DEVELOPMENT OF A COMBINED PHAGE AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVID CONTACTED SOILS

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By

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ABSTRACT

DEVELOPMENT OF A COMBINED PHAGE AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVID-CONTACTED SOILS

CHAN CHEW CHIN

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent for Johne's disease (JD) in ruminants. JD is wide spread in many countries and causes tremendous economical loss to the farming sector. Early diagnosis of JD is crucial for disease control and management. However, this was hampered by the lack of reliable screening tests. Therefore, this project was initiated to develop a combined phage and PCR assay to detect MAP in bovid-contacted soils, a potentially important source for JD transmission that has yet to be explored. Soil samples were collected from cow farms, grazing grounds and non-bovid-contacted grounds from around Kampar, Malaysia. Four methods were evaluated for their feasibility in decontaminating soil samples for analysis by the mycobacteriophage amplification assay: 4% NaOH + 3% SDS method, 4% NaOH + 3% SDS + 2% CTAB method, 2% NaOH + BHI broth method, and 1%, 2% or 3% NaOH + NOA (nystatin-oxacillin-aztreonam) method. Their overall decontamination rates in the

phage indicator plates were 75%, 12.5%, 16.7% and 33.4%, respectively. Among these methods, that with 1% NaOH + NOA was deemed the most suitable for the soil types tested, taking into account the balance between decontamination efficacy and ease of processing. The inhibitory effect of soil on the phage assay observed was shown to be soil type-dependent. Out of a total of 11 soil samples tested, two were positive for mycobacteria in which one was from a bovid-contacted ground. The duplex IS*900*/IS*1081* PCR assay, which targets MAP and *Mycobacterium bovis* (another important bovine pathogen), respectively, was used to identify the target mycobacterial species in positive plaques but none were PCR-positive. Further improvement of the molecular test, especially in the plaque DNA extraction method, is needed. The findings from this project highlight the great potential of the combined assay for MAP detection in soil samples.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotation and citation 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 institution.

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(CHAN CHEW CHIN)

APPROVAL SHEET

This project report entitled " **DEVELOPMENT OF A COMBINED PHAGE AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVID CONTACTED SOILS."** was prepared by CHAN CHEW CHIN and submitted as partial fulfillment of the requirement for degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **CHAN CHEW CHIN** (ID No: **10ADB02936**) has completed this final year project entitled "**DEVELOPMENT OF A COMBINED PHAGE AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVID CONTACTED SOILS.**" under the supervision of 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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(CHAN CHEW CHIN)

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LIST OF ABBREVATIONS

BCG	Bacille Calmette-Guérin
BLASTn	nucleotide-nucleotide Basic Local Alignment Search Tool
$CaCl_2$	calcium chloride
CFU	colony-forming unit (s)
CTAB	centrimoniun bromide
dNTP	deoxyribonucleoside triphosphate
dH2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
E-value	expect value
FAS	ferrous ammonium sulfate
IS	insertion sequence
KH2PO4	potassium dihydrogen phosphate
LAMP	loop-mediated isothermal amplification
LJ	Löwenstein-Jensen
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MgSO4	magnesium sulphate
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodium hydroxide
NCBI	National Center of Biology Information
NOA	nystatin-oxacillin-aztreonam

NTC	no-template control
NTM	non-tuberculosis mycobacteria
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
OGC	OADC-glycerol-calcium
PCR	polymerase chain reaction
PFU	plaque-forming unit (s)
rDNA	ribosomal deoxyribonucleic acid
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
T*aq*	*Thermus aquaticus*
UV	ultraviolet

x g	times gravity
bp	base pair
g	gram
ml	milliliter
µl	microliter
cm	centimeter
M	molar
min	minute(s)
nM	nanomolar
s	second
U	unit
µM	micromolar
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Beef and dairy demand in Malaysia is gradually increasing every year, yet our self-sufficiencies for these produce are 25% and 5%, respectively in 2014 (Lim, 2010). Our nation is heavily relying on meat and dairy import from countries like Australia, New Zealand, United States and India. In 2011, a total of 38,705 tonnes of dairy products were imported into Malaysia (Australia Trade Commission, 2013). This has piled up the country's food import bill and therefore the government is trying to address the issue by constantly emphasizing and giving priority to the agriculture sector (Loh, 2002). In the 2014 Budget, as much as 6 billion ringgit was allocated for implementation and development of the agriculture sector. Local poultry and dairy industries are believed to have been spurred ahead, in line with the effort put in and the high market demand. However, besides requirement on financial support and managerial techniques, knowledge on livestock's diseases remains a daunting barrier for many investors and farmers. The foot-and-mouth disease (FMD), one of the most heard virus-induced bovine illnesses, caused an outbreak in Pahang in 2003, despite constant prevention and vaccination nationwide (Senawi, 2012). Bovine brucellosis and hemorrhagic septicemia are other common diseases affecting the local cattle industry (Jabatan Perkhidmatan Veterinar Negeri Pahang, 2014). Yet, the Johne's disease (JD), a

notorious bovine disease spreading worldwide, is still receiving minimal recognition and attention in Malaysia.

JD is a chronic wasting disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The bacterium mainly targets ruminants such as cattle, sheep, goat, and other hoofed animals. Infection of non-ruminants such as rabbits, foxes, mice and birds has also been reported but they rarely develop severe sickness (Johne's Information Center, 2010). Frequent exposure and close spatial arrangement are contributing factors for the high rate of MAP infection on captive livestock. Infected cattle experiences drop in milk production and subsequent death as infection advances, which make JD one of the main economic impacts that incur great loss in both the dairy and poultry industries worldwide. It was being estimated that about $1.5 billion are lost annually due to JD in the United States (Stabel, 1998 cited in Timms, et al., 2011). The loss is not only due to milk reduction, but also premature culling and replacement cost for infected animals. Many dairy-producing nations have revealed varied degree of JD herd-level prevalence among them. In the United States, at least 2.2% of the dairy farm are affected by JD (United States Department of Agriculture, 2012). More than 50% of the cow farms are being estimated with MAP infection in Europe (Botsaris, et al., 2013), 11-17% in the UK and 55% in the Netherland (Muskens, et al., 2000 cited in Food Standards Australia New Zealand, (FSANZ) 2004). As Malaysia is involved in massive beef and dairy importation, the disease can be brought in occasionally and could result in an outbreak in the unknown future. Indeed, many

studies in Europe have reported the detection of viable MAP cells from pasteurized milk and retailed cheese products in (Patel and Nihir, 2011). Survival of MAP cells through standard food processing techniques is suggested to be due to protection from heat by their clumping and aggregation. Post-treatment contamination by MAP is another possibility (Patel and Nihir, 2011).

Control and detection of JD are impeded by asymptomatic infection of the animals. Calves are easily infected via utero transmission or ingestion of MAP-contaminated manure, milk or feed (Johne's Information Center, 2010). No clinical signs develop before the calves reach the age of two or more. Infected adults facilitate the spread of JD to calves by shedding of organisms in milk (Cocito, 1994). In this case, whole herd or flock of animals will be silently infected. Once clinical signs such as intensive weight loss and diarrhea appear, the animal is severely infected and will eventually die in a cachectic form. Moreover, regardless of controversies, the unclear evidence on the zoonotic potential of MAP in causing Crohn's disease (CD) in human has built up the pressure to establish a rapid and reliable detection test for MAP.

Surveillance of JD infection is never easy and ideally should be conducted as early as possible before the disease spreads widely. Culture remains the gold standard to date for the detection of viable MAP cells in bovine specimen such as feces and milk. However, due to the fastidious nature of MAP, approximately 6-16 weeks of

incubation period is required for observable growth on solid media makes it contamination-prone and time-consuming. In addition, the decontamination process involved in the sample pretreatment lowers the sensitivity of MAP isolation by culture. Use of PCR assay targeting MAP signature sequences like the IS*900* is highly sensitive but cannot facilitate live/dead differentiation. Immunoassays are frequently adopted as methods of choice in MAP detection (Timms, et al., 2011), however the specificity and sensitivity are affected by the variable immunological responses seen during different stages of infection (Stanley, et al., 2007). Hence, development of a rapid detection method with good level of sensitivity and specificity is in dire need.

This project served as a preliminary study to determine the feasibility of a combined phage and molecular assay for detection of MAP in local bovid-contacted soils (Kampar, Perak, Malaysia). While feces and milk are given the prime emphasis as the direct transmission media for MAP, soil contaminated with manure from animals possesses equal hazard in spreading the organism. Soil indeed serves as a good reservoir for MAP despite it being an obligate pathogen. Whittington, et al. (2004) has reported the survival of MAP spiked into pasture soil for over a duration of 55 weeks. Salgado, et al. (2011) stated that MAP-contaminated soil represents an obvious risk to grazing livestock for disease infection and could potentially contaminate nearby water source after a downpour. Ingestion of feed contacted with contaminated soil also provides another route of MAP exposure for cattle.

In this project, soil samples collected from different areas were subjected to various decontamination pretreatments to determine the most suitable treatment for the downstream phage assay. An optimal decontamination treatment must strike a balance between sufficient suppressive effect on competing contaminants and preservation of the viability of target organisms. The decontamination treatments evaluate in this study include: 4% (w/v) NaOH + 3% (w/v) SDS (Ng, 2012); 4% (w/v) NaOH + 3% (w/v) SDS + 2% (w/v) CTAB (Parashar, et al., 2004); 2% (w/v) NaOH + ½ brain heart infusion (BHI) broth; and 1%, 2%, 4% (w/v) NaOH + NOA. While the phage assay works on the basis of infection of MAP cells by mycobacteriophage with subsequent cell lysis and plaque formation on a lawn of sensor cells, the issue with the broad mycobacterial host range of the phage D29 has to be resolved by incorporation of a molecular assay downstream to identify the target mycobacterial species in the resulting plaques. The IS*900* sequence was used as the amplification target due to its specificity to MAP and the presence of 10-20 copies in its genome; the latter would enhance the assay sensitivity (Enosawa, et al., 2003).

The objectives of this projects were:

- To develop a combined phage and molecular assay for detection of viable MAP in bovid-contacted soils.
- To evaluate the effectiveness of different decontamination methods with local soil samples for the phage assay and culture.
- To assess the feasibility of the phage assay in detecting viable mycobacterial cells in soil samples.
- To isolate and preliminary identify the mycobacterial species present in local soil.

CHAPTER 2

LITERATURE REVIEW

2.1 The Genus *Mycobacterium*

2.1.1 General Features

Mycobacterium is the single genus within the family *Mycobacteriaceae*, under the order Actinomycetales. The prefix *myco* suggests the fungus-like and the occasional exhibition of filamentous growth by the organisms of this genus (Tortora, et al., 2010). They are generally described as rod-shaped (slightly curved or straight), aerobic, acid-fast, and non-motile. A high guanine and cytosine(G+C) base content of 62-70% in the genome is another characteristic of *Mycobacterium* (Cocito, et al., 1994).

Mycobacterium is one of the members of the CMN group, which consists of *Corynebacterium, Mycobacterium* and *Nocardia.* Moreover, genera *Rhodococcus*, *Gordona* and *Tsukamurella* are also included in the CMN group.. Members of CMN group are the only microorganisms with the ability to synthesize mycolic acids. Nonetheless, the number of carbon atoms and pyrolysis esters of the mycolic acids and G+C content in DNA, make differentiation among various CMN members possible (Rastogi, et al., 2001).

The multilayered cell wall of mycobacteria resembles more of that in Gram-negative bacteria than the single-layered cell wall in Gram-positive bacteria (Scherr and Liem, 2009). The mycobacterial cell wall contains high proportion of lipids (approximately 30-40% of total weight) (Rastogi, et al., 2001), and is composed of a mycolic acid anchored arabinogalactan outer layer coating the inner peptidoglycan and plasma membrane. Mycolic acids take a vital role in contributing to mycobacterial growth, survival, pathogenicity, and physical and chemical resistance. They form an outer waxy and hydrophobic layer that permits very low permeability of substances into the cell. Nutrient uptake progresses very slowly through this layer, giving rise to the slow growth of mycobacteria, which sometimes takes weeks to establish colonies on solid media (Tortora, et al., 2010). The intrinsic antibiotic resistance of mycobacteria is not solely a factor of the large proportion of unusual lipids in the cell wall, but also due to its highly organised and compact arrangement of the lipids (Rastogi, et al., 2001).

Mycobacteria are previously known to be non-sporulating. However, various evidences are challenging the existence of sporulation in the genus *Mycobacterium*. These include the transformation of *M. tuberculosis* cell into spherical or ovoid cell with thickening cell wall and altered chemical characteristics (Scherr and Liem, 2009); the presence of the ubiquitous actinomycete-specific family of WhiB-like proteins that have role in sporulation (Scherr and Liem, 2009); and the presence of spore-like particles with endospore

features in old cultures of *M. marinum* and *M. bovis* BCG (Ghosh, et al., 2009 cited in Singh, et al., 2010).

2.1.2 Classification of *Mycobacterium* spp.

The categorization of mycobacterial species is made convenient on the basis of their pathogenicity or their growth rate and pigmentation. Clinically, mycobacteria are grouped into obligate pathogens which include *M. tuberculosis, M. leprae, M. bovis;* opportunistic pathogens which include *M. avium, M. simiae, M. xenopi*; and rare pathogens such as *M. smegmatis* and *M. phlei* (Rastogi, et al., 2001). Another similar classification based on mycobacterial pathogenicity divides them into two major groups of tuberculous and non-tuberculous mycobacteria(NTM), with *M. leprae* standing alone as the causative agent of leprosy. NTM include all the environmental opportunistic pathogens and non-pathogenic mycobacterial species.

Runyon (1959 cited in Rastogi, et al., 2001) classified NTM into four groups based on their growth rate and pigmentation, which ranges from pale peach to deep orange color. Groups I to III are slow growers which are further divided into photochromogenic, scotochromogenic, and non-chromogenic species, respectively. Group I photochromogenic species acquire pigmentation in their colonies upon exposure to light, these include *M. kansasii* and *M. marinum.* Group II scotochromogenic species, which exhibit pigmented colonies in the presence or absence of light, include *M. scrofulaceum* and *M. gordonae.* Group III non-

chromogenic species produce no pigmented colonies and include *M. avium*, *M. intracellulare,* and *M. xenopi*. Group IV consists of rapid growers, which include *M. fortuitum* and *M. chelonae*. While slow growers require one or more weeks to develop colonies, rapid growers establish colonies within seven days of incubation.

2.2 *Mycobacterium avium* subsp. *paratuberculosis*

MAP is the etiological agent for Johne's disease. It is a very slow growing and fastidious organism, requiring 6-16 weeks for colonies to develop in culture (Stanley, et al., 2007). MAP has a genome size of 4.4-4.7 Mb in size with G+C content of 66-67% (Cocito, et al., 1994). It is one of the members of the *M. avium* complex (MAC), which also includes *M. avium* subsp. *avium, M. avium* subsp. *silvaticun, and M. avium* subsp. *hominissuis* (Rindi and Garzelli, 2014); high degree of DNA similarity was found among the members of the complex. For instance, MAP shares over 99% DNA homology and identical rRNA sequences with *M. avium* (Manning and Collins, 2001). Discrimination of MAP from other mycobacterial species is made possible by its mycobactin-dependent growth *in vitro*, main host range targeting ruminants, and the presence of 10-20 copies of insertion sequence IS*900* in its genome (Harris and Barlette, 2001; Manning and Collins, 2001; Rastogi, 2001).

Two major strains which partially reveal the host specificity of MAP are identified and termed as: Group I or strain S isolated from sheep; Group II or strain C

isolated from cattle. In recent years, a sub-type of group I was isolated from bison in North America and was classified under Group III or strain B (Whittington, et al., 2001 cited in Rindi and Garzelli, 2014). However, the correlation between strain type and host prevalence is not profound (Dimareli-Malli, et al., 2013). Strain C is the most commonly found and prevalent among wildlife. Strain B recovered from Indian and North American bison demonstrated differences in their genome (Rindi and Garzelli, 2014). To permit differentiation among the MAP strains, the differences in their host range and pathogenicity and the polymorphisms found in IS*900* and IS*1311* were based upon (Dimareli-Malli, et al., 2013; Rindi and Garzelli, 2014).

MAP exhibits strong resilience in the environment despite its obligate pathogen nature. Beumer, et al. (2010 cited in Chiodini and Chamberlin, 2011) reported the detection of MAP in 81% of drinking water and biofilm samples in the United States. Survival duration of 152-246 days of MAP was observed in the fecal samples kept outdoor by Manning and Collins (2001). Besides, Moravkova, et al. (2012) reported a 2% recovery rate of MAP from an infected herd after a year of mechanical cleaning and disinfection.

2.3 Johne's Disease

2.3.1 General Introduction on Johne's Disease

Johne's disease, also known as paratuberculosis, is a chronic intestinal enteritis caused by MAP in ruminants. First reported in 1895, its identity was only clearly defined in 1915 when F.W. Trowt successfully cultivated MAP and produced experimental enteritis from it, which fulfilled the Koch's postulates (Harris and Barletta, 2001). Over the years, JD remains a public concern due to its prevalence and significant effect on the agriculture sector across the world. The latter includes reduction in milk production and meat quality, premature culling of infected animals and the extra veterinary costs incurred. Despite receiving intense monitoring, the levels of JD prevalence documented in various countries are likely to be under-estimated due to the occurrence of subclinical infection and the lack of reliable diagnostic tools.

2.3.2 MAP Transmission and Development of Johne's Disease

Invasion of MAP starts at the host lymphoid tissues via the M cells (Manning & Collins, 2001). The bacterium is then phagocytosed by macrophages and multiplies intracellulary. Production of cytokines leads to the appearance of intestinal granulomas, and this inflammatory process eventually results in thickening and loss of function of the intestinal wall, leading to malnutrition in infected animals (Harris and Barletta, 2001).

Transmission of MAP manifests itself in many forms but the fecal-oral route is the major pathway for the spread of the disease. Shedding of the bacteria by silent carriers creates a highly contaminated environment and raises the exposure frequency of animals to MAP. Young animals or calves are most easily succumbed to infection, attributed to their defective immune system and limited capability in fighting intracellular pathogens (Manning & Collins, 2001). Chronic diarrhea and weight loss are the main symptoms of JD. Clinical signs usually appear after an incubation period of years. In cattle, clinical signs are rarely seen before the infected animal reaches the age of 2-6 years. During advanced stage of the disease, animals become emaciated and threatened to death by dehydration and malnutrition.

2.3.3 Management and Control of Johne's Disease

Due to the inconspicuous nature of JD in the early stage, alerts prompting an early diagnosis of the disease are usually neglected. Prevention and control of JD rely greatly on professional management. Vaccination, albeit its efficiency on disease eradication is not promising, provides partial protection to animals by lowering the shedding of MAP in feces (Harris and Barletta, 2001). Treatment is not a practical means, as specific drugs effectively against the disease is still under development. Calves should be delivered in clean maternity pens and kept away from suspected infected animals and environment. It is ideal to apply restricted control to the nursing of young calves to prevent disease transmission through direct ingestion

of MAP from manure-contaminated udder or milk from infected maternal animals. Good manure management and disposal is an essential requirement to prevent frequent exposure to the disease and avoid excessive accumulation of pathogens in the surrounding environment. Intense scrutiny and assessment should be performed on replacement animals before introducing them into the herd. Culling should be conducted as soon as possible once the animal is diagnosed with the disease. Periodic herd testing, which usually involve serological diagnosis and fecal sample culture, is important for early detection of MAP that would then permits prompt action to be taken.

2.4 Crohn's Disease

CD is an inflammatory bowel disease affecting humans. Major clinical manifestations include abdominal pain, chronic diarrhea and weight loss. Although CD is classified as a benign disease with low mortality rate, it is associated with high morbidity and affects the quality of life of those infected. (Carvalho, et al., 2012). Widespread of CD in the United States, UK, and Northern Europe leads to increasing public awareness and pressure on the disease.

Due to the striking clinical and histopathological similarities between JD and CD, MAP is suspected as the etiological agent for CD. Indeed, the emergence of CD was accompanied by the widespread of JD in the 19th and 20th centuries (Hermon-Taylor, 2009). The hypothesis of the zoonotic potential of MAP was

built on the isolation of MAP from the tissues of CD patients and the detection of MAP DNA in their blood and serum (Juste, 2012). In the study by Carvalho, et al. (2012), sera of CD patients were tested positive for specific antibiotics against MAP via serological test. Cases of antimycobacterial drugs has curing CD patients have also been reported (Carvalho, et al., 2012). All these evidences have contributed to the association of MAP with CD. Cattle products such as milk, cheese and meat are believed to be facilitating the transmission of MAP from cows to humans.

On the contrary, the dispute on the correlation between MAP and CD is ongoing. A review conducted by FSANZ (2004) showed that epidemiological and demographic data did not express a positive corresponding increment of CD incidence in either areas with high JD prevalence or susceptible groups like dairy farmers. Furthermore, the isolation and recovery of viable MAP from CD patients have been inconsistent, and the antimycobacterial drugs prescribed do not specifically target MAP or any mycobacterial species but have broad spectrum against many pathogens.

In general, a consent which suggests that CD is a multi-factorial disease is grounded. MAP is not the only agent responsible for the infection, but seems to be associated with it (FSANZ, 2005; Carvalho, et al., 2012). With no sufficient evidence to ascertain or disprove the possible link between MAP and CD infection,

simply dismissing the potential zoonotic nature of MAP is a bold move not worth to be taken.

2.5 Detection Techniques for MAP in the Laboratory

2.5.1 Acid-fast Smear Microscopy

Mycobacterial cells are poorly stained using the Gram staining technique due to the high lipid content in their cell wall and if stained, produce high staining variable. Microscopy screening for acid-fast bacilli is a preliminary move for rapid revelation of MAP presence in clinical samples. Acid-fastness is a characteristic that describes the retention of the carbolfuchsin dye by the bacteria even after acid-alcohol treatment. Acid-fast bacteria will appear red while non-acid-fast bacteria will be stained blue by the counterstain methylene blue. Two common acid-fast staining techniques are available, the Ziehl-Neelsen and Kinyoun methods. The former is also known as hot staining while the latter is called cold staining. The only difference between these methods is that Ziehl-Neelsen uses heat to push the dye into the cell, while Kinyoun method replaces the heating step by using higher concentration of the carbolfuchsin dye.

Apparently, microscopy screening has little practical use because it does not differentiate MAP from other environmental mycobacterial species and can facilitate live/dead differentiation.

2.5.2 Culture

Isolation and cultivation of MAP from clinical samples is the most definitive method in diagnosing for JD and is an essential step for application of further confirmatory molecular tests (Dimareli-Malli, 2013). Suitable media for MAP culture include the Herrold's egg yolk medium (HEYM), Löwenstein-Jensen (LJ) slant, Middlebrook 7H9 broth, and Middlebrook 7H10 and 7H11 solid media. Addition of mycobactin into the medium is necessary for *in vitro* isolation of MAP.

The long doubling time of MAP for colony establishment has impeded a rapid diagnosis via this method. Automated radiometric liquid culture system is invented to address this problem. This system monitors the synthesis of ^{14}C-labelled product of bacterial metabolism to indicate growth (Manning & Collins, 2007). This method is more sensitive and rapid since the metabolic product is detectable before the target mycobacteria form visible colonies on solid media. However, the liquid culture system has its own limitation. Whittington, et al. (1998) pointed out that identification of MAP is difficult in liquid culture since colony morphology and mycobactin dependency are not observable, and cross-contaminating microbes are not distinguishable.

2.5.3 Immunological Tests

Immunological assays are developed to detect both humoral and cell-mediated immune responses elicited during MAP infection Three standard assays are commonly employed, namely the agar gel immunodiffusion assay, the complement fixation assay, and the enzyme-linked immunosorbent assay (ELISA). Among these assays, ELISA gives the highest sensitivity and specificity, which is more analytically valuable (Manning & Collins, 2001). However, the use of immunological tests for diagnosis is often problematic. Sensitivity of these tests is dependent on the stage of disease progression, and they only exhibit highest level of sensitivity at the later stage of JD when antibody production is intensified along with higher bacterial load (Timms, et al., 2011). Cross-reactions by antibodies of related bacteria of the CMN group, especially the genetically resembled *M. avium*, has impaired diagnostic accuracy (Cocito, et al., 1994). Moreover, vaccinated animals causes interference in immunological tests. Effort has been invested to enhance the specificity of these tests. For instance, a perabsorption step with *M. phlei* antigens is an improvement made in the ELISA assay to eliminate non-specific antibodies against environmental mycobacteria that can potentially cross-react with MAP antigens (Harris and Barletta, 2001).

2.5.4 Molecular-based Identification Methods

Discovery of subspecies-specific genetic sequences permits the identification and differentiation of MAP from other mycobacteria via the conventional polymerase

chain reaction (PCR). The most prominent signature sequence for MAP is IS*900*, which is an insertion sequence related to transposition found uniquely in its genome. Other target genes include the *hsp*X gene, 16SrDNA, F57, ISMAP02 and *hsp*65 (Harris and Barletta, 2001; Timms, et al., 2011). Besides conventional PCR, a new approach termed the loop-mediated isothermal amplification (LAMP) assay was evaluated by Enosawa, et al. (2003) for its usefulness in IS*900*-based detection. This technique was developed by Notomi and collegues (Enosawa, et al. 2003) and offers many advancements compared to conventional PCR, which encompass short amplification duration (usually within 1-2 hours), elimination of the need for thermal cycling as in normal PCR, and closed-tube visualization of amplified DNA via incorporation of a fluorescent dye in the reaction (Enosowa, et al., 2003). Despite the convenience and rapidity of PCR-based detection, its relevance is compromised by difficulty in removing PCR inhibitors from sample extracts, non-specific priming, and lack of live-dead information.

2.6 Mycobacteriophage Amplification Assay

The mycobacteriophage amplification assay is a new, low cost and rapid assay which has been commercialised as a diagnostic tool to detect *M. tuberculosis* in sputum specimens (Stanley, et al., 2007). This assay relies on the infection and replication of mycobacteriaphage to indicate the presence of viable cells in a specimen. In general, this assay is started with mixing of sample with the mycobacteriophage D29 to allow infection. A virucide, ferrous ammonium sulfate

(FAS), is then added for chemical inactivation of exogenous phages, while the phages that have entered the host cells are protected. To detect the infected host cells, sample is mixed with fast-growing sensor cells, *M. smegmatis*, and incubated to form bacterial lawn. The protected progeny phages released as a result of host cell lysis will infect proximal sensor cells and form plaques. Each plaque formed arise from one viable host cell, and hence the analysis is said to be quantitative. The phage assay receives much attention not only because of its rapid turnaround time and ability of live-dead differentiation, but also the potential to be used for the detection of many different mycobacterial species. Due to the broad host range of D29, the phage can infect many mycobacterial species other than *M, tuberculosis*. Stanley, et al. (2007) is the first to report the use of the phage assay to detect MAP in milk samples. A molecular identification test was incorporated downstream to increase the specificity of the phage assay in their study. In this case, each plaque contains a target genome, which allows for genetic identification.

CHAPTER 3

METHODOLOGY

3.1 Experimental Design

The overall experimental design of this project is shown in Figure 3.1

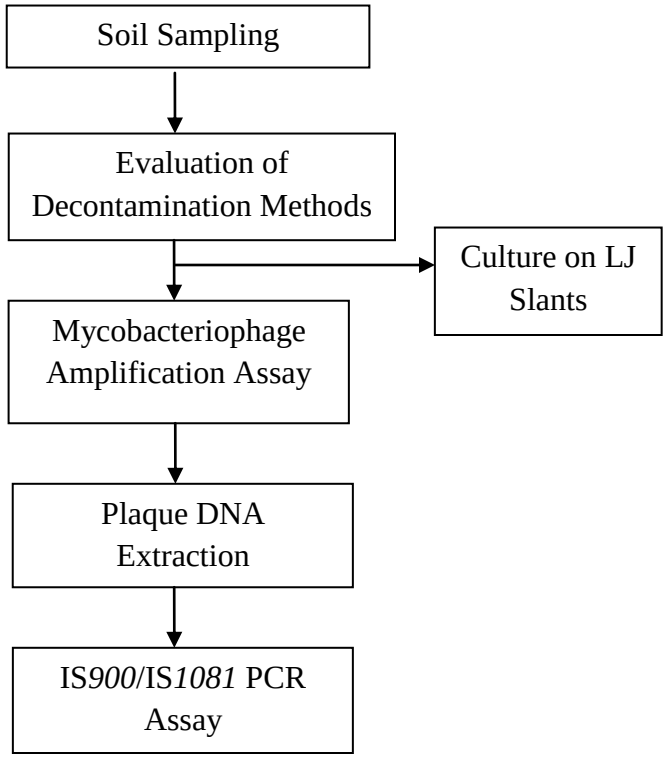


Figure 3.1: Overview of the experimental design for this project.

3.2 Apparatus and Consumables

The apparatus and consumables utilized in this project are listed in Appendix A.

3.3 Preparation of Culture Media

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

3.3.1 Half-strength Brain Heart Infusion Broth with 2% (w/v) NaOH

Half-strength brain heart infusion broth **(**BHI) was prepared freshly before use by dissolving 9.25 g of BHI powder into 500 ml of dH_2O. The autoclaved medium was supplemented with filter sterilized 2% (w/v) NaOH and 0.18% (w/v) saline solution through a 0.22-μm syringe filter.

3.3.2 Löwenstein-Jensen Slants

LJ base was first prepared by mixing 9.325 g of LJ powder and 3 ml of glycerol in 150 ml of dH_2O. The mixture was stirred and boiled until fully homogenized before autoclaving. All further steps were performed in the class 2 biosafety cabinet. Eggs were surface sterilized with 70% (v/v) ethanol and mixed in a sterile beaker. A volume of 250 ml of egg mixture was filtered through a sterile gauze and added to the LJ base. The mixture was stirred on a magnetic stirrer to ensure

homogenous mixing. A volume of 8 ml of the mixture was poured into a sterile 30-ml universal tube each and inspissated in a slanting position at 85℃ for 2 hours. Solidified LJ slants were incubated at 37℃ overnight to check for sterility before storage at 4℃.

3.3.3 Middlebrook 7H9-OADC Broth

Middlebrook 7H9 (M7H9) broth was first prepared by mixing 0.94 g of M7H9 powder and 0.5 g of glycerol in 180 ml of dH_2O. The autoclaved broth was left to cool to room temperature and then supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) before use.

3.3.4 Middlebrook 7H9-OADC-Tween Broth

M7H9 broth was prepared as before (Section 3.3.3) and supplemented with 10% (v/v) OADC and 0.05% (w/v) Tween 80 before use.

3.3.5 Middlebrook 7H9-OGC Broth and Agar

M7H9 broth was prepared as before (Section 3.3.3), while M7H9 agar was prepared with the addition of 3 g of agar powder. Both the broth and agar were supplemented with 10% (v/v) OADC and 1 mM $CaCl_2$ before use.

3.3.6 Middlebrook 7H10 agar

Middlebrook 7H10 agar was prepared by mixing 7.6 g of 7H10 powder and 2.5 g glycerol in 360 ml of dH_2O. The autoclaved medium was left to cool to 55 ℃ and supplemented with 10% (v/v) OADC before use.

3.3.7 Tryptic Soy Agar

Tryptic soy agar (TSA) was prepared by dissolving 40 g of TSA powder in 1000 ml of dH_2O.

3.4 Preparation of Reagents

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

3.4.1 Cetrimonium Bromide Solution, 2% (w/v)

CTAB, cetrimonium bromide solution was prepared by dissolving 2 g of CTAB powder in 100 ml of dH_2O on a magnetic stirrer. The solution was then filter sterilized through a 0.22-µm membrane.

3.4.2 Ferrous Ammonium Sulfate, 50 mM

FAS was freshly prepared before use by dissolving 0.196 g of FAS powder in 10 ml of dH_2O. The solution was then filter sterilized through a 0.22-µm syringe filter.

3.4.3 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) in 200 ml of dH_2O.

3.4.4 Mycobacteriophage Buffer

Mycobacteriophage buffer was prepared by mixing 2 ml of 1 M Tris-Cl (pH 7.6), 20 ml of 1M NaCl, 2 ml of 1M $MgSO_4$, and 0.4 ml of 1 M $CaCl_2$ with dH_2O to a final volume of 200 ml.

3.4.5 NOA Antibiotic Supplement

Nystatin-oxacillin-aztreonam (NOA) antibiotic supplement was prepared by mixing 0.6 mg/ml of oxacillin (dissolved in dH_2O), 3 mg/ml of nystatin (dissolved in methanol), and 9 mg/ml of aztreonam (dissolved in formaldehyde-methanol 1:1 mixture) in equal proportion.

3.4.6 Phosphate Buffer, 0.1 M

Solution of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 were prepared. The 0.1 M phosphate buffer was prepared by mixing 49 ml of 0.2M Na_2HPO_4 with 51 ml of 0.2 M KH_2PO_4. It was then filter sterilized through a 0.22-µm membrane.

3.4.7 Sodium Dodecyl Sulfate Solution, 3% (w/v)

Sodium dodecyl sulphate (SDS) solution was prepared by dissolving 3 g of SDS powder in 100 ml of dH_2O on a magnetic stirrer. The solution was then filter sterilized through a 0.22-µm membrane.

3.4.8 TAE Buffer

TAE buffer was prepared by mixing 242 g of Tris base powder, 57.1 ml of glaciel acetic acid and 37.2 g of ethylenediaminetetraacetic acid (ETDA) powder to a final volume of 1 liter dH_2O. the buffer was diluted 50 times before use.

3.4.9 Tween 80, 10% (w/v)

Tween 80 solution was prepared by dissolving 10 g of Tween 80 in 100 ml of dH_2O and then warmed at 40℃ for 30 min. The solution was filter sterilized through a 0.22-µm membrane and stored at 4℃ away from strong light.

3.4.10 Ziehl-Neelsen Staining Reagents

3.4.10.1 Acid-Alcohol Decolorizer

The decolorizer was prepared by mixing 6 ml of concentrated HCl with 194 ml of 95% (v/v) ethanol.

3.4.10.2 Carbolfuchsin Stain

An amount of 0.3 g of basic fuchsin was dissolved in 10 ml of 95% (v/v) ethanol. A volume of 5 ml molten phenol crystal was mixed with 95 ml of dH_2O. The two solutions were mixed together and stirred on a magnetic stirrer for 1-2 hours and left to settle for three days. The stain was filtered through a filter paper before use.

3.4.10.3 Methylene Blue Counterstain

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

3.5 Soil Sampling

Soil samples were collected using a soil sampling tube at the depth of 3 cm beneath the ground surface. For each sampling location, soil samples were taken

from three spots, which were 3-5 m apart, and mixed together in a single sampling bag. All soil samples were stored at room temperature until processing.

3.5.1 Decontamination of Soil Samples

All soil samples were processed in the same manner before the resulting pellets were subjected to different decontamination treatments. First, 5 g of soil was suspended in 20 ml of dH_2O in a 50-ml Falcon tube and shaken manually for 60 s. The suspension was then centrifuged at 600 x g for 5 min at 4 ℃. A volume of 10 ml of the resulting supernatant was transferred to a new Falcon tube and centrifuged at 8000 x g for 15 min at 4 ℃. The resulting supernatant was discarded and the pellet was subjected to treatments I, II, III, and IV (Sections 3.5.1.1 to 3.5.1.4, respectively).

3.5.1.1 Treatment I

This treatment was based on the method of Ng (2012). The resulting pellet was resuspended in 20 ml of a mixture of 3% (w/v) SDS and 4% (w/v) NaOH. The suspension was separated into two equal portions and incubated for 15 and 30 min, respectively, at room temperature. After incubation, the suspension was centrifuged at 8000 x g for 15 min at 4 ℃. The resulting supernatant was discarded and the pellet was washed twice in 20 ml of dH_2O, and then centrifuged at 8000 x g for 15 min at 4 ℃. Finally, the resulting pellet was resuspended in 1.5 ml of

M7H9-OGC and incubated at 37 ℃ for 18-20 hours to resuscitate chemically stressed mycobacterial cells.

3.5.1.2 Treatment II

This treatment was based on the method of Parashar, et al., (2004) with modifications. The resulting pellet was first processed as in Section 3.5.1.1 until the washing step, and the washing was only performed once. The resulting supernatant was discarded and 20 ml of 2% (w/v) CTAB solution was then added to resuspend the pellet. Treatment durations in CTAB solution for portions A and B were 5 and 15 min, respectively. The suspension was then centrifuged at 8000 x g for 15 min at 4 ℃. The resulting pellet was washed in 20 ml of dH_2O, and then centrifuged as before. Finally, the resulting pellet was resuspended in 1.5 ml of M7H9-OGC and incubated at 37 ℃ for 18-20 hours.

3.5.1.3 Treatment III

A volume of 10 ml of a mixture of half strength BHI broth and 2% (w/v) NaOH was used to resuspend the pellet. The resulting suspension was incubated at 37 ℃ for 18-20 hours and then centrifuged at 8000 x g for 15 min at 4 ℃. The resulting pellet was resuspended in 1.5 ml of M7H9-OGC and then subjected to the phage assay.

3.5.1.4 Treatment IV

The pellet was treated with 3 ml of 1% (w/v) NaOH solution for 15 min. Then, 30 ml of 0.1 M phosphate buffer was added to neutralize the NaOH. The mixture was centrifuged at 8000 x g for 15 min at 4 ℃. The resulting pellet was resuspened in 1.5 ml of M7H9-OGC supplemented with 1.5 µl of NOA and incubated at 37 ℃ for 18-20 hours. The concentrations of 2% (w/v) and 4% (w/v) NaOH were also evaluated.

3.6 Cultivation of Mycobacteria

3.6.1 Isolation of Soil Mycobacteria on LJ Slants

A volume of 100 µl of pre-resuscitation suspension from each decontamination treatment (Sections 3.5.1.1 to 3.5.1.4) was inoculated onto a LJ slant. All LJ slants were incubated at 37 ℃. Physical appearance of and bacterial growth on the slants were observed on daily basis until they fully collapsed; the latter only applied to contaminated slants.

3.6.2 Cultivation of *M. smegmatis*

From the glycerol stock, *M. smegmatis* was cultured by either streaking on M7H10 agar or inoculating 1 ml of the stock into 5 ml of M7H9-OADC-Tween broth in a 50-ml Falcon tube. The broth culture was incubated at 37 ℃ with agitation at 200 rpm for 3-5 days. This starter culture was subcultured into 25 ml

of M7H9-OADC-Tween broth in a 100-ml conical to OD_{580nm} of 0.05. *M. smegmatis* took 2-3 days to develop noticeable colonies on M7H10 agar. In the exponential phase, *M. smegmatis* doubles approximately every 3 hours (Cheah, 2010).

3.6.3 Cultivation of *M. bovis* BCG

From the glycerol stock, *M. bovis* BCG was cultured either streaking on M7H10-OADC agar or inoculating 1 ml of the stock into 5 ml of M7H9-OADC-Tween broth in a 50-ml Falcon tube**.** The broth culture was incubated static at 37℃ for 7-10 days. This starter culture was subcultured into 25 ml of M7H9-OADC-Tween broth in a 100-ml conical flask to OD_{580nm} of 0.05**.** *M. bovis* BCG has a generation time of 24 hours and its culture requires 5-6 days to reach the exponential phase (Cheah, 2010).

3.6.4 Long-term Storage of Soil Mycobacterial Isolate

Stock culture was prepared by emulsifying 2-3 loopful of colonies in 1 ml of a 1:1 mixture of M7H9 broth and 65% (v/v) glycerol solution in a 2-ml cryovial. All glycerol stocks were stored at -80℃.

3.7 Quantification of Mycobacterial Culture

3.7.1 Measurement of Optical Density of Mycobacterial Culture

The optical densities of liquid mycobacterial cultures were measured using a spectrophotometer at the wavelength of 580 nm (OD_{580nm}). Dense cultures ($OD_{580nm} > 1$) were diluted ten-fold in M7H9 broth prior to measurement.

3.7.2 Enumeration of Colony-forming Units

Ten-fold serial dilution was carried out for liquid culture using M7H9 broth as diluent in 1.5-ml Eppendorf tubes. For each dilution, three drops of 20-µl suspension each were plated on M7H10 agar. There were four sets of dilutions on each agar plate. The agar plate was left to dry under laminar air flow and then incubated at 37 ℃ until noticeable colonies formed. The dilution that produced 10-100 colonies was used to calculate the CFU/ml of the test culture.

3.8 Smear Microscopy

3.8.1 Ziehl-Neelsen Staining

For solid culture, a loopful of bacterial colonies was emulsified in a drop of dH_2O on a microscope slide. For liquid culture, a loopful of culture suspension was directly spread onto the slide. The smear was left to air dry and then heat fixed. Then, the smear was flooded with the carbolfuchsin dye and heat was applied until

steam appeared. The smear was left to stain for 5 min. Excess dye was rinsed away with tap water, and the smear was decolorized with acid-alcohol. The slide was rinsed again and then flooded with the counterstain methylene blue and left for 30 s. Following a quick rinse with tap water, the slide was blotted dry and then examined under oil immersion at 1000x magnification.

3.8.2 Gram Staining

Culture smear was prepared as in Section 3.8.1. The crystal violet dye was applied to stain the smear for 1 min. After rinsing with tap water, the smear was flooded with the iodine solution for another minute. The slide was rinsed again with tap water, and the stained smear was then decolorized with alcohol for less than 10 s. Following rinsing, the smear was flooded with the counterstain safranin for 30 s. The slide was rinsed and blotted dry, and then examined under oil immersion at 1000x magnification.

3.9 Mycobacteriophage D29

3.9.1 Preparation of Phage Indicator Plates from *M. smegmatis* Culture

M. smegmatis was cultured in M7H9-OADC broth at 37℃ for 24 hours with agitation at 200 rpm from OD_{580nm} of 0.05. In a 50-ml Falcon tube, 9 ml of molten M7H9-OGC agar was added and maintained at 55℃ in a water bath until use. The *M. smegmatis* culture was left for 30 min to sediment bacterial clumps. Then, 1 ml

of the upper homogenous culture suspension was added to the molten agar, mixed well by inversion and then poured into a Petri dish. Solidified plate were left to dry under laminar air flow prior to use.

3.9.2 Propagation of Phage D29

A volume of 100 µl of phage D29 suspension (approximately 4 x 10^3 PFU/ml) was spread onto a phage indicator plate. The plate was incubated at 37 ℃ for 24 hours. A volume of 10 ml of M7H9-OGC broth was then pipetted onto the plate and incubation was performed as before. After incubation, 5 ml of the plate suspension was filtered twice with 0.22-µm syringe filters. The resulting phage D29 suspension was distributed in 1-ml aliquots into 1.5-ml Eppendorf tubes and then stored at 4 ℃ away from light.

3.9.3 Enumeration of Phage D29

Ten-fold serial dilution of phage D29 suspension was performed in MP buffer in 1.5-ml Eppendorf tubes. For each dilution, three drops of 10-µl phage suspension each were plated onto a phage indicator plate. There were four sets of dilutions on each agar plate. The plates were left to dry under laminar air flow and then incubated at 37 ℃ overnight. The dilution that produced 10-100 plaques was used to calculate the PFU/ml of the test suspension.

3.9.4 Phage Amplification Assay

A volume of 1 ml of test sample was mixed with 500 µl of 10^8 PFU/ml phage D29 in a Bijoux tube. The negative control was performed with 1 ml of M7H9-OGC broth. The tube was gently swirled to mix its content and then incubated at 37℃ for 1 hour for phage infection. A volume of 300 µl of 50 mM FAS was then added to the mixture and vortexed to inactivate exogenous phages. Then, 1 ml of the reaction mixture and 1 ml of *M. smegmatis* lawn culture (prepared as in Section 3.9.1) were added into 9 ml molten M7H9-OGC agar in a 50-ml Falcon tube. The tube content was mixed well by inversion and then poured into a Petri dish. The plate was incubated at 37℃ overnight for plaque formation. The resulting plaques were then enumerated. Theoretically, the number of plaques formed should correspond to the number of viable mycobacterial cells which are present in the test sample.

3.10 Genotypic Identification

3.10.1 Culture DNA Extraction with Boiling Method

Two loopfuls of bacterial colonies were suspended in 200 µl of deionized water in a 1.5-ml Eppendorf tube. The suspension was boiled at 100℃ for 30 min in a heat block and then centrifuged at 16100 x g for 2 minutes. The resulting supernatant was used as template DNA for the 16S rDNA PCR assay.

3.10.2 Plaque DNA Extraction

This was performed as described by Stanley, et al., (2007). A 10-µl pipette tip was used to excise a plaque from a phage assay plate. The excised plaque was transferred into a 1.5-ml Eppendorf tube. A volume of 10 µl of deionised water was added into the tube, which was then heated at 95℃ for 15 min. Following pulse centrifugation, the tube was left at -20℃ for at least 30 min. Then, the tube content was thawed and centrifuged at 16100 x g for 2 min. The resulting supernatant was used as template DNA for the duplex IS*900*/IS*1081* PCR assay.

3.10.3 Universal 16S rDNA PCR Assay

This was performed to identify the bacterial contaminants in LJ cultures that survived the decontamination treatments. Each 25 µl reaction consisted of 5 µl of template DNA, 1x DreamTaq buffer, 250 nM of each forward and reverse primers, 200 µM dNTPs, and 0.625 U of DreamTaq DNA polymerase. The primer set used was designed by K. Halder (unpublished) and the primer sequences were shown in Table 3.1. The PCR was carried out according to the thermocycling profile shown in Table 3.2, with a total of 40 cycles. A no-template control (NTC) and a positive control (*Escherichia coli*) were included in every PCR run.

Table 3.1 : Primers for the universal 16S rDNA PCR assay.

Primer	Nucleotide Sequence	Amplicon Size (bp)	T_m† (℃)
16S-338F	5'-ACTCCTACGGGNGGCNGCA-3'	199	66.6
16S-515R	5'-GTATTACCGCNNCTGCTGGCAC-3'		66.4

†T_m, Melting temperature.

Table 3.2: Thermocycling profile for the universal 16S rDNA PCR assay.

Temperature (℃)	Duration (s)	
95	180	
95	30	x 40 cycles
60	60	
72	60	

3.10.4 *Mycobacterium*-specific PCR Assays

Acid-fast bacterial isolates were subjected to three *Mycobacterium*-specific PCR assays, two targeting the 16S rDNA and the other one targeting the *hsp*5 gene for genotypic identification. Each 25 µl reaction consisted of 5 µl template DNA, 1x DreamTaq buffer, 250 nM or 50 nM of each forward and reverse primers for 16S and *hsp*5 assays, respectively; 200 µM dNTPs, and 0.625 U of DreamTaq DNA polymerase. The primer sequences and thermocycling profile used were shown in Table 3.3 and Table 3.4, respectively. An NTC, a positive control (*M. smegmatis*) and a negative control (*E. coli*) were included in every PCR run. The primer sets

used are MYCO16S designed by Cheah, (2010); Myc16S designed by Singh, et al. (2007); and *hsp*5 designed by Khan, et al. (2004).

Table 3.3 Primers for the *Mycobacterium*-specific PCR assays.

Primer	Nucleotide Sequence	Amplicon size (bp)	T_m (℃)
MYCO16S	5'-GAAACTGGGTCTAATACCG-3'	173	58.0
MYCO16SR	5'-ATCTCAGTCCCAGTGTGG-3'		59.9
Myc16SF	5'-GAGAGTTTGATCCTGGCTCAG-3'	1030	54.0
Myc16Sr	5'-TGCACAACAGGCCACAAGGGA-3'		56.0
hsp5-F	5'-ACCAACGATGGTGTGTCCAT-3'	439	56.8
hsp5-R	5'-CTTGTCGAACCGCATACCCT-3'		57.3

Table 3.4 Thermocycling profile for the *Mycobacterium*-specific PCR assays.

PCR events	MYCO16S		Myc16S		*hsp*5	
	Temp (℃)	Duration (s)	Temp (℃)	Duration (s)	Temp (℃)	Duration (s)
Initial denaturation	95	180	94	180	95	180
Denaturation	95	30	94	60	95	60
Annealing	60	60	62	120	60	60
Extension	72	60	72	120	72	60
Final extension	72		72	600	72	600
Cycles	40		40		35	

3.10.5 IS*900*/IS*1081* Duplex PCR Assay

This duplex PCR assay was performed as described by Stanley, et al., (2007). Each 25-µl reaction consisted of 10 µl of plaque DNA, 1x DreamTaq buffer, 125 nM of each forward and reverse primers, 250 µM dNTPs, 1 mM $MgCl_2$ and 0.5 U of DreamTaq DNA polymerase. The primers for IS*900*/IS*1081* used in the duplex PCR assay were designed by Whittington (1998) and Ahmed (1998), respectively, and shown in Table 3.5. The thermocycling profile is shown in Table 3.6. An NTC, a positive control for IS*1081* (*M. bovis* BCG), and a negative control (*E. coli*) were included in every PCR run.

Table 3.5 Primers for the IS*900*/IS*1081* duplex PCR assay.

Primer	Nucleotide Sequence	Amplicon size(bp)	T_m (℃)
IS*900*			
P90	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'	400	74.5
P91	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'		74.5
IS*1081*			
BW6	5'-CGACACCGAGCAGCTTCTGGCTG-3'	306	64.3
BW7	5'-GTCGGCACCACGCTGGCTAGTG-3'		64.8

Table 3.6: Thermocycling profile for the IS*900*/IS*1081* duplex PCR assay.

Temperature (℃)	Duration (s)	
95	180	
95	30	x 37 cycles
62	60	
72	60	
72	240	

3.10.6 Gel Electrophoresis of PCR Amplicons

PCR amplicons were separated and analyzed on 2% (w/v) agarose gels for the universal 16S rDNA, MYCO16S, and IS*900*/IS*1081* duplex PCR assays. For Myc16S and *hsp*5 PCR assays, 0.9% and 1.0% (w/v) agarose gels were used, respectively. Each well was loaded with 10 µl of PCR reaction and 2 µl of GeneRuler 100-bp DNA ladder was used as marker. Electrophoresis was performed at 80 V for 45 min. The gel was then stained in 0.5 µg/ml ethidium bromide (EtBr) solution for 5 min. After that, the gel was briefly destained in dH_2O for 2 min and then visualized under the ultraviolet (UV) transilluminator.

3.10.7 Gel Purification of PCR Amplicons

PCR amplicons were excised from the gel with a scalpel. The subsequent purification of the amplicon was performed using the Xprep Gel Purification Kit according to the manufacturer's instruction. The concentration and purity (A_{260nm}/A_{280nm}) of the purified amplicons were measured using the nanospectrophotometer. Pure DNA should have A_{260nm}/A_{280nm} ranging from 1.8-2.0 and with concentration more than 10 ng/µl for DNA sequencing.

3.10.8 DNA Sequencing and Analysis

Purified amplicons were sent to Medigene Sdn. Bhd. (Selangor, Malaysia) for sequencing. The resulting sequences for each samples was assessed using the

Sequence Scanner Software 2 (Applied Biosystems) based on sequencing chromatogram prior to analysis. Analysis of DNA sequences was then performed using the nucleotide-nucleotide Basic Local Alignment Tool (BLASTn) available at the National Center of Biology Information (NCBI) website. The identity of the isolates were determined based on the sequence identity with the type strains and expect value (E-value).

CHAPTER 4

RESULTS

4.1 Collection of Soil Samples

4.1.1 Locations of Sampling

A total of 11 soil samples were collected from around Kampar, Malaysia. Locations of sampling include cow farms, bovine grazing grounds, and non-bovid contacted sites (Table 4.1).

Table 4.1: Locations of soil sampling in this study.

Location of Sampling	Soil Sample
Cow farm, Kampung Tersusun Batu Putih	BP-CF
Cow farm, Taman Selatan	TSe-CF
Grazing ground, Eastlake	EL-GG
Grazing ground, Taman Indah	TIn-GG
Grazing ground, Taman Perdana	TPe-GG
Grazing ground, Taman Selatan	TSe-GG
Grazing ground, Taman Suasa	TSu-GG
Grazing ground beside TAR University College	KT-GG
C4 Land, UTAR	C4-U
Dewan Tun Ling Liong Sik, UTAR	GH-U
Library, UTAR	LB-U
Danish residential area	Danish

4.1.2 Visual Appearance of Soil Samples

The visual appearance of the soil samples collected is shown in Figure 4.1. All samples were wet soils with considerable moisture; there was no dry soil in the collection. The soil samples BP-CF, TSe-GG, EL-GG, TSu-GG, LB-U, Danish, and KT-GG were relatively sandier.

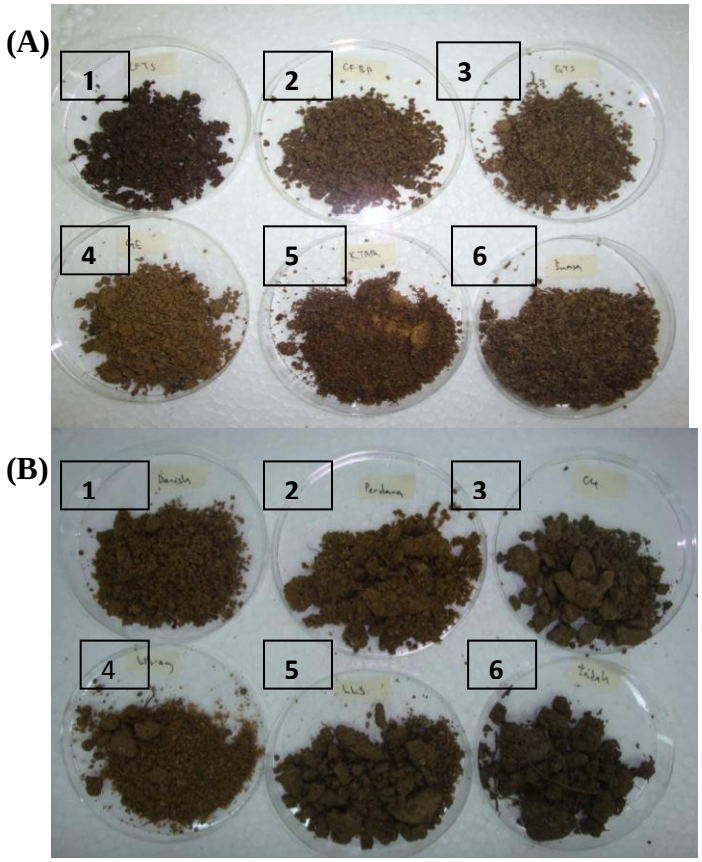


Figure 4.1: Visual appearance of soil samples.

A: 1) TSe-CF, 2) BP-CF, 3) TSe-GG, 4) EL-GG, 5) KT-GG, 6)TSu-GG

B: 1) Danish, 2) TPe-GG, 3) C4-U , 4) LB-U, 5) GH-U, 6) TIn-GG

4.2 Evaluation of Different Methods for Decontamination of Soil Samples for the Phage Assay

Four decontamination methods were evaluated in this study to determine a suitable one for the soil samples studied (Table 4.2). Not all soil samples were tested for each of these methods. Non-bovid contacted soil samples were also assessed in order to determine a decontamination method that can be universally applied to various soil types.

Table 4.2: Chemical composition for different decontamination methods.

Treatment	Chemical Composition
I	3% (w/v) SDS + 4% (w/v) NaOH
II	3% (w/v) SDS + 4% (w/v) NaOH + 2% (w/v) CTAB
III	Half strength BHI broth + 2% (w/v) NaOH
IV	1%, 2% or 4% NaOH + NOA

4.2.1 Treatment I

Overall, treatment I exhibited very low decontamination power, as shown by the high proportion of phage assay plates contaminated (Table 4.3). Only two out of eight plates were free from contaminants. The use of longer duration of treatment, 30 min as opposed to 15 min, resulted in better contamination control. Only two out of four plates were contaminated for the former, but all plates were contaminated for the latter

Table 4.3: Observation of the phage assay plates for soil samples treated with 3% (w/v) SDS + 4% (w/v) NaOH.

Sample[†]	Treatment duration			
	15 min		30 min	
	Plaque formation	Presence of contamination	Plaque formation	Presence of contamination
EL-GG	-	+	-	-
TSe-GG	-	+	-	+
TSu-GG	-	+	-	-
KT-GG	-	+	-	+

[†]Each sample was tested in duplicate.

4.2.2 Treatment II

In order to increase the stringency of decontamination, a solution of 2% (w/v) CTAB was incorporated into treatment I. This method was based on that developed by Parashar, et al. (2004) with minor modifications.

The incorporation of CTAB solution had greatly lowered the contamination rate of the phage assay plates. Only one out of a total of eight plates was found to be contaminated (Table 4.4). The detection of contaminants for TSe-CF sample treated for 30 min and no contamination for that treated for 15 min are anomalous; other results were as expected. A problem was encountered in this method in which a heavy white precipitate was formed when the CTAB solution was added to the resulting pellet after the SDS+NaOH treatment, and it could not be removed by repeated washing (Figure 4.2). The use of higher centrifugal temperature (25 ℃) also gave rise to the precipitation. However, prior washing of the SDS+NaOH-

treated pellet before CTAB treatment solved the problem; no precipitate was observed with this modification to the method.

Table 4.4: Observation of the phage assay plates for soil samples treated with 3% (w/v) SDS + 4% (w/v) NaOH + 2% (w/v) CTAB

Samples[†]	Treatment duration			
	15 min		30 min	
	Plaque formation	Presence of contamination	Plaque formation	Presence of contamination
BP-CF	-	-	-	-
TSe-CF	-	-	-	+
EL-GG	-	-	-	-
TSe-GG	-	-	-	-

[†]Each sample was tested in duplicate.

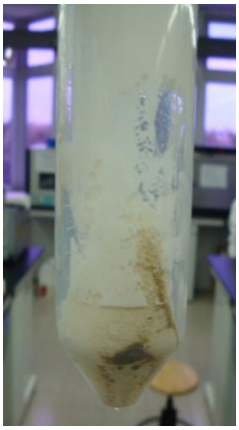
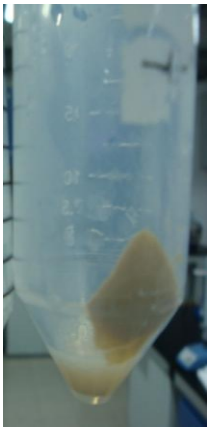
(A)  (B) 

Figure 4.2: Precipitation of decontamination reagents.

A: Formation of heavy white precipitate without a washing step prior to addition of the CTAB solution.

B: No formation of white precipitate with a washing step before the addition of the CTAB solution.

4.2.3 Treatment III

Treatments III and IV were designed in a way that involved lesser steps as compared to treatments I and II. Besides being less laborious and time saving, the use of lesser steps minimizes the risk of target cell loss through the procedures. Most of the contaminants that survived treatments I and II were the endospore-forming Gram-positive bacilli. Even if they were in the spore forms, the use of BHI broth in treatment III would have germinated and rendered them susceptible to killing by the NaOH incorporated in it. This could explain the relatively high level of decontamination accorded by treatment III; only one out of six reactions was contaminated (Table 4.5).

Table 4.5: Observation of the phage assay plates for soil samples treated with half strength BHI broth + 2% (w/v) NaOH

Samples[†]	Observation	
	Plaque formation	Presence of contamination
BP-CF	-	-
TSe-CF	-	-
TSe-GG	-	+
TSu-GG	-	-
GH-U	-	-
LB-U	-	-

[†]Each sample was tested in duplicate.

4.2.4 Treatment IV

The concentration of 1%, 2%, and 4% (w/v) NaOH were evaluated for treatment IV to determine the suitable concentration for decontamination (Table 4.6). The

antibiotic cocktail NOA was used during overnight resuscitation to eliminate the residual contaminants that might have survived the NaOH killing. Table 4.6 showed that no contamination was observed for both 1% and 2% NaOH reactions. Unexpectedly, both the reactions for TSe-GG, which were processed with 4% (w/v) NaOH, were contaminated, these results were thought to be anomalous.

Table 4.6: Observation of the phage assay plates for soil samples treated with 1%, 2% , 4% (w/v) NaOH + NOA

Sample[†]	Observations[‡]					
	1% NaOH		2% NaOH		4% NaOH	
	Plaque	Cont	Plaque	Cont	Plaque	Cont
BP-CF	-	-	-	-	-	-
TSe-CF	-	-	-	-	-	-
TSe-GG	-	-	-	-	-	+

[†]Each sample was tested in duplicate.
[‡]Plaque, plaque formation; Cont, presence of contamination.

4.3 Overall Contamination Rates of Phage Assay Plates

The overall contamination rate for each decontamination method was shown in Table 4.7. Treatment IV with 1% or 2% (w/v) NaOH yielded the lowest contamination rate (0%), followed by treatment II (12.5%), treatment III (16.7%), and treatment I (75%). Treatment IV was therefore used as the routine decontamination method throughout this project.

Table 4.7: Overall contamination rates for all four decontamination methods.

Treatment	Overall contamination rate (%)†
I	75.0
II	12.5
III	16.7
IV	0-33‡

†The overall contamination rate was calculated as follow: number of contaminated phage assay plates ÷ total number of phage assay plates x 100%

‡0-33%, 1%, 2% and 4% (w/v) NaOH + NOA

4.4 Assessment of the Soil Processing Method for the Possible Occurrence of Background Plaques

This test was performed with autoclaved soil samples (complete microbial killing assumed) and no-sample controls processed through the protocol involving treatment IV, the decontamination method chosen for the rest of this study. No background plaques were observed for both the autoclaved samples and no-sample controls with 1% and 2% (w/v) NaOH (Table 4.8). However, false-positive plaques were detected when 4% (w/v) NaOH was used, which further justified the choice of 2% (w/v) NaOH for the routine decontamination method.

Table 4.8: Assessment of the soil processing method for the possible occurrence of background plaques.

Decontamination agents	Presence of plaques	
	Autoclaved soil	No-sample control
1% NaOH + NOA	-	-
2% NaOH + NOA	-	-
4% NaOH + NOA	TNTC[†]	TNTC

[†] TNTC, too numerous to count.

4.5 Evaluation of the Sensitivity of the Phage Assay with Decontaminated Soil Samples

Prior to the test with spiked soil samples, the sensitivity of the phage assay with mycobacterial culture was assessed. An exponential-phase *M. smegmatis* culture (approximately 10^8 CFU/ml) was serially diluted, ten folds at a time, to 10 CFU/ml. These represented samples containing different numbers of target mycobacterial cells, which were then subjected to the phage assay. Following overnight incubation at 37 ℃, five different outcomes were observed in the phage indicator plates: no plaques, countable plaques, too numerous to count (TNTC), confluent lysis and complete lysis. The detection limit of the phage assay with *M. smegmatis* culture was 100 CFU (Table 4.9).

Following this, the sensitivity of the analytical method with artificially spiked soil samples was assessed. Sample LB-U was spiked with approximately 10^2 *M. bovis* BCG CFU and then subjected to treatment IV and subsequently the phage assay. Following overnight incubation, no plaques were observed in the phage indicator

plates. Nevertheless, the size of the test inoculum spiked to each soil sample was a little small to begin with.

Table 4.9: Sensitivity of the phage assay with *M. smegmatis* culture.

CFU/ml tested	PFU/ml recovered[†]
10^8	Complete lysis
10^7	Complete lysis
10^6	Complete lysis
10^5	Confluent lysis
10^4	TNTC
10^3	264
10^2	28
10^1	0
Negative control	0

[†]Complete lysis, lysis of all of the lawn of *M. smegmatis* cells, indicating that more than 10^5 *M. smegmatis* cells were detected (Stanley *et al.*, 2005).
Confluent lysis, lysis of 80-90% of the lawn of *M. smegmatis* cells, indicating that 10^4 to 10^5 *M. smegmatis* cells were detected (Stanley *et al.*, 2005).
TNTC, 10^3 to 10^4 *M. smegmatis* plaques per plate with some merging of individual plaques (Stanley *et al.*, 2005).

4.6 Assessment for Potential Inhibition of the Phage Assay by Soil Residues in Decontaminated Suspensions

The inhibition test was performed by spiking approximately 100 *M. bovis* BCG CFU into each soil suspension at the end of treatment IV. Different samples were tested, which include BP-CF, TSe-CF, TSu-GG and GH-U. The test cells did not undergo the decontamination process, and hence target cell loss or killing of target cells by decontamination agents could be ruled out. In the event of inhibition by

residual soil materials, no plaques would be observed in the phage indicator plates following overnight incubation. Indeed, this was observed for this test (Figure 4.3). It was possible that, for the samples tested, the soil residues might have inhibited the phage assay. However, the presence or the degree of inhibition could be soil-specific and would not apply to all soil types.

(A)

(B)

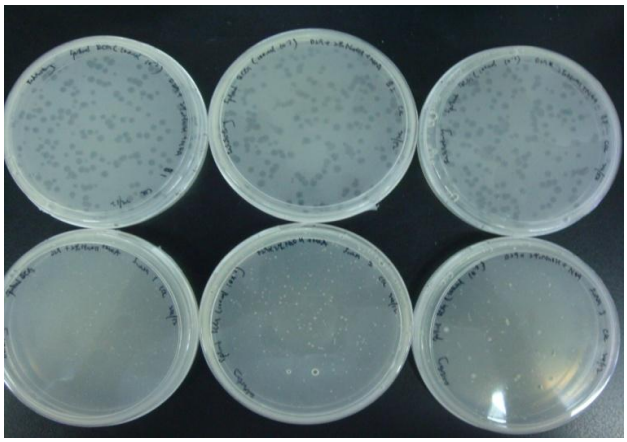


Figure 4.3: Inhibition of the phage assay by soil residues in post-decontamination test suspensions**.**
(A)Top row: Phage assay with *M. bovis* BCG culture (100 CFU; positive controls).
(B)Bottom row: Phage assay with TSu-GG post-decontamination suspension spiked with 100 *M. bovis* BCG CFU.

4.7 Assessment of Decontaminated Soil Samples for the Potential Presence of Mycobacteria via the Phage Assay

A total of 11 soil samples were decontaminated via treatment IV and then subjected to the phage assay; 1% and 2% (w/v) NaOH were compared for the former. For the processing method with 1% NaOH, two samples (TPe-GG and

Danish) were positive for mycobacteria (Table 4.10, Figure 4.4) and four samples were contaminated (Table 4.10). For that with 2% NaOH, contamination was observed in four samples, in which two (KT-GG and TPe-GG) were also contaminated with the 1% NaOH method. The samples TPe-GG and Danish were also positive for mycobacteria with the 2% NaOH method.

The analysis of larger number of soil samples had better represented the actual decontamination strength of treatment IV; the overall contamination rate observed was 33.4% for both 1% and 2% NaOH. The degree of contamination in the plates affected was very minimal and therefore did not affect the formation of a confluent *M. smegmatis* lawn or obscure the observation of target plaques (Figure 4.4). Since the contamination observed for some samples (TIn-GG, GH-U, LB-U and Danish) showed no correlation to the NaOH concentrations used, the concentration of 1% was deemed more suitable at this point. The use of lower NaOH concentration in this case would be less detrimental on the viability of target mycobacterial cells.

Table 4.10: Use of the phage assay to detect mycobacteria in the soil samples decontaminated via treatment IV.

Sample	Observation[†]			
	1% NaOH		2% NaOH	
	Plaque	Cont	Plaque	Cont
BP-CF	-	-	-	-
TSe-CF	-	-	-	-
KT-GG	-	+	-	+
TIn-GG	-	-	-	+
TPe-GG	+	+	+	+
TSe-GG	-	-	-	-
TSu-GG	-	-	-	-
C4 -U	-	-	-	-
Danish	+	+	+	-
GH-U	-	-	-	+
LB-U	-	+	-	-

[†]Plaque, plaque formation; Cont, presence of contamination.

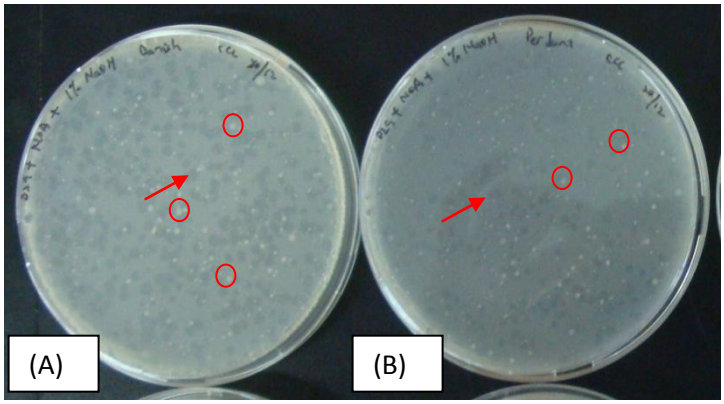


Figure 4.4: Plaque formation on the phage indicator plates of decontaminated soil samples. (A) Danish; (B) TPe-GG. Plaques were shown with red arrows and contaminants circled in red for clarity.

4.8 Use of the Duplex IS*900*/IS*1081* PCR Assay to Identify Target Mycobacterial Species in Phage D29 Plaques

Phage D29 is only *Mycobacterium* genus-specific and therefore the phage assay is not specific for the detection of a particular mycobacterial species. Hence, a PCR assay was combined downstream for specific identification of target mycobacterial species in the D29 plaques. Theoretically, each plaque will contain one genome from a lysed target mycobacterial cell, which can be extracted out for detection by a sensitive PCR assay that targets the multicopy insertion elements. In this study, the duplex IS*900* and IS*1081* PCR assay was used, in which the former is specific to MAP while the latter is specific to *M. bovis*, another potential pathogenic mycobacterial species targeting the bovid (Stanley, et al., 2005)

The plaques observed on the TPe-GG and Danish phage indicator plates (three plaques for each sample) were excised out and the resulting DNA extracts subjected to the duplex PCR assay. However, no target amplicons (400 bp and/or 306 bp, respectively) were detected for these samples upon gel analysis (Figure 4.5). In order to verify that the agar extracts were not inhibitory to PCR amplification, the extracts (from no-plaque regions in the phage indicator plate) were spiked into the reactions with *M. bovis* BCG DNA. As shown in Figure 4.5, bands of the expected size of 306 bp were observed upon gel analysis, indicating that the IS*1081* of *M. bovis* BCG was successfully amplified.

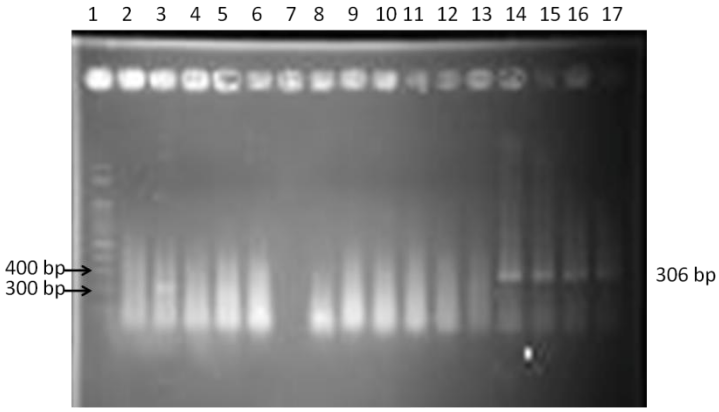


Figure 4.5: Gel analysis of duplex IS*900*/IS*1081* PCR amplicons. Lane 1, GeneRuler 100-bp ladder; lane 2, NTC; lane 3, *E. coli* (negative control); lanes 4 to 6, *M. smegmatis* genomic DNA; lanes 8 to 10, Danish plaque DNA; lanes 11 to 13, TPe-GG plaque DNA; lanes 14 to 16, *M. bovis* BCG DNA + agar extract; lane 17, *M. bovis* BCG DNA (positive control).

4.9 Culture Isolation of Mycobacteria from Decontaminated Soil Samples

4.9.1 Evaluation of Different Decontamination Methods for Culture Isolation

In addition to assessing the decontamination methods (treatments I and II) for detection by the phage assay, their effectiveness for culture isolation of mycobacteria from soil samples was also evaluated. However, none of these methods was effective and almost all the inoculated LJ slants were severely contaminated (Figure 4.6). Only the LJ slant inoculated with EL-GG sample (decontaminated via treatment I) was contaminant-free and yielded Ziehl-Neelsen-positive bacterial colonies. For the contaminated LJ slants, a consistent physical change in their appearance was observed. After two days of incubation, their surface turned dark green in color and subtle cracks were observed. With further

incubation, the slants become pale yellow in color and the cracks became more obvious. Finally, the slants collapsed and liquefied, giving out a foul odor. The inoculation of decontaminated soil samples on LJ slants supplemented with penicillin still resulted in severe contamination, in which the slants collapsed and liquefied. All liquefied slants were screened for the possibility of mixed cultures of mycobacteria and contaminants via the Ziehl-Neelsen staining, but none were positive.

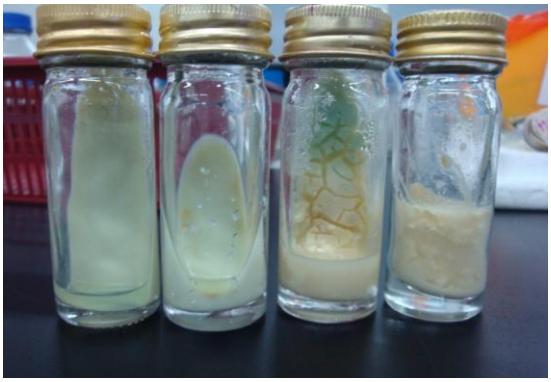


Figure 4.6: Appearance of LJ slants inoculated with decontaminated soil samples. From left to right: 1st and 2nd LJ slants, no signs of contamination; 3rd LJ slant, green coloration and appearance of cracks on contaminated slant; 4th LJ slant, liquefaction of contaminated slant.

4.9.2 Identification of Soil Mycobacterial Isolate

Out of the 11 soil samples processed, only one (EL-GG) was successfully decontaminated and yielded Ziehl-Neelsen-positive bacterial colonies (Figure 4.7). The colonies were small (about 1 mm in size) and smooth in appearance, and they took more than 7 days to develop; the latter would suggest the isolation of a slow-growing mycobacterial species. The isolate also yielded deep orange colonies

when grown in both light and dark conditions, which is a type of pigmentation pattern among certain mycobacterial species referred to as scotochromogens.

The mycobacterial isolate, following subculture on M7H10 agar and genomic DNA extracted from the resulting colonies, was identified using a genotypic method. Three *Mycobacterium*-specific PCR assays, two targeting the 16S rDNA gene and another one targeting the *hsp*5 gene, were separately performed and the resulting amplicons (439, 173 and 1030 bp, respectively) were outsourced for DNA sequencing (Figure 4.8). The BLASTn analysis of the resulting DNA sequences for each PCR assay yielded several possible identities with the highest percentage similarities and lowest E-values (Table 4.11). DNA sequence analysis of amplicons for all the three PCR assays unanimously identified the soil mycobacterial isolate to be that of *M. mantenii* or *M. scrofulaceum*. Both these mycobacterial species are slow growers and scotochromogenic, which are coherent with the culture observations above.

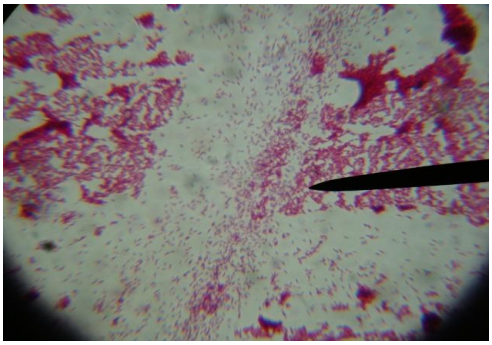


Figure 4.7: Ziehl-Neelsen stain of the soil mycobacterial isolate from EL-GG showing pink acid-fast bacilli

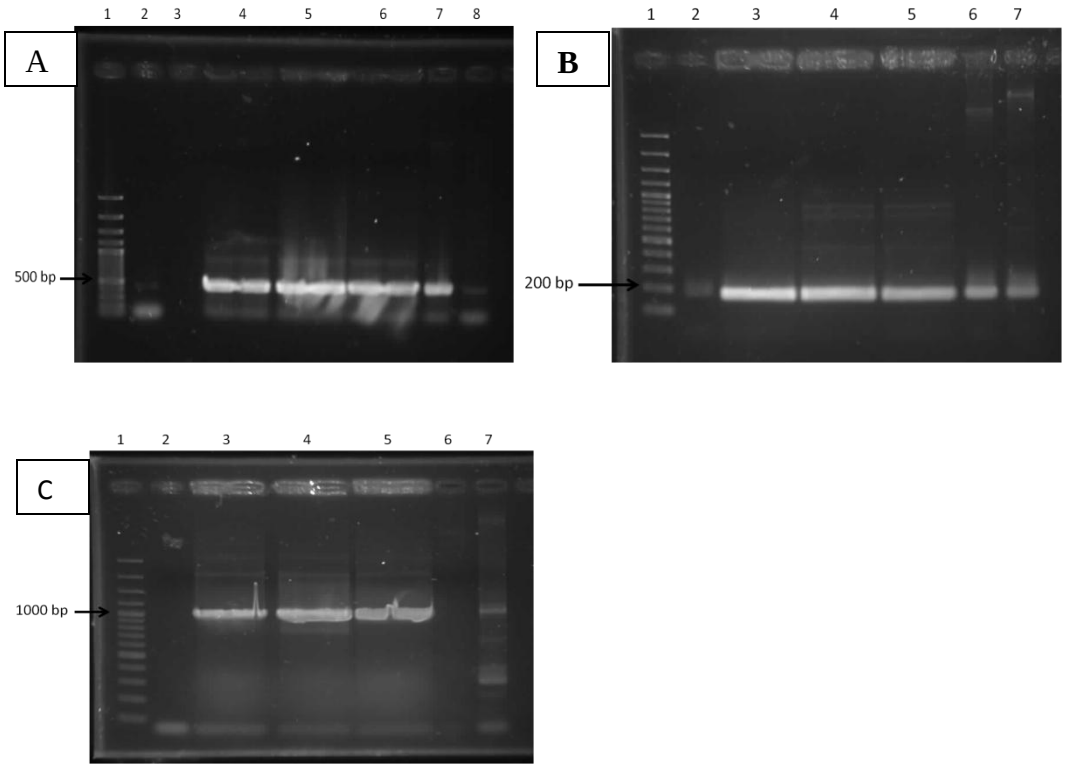


Figure 4.8: Gel analysis of PCR amplicons for the soil mycobacterial isolate. (A) hsp5 PCR assay, (B) MYCO16S PCR assay, (C) Myc16S PCR assay**.**
A: Lane 1, GeneRuler DNA ladder; lane 2, NTC; lane 3, empty; lanes 4, 5 and 6, test isolate; lane 7, *M. smegmatis* (positive control); lane 8, *E. coli* (negative control).
B: Lane 1, GeneRuler DNA ladder; lane 2, NTC; lanes 3, 4 and 5, test isolate; lane 6, *M. smegmatis* (positive control); lane 7, *E. coli* (negative control).
C: Lane 1, GeneRuler DNA ladder; lane 2, NTC; lanes 3, 4 and 5, test isolate; lane 6, *E. coli* (negative control); lane 7, *M. smegmatis* (positive control).

Table 4.11: BLASTn analysis of the DNA sequences of the *hsp5* and 16S amplicons for soil mycobacterial isolate.

PCR assay	BLAST identity	Accession	Score (bits)	Identity (%)	E-value
*hsp*5	GroEL2 (groEL2) gene (*Mycobacterium mantenii* strain FMUNAM08)	JN089411.1	634	95	$2e^{-178}$
	heat shock protein 65 (*Mycobacterium europaeum* strain AFP-0001)	FR682914.1	627	95	$3e^{-176}$
	heat shock protein 65 (*Mycobacterium scrofulaceum* strain KIT 30101)	AY299172.1	627	95	$3e^{-176}$
	heat shock protein 65 (*Mycobacterium scrofulaceum* strain ATCC 19981)	AY299138.1	627	95	$3e^{-176}$
Myc16S	16S ribosomal RNA gene (*Mycobacterium mantenii* strain NLA000800224)	FJ232521.1	904	99	0.0
	16S ribosomal RNA gene (*Mycobacterium paraffinicum* strain 06-839)	GQ153265.1	893	99	0.0
	16S ribosomal RNA gene (*Mycobacterium scrofulaceum* strain ATCC 19981)	NR_025237.1	887	99	0.0
MYCO16S	16S ribosomal RNA gene (*Mycobacterium scrofulaceum* strain ITM091072)	JX119208.1	243	99	$4e^{-61}$
	16S ribosomal RNA gene (*Mycobacterium europaeum* strain AFP-0001)	FR686461.1	243	99	$4e^{-61}$
	16S ribosomal RNA gene *(Mycobacterium mantenii* strain FMUNAM08)	JN049508.1	243	99	$4e^{-61}$

4.9.3 Identification of Bacterial Contaminants in LJ Cultures

The major bacterial contaminants (9 isolates in total) that survived the decontamination process were identified in order to provide better insights on the stringency of the decontamination method to be attempted in future studies. Most of these contaminants are Gram-positive bacilli (Figure 4.9).

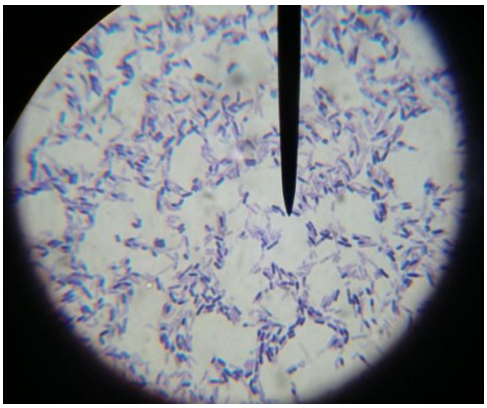


Figure 4.9: Gram stain of isolate B1 from a contaminated LJ culture showing Gram-positive bacilli.

The contaminants were grouped according to their colony types. The selected contaminant for each colony type, following subculture on TSA agar and genomic DNA extracted from the resulting colonies, was identified via 16S rDNA sequencing. Figure 4.10 shows the gel analysis of their resulting 16S PCR amplicons, which were 199 bp in size.

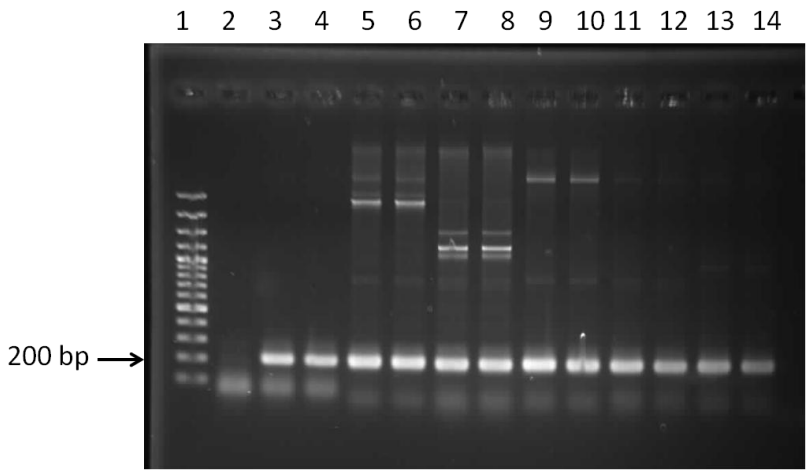


Figure 4.10: Gel analysis of 16S amplicons for bacterial contaminants in LJ cultures.

Lane 1, GeneRuler 100-bp DNA ladder; lane 2, NTC; lanes 3 and 4, isolate A; lanes 5 and 6, isolate B1; lanes 7 and 8, isolate B2; lanes 9 and 10, isolate C; lanes 11 and 12, isolate D; lanes 13 and 14, isolate E.

The BLASTn analysis of the resulting DNA sequences revealed that most of the contaminants belonged to the genus *Bacillus*, while isolates B2, F and I were identified to be those of *Paenibacillus* spp. (Table 4.12). The former corresponds to the observation of Gram-positive bacilli in their Gram-stained smears. The observation of Gram-negative bacilli for the latter questions the reliability of their sequencing results.

Table 4.12: BLASTn analysis of the DNA sequences of the 16S amplicons for bacterial contaminants in LJ cultures.

Contaminant	BLAST identity	Accession	Score (bits)	Identity (%)	E-value
A	16S ribosomal RNA gene (*Bacillus cereus* strain BcTNAU6)	KC540842.1	267	97	$3e^{-68}$
B1	16S ribosomal RNA gene (*Bacillus licheniformis* strain 3389O2)	KF600530.1	261	97	$1e^{-66}$
B2	16S ribosomal RNA gene (*Paenibacillus* sp. RS5-1)	KC117518.1	202	98	$5e^{-49}$
C	16S ribosomal RNA gene (*Bacillus thuringiensis* strain ODB63)	KF771420.1	259	99	$4e^{-66}$
D	16S ribosomal RNA gene (*Bacillus cereus* strain BcTNAU6)	KC540842.1	268	99	$7e^{-69}$
E	16S ribosomal RNA gene (*Bacillus cereus* strain BcTNAU6)	KC540842.1	272	99	$6e^{-70}$
F	16S ribosomal RNA gene (*Paenibacillus azoreducens* strain JN135)	KF687063.1	261	98	$1e^{-66}$
G	16S ribosomal RNA gene (*Bacillus cereus* strain BcTNAU6)	KC540842.1	257	100	$1e^{-65}$
I	16S ribosomal RNA gene (*Paenibacillus* sp. enrichment culture clone 'Bacteriology Laboratory 1')	KF906614.1	265	98	$1e^{-67}$

CHAPTER 5

DISCUSSION

5.1 Sampling and Storage of Soil Samples

The soil samples used in this project can be generally categorized into two groups, bovid-contacted and non-bovid-contacted. The former were obtained from grounds inhabited by bovine animals (cow farms in this study) and their feeding or grazing grounds, with noticeable manures or manure residues on them. The latter were obtained mainly from grounds around the UTAR Perak Campus. Although the former would be more relevant for the study of MAP, the inclusion of other soil types in this project would expand the versatility of the decontamination method developed, since the levels and types of microbiota vary among different soil types (Parashar, et al., 2004).

All soil samples were collected from the depth of 3 cm, as surface samples experience frequent alteration and do not reveal the actual microbial populations in them (Chilima, et al., 2006). The latter is attributed to the influence of environmental stressors such as UV radiation, desiccation, and erosion by the wind, rain water and animal activities. In addition, triplicate soil samples were collected 3-5 m apart from each sampling location and then mixed together in

order to better represent the soil microbial community within the area. If not processed immediately, the soil samples were stored in their collection bags at room temperature back in the laboratory. This was to minimize the detrimental effect on the viability of the microbial populations in them, especially if they were stored at lower temperatures, for instance at 4℃ in the refrigerator (Ng, 2012; Ting, 2012).

5.2 Evaluation of Different Methods for Decontamination of Soil Samples for the Phage Assay

When compared to other biological specimens, such as those that are of clinical or food origin, soil samples undoubtedly harbor higher levels of and more diverse microbial populations. In order for successful detection of mycobacteria in soil samples via culture or the phage assay, prior decontamination is necessary. However, the stringency of the decontamination method used must take into account both its efficacy in eliminating non-target organisms and its effect on the viability of the target mycobacterial species to be detected (Dundee, et al., 2001). Therefore, one of the objectives of this project was to establish a suitable decontamination method for soil samples for subsequent detection of viable MAP cells in them via a combined phage-PCR assay.

The four decontamination methods evaluated (treatments I to IV) gave varied levels of successful decontamination for the soil samples tested. Treatment II,

although gave the lowest contamination rate (12.5%), involved multiple steps, especially those of repeated centrifugation and washing, which might lead to potential target cell loss through the processing and result in a lower sensitivity of detection. Furthermore, the high stringency of this treatment, in which multiple harsh chemicals (NaOH, SDS and CTAB) were used, could significantly affect the viability of target mycobacterial cells. Treatment I, although less stringent, also involved considerable number of processing steps and therefore, the potential for significant target cell loss through the procedure remains a question. In addition, both these decontamination methods were more laborious and time consuming (relatively more for treatment II) than the other two assessed.

In treatment III, the ingenious approach of soil decontamination with NaOH in BHI broth could be effective on endospore-forming contaminants, in which germination in BHI broth during overnight incubation would facilitate their killing by NaOH. Although not the lowest among the methods tested, treatment III yielded a relatively low contamination rate of 16.7%. Although the BHI broth could aid in the recovery of chemically stressed mycobacterial cells, the effect of prolonged NaOH exposure (16-18 hours in this case) on their viability remains to be investigated.

Treatment IV, which involved brief decontamination with NaOH (15 min), also incorporated the NOA antibiotic cocktail in the decontaminated suspension to

enhance its stringency. NOA, at the published concentrations used, has been reported not to affect the viability of *M. tuberculosis*, a mycobacterial species (Albert, et al., 2007; Cheah, 2010). This antibiotic cocktail, which consists of nystatin, oxacillin and aztreonam, has a broad spectrum of antimicrobial activity, targeting fungi, Gram-positive bacteria and Gram-negative bacteria, respectively. Treatment IV, with a relatively low contamination rate of 0-33%, was deemed to be potentially more suitable for subsequent soil decontamination, since it has the shortest turnaround time (approximately 50 min), is less laborious, and requires minimal laboratory resources.

5.3 Assessment of the Soil Processing Method for the Possible Occurrence of Background Plaques

This was performed with autoclaved soil samples and no-soil controls. The former was hypothesized to be free from any viable mycobacteria (and other microbes) that might be present following autoclave killing; however, the potential presence of surviving mycobacterial cells was arguable. For the latter, in the event that plaques were observed in the phage indicator plates upon incubation, the contribution of soil could be ruled out in this case.

In this investigation, different NaOH concentrations (1%, 2% and 4%) for treatment IV were tested. PFUs were observed in the phage indicator plates of 4% NaOH for both autoclaved soil samples and no-soil controls, which were very

likely to be false positives, assuming that autoclaving killed all mycobacteria that might be present. This could be due to the high NaOH concentration used, in which the excessive residues formed could have shielded the exogenous phages from FAS activation, thereby resulting in breakthrough plaques (false positives). Similar observation was reported by Park, et al. (2003) in their study. Therefore, 4% NaOH was eliminated from subsequent testing.

5.4 Evaluation of the Sensitivity of the Phage Assay with Decontaminated Soil Samples

This was performed with soil sample that was spiked with 100 *M. bovis* BCG CFUs. *M. bovis* BCG, instead of *M. smegmatis*, was used as the test mycobacterial model in this investigation as the former was thought to be able to survive the decontamination process better due to the higher content of mycolic acids in its cell envelope, similar to that in MAP (E.S.G. Cheah, personal communication). However, no PFUs were recovered in the phage indicator plate. This could be potentially due to the inhibition of the phage assay by soil residues in the decontaminated suspensions, which justified the subsequent investigation (Section 5.5). The significant loss of small number of target mycobacterial cells used (100 CFUs) through the procedure could be an alternative explanation. Also, artificial spiking does not closely replicate the actual scenario, in which native mycobacteria could be better protected from the harsh effect of decontamination agent(s) via natural interaction with soil particles for the latter.

Nevertheless, the phage assay was shown to be sensitive when tested with *M. smegmatis* culture, in which the detection limit was 10-100 CFU/ml. Similar limits were reported for phage assays with cultures of various mycobacterial species (Stanley, et al., 2005; Cheah, 2010). When compared to the numbers of CFUs tested, the numbers of PFUs recovered were lesser (by approximately 50%), which indicates that a large proportion of viable mycobacterial cells were not infected. McNerney, et al. (2004) suggested that the availability of phage receptors on host cells, metabolic stage of the host and the possibility of abortive infection are some of the important factors influencing the efficiency of phage infection. Therefore, the use of 100 *M. bovis* BCG CFUs in the decontaminated soil suspensions might be too low in cell numbers and stretched the limit of the phage assay (10-100 CFUs). Higher numbers of mycobacterial CFUs should be tested in future studies.

5.5 Assessment for Potential Inhibition of the Phage Assay by Soil Residues in Decontaminated Suspensions

This was performed with decontamination suspensions of soil samples that were spiked with 100 *M. bovis* BCG CFUs each. Control reactions, which were M7H9-OGC broths spiked with the same number of *M. bovis* BCG CFUs each, were also performed. Following overnight incubation, no plaques were observed in the phage indicator plates for the former, suggesting inhibition of the phage assay by soil residues in the test suspensions. Indeed, soil contains a complex mixture of inorganic and organic compounds, cell-free enzymes and various metal elements

that may interfere with the interaction between phage D29 and the target mycobacterial cells. Possible modes of interference include masking of D29 receptors on mycobacterial cells or direct impairment of phage D29 by these compounds (Park, et al., 2003). Only four soil samples were assessed for their inhibitory potentials in this study; the influence of different sample types on the presence and the degree of inhibition, as well as inter-sample variation in the same soil type, warrants further investigation. Nevertheless, the findings from this study showed that soil is not universally inhibitory to the phage assay, as positive PFUs were observed for the samples Danish and TPe-GG.

5.6 Assessment of Decontaminated Soil Samples for the Potential Presence of Mycobacteria via the Phage Assay

Using treatment IV, two NaOH concentrations, 1% and 2%, were used for decontamination of the soil samples. Mycobacteria (positive PFUs) were detected in 2 out of 11 samples for both concentration tested. One of the mycobacteria-positive soil samples was from a bovinegrazing ground, in which the potential presence of MAP or other pathogenic bovine mycobacterial species (e.g. *M. bovis*) remains to be investigated. With regard to the effectiveness of decontamination, both NaOH concentrations yielded the same rate of contamination (33.4%) in their resulting phage indicator plates. However, the degree of contamination in all plates was moderate and did not obscure the formation of the *M. smegmatis* lawn, and observation of plaques for the mycobacteria-positive samples.

Both methods were equally effective in controlling contamination in the phage indicator plates. Between 1% and 2% NaOH, the method with the former was deemed more suitable as it served as a milder decontamination agent and imposed lesser stress on the target mycobacterial cells. Indeed, the concentration of 1% is routinely used for the decontamination of tuberculous sputum specimens for the phage assay, as its effect on the viability of *M. tuberculosis* has been shown to be very minimal (McNerney, et al., 2004; Park, et al., 2003). Although treatment IV with 1% NaOH was deemed suitable for the soil samples in this study, it cannot be universally applied to all soil types. The stringency of decontamination needs to be adjusted based on the levels and diversities of microbial populations in different types of soil, and this imposes challenge to the method development. The interspecies variation among mycobacteria in their tolerance to different decontamination agents, presumably due to differences in the structure of their cell envelope, adds on to this challenge.

5.7 Use of IS*900*/IS*1081* Duplex PCR Assay to Identify 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. Therefore, a species-specific molecular assay is incorporated downstream to identify the target mycobacteria in the resulting D29 plaques. The multicopy insertion sequences are targeted in order to increase the chance of successful amplification of a single target genome within

a positive plaque (Stanley, et al., 2005). The initial intention was to use a LAMP-based assay, which is simpler and quicker in its operation, but was abandoned due to the frequent occurrence of non-specific amplification and false positive fluorescent signals (Tee, H.K., unpublished). A duplex IS*900*/IS*1081* PCR assay was used instead (Stanley, et al., 2005). The latter is specific to the members of the *M. tuberculosis* complex. *M. bovis* BCG (positive control) was PCR-positive while no IS*1081* amplicons were detected for the negative control *M. smegmatis*. MAP type strain was not available, and therefore was not included in the duplex PCR assay as the positive control for the IS*900* reaction.

Out of a total of 6 plaques from the two mycobacteria-positive soil samples (TPe-GG and Danish) tested, none were positive by the duplex PCR assay. The potential of PCR inhibition by agar plug extracts was investigated; this possibility was dismissed as the IS*1081* was successfully amplified in *M. bovis* BCG reactions that were spiked with agar plug extracts. However, several concerns on the sensitivity of plaque DNA amplification were considered, including the ability of single cell genome amplification and also genome loss through the extraction process. Theoretically, there should be only one target mycobacterial genome in a positive plaque and even with a PCR that targets a multicopy gene, the need for single-cell detection might stretch its sensitivity. In fact, low positivity rate (15%) with the plaque DNA PCR-based detection was reported by Cheah (2010) in his study. Development of a more efficient DNA extraction method in combination with a nested PCR should be considered in future studies (Stanley, et al., 2005).

The influence of the media used in the phage assay and time delay in PCR analysis of plaques on the success of target detection should also be investigated (Cheah, 2010). With regard to the former, molecular test has only been applied to plaques generated by the commercial phage assay kit marketed by Biotec Ltd (UK), which was claimed to have been optimized for combined use with molecular test downstream (Cheah, 2010).

The targeting of both IS*900* and IS*1081* in a single PCR assay would enable simultaneous assessment for both MAP and *M. bovis*, another pathogenic mycobacterial species that commonly affects the bovid, in the test specimens. This demonstrates the versatility of the molecular assay in which more specific IS primers can be incorporated (a multiplex PCR assay) for detection of further mycobacterial species that might be clinically relevant to the bovid.

5.8 Evaluation of Different Decontamination Methods for Culture Isolation

All the decontamination methods (treatments I to IV) evaluated were not stringent enough to decontaminate the soil samples tested in this study for culture on LJ slants. Apart from that for one sample, all LJ cultures were severely contaminated in this study. Although treatments I and II were successfully used in two previous studies, respectively (Ng, 2012; Ting, 2012), the findings from this study demonstrated that a single decontamination method cannot be universally applied to all soil types. In this context, the stringency of the decontamination method

required must be ideally customized for a particular soil type, as discussed in Section 5.6.

Most of the contaminants observed in the contaminated LJ cultures were Gram-positive bacteria; only a few were Gram-negative bacteria. The contaminated LJ slants exhibited a consistent change in their physical appearances with incubation. The initial color change was due to the presence of malachite green in the medium. A decrease in the pH by the metabolic activities of Gram-positive contaminants results in deep green zone in the medium, while formation of yellow zone indicates an increase in the pH due to the activities of Gram-negative bacteria (HiMedia Laboratory, 2012). With further incubation, cracks appeared on the contaminated LJ slants, which eventually liquefied and collapsed. These were the results of the actions of proteolytic enzymes secreted by the contaminants into the media (HiMedia Laboratory, 2012).

5.8.1 Identification of Soil Mycobacteria Isolate

Only one out of 11 soil samples was successfully decontaminated (treatment 1) and mycobacteria isolated from it. The isolated mycobacterial species was subjected to the 16S rDNA sequencing-based identification. As shown in Table 4.2, BLASTn analysis has limitation in discriminating closely related species, where all four *M. mantenii*, *M. scrofulaceum, M. paraffinicum* and *M. europaeum* are possible candidates for the analyzed DNA sequences. However, by

considering the BLASTn results for amplicons from the three PCR assays (one that targets the *hsp5* gene and two that target the *Mycobacterium*-specific 16S rDNA) and its growth rate and colony morphology, the isolate was preliminarily identified to be that of *M. mantenii* or *M. scrofulaceum*.

However, in order to confirm the identity of this isolate, a series of biochemical tests must be carried out. These include those that are commonly used for mycobacterial identification, such as the urease, semiquantitive catalase, thermostable catalase, tellurite reduction, nitrate reduction, Tween 80 hydrolysis and urease tests, among others. *M. mantenii* is positive in the semiquantitive catalase and urease tests and negative for tellurite reduction, whereas both *M. paraffinicum* and *M. europaeum* are urease negative (Ingen, et al., 2009; Wang, et al., 2008; Tortoli, et al., 2011). Although *M. scrofulaceum* can give variable results in the urease test, its positivity for tellurite reduction would differentiate it from *M. mantenii* (Wang, et al., 2008).

5.8.2 Identification of Bacterial Contaminants in LJ Cultures of Soil

Genotypic identification results showed that all the bacterial contaminants screened belonged to the genera *Bacillus* and *Paenibacillus*. The endospore-forming nature of the bacterial species of these genera would explain their tolerance to the various decontamination treatments applied. Failure of the penicillin incorporated into the LJ slants in providing second-line decontamination

could be explained by the presence of resistant bacterial strains with the ability to produce β-lactamase to degrade the β-lactam ring of penicillin. Fenselau and colleagues (2008) reported that the β -lactamase gene is evenly found in the genome of many wild-type bacterial species, which include *Bacillus* spp., and this gene is inducible and readily upregulated under selective pressure. Addition of penicillin into the LJ slant was believed to exert this selective pressure and gave rise to the penicillin-resistant *Bacillus* spp. observed in this project.

Some of the contaminant isolates were Gram-negative but were genotypically identified as *Paenibacillus* spp. Indeed, variable Gram stain observations have been reported for species of this genus. Although they have Gram-positive cell wall structure, many *Paenibacillus s*pp. appear Gram-negative under the microscope (Paul, et al., 2009). This observation is even more common when aged culture is examined. Otherwise, in general, *Paenibacillus* spp. have phenotypic resemblance to *Bacillus* spp.

5.9 Future Works

The decontamination method (treatment IV) deemed suitable for the types of soil samples tested in this project needs to be further assessed with larger sample size and more soil types, in order to determine its reliability and versatility, respectively. Although similar observations with *M. tuberculosis* and *M. smegmatis* were assumed for MAP in this study, the specific effects of NaOH and

NOA on MAP should be evaluated. The formation of false positive plaques from interaction between NaOH and FAS warrants further investigation. With regard to this, Park, et al. (2003) included a washing step with M7H9 broth after neutralization with phosphate buffer in their decontamination method, which successfully removed the false positive background caused by NaOH residues observed in their study. This washing step can be added to treatment IV to dismiss the concern on the unanticipated interaction between NaOH and NOA, and possibly alleviating phage assay inhibition by washing off potential inhibitors.

With the use of larger sample size, the postulated varied inhibitory effects on the phage assay among different soil samples and sample types can be better assessed. Study of the kinetics of interaction between phage particle and host cell and the mechanisms of phage deactivation will definitely provide insights on the molecular bases of inhibition and narrow down the scope of screening for possible inhibitory substances present in the soil.

Since the phage assay is not specific to a particular mycobacterial species, there is a concern of rapid-growing mycobacteria (average doubling time of 3 hours), which might be present in the soil, growing and out-competing the slowly growing MAP (doubling time of about 24 hours) during the overnight resuscitation in M7H9-OGC broth. Prolonged decontamination (30 min instead of 15 min) has been reported to be able to inactivate rapid-growing mycobacteria in soil samples,

leaving behind mostly the slow-growing ones (Parashar, et al., 2004). This can be considered for the NaOH treatment step in the method developed in this study, in order to render the downstream phage assay more specific to the latter, in which MAP is one of them.

In order for a more successful detection of target mycobacterial species in phage D29 plaques, development of a more efficient DNA extraction method is in dire need (Cheah, 2010). In addition, modifying the duplex PCR assay into a nested format should be considered for a more sensitive means of single target detection (Stanley et al., 2005). The influence of the media used in the phage assay and time delay in PCR analysis of plaques on the success of target detection should also be investigated.

For culture isolation of mycobacteria from the soil, the genotypic identification results for culture contaminants in this project have prompted for the development of a specific decontamination strategy to eliminate endospore-forming bacterial contaminants, mainly of the genus *Bacillus*. Induced spore germination and use of sporucides such as formaldehyde, glutaraldehyde and o-phthalaldehyde, can be included in the processing treatment to eliminate these spore formers (McDonnell and Russell, 1999). In addition, the incorporation of oxacillin instead of penicillin into LJ slants is expected to offer better antibacterial effect against many bacterial strains that can become easily resistant to penicillin. For reliable identification of

culture mycobacterial isolates, there is a need for both genotypic-based identification and phenotypic identification using a battery of biochemical tests, and the results of both methods simultaneously interpreted.

CHAPTER 6

CONCLUSIONS

The lack of a reliable diagnostic means for JD renders it challenging to control and manage the disease, which causes significant loss to the farming sector annually. The major aim of this project was to develop a combined phage and PCR assay for detection of viable MAP in bovid-contacted soils, a potentially important source for JD transmission that have yet to be explored. Most previous studies focussed on culture isolation of MAP from fecal specimens, and only one study reported the use of the combined phage and PCR assay for MAP detection, but in milk samples.

The robustness of the combined assay relies on an optimal soil decontamination process, which effectively suppresses non-target microbiota while preserving the viability of the target mycobacterial species. An optimal decontamination method was established for the soil types evaluated in this study, which include those from cow farms, bovine grazing grounds and non-bovid-contacted grounds. By using 1% (w/v) NaOH to decontaminate the soil samples and NOA during overnight resuscitation, the overall contamination rate was 33.4%. Although not the lowest, the shorter duration and simpler steps involved justify the choice for this decontamination method. Furthermore, the degree of contamination observed was

moderate if not little, and was insufficient to obscure the observation of plaques in the phage indicator plates.

Although there is no information on the sensitivity of the method with artificially spiked soil samples at this point, the phage assay was demonstrated to be rather sensitive when tested with pure mycobacterial cultures; its detection limit was between 10-100 CFU/ml. With regard to the reliability of the phage assay with soil samples, false positive results were observed but this was resolved by the use of NOA during overnight resuscitation; the exact mechanism involved remains to be investigated. Besides, the inhibitory effect of soil on the phage assay observed was shown to be soil type-dependent, which can be better understood with larger sample size in future studies.

Out of 11 soil samples tested, two were mycobacteria-positive; one of them was from a bovine grazing ground. However, the plaques from their phage indicator plates, when subjected to the duplex IS*900*/IS*1081* PCR, were negative for both targets. The need for a single-cell detection and the efficiency of the plaque DNA extraction method and the sensitivity of the PCR in place need to be assessed. The specificity of the PCR used, especially that for IS*1081*, is another question. The possibility of using a simpler molecular test, such as the LAMP assay, should also be explored.

Besides for the phage assay, the feasibility of the decontamination methods for culture isolation of mycobacteria from soil samples was also assessed. None of the methods tested were suitable for the soil types tested in this study; almost all cultures were contaminated apart from one in which the successfully isolated mycobacterial species was preliminarily identified as either *M. mantenii*or or *M. scrofulaceum* via 16S rDNA sequencing. Some of these decontamination methods have been successfully used by other researchers, and their unsuccessful use in this study demonstrates their non-universal or non-versatile nature when applied to other soil types. Most of the non-target contaminants in the contaminated cultures were preliminarily identified to belong to the tough, endospore-forming genera of *Bacillus* and *Paenibacillus*, which explains their survival through the decontamination treatments. In this context, the choice of decontamination method in future studies should be customized to inclusively target these bacterial species.

In a nutshell, this project has shown that the combined phage and PCR assay has great potential for detection of MAP in bovid-contacted soils, a potentially infectious source for JD with little attention from the veterinary sector. Further improvements on the existing method are necessary for more reliable performance under field settings. The method can also be expanded to be used for the detection of other bovine mycobacterial pathogens.

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APPENDIX A

APPARATUS AND CONSUMABLES

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

Table A1: List of apparatus and their respective manufacturers.

Apparatus	Manufacturer
Autoclave machine	Hirayama
Centrifuge machines	Hettich Zentrifuged, Sigma-Aldrich
Class II biosafety cabinet	Telstar
Fridge and Freezer	Copens Scientific, Pensonic
Gel imaging system	Syngene Bio Imaging
Laminar-flow cabinet	Telstar
Light microscope	Leica CME
PCR thermocycler	Biometra
pH meter	Eutech Instrumental
Shaking incubators	Labnet 211DS, N-Biotek
Spectrophotometer	Genesys 10S UV-VIS
Static incubators	Memmert
Vortex mixer	VELP Scientifica
Water baths	Memmert

Table A2: List of consumables and their respective manufacturers.

Consumable	Manufacturer
Agar agar powder	Bacto
Agarose gel powder	1st Base
Ammonium iron(II) sulfate, 6. hydrate (FAS)	UNI-CHEM
Aztreonam	Amresco
Basic fuschin powder	Merck
Brain infusion broth powder	Conda
CTAB	R&M
Disodium hydrogen phosphate (Na_2HPO_4)	UNI-CHEM
Glycerol 95%	QR ëc
Lowenstein medium base powder	BD
Middlebrook 7H10 agar powder	BD
Middlebrook 7H9 broth powder	BD
Mycobacteriophage D29	University of Leicester*
Mycobacterium bovis BCG	University of Leicester*
Mycobacterium smegmatis MC^2155	University of Leicester*
Nystatin	Bio Basic Canada
OADC	BD
Oxacillin	Bio Basic Canada
Penicillin G, sodium salt	BioBasic
Phenol	Calbiochem
Potassium dihydrogen phosphate (KH_2PO_4)	QR ëc
Sodim dodecyl sulfate	Bendosa
Sodium chloride	Merck
Sodium hydroxide	Merck
Taq polymerase- DreamTaq	Fermentas
Tryptic soy agar powder	Conda
Tween 80	Systerm
Xprep Gel Purification Kit	PKT

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