilli also exhibit heterogeneity population where the cells may be different in sizes and shapes (from coccobacilli to long rods) that depends on the growth condition and age of the culture (Barrera, 2007, Thanky *et al.*, 2007). Thanky *et al.* (2007) reported that extensive heterogeneity occurred during the exponential phase of the growth cycle where the cells were actively participating in cell division and elongation process at this time. They elucidated that as the cells enter the stationary phase, the cells become shorter due to reduction of nutrient in the culture. This causes biosynthetic processes to slow down in the older culture that leads to a mixture of cells.

It has also been reported that the actively multiplying bacilli are strongly acid-fast and tend to form hydrophobic bundles known as serpentine cord formation

(Barrera, 2007). The serpentine cord formation is defined as rope-like aggregates of bacterial cells in position parallel to the long axis of the cord as shown is Figure 1.2.

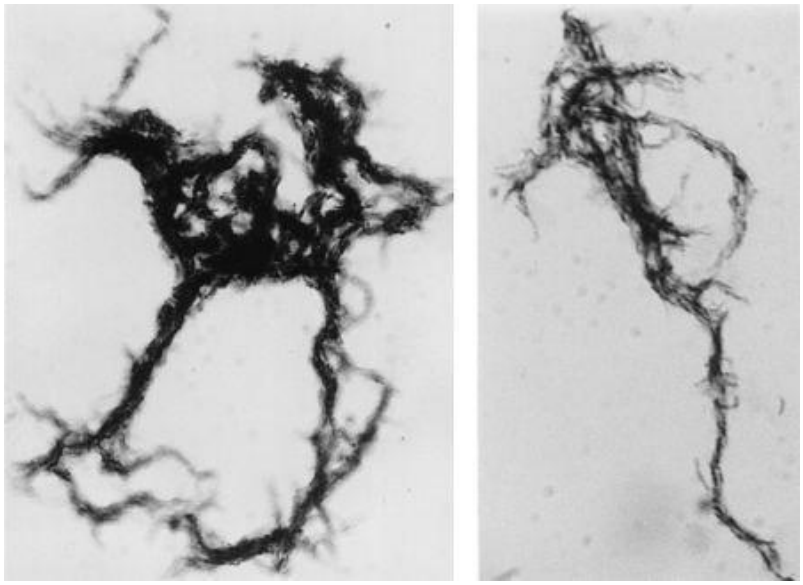

Figure 1.2 Microscopic morphology of *M. tuberculosis* growth exhibiting serpentine cording (Source: Attori *et al.*, 2000).

The cord formation is often associated with virulence of *M. tuberculosis* because this characteristic was found to be very specific (97.3 to 99.5 %) for *M. tuberculosis* (Yagupsky *et al.*, 1990). Several studies had reported that this morphology could be used as a presumptive identification tool to differentiate between *M. tuberculosis* and other mycobacterial species (Yagupsky *et al.*, 1990, Morris and Reller, 1993, González *et al.*, 1998, Attorri *et al.*, 2000, Tu *et al.*, 2003). In a related study, Noll *et al.* (1956) showed that this distinctive microscopic morphology of tubercle cells are attributed to trehalose 6, 6'-dimycolate (TDM) which is an extractable glycolipid made of two mycolic acid molecules and one molecule of disaccharide trehalose which are loosely bound in the outer layer of the

cell wall. It had been reported that this glycolipid displays usual toxicity and virulence factors of *M. tuberculosis* (Behling *et al.*, 1993) as well as contributing to the innate macrophage response during *M. tuberculosis* infection (Indrigo *et al.*, 2002). Moreover, the TDM composition also differs among the strains of mycobacterium due to the difference in the mycolic acid moieties between the strains (Behling *et al.*, 1993).

1.3.3 Colony morphology

Mycobacterium strains are known to behave differently in regard to their gross colonial morphology, the medium and their pathogenicity. For instance, Will *et al.* (1951) reported that on egg medium, virulent tubercle bacilli produce a dry waxy friable colony that crumbles when rubbed with inoculum loops while, avirulent cells produce firm compact colonies that do not crumble and easily emulsify in fluid. They also reported that on sodium oleate agar both virulent and avirulent colonies were wrinkled, flat, spreaded, and corded. On the other hand, the colonies were cream-coloured, buff and rough on the Lowenstein-Jensen (LJ) medium (WHO, 1998, Monteiro *et al.*, 2003). Several studies suggested that these various colonial morphologies are due to the presence of a class of surface antigens so called lipids (Fregnan *et al.*, 1962), glycopeptidolipids (Barrow and Brennan, 1982) or lipooligosaccharides (McNeil *et al.*, 1989).

1.3.4 Growth

Under favorable laboratory conditions, the generation time of *M. tuberculosis* is 18 to 24 hours on stationary cultures and 10 to 12 hours on shake cultures (Harshey and Ramakrishnan, 1977). The clinical specimens were often at lag phase

for three to four weeks before sufficient growth is observed (Saviola and Bishai, 2006). This is extremely slow compared to other cultivable bacteria. Hence, this explains the need for a longer time of incubation to acquire visible colonies *in vitro*. Eventually, this also makes early detection of the disease cumbersome.

The most commonly used mediums for growth assessment of *M. tuberculosis* are egg-based medium (e.g. LJ medium) and agar-based medium (e.g. Middlebrook 7H10 and Middlebrook 7H11 supplemented with glycerol and enriched with oleic acid, albumin, dextrose, and catalase (OADC)) as well as liquid medium such as Middlebrook 7H9 supplemented with glycerol and enriched with albumin, dextrose and catalase (ADC) (Saviola and Bishai, 2006). These media contain a variety of inorganic salts which provide necessary nutrients for the healthy growth of mycobacteria. In addition, these media also contain malachite green to reduce contamination by partially inhibiting other normal floral bacteria. The enrichment media contain albumin which endow protection against toxic agents and therefore, enhances the growth of mycobacterial cells (Middlebrook, 2010).

The growth of *M. tuberculosis* cells is also highly dependent on the physical condition of the environment such as temperature, pH, oxygen concentration, and salinity. In general, *M. tuberculosis* is a mesophile (organism that shows optimum growth at temperature ranging from 25 to 40 °C) and neutrophile (organism that grows best around pH 7) (Madigan and Martinko, 2006, Barrera, 2007). The cells also require oxygen as a final electron acceptor in aerobic respiration. So, the tubercle cells grow most successfully in the tissues rich with oxygen such as in the lungs especially at the well-aerated upper lobes (Barrera, 2007). It is hypothesized that at the lowest oxygen condition the tubercle cells enter the stationary phase and become dormant (Wayne, 1982). A research by Wayne and Lin (1982) showed that

when the *M. tuberculosis* cells were grown in liquid medium without agitation, they adapted to microaerophilic condition (oxygen at a level lower than that in the air) in the sediment and the cells at this sediment did not replicate but were tolerant of anaerobiosis. Hence, this implies that low oxygen concentration plays an important role in mycobacterial dormancy and adaptation to anaerobiosis which perhaps would be a feature of persisting tubercle bacilli (Cunningham and Spreadbury, 1998). Cunningham and Spreadbury (1998) also showed that the tubercle bacilli adapt to low oxygen conditions by developing a thickened cell wall and expression of a unique 16 kilo Dalton protein which confers an advantage to the bacilli during its dormant phase by stabilizing as well as protecting cell structures.

Even though tubercle bacilli grow best at the optimum condition as mentioned earlier, yet it can still withstand condition quite extreme from those environments. For instance, the tubercle bacilli can survive in the mild acidic and alkaline microenvironment. This is because in *in vivo* environment, the cells encounter an acidic environment in the lungs, stomach and macrophage phagosome yet, the cells are able to survive (Cotter and Hill, 2003). Based on this idea, sodium hydroxide, an alkaline solution was used to decontaminate clinical specimens of TB which inactivates the contaminating microfloral bacteria in the sample with only modest inhibitory effects against mycobacteria (Saviola and Bishai, 2006). Besides, the cells can also withstand very low temperature seeing that the stock culture can be preserved at temperature as low as -70 °C for 2.5 to 5 years (Kim and Kubica, 1972, Kim and Kubica, 1973, Kubica *et al.,* 1977). Researchers also found that the cells frozen at this temperature have 100 % viable bacterial suspension, metabolic activities were stable, inoculums was highly reproducible for animal experiments, genetic constituents were maintained, as well as with minimal clonal selection of

undesirable mutants (Kim and Kubica, 1972, Kim and Kubica, 1973, Kubica *et al.*, 1977).

1.3.5 Cell wall: structure and permeability

Structure: Figure 1.3 represents the structure of mycobacterial cell envelope. Unlike other bacteria, the cell envelope of *M. tuberculosis* consists of three main sections: plasma membrane, cell wall and outer capsule like layer (Crick *et al.*, 2001, Barrera, 2007). The uniqueness of the cell envelope of *M. tuberculosis* solely relies on its cell wall architecture as it plays a prominent role in the pathogenicity of the disease (as discussed in Section 1.3.7), immunological reactions of host to mycobacteria and gives the cells acid-fastness characteristics (Barksdale and Kim, 1977, Liu *et al.*, 1999). In addition, many work have also highlighted that the architecture of the cell wall is one of the intrinsic factors that contributes to the drug resistance in *M. tuberculosis* (Rastogi and Goh, 1990, Lisowska *et al.*, 1996, Chatterjee, 1997, Liu *et al.*, 1999, Brennan, 2003, Barkan *et al.*, 2009).

Studies have shown that the cell wall of mycobacteria is composed of three covalently linked major core compounds;

Peptidoglycan: It functions as a backbone of cell wall and protects the microorganism from degradation and is made of alternating units of N-acetylglucosamine and N-glycolyl muramic acid (Chatterjee, 1997, Crick *et al.*, 2001, Alderwick *et al.*, 2007),

Arabinogalactan: It comprised of arabinan and galactan where the arabinan constitutes of branched pentaarabinofuranosyl and galactan consists of 5- and 6-linked galactofuranose (Chatterjee, 1997, Crick *et al.*, 2001, Alderwick *et al.*, 2007), and

Mycolic acid: It is an exquisitely long (up to 90 carbon atoms) α-branched and β-hydroxylated fatty acids and it consists of one-third dry mass of the cell wall (Faller *et al.*, 2004). It is undeniable that mycolic acid is a biologically important component that plays a vital role in viability and cell wall structure (Barkan *et al.*, 2009). It is also involved in the formation of trehalose dimycolate (which is known as cord factor which implicates the pathogenesis of *M. tuberculosis*) and trehalose monomycolate (acts as a precursor for arabinan-linked mycolates) (Chatterjee, 1997, Jackson *et al.*, 2007).

In brief, the arabinogalactan chains covalently bond to cross-linked peptidoglycan via phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units and then the arabinogalactan in turn is esterified to α-alkyl, β-hydroxy mycolic acids (Crick *et al.*, 2001, Alderwick *et al.*, 2007).

On the other hand, observation under transmission electron microscopy (TEM) revealed that the cell wall contains two distinct layers;

(1) **An inner electron-dense layer** of peptidoglycan (Paul and Beveridge, 1992, Takade *et al.*, 2003, Dahl, 2004). The thickness, electron density and appearance of this layer varied depending on the species, growth conditions and methods of preparation for microscopy (Liu *et al.*, 1999).

(2) **An outer electron-transparent layer** containing mycolyl arabinogalactan complex covalently bound to the peptidoglycan (Paul and Beveridge, 1992, Takade *et al.*, 2003, Dahl, 2004). This layer appears to be extremely hydrophobic in nature due to the mycolyl arabinogalactan (Liu *et al.*, 1999). Hence, it is hypothesized that the outer layer could hinder the diffusion of chemotherapeutic agents across the cell wall thus causing drug resistance by exclusion barrier (Rastogi *et al.*, 1981).

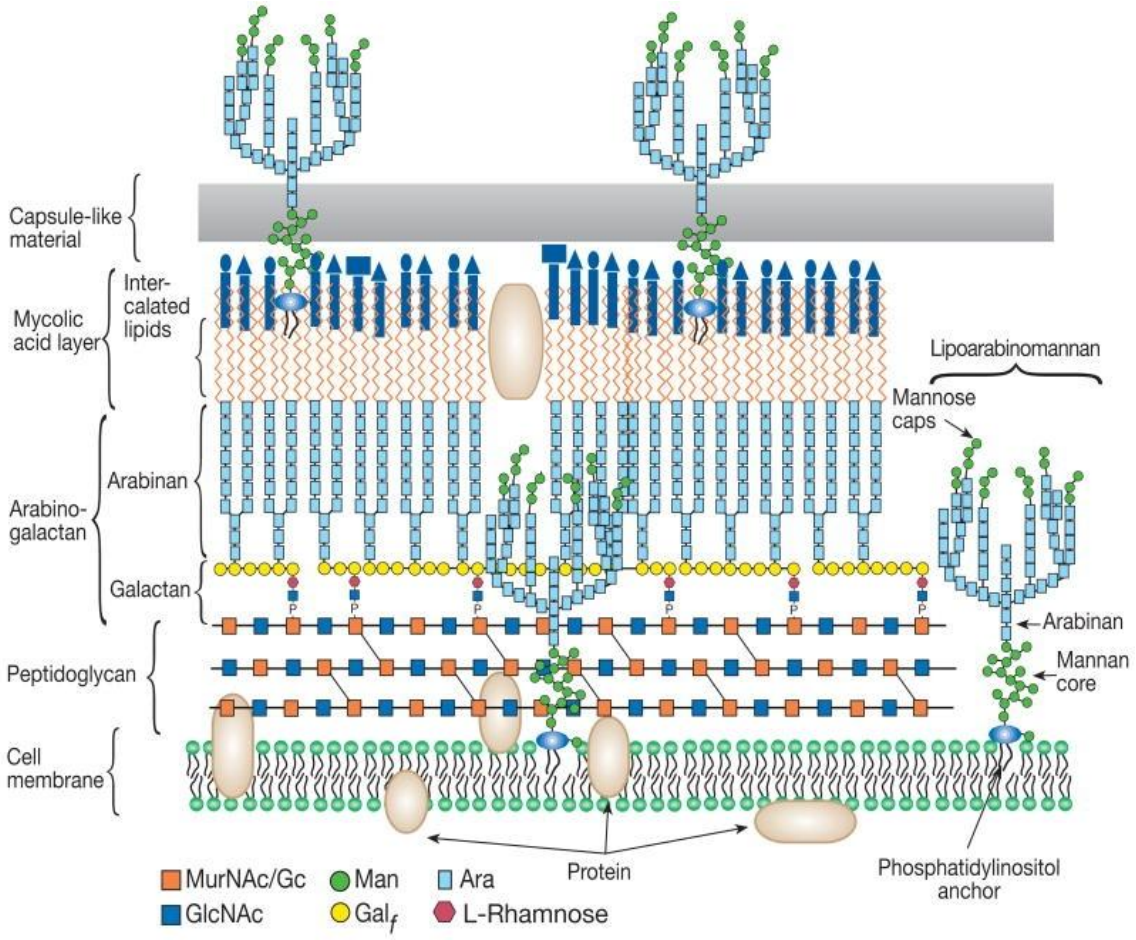

Figure 1.3 Structure of the cell wall of mycobacteria (Source: http://www.ncbi.nlm.nih.gov/books/NBK1945/, 2011)

Permeability: In an earlier review by Minnikin (1982), it was proposed that mycolic acids in the outer cell wall layer were packed side by side in a perpendicular direction to the plane of the surface. It was also suggested that this mycolic acid-containing layer was covered by an outer leaflet, composed of extractable lipids (exclusively of lipopolysacharides) and an inner leaflet, composed of phospolipids. Hence, the whole structure produced an asymmetric lipid bilayer (Minnikin, 1982). It is expected that the inner leaflet to have very low fluidity and mobility that contribute to less permeable outer bilayer membrane (Plésiat and Nikaido, 1992, Yuan *et al.*, 1997, Liu *et al.*, 1999). This assumption was supported by previous findings of Rastogi *et al.* (1990) that drug susceptibility of mycobacterium can be enhanced by inhibitors of cell envelope synthesis such as m-flourophenylalanine (an inhibitor of mycoside-C biosynthesis) and ethambutol (an inhibitor of arabinogalactan). On the basis of this justification several studies (Jarlier and Nikaido, 1994, Faller *et al.*, 2004) suggested that the lipid domain of cell wall apt to be the source of impermeability in mycobacteria as this could act as an effective barrier for penetration of anti-mycobacterial agents.

In 1987, David *et al.* showed that lipophilic drugs such as ansamycin, rifampicin, clofazimine, and pristinamycin were active against *M. avium* strain tested. In subsequent studies by Rastogi *et al.* (1988) and Rastogi and Goh (1990), it was also shown that amphipathic INH derivatives have better efficacy against *M. avium* than the parent hydrophilic compound, INH. They surmised that these derivatives possibly increased the miscibility or liposolubility of the drugs in the lipid outer layer and interact with the amphipathic substance of the outer layer. Thus, this enables the drugs to easily penetrate through the cell wall to make it active against bacteria. In a recent study, Mohamad *et al.* (2004) also reported that a

lipophilic derivative of INH, 1-isonicotinyl-2-nonanoyl hydrazine (INH-C9) had a lower MIC value compared to INH. A similar positive correlation between lipophilicity and the anti-mycobacterial activity was also found among other classes of drug. For instance, Heifets *et al.* (1990) demonstrated that more hydrophobic derivatives of rifamycins, such as rifabutin, rifapentine, CGP-7040, KRM-1648, and T9 have enhanced activity against *M. avium* and *M. tuberculosis*. In addition, Franzblau and White (1990) findings also showed that more hydrophobic flouroquinolones were active against *Mycobacterium leprae*.

On the basis of these antecedents, it is very obvious that the diffusion of lipophilic compounds occurs mainly through the lipid bilayer domains of the outer cell wall layer by solubilised in the amphipathic outer layer. Furthermore, Liu *et al.* (1999) explained that the penetration rate of hydrophobic compounds also depends on the two factors: (1) the compounds should have positive correlation with either theirol/water or octanol/water partition coefficients, and (2) high temperature coefficient because the fluidity of the lipid interior is temperature dependent.

On the other hand, the hydrophilic molecules diffused into the bacteria via the protein channels known as porin (Niederweis, 2003). Jarlier and Nikaido (1990) showed that small hydrophilic nutrient molecules (such as glucose, glycerol, glycine, and leucine) diffuse through the porin channels. They also demonstrated that these porins were neither dependent on the hydrophobicity of the molecules nor the temperature. This again strongly suggests the hydrophilic pathway via porins for permeation of hydrophilic molecules. This observation ascertained by Kartmann *et al.* (1999) who showed that *M. tuberculosis* contains two types of porins which is a 15-kiloDalton (kDa) cation-selective that has short-lived open state and a

more than 60-kDa long-lived open state, which is mimicking the porins from fast-growing mycobacteria.

1.3.6 Antimicrobial susceptibility testing

The diagnosis and the performance of *in vitro* susceptibility tests against clinically significant *M. tuberculosis* endow essential information for effective TB treatment. It also allows early detection of drug resistant TB cases that lead to an initiation of appropriate treatment and surveillance of drug resistance (Martin and Portaels, 2007). In the past, these tests were known as conventional methods. Basically, the conventional methods are laborious and require longer duration to obtain the results. The current drastic development of new technologies and reagents had led to development of various simple and short turnaround time testing methods including phenotypic and genotypic methods.

Conventional methods

The proportion method is a golden standard method in studying the susceptibility of *M. tuberculosis*. Several dilutions of standard bacterial suspension were inoculated on the drug-containing and drug-free (control) plates and incubated. The colonies in both set of plates were then enumerated and proportion of resistance is calculated (Martin and Portaels, 2007). Usually, a strain is considered resistant if the proportion of resistance is higher than 1 %. This method is relatively very simple and cheap, yet cumbersome because it requires longer incubation period (3 - 4 weeks) to obtain the results (Varma *et al.*, 2002).

The Absolute concentration method is conducted via inoculating microorganism at 2×10^3 to 1×10^4 colony forming unit (CFU) into media incorporated with and

without antimicrobial agents. The interpretation of the test is performed after at least 4 weeks of incubation. A strain is categorized as resistant if there is greater than a certain number of CFU at a particular concentration (usually around 20 CFU) (Inderlied, 1991, Martin and Portaels, 2007).

The resistant ratio method is quite similar to absolute concentration method except that another set of tubes are inoculated with a standard *M. tuberculosis* H37Rv strain and the results are interpreted in terms of the ratio of the MIC of drug against tested *M. tuberculosis* strain to the standard *M. tuberculosis* H37Rv (Inderlied, 1991). An isolate considered susceptible if the resistance ratio value 2 or less, while a resistance ratio of 8 or more defines the isolate as resistant (Martin and Portaels, 2007).

The radiometric method is also known as BACTEC Method. This method is based on the ability of mycobacteria to catabolize radioactively labeled ^{14}C fatty acids to carbon dioxide end product that can be detected and quantified by radiorespirometric method (Middlebrook *et al.*, 1977). The major advantage of this method is the capacity to detect drug resistance faster (an average ranged from 4.2 to 6.9 days) compared to other conventional methods (Roberts *et al.*, 1983). However, it requires expensive apparatus set up and proper radioactive waste disposal (Martin and Portaels, 2007).

The mycobacterial growth indicator tube (MGIT). It is performed on the basis of fluorescence detection where Middlebrook 7H9 broth with an oxygen quenching-based fluorescent sensor is used. The growth of organism in the medium will cause the fluorescent dye to illuminate under UV light. This method is shown to be rapid (average 9.9 days), easy to handle and does not require additional costly instrumentation (Pfyffer *et al.*, 1997, Somoskovi and Magyar, 1999).

Genotypic methods

Desoxyribonucleic acid (DNA) sequencing is based on sequencing the gene responsible for certain trait and then comparing the sequence with the reference gene sequence to detect the mutation that gives resistance. Even though this is a rapid method, it cannot be conducted routinely because it is labor intensive and expensive (Hazbon, 2004).

Solid-phase hybridization techniques is based on reverse hybridization of amplified DNA from culture and immobilized on a nitrocellulose or DNA strip and then, the presence of mutation can be visualized by colorimetric reaction in the form of band/s on the strip (De Beenhouwer *et al.,* 1995, Makinen *et al.,* 2006). This is a very rapid method because it does not require the growth of microorganism. The test can be performed directly on the sputum or biopsy samples (De Beenhouwer *et al.,* 1995).

Real time Polymerase Chain Reaction (PCR) techniques. In this method the susceptibility of a strain is detected in regarding to gene mutation with the aid of probes such as TaqMan probe, Fluorescence Resonance Energy Transfer probes, molecular beacons and biprobes (Shamputa *et al.,* 2004). The foremost advantages of this method are the rapidity of the test and lower risk of contamination. Yet, it requires highly skilled personnel and expensive reagents and equipment making this technique not feasible in most laboratories and hospitals (Martin and Portaels, 2007).

Microarrays are based on hybridization of DNA (from samples) to biochips with oligonucleotides immobilized on a solid support such as polyacrylamide gel pads (Gryadunov *et al.,* 2005). As with other genotypic methods, this technique is also not feasible in most clinical mycobacteriology lab especially in third-world countries as it involves high cost and technology (Martin and Portaels, 2007).

Phenotypic methods

The nitrate reductase assay is based on the ability of viable *M. tuberculosis* to reduce nitrate to nitrite in the medium with aid of a chemical reagent. Firstly, the cells will be exposed to a medium incorporated with chemical reagent and antibiotic at a specific concentration for 10 days. The resistant cells that reduce the nitrate cause a pink-red colour to be observed in the medium. The susceptible cells on the other hand will be inhibited where by the colour change will not be observed (Angeby *et al.*, 2002).

Colorimetric methods. The determination of susceptibility is based on the reduction of redox indicators or tetrazolium salts such as alamar blue or tetrazolium salt. The cells will be incubated with drugs *in vitro* in a range of concentration and then followed by the addition of an indicator. The resistant cells will cause the color change of the indicator. On the other hand, the susceptible cells will be inhibited and produce no colour change. The advantages of this method are that it is cheap, high-throughput, rapid, and feasible in any microbiology lab since it does not require any expensive apparatus.

Phage-replication technology. This method is performed with the aid of mycobacteriophage, a lytic virus that is able to infect mycobacteria including *M. tuberculosis* and *Mycobacterium smegmatis*. Basically, the susceptibility is determined via the formation of clear zones known as plaques by the phage infected tubercle cells within the bacterial lawn. This simple test also can be used for the diagnosis of TB (McNerney, 2001).

1.3.7 Transmission and pathogenesis

TB is an air borne disease. The predominant route of transmission of TB is by the small airborne droplets known as droplet nuclei, created through coughing, sneezing, talking, or singing by an untreated person suffering from pulmonary or laryngeal TB. It can also be generated by aerosol treatments, sputum induction, aerosolization during bronchoscopy, and by manipulation of lesions or processing of tissues or secretions in the hospital or laboratory (American Thoracic Society, 2000). These infected droplets can also remain in any indoor space for minute to hours (Knechel, 2009).

Once inhaled, these droplet nuclei are very small enough to reach the alveoli of the lungs where the microorganisms replicate (American Thoracic Society, 2000). This will develop into infection of the respiratory system known as pulmonary TB. The *M. tuberculosis* cells are also able to spread to other organs such as lymphatics, pleura, bone or joints, or meninges and cause extrapulmonary TB (Knechel, 2009).

With regard to pathogenicity, *M. tuberculosis* has shown to infect a variety of immunological cell populations such as macrophages, neutrophil leukocytes, mast cells, dentritic cells, and natural killer cells. Within them, macrophages especially alveolar macrophages are considered as the most important target of *M. tuberculosis* infection (Hernandez-Pando *et al.*, 2007). Hence, the paradigmatic interaction during this infection is briefly discussed in this section.

Once the tubercle bacilli in droplet nuclei reached the alveoli, it is quickly surrounded and engulfed by alveolar macrophages (McDonough *et al.*, 1993). Following that, *M. tuberculosis* resides in the phagosome as an endocytic vacuole (Armstrong and Hart, 1975, Smith, 2003, Warner and Mizrahi, 2007). Phagosome-lysosome fusion will then take place where the cells within the

macrophages will be disclosed to multiple microbicidal mechanisms such as production of both reactive oxygen and nitrogen species, acidic pH, lysosomal enzymes, and toxic peptides to kill or inhibit the growth of mycobacteria (Smith, 2003, Schluger, 2005). If the bacillus is able to survive these initial attacks, it will further multiply within the alveolar macrophages (American Thoracic Society, 2000, Knechal, 2009). At the same time, macrophages also initiate the production of proteolytic enzymes and cytokines as a further attempt to degrade these pathogenic microorganisms. The release of cytokines consequently attracts T lymphocytes to the infected site. This is done by the presentation of *M. tuberculosis* antigens on the surface of macrophages for recognition of T lymphocyte cells (Knechel, 2009). This initial mediated immune response prolongs for 2 to 12 weeks. During this time the tubercle cells also continuously grow until they reach 10^3 to 10^4 in number which is sufficient to elicit cell-mediated immune response (American Thoracic Society, 2000, Knechel, 2009).

In persons with intact cell-mediated immunity, the next defensive step is the formation of granulomas around the *M. tuberculosis* cells (Segovia-Juarez *et al.*, 2004, Schluger, 2005). These granulomas are formed through accumulation of activated T lymphocyte cells and macrophages, which creates a microenvironment that limits the further replication and spread of tubercle cells (American Thoracic Society, 2000, Knechel, 2009). This environment causes destruction of macrophages by apoptosis and produces early solid necrosis at the center of the lesion (Schluger, 2005). However, some *M. tuberculosis* cells are still able to adapt this environment by mobilization of repair or detoxification pathway, or through changing the phenotypic pathways such as protein regulation to enhance their survival (Warner and Mizrahi, 2007). Within two to three weeks time the necrotic quiescence forms a

caseous necrosis with low oxygen level, low pH, and limited nutrient. Thus, this condition limits the growth of bacteria and leads to latency. These lesions might also undergo fibrosis and calcification which controls the tubercle bacilli in a dormant condition (Knechel, 2009). This condition is called latent TB where the disease is not active and infectious thus cannot be transmitted (Jasmer *et al.*, 2002). However, certain diseases could lead to reactivation of these dormant cells such as silicosis, diabetes mellitus and disease associated with immunosuppressant (e.g. HIV infection) as well as through consumption of immunosuppressive drugs such as corticosteroid (American Thoracic Society, 2000, WHO, 2011a).

On the other hand, in a weaker immune system person the granuloma formation fails to trap or contain the bacteria and this causes the necrotic cells to undergo liquefaction. Thus, the bacilli are able to escape and spread to other alveoli and other organs which eventually, will lead to primary progressive TB (Knechel, 2009).

1.3.8 Clinical manifestation (Symptoms and signs)

The pulmonary system is the most common infection site of TB (known as pulmonary TB) involving lung parenchyma. It mainly occurs through the aerosol route and the symptoms are persisting cough, chest pain, sputum production, fever, night sweats, and hemoptysis (blood stained sputum) in severe conditions (Saviola and Bishai, 2006). Other signs of pulmonary TB include loss of appetite and weight, dyspnoea (difficulty in breathing), chest pain, and hoarseness of voice as well as abnormalities in the chest x-ray (Ministry of Health Malaysia, 2002).

Extrapulmonary TB is tubercle infection that occurs outside the lungs (Davies, 2011). The risk of extrapulmonary TB is very high among immune

suppressed individuals especially patients with HIV infection and also among immunocompromised individuals such as in very young or old individuals (Saviola and Bishai, 2006). The main reason for this scenario is the failure of their immune response to contain tubercle cells which enables hematogenous dissemination and consequently leads to involvement of single or multiple non-pulmonary sites of infection (American Thoracic Society, 2000). There are only about 15 % probability of extrapulmonary TB in immunocompetent person and it is also attributed to reactivation from latent extrapulmonary (Saviola and Bishai, 2006, Knechel, 2009).

One of the prominent constraints of extrapulmonary TB is the diagnosis. This is because it involves rarely accessible sites of infection and small numbers of bacilli which makes diagnosis difficult (American Thoracic Society, 2000). The systemic symptoms of extrapulmonary TB are often non-specific, including lassitude, anorexia, fever, and drastic weight loss (Ministry of Health Malaysia, 2002). Usually, the specific feature of extrapulmonary disease depends on the organs involved. The commonest sites of infection and its clinical manifestations are summarized as follows:

1) Disseminated TB or military TB is considered as a most fatal form of extrapulmonary TB due to the infection in the blood (Knechel, 2009). Hence, the bacilli will spread throughout the body as the infected blood goes and lead to multi organ infection (Wang *et al.*, 2007). The diagnosis of disseminated TB is also very complicated because the clinical manifestations are very confounding and nonspecific. Common symptoms would be fever, night sweats, abdominal pain, cough, weight loss, and aneroxia. The conditions of the organs involved are hepatomegaly, lymphadenopathy, and draining sinuses (Andres and Tan-Alora, 2001).