EVALUATION OF THE MYCOBACTERIOPHAGE AMPLIFICATION ASSAY FOR DETECTION OF *MYCOBACTERIUM BOVIS* BCG CELLS EXPOSED TO DIFFERENT STRESSES *IN VITRO*

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MAY 2013

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By

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A project report submitted to the Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

in partial fulfillment of the requirements for the degree of

Bachelor of Science (Hons) Biotechnology

May 2013

ABSTRACT

EVALUATION OF THE MYCOBACTERIOPHAGE AMPLIFICATION ASSAY FOR DETECTION OF *MYCOBACTERIUM BOVIS* BCG CELLS EXPOSED TO DIFFERENT STRESSES *IN VITRO*

TAY YII HAN

Tuberculosis (TB) remains one of the most severe contagious diseases that threatens one-third of the world population. The causative agent for this disease is *Mycobacterium tuberculosis* (Mtb). Mtb is expectorated in tiny aerosol droplets that are able to remain suspended in the air for hours. They are then exposed to various stresses in the environment before being inhaled by another host. Current laboratory diagnostic methods are either slow or low in sensitivity. The phage amplification assay, a relatively new diagnostic method, is able to generate result in a short time and is highly sensitive. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) was used as model in this project to evaluate the efficiency of this assay in detecting the stressed mycobacterial cells that simulate Mtb bacilli in tuberculous droplet nuclei. Exponential-phase *M. bovis* BCG cells were exposed to various stresses *in vitro* and the deviation in plaque formation was compared in plaque-forming unit (PFU). The results showed that the number of PFU reduced significantly after exposure to desiccation, nutrient starvation, ultraviolet radiation and chemical stress. In this project, the effects of *M. smegmatis* supernatant (containing resuscitation-promoting factors) on stressed *M. bovis* BCG cells were

also investigated. Rpfs are a family of proteins that have been reported to possess the ability to resuscitate stressed mycobacterial cells. Only exposure to ultraviolet radiation and chemical stress showed a slight increment in PFU. In general, there was no significant effect on PFU after supernatant treatment. All results obtained were unable to determine whether the cells had died or still remained viable as colony-forming unit (CFU) assay was unsuccessful due to contamination problems. Future works that can be carry out include obtaining all PFU/CFU ratios for all stresses to determine the culturability and phage infectivity of stressed cells, use of *M. bovis* BCG supernatant, and evaluation of the phage amplification assay on other stresses.

ACKNOWLEDGEMENTS

I would to like express my gratitude and big thank you to all the people who helped and supported me during the process of my project:

- my supervisor, Dr. Eddy Cheah Seong Guan, who gave great supports, patience and his precious time to guide me throughout this project.
- my beloved family, who willing to listen to my complaints and grumbles whenever I was stressed.
- the laboratory assistants, Ms. Luke, Ms. Woo and Mr. Loke Wee Leiam, for their technical supports and willing to lend centrifuge machine in the chemistry laboratory for me.
- my group mates, Tan Yee Thin, Tan Poh Suan and Cheng Yi Wen, who always been helpful and supportive throughout the project.
- all the unmentioned ones, who had helped and encouraged me all the way during this project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(TAY YII HAN)

APPROVAL SHEET

This project report entitled **"EVALUATION OF THE MYCOBACTERIOPHAGE AMPLIFICATION ASSAY FOR DETECTION OF *MYCOBACTERIUM BOVIS* BCG CELLS EXPOSED TO DIFFERENT STRESSES *IN VITRO*"** was prepared by TAY YII HAN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **TAY YII HAN** (ID No: **09ADB04114**) has completed this final year project entitled **"EVALUATION OF THE MYCOBACTERIOPHAGE AMPLIFICATION ASSAY FOR DETECTION OF *MYCOBACTERIUM BOVIS* BCG CELLS EXPOSED TO DIFFERENT STRESSES *IN VITRO"*** supervised by Dr. Eddy Cheah Seong Guan (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

Yours truly,

_____

(TAY YII HAN)

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LIST OF ABBREVIATIONS

AFB	acid-fast bacilli
BCG	Bacille Calmette-Guérin
$CaCl_2$	calcium chloride
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit
dH_2O	distilled water
DNA	deoxyribonucleic acid
FAS	ferrous ammonium sulphate
HCl	hydrochloric acid
HIV	human immunodeficiency virus
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani
MAPTB	Malaysian Association for the Prevention of Tuberculosis
$MgSO_4$	magnesium sulfate
MP	mycobacteriophage
Mtb	*Mycobacterium tuberculosis*
NaCl	sodium chloride
NaOH	sodium hydroxide
NIH	National Institutes of Health

NLM	National Library of Medicine
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
OGC	OADC-glycerol-calcium
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque-forming unit
Rpf	resuscitation-promoting factors
TB	tuberculosis
Tris-HCl	tris (hydroxymethyl) aminomethane-hydrochloric acid
UTAR	Universiti Tunku Abdul Rahman
UV	ultraviolet
UV/Vis	ultraviolet/visible
WHO	World Health Organization
ZN	Ziehl-Neelsen
℃	degree Celsius

LIST OF UNITS OF MEASUREMENTS

µl	microliter
µm	micrometer
µM	micromolar
cm	centimeter
g	gram
g	gravity
h	hour
min	minute
ml	milliliter
M	molar
mM	millimolar
mm	millimeter
nm	nanometer
rpm	revolutions per minute
s	second
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) remains one of the most severe contagious diseases that threatens one-third of the world population; it continues to be the leading cause of human mortality in some countries nowadays (Eltringham *et al*., 1999). The causative agent of TB is *Mycobacterium tuberculosis* (Mtb), a slow-growing mycobacterium that can remain dormant in its host. Globally, an average of 9 million new TB cases and 2 million deaths are reported every year (Yew and Leung 2009). In Malaysia, the Malaysian Association for the Prevention of Tuberculosis (MAPTB) has estimated an average of 14,000 TB cases reported in each state every year (MAPTB 2012a).

Mtb is an airborne pathogen. An active TB patient can infect more than 10 people a year by disseminating Mtb into the surrounding air through coughing, sneezing, laughing and speaking, especially in overcrowded places with poor ventilation, such as prisons, nursing homes and hospitals (CDC 2011a). The expectorated tiny aerosol droplets containing Mtb are 1-5 μm in size and are able to remain suspended in the air for minutes to hours before being inhaled by another individual (Wells 1934). TB can cause serious infection, but it is not incurable. TB can be cured by taking drugs or antibiotics for up to six months to completely eliminate the Mtb, including dormant Mtb, from the patient's body.

Due to the ability of Mtb to remain dormant in the human host and their serious pathological consequences, early and rapid diagnosis of TB is extremely important for timely treatment and public health control. Current diagnostic techniques used for the detection of Mtb are smear microscopy, culture and molecular methods (Shenai *et al*., 2002). However, these methods have their limitations. Smear microscopy lacks specificity as it detects all acid-fast bacilli. It is also unable to differentiate between viable and nonviable cells. Culture is time-consuming and is prone to contamination; while conducting molecular techniques are much more expensive than other diagnostic methods.

Following the emergence of drug-resistant TB, it is necessary to develop an effective diagnostic method that is convenient for extensive implementation around the world. The phage amplification assay is one of the new TB diagnostic techniques that is currently under investigation. Mycobacteriophages, especially the lytic phage D29, are employed in this technique for specific detection of viable mycobacterial cells (David *et al*., 1980). The phage amplification assay results can be obtained within 48 hours. Plaques formed are indicative of the infection of mycobacterial cells by mycobacteriophages, which can be easily observed on phage indicator plates (Prakash *et al*., 2009). As compared to other diagnostic techniques (culture and molecular techniques), the phage amplification assay is relatively sensitive, specific, cost-effective, rapid and it can provide live-dead differentiation of mycobacterial cells.

When Mtb are expelled from the host, they are likely to encounter some environmental stresses before reaching another host. Stresses are defined as the unfavorable or tensed conditions that threaten the survival of Mtb. External stresses include desiccation and exposure to ultraviolet (UV) radiation while internal stresses include oxidative stress and starvation. In general, Mtb has strong resistance towards the host's immune system due to its thick mycolic cell wall (Saviola 2010). They are able to resist environmental stresses and survive under harsh environments.

From the definition of stress, bacterial cells that have entered stationary phase can be considered as being stressed, in which depletion of nutrients and limitation of space could be the factors that lead to the decrease in cell number. Cheah (2010) compared the plaque-forming unit (PFU) to colony-forming unit (CFU) ratio (PFU/CFU ratio) of exponential-phase mycobacteria with those for stationary-phase mycobacteria. The results showed that the latter yielded lower PFU/CFU ratio. Saviola (2010) mentioned that mycobacteria can be inactivated following exposure to UV light and desiccation.

An investigation on how these stresses might affect the detection of exposed mycobacterial cells by the phage assay is essential for the improvement of this diagnostic technique. By using the phage amplification assay, the presence of Mtb in respiratory specimens can be detected and then hypothesized number can be estimated. In this project, it was suspected that mycobacterial cells that were exposed to physical and chemical stresses might show disparities on the detection

by the phage amplification assay; this includes both their abilities to survive and to be infected by mycobacteriophages.

Resuscitation-promoting factors (Rpf) are a family of proteins secreted by mycobacteria that play an essential role in mycobacterial growth (Mukamolova *et al*., 2010). Previous research showed that these proteins are able to restore the culturability of mycobacteria from a dormant state into an active state (Kana *et al*., 2007), and this probably explains their role in reactivation of latent TB. Rpf proteins are able to hydrolyse the mycobacterial cell walls, similar to enzymatic activities of lysozymes (Cohen-Gonsaud *et al*., 2005). In this project, the ability of Rpf proteins to stimulate restoration of mycobacterial cells back into the active metabolic state after exposure to different stresses was also investigated.

Cultivation of Mtb in the laboratory is a high-risk task, as they are easily transmitted through the air. To study TB, *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) is a commonly-used model. With the loss of its virulence, *M. bovis* BCG is currently used for vaccination to effectively protect children from TB (WHO 2013a). In this project, *M. bovis* BCG cells were exposed to various physical and chemical stresses *in vitro* (growth in stationary phase, nutrient starvation, desiccation, exposure to UV light, exposure to dark condition and chemical stress), and then their detection by the phage assay assessed. The second part of this project involved the evaluation of the effect of *M. smegmatis* culture supernatant (containing Rpfs) in resuscitating stressed mycobacterial cells for detection by the phage assay. The mode of assessment was to investigate the

differences in PFU counts before and after each stressor treatment. CFU plating assays were performed in parallel to assess for culturability, but frequent contamination of CFU plates hampered this process.

The aims and objectives of this project were:

I. To evaluate the ability and efficiency of the phage amplification assay to detect *M. bovis* BCG cells exposed to various physical and chemical stresses *in vitro.*

II. To investigate the effect of *M. smegmatis* supernatant on the detection of *M. bovis* BCG cells exposed to various physical and chemical stresses by the phage amplification assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycobacteria in general

Mycobacteria are categorized under the genus *Mycobacterium*, further grouped under its family Mycobacteriaceae and order Actinomycetales (Rastogi *et al*., 2001). The genus *Mycobacterium* was first introduced in 1896, in which "myco-" generally refers to fungus and descriptively refers to the mold-like pellicles observed on the surface of liquid medium during growth (Gangadharam and Jenkins 1998).

Mycobacteria possess some distinct characteristics, which are acid-fast, with thick mycolic cell wall and slow growth rate (Rastogi *et al*., 2001). Generally, mycobacteria can be classified into rapid-growing and slow-growing species, of which the former still grow comparatively slower than many other bacteria (Falkinham III 2009). With generation time of approximately 2 to 6 hours, rapid-growing mycobacteria require less than 7 days to form observable colonies under optimal growth conditions (Rastogi *et al*., 2001). Rapid-growing mycobacteria are commonly non-pathogenic, such as *M. smegmatis* and *M. phlei*. However, these mycobacteria are still able to cause infections in immuno-compromised patients (e.g. human immunodeficiency virus (HIV) patients), those with pre-existing lung diseases, alcoholics and smokers (Fowler *et al*., 2006). Slow-growers, such as Mtb and *M. bovis*, are strict pathogens that cause diseases in their hosts under any

circumstances. These mycobacteria require approximately 15 to 28 days to form observable colonies, with generation time of 12 to 24 hours (Rastogi *et al*., 2001).

Mycolic acids are present in the mycobacterial cell wall. They are complex and branched fatty acids with large number of carbon atoms and consist of multiple different types of functional groups (Leray 2012). The lipid-rich cell wall contributes to its high hydrophobicity, which allows surface adherence and aerosolization that assist them to survive under low-nutrient environments (Falkinham III 2009). Previous studies showed that the thick mycobacterial cell wall is the main cause leading to their slow growth (Brennan and Nikaido 1995). Mycobacteria such *M. avium* are resistant to therapeutic drugs due to the low permeability of their thick cell wall (Rastogi and Barrow 1994).

2.2 Tuberculosis

2.2.1 Statistics

TB is a contagious and infectious lung disease caused by Mtb. It is disseminated through the air. The resurgence of TB worldwide had turned it into a considerable public health concern, especially in countries with high prevalence of HIV infection, such as the sub-Saharan Africa (Andrews *et al*., 2007).

As one of the leading infectious causes of human mortality, TB is estimated to affect one-third of the world's population, with majority of people being latently infected (WHO 2013b). There were 8.7 million of TB cases reported and

approximately 1.4 million people died in 2011 worldwide (WHO 2011a). In Malaysia, there were more than 20,000 of TB cases reported in 2011, which rose approximately 7% from the previous year. TB patients ranged from age 15 to 54, which accounted for more than three quarters of the total TB cases reported (Lee 2012).

2.2.2 Transmission

TB mainly spreads through the air when a patient with active disease coughs, laughs, speaks and sneezes. Thousands of aerosol droplets containing Mtb are formed and expelled into the surrounding air and transmitted to other individuals. These droplets with the size of 1-5 μm in diameter are small enough to be inhaled by others (Wells 1934). Most of the time, Mtb enter their hosts through respiratory route and mainly infect the lungs. Under certain circumstances, these bacteria can disseminate to other parts of the body and cause infections. Any organs can be infected since Mtb is able to express its virulence in many organs, thus causing extra pulmonary diseases (Hopewell and Jasmer 2005).

2.2.3 Latent infection

Mtb is an obligate pathogen that can only survive within a viable host on which their existence is totally dependent (Fenton and Vermeulen 1996). Infection by Mtb can lead to disease but in most cases, latent infection results. However, the host's immune system is still able to kill these pathogens after their invasion. Infection and disease will only develop when weakened immune system fails to control the invasion of Mtb and it starts to reproduce (WHO 2009). Latent

infection refers to a state in which the bacteria are in a "sleeping" or dormant state. Under this state, Mtb experience low metabolic activity and can survive for long period of time without division (Kell *et al*., 1995). However, they might "wake up", enter the active growing state and develop active disease. Selwyn *et al*. (1989) reported that 5-10% of latent infection would reactivate and develop into active TB. Vaccination is used extensively for preliminary prevention of TB infection. The live vaccine used is known as BCG, a weakened variant of *M. bovis* that has lost its virulence (WHO 2013a).

2.2.4 Symptoms

Lungs are the most commonly infected organ by Mtb. The most obvious characteristic symptom of active TB is vigorous and persistent coughing, in which blood is sometimes present in the sputum expectorated (MAPTB 2012b). The latter is known as hemoptysis, which often happens due to erosion of the bronchial artery (Hopewell and Jasmer 2005).

Most of the times, a healthy person usually shows no symptoms when infected as the immune system is able to "wall off" the infection (WHO 2013c). Other than chronic coughing that lasts for weeks, a patient with active TB develops other symptoms such as night sweats, difficulty in breathing, weakness, tiredness, loss of weight, appetite loss, anorexia, fever and chest pain (WHO 2013c; MAPTB 2012b).

2.3 Diagnosis of TB

2.3.1 Acid-fast bacilli smear microscopy

Mycobacteria are able to resist decolorization by acid-alcohol following staining with carbolfuchsin and are therefore stained pink. According to CDC (1994), the results of acid-fast bacilli (AFB) smear microscopy are used to determine whether respiratory isolation of patients with active disease can be discontinued. Smear microscopy acts only a preliminary determination of the presence of AFB and its confirmation of TB is not 100%.

AFB microscopy allows rapid detection of Mtb in sputum but does not distinguish between viable and non-viable mycobacterial cells. False positive results might occur frequently, as smear microscopy is unable to distinguish among different mycobacterial species, such as between the pathogenic Mtb and the non-pathogenic *M. smegmatis* (Deysel 2008). In other words, positive AFB isolated from TB-suspected patient may not be Mtb. Lack of specificity and sensitivity are the main limitations of AFB smear microscopy for the diagnosis of TB (Mole and Maskell 2001).

2.3.2 Culture

Culture examination of Mtb from sputum is the 100% confirmation for the diagnosis of TB (CDC 2011b). It is known as the "gold standard" for active TB diagnosis, in which different antibiotic tests can be carried out to identify the specific strain of TB that infects the patient.

Mtb can be cultured in solid or liquid media, in which the former allows colony morphology to be examined so that preliminary identification can be made (Frieden *et al*., 2003). However, it is not possible for Mtb to be present in every sample collected from an infected patient, especially when the sample is non-pulmonary. Culture method is also complicated and troublesome because of the slow growth rate of Mtb. Culture method is highly sensitive but it is time-consuming and is prone to contamination (Thornton *et al*., 1998).

2.3.3 Clinical diagnosis

Most clinical diagnoses are available in normal clinics or hospitals without the requirement for tedious laboratory analyses.

TB blood test is also known as interferon-gamma release assay (IGRA), which tests for the immune response mounted against Mtb infection sensitized T cells detectable in the blood sample (CDC 2011c). IGRA test cannot identify whether the TB infection is latent or active. TB blood test result is available in 24 hours and is not affected by previous BCG vaccination (CDC 2011c). However, according to WHO (2011b), TB blood test is unreliable and often leads to misdiagnosis because the immune responses mounted vary in different people. Tuberculin skin test (TST) involves injecting a substance known as tuberculin or purified protein derivative (PPD) under the skin of the patient, followed by observation of the reactions after 48 hours (NLM and NIH 2013). Swelling, harden, raised area of the skin at the injection site is a positive reaction which

indicate the presence of Mtb, but is unable to differentiate between latent and active TB infection (CDC 2012).

Chest radiography or chest X-ray can be used for diagnosing TB infection by checking the abnormalities of TB-infected lungs, such as tubercles formation and inflammation in the lung tissues. However, Kumar *et al*. (2005) reported that the sensitivity and specificity of chest radiography are unsatisfactory for pulmonary TB diagnosis. The abnormalities showed by chest X-ray might be due to other diseases that produce similar appearance. Other than that, the infected lungs may not show any abnormalities at the early stage of the infection and this can lead to misdiagnosis. Therefore, TST and chest radiography are often coupled with AFB microscopy and culture method for more accurate TB diagnosis (Dorman 2010).

2.3.4 Molecular techniques

Molecular techniques commonly used for diagnosis of TB including polymerase chain reaction (PCR), DNA sequencing, loop-mediated isothermal amplification (LAMP) assay and nucleic acid probe tests. The sensitivity of molecular methods is high, probably approaching 95-98% (Frieden *et al*. 2003).

PCR is the most common molecular techniques used in TB diagnosis. Purified DNA collected from clinical sputum is specifically amplified by PCR and result is usually available in 2 hours (Garberi *et al*., 2011). PCR method can be coupled with DNA sequencing to accurately identify the mycobacteria species present in the sputum (Seetha *et al*., 2009). Previous studies reported that sputum PCR can

be used for detection of genetic changes in Mtb, for instance those that lead to drug resistance (Winetsky *et al*., 2012).

PCR is highly sensitive and is able to amplify small amounts of DNA extracted from acid-fast-positive sputum (Davis *et al*., 2011). Culture method is usually carried out to increase the Mtb cell number before PCR is conducted. Similarly, the loop-mediated isothermal amplification (LAMP) assay makes use of extensive amplification of nucleic acids, in which the result can be obtained within one hour (Iwamoto *et al*., 2003).

The disadvantages of molecular techniques are their high cost and the requirement for sophisticated laboratory equipments. According to WHO (2006), TB diagnosis costs more than US$ 1 billion every year, but yet there are still million of TB cases left undiagnosed in developing countries. Many countries with high TB burden are poor and are unable to afford expensive molecular diagnosis (Neonakis *et al*., 2011).

2.3.5 Phage amplification assay

Since the early of the 21st century, scientists had sought for the application of lytic mycobacteriophages as tools for rapid detection of Mtb, a diagnostic method known as the phage amplification assay. In general, mycobacteriophages are viruses that specifically infect mycobacteria. They can be classified into lytic phages and temperate phages, such as D29 and L5, respectively. Most of these phages can infect both rapid- and slow-growing mycobacterial species. The lytic

phage D29 is commonly chosen to perform mycobacteriophage amplification assay because it replicates immediately after infection without integrating their DNA into the host's genome. D29 is a double-stranded DNA phage that possesses wide host range and high host specificity, as it does not extend its host range beyond a single genus (Hatfull 2000). Previous studies reported that Mtb is able to be infected by mycobacteriophage D29, with the lysis of bacterial cells resulting in clear areas called plaques formed on the bacterial lawn after 24-hour incubation at 37^{o}C (McNerney *et al*., 2004).

The general principle of the phage amplification assay is based on the infection by mycobacteriophage to indicate the presence of viable host bacteria by enumerating the number of plaques formed by the progeny phage particles after overnight incubation (Alcaide *et al*., 2003; Figure 2.1). Phage D29 particles replicate and accumulate inside the host, using their host's energy, ribosomes and other resources (Todar 2008). Ferrous ammonium sulphate (FAS), a specific virucide, initiates the virucidal action to eliminate all exogenous phages that are not involved in infection. Progeny phages are released following lysis of the infected host cells and these then infect the sensor cells (commonly *M. smegmatis*). This result in formation of plaques and the number of plaques formed corresponds to the number of viable mycobacterial cells originally present in the sample (McNerney *et al*., 2004). *M. smegmatis* is chosen as sensor cells as its lytic cycle can be completed within 90 min, enabling results to be obtained within 48 h as compared to 13 h for Mtb (McNerney 1999).

Plaques formed are indicative of the infection of viable mycobacteria by mycobacteriophage, which can be easily analyzed by naked eyes (Prakash *et al*., 2009). Albay *et al*. (2003) reported that the sensitivity of this method approached 100 bacilli per ml with sputum specimens. Therefore, phage amplification assay is said to be well suited in developing countries as compared to other diagnostic techniques because it is highly sensitive, specific, cost-effective, rapid, and can provide live-dead differentiation in detecting the presence of mycobacteria.

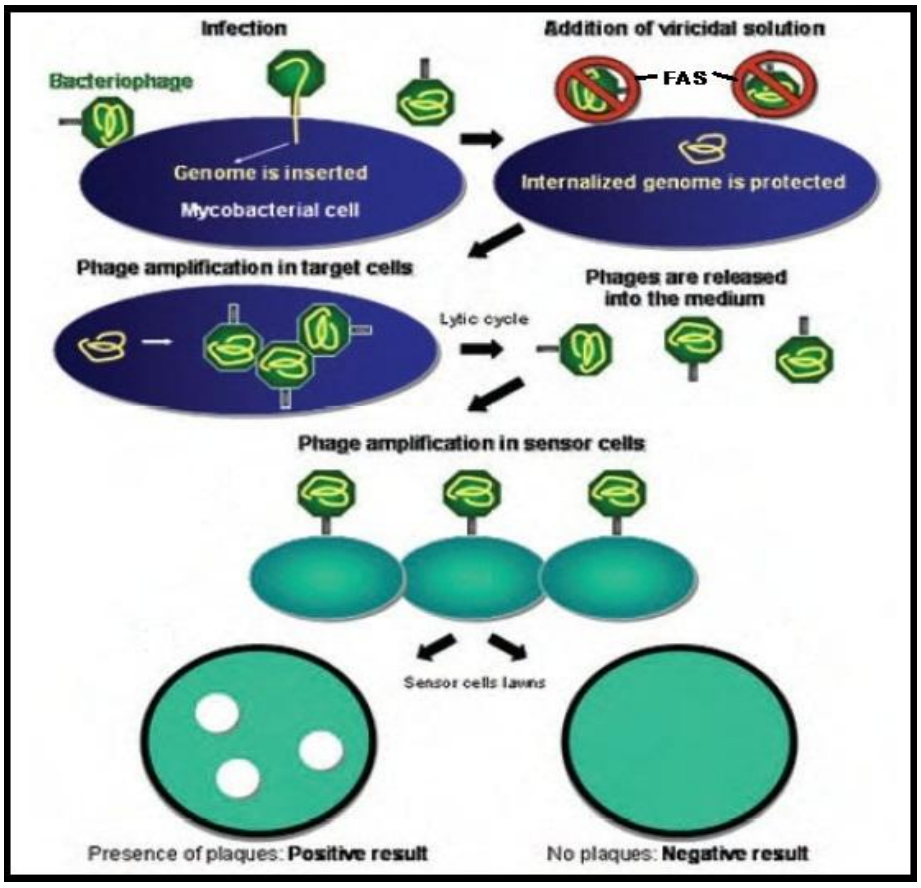

Figure 2.1 Principle of the mycobacteriophage amplification assay. Reproduced from the journal article by Hazbón (2004).

2.4 General responses of mycobacteria towards stresses

2.4.1 Thick mycobacterial cell wall acts as protective barrier

The term "stresses" refers to any terrible, unfavorable or tension conditions that threaten the survival of mycobacteria. Mtb is airborne and is transmitted through air to another host. When they are expelled from the host, these bacteria can stay alive, floating around for minutes to hours in the environment (Saviola 2010). However, they are exposed to external stresses, such as desiccation and UV radiation and internal stresses, such as oxidative stress and starvation.

Mycobacteria have the extraordinary capability to survive better than other bacteria due to their thick mycolic cell wall (Saviola 2010). Mtb apparently have unique defense strategies to resist against harsh environments, for instance, the killing mechanisms of macrophages in the host immune system (Gupta and Chatterji 2005). Trehalose dimycolate, a glycolipid cord factor present within the cell wall, constitutes the cell's physical barrier that protects it from damage (Saviola 2010).

2.4.2 Gene regulation

Specific gene induction is a common response of mycobacteria when facing stresses. Mycobacteria can regulate different genes and produce proteins under certain conditions by inducing or inhibiting some biochemical pathways (Saviola 2010). Regulating useful mycobacterial resources, such as RNAs, allows mycobacteria to withstand stressful environments in a more effective way. Ojha *et al*. (2000) reported that mycobacteria possess an adaptive mechanism known as

the "stringent response", specifically when mycobacteria encounter nutritional stress. The stringent factor, guanosine tetraphosphate, is accumulated during stringent response and it down-regulates rRNA and tRNA synthesis by targeting the RNA polymerase (Cashel *et al*., 1996; Chatterji *et al*., 1998).

Previous studies showed that guanine nucleotides are involved in this stress resistant mechanism in certain mycobacteria, for instances Mtb, *M. leprae* and *M. smegmatis* (Ojha *et al*., 2000; Avarbock *et al*., 1999; Lee and Colston 1985). Common genes that are usually down-regulated when mycobacteria encounter stresses are those encoding ribosomal proteins and lipid biosynthetic enzymes (Chatterji and Ojha 2001).

2.4.3 Entry into stationary phase

Accumulation of toxic compounds and unfavorable growth conditions can cause bacteria to enter stationary phase (Kjelleberg 1993). Smeulders *et al*. (1999) and Wallace (1961) reported that bacterial cells that reduce their division rate would have become more resistant to external stresses, such as osmotic stress and high temperature. Latent Mtb bacilli isolated from mice possess strong resistance to high temperature *in vitro* during the stationary phase (Wallace 1961).

Mycobacteria are able to sense very limited concentration of carbon source, such as glycerol, before it completely runs out. Thereby, they initiate a "shut down" of growth and enter the stationary phase, allowing bacterial cells to survive without sufficient nutrient for up to more than one year (Smeulders *et al*., 1999).

2.4.4 Entry into dormant state

Previous studies reported that Mtb cells would possibly enter a "non-culturable" state after they entered the stationary phase for some times (Kaprelyants and Kell 1993; Kaprelyants *et al*., 1994; Shleeva *et al*., 2002). Mtb would enter dormancy when encountered with stresses and their shapes might change (Deb *et al*., 2009).

Mtb cells present in lung lesions of infected host show different growing morphologies and staining properties as those growing *in vitro* (Nyka 1967). When Mtb is starved *in vitro*, they show similar properties as above, in which they appear in small spherical shape instead of rod shape, and they are not acid-fast (Nyka 1967). However, Mtb is not dying under those conditions. Studies showed that they are able to retain their viability and virulence for up to two years during starvation and can resuscitate rapidly to their normal morphologies once fresh nutrient medium is supplied (Nyka 1974).

2.5 Resuscitation-promoting factors

Mtb and other actinobacteria possess the ability to secrete a family of proteins called Rpfs that are responsible for mycobacterial growth (Kell and Young 2000; Mukamolova 2010). Mtb have *rpfA-E* genes that encode for five types of Rpf proteins which are collectively dispensable for Mtb growth *in vitro* (Kana *et al*., 2007). Mtb that remain dormant inside the host's body can regain their virulence and restore their culturability (Kana *et al*., 2007). After that, they will start to reproduce and cause active disease to their host. Previous studies showed that *M.*

smegmatis Rpf proteins are proved to have resuscitation effect on "non-culturable" cells, reactivating them for growth.

Rpf proteins are believed to be involved in chronic TB infection, possessing muralytic activities similar to those of lysozymes. They break off or cleave the thick cell wall of mycobacteria, promoting multiplication and expression of virulence (Mukamolova *et al.*, 2010). Other than that, Rpf proteins are also believed to play important role in re-stimulating the growth and multiplication of dormant and stressed mycobacteria, such as nutrient-starved mycobacteria, with active concentrations in the picomolar or subpicomolar range (Mukamolova *et al*., 2002).

Smeulders *et al*. (1999) stated that nutrient-starved bacterial cells need specific signals to allow resuscitation, which are possibly the Rpf proteins secreted by that particular mycobacterium itself. Mukamolova *et al*. (2002) reported that Rpf proteins from Mtb did show cross-species cell activation, regardless of whether they are slow-growing or rapid-growing mycobacterial species. Previous *in vivo* studies showed that Rpf supports Mtb survival and revives dormant Mtb leading to disease reactivation, but its influence towards human infection is still currently unknown (Mukamolova *et al*., 2002).

CHAPTER 3

METHODOLOGY

3.1 Experimental design

The overview of the experimental design of this project is summarized in Figure 3.1.

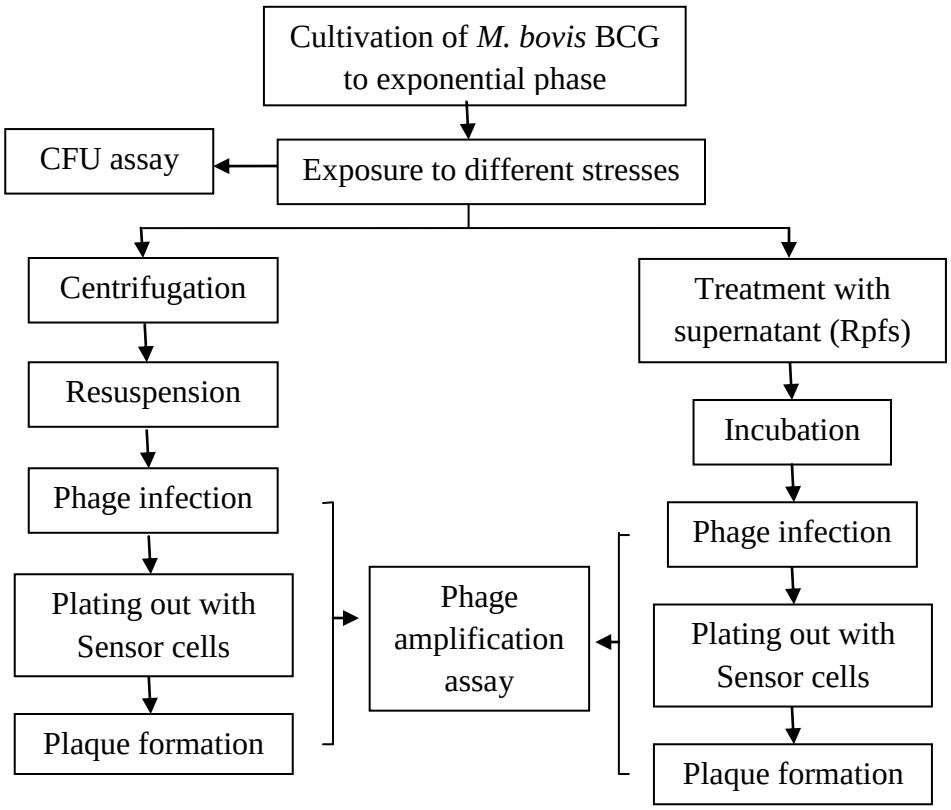

Figure 3.1 Overview of the experimental design of this project.

3.2 Apparatus and consumables

Apparatus and consumables used in this project are listed in Appendix A.

3.3 Preparation of culture media

All media were sterilized by autoclaving at 121℃ for 15 min unless otherwise stated.

3.3.1 Luria-Bertani agar

Luria-Bertani (LB) agar was prepared by dissolving 16.0 g of LB agar powder in 400 ml of distilled water (dH_2O).

3.3.2 Middlebrook 7H9-OADC-Tween broth

Middlebrook 7H9 broth was prepared by mixing 0.94 g of Middlebrook 7H9 broth powder with 0.5 g of glycerol and dissolving them in 180 ml of dH_2O. The autoclaved medium was supplemented with 10% v/v OADC and 0.05% w/v Tween 80 before use.

3.3.3 Middlebrook 7H9-OADC broth

Middlebrook 7H9 broth was prepared as described in Section 3.3.2. The medium was supplemented with 10% v/v OADC before use, without the addition of Tween 80. Tween 80 was excluded as it inhibits the interaction and adsorption mycobacteriophages to mycobacterial cells (Cheah 2010). This medium is used for cultivation of *M. smegmatis* lawn culture.

3.3.4 Middlebrook 7H9-OGC broth

Middlebrook 7H9 broth was prepared as described in Section 3.3.2, without the addition of Tween 80. The medium was supplemented with 10% v/v OADC and 1

mM $CaCl_2$ before use. The $CaCl_2$ solution provides calcium ions to promote adsorption of mycobacteriophages to mycobacterial cells during phage infection (Sellers *et al*., 1962).

3.3.5 Middlebrook 7H10 agar

Middlebrook 7H10 agar was prepared by mixing 7.6 g of Middlebrook 7H10 agar powder and 2.5 g of glycerol in dH_2O to a final volume of 360 ml. The autoclaved agar was cooled to 55^{o}C and was supplemented with 10% v/v OADC before use.

3.4 Preparation of reagents

All reagents were sterilized by autoclaving at 121 ℃ for 15 min unless otherwise stated.

3.4.1 Acid-alcohol

Acid-alcohol was prepared by mixing 3 ml of concentrated HCl with 97 ml of 95% v/v ethanol solution.

3.4.2 Carbolfuchsin stain

An amount of 0.3 g of basic fuchsin powder was dissolved in 10 ml of 95% v/v ethanol and 5 ml of heat-melted phenol crystals was dissolved in 95 ml of dH_2O. Both solutions were mixed and heated for 30 min on a hot plate. The mixture was left to stand for 2-5 days and then filtered with filter paper before use.

3.4.3 Ferrous ammonium sulphate

The virucide ferrous ammonium sulphate (FAS) for phage D29 was always prepared fresh before use in the phage amplification assay. FAS was prepared by dissolving 0.39 g of FAS powder in 20 ml of dH_2O, yielding the final concentration of 50 mM. It was filter sterilized using a 0.2-µm syringe filter before use.

3.4.4 Glycerol solution, 65% v/v

Glycerol solution was prepared by mixing 162.5 g of glycerol, 20 ml of 1 M $MgSO_4$ and 5 ml of 1 M Tris-HCl (pH8) in dH_2O to a final volume of 200 ml.

3.4.5 Methylene blue stain

The methylene blue stain was prepared by dissolving 0.3 g of methylene blue chloride powder in 100 ml dH_2O.

3.4.6 Mycobacteriophage buffer

Mycobacteriophage (MP) buffer was prepared by mixing together 4 ml of 1 M Tris-Cl (pH 7.6), 40 ml of 1 M NaCl, 4 ml of 1 M $MgSO_4$, 0.8 ml of 1 M $CaCl_2$ and 351.2 ml of dH_2O.

3.4.7 Phosphate buffer, 67 mM, pH 6.8

The stock solutions 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 were first prepared. Phosphate buffer was prepared by mixing both KH_2PO_4 and K_2HPO_4 with dH_2O to their final concentrations of 34 mM and 33 mM, respectively.

3.4.8 Phosphate-buffered saline

Phosphate buffered saline (PBS) was prepared by dissolving one PBS pellet in 100 ml of dH_2O.

3.4.9 Sodium hydroxide solution, 2% w/v

Sodium hydroxide solution was prepared by dissolving 0.2 g of sodium hydroxide pellets in 10 ml of dH_2O. The solution was always prepared fresh and filter sterilized using a 0.2-µm syringe filter before use.

3.4.10 Tween 80, 10% w/v

Tween 80 solution was prepared by dissolving 10 g of Tween 80 in 100 ml dH_2O. The solution was warmed at 40^oC for 30 min and filter sterilized using a 0.2-µm syringe filter. The solution was stored at 4^oC away from light.

3.5 General methods

3.5.1 Cultivation of mycobacteria culture

3.5.1.1 Preparation of culture stocks for long-term storage

Both *M. smegmatis* and *M. bovis* BCG were grown to the exponential phase. Stock cultures were prepared by mixing the bacterial cultures with 65% v/v glycerol solution in 1:1 ratio. The cell suspension was then aliquoted into 1.5-ml cryovials in 1-ml aliquots. The glycerol stocks were then stored at -80^oC for long-term storage.

3.5.1.2 Cultivation of *M. bovis* BCG

An aliquot of *M. bovis* BCG glycerol stock was thawed at room temperature and then inoculated into 5 ml of Middlebrook 7H9-OADC-Tween broth in a 50-ml centrifugation tube. The culture was incubated static at 37^{o}C until it reached the OD_{580nm} of approximately 0.7. The resulting *M. bovis* BCG culture was then subcultured into 25 ml of Middlebrook 7H9-OADC-Tween broth in a 100-ml conical flask to OD_{580nm} of approximately 0.05. The culture was incubated under the same condition to the exponential phase (about 3 days).

3.5.1.3 Cultivation of *M. smegmatis*

An aliquot of *M. smegmatis* glycerol stock was thawed at room temperature and then inoculated into 25 ml of Middlebrook 7H9-OADC-Tween broth in a 100-ml conical flask. The culture was incubated at 37^{o}C with shaking at 200 rpm to reach OD_{580nm} of 1.0-1.5. The resulting culture was then subcultured into 25 ml of Middlebrook 7H9-OADC-Tween broth in a 100-ml conical flask to OD_{580nm} of approximately 0.05. The culture was incubated under the same condition for about 24 h to reach the exponential phase.

3.5.1.4 Preparation of *M. smegmatis* lawn culture

M. smegmatis lawn culture was prepared as described in Section 3.5.1.3. The medium used for cultivation was Middlebrook 7H9-OADC broth without the addition of Tween 80, as described in Section 3.3.3.

3.5.1.5 Measuring optical density of cultures

The optical density (OD) of mycobacterial cultures were measured spectrophotometrically at the wavelength of 580 nm (Sartain *et al*., 2011). A volume of 1 ml of culture was transferred to a cuvette for measurement. A ten-fold dilution would be performed for dense cultures with OD_{580nm} of more than 1.

3.5.1.6 Enumeration of colony-forming units

Ten-fold serial dilutions were performed on a liquid culture in 450-µl aliquots of Middlebrook 7H9 broth in 1.5-ml microcentrifuge tubes. Each dilution was plated out by dropping three drops of 20 µl on a Middlebrook 7H10 agar plate. The plates were incubated at 37^{o}C for 2-3 days for *M. smegmatis* and at least 2 weeks for *M. bovis* BCG, for colonies to become observable. The dilution that yielded 10-100 colonies was used to calculate the CFU/ml of the test culture.

3.5.1.7 Preparation of *M. smegmatis* culture supernatant

Mycobacterial supernatant contains Rpf proteins that were required in this project. *M. smegmatis* with the initial OD_{580nm} of approximately 0.05 was cultured in Middlebrook 7H9-OADC broth for 20 h at 37^{o}C with shaking at 200 rpm. The culture was then centrifuged at 3000 *g* for 15 min and the resulting supernatant was filter sterilized using a 0.2-µm syringe filter. An aliquot of the filtered supernatant was incubated at 37^{o}C for 24 h to check for presence of carry-over mycobacterial cells.

3.5.2 Ziehl-Neelsen acid-fast staining

A loopful of liquid culture was smeared onto a microscope slide and then heat-fixed. For solid culture, a loopful of colonies was smeared in a drop of dH_2O on the slide. The smear was first stained with the carbolfuchsin stain. The underside of the slide was gently heated by passing a flame under the staining rack until steam was observed and then the smear was left to stain for 5 min.

The carbolfuchsin stain was gently washed away with running tap water until no color appeared in the effluent. The smear was then decolorized with acid-alcohol until the stained smear appeared faintly pink and the effluent was clear. The smear was then flooded with the methylene blue counterstain for about 30 s and then rinsed with tap water. The slide was blotted dry with a filter paper and then examined under the light microscope at 1000× magnification.

3.5.3 Mycobacteriophage D29

3.5.3.1 Propagation

Firstly, phage indicator plates were prepared by plating out 1 ml of *M. smegmatis* lawn culture with 9 ml of molten Middlebrook 7H9-OGC agar each. A volume of 100 µl of phage D29 suspension was spread on the surface of an indicator plate and incubated at 37^{o}C overnight. Plaques were observed on the *M. smegmatis* lawn following incubation. A volume of 10 ml of Middlebrook 7H9-OGC was pipetted onto each plate and they were further incubated overnight at 37^{o}C. The resulting suspension was then transferred into a 50-ml centrifugation tube and then

filtered twice using 0.45-µm syringe filters. The resulting phage suspension was aliquoted into 1.5-ml microcentrifuge tubes in 1-ml aliquots and stored at 4^{o}C away from light.

3.5.3.2 Enumeration

A ten-fold serial dilution was performed on a phage D29 suspension in 450-µl aliquots of MP phage buffer in 1.5-ml microcentrifuge tubes. Each dilution was plated out by dropping three drops of 10 µl on an indicator plate (two dilutions per plate). The plates were incubated at 37^{o}C overnight. The plaque-forming unit (PFU) was calculated from the dilution that yielded 10-100 plaques.

3.5.3.3 Phage amplification assay

M. bovis BCG culture was centrifuged at 3000 *g* for 15 min and the resulting pellet was resuspended in Middlebrook 7H9-OGC broth. A volume of 1 ml of the suspension was transferred into a 7-ml Bijoux bottle, followed by the addition of 500 µl of phage D29 suspension (10^{8} PFU/ml). The mixture was then incubated at 37^{o}C for one hour to allow phage infection of *M. bovis* BCG cells. A volume of 300 µl of 50 mM FAS solution was added after the incubation to eliminate all the exogenous phages that were not involved in the infection and the mixture was briefly vortexed. If dilutions were required, it would be performed at the point using Middlebrook 7H9-OGC broth. A volume of 1 ml of the mixture and 1 ml of the lawn culture (as described in Section 3.5.1.4) were added to 9 ml of molten Middlebrook 7H9-OADC agar. The resulting mixture was mixed well and then

poured into a Petri dish. After the agar had solidified, the plate was incubated at 37^{o}C overnight. The number of plaques formed on the plate was then enumerated.

3.6 Exposure of *M. bovis* BCG cells to various stresses *in vitro*

3.6.1 Growth in stationary phase

M. bovis BCG culture was performed as described in Section 3.5.1.2. The culture was incubated at 37^{o}C for approximately 15 days for *M. bovis* BCG cells to enter the stationary phase.

3.6.2 Desiccation

A volume of 100 µl of exponential-phase *M. bovis* BCG culture suspension was spotted onto a sterilized cover slip (sterilized by 70% v/v ethanol and passing through flames) and placed inside a Petri dish. The Petri dish was left opened under laminar air flow for at least one hour until the culture evaporated completely. The cover slip was then transferred into 10 ml of Middlebrook 7H9-OGC broth in a 50-ml centrifuge tube and vortexed for 3 min. For the control, the same procedures were performed, except that the Petri dish was left closed and sealed with laboratory sealing film to prevent desiccation of the spotted culture suspension.

3.6.3 Nutrient starvation

A volume of 10-20 ml of exponential-phase *M. bovis* BCG culture was transferred into a 50-ml centrifugate tube and centrifuged at 3000 *g* for 15 min. The resulting

supernatant was discarded and then the pellet was resuspended in the same volume of PBS solution. Centrifugation was repeated twice to allow complete removal of nutrients from the previous medium. *M. bovis* BCG cells were starved for one week at 37^{o}C. For the control, the same procedures were performed except that Middlebrook 7H9-OADC-Tween broth was used to resuspend the pellet.

3.6.4 Exposure to ultraviolet radiation

A volume of 3 ml of exponential-phase *M. bovis* BCG culture was transferred into a Petri dish. The culture was exposed to UV radiation (wavelength of 302 nm) under laminar air flow for 30 min. For the control, the Petri dish was wrapped with aluminum foil before exposure to UV light.

3.6.5 Exposure to light versus dark conditions

A volume of 10 ml of exponential-phase *M. bovis* BCG culture was transferred into a Petri dish. For dark condition, the Petri dish wrapped with aluminum foil to avoid exposure to light. For light condition, the Petri dish was left unwrapped for exposure to room light. Both conditions were performed for 24 h.

3.6.6 Chemical stress of NaOH decontamination

A volume of 1 ml of exponential-phase *M. bovis* BCG culture was transferred into a 50-ml centrifuge tube, followed by addition of 1 ml of 2% w/v NaOH solution. The mixture was vortexed for less than 30 s and left at room temperature for 15 min. The mixture was then neutralized with 20 ml of 67 mM phosphate buffer and centrifuged at 3000 *g* for 15 min, followed by resuspension of the resulting pellet

in 1 ml of Middlebrook 7H9-OGC broth. The tube contents were incubated at 37^{o}C for 24 h to resuscitate chemically-stressed cells. For the control, the 2% w/v NaOH was replaced with deionized water.

3.7 Treatment of stressed *M. bovis* BCG cells with *M. smegmatis* culture supernatant

A volume of 1 ml of stressed *M. bovis* BCG culture was transferred into a 50-ml centrifuge tube, followed by addition of 1 ml of filter sterilized *M. smegmatis* culture supernatant (containing Rpf proteins). The mixture was then incubated at 37^{o}C for 24 h. The treated *M. bovis* BCG culture was then centrifuged at 3000 *g* for 15 min and resuspended in 2 ml of Middlebrook 7H9-OGC broth. For the control, the culture supernatant was replaced with Middlebrook 7H9-OADC broth.

3.8 Statistical analysis

All results obtained were subjected to one-tail paired *t* test.

CHAPTER 4

RESULTS

4.1 Exposure of *M. bovis* BCG cells to various stresses *in vitro*

Each stress condition was conducted using exponential-phase *M. bovis* BCG culture, with incubation duration of 3 days (approximately 70 hours), unless otherwise stated.

4.1.1 Growth in stationary phase

Exponential-phase and stationary-phase *M. bovis* BCG cultures were subjected to phage amplification assay and their PFU/CFU ratios were compared in Table 4.1. The ratios obtained in stationary-phase culture were always lower than those in exponential phase culture by approximately 2-fold in average. Dilutions were performed in order to get countable plaques for PFU counting (Figure 4.1)

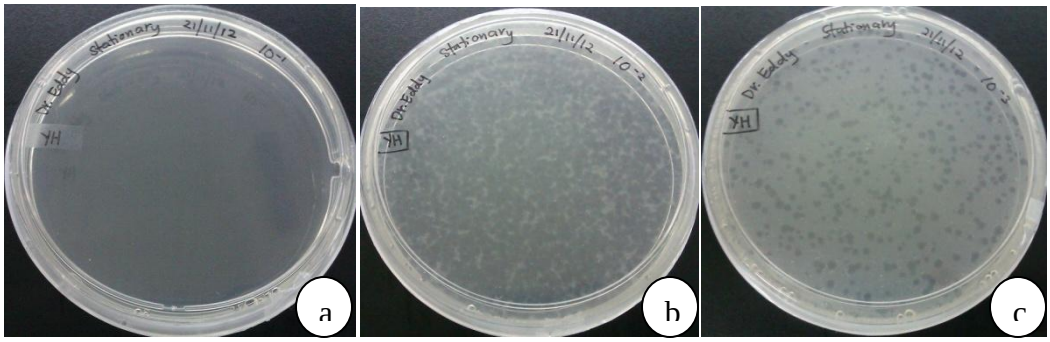

Figure 4.1: Indicator plates from phage amplification assay. a) Complete lysis; b) confluent lysis; and c) countable plaques.

Table 4.1: Results from exponential-phase and stationary-phase *M. bovis* BCG.

Exponential phase			Stationary phase		
PFU/ml	**CFU/ml**	**PFU/CFU ratio (%)**	**PFU/ml**	**CFU/ml**	**PFU/CFU ratio (%)**
Confluent lysis	4×10^6	-	Complete lysis	5×10^7	-
Confluent lysis	4×10^5	-	Confluent lysis	5×10^6	-
4×10^3	4×10^4	10	Confluent lysis	5×10^5	-
342	4×10^3	8.6	1.2×10^3	5×10^4	2.4
20	400	5	205	5×10^3	4.1
0	40	-	20	500	4
0	4	-	2	50	3.6
0	-	-	0	5	-

CFU assays were carried out in drop-plate method as described in Section 3.5.1.6. The dilutions that yielded 10-100 colonies were used to calculate the CFU/ml of the test culture (Figure 4.2).

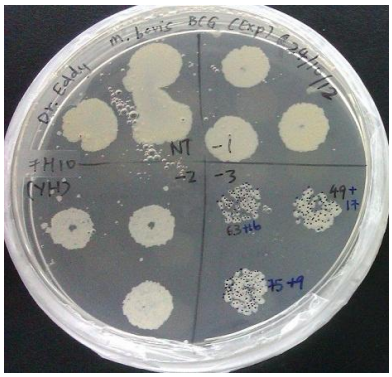

Figure 4.2: Plate from exponential phase CFU plating assay.

4.1.2 Desiccation

Desiccation is one of the common stresses that might be encountered by mycobacteria when they are in the environment. Exponential-phase *M. bovis* BCG culture was spotted onto sterilized cover slips and exposed for desiccation under laminar air flow for about 2 h. The PFU counts for desiccated mycobacterial cells decreased significantly by approximately 100-fold relative to those for the control ($P < 0.05$; Table 4.2).

Table 4.2: PFU counts of desiccated *M. bovis* BCG suspension and control.

Control			Stressed		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	9.00×10^4		**1**	5.40×10^3	
2	8.46×10^4	1.15×10^5	**2**	7.20×10^3	4.80×10^3
3	1.71×10^5		**3**	1.80×10^3	

4.1.3 Nutrient starvation

Exponential-phase *M. bovis* BCG culture was starved for one week in phosphate-buffered saline solution. Their phage infectivity was assessed based on the number of PFUs formed following the phage assay. PFU counts showed that there was approximately 100-fold reduction after the starvation ($P < 0.05$; Table 4.3).

Table 4.3: PFU counts of starved *M. bovis* BCG suspension and control.

Control			Stressed		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	1.51×10^7		**1**	7.20×10^4	
2	1.64×10^7	1.28×10^7	**2**	5.40×10^5	2.52×10^5
3	7.00×10^6		**3**	1.44×10^5	

4.1.4 Exposure to ultraviolet radiation

M. bovis BCG culture was exposed to UV radiation with a wavelength of 302 nm for 30 min. Their phage infectivity was assessed based on the number of PFUs formed following the phage assay. An approximately 10-fold reduction on PFU counts was observed after the UV exposure ($P < 0.05$; Table 4.4).

Table 4.4: PFU counts of UV-exposed *M. bovis* BCG suspension and control.

Control			Stressed		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	4.95×10^4		**1**	2.09×10^4	
2	5.74×10^4	5.38×10^4	**2**	1.44×10^3	8.77×10^3
3	5.44×10^4		**3**	3.96×10^3	

4.1.5 Chemical stress during NaOH decontamination

Exponential-phase *M. bovis* BCG culture was subjected to NaOH treatment for 15 min. Chemically-stressed *M. bovis* BCG cells were evaluated for their phage infectivity through the phage amplification assay. The PFU counts of chemically-stressed *M. bovis* BCG culture showed approximately 10-fold reduction than those unstressed culture ($P < 0.05$; Table 4.5).

Table 4.5: PFU counts of chemically-stressed *M. bovis* BCG suspension and control.

Control			Stressed		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	3.73×10^5		**1**	6.30×10^4	
2	4.79×10^5	4.34×10^5	**2**	3.24×10^4	4.44×10^4
3	4.50×10^5		**3**	3.78×10^4	

4.1.6 Exposure to light and dark conditions

Exponential-phase *M. bovis* BCG culture was exposed to both light and dark conditions in the room for 24 h and the phage amplification assay was performed. The PFU counts of dark-exposed culture showed only a slight reduction which was considered insignificant ($P > 0.05$; Table 4.6).

Table 4.6: PFU counts of exposed *M. bovis* BCG suspension and control.

Exposed to light			Exposed to dark		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	4.88×10^4		**1**	2.84×10^4	
2	3.38×10^4	3.98×10^4	**2**	3.55×10^4	2.45×10^4
3	3.69×10^4		**3**	9.54×10^3	

4.2 Treatment of stressed *M. bovis* BCG cells with *M. smegmatis* culture supernatant

M. smegmatis supernatant (containing Rpfs) was used in this project, which is believed to have muralytic activity that may resuscitate dormant mycobacterial cells back to their active state by stimulating their growth (Mukamolova *et al*., 2010). All stressed *M. bovis* BCG cultures were subjected to supernatant treatment except for those tested for light and dark conditions. Leftover supernatant was incubated overnight at 37^{o}C to check for carry-over cells that may cause false-positive effect on supernatant-treated *M. bovis* BCG culture. Figure 4.3 showed the supernatant was compared with 24-h-incubated *M. smegmatis* culture.

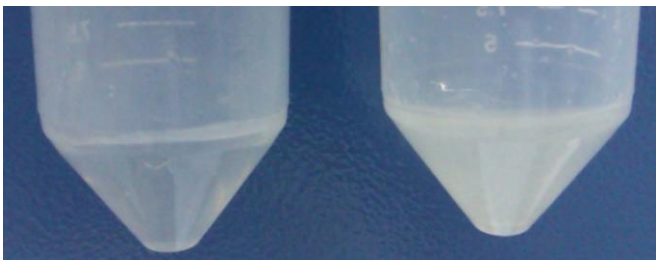

Figure 4.3: Check for carry-over mycobacterial cells from *M. smegmatis* supernatant. Left: overnight-incubated *M. smegmatis* supernatant (clear); right: overnight-incubated *M. smegmatis* culture (turbid).

4.2.1 Growth in stationary phase

The PFU counts of control and supernatant-treated of stationary-phase *M. bovis* BCG culture are shown in Table 4.7. The results showed that the PFU counts of supernatant-treated culture were relatively lower than that for the control, but the reduction was insignificant ($P > 0.05$; Table 4.7).

Table 4.7: PFU counts of supernatant-treated stationary-phase *M. bovis* BCG suspension and control.

Control			Supernatant treatment		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	3.10×10^6		**1**	9.2×10^5	
2	2.21×10^6	2.54×10^6	**2**	2.36×10^6	2.04×10^6
3	2.32×10^6		**3**	2.84×10^6	

4.2.2 Desiccation

The PFU counts of control and supernatant-treated of desiccated *M. bovis* BCG culture are shown in Table 4.8. The PFU counts obtained from for the supernatant-treated desiccated culture was slightly lower than that for the control (Table 4.8). The PFU count was reduced by approximately 25 % but the reduction was insignificant ($P > 0.05$).

Table 4.8: PFU counts of supernatant-treated desiccated *M. bovis* BCG suspension and control.

Control			Supernatant treatment		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	1.37×10^4		**1**	8.10×10^3	
2	1.04×10^4	1.22×10^4	**2**	8.64×10^3	9.03×10^3
3	1.26×10^4		**3**	1.04×10^4	

4.2.3 Nutrient starvation

The PFU counts of control and supernatant-treated of starved *M. bovis* BCG culture are shown in Table 4.9. The PFU counts of culture did not increase after the supernatant treatment. Interestingly, it dropped by approximately 10-fold compared to the control, but it was still considered an insignificant reduction ($P > 0.05$).

Table 4.9: PFU counts of supernatant-treated starved *M. bovis* BCG suspension and control.

Control			Supernatant treatment		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	7.20×10^3		**1**	2.90×10^4	
2	1.04×10^4	4.19×10^4	**2**	3.24×10^3	1.17×10^3
3	1.08×10^5		**3**	2.88×10^3	

4.2.4 Exposure to ultraviolet radiation

The PFU counts of control and supernatant-treated of exposed *M. bovis* BCG culture are shown in Table 4.10. The supernatant did show some effect on the PFU as the plaque count was approximately doubled after supernatant treatment. However, the increment was not significant ($P > 0.05$). The number of PFU counts obtained was relatively lower than those from other tests.

Table 4.10: PFU counts of supernatant-treated UV-exposed *M. bovis* BCG suspension and control.

	Control			**Supernatant treatment**	
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	538		**1**	599	
2	20	253	**2**	43	402
3	202		**3**	563	

4.2.5 Chemical stress during NaOH decontamination

The PFU counts of control and supernatant-treated of chemically-stressed *M. bovis* BCG culture are shown in Table 4.11. The plaque count was approximately doubled after supernatant treatment, indicated that there was some effects shown by the Rpf proteins. However, the increment was not significant ($P > 0.05$) because of the inconsistent plaque count obtained from the triplicates for both controls and supernatant-treated samples. The number of PFU counts obtained was relatively lower than those from other tests.

Table 4.11: PFU counts of supernatant-treated chemically-stressed *M. bovis* BCG suspension and control.

Control			Supernatant treatment		
Replicates	**PFU/ml**	**Average**	**Replicates**	**PFU/ml**	**Average**
1	90		**1**	45	
2	2	32	**2**	162	70
3	5		**3**	4	

4.3 Confirmation of pure *M. bovis* BCG cultures

Contamination was the major problem encountered and made the works became more challenging when this project was carried out. Therefore, various ways to identify and confirm pure *M. bovis* BCG cultures were carried out.

4.3.1 Ziehl-Neelsen method for acid-fast staining

ZN staining was always performed to check the purity of mycobacterial cultures because acid-fastness is one of the distinguishable characteristics of mycobacteria. They retain the reddish-pink carbolfuchsin stain during ZN staining, while non-mycobacterial cells retain the counterstain methylene blue. *M. bovis* BCG cells tend to clump together as viewed under the light microscope when compared to *M. smegmatis* cells. Pure *M. bovis* BCG cultures showed only pink-stained cells without any blue-stained cells. *M. smegmatis* also appeared as rod-shaped cells under the light microscope as ZN staining is unable to differentiate among

Mycobacterium species. The only difference is that exponential-phase *M. smegmatis* cells are longer and thinner compared to *M. bovis* BCG cells in the same growth phase (Figure 4.4).

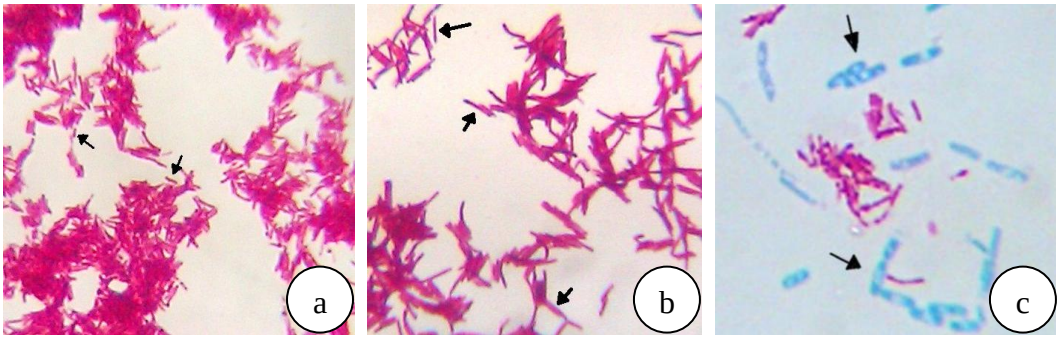

Figure 4.4: Cell morphologies of mycobacteria (1000×). a) *M. bovis* BCG Glaxo; b) *M. smegmatis* MC^2155; c) contaminated *M. bovis* BCG culture.

False contamination refers to the observation of blue-stained artifacts after ZN staining (Figure 4.5). These artifacts could be easily mistaken as contaminants if not interpreted carefully.

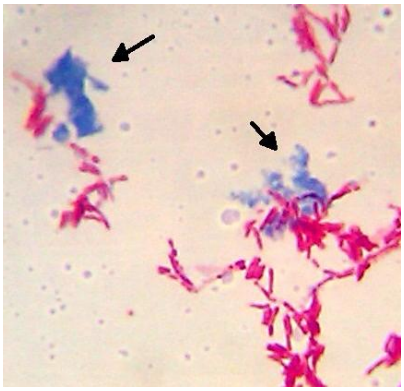

Figure 4.5: Blue-colored artifacts found in *M. bovis* BCG culture (1000×).

4.3.2 Measurement of optical density

In this project, the OD of *M. bovis* BCG cultivated in Middlebrook 7H9 broth that reached the exponential phase (about 3 days) usually fell into the range of 0.3-0.4. Mixed culture usually gave higher OD due to the fast growth of non-*Mycobacterium* species, leading to higher turbidity which could be easily observed in a *M. bovis* BCG broth culture. This included the cultures that were cross-contaminated by *M. smegmatis*, which took only 24 h to reach exponential phase with the OD of 1.0-2.5.

4.3.3 Colony morphology

M. bovis BCG culture took at least two weeks for colonies to be observable on Middlebrook 7H10 agar plate, but *M. smegmatis* took only two days. Colonies of *M. bovis* BCG were creamy-white in color, with domed, thick, rough and rugose surface and irregular margins, while colonies of *M. smegmatis* were moist, raised, but with wrinkled surface (Figure 4.6). Their colony appearances and growth rates are different and cross-contamination can be identified easily.

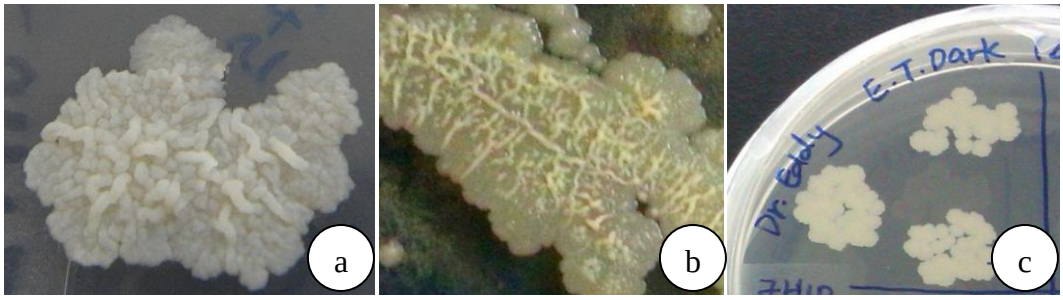

Figure 4.6: Morphologies of mycobacterial colonies grown on Middlebrook 7H10 agar. a) *M. bovis* BCG Glaxo; b) *M. smegmatis* MC^2155; c) contamination of *M. bovis* BCG CFU plate by *M. smegmatis*.

4.3.4 Size of plaques formed in the phage amplification assay

After phage amplification assay, plaques formed on the indicator plates appeared in different sizes for different test mycobacterial species used. *M. bovis* BCG cultures infected by phage D29 tend to show relatively smaller plaques compared to *M. smegmatis* after two days of incubation (Figure 4.7). If the plaque sizes were larger than the expected plaque size for *M. bovis* BCG, the culture was most probably contaminated by *M. smegmatis*.

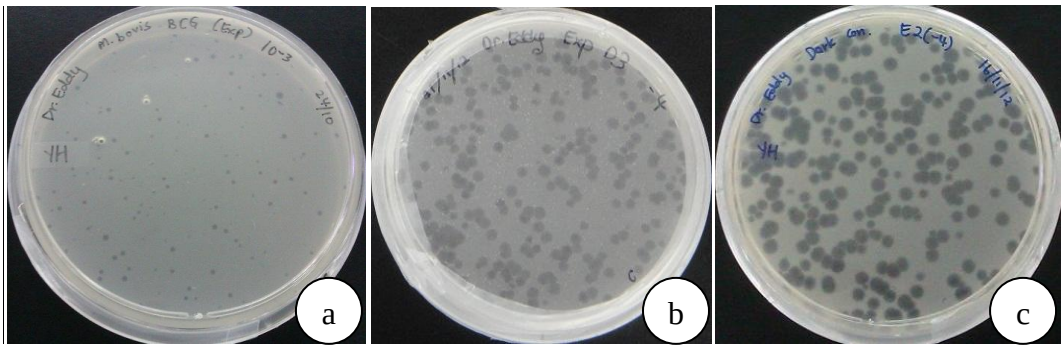

Figure 4.7: Phage indicator plates showing different plaque sizes from infection of different mycobacterial species. a) *M. bovis* BCG; b) *M. smegmatis*; c) contamination of *M. bovis* BCG phage amplification assay by *M. smegmatis*.

4.3.5 Other contaminants

Contamination during CFU plating was one of the major problems encountered in this project. Common contaminants were bacteria and fungi which easily covered up the area of drops of *M. bovis* BCG cell suspension before they could develop into observable colonies (Figure 4.8).

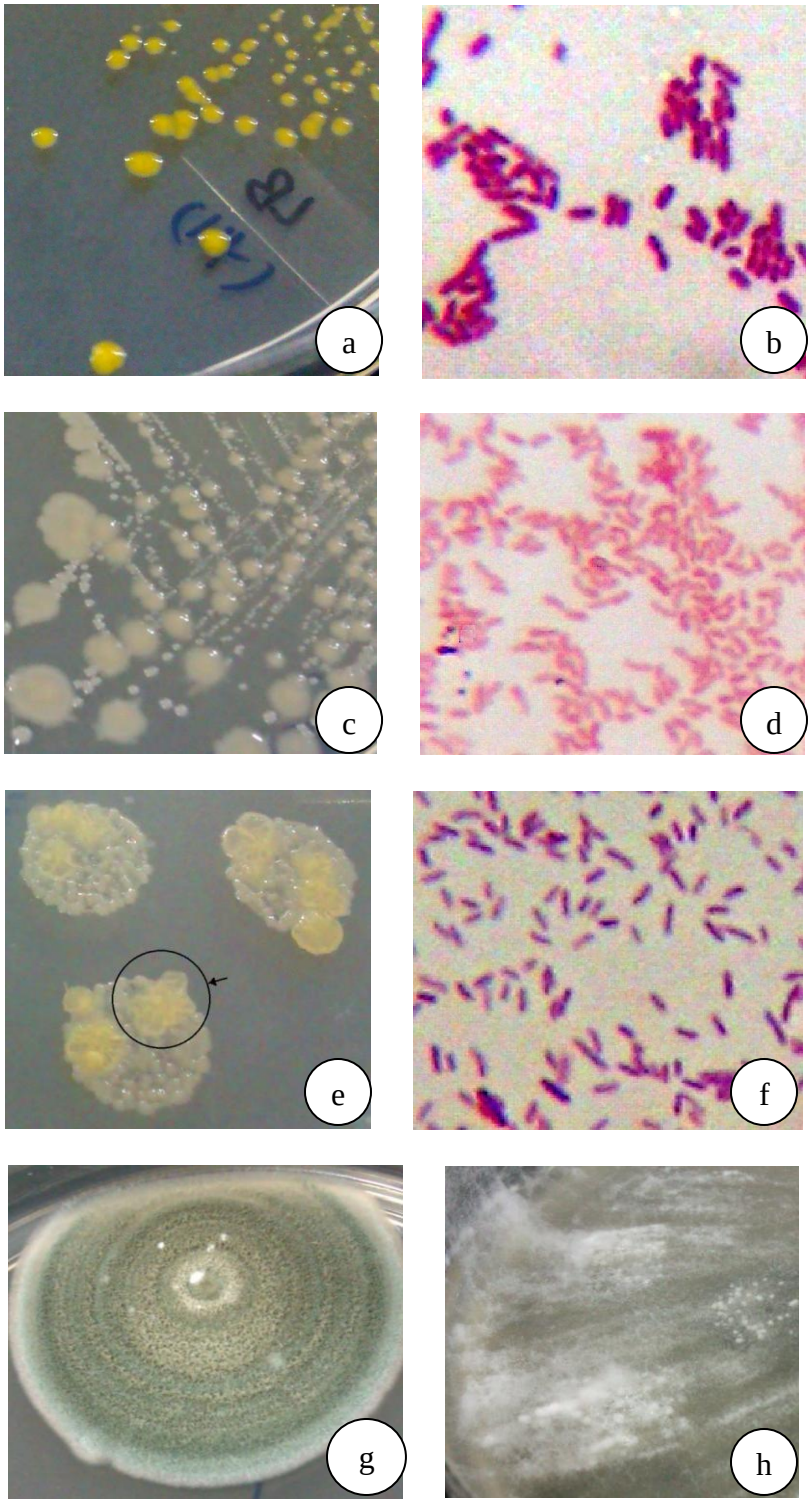

Figure 4.8: Contaminants encountered in CFU plating assay. a) and b) Yellow-colored bacterial colonies and cell morphologies (1000×); c) and d) creamy-white-colored bacterial colonies and cell morphologies (1000×); e) and f) rough bacterial colonies and cell morphologies (1000×); g) greenish fungus; h) white cotton-like fungus.

CHAPTER 5

DISCUSSION

5.1 Exposure of *M. bovis* BCG cells to various stresses *in vitro*

In this project, *M. bovis* BCG was used as a non-pathogenic surrogate model for pathogenic Mtb. The efficiency of the phage amplification assay was evaluated as a diagnostic method to detect stressed Mtb cells in tuberculous droplet nuclei. The phage infectivity and culturability of mycobacterial cells were assessed through PFU and CFU counts respectively after exposure to different stresses, such as desiccation, nutrient starvation and exposure to UV. CFU assay was initially planned to carry out for every stress, but contaminations led to failure to obtain CFU counts. Therefore, the culturability of *M. bovis* BCG cells after exposure to the different stresses tested remains unknown.

5.1.1 Growth in stationary phase

Stationary-phase *M. bovis* BCG culture always yielded lower PFU/CFU ratios than exponential-phase culture ($P > 0.05$). The reduction of PFU/CFU ratios for stationary-phase culture was approximately 2-fold compared to that for exponential-phase culture. Similar results were shown by Cheah (2010), in which the PFU/CFU ratio of *M. bovis* BCG culture reduced 2- to 3-fold after the cells entered stationary phase.

It had been suggested that mycobacterial cells will thicken their cell wall when they enter stationary phase and achieve low metabolic rate which contributes to higher adaptation towards stress (Dahl *et al*., 2005; Hampshire *et al*., 2004; Shleeva *et al*., 2004). Stationary phase is considered as one of the stresses because depletion of nutrients and accumulation of toxic compounds threaten the survival of mycobacterial cells (Kjelleberg 1993). The lower PFU/CFU ratio indicated that the culturability of stationary-phase cells was retained while their phage infectivity was reduced. In other words, the cells became less detectable by phage, although they were still alive. Therefore, the thickening of mycobacterial cell wall affects their susceptibility towards infection by mycobacteriophage (Dusthackeer *et al*., 2008; Piuri and Hatfull 2006).

5.1.2 Desiccation

The PFU count of desiccated *M. bovis* BCG culture dropped by approximately 100-fold relative to that for the control ($P < 0.05$). This indicates that the desiccated cells became less infected by phage. The results obtained from another experimenter using *M. smegmatis* as model also showed significant reduction in PFU count after desiccation. This could be possibly due to the presence of the specialized cell wall of mycobacterial cells that plays an important role in its survival under stressful conditions, including desiccation (Jarlier and Nikaido 1990). Desiccation is one of the most commonly-encountered stresses when aerosol droplets containing Mtb are rapidly evaporated during transmission from host to host in aerosol droplets.

The CFU counts of *M. smegmatis* from another experimenter showed approximately 10-fold reduction, which dropped more than their respective PFU counts. This means that the culturability of mycobacterial cells was more affected by the desiccation stress then their phage infectivity. If these results were comparable, then it would be expected to obtain similar results for desiccated *M. bovis* BCG culture. The mycobacterial cells may possibly enter dormancy when encounter desiccation (Anuchin *et a*l., 2009). However, if the stressful conditions are too harsh for the cells to generate their survival system, they would probably lose their culturability (Mukamolova *et al*., 2003).

5.1.3 Nutrient starvation

The PFU count of nutrient-starved *M. bovis* BCG culture dropped by approximately 100-fold relative to that for the control ($P < 0.05$). This indicates that the phage infectivity of the cells was significantly affected after nutrient starvation. It could be possible that the cells became dormant to resist the unfavorable environment during starvation and undergone thickening of their cell wall, which eventually affected their susceptibility to be infected by D29.

There was no previous study on the effect of nutrient starvation on phage infectivity of mycobacterial cells. Nyka (1974) reported the effect of the duration of starvation towards the resuscitation of mycobacterial cells in fresh medium and their acid-fastness. Gupta and Chatterji (2005) reported the mechanism "stringent response" and the gene regulation in starved mycobacterial cells. It was initially

hypothesized in this project that nutrient starvation might negatively affect the culturability and phage infectivity of starved *M. bovis* BCG cells.

According to the results from another experimenter using *M. smegmatis* as model, CFU counts of starved cells dropped more significantly (approximately 10-fold) than PFU counts. If these results were applicable to *M. bovis* BCG, then this indicates that the culturability of the cells was more affected than the infectivity by phage after starvation.

5.1.4 Exposure to ultraviolet radiation

PFU count was significantly reduced by approximately 10-fold for the UV-exposed *M. bovis* BCG culture relative to that for the control ($P < 0.05$). This indicates that the exposed cells were dead or became less detectable by phage. According to previous studies, the exposure to UV radiation had been use for disinfection of drinking water to inactivate potential pathogenic mycobacteria, for example *M. avium* complex, *M. intracellulare* and *M kansasii* (Altic *et al*., 2007; Guerrero-Beltra ń and Barbosa-Ca ńovas 2004). Exposure to UV will disrupt the chemical bonds and induce thymine-dimer formation during the DNA replication process (Kubin *et al*., 1982; LeChevallier and Au 2004). Other than that, UV radiation has been reported to have significant lethal effect on mycobacteria under short-wavelength (< 280 nm) (LeChevallier and Au 2004). Therefore, it was initially hypothesized that the exposure to UV radiation might kill the mycobacterial cells and leads to drop in both PFU and CFU counts.

However, the wavelength used in this project was 302 nm, which was suspected to induce dormancy in mycobacterial cells rather than killing them. This can be observed from the data obtained from another experimenter using *M. smegmatis* as model, in which the PFU counts dropped by approximately 5 folds and the CFU counts dropped by approximately 10 folds. This indicates that the some of the UV-exposed cells were still alive, but they became non-culturable, as mycobacteria possess strong resistance towards UV radiation, which is 2-10 times stronger than other non-mycobacterial species (Lee *et al*., 2010). Therefore, it is possible that some of the mycobacterial cells were killed by exposure to UV radiation but some of them entered dormancy. The culturability of the mycobacterial cells was more affected than their phage infectivity after exposure to UV.

5.1.5 Chemical stress during NaOH decontamination

The chemically-stressed *M. bovis* BCG culture showed significant reduction in PFU count by approximately 10-fold. The chemical stress refers to the decontamination procedure on sputum specimens to kill all contaminants in order to recover mycobacteria which is routinely performed in clinical microbiology laboratories (Coulter and Charache 2010). The most commonly-used decontamination agent is the 2% w/v NaOH solution. It is effective in killing contaminants, but could be potentially toxic to mycobacteria at the same time. A percentage of as high as 90% of mycobacteria present in a sputum specimen can be killed during the decontamination process (Coulter and Charache 2010). Therefore, the decrease in PFU counts might be due to the mycobacterial cells

became less detectable by D29, or they were being killed during the NaOH treatment. It could be possible that resuscitation in Middlebrook 7H9 medium at 37^{o}C for 24 h was inadequate to recover the phage infectivity of chemically stressed *M. bovis* BCG cells.

According to the results obtained by another experimenter, the chemically-stressed *M. smegmatis* cells showed approximately 10-fold reduction in CFU count. If these results could be applied to *M. bovis* BCG, then it would be expected that the phage infectivity of *M. bovis* BCG cells would be superior to their culturability following NaOH decontamination. The reduction in both PFU and CFU counts of chemically-stressed *M. smegmatis* might possibly indicate that the culturability of the cells was more affected than their phage infectivity. Cheah (2010) reported similar results in which both PFU and CFU counts of *M. bovis* BCG decreased after NaOH treatment. It is possible that some of the cells were killed.

5.1.6 Exposure to light and dark conditions

PFU count was slightly reduced for the dark-exposed *M. bovis* BCG culture relative to that exposed to light condition ($P < 0.05$). Different from the other stressors, the reduction in plaque count of dark-exposed *M. bovis* BCG culture was not significant. Generally, the growth of mycobacteria is not affected by exposure to light or dark conditions, but their pigmentation does. The effect of dark condition was carried out because transmission of Mtb commonly occurs in crowded and dimmed places, for instances, prison, nursing homes and refugee

camps. Therefore, the effect of exposure to dark condition should be taken into account.

However, the infection of *M. bovis* BCG by phage D29 was found to be not influenced by the exposure to light or dark conditions due to the insignificant change in the PFU count after exposure. Similar result was obtained by another experimenter, in which both the PFU and CFU count of *M. smegmatis* remained after exposure to light and dark conditions. Thus, this stress was excluded from further study on the effect of supernatant treatment (Rpf treatment).

5.2 Effect of *M. smegmatis* supernatant on stressed *M. bovis* BCG cells

Generally, the PFU count of stressed *M. bovis* BCG cells obtained in this project did not show any significant changes after the treatment with *M. smegmatis* supernatant (containing Rpfs) for 24 h at 37^{o}C. Out of the five stresses, only UV-exposed and chemically-stressed *M. bovis* BCG cultures showed a little increment in PFU counts after supernatant treatment relative to the control, but they were not significant. In contrast, the PFU count for other stresses (growth in stationary phase, desiccation and nutrient starvation) decreased after the supernatant treatment. Similar results were obtained from another experimenter, in which only the PFU count for desiccated *M. smegmatis* showed a significant 2-fold increment and other stresses showed no effect after the supernatant treatment.

Initially, it was hypothesized that the PFU and CFU counts of stressed *M. bovis* BCG cells would increase after the supernatant treatment. Kell and Young (2000) and Mukamolova (2010) reported that Rpf proteins are believed to play an important role in promoting multiplication and expression of virulence by their muralytic activities, similar to those of lysozymes that cleave the thick cell wall of dormant mycobacterial cells. However, the overall results showed that the phage infectivity of the stressed *M. bovis* BCG cells was not recovered following treatment with *M. smegmatis* supernatant.

The phage infectivity of stressed *M. bovis* BCG cells did not recover, but their culturability remains unknown. The data from another experimenter showed that CFU counts of stressed *M. smegmatis* cells slightly increased for all stresses following treatment with supernatant. If these results were applicable to *M. bovis* BCG, then it would be expected that the stressed *M. bovis* BCG cells might slightly regain their culturability after the supernatant treatment. Previous studies did show that *M. smegmatis* supernatant has resuscitation effect on "non-culturable" mycobacterial cells to reactivate them to grow. Therefore, it can be concluded that supernatant treatment might only give effect on culturability but not phage infectivity.

Several reasons have been proposed according to the results obtained from supernatant treatment in this project. Mukamolova *et al*. (2002) reported that Mtb Rpf proteins showed cross-species cell activation, regardless whether they are slow-growing or rapid-growing mycobacterial species. However, the *M.*

smegmatis supernatant might have no effect on cross-species resuscitation. This can be shown with the results obtained from another experimenter when desiccated *M. smegmatis* cells did show significant increase in PFU count, but not in desiccated *M. bovis* BCG cells. Therefore, it was suggested that stressed *M. bovis* BCG cells might recover their phage infectivity if treated with *M. bovis* BCG supernatant rather than *M. smegmatis* supernatant. Another factor is the quality of *M. smegmatis* supernatant. Previous studies used supernatant containing Tween 80, but the supernatant used in this project were prepared in Tween-free media. This might be one of the reasons that caused deviations in the results for supernatant-treated stressed mycobacterial cells. However, the effect of Tween on the quality of mycobacterial supernatant is yet to be investigated. Besides that, it could be possible that *M. smegmatis* supernatant is toxic to *M. bovis* BCG cells and caused some PFU counts to drop after the treatment. Another reason proposed is the insufficient duration of resuscitation during supernatant treatment. The stressed mycobacterial cells might be unable to regain their culturability and phage infectivity within 24 h of incubation with supernatant. However, the effect of supernatant on stressed *M. bovis* BCG cells requires further investigation.

5.3 Confirmation of pure *M. bovis* BCG cultures

Contamination was a major problem encountered in this project due to the slow growth rate of *M. bovis* BCG. Contaminations were identified in various ways to confirm that pure *M. bovis* BCG cultures were obtained. Unlike non-

mycobacterial contaminants that could be identified easily, identification of contamination by *M. smegmatis* was a challenging task in this project.

5.3.1 Ziehl-Neelsen method for acid-fast staining

ZN staining was always performed to check the purity of mycobacterial cultures because acid-fastness is one of the distinguishable characteristics of mycobacteria. However, ZN staining is unable to distinguish the species within the genus *Mycobacterium*, as all mycobacteria possess acid-fastness property (Hale *et al*., 2001; Singh *et al*., 2010). ZN staining is known as "hot staining" that uses heat to force the phenicated carbolfuchsin stain to penetrate the waxy mycobacterial cell wall (Hussey and Zayaitz 2011). They retain the reddish-pink carbolfuchsin stain while non-mycobacterial cells are counterstained by methylene blue.

Although *Mycobacterium* species show similar morphologies under the light microscope, there are still some features to differentiate *M. bovis* BCG from *M. smegmatis* in this project. *M. bovis* BCG cells were always observed as clumps under the light microscope due to the higher proportion of mycolic acids in their cell wall and therefore higher hydrophobicity (Lambrecht *et al*., 1988). *M. smegmatis* cells also possess tendency to clump, but not as much as *M. bovis* BCG cells. Cultures that were contaminated by exponential-phase *M. smegmatis* cells showed longer and thinner cells morphology compared to *M. bovis* BCG cells. However, this comparison was not reliable because the morphology of stationary-phase *M. smegmatis* cells (incubated approximately two days) resembled exponential-phase *M. bovis* BCG cells (incubated approximately three days).

Stationary-phase mycobacterial cells tend to become shorter and the morphological variations were according to the surrounding growth conditions (Chauhan *et al*., 2006; Farnia *et al*., 2010; Velayati and Farnia 2012; Vera and Rettger 1940; Young *et al*., 2005).

5.3.2 Measurement of optical density

Commonly, the OD of exponential-phase *M. bovis* BCG cultures fell in the range of 0.3-0.4. Similar to Mtb, *M. bovis* BCG always gave low OD values due to its slow growth with a generation time of approximately 24 hours. *M. bovis* BCG culture usually takes 3 days to enter the exponential phase and 9 days to enter the stationary phase. This considerable long period of incubation rendered the culture to contamination, including contamination by *M. smegmatis*. *M. smegmatis* has a generation time of 3 hours, which is much slower than other non-mycobacterial species, but relatively faster than *M. bovis* BCG (Bettencourt *et al*., 2010). If the OD exceeded 0.4, most probably the culture had been contaminated. Contamination by non-mycobacterial species usually gave OD of more than 1.0 and contamination by *M. smegmatis* usually gave OD of about 0.7. This method is reliable to be used to check purity of the mycobacterial culture together with ZN staining to ensure that pure culture is obtained (Meyers *et al*., 1998).

5.3.3 Colony morphology

When the CFU assay was carried out, colonies of *M. bovis* BCG were easily being covered up by non-mycobacterial contaminants. These contaminants could be easily identified through their colony morphologies. *M. smegmatis* contaminants

could be identified through their growth rate. *M. bovis* BCG is a slow-grower that may take weeks for colony formation, while *M. smegmatis* is able to produce observable colonies in two days (Hett and Rubin 2008). The cross-contamination by *M. smegmatis* might be caused by the sharing of laminar air flow with another experimenter that was working with *M. smegmatis* culture. To overcome contamination problems, some precautions should be carried out when conducting CFU assay. These include use of filter micropipette tips and the availability of dedicated cabinet and incubator for work with *M. bovis* BCG culture.

Although the colony morphologies of *M. smegmatis* and *M. bovis* BCG exhibit some similarities, they are somehow different from each other (Figure 4.6). Chuda *et al*. (2012) reported that the colonies of *M. bovis* BCG appear dome-shaped with thick, rough and rugose surface, irregular margin and are grayish-white in color. Previous studies reported that *M. smegmatis* colonies appeared as relatively smooth, moist, raised, but with wrinkled surface on Middlebrook 7H10 agar (Smeulders *et al*., 1999; Mart ńez *et al*., 1999). These morphologies were consistent with those observed in this project.

5.3.4 Size of plaques formed in the phage amplification assay

Throughout this project, plaques formed from the infection of *M. bovis* BCG by D29 were always smaller than those for *M. smegmatis* (compared to phage assay plates from other experimenter). Garza *et al*. (2002) reported that plaques from infection of *M. bovis* BCG were smaller than those for other mycobacterial species. Average diameter of *M. bovis* BCG plaques was about 1 mm, while most *M.*

smegmatis plaques were more than 2 mm in diameter. It is possible that the plaque size might be affected by the growth rate and virulence of the target mycobacterial species. However, this assumption requires further investigation to study the relationship between mycobacterial species with their burst size. The plaque size obtained in the indicator plates that was larger than expected might be possibly cross-contaminated by *M. smegmatis* when conducting the experiment.

5.4 Potential future works

5.4.1 Complete set of CFU results for the stresses evaluated

The complete data for PFU/CFU ratios of all stresses and supernatant treatments should be obtained in the future so that a clearer idea of relationship between the culturability and the phage infectivity of stressed *M. bovis* BCG cultures can be evaluated. The use of Middlebrook 7H11 agar and antibiotics can be considered to effectively inhibit contaminants by selectively allowing only he growth of the desired mycobacterial species.

5.4.2 Evaluation of the effect of other stresses

Other internal stressors such as oxidative stress, osmotic stress, heat stress, exposure to acidic conditions and exposure to different temperatures can be encountered by Mtb inside the host. When the droplet nuclei containing Mtb are inhaled, these are the major stressors encountered by Mtb *in vivo* (Wu *et al*., 2007). Therefore, it is necessary to evaluate the effectiveness of phage amplification assay as diagnostic method to detect the presence of these Mtb.

5.4.3 Evaluation on Mtb

The experiment can be conducted using a non-pathogenic, class-2 Mtb strain known as H37Ra. It possesses relatively more similar properties with pathogenic Mtb than either *M. bovis* BCG or *M. smegmatis*. Results obtained from this model could be different from those obtained using *M. bovis* BCG.

5.4.4 Treatment of stressed *M. bovis* BCG culture with *M. bovis* BCG supernatant

It is possible that *M. bovis* BCG supernatant might show positive effect on stressed *M. bovis* BCG cells. Therefore, the experiment can be conducted using *M. bovis* BCG supernatant instead of *M. smegmatis* supernatant.

5.4.5 Use of a better protocol for UV exposure

The protocol of UV exposure should be reinvestigated so that the mycobacterial cells are directly exposed to sunlight rather than light from a UV lamp. A suitable material should be investigated so that it allows penetration of UV radiation but prevents desiccation and contamination.

CHAPTER 6

CONCLUSION

In this project, *M. bovis* BCG was used as a non-pathogenic surrogate model, and exposed to various physical and chemical stresses *in vitro* to mimic the circumstances of tuberculous droplet nuclei. Preliminary results showed that desiccation, nutrient starvation, exposure to UV and chemical stress led to significant reduction in PFU counts, while exposure to light and dark conditions showed no significant effect on PFU counts. By comparing the PFU/CFU ratio of stationary-phase cells that for exponential-phase cells, the culturability of stationary-phase cells was more affected than their phage infectivity. However, for other stressors, it was not possible to determine whether the cells had died or were still viable because CFU assays failed due to contamination problem. Data from another experimenter using *M. smegmatis* as model showed that the reduction in CFU count was much more significant (approximately 10 folds) than the reduction in PFU counts (less than 10 folds). If these results were comparable, then it would be expected that the phage infectivity of *M. bovis* BCG cells would be superior to their culturability. The second part of the project was to investigate the effect of *M. smegmatis* supernatant on the detection of stressed *M. bovis* BCG cells by the phage amplification assay. Preliminary results showed that *M. smegmatis* supernatant treatment had no significant effect on PFU count of stressed *M. bovis* BCG. Interestingly, for some stresses, the PFU counts dropped after supernatant treatment. Out of the five tests, only the treatment of UV-

exposed and chemically-stressed *M. bovis* BCG cells with supernatant showed an insignificant increase in PFU counts relative to the controls. The effect of supernatant may be influenced by its quality, potential toxicity and duration of treatment. Further investigation should be carried out using *M. bovis* BCG supernatant on stressed *M. bovis* BCG cells because *M. smegmatis* supernatant might had no effect on cross-species resuscitation. Effect of supernatant on both phage infectivity and culturability of stressed mycobacterial cells can be further investigated. Therefore, the complete data for PFU/CFU ratios of all stresses and supernatant treatments should be obtained in the future.

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Appendix A

APPARATUS AND CONSUMABLES

The following are the lists of apparatus and consumables used in this project:

Table A1: List of apparatus and their respective manufacturers.

Apparatus	Manufacturer
Autoclave machine	Hirayama
Centrifuge machine	Hettich Zentrifugen
Deionized water dispenser	Sartorius Stedim Biotech
Incubators	Memmert
Laminar air flow	Telstar
Micropipette (10 µl, 100 µl, 1000 µl)	Eppendorf
Microscope	Leica
Spectrophotometer	Genesys
Syringe	Cellotron
Syringe filter (0.2 µm)	Nalgene
Ultraviolet lamp	UVP
Vortex machine	Velp
Water bath	Memmert

Table A2: List of consumables and their respective manufacturers.

Consumables	Manufacturer
Bactor agar powder	BD
Basic fuchsin	Merck
Calcium chloride (CaCl2)	Gene Chem
Di-potassium hydrogen phosphate (K_2HPO_4)	HmbG Chemicals
Ethanol	Labmart
Glycerol	QR äC
Hydrochloric acid (HCl)	Merck
Luria-Bertani (LB)	Merck
Magnesium sulphate ($MgSO_4$)	QR äC
Methylene blue	Systerm
Middlebrook 7H9 powder	BD
Middlebrook 7H10 powder	BD
Oleic acid-albumin-dextrose-catalase (OADC)	BD
Phenol	Merck
Phosphate buffered saline	Sigma
Potassium di-hydrogen phosphate (KH_2PO_4)	QReC
Sodium chloride (NaCl)	Merck
Sodium hydroxide (NaOH)	Merck
Tween 80	Systerm