# DEVELOPMENT OF A MASK SAMPLING METHOD FOR COLLECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPIRATORY DROPLETS

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# DEVELOPMENT OF A MASK SAMPLING METHOD FOR COLLECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPIRATORY DROPLETS

By

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## ABSTRACT

# DEVELOPMENT OF A MASK SAMPLING METHOD FOR COLLECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPIRATORY DROPLETS

**TEE HAN KANG**

*Mycobacterium tuberculosis* (Mtb) is transmitted via the airborne route in the respiratory droplets expectorated by active pulmonary tuberculosis (TB) patients. To date, there is still lack of an established method for sampling respiratory aerosol droplets and measuring the Mtb concentration in them. Current medical practice continues to rely heavily on the acid-fast bacilli (AFB) smear status to gauge the infectiousness of TB patients. However, several limitations have been reported that challenge its reliability. In this project, a mask sampling approach was developed as the potential alternative to AFB smear microscopy for the assessment of TB infectivity. *Mycobacterium smegmatis* was used as the non-pathogenic surrogate model for Mtb throughout this study. Surgical masks were artificially spiked with *M. smegmatis* culture and then subjected to the on-mask phage assay. The detection limit observed was 100-1,000 CFUs, which is $10^4$ to $10^5$ folds higher than the method of PCR on mask eluates. In order to identify the target mycobacterial species in the resulting plaques, a PCR assay targeting the

multicopy Mtb complex-specific IS*1081* was performed downstream. However, it was unsuccessful in several trials, possibly due to inhibition by substances that were co-extracted with the target DNA from the phage plaque. Evaluation with masks worn by healthy subjects that were voluntarily coughed upon showed that the use of an antibiotic cocktail was efficient in completely preventing contamination of the phage assay by rapid-growing microbiota. Further studies with healthy volunteers also demonstrated that culturable respiratory aerosols were mainly sampled in the middle section of the mask and coughing, when compared to talking, yielded higher level of culturable aerosols on average. With further improvements and optimizations, the mask sampling approach could potentially be more reliable than AFB smear status in gauging TB infectiousness. This method could facilitate better understanding of TB transmission and provide a new platform for TB diagnosis.

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Most important of all, a big thank you to my family and friends, especially my parents, for always be there as a harbor for me to shelter my soul. My life will be totally different as what I have today without you all. I love you all.

## DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations in 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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(TEE HAN KANG)

## APPROVAL SHEET

The project report entitled **"DEVELOPMENT OF A MASK SAMPLING METHOD FOR COLLECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPIRATORY DROPLETS"** was prepared by TEE HAN KANG and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Microbiology at Universiti Tunku Abdul Rahman.

Approved by

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**PERMISSION SHEET**

It is hereby certified that TEE HAN KANG (ID No: 10ADB04933) has completed this final year project entitled **"DEVELOPMENT OF A MASK SAMPLING METHOD FOR COLLECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPIRATORY DROPLETS"** supervised by Dr. Eddy Cheah Seong Guan from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(TEE HAN KANG)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFB | acid-fast bacilli |
| BCG | Bacille Calmette-Guerin |
| $CaCl_2$ | calcium chloride |
| CDC | Centers of Disease Control and Prevention |
| CFU | colony-forming unit |
| dNTP | deoxyribonucleoside triphosphate |
| $dH_2O$ | distilled water |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| FAS | ferrous ammonium sulfate |
| HCl | hydrochloric acid |
| HIV | human immunodeficiency virus |
| IS | insertion sequence |
| LAMP | loop-mediate isothermal amplification |
| M7H9 | Middlebrook 7H9 |
| M7H10 | Middlebrook 7H10 |
| $MnCl_2$ | Manganese chloride |
| $MgSO_4$ | magnesium sulfate |
| MP | mycobacteriophage |
| Mtb | *Mycobacterium tuberculosis* |
| NaCl | sodium chloride |
| NTC | no-template control |
| NTM | non-tuberculous mycobacteria |
| OADC | oleic acid-albumin-dextrose-catalase |
| OD | optical density |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PFU | plaque-forming unit |
| Rpf | resuscitation-promoting factor |
| TAE | Tris-acetate-EDTA |
| TB | tuberculosis |
| TSA | trypticase soy agar |
| UTAR | Universiti Tunku Abdul Rahman |
| UV | ultraviolet |
| WHO | World Health Organization |

| | |
|---|---|
| bp | base pair |
| cm | centimeter |
| fg | femtogram |
| g | gram |
| M | molar |
| rpm | revolutions per minute |
| ml | milliliter |
| mM | millimolar |
| µl | microliter |
| µM | micromolar |
| ng | nanogram |
| pg | picogram |
| U | enzyme unit |
| v/v | volume per volume |
| w/v | weight per volume |
| x *g* | times gravity |

# CHAPTER 1

## INTRODUCTION

Tuberculosis (TB) is a lethal and infectious disease caused by *Mycobacterium tuberculosis* (Mtb). It typically infects the lungs (pulmonary TB), however it can infect other internal sites as well, causing extrapulmonary TB. TB remains as the second leading cause of death from infectious disease worldwide, with estimation of about one-third of the global population being infected (CDC, 2014). According to the World Health Organization (WHO, 2014), there were 8.6 million people who suffered from this illness and 1.3 million deaths in 2012. This resurgent disease is largely attributed to immigration from countries with high prevalence of TB, co-infection with HIV, social problems and dismantling of TB services (Nissapatorn *et al.*, 2007). In Malaysia, the TB incidence has risen to 18,000 cases in 2010 from 16,000 cases in 2008. In 2011, there were 20,000 cases reported with 1,600 fatalities, and about 12% to 13% of TB patients were foreign workers (The Star Online, 2013). The substantial influx of the immigrant populations into the community has contributed to one of the postulated factors to be elucidated relating to TB.

TB is transmitted by Mtb through the airborne route. It is currently spreading at the rate of one person per second (WHO, 2014). This contagious disease is spread

by TB patient who expels the bacteria by, for example, coughing. Those who inhaled the bacteria will get infected, 10% will progress to TB disease, whereas 90% will develop asymptomatic latent infection. People who are ill with TB can infect up to 10-15 other people through close contact over the course of a year (WHO, 2014). The determinants of TB transmission from one person to another depends on several factors, which include anti-tuberculous chemotherapy, expiratory maneuvers, duration and proximity of exposure and exposure due to close ventilation among others (Cheah, 2010). The resurgence of TB worldwide and close association with HIV have prompted increased global attention and concern on this serious health dilemma. The effort to reduce global TB burden focuses on prevention of disease transmission, which it is halted by the limited knowledge of relationship between tuberculous aerosol output and infectiousness. In addition, the inability to diagnose and treat TB in a timely manner remains as major obstacle in controlling the its spread (Molicotti, 2014).

TB diagnosis continues to rely heavily on the detection of acid-fast bacilli (AFB) via microscopic examination and culture. The AFB smear microscopy is used to gauge the infectiousness of TB patients as well. The presence of Mtb in respiratory sample is determined by using acid-fast staining, or more specifically Ziehl-Neelsen staining. The AFB smear test is rapid, easy to perform and cheap. It is normally performed with the sputum specimen collected from the patient, which is stained and directly examined for the presence of Mtb. It is generally known that AFB smear-positive patients are more infectious than smear-negative

ones; however the reason was poorly understood (Shaw and Wynn-Williams, 1954). The reliability and sensitivity of the AFB smear microscopy in gauging TB infectivity was challenged by Catanzaro (1982) and Behr (1999) as TB transmission from AFB smear-negative patients was observed. Furthermore, AFB smears are often showed negative when TB and HIV are comorbid (Maartens and Wilkinson, 2007). Consequently, the sputum culture method is essential for diagnostic confirmation and antimicrobial susceptibility testing. Nonetheless, it is restricted by the slow growth of Mtb which can take three weeks or longer to show visible growth. Several studies attempted to directly collect and quantify airborne Mtb in respiratory droplets expectorated by TB patients. However, most of these methods suffered from various limitations. The use of PCR-based method is unable to provide distinction between live and dead cells, the use of animal models is unethical and cost prohibitive, and the use of culture is time consuming and prone to contamination (Cheng, 2012). Therefore, a more reliable method for sampling and direct quantification of Mtb in respiratory aerosols is urgently needed for TB infectivity study.

In this project, a novel approach based on surgical face mask was developed to collect and quantify Mtb in respiratory droplets from TB patients. In a nutshell, this proposed sampling approach is economical and easy to perform (Cheah, 2010). The mask sampling obviates the need for any sophisticated equipment. It only needs face masks which are universally available in healthcare center, pharmacies and departmental stores. By wearing mask, it confers additional

protection to healthcare staff and other patients, and minimizes TB transmission. Willeke and his co-investigators (1996) demonstrated that Mtb is less likely to penetrate through the surgical face mask. This finding further convinced that the face mask is suitable for sampling of Mtb in respiratory aerosol due to its high bacterial retention efficiency. In this project, detection of target mycobacterial cells on mask was attempted by a combined assay which consists of mycobacteriophage amplification assay and a molecular assay downstream. Initially, the Loop-mediated isothermal amplification (LAMP) assay was attempted as the molecular mean to detect the target mycobacterial DNA in phage plaques; however the high occurrence of false positivity has challenged the reliability of this assay. A conventional PCR assay was then attempted. Besides facilitating a timely diagnosis of TB, this advocated combined assay has the added advantage of being more reliable for Mtb detection.

The findings from the study would pose great importance to better understanding of TB transmission and infectivity. The overall objective of this study was:

1. To develop a mask sampling method for sampling and quantification of Mtb organisms in respiratory droplets expectorated by active pulmonary TB patients.

The specific objectives of this study were:

1. To assess the potential of a combined phage amplification and molecular assay to detect and identify mycobacterial cells spiked onto face masks.

2. To investigate the presence of different amounts of normal microbiota in healthy individuals and to develop an effective decontamination method that can be applied to clinical mask samples.

3. To study the level and distributions of culturable respiratory aerosols on face masks during different expiratory maneuvers

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Mycobacteria in General

### 2.1.1 The Genus *Mycobacterium*

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* that belongs to the phylum Actinobacteria. Microscopic examination of mycobacterial cells reveals slightly curved, rod-shaped bacilli, with size ranging from 0.2 to 0.6 µm by 1 to 10 µm (Shimeld and Rodgers, 1999). Mycobacterium is aerobic, non-spore forming (except for *M. marinum*) and non-motile. The presence of unique lipids (mycolic acids) in its cell wall gives rise to the distinctive feature of acid-fastness. This unique feature allows mycobacterial cells to be stained red by the carbolfuchsin dye and resist subsequent decolorization by acid-alcohol. The slow growth of mycobacteria is often related to the low permeability of their cell walls to hydrophilic nutrients. When growing on solid media, mycobacteria normally form tight, compact and wrinkled colonies. This morphology is largely attributed to the high lipid content and hydrophobic nature of the cell surface, causing them to stick together (Madigan *et al.*, 2006).

Medically, mycobacteria can be categorized into two major groups based on the ability to cause TB (Murray *et al*., 2003). The Mtb complex, as suggested by the name, is a group that consists of etiological agents of TB, which include Mtb, *M. bovis*, *M. africanum*, *M. canetti*, *M. microti*, *M. caprae*, and *M. pinnipedii*. The other group, the non-tuberculous mycobacteria (NTM), consists of species that do not cause TB, for example *M. avium* complex, *M. fortuitum, M. abscessus, M. kansasii*, *M. scrofulaceum, M. simiae* and *M. ulcerans.*

### 2.1.2 *Mycobacterium tuberculosis*

In 1882, a German microbiologist, Robert Koch, discovered the causative agent of TB, which is Mtb (Cambau and Drancourt, 2014). Mtb is a slender, strongly acid-fast, rod-shaped bacterium. It is genetically diverse, which results in significant phenotypic distinctions among clinical isolates (Gagneux, 2009). Different strains of Mtb were reported to be associated with different geographical regions. It grows at 37℃ and requires enriched or complex media for growth in the laboratory. It is usually characterized by its slow growth, with a mean generation time of 12-24 hours. Dry, rough, buff-colored colonies normally appear on solid medium after 3-6 weeks of incubation (Ryan and Ray, 2010). Due to its highly hydrophobic lipid-laden cell envelope, Mtb is relatively resistant to desiccation, most disinfectants, and also acids and alkalis. However, it is heat-labile and susceptible to UV inactivation.

## 2.2 Tuberculosis in General

### 2.2.1 Pathogenesis of Tuberculosis

TB infection is a consequence of host-pathogen interaction that can last for decades. When a patient with active TB expectorates, aerosol droplets loaded with Mtb will be generated and propelled into the air and remain suspended over long distance and for extended period. If these droplets are inhaled by another person, there is a 5% chance of direct development of active TB disease and 95% chance of latent infection (Koul *et al*., 2011; Ahmad, 2011). The latter is asymptomatic as the Mtb inhaled remains inactive due to restriction of by the host immune response. However, this dormant Mtb can be reactivated due to certain factors, such as immunosuppression and co-infection with HIV. Individuals with active TB disease are infectious, spreading Mtb through the airborne route, normally via coughing. As the disease progresses, sputum will be produced during coughing, which later on contains blood (hemoptysis) (Ryan and Ray, 2010). As it goes on, fever, malaise, fatigue, sweating and weight loss will develop and exacerbate the condition.

### 2.2.2 Clinical Diagnosis of Tuberculosis

Clinical diagnosis of TB is via chest radiography and the tuberculin skin test (TST). Chest radiographic findings suggestive of TB include upper-lobe infiltrates, cavitary infiltrates, and hilar or paratracheal adenopathy (Meya and McAdam, 2007). However, chest radiography can only localize the site of pathology without

confirming the etiology (Tsai *et al.*, 2013). The presence of these radiological lesions may strongly suggest TB, although they are not necessarily caused by TB. On the other hand, TST is generally used to screen for latent TB infection. It is administered by intradermal injection of tuberculin, a purified protein derivative (PPD), in the forearm. After injection, a pale elevation will be formed on the skin as a result of wheal formation, measuring about 6-10 mm in diameter (CDC, 2012a). The test reaction is read after 48-72 hours. The diameter of the induration is measured and then interpreted based on the risk groups of the tested individuals.

### 2.2.3 Treatment of Tuberculosis

The objectives of TB treatment are cure without relapse, to halt transmission, to avoid death and to prevent the emergence of drug resistance (Frieden *et al*., 2003). Mycobacteria are inherently resistant to many antimicrobial agents due to the impermeability of their lipid-rich cell wall. The antimicrobial agents that are effective against Mtb are divided into first-line and second-line drugs. First-line agents such as isoniazid and rifampicin are more preferred. Meanwhile, second-line agents are reserved for use when the Mtb strains are resistant to the first-line agents. TB treatment consists of two phases, namely the intensive and continuation phases (Pardeshi, 2010). For the initial intensive phase, the treatment is designed to kill actively growing bacilli. Isoniazid, rifampicin, ethambutol and pyrazinamide are both used in this phase. The continuation phase aims to

eradicate the remaining bacilli, thereby reducing the numbers of treatment failures and disease relapses.

### 2.2.4 Vaccination against Tuberculosis

BCG vaccine is a live attenuated vaccine developed by Albert Calmette and Camille Guérin. It was originally derived from *M. bovis* that was attenuated by repeated subculture. It is normally given as a single intradermal injection at the deltoid region of the arm. The protective efficacy of this vaccine ranges from ineffective to 80%. This variability in efficacy is attributed to some factors, including strain-specific immunogenicity, age at vaccination, genetic differences between populations and host co-infection by parasites (Newton *et al.,* 2008). Consequently, it has been reported by Aagaard *et al*. (2009) and Horvath and Xing (2013) of its limited effects against adult pulmonary TB. As a double-edged sword, BCG vaccine poses some side effects to the users such as painful urination, swollen lymph nodes, upset stomach and fever (MedlinePlus, 2013).

### 2.2.5 Current Challenges for Tuberculosis

Multidrug-resistant TB (MDR-TB) results from either primary infection with drug-resistant Mtb or development of resistant Mtb strains in the course of case treatment when non-optimal regimens or durations are used (Koul *et al.,* 2011). MDR-TB strains are resistant to the two most important first-line drugs, isoniazid

and rifampicin. Besides that, a more severe form of drug-resistant TB, the extensively drug-resistant TB (XDR-TB), is caused by Mtb strains that are not only resistant to the first-line drugs, but are also resistant to fluoroquinolone and any of the second-line drugs (CDC, 2012b).

HIV and TB infections form a lethal partnership; the devastating association between AIDS and TB has aggravated the global TB burden. HIV weakens cell-mediated immunity by destroying CD4-positive T lymphocytes and promotes the progression from latent TB to active TB (Nachega and Chaisson, 2000). With HIV co-infection, the risk of reactivation of latent TB infection is increased by 200-300 times (Ryan and Ray, 2010).

## 2.3 Expectoration of Mtb in Aerosol Droplets and TB Transmission

The idea that TB is spread by specific germs was proposed by Budd (1867) based on the epidemiology of the disease in the community. This was proven later after Mtb was discovered by Robert Koch. Mtb-laden aerosol droplets are generated and expectorated into the air by active pulmonary TB patients through, for example, coughing, sneezing, singing or talking. The moisture in the aerosol particles evaporates, leading to formation of droplet nuclei, which are 1-5 μm in diameter (CDC, 2013). Kawada *et al.* (2008) claimed that the respiratory aerosols produced by active TB patients during normal breathing do not act as vectors for the spread of Mtb. The aerosolization of respiratory secretion was first attributed

to the airflow turbulence of inhaled air. It was later proven that the turbulent airflow is only limited to the upper airways (Johnson and Morawska, 2009). Recently, the bronchiole fluid film burst model, which was proposed by Houspie *et al.* (2011), suggested that aerosolization was due to the fluid bubbles present in the bronchioles during inhalation.

Depending on environmental conditions, the droplet nuclei may remain suspended in the air for a few hours and can even travel over a long distance. Xie *et al.* (2009) proposed that larger droplet nuclei may rapidly settle out of the air and facilitate disease transmission in close proximity, and vice versa. Recently, Jones-López *et al.* (2013) reported that contacts with patients who produced higher Mtb CFUs were more likely to be infected compared to those that produced lower CFUs. After the Mtb-containing droplet nuclei are inhaled by another person, they will traverse the mouth or nasal passages, upper respiratory tract, and finally the bronchi to reach the alveoli (CDC, 2013). Consequently, it may give rise to either latent infection or active disease.

## 2.4 Laboratory Diagnosis of Tuberculosis

### 2.4.1 AFB Smear Microscopy

Early TB diagnosis relies heavily on microscopic examination of clinical samples. It is easy to perform, rapid and economical for demonstration of the possible presence of Mtb. The most common staining technique used is the Ziehl-Neelsen

(ZN) acid-fast stain. This stain allows AFB to be stained red against a blue background, which consists of host cells and other bacterial cells. Another staining technique is the Kinyoun method, in which high concentration of the carbolfuchsin dye is used to penetrate the thick, hydrophobic mycobacterial cell wall. In addition, AFB can also be examined by fluorescence microscopy using specific fluorescent dyes, such as auramine and rhodamine. AFB smear microscopy has some limitations such as inability of live/dead differentiation and low sensitivity. According to Tsai *et al*. (2013), the ZN stain can only reveals AFB if the sample contains greater than 10,000 bacilli per ml.

### 2.4.2 Mtb Isolation and Culture

Culture remains the most sensitive method of Mtb detection in clinical samples. It can detect as few as 10-100 bacilli per ml of clinical specimen, which is way more sensitive than AFB smear microscopy (Lighter and Rigaud, 2009). The solid media used to isolate Mtb can be divided into two categories: egg-based media (Löwenstein-Jensen) and agar-based media (Middlebrook 7H10 and Middlebrook 7H11). Both contain malachite green to suppress the growth of contaminating fungi and bacteria. The latter are preferred when it comes to observation of colony morphology and colony enumeration. Liquid media, such as the Middlebrook 7H9 broth, support the growth of the Mtb complex better than solid media with an increased recovery of positive cultures and shorter time to positivity (Hanna, 2004). Automated broth-based mycobacterial culture systems, such as the

BACTEC MGIT 460, are also commercially available. Culture-based methods are limited by the slow growth of mycobacteria, which is impractical for timely diagnosis and increases the risk of culture contamination.

### 2.4.3 Mycobacteriophage Amplification Assay

The mycobacteriophage amplification assay is an established method that utilizes phage amplification to reflect the presence of viable mycobacterial cells in clinical specimens. The concept of this method stems from the fact that bacteriophage replication is hundreds of times faster than that for bacteria. By incorporating this advantage in diagnosis, it will greatly enhance TB detection with much shorter turnaround time. Mycobacteriophage D29 is used due to its broad host range, in which it can infect many different mycobacterial species (Rybniker *et al*., 2006). For the method, decontaminated clinical specimens containing Mtb are mixed with phage D29 and infection is allowed to proceed for one hour. After that, a virucidal agent, ferrous ammonium sulfate (FAS), is added to kill the exogenous phages that have not infected the target mycobacterial cells. To detect the protected phages inside the target cells, non-pathogenic, rapid-growing *M. smegmatis* cells (sensor cells) are added to the reaction, after which it was plated out. During overnight incubation, the infected target cells will lyse and release the protected phages inside. The released phages will then form plaques by infecting the surrounding *M. smegmatis* cells (bacterial lawn). The number of plaques formed would indicate the number of mycobacterial cells that are present

in the test sample (Barman and Gadre, 2007). The sensitivity of this assay with sputum specimen was reported to be about 100 bacilli per ml (Albay *et al.,* 2003; McNerney *et al.,* 2004). Nonetheless, the phage assay is limited in its specificity as the phage D29 used is not specific for the Mtb complex and can cross-react with NTM.

### 2.4.4 Nucleic Acid Amplification Techniques

#### 2.4.4.1 PCR Detection

Nucleic acid amplification techniques **(**NAATs) have been developed for a more rapid and sensitive identification of Mtb directly in specimens or in culture isolates. However, it does not replace the AFB smear microscopy and culture for laboratory diagnosis, mainly due to variations in methodology and limited accuracy. The advent of the polymerase chain reaction (PCR) has opened new possibilities for diagnostic mycobacteriology. The DNA sequence most frequently targeted is IS*6110,* which is the signature sequence of Mtb (Sjöbring *et al.,* 1990). Another DNA sequence, the IS*1081,* has been used to differentiate the Mtb complex from the NTM (Wards *et al.,* 1995; Ahmed, 1998). FDA has approved two commercial PCR assays, which are the Amplified Mtb Direct Test (Gen-Probe) and Amplicor Mtb Test (Roche). Nevertheless, these two PCR assays have yet to be universally applied in clinical diagnosis due to their potential high false-positive rates (Lighter and Rigaud, 2009).

### 2.4.4.2 Loop-mediated Isothermal Amplification Assay

A newer NAAT termed the LAMP assay, developed by Notomi *et al.* (2000), amplifies DNA with high specificity, simplicity, and rapidity under isothermal conditions. This assay employs the *Bst* polymerase, which is able to perform autocycling strand displacement DNA synthesis. Without thermal cycling, it is relatively much faster compared to conventional PCR. Besides that, the use of four primers that recognize six distinct regions on the target DNA contributes to its high specificity (Figure 2.1). The final products are stem-loop DNAs with inverted repeats of the target and cauliflower-like structures with multiple loops (Minami *et al.*, 2006).

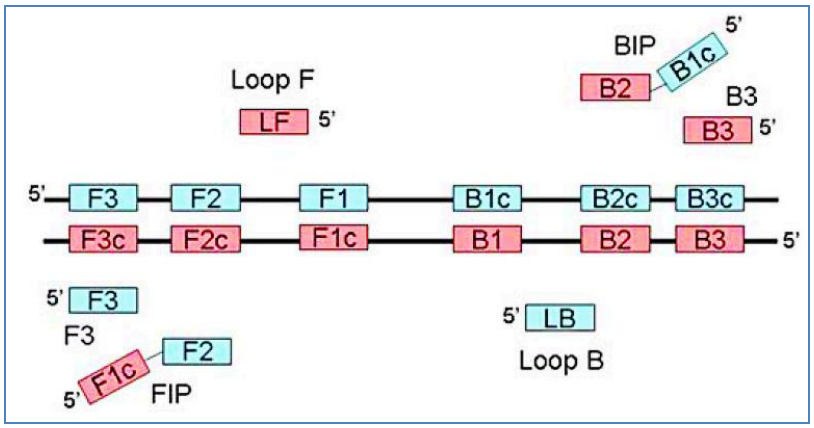


**Figure 2.1**: Schematic representation of the LAMP primers. The 3' and 5' ends of the forward inner primer (FIP) are designed to be complementary to the F2c and F1c regions on the target DNA. Meanwhile, the 3' and 5' ends of the backward inner primer (BIP) are designed to be complementary to the B2c and B1c regions. The outer primers (F3 and B3) are designed to be complementary to the F3c and B3c regions, respectively. The loop primers consist of sequences that are complementary to the single-stranded loop regions on the dumbbell-like starting material (Reproduced from Mori and Notomi, 2009).

Two visual detection formats can be used to detect the amplified products, which are based on turbidity and fluorescence, respectively. During amplification, large amounts of a by-product, magnesium pyrophosphate, are produced, which are observed as white precipitates. The resulting turbidity provides the distinction of whether DNA amplification takes place or not (Mori *et al.,* 2001). Since the amount of precipitates produced correlates with the amount of DNA synthesized, this approach can be made quantitative to measure the amount of targets in the sample. Meanwhile, two dyes, SYBR Green (Iwamoto *et al.,* 2003) and calcein (Tomita *et al.,* 2008), have been used in fluorescence detection of amplified products. The former binds to double-stranded amplicons and emits fluorescence, whereby negative reactions remain orange in color. For the latter, manganese ions bind to calcein dyes to quench the fluorescence in the initial reaction. As amplification proceeds, the pyrophosphates produced will bind to the manganese ions. The depletion of manganese ions in the reaction mixture unmasks the calcein dyes, leading to fluorescence emission.

## 2.5 Sampling and Quantification of Mtb in Respiratory Droplets

### 2.5.1 Use of Animal Models

Flugge pioneered the use of animal models for Mtb transmission study by infecting guinea pigs with Mtb in the expectorated aerosols of TB patients (Fennelly, 2007). At that time, he erroneously interpreted that TB was transmitted

by large amount of respiratory droplets. However a few years later, his protégé, Richard Riley, has proven that Mtb is transmitted through the airborne route.

Riley *et al.* (1959), in their study, constructed an animal exposure chamber which consisted of 36 cages, with about 4-5 guinea pigs housed in each of them. The chamber was connected to a TB ward occupied by patients with active pulmonary TB, through a closed circuit ventilating system. The ward air was vented continuously to the animal chamber for two years. All animals were tuberculin-tested at monthly intervals and those with positive reactions were removed for autopsy. A total of 71 out of 156 guinea pigs were infected with TB. Among those infected, 20 cases showed no pulmonary tubercle but their hilar lymph nodes were infected. They therefore suggested that Mtb may be occasionally carried to the lymph nodes without leaving any macroscopic evidence in lungs. This sampling study was used by Escombe *et al.* (2007) in their study on TB and HIV co-infection. This animal-based sampling approach was reported to be impractical due to the its prohibitive cost and the requirement for ethical approval and specially constructed facilities.

### 2.5.2 Air Filtration Coupled to PCR for Detection of Airborne Mtb

An attempt was performed by Mastorides *et al.* (1999) to detect the presence of Mtb droplet nuclei in the air of a TB ward. They combined the techniques of air filtration and PCR method to detect aerosolized Mtb in a negative pressure isolation room occupied by TB patients. The air from the respiratory isolation

room was filtered through a 0.2-μm polycarbonate membrane filter over six-hours period. After sampling, the membrane filters were minced and then sonicated for DNA extraction. The resulting DNA sample was analyzed by PCR. Despite that Mtb complex was successfully detected for 6 out of 7 culture-positive patients, none of the three culture-negative patients were flagged as positive. Among the 6 PCR-positive patients, one was AFB smear-negative. The modified version of this approach was used by Chen and Li (2005), in which they successfully demonstrated that the airborne Mtb level in the respiratory isolation room correlated with the clinical status of the TB patients studied.

The use of filtration/PCR detection method is extremely sensitive and provides rapid quantification of airborne Mtb. Nonetheless, it is prone to assay inhibition and is unable to differentiate between viable and dead cells.

### 2.5.3 Culture via the Cough Aerosol Sampling System

Fennelly *et al.* (2004) developed a method to sample culturable Mtb in respiratory expectorate of TB patients and to measure the size distribution of the aerosols produced. They developed the cough aerosol sampling system (CASS) which contains two Andersen-six stage cascade impactors, for this viable air sampling. The impactors were loaded with six Middlebrook 7H11 agar plates in stack. The recruited patients were requested to cough into the chamber for 5 minutes (Figure

2.2). The agar plates were incubated and checked weekly, for any visible Mtb colonies over 6-10 weeks. The identities of the colonies were then confirmed by nucleic acid probes and restriction fragment length polymorphism (RFLP).

Positive cultures were detected in 4 out of 16 subjects with smear-positive pulmonary TB. These four patients showed a rapid decline of in the numbers of viable Mtb aerosols within three weeks of antituberculous therapy (Fennelly *et al.*, 2004). Moreover, Fennelly and his co-investigators also found a correlation between cough frequency and the amount of tuberculous aerosols produced by the patients. They suggested that individuals who are closer to a coughing TB patient are probably exposed to larger amounts of Mtb aerosols than those who are further away (Fennelly *et al.*, 2007). Despite that various sizes of Mtb aerosols were detected, most particles were in the respirable range.

Direct sampling of respiratory Mtb aerosols provides short-term evaluation of infectious aerosol production. However, it requires long incubation period, leading to delay in obtaining results. Besides that, fungal contamination and desiccation of media further compromise the usefulness of this approach.

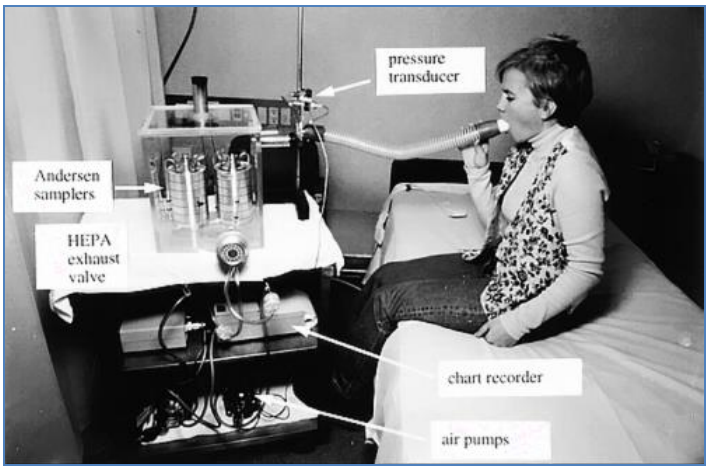


**Figure 2.2:** Cough aerosol sampling system showing a child coughing into the sampling chamber through the inlet tubing.

### 2.5.4 Use of Surgical Face Mask

Cheah (2010) developed a novel mask sampling approach to collect and quantify the Mtb aerosols expectorated directly by active pulmonary TB patients. Mask sampling was coupled to the phage assay downstream to detect and quantify live Mtb cells expectorated by smear-positive TB cases. The "Kimberly-Clark" face masks were used in the study. Following sampling, the contaminated mask samples were processed by the phage assay in two ways, one involved the detection of Mtb cells in mask eluates while another one involved direct detection of Mtb cells on the masks. The findings show that the latter has higher sensitivity than the former. In addition, the study also showed that higher amounts of Mtb aerosols were expectorated by TB patients in the morning than in the evening, and most of the Mtb cells detected were predominantly sampled in the middle section of the masks.

Another mask sampling was attempted by Huynh *et al.* (2008) to directly detect respiratory viruses in exhaled aerosols. In this study, mask sampling was performed on 20 patients with cold symptoms and the mask DNA extracts were then subjected to a multiplex PCR assay. Rhinovirus, influenza A virus and parainfluenza virus were successfully detected in some of the mask samples. The findings from this study also suggested that different expiratory maneuvers might generate airborne particles of various sizes, which might carry viruses and result in transmission.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Apparatus and Consumables

The apparatus and consumables used in this project are listed in Appendix A along with their respective manufacturers.

## 3.2 Preparation of Culture Media

All culture media prepared were autoclaved at 121 ℃ for 15 minutes prior to use, unless otherwise indicated.

### 3.2.1 Middlebrook 7H9-OADC Broth

M7H9 broth was prepared by dissolving 1.88 g of M7H9 broth powder and 1 g of glycerol in $dH_2O$ to a final volume of 360 ml. The autoclaved broth was supplemented with 10% v/v OADC before use.

### 3.2.2 Middlebrook 7H9-OADC-Tween Broth

M7H9 broth was prepared as before (Section 3.2.1) and supplemented with 10% (v/v) OADC and 0.05% (w/v) Tween 80 before use.

### 3.2.3 Middlebrook 7H9-OGC Broth and Agar

M7H9-OADC broth was prepared as before (Section 3.2.1), with the addition of 1 mM $CaCl_2$ prior to use. M7H9-OGC agar was prepared as with the broth but with the addition of 1.5% (w/v) of agar powder.

### 3.2.4 Middlebrook 7H10 Agar

M7H10 agar was prepared by dissolving 7.6 g of M7H10 agar powder and 2.5 g of glycerol in $dH_2O$ to a final volume of 360 ml. The agar was boiled for 5 minutes before autoclaving. The agar was supplemented with 10% (v/v) OADC before use.

### 3.2.5 Peptone Broth, 1% (w/v) with Tween 80 Reagent, 1% (w/v)

An amount of 4 g of peptone broth powder was dissolved in $dH_2O$ to a final volume of 360 ml. The autoclaved broth was then supplemented with 40 ml of 10% (w/v) Tween 80 prior to use.

### 3.2.6 Trypticase Soy Agar

TSA was prepared by dissolving 32 g of TSA powder in $dH_2O$ to a final volume of 800 ml.

## 3.3 Preparation of Reagents

All reagents prepared were and autoclaved at 121℃ for 15 minutes prior to use unless otherwise indicated. Some reagents were filter-sterilized through 0.22 µm membrane filter unless otherwise indicated.

### 3.3.1 Acid-alcohol

Acid-alcohol was prepared by mixing 194 ml of 95% (v/v) ethanol with 6 ml of concentrated HCl.

### 3.3.2 Calcein solution, 25 µM

An amount of 3.89 mg of calcein powder was dissolved in 1 ml of 0.1 M NaCl. After that, 50 µl of the calcein solution was mixed with 450 µl of 12.5 µM $MnCl_2$. The mixture was stored at -20℃ away from light.

### 3.3.3 Carbolfuchsin Stain

An amount of 0.6 g of basic carbolfuchsin powder was dissolved in 20 ml of 95% (v/v) ethanol. Then, 10 ml of molten phenol crystals was mixed with 190 ml of $dH_2O$. Both of the solutions were mixed together and allowed to stand for a few days. The mixture was then filtered through a filter paper and stored away from light.

### 3.3.4 Ferrous Ammonium Sulfate, 200 mM

FAS was prepared freshly before use by dissolving 0.784 g of FAS powder in $dH_2O$ to a final volume of 10 ml. The solution was then filter-sterilized before use.

### 3.3.5 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 (pH 8) with $dH_2O$ to a final volume of 200 ml.

### 3.3.6 Methylene Blue Stain

An amount of 0.6 g of methylene blue chloride powder was dissolved in $dH_2O$ to a final volume of 200 ml.

### 3.3.7 Mycobacteriophage Buffer

A volume of 2 ml of 1 M Tris-HCl (pH 7.6), 20 ml of 1 M NaCl, 2 ml of 1 M $MgSO_4$ and 0.4 ml of 1 M $CaCl_2$ were mixed with $dH_2O$ to a final volume of 200 ml.

### 3.3.8 NOA Antibiotic Cocktail Antimicrobial Supplement

NOA stock was prepared by mixing 11.25 mg/ml nystatin (in methanol), 153.5 mg/ml oxacillin (in $dH_2O$), and 9 mg/ml aztreonam (in formamide-methanol 1:1 mixture) in equal proportions.

### 3.3.9 Tris-acetate-EDTA Buffer

Tris-acetate-EDTA **(**TAE) buffer was prepared by dissolving 242 g of Tris base, 37.2 g of EDTA and 57.1 ml of glacial acetic acid in $dH_2O$ to a final volume of 1 L. The buffer was diluted 50 times before use.

### 3.3.10 Tween 80, 10% (w/v)

Tween 80 (10% w/v) was prepared by mixing 10 g of Tween 80 stock with $dH_2O$ to a final volume of 100 ml. It was warmed at 40 ℃ in a water bath for 30 minutes, filter-sterilized and stored at 4 ℃ away from light.

## 3.4 General Methods

### 3.4.1 Optical Density Measurement of Cultures

The optical density (OD) of 1 ml of mycobacterial culture was measured at the wavelength of 580 nm using the spectrophotometer. M7H9 broth was used as the blank. Dense cultures ($OD_{580nm}$ > 1.0) were diluted ten-fold prior to the measurement.

### 3.4.2 Cultivation of *M. smegmatis*

*M. smegmatis* culture was grown by streaking a loopful of a single colony from a previous culture plate or thawed glycerol stock onto M7H10 agar. The plate was incubated at 37℃ for about three days until visible colonies were observed. For liquid culture, a few loopfuls of colonies were emulsified in M7H9 broth and the $OD_{580nm}$ of the resulting suspension was measured. The suspension was then used to inoculate 10 ml or 20 ml of M7H9 broth to an $OD_{580nm}$ of 0.05 in a 50-ml centrifuge tube and 100-ml conical flask, respectively. It was then incubated at 37℃ with shaking at 200 rpm for about 15-18 hours to reach the exponential phase. *M. smegmatis* cells will double every three hours in this phase (Cheah, 2010).

### 3.4.3 Cultivation of *M. bovis* BCG

*M. bovis* BCG was grown by inoculating 1 ml of a thawed glycerol stock into 5 ml of M7H9-OADC-Tween broth in a 50-ml centrifuge tube. It was incubated static at 37 ℃ for about 10 days to reach an $OD_{580nm}$ of approximately 1. It was then subcultured into 25 ml of M7H9-OADC-Tween broth to an $OD_{580nm}$ of 0.05. The culture was incubated at 37 ℃ for about 3-4 days to reach the exponential phase. The doubling time of exponential *M. bovis* BCG cells is about 24 hours (Cheah, 2010).

### 3.4.4 Preparation of Glycerol Stock Cultures for Long-term Storage

Stock cultures of mycobacteria (*M. smegmatis and M. bovis* BCG) were prepared by mixing exponential-phase culture suspension with 65% (v/v) glycerol solution in 1:1 ratio. The mixture was then distributed in 1-ml aliquots into cryovials and then stored at -80 ℃.

### 3.4.5 Preparation of *M. smegmatis* Culture Supernatant

*M. smegmatis* culture was prepared as before (Section 3.4.2) except that it was grown for 20 hours. The culture was then centrifuged at 3,000 x *g* for 15 minutes and the resulting supernatant was filter-sterilized through a 0.22 μm membrane filter. The cell-free supernatant was mixed with M7H9-OGC broth in 1:1 ratio before use.

### 3.4.6 Enumeration of Colony-forming Units

The drop plate method (Hoben and Somasegaran, 1982) was used to enumerate the number of colony-forming units (CFUs) in test samples. Firstly, ten-fold serial dilutions were performed in 450 µl of M7H9 broth in 1.5-ml Eppendorf tubes. Three 20-µl drops for each dilution were spotted onto M7H10 agar. The drops were left to dry and then the plate was incubated at 37℃ until visible colonies could be observed. The dilution that yielded about 10-100 CFUs was used to determine the CFU/ml of the test sample.

### 3.4.7 Ziehl-Neelsen Acid-fast Staining

A loopful of bacterial culture was smeared onto a microscope slide. The smear was then heat-fixed. Carbofuchsin stain was first applied onto the smear with mild heating until steam was released. After leaving for 5 minutes, the smear was rinsed with tap water and then decolorized by acid-alcohol. The slide was rinsed with tap water again and then flooded with the counterstain methylene blue. After 1 minute, the slide was again rinsed with tap water and then blotted dry. The smear was examined under oil immersion at 1,000x magnification.

## 3.5 Mycobacteriophage D29

### 3.5.1 Preparation of Phage Indicator Plates

*M. smegmatis* culture was grown in M7H9-OADC broth for about 24 hours at 37℃ with shaking. The culture was left to stand for 30 minutes to sediment the mycobacterial clumps. An amount of 1 ml of the upper homogenous cells suspension was mixed with 9 ml of molten M7H9-OGC agar (maintained at 55℃) and then poured into a Petri dish. All phage indicator plates were left to dry under laminar air flow for about 15 minutes before use.

### 3.5.2 Phage Propagation

A volume of 100 µl of phage D29 suspension at 4 x $10^3$ plaque-forming units (PFUs)/ml was spread onto a phage indicator plate. The plate was then incubated overnight at 37℃ to yield large number of plaques. Then, 10 ml of M7H9-OGC broth was pipetted into the plate and then further incubated as before. The resulting suspension was filtered through a 0.45-µm syringe filter. Finally, the cell-free phage suspension was distributed in 1-ml aliquots into 1.5-ml Eppendorf tubes. The aliquots were stored at 4℃ away from light.

### 3.5.3 Enumeration of Plaque-Forming Units

Phage suspension was ten-fold serially diluted in aliquots of 450 µl of MP buffer each. Three 10-µl drops of each dilution were spotted onto the phage indicator

plates. The plates were left to dry and then incubated at 37℃ overnight. The dilution that yielded 10-100 plaques was used to enumerate the PFU/ml of the phage suspension.

### 3.6 Artificial Contamination of Face Masks with Mycobacterial Cells

Surgical face masks that are easily available in Malaysia were used in this project (Figure 3.1). It is made up of spun-bonded polypropylene and consists of three layers: the outer shell, the middle filter layer and the inner cover web. The face mask was first cut into 5 cm x 5 cm segments. Each side of the mask was treated with UV radiation for 30 minutes in the laminar airflow cabinet. Ten 50-µl drops of exponential-phase mycobacterial cultures were pipetted onto the inner cover web and then left to dry under laminar air flow for about four hours. Negative control was performed with mask applied with M7H9-OGC broth. The dry mask segment was transferred to a 30-ml universal tube and then 20 ml of M7H9-OGC broth added. The mycobacterial cells on the mask were released by vortexing for two minutes. Following this, both the mask and the resulting suspension were subjected to the phage assay.


**Figure 3.1:** The commercial surgical face mask used in this study. *(Image was adapted from the China Medical Device website.)*

### 3.7 Direct Mask Analysis by the On-mask Phage Assay

Mycobacteriophage D29 was added to the tube containing mask and suspension to 3 x $10^7$ PFU/ml, as depicted in Figure 3.2. The reaction mixture was mixed gently and then incubated at 37 ℃ for 1 hour for phage infection. FAS was then added to the reaction mixture to a concentration of about 8.3 mM to kill exogenous phages. After 5 minutes, 1 ml of reaction mixture was pipetted into 9 ml of molten M7H9-OGC agar along with 1 ml of *M. smegmatis* lawn culture. The mixture was swirled gently and then poured into a Petri dish. The solidified plate was incubated overnight and then examined for formation of visible plaques, followed by enumeration.

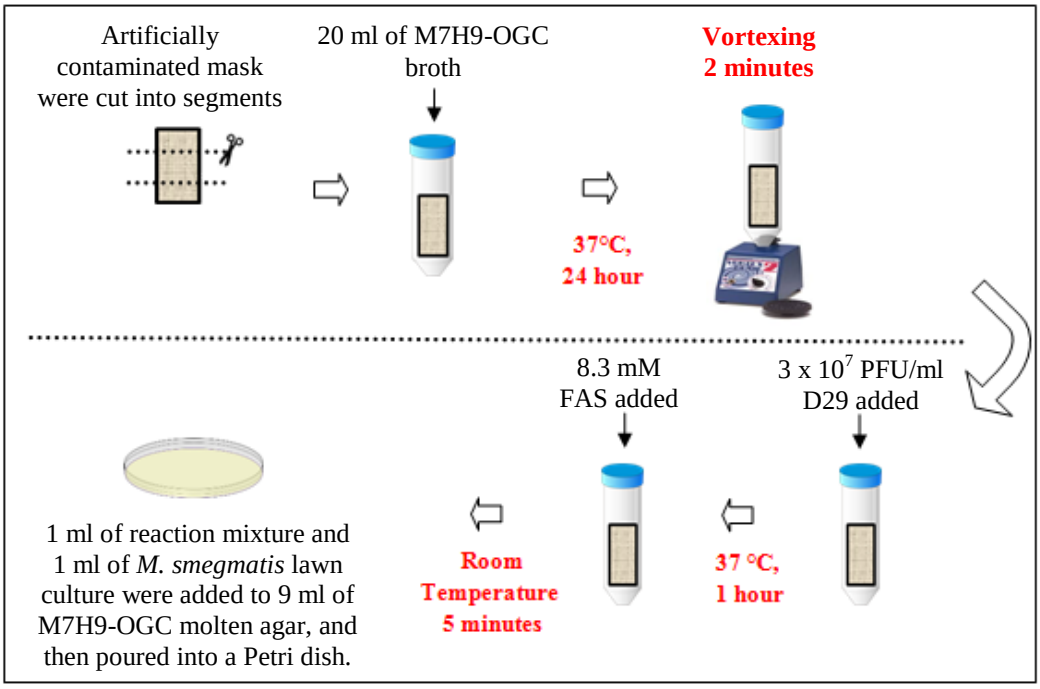


**Figure 3.2:** On-mask phage infection of artificially contaminated mask sample.

## 3.8 Use of Mask Sampling to Study the Expectoration of Respiratory Droplets by Healthy Subjects.

### 3.8.1 Mask Sampling with Healthy Subjects

This study was performed with healthy subjects to assess the distribution of respiratory aerosols on face masks for different expiratory maneuvers. The subjects were requested to wear the face masks provided and perform 1-minute coughing, 1-minute reading and 1-minute breathing. Following this, the masks were stored in a plastic container each, which was pre-sanitized with 70% (v/v) ethanol until processing. Negative control was performed with clean and unworn mask.

### 3.8.2 Processing of Worn Masks from Healthy Subjects

The ear loops of worn face mask were trimmed off and excluded from processing. The mask was cut and split into three segments: middle segment (M, 9 cm x 10 cm), left and right segments (LandR, each of 9 cm x 3.5 cm). The middle segment was transferred to a 50-ml centrifuge tube while left and right segments were processed together in another tube. Each tube was filled with 40 ml of 1% (w/v) peptone + 1% (w/v) Tween 80. All tubes were then vortexed horizontally for 2 minutes. After that, 100 µl of the resulting suspension from each tube was spread onto TSA agar in duplicate. The plates were incubated at 37℃ for 2-3 days and the colonies formed were enumerated. The morphology of the number of colonies was also briefly assumed. All data obtained in this test were analysed by the two-tailed paired t test as required.

### 3.8.3 NOA Decontamination of Worn Masks

The mask processing protocol depicted in Section 3.7 was modified to include the use of the NOA antibiotic cocktail to control contamination. The mask was immersed in 40 ml of M7H9-OGC broth and resuscitated at 37℃ for 24 hours before vortexing. A published concentration of NOA (nystatin 10 µg/ml, oxacillin 2 µg/ml and aztreonam 30 µg/ml) was added to the M7H9-OGC broth while an increased concentration (nystatin 37.5 µg/ml, oxacillin 512 µg/ml and aztreonam 30 µg/ml) was incorporated into the final phage indicator plate.

## 3.9 Molecular Detection of Mycobacteria in Mask Samples

### 3.9.1 DNA Extraction from Mycobacteria in Mask Eluates

Mycobacterial cells were released from the artifically contaminated mask samples by sonication for 3 minutes. After that, the mask segment was removed and the mask eluate was centrifuged at 4,020 x *g* for 15 minutes. The resulting pellet was resuspended in 1 ml of deionized water. The suspension was then transferred to a 1.5-ml Eppendorf tube for another centrifugation at 13,300 x *g* for 2 minutes. The resulting pellet was resuspended in 50 µl of deionized water, boiled for 5 minutes and then centrifugated at 13,300 x *g* for 2 minutes. The resulting supernatant was subjected to the IS*1081* PCR (Section 3.9.4).

### 3.9.2 Plaque DNA Extraction

Plaque DNA was extracted as described by Stanley *et al.* (2007). Plaque was excised from the phage indicator plate using a 10-µl pipette tip. It was then transferred to a 1.5-ml Eppendorf tube and then 10 µl of deionized water added. Tube content was heated at 95℃ for 5 minutes, pulse centrifuged and then incubated at -20℃ for at least 15 minutes. Prior to use, the tube content was thawed and centrifuged at 13,300 x *g* for 2 minutes. The resulting supernatant was subjected to molecular analysis downstream.

### 3.9.3 Mtb complex-specific 16S rDNA LAMP Assay

The Mtb complex-specific 16 rDNA LAMP assay was carried out in 25-µl reaction volume consisting of 1x isothermal amplification buffer, 6 mM $MgSO_4$, 0.8 M betaine, 1.4 mM dNTPs, 0.2 µM each for F3 and B3, 1.6 µM each for FIP and BIP, 0.8 µM each for FLP and BLP (forward and reverse loop primer, respectively), 8 U of *Bst* DNA polymerase, 25 µM calcein and template DNA.

Amplification was performed at 65˚C for 40 minutes in a heating block. The reaction was then terminated at 80˚C for 5 minutes in a water bath. The reaction tubes were finally visualized under the UV transilluminator. Green fluorescence would indicate a positive reaction while negative reaction would remain orange in color. The LAMP assay was designed and developed by Pandey *et al.* (2008); the primer sequences are shown in Table 3.1.

**Table 3.1:** Sequences of Mtb complex-specific 16S rDNA LAMP primers.

| Primer | Sequence |
|---|---|
| MTB-FIP | 5'- CAC CCA CGT GTT ACT CAT GCA AGT CGA ACG GAA AGG TCT -3' |
| MTB-BIP | 5'- TCG GGA TAA GCC TGG ACC A mCA AGA CAT GCA TCC CGT -3' |
| MTB-F3 | 5'- CTG GCT CAG GAC GAA CG -3' |
| MTB-B3 | 5'- GCT CAT CCC ACA CCG C -3' |
| MTB-FLP | 5'- GTT CGC CAC TCG AGT ATC TCC G -3' |
| MTB-BLP | 5'- GAA ACT GGG TCT AAT ACC GG -3' |

### 3.9.4 IS*1081* PCR Assay

The IS*1081* PCR assay was performed as described by Wards *et al.* (1995). The primers used were designed by Ahmed *et al*. (1998) and are listed as listed in Table 3.2. They target a 306-bp region of IS*1081*, which is the signature sequence of Mtb complex. A 25-µl reaction mixture was prepared as shown in Table 3.3. The PCR assay was carried out according to the profile shown in Table 3.4; the cycling was repeated 35 times. A non-template control (NTC) was included in every PCR run. Meanwhile, *M. smegmatis* and *M. bovis* BCG DNAs served as negative and positive controls, respectively.

**Table 3.2:** Oligonucleotide primers used in the IS*1081* PCR assay.

| Primer | Sequence | Position (bp) |
|---|---|---|
| BW6 | 5'- CGA CAC CGA GCA GCT TCT GGC TG -3' | 405-427 |
| BW7 | 5'- GTC GGC ACC ACG CTG GCT AGT G -3' | 710-689 |

**Table 3.3:** Components of the IS*1081* PCR assay.

| Components | Amount (µl) | |
|---|---|---|
| | Plaque DNA | Genomic DNA |
| Buffer, 10x | 2.5 | 2.5 |
| Primer BW6, 12.5 µM | 1 | 1 |
| Primer BW7, 12.5 µM | 1 | 1 |
| dNTP mix, 10 µM | 0.5 | 0.5 |
| *Taq* DNA polymerase, 5 U/µl | 0.25 | 0.25 |
| $MgCl_2$, 50 mM | 0.25 | 0.25 |
| Formamide, 100% | 0.6 | 0.6 |
| Deionized water | 8.9 | 16.9 |
| Template DNA | 10 | 2 |
| **TOTAL** | 25 | 25 |

**Table 3.4:** Profile for the IS*1081* PCR assay

| Temperature (℃) | Duration (s) |
|---|---|
| **95** | 180 |
| **95** | 60 |
| **68** | 90 |
| **72** | 120 |
| **72** | 600 |

### 3.9.5 Gel Analysis of PCR Amplicons

PCR amplicons were electrophoresed on 1.5% (w/v) agarose gel. A volume of 10 µl of amplicons for each sample was analyzed along with 2 µl of GeneRuler 100-bp DNA ladder on the gel. Gel electrophoresis was performed at 85 V for 45 minutes. The gel was then stained with ethidium bromide for about 10 minutes and then destained with $dH_2O$ for two minutes. Finally it was visualized under the UV transilluminator.

# CHAPTER 4

# RESULTS

## 4.1 Evaluation of the Phage Assay for Direct Detection of Mycobacterial Cells on Face Masks

The feasibility of the phage assay to directly detect mycobacterial cells sampled on the face masks was investigated. This was done by spiking known amounts of *M. smegmatis* CFUs onto 5-$cm^2$ mask segments, which were then subjected to the phage assay. Following overnight incubation at 37 ℃, four possible outcomes could be observed in the phage indicator plates, as shown in Figure 4.1.

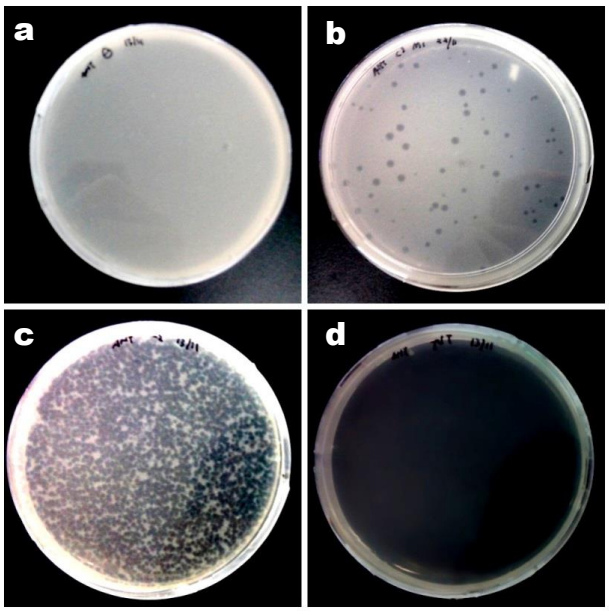


**Figure 4.1:** Different plaque densities in the phage indicator plates following overnight incubation at 37 ℃. a) No plaque; b) Countable plaque; b) Confluent lysis; d) Complete lysis.

### 4.1.1 Assessment of the On-mask Phage Assay for Potential Occurrence of Background PFU Counts

This was carried out to investigate the possibility of interference of FAS inactivation by released mask materials or the trapping of exogenous phages among the mask layers, which would lead to the observation of false positive (background) PFUs and affect the reliability of the assay. Four non-spiked mask segments were subjected to the on-mask phage assay; a *M. smegmatis*-spiked mask segment was also included as the positive control. As shown in Table 4.1, only one PFU was observed in a single replicate of one out of the four mask segments tested, which could have happened by chance. These findings suggest that the on-mask phage assay is not prone to the occurrence of background PFUs and the results obtained are reliable true positives.

**Table 4.1:** Assessment of the on-mask phage assay for the potential occurrence of background PFUs.

| | **Spiked Mask Segment** | **Non-spiked Mask Segments** | | | |
|---|---|---|---|---|---|
| | | **I** | **II** | **III** | **IV** |
| **Number of PFU** | Complete Lysis[†] | Not Detected | Not Detected | 1 | Not Detected |
| | Complete Lysis | Not Detected | Not Detected | Not Detected | Not Detected |

- Spiked mask segment (positive control) contained about $10^8$ *M. smegmatis* CFUs. Each mask reaction was plated out in duplicate.

[†]According to Stanley *et al.* (2007), complete lysis of *M. smegmatis* lawn culture indicates that more than 1 x $10^5$ *M. smegmatis* cells were detected.

### 4.1.2 Penetration of Spiked Mycobacterial Cells through the Face Mask

This was investigated to determine whether the face mask could be processed intact or needed to be processed in separated layers. In the case that significant amount of spiked mycobacterial cells penetrated to the subsequent layers, the latter would apply. Referring to Table 4.2, the results show that most of the spiked *M. smegmatis* cells were retained on the inner layer of the mask, resulting in complete lysis of the *M. smegmatis* lawn. A relatively small number of *M. smegmatis* cells were detected in the middle layer, and even lesser in the outer layer. Hence, the degree of penetration was negligible and all mask segments were processed intact in subsequent experiments.

**Table 4.2:** Penetration of spiked *M. smegmatis* cells through face masks.

| Mask | | | PFUs/Mask Segment |
|---|---|---|---|
| **Intact Mask** | Rep. 1 | | Complete Lysis |
| | Rep. 2 | | Complete Lysis |
| **Separated Mask** | Rep. 1 | Inner | Complete Lysis |
| | | Middle | 100 |
| | | Outer | 53 |
| | Rep. 2 | Inner | Complete Lysis |
| | | Middle | 6 |
| | | Outer | 6 |

- Each 5-$cm^2$ mask segment was spiked with about $10^8$ *M. smegmatis* CFUs. Complete lysis of the *M. smegmatis* lawn indicates that more than $10^5$ *M. smegmatis* cells were detected (Stanley *et al*. 2007).

### 4.1.3 Evaluation of the Sensitivity of the On-mask Phage Assay with Artificially Spiked Mask Samples

The sensitivity of the on-mask phage assay was assessed with 5-$cm^2$ mask segments that were spiked with different amounts of *M. smegmatis* cells in the range of 10 to $10^8$ CFUs. Its detection limit was determined to be between 100 to 1,000 CFUs per mask segment, which is only 10-100 folds lower than that for phage assay with *M. smegmatis* culture (Table 4.3). The lower sensitivity for the on-mask phage assay was shown to be related to the poorer PFU recovery efficiencies, which ranged from 0.32% to 7%, compared to those for phage assay with *M. smegmatis* culture, which ranged from 26% to 28%. Therefore, the on-mask phage assay was deemed to be quite sensitive to be used as a means of clinical diagnosis.

**Table 4.3:** Evaluation of the sensitivity of the phage assay.

(a) On-mask phage assay with *M. smegmatis*-spiked 5-$cm^2$ mask segments.

| ***M. smegmatis* CFUs Tested Per Mask Segment** | **Phage D29 PFUs Recovered Per Mask Segment** |
|---|---|
| $10^8$ | Complete Lysis [a] |
| $10^7$ | Confluent Lysis [b] |
| $10^6$ | Near Confluent Lysis |
| $10^5$ | 320 |
| $10^4$ | 17 |
| $10^3$ | 13 |
| $10^2$ | 7 |
| 10 | Not Detected |
| Negative control[†] | Not Detected |

[a] Complete lysis, more than $10^5$ *M. smegmatis* cells were detected.
[b] Confluent lysis, about $10^4$ *M. smegmatis* cells were detected.
[†] Negative control was performed with a clean, non-spiked on mask segment.

(b) Phage assay with *M. smegmatis* culture.

| ***M. smegmatis* CFUs Tested Per ml** | **Phage D29 PFUs Recovered Per ml** |
|---|---|
| $10^8$ | Complete Lysis [a] |
| $10^7$ | Complete Lysis |
| $10^6$ | Complete Lysis |
| $10^5$ | Near Complete Lysis |
| $10^4$ | Confluent Lysis [b] |
| $10^3$ | 264 |
| $10^2$ | 28 |
| 10 | Not Detected |
| No Mask | Not Detected |

### 4.1.4 Evaluation of NOA Decontamination of Contaminated Masks Prior to the Phage Assay

Contamination of the phage assay by rapid-growing microbiota organisms that are co-sampled on the face masks could obscure the development and observation of plaques in the phage indicator plates. The use of the NaOH-NALC treatment, the conventional decontamination method applied to tuberculous specimens, has been reported to affect the sensitivity of the phage assay, besides being time consuming (Cheah, 2010). The incorporation of NOA (antimicrobial cocktail consists of nystatin, oxacillin and aztreonam) into the phage assay, published concentration during overnight incubation of mask sample (Albert *et al.*, 2007) and an increased concentration in the phage indicator plate, was employed in the method of Cheah (2010) to control contamination by rapid-growing microbiota.

The latter means of decontamination was assessed for the model of face mask used in this study. Five healthy subjects were asked to put on a mask each and

instructed to cough voluntarily onto it for one minute. The mask samples were then subjected to the on-mask phage assay as described in Section 3.8.3. The final reaction mixture was plated out in duplicate for each sample. After overnight incubation, no contamination was observed in all the phage indicator plates (Table 4.4). Surprisingly, PFUs were yielded for some mask samples, in the range of 1 to 48 PFUs per mask segment. In order to check if the incorporation of NOA led to background PFUs, a test was carried out with two sets of clean masks, one with NOA included in the phage assay and the other without (control). This was performed in a separate laboratory area to rule out the possibility of cross-contamination. Following overnight incubation, only one replicate reaction of one of the NOA-exposed masks yielded two PFUs, while all other phage indicator plates were clear. The former observation was of little concern and could have occurred by chance.

**Table 4.4:** Effect of NOA decontamination of contaminated face masks from five healthy subjects.

| Subject no. | Mask Segment[†] | Average PFUs/plate | Presence of Contamination |
|---|---|---|---|
| **1** | M | 4 | Not Detected |
| | L&R | 2 | Not Detected |
| **2** | M | 0 | Not Detected |
| | L&R | 0 | Not Detected |
| **3** | M | 48 | Not Detected |
| | L&R | 6 | Not Detected |
| **4** | M | 24 | Not Detected |
| | L&R | 0 | Not Detected |
| **5** | M | 1 | Not Detected |
| | L&R | 0 | Not Detected |

[†] 'M', middle segment; 'L&R', left and right segments.

### 4.1.5 Effect of Storage of Face Masks on the Detection of Mycobacterial Cells by the On-mask Phage Assay

At times, clinical mask samples will not be able to be processed immediately upon their receipt, for instance if they arrive at the end of the working day or working week. In this case, the mask samples will normally be stored at 4 ℃ until processing. An experiment was performed to assess the effect of this storage on detection of mycobacterial cells on them by the phage assay. Three sets of 5-$cm^2$ mask segments were spiked with $10^5$ *M. smegmatis* CFUs each; one set was processed immediately (control) and the other two were stored at 4 ℃ and at room temperature overnight, respectively, and then processed. Each set of masks was tested in duplicate. As shown in Figure 4.2, the numbers of PFUs recovered from the masks stored at 4 ℃ overnight decreased by more than 50% relative to those that were immediately processed. Interestingly, PFU recovery from masks stored at room temperature overnight showed about five-fold decline compared to those stored at 4 ℃.

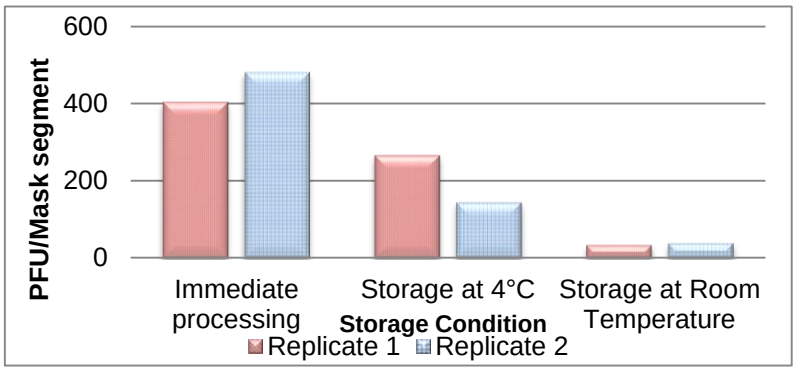


**Figure 4.2:** Effect of storage of spiked mask segments on the detection of *M. smegmatis* cells on them by the phage assay.

During overnight storage at 4 ℃, mycobacterial cells could become dormant as a result of exposure to stresses such as low temperature, desiccation and nutrient starvation, potentially rendering them invulnerable to phage infection (Tan, 2013). The mycobacterial resuscitation-promoting factors (Rpfs) have been demonstrated to be able to revive dormant or inactive mycobacterial cells *in vitro* (Cheah, 2010; Mukamolova *et al.*, 2010; Tan, 2013). Therefore, another experiment was carried out to investigate if prior treatment of stored masks with mycobacterial culture supernatant (containing Rpfs) could improve the detection of mycobacterial cells on them by the phage assay. Three sets of 5-$cm^2$ mask segments with $10^6$ *M. smegmatis* CFUs each; one set was processed immediately after overnight storage at 4 ℃ (control), and the other two were treated with M7H9-OADC broth and *M. smegmatis* culture supernatant, respectively, at 37 ℃ for 3 hours following storage and then subjected to the phage assay. Each set of masks was tested in duplicate. Figure 4.3 shows that higher numbers of PFUs were recovered from mask segments treated with M7H9-OADC broth and culture supernatant, as compared to the one immediately processed after 4 ℃ storage. For the former, treatment in M7H9-OADC broth yielded slightly higher PFUs than in culture supernatant.

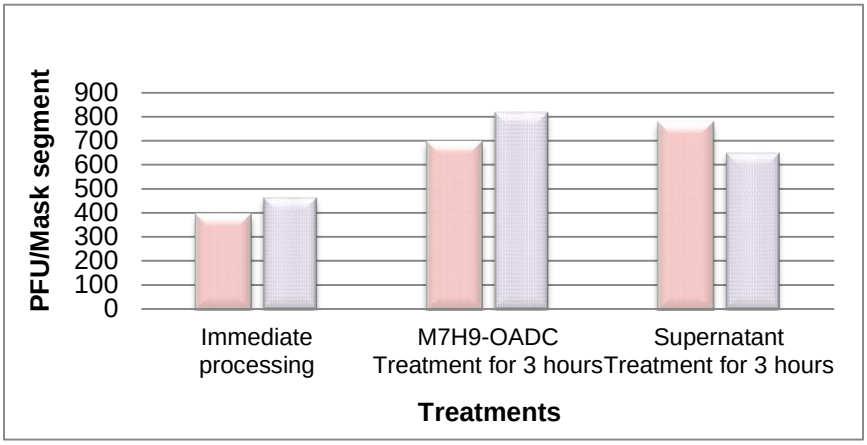


**Figure 4.3:** Effect of treatment of stored mask segments with M7H9-OADC broth and *M. smegmatis* culture supernatant at 37 ℃ for 3 hours on the detection of *M. smegmatis* cells on them by the phage assay.

## 4.2 Evaluation of the Feasibility of a Molecular Assay for Detection of Target Mycobacterial Species in Phage D29 Plaques

Due to the nature of the mycobacteriophage (D29) used, the phage assay is not specific for a particular mycobacterial species. In order to verify the identity of positive plaques observed in the phage indicator plates, a specific molecular assay was incorporated downstream of the phage assay, as performed by Stanley *et al.* (2005). Theoretically, there should be one genome from a lysed target mycobacterial cell in a positive plaque and by targeting a multicopy gene like the insertion sequence (IS), detection by a molecular assay is possible. The signature IS elements for the Mtb complex include the IS*6110* and IS*1081*; the former is present in 1-20 copies in the Mtb genome while the latter is present in 5-6 copies in the *M. bovis* BCG genome. In this study, the latter was used to assess the feasibility of this combined approach.

### 4.2.1 Feasibility of the Mtb Complex-specific 16S LAMP Assay

The specificity of Mtb complex-specific 16S LAMP assay (Pandey *et al.*, 2008) was initially evaluated with culture DNA extracts of *M. bovis* BCG (target), *M. smegmatis* and *E. coli*. As expected, the *M. bovis* BCG reaction showed an intense green fluorescence that indicates a positive result while the *E. coli* and NTC reactions remained orange (Figure 4.4). However, the *M. smegmatis* reaction was anomalously positive in this LAMP assay. Repeated trials were hampered by higher levels of false positivity; the NTC and both the non-target reactions (*M. smegmatis* and *E. coli*) showed intense green fluorescence in almost every run. Several attempts to troubleshoot via the use of new stocks of LAMP reagents (primers, dNTPs, betaine, calcein and deionized water) and dedicated areas and pipettes to perform the assay were unsuccessful. Therefore, the LAMP assay was abandoned and focus was shifted to the conventional PCR assay in further investigations.

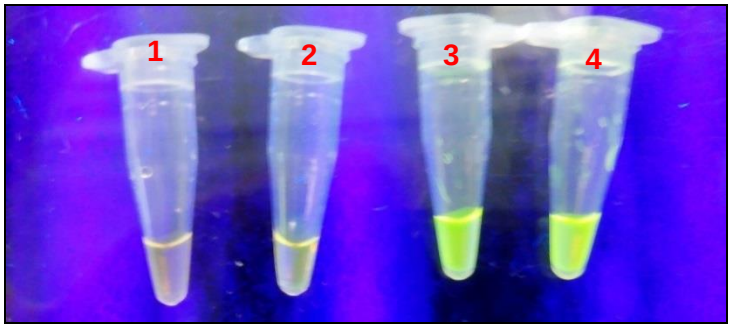


**Figure 4.4:** Visual assessment of Mtb complex-specific 16S LAMP reactions via calcein fluorescence. Tube 1, NTC; tube 2, *E. coli* DNA; tube 3, *M. smegmatis* DNA; tube 4, *M. bovis* BCG DNA.

### 4.2.2 Feasibility of the Conventional IS*1081* PCR Assay

The IS*1081* PCR assay (Wards *et al.*, 1995; Ahmed *et al.*, 1998) was initially assessed for its specificity with culture DNA extracts. The concentrations of DNA extracts tested were in the range of 1-10 ng/µl. Among the PCR reactions, 306-bp amplicons were observed on the gel for those of the Mtb complex (*M. bovis* BCG, *M. africanum*, Mtb CDC 1551 and Mtb CH) (Figure 4.5). No bands were observed for the NTC and *M. smegmatis* (non-target) reactions. Unexpectedly, both the *E. coli* and *S. aureus* (non-targets) reactions yielded faint 306-bp bands on the gel.

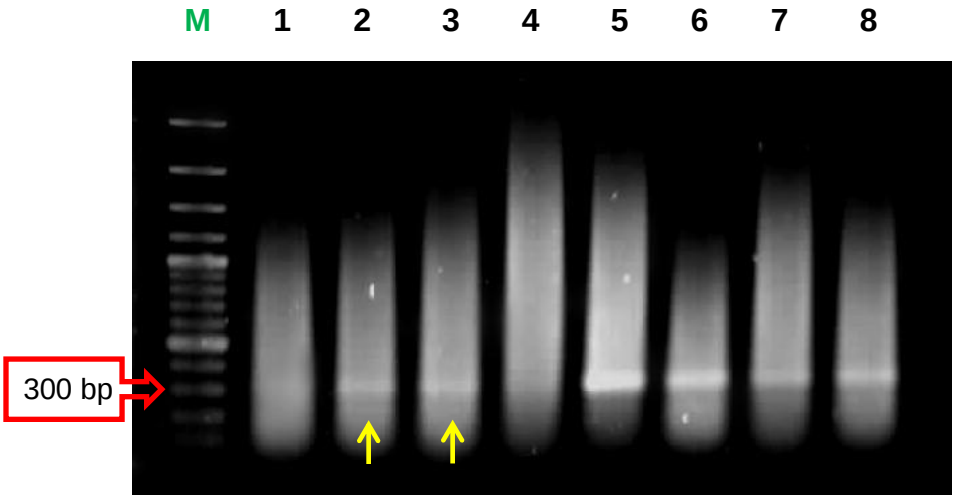


**Figure 4.5:** Gel analysis of IS*1081* PCR reactions in the assay specificity test. Faint bands are denoted by yellow arrows for clarity.

Lane M: GeneRuler 100-bp DNA ladder; lane 1: NTC; lane 2: *E. coli* DNA; lane 3: *S. aureus* DNA; lane 4: *M. smegmatis* DNA; lane 5: *M. bovis* BCG DNA; lane 6: *M. africanum* DNA; lane 7: Mtb CDC 1551 DNA; lane 8: Mtb CH DNA.

The IS*1081* PCR assay was next assessed for its sensitivity with different quantities of *M. bovis* BCG DNA from 2200 pg to 0.0022 pg. The assay was shown to be sensitive in which the target DNA was detected down to 0.0022 pg, which corresponds to about 4.4 genome equivalents (Figure 4.6).

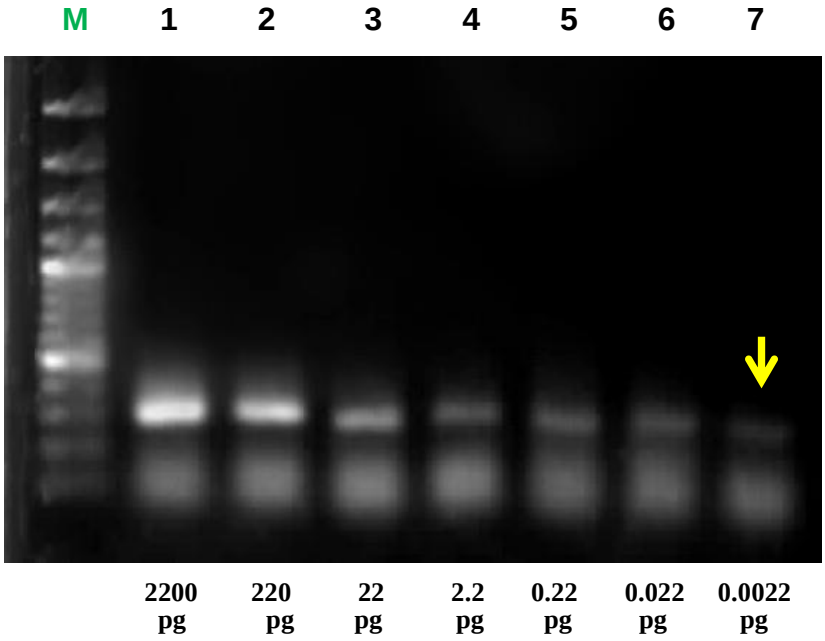


**Figure 4.6:** Gel analysis of *M. bovis* BCG IS*1081* amplicons in the assay sensitivity test. Faint bands are denoted by yellow arrows for clarity.

Lane M: GeneRuler 100-bp DNA ladder; lane 1, 2200 pg of DNA; lane 2, 220 pg of DNA; lane 3, 22 pg of DNA; lane 4, 2.2 pg of DNA; lane 5, 0.22 of pg DNA; lane 6, 0.022 pg of DNA; lane 7, 0.0022 pg of DNA.

### 4.2.3 Detection of Target Mycobacterial Species in Phage D29 Plaques

The IS*1081* PCR assay was then evaluated for its feasibility in detecting the single target *M. bovis* BCG genome in a positive D29 plaque. A total of 8 plaques

from the phage assay with *M. bovis* BCG culture and *M. bovis* BCG-spiked mask were screened but none were positive by the IS*1081* PCR (Figure 4.7; Lanes 6-9 and 10-13, respectively). The use of new stocks of reagents to repeat the PCRs still resulted in negative reactions.

The potential inhibition of the PCR reactions by interfering substances that were co-extracted along with the plaque DNA was then investigated. PCR reactions with *M. bovis* BCG DNA were spiked with non-plaque plate extracts in a ratio of 1:4 of the template volume (2 µl of DNA + 8 µl of plate extract); the plate extracts were prepared using the same extraction method as the plaque DNA extracts. From Figure 4.7, no 306-bp target amplicons were observed for reactions containing mixtures of *M. bovis* BCG DNA and plate extracts (Lanes 4 & 5), similar to those with plaque DNA extracts (Lanes 6-13). The positive control, reaction with culture *M. bovis* BCG DNA extract, yielded a bright band of 306 bp in size (Lane 3). Therefore, there is preliminary evidence to suggest that the interfering substances that are co-extracted along with the plaque DNA are inhibitory to PCR, at least in this study.

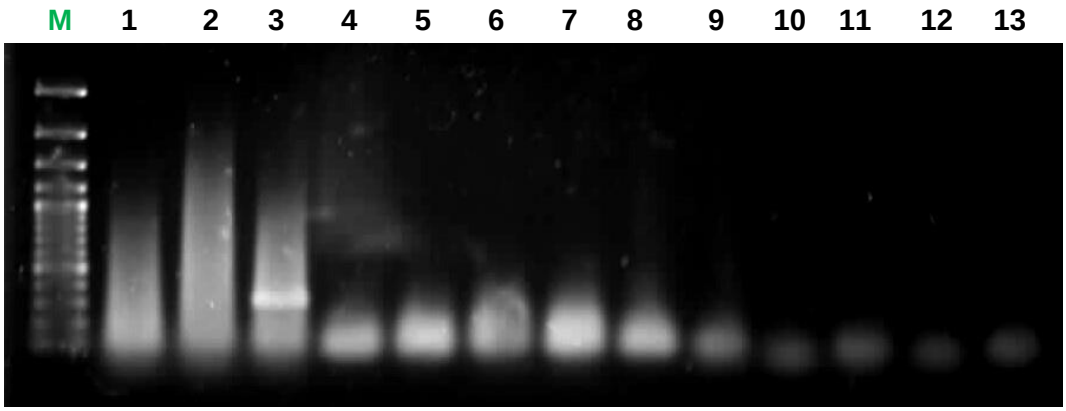


**Figure 4.7:** Gel analysis of IS*1081* PCR reactions with extracts from *M. bovis* BCG phage indicator plates.

Prescreening inhibitory analysis of phage indicator agar extract on *M. bovis* BCG DNA amplification and plaque DNA detection.

Lane M, GeneRuler 100-bp DNA ladder; lane 1, NTC; lane 2, *M. smegmatis* DNA; lane 3, *M. bovis* BCG DNA; lanes 4 & 5, *M. bovis* BCG DNA + plate extract; lanes 6-9, *M. bovis* BCG plaque DNA from phage assay with culture; lanes 10-13, *M. bovis* BCG plaque DNA from phage assay with spiked mask.

## 4.3 Evaluation of the Sensitivity of Molecular Detection of Mycobacterial Cells in Mask Eluates

In addition to the on-mask phage assay, the method of molecular analysis of mask eluates was also evaluated for its feasibility in detecting mycobacterial cells sampled on face masks. The conventional IS*1081* PCR assay was used as the molecular detection method. The sensitivity of this method was assessed with 5-$cm^2$ mask segments that were spiked with different amounts of *M. bovis* BCG cells in the range of 10 to $10^7$ CFUs. Only one replicate was performed for each CFU amount. The mask samples were then subjected to sonication to elute the *M. bovis* BCG cells applied onto them. Target cells in the resulting eluates were concentrated via centrifugation and then subjected to DNA extraction with a

simple boiling method. Finally, the crude DNA extracts were analysed via the IS*1081* PCR assay. Upon gel analysis, the 306-bp target band was only observed for the PCR reaction of the mask spiked with $10^7$ *M. bovis* BCG CFUs (data not shown), which implies that the overall method was $10^4$ to $10^5$ folds less sensitive relative to the on-mask phage assay. The extremely poor sensitivity of this method limits its usefulness and reliability for clinical diagnosis.

## 4.4 Assessment of Levels and Distributions of Culturable Respiratory Aerosols on Face Masks of Healthy Subjects with Different Expiratory Maneuvers

A pilot study was conducted with healthy subjects to assess the levels and distributions of culturable respiratory aerosols on the face masks during different expiratory activities, in which the findings of the former would be useful for investigation on TB infectivity. Ten healthy subjects, who are students of the Faculty of Science, UTAR, took part in this study; there were five subjects for each gender. The subjects were asked to put on three face masks: one for one-minute coughing, one for one-minute reading (to simulate talking) and one with no expiratory activity for one minute (control). Each worn mask was cut into the middle segment (M) and the left and right segments (L&R). Both M and L&R segments were then processed separately to recover the sampled CFUs as depicted in Section 3.8.

Although it was not possible to assess the exact distribution of CFUs on the face mask, it did show a skewed, predominant distribution in its M segment (Figure 4.8). For most of the subjects, more CFUs were detected in the M segment of their face masks compared to the L&R segments. Among the expiratory activities performed, the difference in the level of CFUs recovered between the M and L&R segments was statistically significant for coughing; higher CFUs were recovered from the M segment in this case ($P < 0.05$). Overall, the findings from this study also showed that more CFUs were detected in the face masks from the male subjects than those from the female ones ($P < 0.05$).

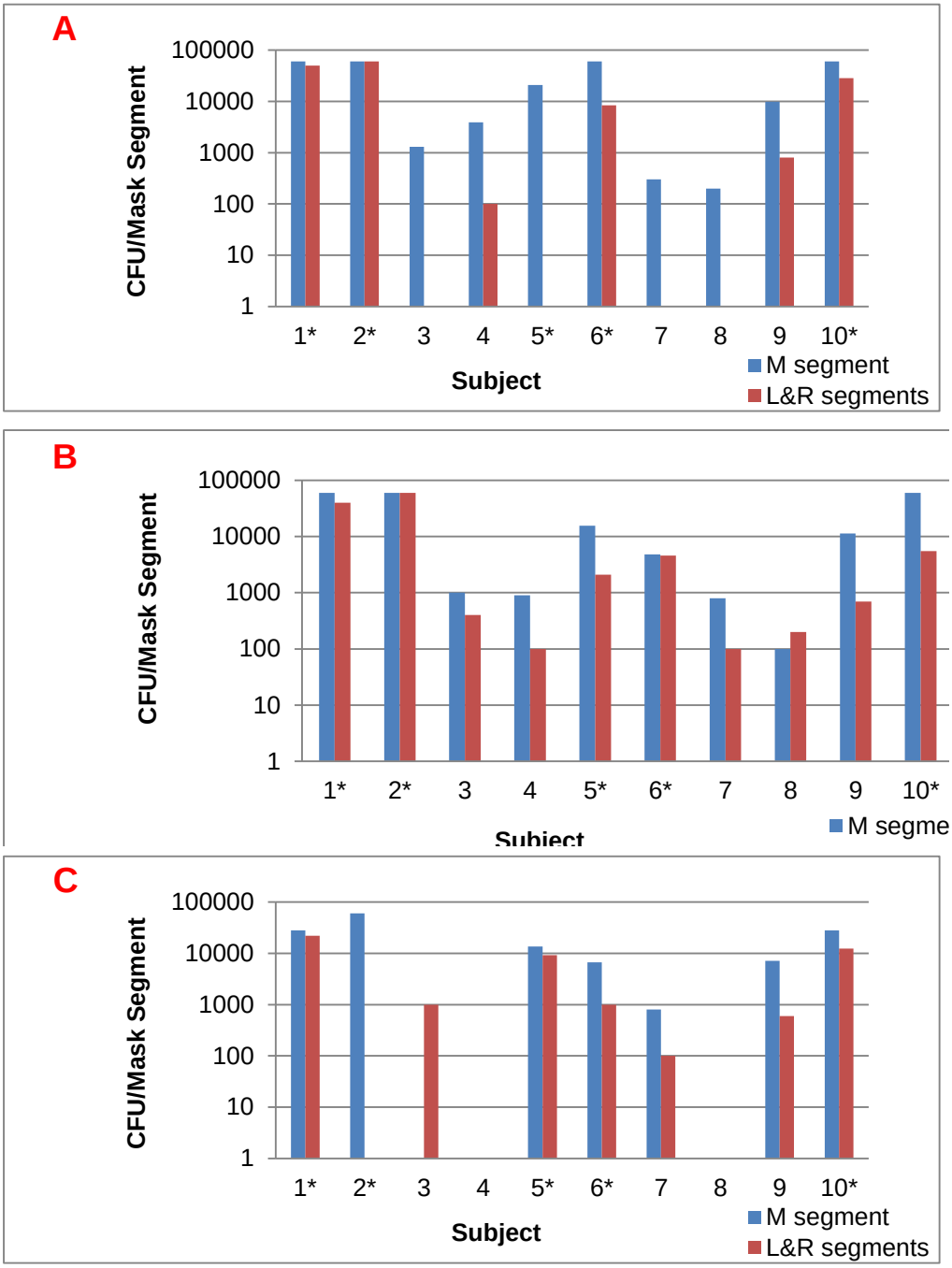


**Figure 4.8:** Distribution of CFUs on face masks of healthy subjects: (A) One-minute coughing; (B) One-minute reading; (C) One-minute no expiratory activity.

- '*' denotes male subject.
- The charts were displayed in logarithmic scale due to large differences among some data.

Based on the CFU counts of the M segments, this study demonstrated coughing yielded more CFUs than reading and control for most of the subjects (Figure 4.9). However, the difference in the CFU count between coughing and reading was not statistically significant ($P > 0.05$); the difference between coughing and no expiratory activity was significant ($P < 0.01$). It is noteworthy that the types of bacterial colonies recovered from male subjects were more diverse compared to those from female subjects; the former yielded two to three types of colonies on average (data not shown) while the latter yielded maximum two colonies.

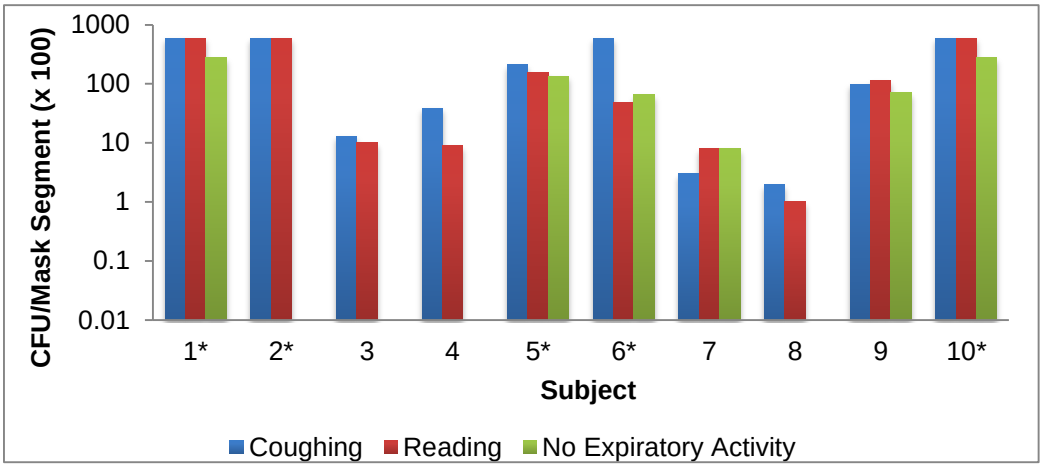


**Figure 4.9:** Distribution of CFUs on the middle segment of face masks of healthy subjects during different expiratory activities.

- '*' denotes male subject.

# CHAPTER 5

# DISCUSSION

## 5.1 Evaluation of Phage Assay for Direct Detection of Mycobacterial Cells on Face Masks

### 5.1.1 Assessment of the On-mask Phage Assay for Potential Occurrence of Background PFU Counts

Out of the four mask segments (duplicate plate each), only one background PFU was yielded in one plate. Hence, there is no concern on the occurrence of high level of false positive (background) PFUs that might interfere with the reliability of the on-mask phage assay. It is noteworthy that the observation was contrary to that reported by Cheah (2010) with the TECNOL Standard Surgical Masks from Kimberly-Clark, in which high background PFUs (more than 30 PFUs per plate) were observed. This could be explained by the possible difference in the composition between these two mask models. The masks used in the study possibly rendered weak adherence of exogenous phage particles to them, thus making the phages more susceptible to FAS inactivation and therefore resulted in low occurrence rate of false positive PFUs.

### 5.1.2 Penetration of Spiked Mycobacterial Cells through the Face Mask

Most of the artificially spiked *M. smegmatis* cells were retained on the inner layer of the surgical mask with very little penetrated to the middle layer and even lesser to the outer shell. This was evident from the very low percentage of PFUs recovered from the middle layers, which ranged from 0.0004% to 0.000006%, and outer layers, which ranged from 0.00005% to 0.000006%. A similar study by Cheng (2013) showed that most mycobacterial cells were retained in the middle layer with no penetration to the outer layer.

Chen *et al.* (1994) reported that the filter efficiency of surgical mask was more than 97% against particles less than 1 μm in aerodynamic diameter on average. This was further supported by Willeke *et al.* (1996) in which they showed that rod-shaped bacteria like Mtb have low penetration through respiratory filter compared to spherical bacteria of the same aerodynamic equivalent diameter.

Penetration of mycobacteria-containing aerosol particles through respiratory filter depends on their flow rate and the relative humidity of the surrounding environment, as shown by McCullough *et al.* (1997) in which they used *M. abscessus* as a surrogate model for Mtb in their investigation. Application of mycobacterial cell suspension onto face masks by spiking in this study did not exactly simulate the expulsion of respiratory aerosols onto masks by TB patients during respiratory maneuvers; the former involved lower flow rate. Thus, the use

of a nebulizer to apply mycobacterial cell suspension onto face masks would have better simulated the latter.

### 5.1.3 Evaluation of the Sensitivity of the On-mask Phage Assay with Artificially Spiked Mask Samples

The current study demonstrated that the phage assay is relatively sensitive for the detection of mycobacterial cells applied onto the masks. The detection limit ranged from 100 to 1,000 CFUs per mask segment, which is similar to that for phage assay with mycobacterial cultures. Barman and Gadre (2006) reported that the analytical sensitivity of the phage assay was about 94% relative to AFB smear microscopy. On the other hand, Albay *et al.* (2003) also demonstrated that the phage assay has higher sensitivity compared to PCR approach, with respect to the BACTEC 460 automated culture system as the gold standard. Although the sensitivity of culture-based methods can approach less than 10 cells per ml of specimen, the fact that they are extremely slow (more than three weeks to obtain results) and prone to contamination limit their reliability.

### 5.1.4 Evaluation of NOA Decontamination of Contaminated Masks Prior to the Phage Assay

The NOA antimicrobial supplement appeared to have broad antimicrobial spectrum against the contaminants sampled on the face mask. The background PFUs detected for some mask samples were shown not to be due to incorporation

of NOA; both clean mask samples processed with and without NOA, respectively, did not yield any plaque. The PFUs in the former were highly likely due to laboratory cross-contamination, as the respective subjects were working with mycobacterial cultures and sampling was performed in the laboratory. However, this occurrence was a blessing in disguise, in which the NOA formulation was shown to be not completely detrimental to mycobacterial cells. Albert *et al*. (2007) have demonstrated that the incorporation of NOA had no significant effect on the number of plaques formed when mycobacterial cells were detected. Hence, the use of NOA antimicrobial supplement in the phage assay will enable higher levels of interpretable results to be obtained during mask analysis, without impairing the assay sensitivity and reliability.

### 5.1.5 Effect of Storage of Face Masks on the Detection of Mycobacterial Cells by the On-mask Phage Assay

After overnight storage at 4 ℃, the mask segments spiked with *M. smegmatis* cells resulted in about 50% decline in PFU counts. Sula *et al.* (1960) demonstrated that *M. bovis BCG* stored at 2-4 ℃ lost 17% of viability after one week and 67% after four weeks. A five-fold drop in PFU counts shown by storage at room temperature compared to storage at 4 ℃ in this study suggested that most mycobacterial cells became non-viable in the former. The great loss in mycobacterial viability was attributed to the change in the relative humidity and the temperature under the condition in which the masks being stored (Brosseau *et al.*, 1997; Tang, 2009).

During storage at 4 ℃, the mycobacterial cells were exposed to various stresses, such as cold, desiccation and nutrient starvation (Tay, 2013). These stresses might have inactivated the mycobacterial cells on masks, rendering them invulnerable to phage infection. Desiccation might have also caused mycobacterial cells to adhere more firmly to the masks, making it difficult for them to be detached from the masks.

Incubation of *M. smegmatis*-spiked mask segments after storage showed a PFU decline to about 600-800 PFUs compared to confluent lysis (about $10^4$-$10^5$ PFUs) yielded from immediately processed mask segments. The *M. smegmatis*-spiked mask segments incubated in M7H9-OADC broth at 37 ℃ post-storage resulted in slightly higher PFU recovery compared to those incubated in *M. smegmatis* culture supernatant treatment (containing Rpfs). Rpfs, the proteins secreted by mycobacteria to stimulate mycobacterial growth (Mukamolova *et al.*, 2010), were initially thought to have positive effects on the phage detection of low-temperature-stored cells, but this was proven otherwise. Temperature-assisted resuscitation showed to be better in this case. Previous studies showed that treatment with mycobacterial culture supernatant had positive effects on mycobacterial culturability; however its effect on phage infectivity remains unknown. The detrimental effect of culture supernatant in this study could be possibly due to the presence of certain metabolites that may be toxic to the *M. smegmatis* cells tested; in this case, the growth phase in which it is harvested from

may play an important role (E.S.G. Cheah, personal communication). Additionally, the duration of treatment with supernatant might be insufficient; this remains to be evaluated (Tay, 2013).

Reponen *et al.* (1999) had performed a similar experiment to test the survivability of mycobacteria on respiratory filters. They tested three possible treatments which include nutrient-free medium, human saliva (to simulate the real scenario) and nutrient broth under optimal storage condition for the mycobacterial growth. They reported that mycobacteria could survive on respiratory filters for about three days if the storage condition was applied, but they were not able to grow.

## 5.2 Evaluation of the Feasibility of a Molecular Assay for Detection of Target Mycobacterial Species in Phage D29 Plaques

### 5.2.1 Feasibility of the Mtb Complex-specific 16S LAMP Assay

Since the phage assay is unable to provide distinction between the Mtb complex and NTM species, the LAMP assay was performed as downstream molecular assay to further identify the lysed target mycobacterial cell within the phage D29 plaque. The LAMP assay was first attempted to assess its specificity by using culture DNA extracts. The LAMP assay assessed targets the 16S rDNA of Mtb complex and calcein fluorescent-based detection was employed. Unfortunately, this approach was hampered with the frequent occurrence of false positive fluorescent signal in the absence of target DNA amplification. This could be due

to unstable binding of mangenese ions to calcein dye molecule at the beginning of the reaction, resulting in the fluorescence of the latter not being quenched. However, this was contradicted by the observation of smeared bands, presumably amplified products, following gel electrophoresis.

Frequent occurrence of false positive fluorescent signal has also been reported by Hong *et al.* (2012) and other researchers performing similar LAMP assays, which was explained to be mainly attributed to cross-contamination. In the current study, there is lack of evidence that cross-contamination was responsible for the false-positive signal observed. In this case, the incorporation of UNG in the LAMP assay reaction would have checked for this (Hsieh *et al*., 2014.)

The WHO Expert Group Meeting ( WHO, 2013) reported that the performance of the LAMP assay can be influenced by the humidity of the environment in which it is performed. In settings with high heat and humidity (as in Malaysia), there is a propensity of non-specific DNA amplification, giving rise to false positive fluorescent signal. Another possible factor reported is the reaction volume, in which the risk of false positivity is reduced by the use of larger volume; in this case, reaction volume of 30-35 µl is recommended.

### 5.2.2 Feasibility of the Conventional IS*1081* PCR Assay

As the LAMP assay did not work, a conventional PCR assay was assessed instead for its potential to detect target mycobacterial DNA in phage D29 plaques. The PCR assay chosen targets the IS*1081* region, a signature sequence of the Mtb complex which is present in 5-6 copies in the genome (van Soolingen *et al.*, 1992), thereby contributing to an increased sensitivity of the assay (Stanley *et al.*, 2007). The initial test with different bacterial species (targets and non-targets) demonstrated the moderate specificity of the IS*1081* PCR assay in detecting the members of the Mtb complex.

Faint 306-bp bands were also observed for the negative controls, which consisted of *S. aureus* and *E. coli* DNA. The IS*1081* sequence was reported to bear some resemblance to that of IS*256* in *S. aureus*, which possibly explains the non-specific amplification of the former (Collins and Stephens, 1991). Meanwhile, the positive PCR amplification for the latter was believed to be due to cross-contamination between reactions. Another negative control, *M. smegmatis*,which is more closely related to the Mtb complex, was not amplified. Nevertheless, the non-specific amplification in the *S. aureus* and *E. coli* reactions should not be a concern since the preceding phage assay specifically detects mycobacterial species. Therefore, this IS*1081* element is of considerable practical value as PCR target in detecting Mtb complex in D29 plaques.

The sensitivity of the IS*1081* PCR assay was also assessed and its detection limit was shown to be as low as about 2.2 fg, which corresponding to 4.4 genome equivalents. Wards *et al.* (1995) also reported similar level of sensitivity (about 1 fg) with their IS*1081* PCR assay using another set of primers. However, the sensitivity of the assay could be lower with plaque DNA extracts, taking into account the possible presence of PCR inhibitors and target DNA loss via the extraction procedure; this remains to be investigated.

### 5.2.3 Detection of Target Mycobacterial Species in Phage D29 Plaques

Attempts to detect target mycobacterial DNA in D29 plaques by the IS*1081* PCR assay were unsuccessful. This could be possibly explained by the inhibition of PCR by interfering substances in the agar plug that were released along with mycobacterial DNA. Indeed, the PCR amplification of *M. bovis* BCG DNA was inhibited in the presence of artificially spiked non-plaque lawn extract.

As previously reported by Bessetti (2007) and Eilert and Foran (2009), calcium ions ($Ca^{2+}$) are common PCR inhibitors that should be removed before the amplification process. In this case, the residual $CaCl_2$ released from the agar plug during plaque DNA extraction could have inhibited the PCR. $Ca^{2+}$ could compete with magnesium ions ($Mg^{2+}$) for binding to the *Taq* polymerase, thereby inhibiting the PCR reaction (Al-Soud and Rådström, 2001). This competitive inhibition is illustrated in Figure 5.1. Therefore, the use of an increased $Mg^{2+}$

concentration to overcome the inhibition effect of $Ca^{2+}$ should definitely be considered in future trials.

Another possible reason for the unsuccessful PCR amplification of the plaque DNA was the high chance of target DNA loss through the extraction procedure, since there should only be one copy of target genome in a positive plaque.

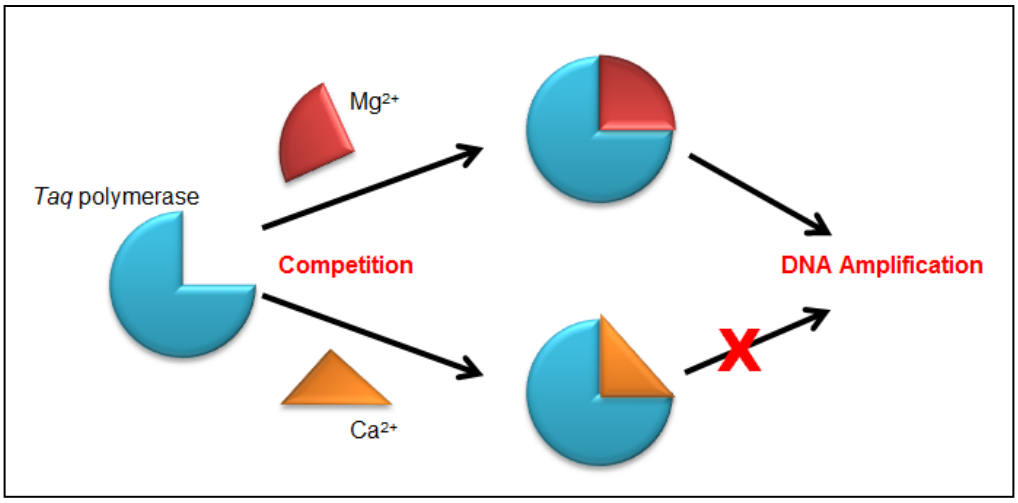


**Figure 5.1:** Competitive inhibition exhibited by calcium ions to inhibit DNA amplification during PCR.

## 5.3 Evaluation of the Sensitivity of Molecular Detection of Mycobacterial Cells in Mask Eluates

In addition to the on-mask phage assay, PCR-based detection of mycobacterial cells in mask eluates was also attempted in this study for comparison. The IS*1081* PCR assay was used in this investigation. However, this method was shown to be

low in sensitivity; the detection limit was $10^7$ CFUs spiked onto the mask, which is $10^4$-$10^5$ folds lower than that for the on-mask phage assay. Even though the fraction of sample volume analyzed for the former is ten folds higher than the latter (50% vs. 5%), this did not translate into better sensitivity. In the study of Cheah (2010), the sensitivity of detection of mycobacterial cells in mask eluates by phage assay has been reported to approach less than 100 CFUs spiked on the mask.

The poor performance of PCR analysis of mask eluates was attributed to the need to elute the mycobacterial cells applied onto the masks, in which poor elution efficiency has been reported for the elution method used (Cheng, 2013). Furthermore, the multiple centrifugation steps involved would have led to significant target cell loss and the simple boiling method used to release DNA from the cells could be inefficient due to the thick and waxy nature of the mycobacterial cell envelope (Käser *et al.*, 2009) and their tendency to clump (Wang and Tay, 1999). The simple boiling method is rapid and easy to perform, however it is normally associated with low yields of DNA (Tarhan *et al.*, 2009). The use of a more vigorous DNA extraction method should be considered, but the multiple steps that are normally involved would lead to further target loss.

## 5.4 Investigation of Levels and Distribution of Culturable Respiratory Aerosols on Mask with Different Expiratory Maneuvers

The recovery of CFUs from the mask samples of healthy subjects, which were exposed to different expiratory maneuvers, revealed that most bacteria were sampled in the middle (M) segments. Cheah (2010) also reported similar observation. This was as expected since the middle segment of the mask covers the wearer's nose and mouth, which are the exit portals for respiratory droplets. Therefore, with further supporting data, selective processing of the frequently contaminated area on the mask (the middle segment in this case) can be considered, which would be more economical with regard to processing time and cost.

The quantities of culturable aerosols generated with different expiratory maneuvers (coughing and talking) by healthy subjects were also briefly assessed. Overall, coughing yielded the highest level of culturable aerosols, which could be explained by the strong expiratory strength and the more vigorous motion of the mucociliary escalator involved in this expiratory maneuver (Park *et al.*, 2010). This could mean that, among active TB cases, coughing is more superior than talking when it comes to the quantity of Mtb-containing respiratory droplets expectorated, and hence a more infectious mode of disease transmission.

Another observation during this investigation was that the male subjects expectorated more culturable aerosols onto the masks than the female ones did. This could be explained by the differences in the maximal expiratory pressure (MEP) and peak expiratory flow (EPF) between the two genders. Freitas *et al.* (2010) demonstrated that MEP and PEF, are 17.2 $cmH_2O$ and 139.5 L/min higher, respectively, in male on average. Therefore, a longer mask sampling duration for female TB cases might need to be considered in order to obtain more comprehensive and comparable data with minimal underestimation.

## 5.5 Future Works

The use of nebulizer to spike mycobacterial cells onto face mask may better simulate the expectoration of Mtb-containing respiratory droplets by active TB cases, than the simple pipetting did. In fact, a nebulizer can produce tiny aerosolized droplets of mycobacterial suspension, which allows them to be evenly dispersed and deposited onto the mask.

The specificity issue with the IS*1081* PCR assay should be addressed. In this case, the incorporation of enhancers (e.g. formamide or dimethyl sulfoxide) into the PCR reaction and/or the use of higher annealing temperature could be considered (Chakrabarti and Schutt, 2001; Hwang *et al.*, 2003). To add specificity to the assay, a restriction fragment length polymorphism (RFLP) analysis can be carried out downstream to further identify the amplified products (Park *et al.*, 2000). If

these attempts were unsuccessful, design of a new set of IS*1081* primers for a more specific PCR assay should be considered. However, it will be more ideal if a LAMP assay can be used as the means of molecular-based detection instead. Use of hydroxynaphthol blue (HNB) fluorescent dye to replace calcein dye in future study could be considered to reduce non-specific amplification and enhances detection sensitivity in reaction (Das *et al*., 2012).

The failure in amplifying target mycobacterial DNA in phage D29 plaques in this study also warrants further investigation. This should focus on the potential of assay inhibition by interfering substances from the phage indicator medium and the efficiency of the DNA extraction protocol in place (Wards *et al.*, 1995). The former, if shown to be true, can be addressed via the simple use of higher concentration of $Mg^{2+}$ in the amplification reaction.

Most importantly, the on-mask phage assay should be attempted on clinical mask samples from TB patients, which could be made possible via collaboration with local hospitals and a research institution that has a Biosafety Level 3 (BSL-3) laboratory. The versatility of the mask sampling approach for other airborne infectious diseases (e.g. influenza and SARS) should definitely be explored in the distant future.

# CHAPTER 6

# CONCLUSION

A mask sampling approach coupled to a combined phage-PCR assay was developed in this study to be used for the assessment of infectivity of TB cases. The phage assay is simple to perform and obviates the need for cumbersome and sophisticated equipment. It can yield results as quick as 48 hours upon specimen receipt (Krishnamurthy *et al.*, 2002). The combination with a PCR assay downstream enables specific detection of Mtb and live/dead differentiation to be achieved simultaneously. The phage assay was demonstrated to be relatively sensitive in detecting mycobacterial cells applied onto masks, with detection limit of 100-1,000 CFUs, which is $10^4$ to $10^5$ folds compared to the method of PCR on mask eluates. The low sensitivity of the latter was attributed to poor elution of mycobacterial cells from the mask and potentially PCR inhibition by mask materials that might be co-eluted.

In order to identity the target mycobacterial species in plaques post-phage assay, a PCR assay targeting the multicopy IS*1081*, a signature sequence of the Mtb complex, was performed downstream. The initial intention to use a LAMP-based assay instead was hampered by the frequent occurrence of non-specific amplification and false positive fluorescent signals, which could be due to the

nature of the detection chemistries used or the influence of environmental factors. The conventional IS*1081* PCR was moderately specific to the Mtb complex. Several attempts with this PCR assay to detect the target mycobacterial species in phage D29 plaques were unsuccessful, possibly due to the presence of PCR inhibitors in the agar plug that might be co-extracted along with the target DNA. In addition, the need to detect a single target genome from a positive plaque stretches the limit of this method. In this case, there is a high tendency of target DNA loss through the extraction process (Cheah, 2010), and therefore a more efficient extraction method is in dire need.

Evaluation with masks worn by healthy subjects that were voluntarily coughed upon showed that the use of the NOA antimicrobial supplement was effective in completely preventing contamination of the phage assay by rapid-growing microbiota. A few studies reported that the incorporation of NOA into the phage assay does not affect its sensitivity and reliability (Albert *et al*., 2007). Further studies with subjects also demonstrated that culturable respiratory aerosols were mainly sampled in the middle segment of the mask, which could support the idea of selective processing of this mask area in order to minimize the processing time and the laboratory resources to be consumed. Besides that, coughing, when compared to talking, yielded higher level of culturable aerosols on average, which could indicate that it is a more vigorous mode of airborne spread of Mtb-containing droplet nuclei among TB cases. A study with the healthy subjects also showed that there was a gender difference in the level of culturable aerosols

expectorated. It was found that this was lower for females on average, and could therefore translate into the need for longer sampling duration in order to obtain reliable, comparative data.

With further improvements and optimizations, the mask sampling approach has great potential to be used for the assessment of TB case infectivity, which could potentially be more reliable than AFB smear status. The data obtained could facilitate better understanding of the nature of TB transmission, particularly on the varied level of infectiousness among different TB cases. In the distant future, the versatility of the mask sampling method with other infectious airborne diseases, such as influenza and SARS, should definitely be explored.

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# APPENDIX

## APPARATUS AND COMSUMABLES

The lists of apparatus and consumables used in this project are as follows:

**Table A1:** List of apparatus and their manufacturers.

| Apparatus | Manufacturer |
|---|---|
| Autoclave machine | Hirayama |
| Centrifuge machines | Hettich Zentrifuged, Sigma-Aldrich |
| Class II biosafety cabinet | Telstar Bio II A |
| Fridge and freezer | Copens Scientific, Pensonic |
| Gel imager | Sygene Bio Imaging |
| Heat block | Major Scientific elite |
| Laminar air flow cabinet | Gelman Class 100 Series |
| Mini centrifuge | Profuge/ Labogene |
| PCR thermocycler | Kratec |
| pH meter | Eutech Instrumental |
| Shaking incubator | Labnet 211DS, N-Biotek |
| Sonicator | Power Sonic 405 |
| Spectrophotometer | Genesys 10S UV-VIS |
| Static incubator | Memmert |
| Vortex mixer | Stuart |
| Water bath | Memmert |

**Table A2:** List of consumables and their manufacturers.

| Consumable | Manufacturer |
|---|---|
| Agar-agar powder | BD |
| Agarose powder | Vivantis |
| Aztreonam | Sigma-Aldrich |
| Basic fuchsin | Merck |
| Calcein powder | Sigma-Aldrich |
| Calcium chloride | R & M Chemicals |
| Crystal violet | Merck |
| DNA ladder | Fermentas |
| dNTPs | Fermentas |
| DreamTaq DNA polymerase and PCR buffer | Fermentas |
| Ethanol | Labmart |
| Ethidium bromide | Bio Basic |
| Ferrous ammonium sulfate (FAS) | UNI-CHEM |
| Ethylenediaminetetraacetic acid (EDTA) | Systerm |
| Glycerol | QR ëC |
| Iodine | Merck |
| Magnesium chloride ($MgCl_2$) | QR ëC |
| Magnesium sulfate ($MgSO_4$) | QR ëC |
| Methylene blue | Systerm |
| Methylformamide | Lab-Scan |
| Middlebrook 7H10 agar powder | BD |
| Middlebrook 7H9 broth powder | BD |
| Mtb CDC1551 genomic DNA | University of Leicester ** |
| Mtb CH genomic DNA | University of Leicester ** |
| Mycobacteriophage D29 | University of Leicester ** |
| *Mycobacterium africanum* genomic DNA | University of Leicester ** |
| *Mycobacterium bovis* BCG Glaxo | University of Leicester ** |
| *Mycobacterium smegmatis* $MC^2155$ | University of Leicester ** |
| Nystatin | Sigma-Aldrich |
| OADC | BD |
| Oxacillin | Sigma-Aldrich |
| Peptone broth powder | BD |
| Safranin | Merck |
| Sodium hydroxide (NaOH) | Merck |
| Syringe filter (0.2 µm) | Pall Life Science |
| Syringes | Cellotron |
| Tris | MP Biomedicals |
| TSA agar powder | BD |
| Tween 80 | Systerm |

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