A SIMPLE *IN VITRO* BIOASSAY PROTOCOL AGAINST LATENT PHASE MYCOBACTERIAL CELLS

LAI HUNG WEI

UNIVERSITI SAINS MALAYSIA

2016

A SIMPLE *IN VITRO* BIOASSAY PROTOCOL AGAINST LATENT PHASE MYCOBACTERIAL CELLS

By

LAI HUNG WEI

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Microbiology with Honours

JUNE 2016

ACKNOWLEDGEMENTS

I owe the completion of this thesis to my supervisor, Dr Suriyati Mohamad and my co-supervisor, Assoc. Prof. Dr Pazilah Ibrahim. The entire progress of this project until the submission of this thesis would not be successful without their encouragement, guidance and support. I would like to take this opportunity to express my uppermost gratitude to them for their willingness to sacrifice their time, money and energy on me with patience, dedication and kindness throughout my entire project. It is an honour to be one of their students.

I would also like to thank all the staff of School of Biological Sciences especially Madam Nurul Huda, Mr. Khairul Nasiruddin and Mr. Muttalib Ismail for their continuous time and effort in providing technical support to me in terms of methodology, preparation of media, and sterilization of materials. Many thanks to my fellow seniors and friends: Ms. Liyana Jamaluddin, Ms. Azirah Inez, Ms. Rasyidah Asilah, Ms. Nur Najihah Ismail, Mr. Chong Zhen Yee and Mr. Benedict Lian Shi Xiang for their assistance, guidance and professional advice during the course of this project.

Last but not least, credits should also be given to my family and friends. Without their constant support, love and tolerance, completing my master thesis would certainly remain a dream. Each and every one mentioned above are my inspirations in the completion of my master research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVATIONS

µL	: Microlitre
°C	: degree Celsius
ADC	: Albumin/ Dextrose/ Catalase
AIDS	: Acquired immunodeficiency syndrome
ATP	: Adenosine triphosphate
$BaCl_2$	: Barium chloride
CFU	: Colony forming unit
CO_2	: Carbon dioxide
g	: Gram
HIV	: Human immunodeficiency virus
HSR	: Headspace ratio
L	: Litre
MABA	: Microplate alamar blue assay
MB7H10	: Middlebrook 7H10
MB7H9	: Middlebrook 7H9
MDR	: Multiple drugs resistant
MHC	: Major histocompatibility complex
MIC	: Minimum inhibitory concentration
mL	: Millilitre
MTT	: 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide
NAD	: Nicotinamide adenine dinucleotide
NADH	: Nicotinamide adenine dinucleotide (reduced)
O_2	: Oxygen

OADC	: Oleic Acid/ Albumin/ Dextrose/ Catalase
PBS	: Phosphate Buffered Saline
p mole	: Pico mole
SEM	: Scanning electron microscope
TB	: Tuberculosis
TEMA	: Tetrazolium microplate assay
WHO	: World Health Organization

LIST OF APPENDICES

PROTOKOL MUDAH BIOASAI *IN VITRO* TERHADAP SEL MIKOBAKTERIA FASA PENDAM

ABSTRAK

Pembangunan ubat-ubatan baru terhadap tuberkulosis (TB) terencat disebabkan oleh kompleksiti protokol makmal asai pendam *in vitro*. Oleh itu, tujuan utama kajian ini adalah untuk menambahbaik protokol TB pendam yang mudah dengan memanipulasi parameter-parameter pertumbuhan biasa dengan menggunakan empat spesies *Mycobacterium* tumpang, iaitu *M. smegmatis, M. fortuitum, M. kansasii,* and *M. tuberculosis* H37Ra. Pengoptimuman pertumbuhan fasa awal dengan memanipulasi ketersediaan nutrien, keadaan pengeraman, kandungan O_2, dan pengudaraan sistem kultur telah dijalankan dalam sistem kultur kelompok selama 10 - 12 hari. Hasil kajian menunjukkan bahawa penambahan 10% suplemen pengayaan dalam sistem kultur yang statik pada persekitaran sub-anaerobik 4% CO_2, dan kandungan O_2 sebanyak 0.25 nisbah "headspace" menyebabkan sel mikobakteria memasuki fasa pertumbuhan pendam. Manipulasi pertumbuhan ini juga melanjutkan tempoh fasa pendam kepada 5 - 6 hari berbanding dengan sel kawalan yang menjalani pertumbuhan normal. Replikasi parameter yang telah dioptimumkan dalam sistem piring mikro 96-perigi untuk digunakan dalam asai pelbagai sampel, didapati berupaya menghasilkan tempoh fasa pendam yang lebih panjang. Penilaian kadar metabolisme sel-sel fasa pendam yang diukur melalui paras adenosina 5'-trifosfat dengan menggunakan asai luciferase biopendarkilau telah menunjukkan penurunan dalam nilai-nilai pendarkilau sebanyak 1.6 – 2.3 kali ganda berbanding spesies *Mycobacterium* dalam fasa log. Pemerhatian

morfologi struktur luar sel pendam di bawah mikroskop elektron imbasan menunjukkan bahawa sel ini dalam keadaan sihat dan utuh tanpa pembentukan septat. Ini menunjukkan bahawa mereka masih hidup tetapi tidak menjalani replikasi aktif. Akhir sekali, protokol untuk aktiviti terhadap mikobakteria fasa pendam telah ditambahbaik dengan menyesuaikan prosedur asai kolorimetri piring mikro dengan menggunakan tetrazolium bromida sebagai reagen redoks. Protokol ini diuji dengan menggunakan drug TB yang telah diketahui. Aktiviti sel pendam ini dinilai berdasarkan nilai kepekatan perencatan minimum drug itu dan dibandingkan dengan aktiviti sel fasa log. Protokol baru yang dihasilkan untuk mengukur kerentanan drug terhadap spesies *Mycobacterium* pendam telah berjaya ke tahap tertentu, yang boleh dilihat dari segi perbezaan nilai MIC antara fasa log dan pendam *M. smegmatis* dan *M. fortuitum*. Walau bagaimanapun, protokol itu tidak menunjukkan aktiviti yang sama terhadap *M. tuberculosis* H37Ra pendam. Ini mencadangkan penambahbaikan protokol asai ini masih diperlukan. Kesimpulannya, hasil kajian ini menunjukkan bahawa parameter pertumbuhan normal pada kadar yang sesuai memang boleh mendorong sel mikobakteria memasuki fasa pertumbuhan pendam yang lebih panjang. Oleh itu, dengan menggunakan simulasi ini, suatu protokol makmal yang mudah dapat dikembangkan untuk menyukat aktiviti ejen berpotensi terhadap TB pendam.

A SIMPLE *IN VITRO* BIOASSAY PROTOCOL AGAINST LATENT MYCOBACTERIAL CELLS

ABSTRACT

Development of new drugs against latent tuberculosis (TB) is partly hindered by the complexity of laboratory protocol for dormant *in vitro* assay. Therefore, the main aim of this study was to improvise a simple protocol for latent TB by manipulating normal growth parameters using four surrogate *Mycobacterium* species: *M. smegmatis, M. fortuitum, M. kansasii,* and *M. tuberculosis* H37Ra. Initial growth optimizations by manipulating nutrient availability, incubation condition, O_2 content, and aeration of the culture system were carried out in a batch-culture system over 10 - 12 days. The results showed that an addition of 10 % enrichment supplement in combination with a static culture system in sub-anaerobic environment of 4 % CO_2, and an O_2 content of 0.25 headspace ratio induced the mycobacterial cells to enter latent phase of growth. These growth manipulations also extended the duration of latent phase to 5 - 6 days compared to the control cells undergoing normal growth. Replication of the optimized parameters in a 96-well microplate system for use in the assay of multiple samples produced longer duration of latent phase. Assessment of the metabolic rate of the latent phase cells by measuring the adenosine 5'-triphosphate levels using luciferase bioluminescence assay showed a decline in luminescence values of 1.6 – 2.3 folds compared to the log phase of the *Mycobacterium* species. Observation of the external structural morphology of these dormant cells under a scanning electron microscope showed that these cells were healthy and intact but lacked septate formation, indicating that they were alive but

not undergoing active replication. Finally, the protocol for activity against latent phase mycobacteria was improvised by adapting the procedure of colorimetric microplate assay using tetrazolium bromide as a redox reagent. The protocols were tested using known TB drugs. The results based on minimum inhibitory concentration (MIC) values of the drugs were compared to their activity against log phase cells. The newly established protocol for measuring drug susceptibility against dormant *Mycobacterium* species were successful to a certain extent, which can be seen by the difference in MIC value between log and latent phase culture of *M. smegmatis* and *M. fortuitum*. However, the protocol did not correspond well against dormant *M. tuberculosis* H37Ra, suggesting further improvement of the assay protocol is still required. In conclusion, the findings in this study showed that normal growth parameters at the right proportion could indeed induce mycobacterial cells to enter a longer latent phase of growth and using such simulation, it is possible to develop a simple laboratory protocol to test the activity of potential agents against latent TB.

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) was first discovered by Robert Koch in 1882. It accounts for a major cause of morbidity and mortality among infectious diseases (Dye *et al.,* 1999). According to the World Health Organization (WHO, 2015b), one third of the world's population is infected with TB. In 2014, 9.6 million new TB cases were reported with 1.1 million deaths (WHO, 2015b). The standard treatment regimen for TB requires a combination of three to four drugs over a long period of six to nine months (WHO, 2010). The emergence of multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) strains further complicate the TB management leading to more incidence cases and number of deaths. The global TB burden worsens even more as the human immunodeficiency virus (HIV) is known to fuel the epidemic of TB (Zhang *et al.,* 1994). In addition, the increasing occurrence of latent or dormant TB, whereby the TB bacilli are able to survive inside the host's primary immune cells under stress conditions, makes the treatment goal even more difficult to achieve (Gupta & Bhakta, 2012).

Various *in vivo* and *in vitro* models have been proposed to study latent TB (Tufariello *et al.,* 2003). The best animal model would be the model that could contain very small amount of TB bacteria in it for years while maintaining its latent phase, in which, sudden reactivation is infrequent and could be reactivated when immunodeficiency occurs (Tufariello *et al*., 2003). However, *in vivo* models such as mice, have proven to be difficult because the immune response does not reflect a

latent infection in human (Tufariello *et al*., 2003). Mice models are also quite resistant towards development of TB as they could control the infection with strong but stable tissue burdens in themselves (Orme & Collins, 1994). Another example of animal model, guinea pig, had failed to establish a prolonged latent state of TB although these animals are highly susceptible to TB infection (Wiegeshaus *et al.*, 1989). In addition to the above disadvantages, animal models are less affordable in comparison to the usage of *in vitro* models such as growth in liquid and on solid media. Current *in vitro* models such as hypoxia and nutrient starvation models had also failed to give an exact simulation of TB infection in the lungs, but they are comparatively affordable (Betts *et al.*, 2002). In these models, many physiologic assays are involved in order to maintain the dormant state, in addition to labourious procedures and the dangerous handling of the slow-growing and highly pathogenic *M. tuberculosis* (Wayne & Hayes, 1996). These limitations further increase the urgency to establish new *in vitro* models, which should be simpler, faster, and safer. Therefore, this study was concerned with establishing a simple bioassay protocol using an *in vitro* growth model by utilizing simple laboratory manipulations with four different surrogate *Mycobacterium* species. Variables including nutrient contents, oxygen (O_2)/carbon dioxide (CO_2) availability, temperature and pH were used to induce latent TB with minimal metabolic rate.

1.1 AIM AND OBJECTIVES

The main aim of this research was to simulate latent tuberculosis (TB) phase utilizing simple *in vitro* bioassay protocols using four *Mycobacterium* species, namely, *M. smegmatis, M. fortuitum, M. kansasii* and *M. tuberculosis* H37Ra. These species were selected as the test organisms because they are common surrogate

organisms of the highly pathogenic etiologic agent of TB, *M. tuberculosis.* Since they are not pathogenic, the research can be carried out in a normal Risk Level 2 laboratory. The specific objectives were:

1. To determine the parameters that will cause *M. smegmatis, M. fortuitum, M. kansasii* and *M. tuberculosis* H37Ra to enter latent phase using simple laboratory manipulations.
2. To develop laboratory procedures for maintaining latent growth phase in 96-well microplate.
3. To determine the correlation of growth rate and metabolic rate in latent phase.
4. To evaluate the latent bioassay protocol using known drugs.

CHAPTER 2

LITERATURE REVIEW

2.1 DEFINITION OF TERMINOLOGIES

A few terminologies are used in this thesis to describe the characteristics of the tuberculosis (TB) disease, mycobacterial cells and the phases in their growth cycle. These terminologies are as follows: latent TB disease, lag phase, log phase, stationary phase, dormant bacterial cells, and latent phase. Latent TB disease is defined as a condition, in which an individual is infected with *Mycobacterium tuberculosis*, but does not exhibit any signs or symptoms of active TB disease (Hauck *et al.*, 2009). Lag phase of microbial growth is defined as the period between inoculation of bacterial culture and the time of the commencement of its maximum rate of growth (Black, 2008). Log phase of microbial growth is the exponential phase of growth of a microorganism (Zwietering *et al.*, 1990). Stationary phase is defined as the period when the number of bacterial cells stops increasing, whereby the rate of cell death is equivalent to the rate of cell growth (Zwietering *et al.*, 1990; Kolter *et al.*, 1993). Dormant bacterial cells are those that are in an inactive condition where their growth and metabolic processes are halted (Biology Online Dictionary, 2005). Latent phase cells are those being in condition of biological rest or inactivity characterized by cessation of growth or development and the suspension of many metabolic processes (English-Spanish Dictionary, 2012). The latent mycobacterial cells are actually in the state of stationary phase whereby they remain dormant in the host tissues (Flynn & Chan, 2001). In this thesis, these mycobacterial cells are referred to as latent phase cells for easy description.

2.2 TUBERCULOSIS

2.2.1 Overview

Human beings have been plagued with TB for thousands of years (Daniel *et al.*, 1994). The seriousness of this epidemic is proven when WHO declared TB as a global health emergency in 1993 (WHO, 2002). Until today, it is still the single top infectious disease, which causes more death in humans, despite the extensive control measures that had been carried out since decades ago (Wayne, 1994).

There are three types of TB, namely, pulmonary (PTB), extra-pulmonary (EPTB) and latent TB (Dugdale, 2011). PTB is the most common type of TB disease. As *M. tuberculosis* can float in the air and stay there for several hours, an individual will get infected if he or she inhaled the droplets expelled from an infected individual. Symptoms of PTB include coughing, fever, excessive sweating, weight loss and fatigue. Individuals with PTB will be required to stay home and avoid close contacts with others until they are no longer contagious (Dugdale, 2011). TB can also be classified into primary and post-primary forms. Primary TB patients are patients that are not previously exposed to *M. tuberuculosis* infection (American Thoracic Society and Centers for Disease Control and Prevention (ATSCDCP), 2000). It is more commonly found in infants and children. On the other hand, post-primary TB usually occurs after one to two years prior to initial infection. Reactivation of these tubercle bacteria is usually associated with a weak immune system (ATSCDCP, 2000).

EPTB is not as common as PTB (Lee, 2015). In EPTB, TB infection occurs in various organs such as lymph node, bones and other organs than lungs. Diagnosing EPTB is extremely hard, as clinical samples obtained from relatively inaccessible sites might be paucibacillary, which might affect the sensitivity of

diagnostic tests. This is important as the conclusive diagnosis of TB is to isolate *M. tuberculosis* by culture from patients' samples (Lee, 2015).

Unlike PTB, latent TB infection does not show any evidence of clinical symptoms as manifested by active TB (Mack *et al*, 2009). *M. tuberculosis* antigens do not stimulate an immune response in the body, but instead remain dormant (Mack *et al*, 2009). Patients with latent TB infection usually converts to active TB disease within five years after infection. Latent TB patients are not infectious as long as the mycobacteria is not activated (Comstock *et al*, 1974).

TB is actually a disease that can be cured and prevented. In fact, TB case rates have shown gradual decrease in many parts of the world (WHO, 2015b). However, limitation of available resources, disruption of societal activities and the dispersion of HIV epidemic have hindered preventive and treatment measures and thus, fuelled the increase of local and regional TB cases (Frothingham *et al.*, 2005).

2.2.2 Epidemiology of tuberculosis

In 2013, it was estimated that nine million people were infected with TB with 1.5 million deaths. Out of these total number, around 360,000 individuals were patients with acquired immunodeficiency syndrome (AIDS) (Baddeley *et al.*, 2014). The global number of cases of new and relapse TB were about 6.1 million in 2014 (WHO, 2015b). It was estimated that there were 133 TB infection per 100, 000 people worldwide. Geographically, the Asian and African regions account for the highest rate of TB incidences and deaths per capita (Figure 2.1). The rate of TB mortality in South East Asia also shows a worrying figure, killing 24 per 100, 000 population (WHO, 2015b).

HIV patients, due to their weak and compromised immune system, are 26 times more likely to develop TB disease compared to HIV negative individuals (WHO, 2015b). Although the number of death caused by HIV-associated TB shows a 32 % decline over 10 years, TB death among HIV patients still contributes for 25 % of all TB death worldwide. This shows that HIV-associated TB still remains as an enormous burden of preventable death and ill-health (WHO, 2015b).

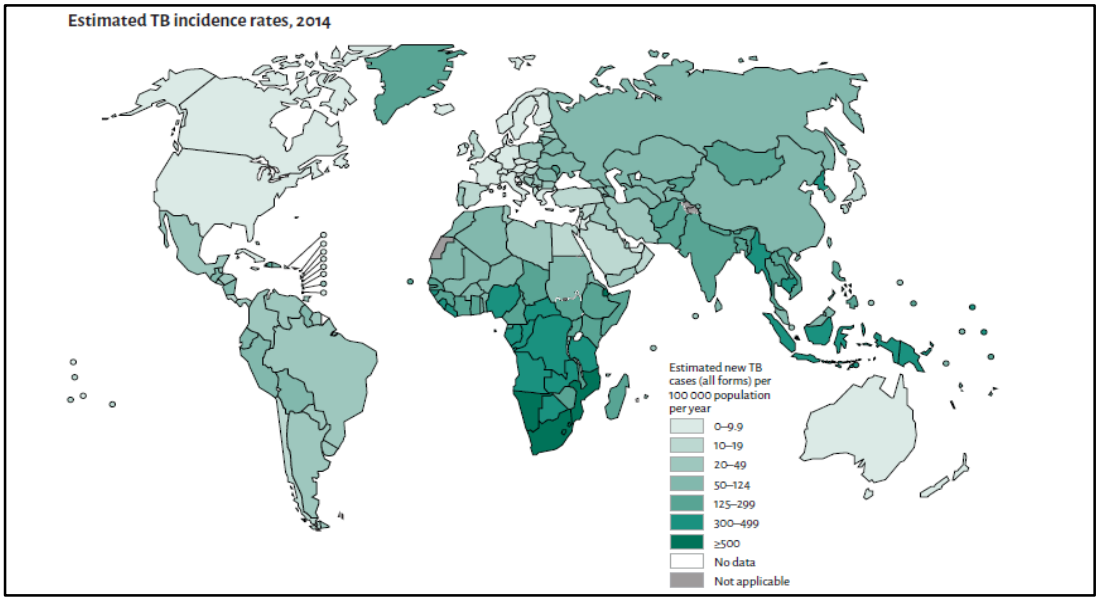

Figure 2.1. Global incidence of tuberculosis, 2014.
(WHO, 2015b)

Statistics show that TB infection in Malaysia is slightly better compared to its neighbouring countries. According to the Malaysian TB dataset, HIV positive associated TB cases in Malaysia is only 2.1 %, which is much lower than most countries around the globe (Malaysia Health Management and Disease Control, 2012). The number of new and relapse TB cases in 2014 was 24,054 cases (103 per 100, 000 people), which is also slightly lower than the global figure (133 per 100,000 population) (WHO, 2015b). Mortality rate of HIV negative TB patients in Malaysia is 8 %, which is also much lower than the global figure (16 %).

Although recent statistical data shows a slight hope in future eradication of TB, however, the percentage of newly arisen MDR-TB cases from treated TB patients had increased to 20.5 % in year 2013 (Baddeley *et al.*, 2014). Twelve percent of newly recorded TB cases globally are found to be MDR-TB patients (Figure 2.2). The figure is even higher in Malaysia, which is 37 % of all local recorded cases (WHO, 2015b). MDR-TB is very dangerous as the treatment success rate is only 37 % compared to 88 % for pulmonary TB (WHO, 2015b). Hence, immediate actions and measures need to be taken to prevent the continuous rise of TB patients on the global scale.

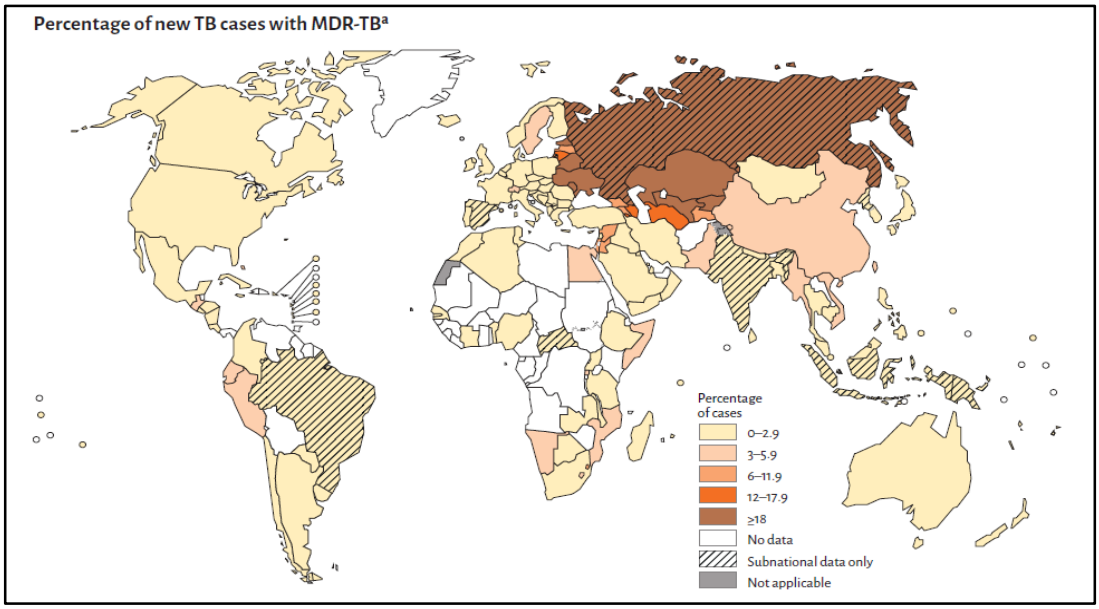

Figure 2.2. Global incidence of multiple drug-resistant tuberculosis, 2014. (WHO, 2015b)

2.2.3 The tubercle bacteria

Mycobacterium tuberculosis, the etiologic agent of TB is an intracellular pathogen that appears as long and straight rod-shaped cell (Falkinham, 2002; Tufariello *et al.*, 2003). The mycobacterial cells could not form any capsules, flagella,

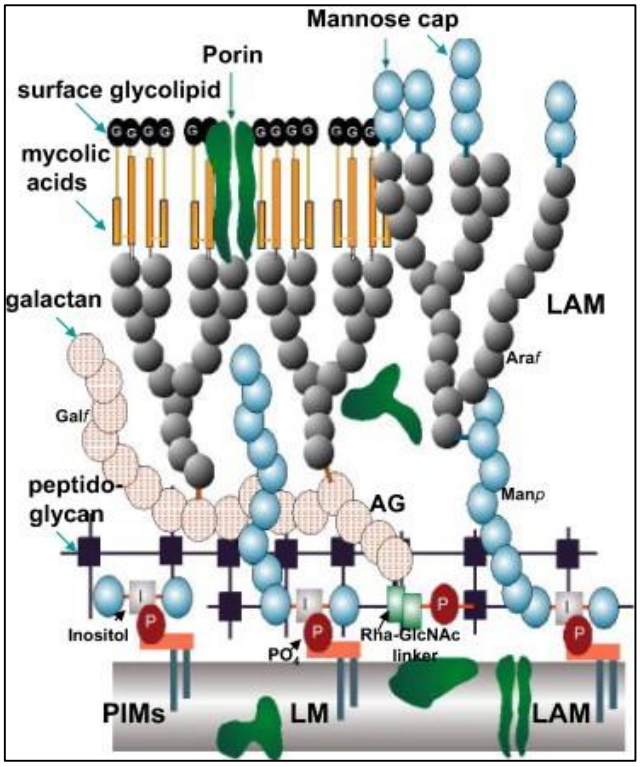

Figure 2.3. Schematic representation of the mycobacterial cell wall.
(Quesnaiux *et al.*, 2004)

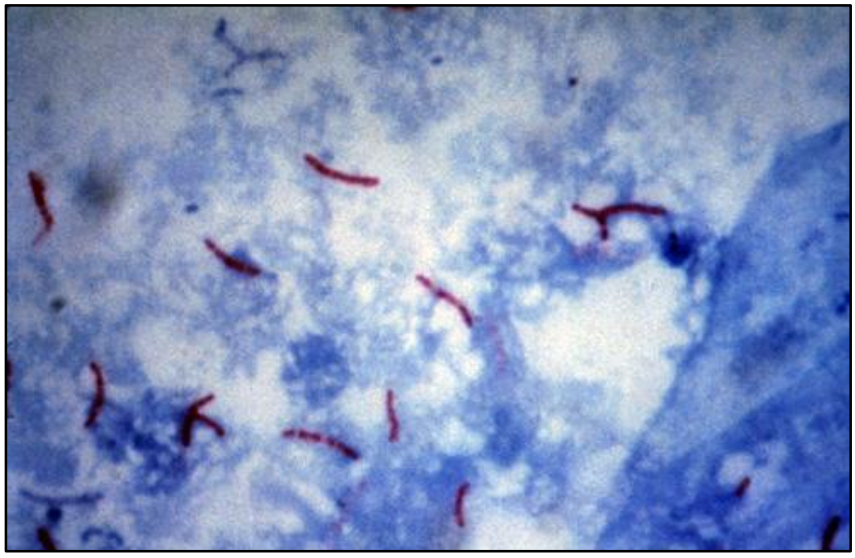

Figure 2.4. Acid-fast stain of *Mycobacterium tuberculosis.*
(Kubica, 1979)

exotoxins or spores but have a waxy cell wall, which is made up of mycolic acid (Figure 2.3) (Colijn *et al.*, 2009). *M. tuberculosis* exhibits cord factors from *in vitro*-grown colonies, which are also known to cause the mycobacteria to form long strands of cells (Talaro & Chess, 2011; Todar, 2011). These characteristics contribute to the acid-fastness property (Figure 2.4) of the cells and cause an increase in virulence by preventing killing by lysosome of the macrophages (Hunter *et al.*, 2006). Acid-fast bacteria are impermeable to certain dye and stains. However, they cannot be decolorized by heating or treatment with acidified organic compounds once they are being stained (Todar, 2011). Mycobacterial cell wall consists of an inner layer and outer layer (Hett & Rubin, 2008). The inner layer composed of covalently linked-peptidoglycan (PG), arabinogalactan (AG) and mycolic acid (MA), which are highly hydrophobic and forms a lipid barrier around the mycobacterial cell, thus affecting mycobacterial cells' permeability and staining properties (Brennan & Nikaido, 1995; Draper, 1998; Todar, 2011). The outer layer consists of free lipids and proteins and some non-covalently associated glycolipids (GPLs) such as lipoarabinomannan (LAM), phospholipids including phosphatidylinositol mannosides (PIMs) and other GPLs specific to mycobacteria. LAM is known to play a major role in contributing virulence properties of mycobacterial disease (Chatterjee, 1997).

M. tuberculosis is known as an obligate aerobe, where it grows optimally in the well-aerated upper portion of the lungs (Todar, 2011). However, it is also a facultative intracellular parasite, which resides in human macrophages as its primary habitat, by the transmission of airborne particles (Talaro & Chess, 2011; Todar, 2011). *M. tuberculosis* has a long generation time of 15 - 20 hours and is highly resistant to drying such that the organism could survive for eight months in fine

aerosol particles (Talaro & Chess, 2011; Todar, 2011). On the other hand, it does not live only in human habitats as there is also evidence of *M. tuberculosis* being found in wastewater (Hartsman *et al.,* 2006). *M. tuberculosis* is highly pathogenic and has a very low infectious dose, which often intensifies the infection process (Kelly *et al.,* 1996).

2.2.4 Pathophysiology of tuberculosis

Tuberculosis is transmitted via airborne particles known as droplets nuclei (WHO, 2010). These droplets are produced when pulmonary TB patients cough, sneeze, shout, speak or spit. Transmission of TB bacteria is highly dependent on the concentration of infectious airborne droplets, exposure duration, and physical condition of the exposed individual and environmental factors (Malaysia Health Management and Disease Control, 2012).

These droplets enter the airway when inhaled and are trapped in the upper parts of the airways by mucus-secreting goblet cells, which are then removed from the body (Frieden *et al.,* 2003). However, some bacteria in the droplets can bypass this barrier and reach the alveoli, whereby they are instantly engulfed by macrophages (ATSCDCP, 2000; Frieden *et al.,* 2003). Phagocytosis by macrophages triggers a chain of events that results in the successful control of the infection, which is soon followed by latent infection that may progress into an active disease later on. This phenomenon is known as primary progressive TB (ATSCDCP, 2000). The subsequent outcome is determined by the strength of the host immune system and the virulence of the invading mycobacteria (van Crevel *et al.,* 2002; Guyot-Revol *et al.,* 2006).

The mycobacterial cells continue to proliferate slowly inside the macrophages (ATSCDCP, 2000). Macrophages continue to produce proteolytic enzymes and cytokines in an attempt to digest the mycobacterial cells (van Crevel *et al.,* 2002). T-lymphocyte is then stimulated by these cytokines and form lesions of granulomas around the *M. tuberculosis* cells (Rosenkrands *et al.,* 2002; van Crevel *et al.,* 2002). A microenvironment is created in a granuloma that limits the proliferation and transmission of the mycobacteria (Frieden *et al.,* 2003; Nicod, 2007). Macrophages are killed in this microenvironment and produce solid necrosis in the lesion. However, these mycobacterial cells are able to adapt to the condition and survive. The environment changes into caseous necrosis, where O_2 levels and nutrient levels are at extremely low concentrations, associated with a low pH. Growth of these bacilli are restricted and enter dormant stage (Dheda *et al.,* 2005).

The lesions will undergo fibrosis and calcification, which will contain all the bacilli inside these healed, dormant lesions in individuals with adequate immune system (Dheda *et al.,* 2005). Lesions in individuals with weak immune system or immunodeficient patients will undergo liquefaction, where the internal contents of the lesions including mycobacteria, flow out and enter bronchus and nearby blood vessels (Dheda *et al.,* 2005). The individual is now known to have primary progressive TB and is likely to infect other people (Li *et al.,* 2002; Frieden *et al.,* 2003; Dheda *et al.,* 2005).

2.2.5 Diagnosis of tuberculosis

The most commonly used method in TB diagnosis is the Mantoux skin test (WHO, 2003). This tuberculin screening test (TST) is the first practical diagnostic

test introduced by von Pirquet in 1909 (Goldstein, 2002). It is carried out by injections into the skin of patients. This intracutaneous injection is administered as Mantoux test for clinical screening and analysis. A volume of 0.1 mL of purified protein derivative (PPD) solution is injected into the patient's arm and a wheal formation could be observed after two to three days. The diameter of the induration that transversed the long axis of forearm is measured after 48 hours using a specialized ruler (Goldstein, 2002). The strength of this test is that repeating tuberculin skin testing on a tuberculin–negative patients who have no infection history of *M. tuberculosis* would not induce a TST-false-positive reaction (Dacso, 1990).

In some cases, the reactivity of human body towards TST disappears with time. TST will be administered to boost reaction of body to future TST administration (Menzies, 1999). Two step tests are utilized to avoid misinterpretation of the booster as an infection. A TST will be repeated after one to three weeks if the first TST shows a negative result. Two step tests are common for individuals such as health care workers who have not taken a test in the last 12 months but will undergo regular testing in the future (Thompson *et al.,* 1979). TST also works for latent TB infection (ATSCDCP, 2000).

Traditionally, it is believed that chest radiographic expression of primary TB are mediastinal lymph node enlargement, lower lobe lesions, and pleural effusions, whereas, upper lobe lesions, cavitation, and fibrosis express the post-primary forms (Lee *et al.,* 1993; Lee *et al.,* 2000; Jeong & Lee, 2008). However, it is actually relatively hard to distinguish between primary and post-primary TB using a chest radiograph, since their features might overlap. Furthermore, confirming the diagnosis is much more crucial than determining the sub-type as it allows appropriate clinical

management (Burrill *et al.,* 2007). It is also important to note that chest radiographs are not definitive test of PTB and sputum smear test and isolation of the etiologic organism have to be carried out (Burrill *et al.,* 2007). Sputum smear test has been the primary method to identify PTB infection in most low and middle-income countries as it is an easy-to-carry-out, cost-efficient and rapid technique (Hopewell *et al.,* 2006). The downside of this technique is that it fails to detect the presence of the mycobacteria with a bacteria load of less than 10,000 organism per mL of sputum sample (Desikan, 2013). It is also ineffective in detecting EPTB, paediatric TB and in TB cases with HIV infection (Perkins, 2000).

The definitive TB diagnosis requires successful and proper isolation of TB pathogen from appropriate specimens (Fillioil *et al.*, 2003). Specimens are collected depending on the site of the disease such as lungs, pus, urine, stool and body fluids. BACTEC 460 (Becton Dickinson Instrument System, Sparks, MD) is developed as an automatic radiometric system for the identification of mycobacteria (Garg *et al.,* 2003). However, the price of the BACTEC system and the cost of radioactive waste disposals is high and unaffordable for most healthcare centres.

Drug-susceptibility test should also be carried out upon successful isolation of the mycobacteria (Heifets & Cangelosi, 1999). These test are usually carried out using first-line and second-line anti-TB drugs on agar plates and requires around two weeks to obtain definitive results.

Recent findings have introduced new TB diagnostic methods such as serological test, usage of polymerase chain reaction (PCR) and nucleic acid amplification tests. Serological test is based on the detection of antibodies to *M. tuberculosis* antigens, thus, it is able to give simple and rapid diagnosis at a very low cost. However, serological test results are often inaccurate and imprecise for clinical

diagnostics, and thus, limiting their effectiveness (Steingart *et al.*, 2012). On the other hand, although PCR and nucleic acid amplifications tests are able to give a more accurate result, their usage remained limited among low-income countries as these tests required highly trained personnel for handling and incur high cost (Steingart *et al.*, 2012).

2.2.6 Treatment and prevention of tuberculosis

According to WHO, an ideal anti-TB drug should not only be cheap and of low toxicity, but it also must possess the ability to contribute a strong bactericidal activity and sterilizing activity towards metabolically active and semi-dormant bacilli (WHO, 1991). The drug must also be able to inhibit the development of resistant bacilli. However, no known chemotherapeutic agent contains all of the mentioned properties, whereby, its anti-tubercular activity is significant enough to act on its own (WHO, 1991). Thus, a six-month chemotherapy treatment composed of at least four first-line anti-TB drugs has been established, such as isoniazid, rifampicin, pyrazinamide and ethambutol (WHO, 2010). All four drugs are given to the TB patients for the first two months of the intensive phase treatment while rifampicin and isoniazid are continued for the next four months of continuation phase. The dosing frequency of these drugs varies according to the patient's condition. The recommended doses of each first-line anti-TB drugs for adults are as shown in Table 2.1 (WHO, 2010).

Treatment of new TB patients with high level of isoniazid resistance would not be effective even with the six-month chemotherapy (Menzies *et al.*, 2009). In this case, patients may receive isoniazid, rifampicin, ethambutol and pyrazinamide

during the intensive phase of treatment and isoniazid, rifampicin and ethambutol as alternative therapeutical regimen in the continuation phase (WHO, 2010).

MDR-TB is extremely difficult to be treated. The recommended regimen for MDR-TB is to use at least four second-line anti-TB drugs, such as capreomycin, kanamycin and amikacin, together with pyranzinamide for 20 months (WHO, 2003). However, second-line drugs are less effective and more toxic to the human body. A shorter regimen for treating MDR-TB has been established recently, where gatiofloxacin, clofazimine, ethambutol and pyrazinamide are administered throughout the nine month treatment period, together with high dose of isoniazid, prothionamide and kanamycin for the first four months of intensive phase (Van Deun *et al.,* 2010). However, patients who show good progress during the treatment period, might have a higher risk in acquiring drug resistance and relapse with TB in the future (WHO, 2015b).

WHO has aimed to reduce the number of deaths caused by TB by 95 % and a 90 % reduction in TB cases by year 2035 (WHO, 2015b). WHO is currently formulating a comprehensive approach in improving TB diagnostic, treatment and prevention. Future TB vaccine research would be focusing on prevention of infection, prevention of disease and prevention of recurrence (Tuberculosis Vaccine Development, 2015). The currently used Bacille Calmette-Guérin (BCG) vaccine was first administered onto human by Albert Calmette and Camille Guérin in 1921 (Behr & Small, 1999). It is the most widely used vaccine until today (Tuberculosis Vaccine Development, 2015). Studies showed that BCG vaccine can reduce TB risk by 50 % but the period of protection could not be calculated (Colditz *et al*, 1994). On the other hand, despite the introduction of the newly developed DNA-subunit or

Table 2.1. Recommended doses of first-line anti-tuberculosis drugs for adults.
(WHO, 2010)

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

Notes:

1. Daily (rather than three times weekly) intensive-phase dosing may help to prevent acquired drug resistance in TB patients starting treatment with isoniazid resistance.

2. Acceptable alternative provided that the patient is receiving directly observed therapy and is not living with HIV or living in an HIV-prevalent setting.

3. WHO no longer recommends omission of ethambutol during the intensive phase of treatment for patients with non-cavitary, smear-negative PTB or EPTB who are known to be HIV-negative. In tuberculous meningitis, ethambutol should be replaced by streptomycin.

4. Patients aged over 60 years may not be able to tolerate more than 500–750 mg daily, so some guidelines recommend reduction of the dose to 10 mg/kg per day in patients in this age group. Patients weighing less than 50 kg may not tolerate doses above 500–750 mg daily.

protein vaccine and attenuated *M. tuberculosis* vaccine, their effectiveness on TB vaccination still requires many improvements and improvising (Martin *et al.,* 2006). Another major problem in TB vaccine development is due to the lack of correlation between immunological data and duration of vaccine protection (Tuberculosis Vaccine Development, 2015).

2.3 Challenges in latent tuberculosis management

Tuberculosis pathogenesis is commonly caused by an exogenous (recently acquired) infection or occasionally an endogenous (reactivation of latent TB) infection (van Rie *et al.,* 1999; Lillebaek *et al.,* 2002). Most people develop latent TB when they are infected with dormant *M. tuberculosis* (Tufariello *et al.,* 2003). Latent TB is generally non-infectious. However, patient with latent TB infection has a chance of 5 - 15 % of developing active pulmonary TB disease and become highly infectious (WHO, 2015b).

Latent TB remains a big problem in TB disease management due to the fact that latent bacilli had proven to be difficult to kill (Karakousis *et al.,* 2008). This is because conventional drugs for *M. tuberculosis* can only attack and kill them in their active replication state but not in their latent phase (Sharbati-Tehrani, 2005). In 2010, it was estimated that around 10 to 15 million individuals in the United States are affected with such latent infection (Shiloh & Champion, 2010).

Initial infection normally does not cause disease as most of the tubercle bacilli are being cleared away by the body's immune system (Murray *et al.,* 1991). However, some tubercle bacilli can enter as asymptotic, inactive cells and remain in latent phase for decades before a relapse occurs (Murray *et al.,* 1990). Some patients show calcified (healed) lesions in their lungs, which is believed to contain dormant

TB bacilli, with the possibilities to develop into a clinical, active TB later in life and the pathology pattern is often very similar to active TB (Kassa *et al.,* 2012).

Under normal conditions, peptide complex and major histocompability complex (MHC) class II antigens are formed by processing the mycobacterial antigens in phagosomes (Ramachandra *et al*., 2001). However, in a latent TB infection, the mycobacterial cells are capable of inhibiting the antigen presentation process in human body immune response, causing some *M. tuberculosis* cells not eliminated even after being engulfed by phagosomes (Ramachandra *et al.,* 2001). It was found that the level of peptide complex in phagosome containing live bacilli is higher than those containing dead mycobacterial cells, suggesting that live mycobacteria could attenuate or retard the MHC class II antigen processing mechanism and thus, preventing maturation of phagosomes (Noss *et al.,* 2001). Studies have shown that the interference starts almost immediately after the mycobacteria are being engulfed (Noss *et al.,* 2001). This suggests that *M. tuberculosis* could alter the MHC class II antigen processing pathway during its infection (Noss *et al.,* 2001).

Study on latent TB is vital as the mycobacteria could remain hidden in the body without generating any symptoms on the patient and could be reactivated any time (Parrish *et al.,* 1998). The risk of being infected by TB would increase if the person is co-infected with HIV (Flynn & Chan, 2001). As latent TB has the tendency to develop into a MDR-TB, an effective cure against them remains a big health problem (Karakousis *et al.,* 2008). Therefore, it is vital to establish laboratory models that could allow us to study this disease to stop its spread.

2.4 STRATEGIES FOR LATENT TUBERCULOSIS RESEARCH

2.4.1 Target organisms

Ideally, the target organism should be the etiologic agent, *M. tuberculosis,* itself. However, it is extremely slow growing and highly pathogenic, making research on these mycobacterial cells to be extremely dangerous, time consuming and expensive (Forrellad *et al.,* 2013). Thus, it is wiser to utilize surrogate mycobacteria organisms for research, particularly, in laboratories without biosafety level three facility.

Surrogate organism is defined as organism that is utilized to study the fate of a pathogen in a specific environment (Sinclair *et al.,* 2012). **S**urrogate mycobacteria species, such as *M. smegmatis* and *M. fortuitum* are classified under the same genus as *M. tuberculosis* and are widely used in molecular analysis of *M. tuberculosis.* The surrogate organisms selected in this research have faster growth rate and low virulence compared to *M. tuberculosis* (Rao *et al.,* 2001). Most surrogate mycobacteria are environmental opportunist, with no evidence of spread of disease in human is found (Wallace, 1994, Bryant *et al.,* 2013).

Mycobacterium smegmatis

The advantage of using *M. smegmatis* as a surrogate organism is that it is safe to handle because there are no cases of *M. smegmatis* infection reported since its discovery in 1884 (Pierre-Audigier *et al.,* 1997). Despite a clear understanding of its genome sequence, very little is known about its growth-controlling-mechanism (Draper, 1998). *M. smegmatis* is considered as a rapid grower among *Mycobacterium* species due to its relatively short generation time of 3 to 6 hours (Klann *et al.,* 1998). This short generation time largely shortens the duration on research as compared to

M. tuberculosis. *M. smegmatis* shows close resemblance to *M. tuberculosis* in its response to anaerobiosis and substrate deprivation, which they share a number of genes required in adaption to stress condition (O'Toole *et al.*, 2003). On the other hand, *M. smegmatis* is also able to oxidize carbon monoxide under aerobic condition, like *M. tuberculosis* (King, 2003).

Mycobacterium fortuitum

Although *M. fortuitum* is classified as a rapid grower among *Mycobacterium* species, it is actually a slow growing organism because it requires 3 to 5 days to form liquid culture with high turbidity or colonies on solid medium at 37 ºC (Smith, 1976). This low growth rate also means that the mycobacteria die off very slowly under natural conditions, a phenomenon similar to *M. tuberculosis* (Soini & Musser, 2001). Unlike *M. tuberculosis*, it is an environmental opportunistic mycobacteria (Wallace, 1994). However, it is always mistaken as a saprophyte (Wallace, 1994). It is reported that *M. fortuitum* is responsible for most diseases caused by rapidly growing mycobacteria especially infections that incurred due to cardiac bypass surgery (Wallace *et al.*, 1989; Wallace, 1994). However, no proof of any person-to-person spread had been discovered, making them safe to work with (Wolinsky, 1979).

Mycobacterium kansasii

Mycobacterium kansasii is known as a slow growing bacteria, like *M. tuberculosis*. It grows well within 32 ºC to 42 ºC, but could not grow at temperature above 42 ºC (Falkinham, 1996). It is an opportunist pathogen, which could only cause lymphadenitis and various infections to skin, respiratory system

and connective tissue in individuals with weak immune system (Wolinsky, 1979). Like *M. tuberculosis, M. kansasii* is reported to be able to adapt to *in vitro* growth under microaerophilic condition with ease. Up to today, no evidence of human-to-human transmission has been found for *M. kansasii* infection and thus, become a safer option in *M. tuberculosis* research (Powell & Steadham, 1981).

***Mycobacterium tuberculosis* H37Ra**

Serial passage of generations of *M. tuberculosis* H37 through media culture with different pHs had gave rise to two forms of mycobacteria strains: the virulent *M. tuberculosis* H37Rv and the attenuated *M. tuberculosis* H37Ra (Steenkeen *et al.*, 1934). *M. tuberculosis* H37Ra is an attenuated tubercle bacillus closely related to the virulent type strain *M. tuberculosis* H37Rv (Brosch *et al.,* 1999). *M. tuberculosis* H37Ra is the best surrogate for the virulent H37Rv strain in primary screening assay, as it has less biosafety concerns and lower requirement of biocontainment level (Collins & Franzblau, 1997).

2.4.2 *In vivo* animal models

Various laboratory *in vivo* models have been used to help scientists study latent TB such as mouse, guinea pig and rabbit models (Tufariello *et al.*, 2003). Each model has its own respective advantages over one another. Mouse model, as the most commonly used model, has the most economical value and uses easily available immunogenic reagents (Orme *et al.,* 1994). Mice are relatively resistant to TB development, allowing studies on establishing latent TB infection to be carried out easily (Miyazaki *et al,* 1999). The Cornell mouse model developed by McCune in Cornell University in the 1950s is the most accepted mouse model for studying

TB dormancy (Miyazaki *et al,* 1999). The mice are treated with anti-TB drugs such as isoniazid and pyrazinamide to kill actively replicating TB bacilli following the infection of high dose of *M. tuberculosis*. However, active infection relapsed in one-third of the mice after stopping the chemotherapy for three months (Miyazaki *et al*, 1999). Relapse occurs in almost all mice when immunosuppressant therapy is applied to the mice. Surprisingly, the bacilli recovered from these TB relapsed mice are acid fast and completely susceptible to isoniazid and pyrazinamide, suggesting that dormant bacilli do not develop a resistance to drugs at the genetic level but only phenotypically resistance to drugs in their dormant state (Gupta & Katoch, 2005). Although mouse model plays a large role in understanding TB latency and reactivation, it lacks consistency and stability, and do not resemble host immunity-induced latent TB infection as the mouse does not develop a well-formed granuloma and lacks the formation of caseous necrotic centre as in humans (Phyu *et al.,* 1998).

Latent infection could not be established in a guinea pig model due to the animals' high susceptibility to *M. tuberculosis* (Ordway *et al.,* 2008). A recent study with reproducible results proved the existence of dormant TB bacilli in the lung lesions of guinea pigs, but the particular strain, *M. tuberculosis* 18b, which is a streptomycin-auxotrophic mutant, is available only to certain laboratories (Lenaerts *et al.,* 2007). The most important part of this model is that, it could develop a strong delayed-type hypersensitivity response, which closely resembles human system (Dannenberg & Collins, 2001). The main limitations with this model is the unavailability of immunological agent and higher cost compared to mouse model (Gupta & Katoch, 2005).

Unlike the mouse model, the rabbit model is able to replicate the formation of caseous necrosis centre, which represents the clinical condition in humans (Converse *et al.,* 1996). Rabbit is utilized for studying latent TB infection due to its high resistance to *M. tuberculosis*, which often causes TB infection to stay dormant within the rabbit (Manabe *et al.,* 2008). In their research, the lungs of rabbits were infected with the mycobacteria by the aerosol route. Samples were taken from infected rabbits' lung at fixed interval to observe the growth of the mycobacteria. Initially, growth of these bacilli are observable at the infection sites. However, no growth of bacilli could be observed in these samples after the fifth week of infections. Although no bacilli were found after four months of infection, some rabbits develop active TB at the sixth month (Manabe *et al.,* 2008). These results have suggested that latent TB infection and its reactivation in these rabbits occur in similar fashion with humans (Manabe *et al.,* 2008). However, *in vivo* research using rabbit model have been limited due to the lack of available immunological reagents, the extensive space and high cost required for breeding rabbits compared to mouse or guinea pig models (Gupta & Katoch, 2005).

2.4.3 Laboratory *in vitro* models

Carbohydrate, lipids, nitrogen metabolism and oxidative phosphorylation have been used to study the metabolism of mycobacteria in *in vitro* models to further understand the physiology of mycobacteria by limiting oxygen supply in order to induce latent TB (Wayne & Lin, 1982; Masood, 1985). However, when actively growing *M. tuberculosis* in aerated culture was abruptly transferred to anaerobic condition, it died rapidly with a half-life of only 10 hours (Wayne & Lin, 1982). Nevertheless, mycobacteria was found to adapt to microaerophillic conditions when