DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVINE FAECES

By

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ABSTRACT

DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVINE FAECES

JOSHUA ANG XIN DE

Johne's disease (JD), a chronic intestinal disease among ruminants, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Reduced milk production in and death of infected animals incur a huge economical loss to the farming sector. To date, there is still lack of a reliable diagnostic method for JD and this poses a challenge to its control. To address this, a combined phage and PCR assay was developed in this project for detection of MAP in bovine faecal samples. Prior to the mycobacteriophage amplification assay, faecal samples were decontaminated to prevent non-mycobacterial contaminants from obscuring the observation of plaques formed. Out of the various decontamination methods tested, the method with 2% (w/v) NaOH + NOA (nystatin-oxacillin-aztreonam) had produced 0% contamination in all the ten faecal samples tested; it was therefore deemed effective (optimised method) in this study. The use of 2% NaOH + incorporation of penicillin (50 U/mL) into Löwenstein-Jensen slant was also

proven the best among the decontamination methods evaluated for culture of mycobacteria from bovine faeces as only the faecal samples decontaminated with this method yielded mycobacterial colonies. Potential false positivity, false negativity, and the sensitivity of the phage assay in detecting mycobacterial cells in decontaminated samples was assessed and it was found that the assay would not contribute to any false positive or false negative results. The sensitivity of the assay, on the other hand, could not be determined as the actual condition of mycobacteria within the faecal samples could not be simulated. With this being said, it could be safely assumed that the plaques recovered from the assay were from genuinely infected target mycobacterial cells. 40% of faecal samples decontaminated with the 'optimised method' were found to be mycobacteria-positive. However, when a proportion of plaques recovered (32 in total) were tested via the PCR assay for the presence of either IS*900* or IS*1081* (MAP and *M. tuberculosis* complex-specific respectively), none had shown any positive results. This prompts for further investigation on the reason behind this negativity and perhaps development of methods to improve the PCR assay. It is the belief of this study that once the PCR assay is optimized, the combined assay would be in the near future, a widely used diagnostic method for the detection of JD in bovine species.

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"The difficulties that God put in your life today will make your shoulder stronger to carry greater weights in the future"

That was what you told me. Jophy, I am sorry for always being unavailable when you needed me. Thank you for standing by my side through thick and thin.

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DECLARATION

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

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(JOSHUA ANG XIN DE)

APPROVAL SHEET

This project report entitled "**DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVINE FAECES**" was prepared by JOSHUA ANG XIN DE and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **JOSHUA ANG XIN DE** (ID No: **10ADB07125**) has completed this final year project entitled "**DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND PCR ASSAY FOR DETECTION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVINE FAECES**" under the supervision of Dr. Eddy Cheah Seong Guan (Supervisor) from the Department of Biological Science, Faculty of Science.

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

Yours truly,

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(JOSHUA ANG XIN DE)

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LIST OF ABBREVIATIONS

BCG	Bacille Calmette-Guérin
BHI	Brain heart infusion
$CaCl_2$	calcium chloride
CD	Crohn's disease
CFU	colony-forming unit(s)
dH_20	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAS	ferrous ammonium sulphate
HCl	hydrochloric acid
HPC	Hexyldecylcetylpyridinium chloride
IS	insertion sequence
JD	Johne's disease
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani
LJ	Löwenstein-Jensen
M7H9	Middlebrook 7H9
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MTC	*Mycobacterium tuberculosis* complex
NaCl	sodium chloride
NaOH	sodium hydroxide
NOA	nystatin-oxacillin-aztreonam
NTC	non-template control

NTM	nontuberculous mycobacteria
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
PCR	polymerase chain reaction
PFU	plaque-forming unit(s)
RNA	ribonucleic acid
TNTC	too numerous to count
UV	ultra violet

x g	times gravity
bp	base pair
g	gramme
mL	milliliter
µL	microliter
cm	centimeter
µm	micrometer
M	molar
mM	millimolar
µM	micromolar
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Mycobacterium is categorised within the phylum Actinobacteria, under the family *Mycobacteriaceae*. Due to the complications that have arisen from tuberculosis (TB), a lethal and highly infectious disease, mycobacteriology has become the focal point for research in the field of medical microbiology. The members of the *Mycobacterium tuberculosis* complex (MTC), which include *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium canettii, Mycobacterium microti, and Mycobacterium pinnipedii*, are the causative agents for TB (Ueyama, et al., 2013).

However, the genus *Mycobacterium* does not consist of only the MTC but also the non-tuberculous mycobacteria (NTM), which are also known as the atypical mycobacteria. The NTM include pathogens that can survive in diverse natural habitats and they are also capable of infecting a wide range of hosts (Briancesco, et al., 2013). In fact, the NTM are found in at least half of the potable water samples tested by Shin, et al. (2006) and Fernandez-Rendon, et al. (2011).

The subject of this research, namely *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is an NTM. MAP is the aetiological agent for Johne's disease (JD) in ruminants. Albeit decades of debate on whether it is the causative agent for Crohn's disease (CD) in human, the dispute still carries on due to the inconsistent findings of various researchers (Grant, 2005; Hulten, et al., 2001; Sechi, et al., 2005). Whether it is zoonotic or not, efforts to cease the continual spreading of JD must be intensified as 84.3% of cattle intestinal samples were tested MAP-positive by Pribylova, et al. (2011).

JD is a chronic enteric infection that affects primarily the small intestine of ruminants. Owing to the facts that JD is transmissible via multiple routes and reliable methods for diagnosis, treatment or prevention are non-existent, the disease is penetrating into herds of both wild and domestic ruminants globally at an alarming rate (Chiodini, 2000; Carta, et al., 2012). As MAP is being perceived as a foreign material by the macrophages in the animal, it forms a granuloma at the infected site (most of the time, the intestinal tract) as more macrophages are being deposited in the site. Eventually, the deposition of MAP at the infected site reaches congestion and the congested mucosal epithelium cells are discharged along with the faeces.

The progression of the disease is characterised by decreased milk production, diffused oedema, anaemia, emaciation, high amount of MAP shed in the stools (as much as 10^{12} bacilli per animal daily), and eventually the ruminant will die in a cachectic state. Additionally, it was shown by Gonda, et al. (2007) that on

average, JD-infected cows produced 303.9 kg less milk/lactation, 11.5 kg less fat/lactation, and 9.5 kg less protein/lactation compared to healthy cows. In another report by Ott, et al. (1999), JD costs an average reduced productivity of USD 200-250 million to the US dairy industry annually.

Spreading of the disease will gradually become uncontrollable once MAP is heavily shed in the stools of ruminants at later stages of JD. The MAP in the stools will come into contact with the grazing ground which will then be passed on to the grazers nearby through the faecal-oral route (Singh and Vihan, 2004). However, before any further investigations on the control of JD transmission can be done, it is crucial to first address the problems faced in detecting MAP in the faeces.

The laboratory methods currently used to detect MAP in the faeces include acid-fast bacillus (AFB) smear microscopy, culture, polymerase chain reaction (PCR) targeting the MAP-specific IS*900* sequence found in MAP, and antigen detection assays (Ayele, et al., 2004; Jungersen, et al., 2012; Pavlik, et al., 2000; Timms, et al., 2011). However, each of the methods has its own limitations, rendering them unreliable to be used on faecal samples.

It has been estimated by Allen (1923) that there are approximately 5 million bacteria per gramme of fresh cow faeces while 1 billion bacteria per gramme in those which had dried on the cow's body at near body temperature. With the

presence of microbiota in cow faecal samples, it poses a severe threat to the ability of any methods to stain and view MAP under the microscope, let alone the possibility of any decontamination method to obtain a pure culture of MAP. Additionally, it takes up to 18 weeks for MAP colonies to become observable regardless of the types of culture medium used; which is rather lengthy for a diagnostic process.

The molecular approach using PCR to amplify the IS*900* sequence in MAP, which is a MAP-specific multicopy insertion sequence (IS), seems one of the more promising methods in detecting MAP in cattle milk (Donaghy, et al., 2008). In faeces, however, it is proven otherwise. It has been reported that faecal materials contain PCR inhibitors which could lead to false positive results (Enosawa, et al., 2003). The inability of the approach to differentiate between viable and non-viable cells is also one of its limitations.

One of the drawbacks of the enzyme-linked immunosorbent assay (ELISA), which is a type of immunological test for MAP, is the reduced specificity that results from cross-reaction with environmental mycobacterial species (Nagata, et al., 2013). High genetic relatedness between MAP and other mycobacterial species is the contributor to the difficulty of finding an antigen that could specifically detect MAP without any cross-reactivity (Nielsen and Toft, 2008). Whitlock, et al. (2000) concluded that only repeated serial testing of cattle using ELISA can provide data to determine the true infection rate of an infected herd.

This project aimed to develop a method that can reliably and effectively detect MAP in cow faeces and perhaps becoming the conventional method in the future. Faecal samples were collected from various locations in Kampar and decontaminated with methods similar to that used by Gwóźdź (2006) with several modifications to recover viable MAP cells. The mycobacteriophage amplification assay as described by Stanley, et al. (2007) was used to shorten the time of detection from a few weeks to 48 hours. D29 is a mycobacteriophage used in the phage assay and it infects only microorganisms from the genus *Mycobacterium*. Infected mycobacterial cells will be lysed to form transparent plaques on the agar. Theoretically, each plaque represents one single viable mycobacterium that is present in the cow faeces. A PCR assay targeting the IS*900*, which is present in multiple copies in the MAP genome was conducted to differentiate MAP from other mycobacteria that might be present in the sample.

Hence, this project has the following objectives:

I. To develop a combined phage and molecular assay in detecting viable MAP in bovine faeces
II. To compare and identify the most suitable decontamination method for bovine faecal samples which would be tested for the presence of MAP
III. To investigate the possible occurrences of false positivity and/or false negativity which could affect the results of the combined assay

CHAPTER 2

LITERATURE REVIEW

2.1 *Mycobacterium* in General

2.1.1 Origin of Its Name

Being classified under the phylum *Actinobacteria*, the genus *Mycobacterium* consists of microbes which are thin, non-motile, non-spore forming, rod-shaped bacilli which appear bent or curved (Percival and Williams, 2014). Mycobacterial cells are pleomorphic, which means they are able to appear in shapes ranging from coccoid form to long slender rods. The Latin prefix 'myco-' in the name means both fungus and wax, reflecting the fungal-like morphology of mycobacteria in liquid culture and the waxy compound in their cell wall, respectively.

2.1.2 Classification of Mycobacterial Species

By having growth rates that vary, scientists were able to utilise this criterion to categorise mycobacterial species into two large groups, namely the slow growers and the rapid growers (Brooks, et al., 2013). A slow grower requires more than seven days to form observable colonies on solid culture medium while a period of lesser than seven days is needed for a rapid grower. An example of a rapid grower is *M. smegmatis*. MAP, as a matter of fact, is one of

the slowest growing mycobacterial species among the slow growers (Dow, 2012).

Based on the ability to produce pigments, mycobacterial species can be categorised into photochromogens (species which form pigmented colonies when grown in the presence of light), scotochromogens (species which produce deep yellow to orange colonies when grown in the presence or absence of light), and the nonchromogens (species which do not produce any pigments).

2.1.3 Mycobacterial Cell Wall

Despite the many distinctive features each mycobacterial species seems to possess, there remains a unifying characteristic within the genus itself; one which separates them from other bacterial species – the special composition of their cell wall. The mycobacterial cell wall is made up of a three-component superpolymer consisting of peptidoglycan (the inner layer), arabinogalactan (the middle layer), and mycolic acid (the outer layer) (Barry, 2001). In the case of *M. tuberculosis*, the mycolic acid, which is a type of fatty acid is the main contributing factor to the impermeability of its cell wall to antibiotics and to the body immune system (Bhamidi, et al., 2012). Not only does the mycolic acid help in elevating mycobacterial resistance against various substances which are bactericidal and bacteriostatic, the reduced permeability of the cell

wall to various solutes renders mycobacteria to grow slower than other bacterial species.

Mycolic acid exists not only among the species in the genus *Mycobacterium* but also those in the genera *Nocardia*, *Rhodococcus*, and *Corynebacterium*. It is reported by Verschoor, et al. (2012) that mycolic acid in *Mycobacterium* have the longest carbon chain (approximately 60-90 carbons) among the other genera and that the mycolic acid is an essential component for cell viability only among *Mycobacterium* spp. Due to the high lipid content in its cell wall, mycobacterial cells can retain the carbolfuchsin dye even after decolourisation with acidified alcohol during the Ziehl-Neelsen (ZN) staining, and therefore said to be acid-fast. An acid-fast microbe is stained purplish-red in this staining.

2.2 *Mycobacterium avium* subsp. *paratuberculosis*

MAP is a species grouped under the *M. avium* complex (MAC). Two principal species, *M. avium* and *M. intracellulare*, are included in this complex (Balseiro, et al., 2011). *M. avium* however can be subdivided into four sub-species, namely *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis*, and MAP.

2.2.1 Distinctive Features of MAP

MAP is easily distinguishable from other mycobacteria through one main feature it possesses – the presence of IS*900*. IS*900* is a unique insertion element which is found to be present only in MAP (Green, et al., 1989). Approximately 14-18 copies of the IS*900* gene, which is 1451-bp-long each, can be found in the MAP genome. In fact, a great number of researchers have used this gene as a target sequence for PCR to detect MAP in various samples (Englund, 2003; Kralik, et al., 2011; Millar, et al., 1996; Vansnick, et al., 2007).

2.2.2 Paratuberculosis Strains

MAP, the causative agent of JD, is found to consist of at least 3 strains, each strain classified according to its host preference. Many have tried to classify MAP into Type I, Type II, and Type III strains, which are the sheep strain, cattle strain, and intermediate strains, respectively (Borrmann, et al., 2011; Juan, et al., 2006). Despite of this classification there are still rare cases of cross infection (Abendaño, et al., 2013b). Some other studies have grouped the strains into strain S, strain C, and strain B, each representing the sheep strain, the cattle strain, and the bison strain, respectively. The bison strain was identified following the discovery of a different polymorphism pattern in the IS*1311* gene of MAP which infected bison (Kaur, et al., 2011; Whittington, et al., 2001). Marsh and Whittington (2007) have identified 11 single nucleotide polymorphisms (SNPs) in eight genes, namely *hsp65*, *sodA*, *dnaA*, *dnaN*, *recF*, *gyrB*, *inhA*, and *pks8*, in the S and C strains.

2.2.3 Pathogenicity of MAP

Studies to understand the possible reasons of the prevalence of MAP and its dissemination among ruminants are being done unceasingly over the past decades. Apart from the composition of the mycobacterial cell wall that renders it resistant to various harsh environments, a number of factors have been discovered to aid in the extraordinary persistence of MAP even when facing the ruminant's immune system. The gene *pstA* that promotes the formation of biofilm has long been known to be involved in the virulence of MAP. Taking another step forward based on this presumption, Wu, et al. (2009) have proposed a novel cell wall component that might have contributed to the pathogenicity of MAP. Periasamy, et al. (2013) on the other hand have observed in their study a yet unknown mechanism adapted by MAP to induce apoptosis and necrosis in bovine macrophages. Results from Souza, et al. (2006) indicated that MAP may have exploited the mitogen activated protein kinase pathway to circumvent the antimycobacterial effects of bovine monocytes.

On top of all these, importantly, MAP is the causative agent of Johne's disease in ruminants and the zoonotic potential of it to cause CD in human has yet to be established. Taken together, the effect of MAP infection on both health and the economy should not be underestimated.

2.3 Johne's Disease

Paratuberculosis, also known as JD, is an infectious disease which causes chronic enteritis to ruminants worldwide. Having no reliable means of diagnosis, treatment, or prevention, it is considered by many the most serious infectious disease now damaging the world's cattle, goat, and sheep industries at an alarming rate.

2.3.1 Epidemiology and Development of Johne's Disease

According to Cocito, et al. (1994) and Crossley, et al. (2005), JD progresses through three clinical stages:

I. Stage I - Subclinical in which the infected animal is asymptomatic and no MAP is being shed in the faeces
II. Stage II - Subclinical excretory phase in which the infected animal is still asymptomatic but significant number of MAP is being shed in the faeces, as its concentration within the intestinal mucosa of the ruminant is increasing progressively
III. Stage III - Clinical and excretory phase in which JD is symptomatic (weight loss and diarrhoea which are unresponsive to antibiotic treatment) and heavy shedding of MAP in the faeces occurs

Due to the increasing amount of MAP being shed into the faeces, it becomes apparent that the disease is transmitted mainly by the faecal-oral route in which pasture contaminated with MAP is ingested by healthy cows. The medium of

transmission is not limited to only pastures but includes bedding, maternity pen surfaces, udders, or feeding utensils that have come into contact with MAP-contaminated faeces (Sweeney, 2011). Eisenberg, et al. (2010) have suggested that MAP can be spread throughout the internal and external environments of a dairy barn via bioaerosols. JD has also been found to be vertically transmitted by infected cows to the foetus in their womb. This is supported by the finding of Ayele, et al. (2004) that MAP in the gastrointestinal tract can infiltrate the reproductive organs of infected bovines. This indicates that contaminated semen represents a source of infection. Furthermore, cows may shed MAP directly into milk or colostrums, so consumption of these MAP-infected products by calves can also result in infection.

2.3.2 Impacts of Johne's Disease on The Farming Industry

According to the Economist Intelligence Unit Limited (2005), meat and milk consumption among Malaysians are estimated to expand slowly from 2005 to 2009. On average, a Malaysian was expected to consume as much as 59 litres of milk in 2009, signifying that JD will undoubtedly cause economic losses if it starts to affect local herds. Although no studies have yet to be performed in Malaysia, the effect of JD on the farming industry is apparently severe in the US (Gonda, et al., 2007; Ott, et al., 1999). In-herd prevalence was shown to be fairly consistent irrespective of the region affected. Through different methods of determining the in-herd prevalence of JD, the disease has been documented to prevail in 10% - 20% in herds found in the US, Europe, Denmark, Belgium, Eastern Canada, and Veneto (Boelaert, et al., 2000; Ferrouillet, et al., 2009;

Kreeger, 1991; McKenna, et al., 2004; Nielsen and Toft, 2009; Okura, et al., 2010; Pillars et al., 2011). Herd-level prevalence (percentage of JD-infected herds in a specified region) on the other hand is found to be >50% in all the investigations conducted (Lombard, et al., 2007; Nielsen and Toft, 2009; Pozzato, et al., 2011). New discoveries by Shaughnessy, et al. (2013) suggested MAP-infected rabbits in herds as a reservoir of MAP, leading to the high persistence of JD among livestocks while Carta, et al. (2013) ventured into the possibility that infected wildlife may be the source of infection instead. It is worrying however, with so many evidences put forward, Malaysia has yet to conduct any studies on JD.

2.3.3 Current Strategies for Controlling Johne's Disease

On contrary to the lack of attention to JD in Malaysia, the Europeans, Australians, and Americans are paying much effort to stop the spreading if not eradication of the disease. Strategies such as culling, vaccination, and herd management practices are in place in the farming sector (Juste, 2012; Windsor, 2013). The latter is deemed to be financially the most practical strategy in controlling JD transmission (Johnson-Ifearulundu and Kaneene, 1998; Ridge, et al., 2010). In fact, Australia has had national control and assurance programme since 1996 to help identify and control JD infection (Kennedy and Allworth, 2000). Nevertheless, JD will continue to prevail in cattle herds until a reliable means for early diagnosis is made available in the near future.

2.4 Crohn's Disease

CD is an inflammatory bowel disease that is capable of affecting any parts of the human gastrointestinal tract; it is an autoimmune disorder. It causes dybiosis, a breakdown in the balance of 'harmful' and 'protective' intestinal bacteria (Man et, al., 2011). This chronic condition has no medical cure currently and commonly requires a lifetime of medication. In a report by Bandzar, et al. (2013), approximately 1.4 million of Americans are thought to be affected by CD, out of which 10% of them are under 18 years old. Although only an average of 1.37 inflammatory bowel disease patients per 100,000 population were found in a study by Ng, et al. (2013), CD should not be overlooked as an insignificant disease, as a trend towards an increased diagnosis of CD is being observed in the Asia Pacific region (Wei, et al., 2011).

2.4.1 Clinical Manifestations of Crohn's Disease

Mishina, et al. (1996) and Greenstein (2003), who are in favour of MAP as the aetiological agent of CD, have suggested two forms of clinical manifestations to classify CD in patients, namely the perforating and non-perforating types; the previous being characterised by its aggressiveness while the latter by its indolence. This classification was proposed as these two forms would be analogous to the two forms of leprosy, which is caused by *M. leprae* (Greenstein and Greenstein, 1995). Be it the perforating or non-perforating form, the common symptoms of CD range from weight loss, abdominal pain, reduced appetite, perianal discomfort, to growth impairment and delayed sexual maturation (Bandzar, et al., 2013; Schall, et al., 2002).

2.4.2 Paratuberculosis as Possible Aetiological Agent of Crohn's Disease

The absence of an aetiological agent for CD makes the disease difficult to prevent or cure. Several possibilities of the cause of CD have since its discovery been suggested to enable researchers to understand the disease from an appropriate perspective. These hypotheses include CD being an autoimmune disease due to the pathogenic effect of cytokines, persistent measle virus, and mycobacteria, particularly MAP (Mishina, et al., 1996; Wakefield, et al., 1995).

Although controversial findings on the zoonotic potential of MAP are still being reported, Greenstein (2003) in his review article have however listed a number of sound refuting points to support the possibility of MAP in CD being a zoonosis. The points given in the article include two case reports proving the pathogenicity of MAP in two patients, the presence of MAP in milk, and the extraordinary resistance of MAP against normal chlorine concentration in potable water. Some have detected the presence of the MAP in the tissues of patients with CD and some have found otherwise (Baksh, et al., 2004; Hulten, et al., 2001; Naser, et al., 2000; Sechi, et al., 2005).

2.5. Laboratory Diagnostic Tools for Johne's Disease

There are four main methods currently used to detect MAP in specimens of bovine origin: 1) microscopy; 2) culture; 3) molecular techniques and 4) immunological techniques.

2.5.1 Acid-Fast Bacilli Smear Microscopy

Since its introduction by two Greek doctors in the 18th century, the ZN stain, also known as the acid-fast stain, has been the prominent staining method used to distinguish mycobacteria from other microbes when viewed under the microscope. Shapiro and Hanscheid (2008) suggested that the viewing of ZN-stained slides by fluorescence may increase the detectability of mycobacteria. Fluorescent staining of *M. tuberculosis* with auramine O was also recommended to improve the sensitivity of the method in detecting mycobacteria.

2.5.2 Culture

In normal practices, samples are decontaminated to exterminate non-target microbes, leaving only viable MAP to obtain a pure culture for detection. Culture media used are different from the ones used for most bacteria due to the distinct nutritional requirements of mycobacteria. A variety of solid and liquid media such as the Herrold's egg yolk medium (HEYM), BACTEC medium, Löwenstein-Jensen (LJ) slant, Middlebrook 7H9 (M7H9) broth, Middlebrook 7H10 agar, Middlebrook 7H11 agar, and Dubos broth have been documented to work well for the isolation of MAP (Bower, et al., 2010; Donaghy, et al., 2008; Gwóźdź, 2006; Patel and Shah, 2011; Sivakumar, et al., 2005). Components such as mycobactin J, supplement containing the mixture of oleic acid, albumin, dextrose, and catalase (OADC), oxalic acid, and antibiotic cocktails were added into some of these culture media to elevate

selectivity towards MAP (Dimarelli-Malli, et al., 2013; Fiorentino, et al., 2012; Pavlik, et al., 2000; Pozzato, et al., 2011).

2.5.3 Molecular techniques

Rapid development of molecular techniques to detect MAP in various types of specimens has been observed in the past few decades. Results of various studies have produced a list of MAP-specific sequences which includes the F*57*, IS*Mav2*, *hspX*, genes 251 and 255, IS*Mpa1*, IS*MAP0*, IS*900* and IS*1311* (Castellanos, et al., 2012; Strommenger, et al., 2001). Although these sequences are MAP-specific, some of them which include the ISMpa1 and ISMav2 are also found in other species of the *Mycobacterium* family (Mobius, et al., 2008; Olsen, et al., 2004). Compared to the IS*900* sequence which exists in 14-18 copies in the MAP genome however, the rest are used less frequently in molecular assays for the detection of MAP due to their low copy numbers in the genome (Paustian, et al., 2004).

Modified nucleic acid amplification assays such as the real-time PCR, duplex and multiplex PCR, and the loop-mediated isothermal amplification (LAMP) assay that target the IS*900* gene have shown to be promising in detecting MAP in cultures or directly in specimen (Enosawa, et al., 2003; Kaur, et al., 2011; Kralik, et al., 2011). Yet, molecular-based detection seems to be an unreliable mean to detect MAP in faecal samples as significant cases of false negative have been repeated (Singh, et al., 2007). Cook and Britt (2007) have suggested

that DNA extraction protocols, sampling protocols, and PCR conditions may possibly be the factors causing the false negatives.

2.5.4 Immunological methods

Owing to the fact that ELISA, although a highly specific method to detect MAP in various sample types; is flawed in its significantly low sensitivity level which never exceeds 60% in the studies reviewed (Sockett, et al., 1992; Whitlock, et al., 2000), other attempts becomes all the more important as they might well be better alternatives for MAP detection in the future.

Wang, et al. (2013) have documented in their studies regarding the increase of certain immunological factors in macrophages of JD-infected cattle. The elevated factors include: 1) the enhanced expression of the CD80 and CD86 genes, which code for molecules belonging to the immunoglobulin superfamily, and 2) the over-secretion of IFN-γ and TNF-α, which are macrophage activating factors. Studies have also been done to understand the mechanism of infection of MAP and the reason behind its extraordinary resistance towards bovine immunity. Plattner and Hostetter (2011) and Sohal, et al. (2008) have focused on the effects of $\gamma\delta$ T cells in MAP infection; Abendano, et al. (2013a) have reviewed on works investigating the change in the expression of the cytokines IL-10 and IL-6 as a result of MAP infection; while Woo, et al. (2009) have considered the possibility of ATP elimination in aiding the intracellular survival of MAP.

2.5.5 Combined Phage and Molecular Assay for MAP Detection

The phage assay was originally developed for detection of *M. tuberculosis* directly in tuberculous sputa (McNerney, et al., 1998; McNerney, et al., 2004; Park, et al., 2003). The lytic mycobacteriophage D29 used in this assay infects both fast- and slow-growing mycobacterial species. Based on genome sequencing data, Ford, et al. (1998) have highlighted the implications of phage evolution between L5 and D29, with the deletion of a 36 kb repressor gene in the latter. The deleted gene accounts for the inability of D29 to form lysogens.

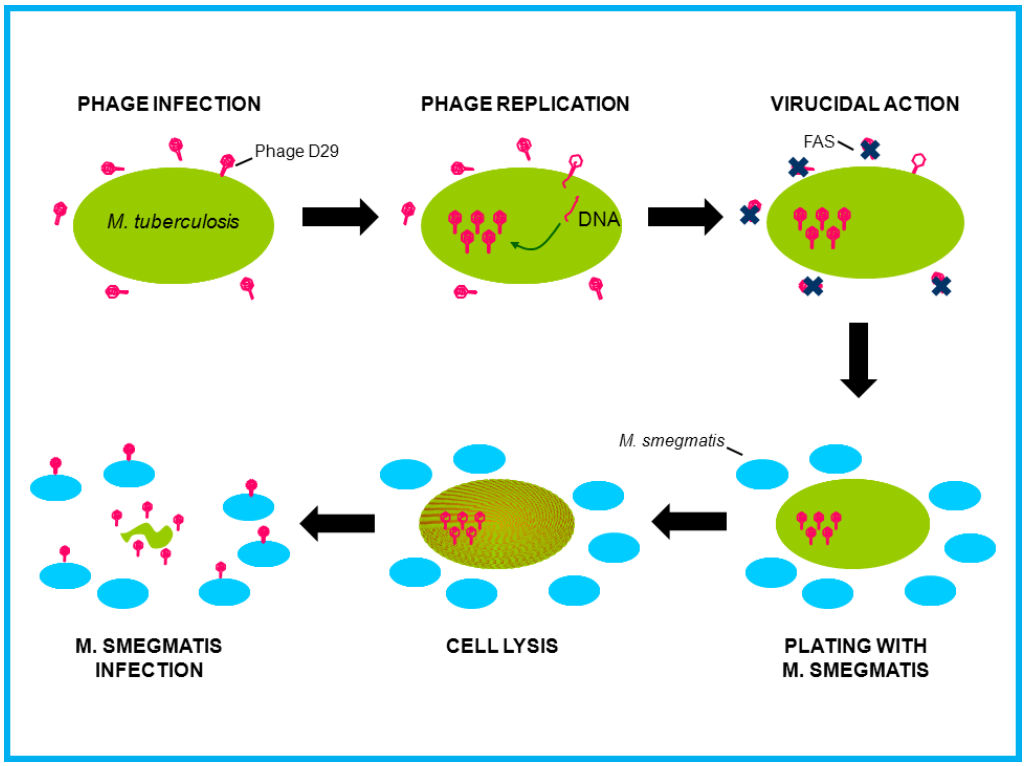

Figure 2.1: Principles of the phage amplification assay. (Cheah, 2010)

As illustrated in Figure 2.1, the phage assay starts with the phage infection process in which D29 is added into the samples which is suspected to contain

mycobacteria. Once infected, time is given for the phage to replicate within the target cells. The virucide ferrous ammonium sulphate (FAS) is then added to the sample before target cell lysis. The role of FAS is to inactivate the extracellular phages that did not infect the target cells. The reaction mixture is then plated out along with *M. smegmatis*, a fast-growing mycobacterial species which acts as sensor cells. As the infected target cells lyse during incubation, sensor cells surrounding the target cells will also be infected. After successive rounds of infection and cell lysis, transparent observable plaques will be formed on the plate. Botsaris, et al. (2013) and Stanley, et al. (2007) have pioneered the specific and quantitative detection of MAP in milk samples through a combined phage-PCR assay with the help of a commercial phage assay (*FASTPlaque*TB) described in their studies. Plaque formed from the phage assay was excised and the plaque DNA was extracted before being subjected to a MAP-specific PCR. Theoretically, one plaque formed on the plate will contain a genome from a lysed target cell as only one D29 is allowed to infect one target cell at a time. Hence, the sample could be concluded to be MAP-positive if the DNA extracted from the plaques is confirmed to contain MAP-specific sequences.

2.6 Bovine Faecal Decontamination Methods

The fact that contaminants obscure the observation of plaques prompts for a solution especially when faecal samples are dealt with. Chemical agents such as oxalic acid, NaOH, benzalkonium chloride, hexyldecyletylpyridinium chloride (HPC), trisodium phosphate, sulphuric acid, and antiformin have been

used in various studies to decontaminate faecal samples prior to culture (Gwóźdź, 2006; Ridge, 1993; Whipple, et al., 1991). Amphotericin B, nalidixic acid, cocktail of vancomycin + amphotericin + nalidixic acid, cocktail of nystatin + oxacillin + aztreonam (NOA), and cocktail of polymyxin B + amphothericin + nalidixic acid + trimethoprim + azlocillin (PANTA) are the examples of antibiotics or antibiotic mixtures added into culture media to kill the residual contaminants that survived the decontamination process.

CHAPTER 3

METHODOLOGY

3.1 Experimental Design

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

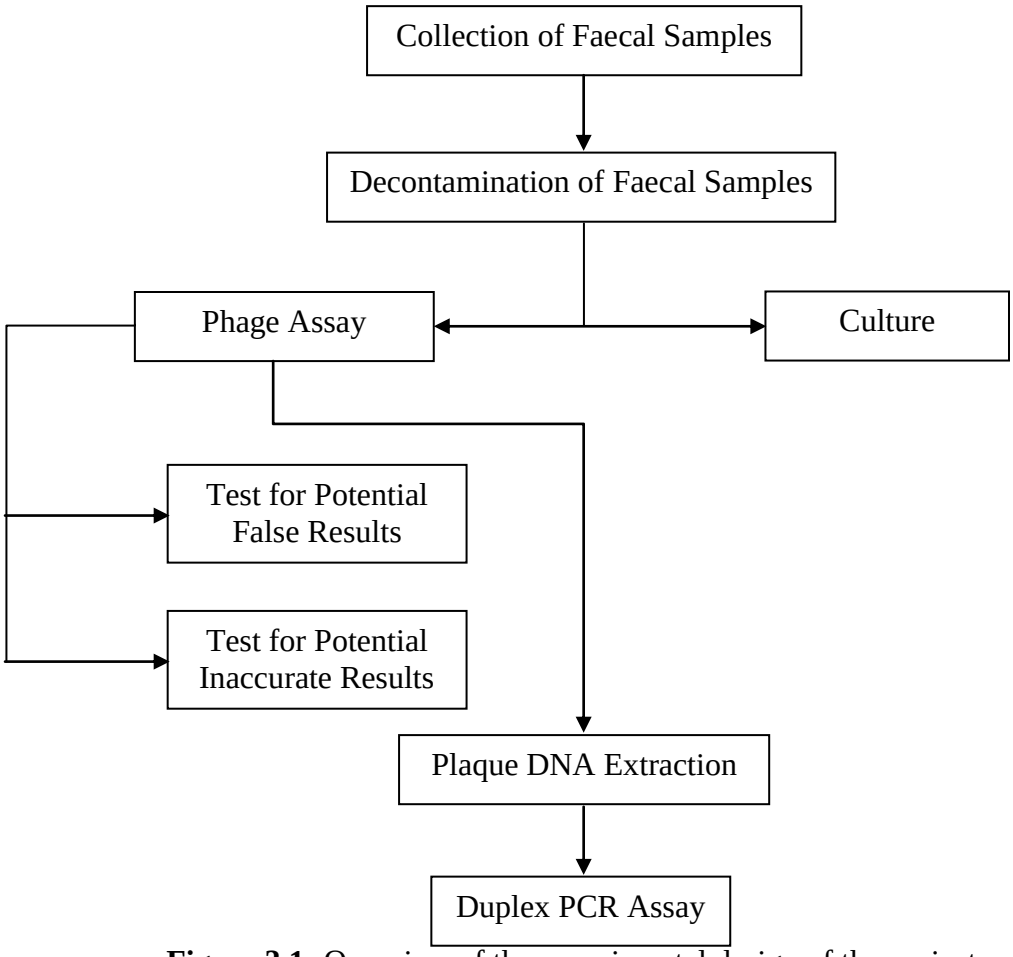

Figure 3.1: Overview of the experimental design of the project.

3.2 Apparatus and Consumables

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

3.3 Preparation of Culture Media

All media were sterilized by autoclaving at 121ºC for 15 minutes unless otherwise stated.

3.3.1 Löwenstein-Jensen Slants

An amount of 9.325 g of (LJ) medium base and 3 mL of glycerol were dissolved in dH_2O to a final volume of 150 mL. The mixture was stirred and boiled for 1 minute and then autoclaved. LJ base with antibiotic was prepared as described above, with the addition of 50 U/mL penicillin G post-autoclaving. The following steps were performed aseptically in the Class 2 microbiological safety cabinet. A volume of 250 mL of eggs was homogenised in a sterile beaker and then filtered through a gauze. The resulting filtrate was mixed with the autoclaved LJ base. Following thorough mixing, the mixture was poured into 30-mL universal bottles in 8-mL aliquots. Then, the media were inspissated at 85ºC for 2-3 hour in slanted position. The LJ slants were then incubated at 37ºC overnight to check for the presence of any contamination.

3.3.2 Luria-Bertani Broth and Agar

(LB) broth was prepared by mixing 10 g of LB broth powder with dH_2O to a final volume of 400 mL. LB agar was prepared by mixing 16 g of LB agar powder with dH_2O to a final volume of 400 mL.

3.3.3 Middlebrook 7H9-OADC Broth

M7H9 broth was prepared by mixing 0.94 g of M7H9 broth powder and 0.5 g of glycerol in dH_2O to a final volume of 180 mL. After autoclaving, the broth was allowed to cool to room temperature and then supplemented with 10% (v/v) OADC before use.

3.3.4 Middlebrook 7H9-OADC-Tween Broth

M7H9 broth was prepared as described above, with the addition of 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 described above, with the addition of 10% (v/v) OADC and 1 mM $CaCl_2$ before use. M7H9-OGC agar was prepared as described for the broth, with the addition of 1.5% (w/v) agar powder.

3.3.6 Middlebrook 7H10 Agar

Middlebrook 7H10 agar was prepared by mixing 7.6 g of Middlebrook 7H10 agar powder and 2.5 g of glycerol with dH_2O to a final volume of 360 mL. The agar was boiled for 5 minutes to dissolve the powder prior to autoclaving. The agar was supplemented with 10% (v/v) OADC before use.

3.4 Preparation of Reagents

All reagents were sterilised by autoclaving at 121ºC for 15 minutes unless otherwise stated.

3.4.1 Acid-alcohol

Acid-alcohol was prepared by mixing 3 mL of concentrated HCl with 97 mL of 95% (v/v) ethanol.

3.4.2 Carbol Fuchsin Solution

Carbol fuchsin stain was prepared by mixing a solution of 0.3 g of basic fuchsin in 10 mL of 95% (v/v) ethanol with a solution of 5 mL of molten phenol crystals in 95 mL of dH_2O. The resulting mixture was left to stand for two days and then filtered through a filter paper before use.

3.4.3 Ferrous Ammonium Sulphate, 50 mM

FAS was prepared fresh before use by dissolving 0.196 g of FAS powder in 10 mL of dH_2O. The solution was filter-sterilised through a 0.22-μm syringe filter.

3.4.4 Hexyldecylpyridinium Chloride or Sodium Hydroxide in Half-Strength Brain Heart Infusion Broth

(HPC) in half-strength BHI broth at 0.75%, 0.85%, and 0.95% (w/v) were prepared fresh by mixing 0.375 g, 0.425 g, and 0.475 g of HPC powder respectively, and 0.925 g of BHI powder with dH_2O to a final volume of 50 mL. Each solution was boiled to allow dissolution of the powder before autoclaving.

NaOH in half-strength BHI broth at 1%, 2%, 4%, and 0.95% (w/v) were prepared fresh by mixing 0.5 g, 1 g, 2 g, and 4 g of NaOH pellet respectively, and 0.925 g of BHI powder with dH_2O to a final volume of 50 mL.

3.4.5 Methylene Blue Solution

Methylene blue solution was prepared by dissolving 0.3 g of methylene blue chloride in 100 mL of dH_2O. The solution was filtered through a filter paper before use.

3.4.6 Mycobacteriophage Buffer

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

3.4.7 Nystatin-Oxacillin-Aztreonam Antibiotic Cocktail

NOA^H was prepared by mixing 11.25 mg/mL nystatin (dissolved in methanol), 153.5 mg/mL oxacillin (dissolved in sterile distilled water), and 9 mg/mL aztreonam (dissolved in formamide-methanol 1:1 mixture) in equal proportions. This NOA^H cocktail was incorporated into the phage indicator agar.

NOA^L was prepared in the same way as described above by mixing 3 mg/mL nystatin, 0.6 mg/mL oxacillin, and 9 mg/mL aztreonam in equal proportions. The final concentrations were 3, 200, and 4.5 times, lower than the NOA^H concentrations, respectively. The NOA^L cocktail following the published concentration and was incorporated into the suspension during overnight resuscitation (Albert, et al., 2007; Biotec Laboratories, 2005).

3.4.8 Saline Solution, 0.9% (w/v)

Saline solution at 0.9% (w/v) was prepared by dissolving 0.45 g of NaCl in dH_2O to a final volume of 50 mL and then vortexed to ensure that a

homogenous solution was obtained. The solution was filter-sterilised through a 0.22-µm syringe filter.

3.4.9 Tris-acetate-EDTA Buffer

Tris-acetate-EDTA (TAE) buffer was prepared by mixing 242 g of TRIS base, 37.2 g of EDTA, and 57.1 mL of glacial acetic acid with dH_2O to a final volume of 1 litre. The buffer was diluted 50 times with dH_2O before use.

3.4.10 Tween 80, 10% (w/v)

Tween 80 solution at 10% (w/v) was prepared by dissolving 10 g of Tween 80 in dH_2O to a final volume of 100 mL and then warmed in the water bath at 40ºC for 30 minutes. The solution was filter-sterilised through a 0.22-µm syringe filter and then stored at 4ºC away from strong light.

3.5 Cultivation of Mycobacteria

3.5.1 Cultivation of *M. bovis* BCG

Cultivation of *M. bovis* BCG was carried out by thawing two 1-mL aliquots of *M. bovis* BCG glycerol stocks and each of the aliquots was inoculated into 5 mL of M7H9-OADC-Tween broth in a 50-mL Falcon tube. Cultures were incubated statically at 37ºC; it took about 10 days to reach an OD_{580nm} of 1. *M. bovis* BCG was subcultured from one of these starter cultures into 25 mL of M7H9-OADC-Tween broth in a 100-mL conical flask to an OD_{580nm} of 0.05,

and grown at 37ºC for 3-4 days. According to Cheah (2010), *M. bovis* BCG doubles approximately every 24 hours in the exponential phase.

3.5.2 Cultivation of *M. smegmatis*

Cultivation of *M. smegmatis* was carried out by streaking a loopful of glycerol stock or a single colony from previous plate culture onto Middlebrook 7H10 agar. For liquid culture, 1 mL of a thawed glycerol stock was inoculated into 5 mL of M7H9-OADC-Tween broth in a 50-mL Falcon tube. Both solid and liquid cultures were grown at 37ºC for 2-3 days, the latter with shaking at 200 rpm. The solid cultures were kept as stocks while the resulting liquid culture was subcultured into 25 mL of M7H9-OADC-Tween broth in a 100-mL conical flask to an OD_{580nm} of 0.05 and grown as before. According to Cheah (2010), *M. smegmatis* doubles approximately every 3 hours in the exponential phase.

3.6 Purity Check and Enumeration of Mycobacterial Cultures

3.6.1 Measuring the Optical Density of Mycobacterial Cultures

The optical density of mycobacterial cultures was measured with a spectrophotometer at the wavelength of 580 nm (OD_{580nm}). Dense cultures ($OD_{580nm} > 1.0$) were diluted ten-fold with M7H9 broth before measurement.

3.6.2 Ziehl-Neelsen Acid-fast Staining

A smear was prepared by emulsifying a loopful of colonies in a drop of dH_2O or spreading a loopful of liquid culture on a clean slide. The smear was then heat-fixed by briefly heating the slide until the smear dried. Subsequently, the slide was flooded with the carbol fuchsin dye and heated until steam was observed and then left for 5 minutes before rinsing with tap water. Next, the stained smear was decolourised with acid-alcohol for a few seconds and then rinsed with tap water again. Finally, the smear was counterstained with the methylene blue dye for 1 minute and then rinsed with tap water. The stained smear was examined under oil immersion at 1000x magnification.

3.6.3 Gram Staining

Bacterial smear was prepared as described in Section 3.5.2. The smear was stained with the crystal violet dye for 1 minute and then rinsed with tap water. Subsequently, the slide was flooded with the iodine solution for 1 minute and then rinsed again with tap water. Then the stained smear was decolourised with a few drops of alcohol for not more than 10 seconds and then rinsed with tap water. Finally, the smear was counterstained with the safranin dye for 1 minute and then rinsed again with tap water. The stained smear was examined under oil immersion at 1000x magnification.

3.6.4 Enumeration of Colony-forming Units

Colony-forming unit (CFU) enumeration was carried out using the drop plate method (Hoben and Somasegaran, 1982). A ten-fold serial dilution of a culture was performed with 450-µL aliquots of M7H9 broth in 1.5-mL Eppendorf tube until a dilution factor of 10^7 was achieved. Then, three 20-µL drops were plated out for each dilution on Middlebrook 7H10 agar (four dilutions per plate). Agar plates were left to dry under laminar air flow and then incubated at 37ºC until single colonies became visible. This took about 2-3 days for *M. smegmatis*. The dilution that produced 10-100 colonies was used to calculate the CFU/mL of the test culture.

3.7 Mycobacteriophage D29

The following methods were performed according to the methods described by Cheah (2010).

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

M. smegmatis culture was grown in M7H9-OADC broth at 37ºC with shaking at 200 rpm for 24 hours. The culture was left to stand for at least 30 minutes to sediment mycobacterial clumps. Then, 1 mL of the upper homogeneous culture suspension was added to 9 mL of molten M7H9-OGC agar (cooled to 55ºC) in a 50-mL Falcon tube. The tube was inverted a few times to ensure adequate mixing before pouring its content into a Petri dish. All phage indicator plates

were left to dry for 10-15 minutes under laminar air flow before use. The plates were stored at 4ºC and used within one week.

3.7.2 Propagation of Phage D29

A volume of 100 µL of phage D29 suspension (approximately 4 x 103 PFU/mL) was pipetted and spread onto phage indicator plate (Section 3.7.1). The plate was incubated at 37ºC for 24 hours. Numerous plaques were formed on the indicator plate following incubation. Then, 10 mL of M7H9-OGC broth was pipetted onto the plate. Following overnight incubation, 5 mL of the phage suspension was transferred into a 50-mL Falcon tube and then filtered twice with 0.45-µm syringe filters. Finally, the phage suspension was distributed in 1-mL aliquots into 1.5-mL Eppendorf tubes and stored at 4ºC away from strong light.

3.7.3 Enumeration of Phage D29 Titre

Ten-fold serial dilutions of a phage D29 suspension were performed in 450-µL aliquots of mycobacteriophage buffer in 1.5-mL Eppendorf tubes until the dilution factor of 10^9. Then, three 10-µL drops of each dilution were spotted onto a phage indicator plate (two dilutions per plate). The plates were left to dry under laminar air flow and then incubated at 37ºC for 24 hours. The dilution that yielded 10-100 plaques was chosen to enumerate the PFU/mL of the phage D29 suspension.

3.7.4 Mycobacteriophage Amplification Assay

A volume of 500 μL of phage D29 suspension (108 PFU/mL) was added to 1 mL of test suspension in a 7-mL Bijoux tube. For the negative control, the sample was replaced with 1 mL of M7H9-OGC broth. The tube was gently swirled to mix its content and then incubated at 37ºC for an hour for phage infection. After incubation, 300 μL of 50 mM FAS was added and the tube content was mixed by vortexing. FAS was used as the virucide in the phage assay to kill exogenous phage D29 particles that did not tranfect the target cells (excess phages). Subsequently, 1 mL of the tube content and 1 mL of *M. smegmatis* lawn culture (Section 3.7.1) were pipetted into 9 mL of molten M7H9-OGC agar (cooled to 55ºC) in a 50-mL Falcon tube. A volume of 100 μL of NOA^H was also added to the tube content for the optimised decontamination method (Table 3.1). The tube was inverted three times to mix its content before pouring into a Petri dish. Finally, the plate was incubated at 37ºC for up to 24 hours for visible plaque formation. The plaques that were formed after the incubation were enumerated.

3.8 Collection of Faecal Samples

Faecal samples were collected from cattle farms, fields, and roadsides in Kampar, Malaysia. A sterile spatula was used to scoop fresh and wet bovine faeces into a sterile 50 mL Falcon tube. Whenever a new pile of faeces was to be collected, the spatula was wiped with a piece of tissue and decontaminated with 70% (v/v) ethanol beforehand. All faecal samples were stored at 4ºC until processing. The areas where the faecal samples were collected included the

road beside UTAR East Gate (EG; three samples), cattle farm in Kampung Batu Tersusun Batu Putih (BP; two samples), cattle farm in Taman Mahsuri (TM; one sample), roadside around KTM station (KK; two samples), and the roadside of Sekolah Kebangsaan Sentosa (SS; six samples). The alphabetical labels are referred as such from this point onward.

3.9 Decontamination of Faecal Samples

An amount of 4 g of faeces was transferred into a 50-mL Falcon tube and then 20 mL of dH_2O added to resuspend the faeces. The tube was agitated on a horizontal shaker at 200 rpm for 30 minutes and then left for 30 minutes to sediment large faecal particles. After that, 10 mL of the resulting supernatant was pipetted into a new 50-mL Falcon tube and centrifugation was performed at 2000 x g for 15 minutes at 4°C. The resulting pellet was then treated with a mixture of 2 mL of 0.9% (w/v) saline solution and 8 mL of decontaminating agent (HPC or NaOH) dissolved in half-strength BHI broth. Several treatment durations were tested. The suspension was centrifuged at 2000 x g for 30 minutes at 4°C and the resulting pellet resuspended in 1.5 mL of M7H9-OGC broth with or without 15 µL of NOA^L. The suspension was incubated at 37°C for 24 hours to allow resuscitation of chemically stressed mycobacterial cells that might be present in the sample. Finally, 100 µL of the suspension was inoculated onto an LJ slant with or without 50 U/mL of penicillin while 1 mL of it was proceeded to the phage assay as described in Section 3.7.4. Table 3.1 gives a detailed description on the manipulated variables of numerous decontamination methods tested.

Table 3.1: Various parameters of different decontamination methods for bovine faecal samples for downstream analysis by the phage assay.

Decontamination method	Decont. agent	Concentration of decont. agent (%)	Treatment duration (hour)	Presence of NOA	
				Overnight resuscitation (NOA^L)	Phage indicator plate (NOA^H)
DM1	HPC	0.75, 0.85, 0.95	18	-	-
DM2	NaOH	1, 2, 4, 8	18	-	-
DM3	NaOH	1, 2, 4, 8	2	-	-
DM4	NaOH	2	0.25	+	-
DM5	NaOH	2	2	+	-
DM6	NaOH	1, 2, 4, 8	0.25	+	+

Decont. – Decontamination; '+' – Present; '-' – Absent

3.10 Detection of Target Mycobacterial DNA in Phage D29 Plaques

3.10.1 Plaque DNA Extraction

Plaque DNA extraction was carried out according to the method described by Stanley, et al. (2007). Firstly, 10-µL pipette tips were used to excise plaques from the phage indicator plates. The excised plaques were transferred into a 1.5-mL Eppendorf tube each, to which 10 µL of deionised water was added. The tube content was heated at 95ºC for 15 minutes and then pulse centrifuged. The tube was left at -20ºC for at least 30 minutes. The tube was then thawed and centrifuged at 16100 x g for 2 minutes. The resulting supernatant was subjected to the duplex IS*900*/IS*1081* PCR assay (Section 3.8.2).

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

The duplex IS*900*/IS*1081* PCR assay was carried out in 25-µL reaction containing 10 µL of plaque DNA extract, 75 ng of each primer IS*900*F, IS*900*R, IS*1081*F, and IS*1081*R, 1x DreamTaq buffer, 250 µM dNTPs, 1 mM $MgCl_2$ and 0.5 U of DreamTaq DNA polymerase (Stanley, et al., 2007). PCR was performed as follows: 1) 95ºC for 3 minutes to activate the *Taq* DNA polymerase; 2) 37 cycles of 95ºC for 30 seconds, 62ºC for 30 seconds, and 72ºC for 1 minute and 3) final extension at 72ºC for 4 minutes. Table 3.2 shows the primer sequences and the lengths of the amplified products. The primer pairs used to amplify both IS*900* and IS*1081* were designed by Whittington, et al. (1998) and Ahmed, et al. (1998), respectively.

Table 3.2: Primer sequences and length of their corresponding amplified products for PCR assay.

Primer	Sequence (5' – 3')	Length of product (bp)
IS*1081*F	CGCACCGAGCAGCTTCTGGCTG	306
IS*1081*R	GTCGCCACCACGCTGGCTGTG	
IS*900*F	GTTCGGGGCCGTCGCTTAGG	400
IS*900*R	GAGGTCGATCGCCCACGTGA	

3.10.3 Gel Analysis of PCR Amplicons

The amplicons from the duplex PCR were separated on 2% (w/v) agarose gels. A volume of 15 µL for each reaction was analysed on the gel along with 2.5 µg of GeneRuler 100-bp DNA ladder. Electrophoresis was performed at 80 V for 30 minutes. Then the gel was stained with 0.5 µg/mL ethidium bromide for 10 minutes and then destained with dH_2O for 2 minutes before viewing under the UV transilluminator.

CHAPTER 4

RESULTS

4.1 Physical Appearance of Bovine Faecal Samples

Of all the samples collected which appeared brown, damp, and firm, there was one particular sample from Kampung Tersusun Batu Putih (BP) which appeared dark brown and liquefied. An example of the appearance of a brown, damp, and firm faeces is shown in Figure 4.1.

Figure 4.1: Appearance of freshly-shed faeces on sandy ground.

4.2 Evaluation of Various Decontamination Methods for Detection of MAP in Bovine Faeces

4.2.1 Detection via the Phage Assay

Various parameters of the decontamination methods were tested to obtain an optimal method which could successfully decontaminate faecal samples in the shortest possible time and at the same time allow growth of the *M. smegmatis* lawn. Faecal samples which were not decontaminated prior to the phage assay expectedly exhibited heavy contamination which would have masked any plaques that had been formed (Figure 4.2).

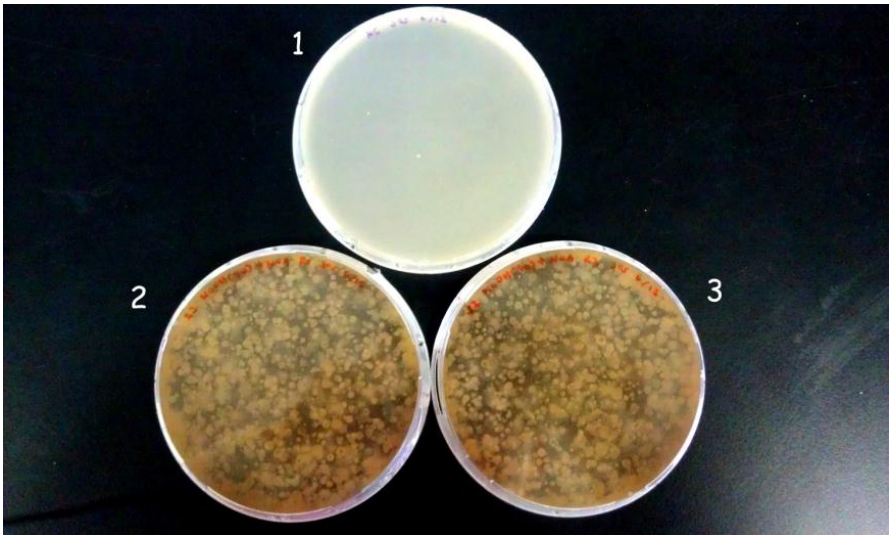

Figure 4.2: Appearance of the phage indicator plates for non-decontaminated bovine faecal samples. 1) Phage assay negative control; 2) and 3) Heavy contamination masking plaques if any were formed.

Figure 4.3 illustrates the process of optimising the decontamination method into the final 'optimised method', which could meet the criteria stated above for the phage assay.

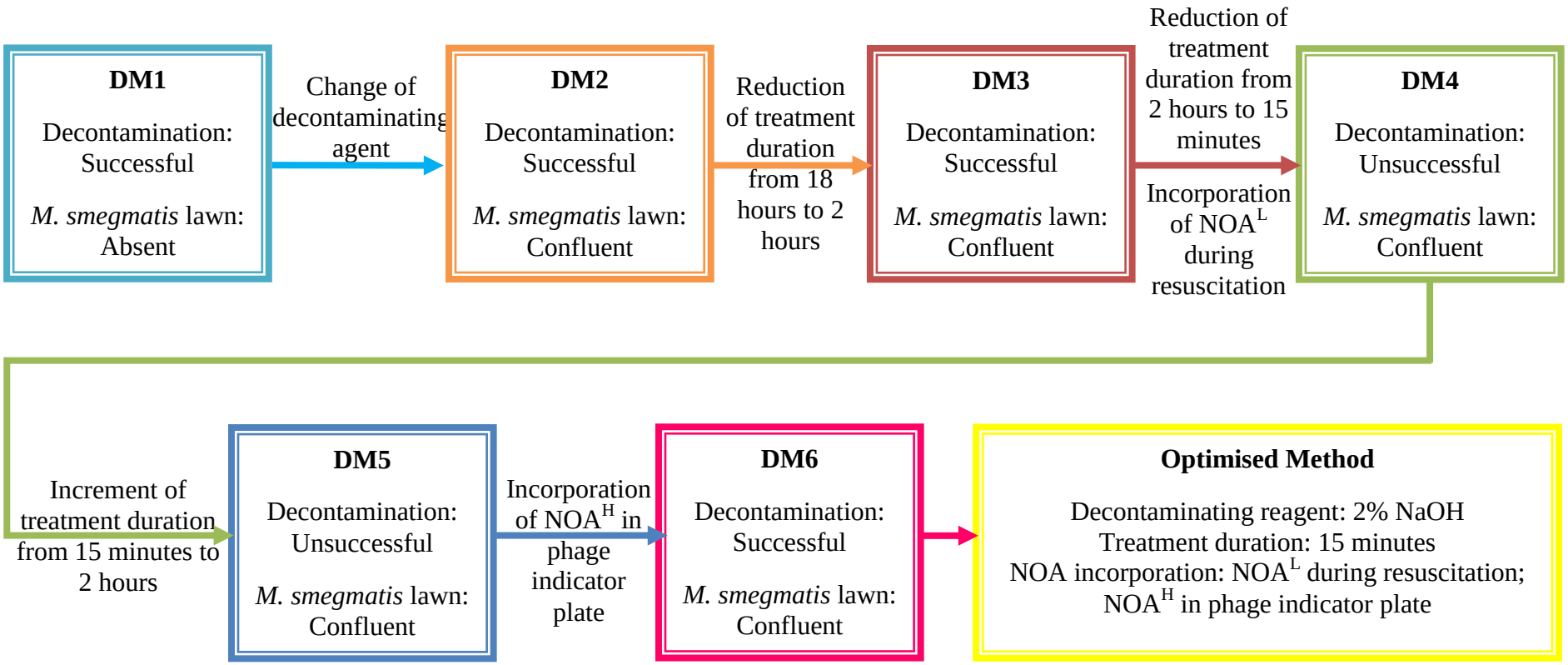

Figure 4.3: The process of optimisation of decontamination method. Successful – No contaminants which interfered with the observation of the plaques formed; Unsuccessful – Presence of contaminants which interfered with the observation of the plaques formed.

HPC used at concentrations of 0.75%, 0.85%, and 0.95% were proven to be able to decontaminate faecal sample EG1 for the phage assay as contaminants were absent in all plates. In a total of six phage indicator plates tested (two for each HPC concentration), 0% contamination rate was achieved. The *M. smegmatis* lawn, however, was not visible, therefore causing no plaques to be observable, if any were present. Figure 4.4 illustrates the difference in appearance between a phage indicator plate with confluent *M. smegmatis* lawn which appeared rather opaque and one without confluent *M. smegmatis* lawn which appeared rather translucent.

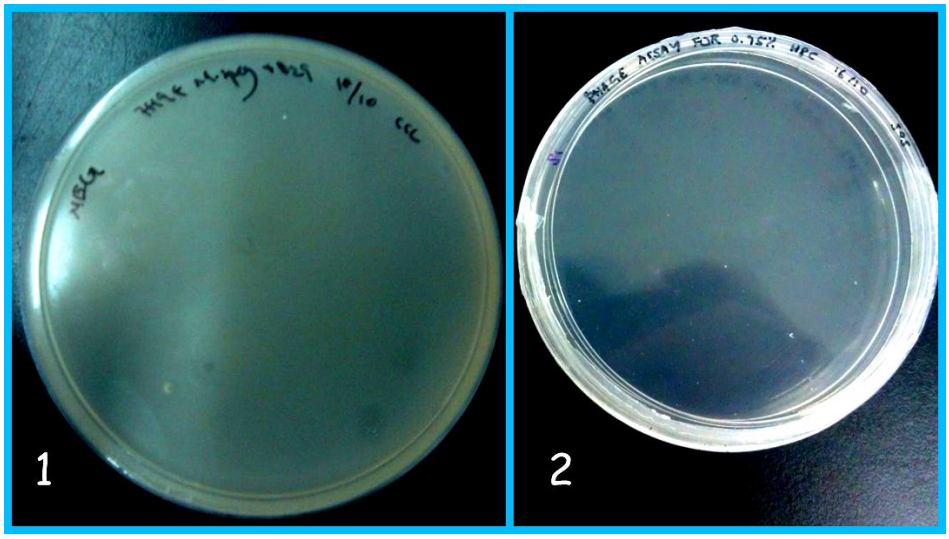

Figure 4.4: Appearance of the phage indicator plates. 1) Presence of confluent *M. smegmatis* lawn; 2) Absence of confluent *M. smegmatis* lawn

The absence of *M. smegmatis* lawn in the phage indicator plates of faecal samples decontaminated with HPC prompted its substitution with NaOH. Results were clearly promising as phage indicator plates for all NaOH-treated samples (excluding those with heavy contamination) showed confluent growth of the *M. smegmatis* lawn, regardless of the methods (DM2 to DM6) tested. However, the use of 1% NaOH in DM3 appeared to be insufficient in decontaminating faecal samples for the phage assay (Table 4.1). The failures of DM4 and DM5 to effectively decontaminate the faecal samples were verified as all of the phage indicator plates from trials 9 to 14 were all heavily contaminated. For all the decontamination methods assessed, the phage indicator plates for most of the samples exhibited either TNTC or 0 PFU, except for those decontaminated with 1% and 2% NaOH of DM2 (sample BP1) and 1% NaOH in DM6. For the former, 1 and 243 PFUs were yielded, respectively, while for the latter, the duplicate reactions produced 183 and 62 PFUs, respectively.

Table 4.1: Assessment of the phage indicator plates for bovine faecal samples subjected to various decontamination methods for the presence of contamination and PFUs.

Number*	Method	Sample	NaOH concentration (%)	Contamination	Number of PFU(s)[†]
1	DM2	BP1	1	Absent	1
2			2	Absent	243
3			4	Absent	Unsure
4			8	Absent	TNTC
5	DM3	TM1	1	Present	0
6			2	Absent	TNTC
7			4	Absent	0
8			8	Absent	0
9	DM4	EG2	2	Present	0
10		EG3	2	Present	0
11	DM5	BP1	2	Present	0, 0
12		EG2	2	Present	0, 0
13	DM6	TM1	1	Absent	183, 62
14			2	Absent	TNTC, TNTC
15			4	Absent	0, 0
16			8	Absent	0, 0

[†]TNTC – Too numerous to count (10^3-10^4 PFUs per plate with some merging of individual plaques); Unsure – The appearance of the medium was not indicative of whether the lawn was completely lysed or no plaques were formed.

*Faecal samples for trials 11 to 16 were tested in duplicates.

4.2.2 Detection via Culture on Löwenstein-Jensen Slants

Both DM1 and DM2 were evaluated for their feasibilities in decontaminating bovine faecal samples for culture isolation on LJ slants; the effect of incorporation of penicillin (50 U/mL) in the slants was also assessed for the latter. The presence of contamination in the LJ cultures, due to the growth of rapid-growing non-acid-fast microbes, was assessed by observing the physical changes in the slants. Contaminated LJ slants would exhibit gradual increase in the intensity of the colour of malachite green, and would eventually liquefy and collapse with the release of foul smell, as observed in this study (Figures 4.5 and 4.6).

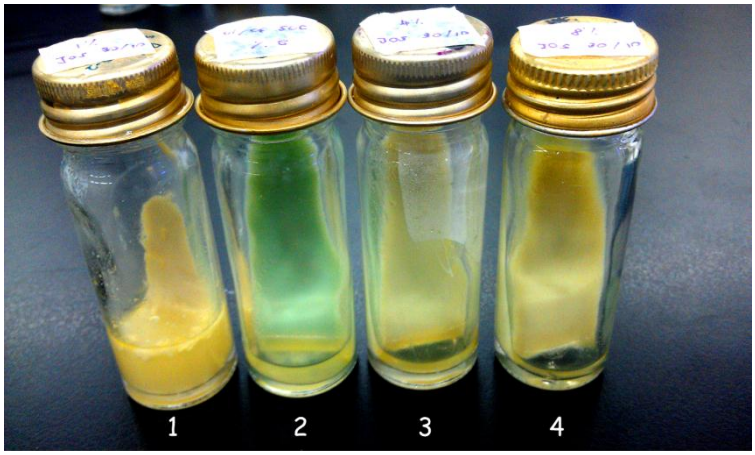

Figure 4.5: Appearance of LJ slants without penicillin inoculated with BP1 which was subjected to DM1 at day 7 of incubation. 1) Yellow and moderate liquefaction; 2) Green and firm; 3) and 4) Yellow and firm.

Figure 4.6: Appearance of LJ slants with penicillin inoculated with BP1 which was subjected to DM2 at day 7 of incubation. 1) Complete liquefaction; 2), 3), and 4) Yellow and firm.

With DM1, the use of 0.95% HPC seemed to be more effective in decontaminating the test faecal sample (EG1) compared to the other HPC concentrations tested (Table 4.2). At day 14 after inoculation, only its slant remained firm while the others were either completely or partially liquefied. The colonies that grew on this slant were ZN-positive but non-acid-fast contaminants (blue cocci) were also observed among the acid-fast bacilli (image 1, Figure 4.7).

However, when being compared to treatment with NaOH, HPC in general could be concluded to be a least effective decontaminating agent. As much as 75% of the LJ slants inoculated with faecal samples decontaminated with NaOH were still yellow and firm after day 14 of inoculation, while most of those decontaminated with HPC had experienced certain degrees of liquefaction by this time (Tables 4.2 and 4.3). Complete liquefaction within two weeks of inoculation proved that 1% NaOH is insufficient in eliminating

non-target microbes. Mold-like growth, which was not stained by both the Gram stain and ZN stain, was observed in slant 8 within the first two weeks (data not shown).

For the surviving LJ slants, those inoculated with the faecal samples decontaminated by 4% and 8% NaOH were hardened and dried up in the third week, though the media remained yellow in colour. Isolated colonies from slant 6 in Table 4.3, which were tested to consist of relatively pure acid-fast bacilli (image 2, Figure 4.7), had non-pigmented morphology on its slant. This could imply that the treatment with 2% NaOH for 18 hours and the inoculation of the treated sample onto penicillin-supplemented LJ slants was probably the best among the other methods for culture isolation of mycobacteria from bovine faecal samples.

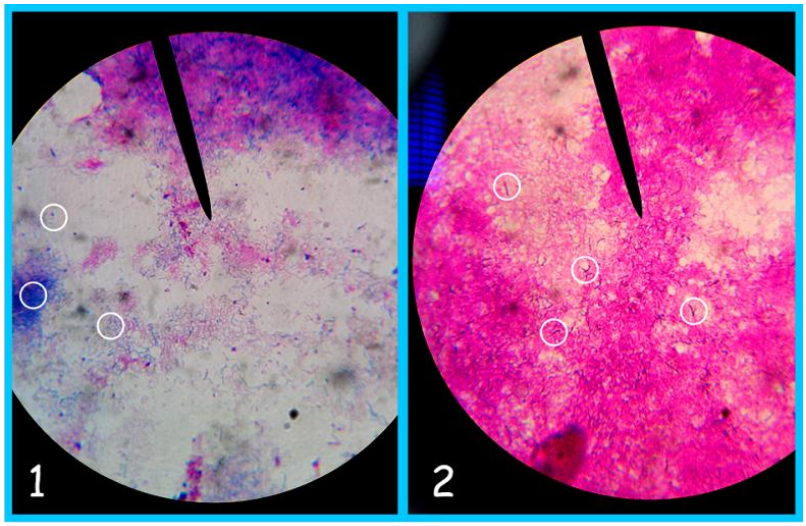

Figure 4.7: ZN-stained isolates from LJ slants observed under oil immersion (1000X). 1) Isolates from EG1 decontaminated with 0.95% HPC; 2) Isolates from BP1 decontaminated with 2% NaOH with penicillin added to the LJ slant.

*White circles in 1) and 2) represent blue-coloured, coccus-shaped contaminants and red coloured rod-shaped mycobacterial cells, respectively.

Table 4.2: Visual appearance of LJ slants inoculated with EG1 which was subjected to DM1.

No.	HPC (%)	Visual appearance of LJ slants[†]		
		Day 1	Day 7	Day 14
1	0.00	Green and firm	Yellow and completely liquefied	Discarded
2	0.75	Slightly greenish and firm	Yellow and slightly liquefied	Yellow and completely liquefied
3	0.85	Yellow and firm	Slightly greenish and firm	Slightly greenish and liquefied
4	0.95	Yellow and firm	Slightly greenish and firm	Green and firm

[†]Discarded – LJ slant liquefied and collapsed before day 14.

Table 4.3: Visual appearance of LJ slants with and without penicillin inoculated with BP1 which was subjected to DM2.

No.	NaOH (%)	Penicillin	Visual appearance of LJ slants[†]		
			Day 1	Day 7	Day 14
1	1	-	Green and firm	Yellow and moderately liquefied	Discarded
2	2	-	Green and firm	Green and firm	Yellow and firm
3	4	-	Yellow and firm	Yellow and firm	Yellow and firm
4	8	-	Yellow and firm	Yellow and firm	Yellow and firm
5	1	+	Green and firm	Grayish and completely liquefied	Discarded
6	2	+	Yellow and firm	Yellow and firm	Yellow and firm
7	4	+	Yellow and firm	Yellow and firm	Yellow and firm
8	8	+	Yellow and firm	Yellow and firm	Yellow and firm

[†]Discarded – LJ slant liquefied and collapsed before day 14; '+' – Present; '-' – Absent.

4.3 Assessment for the Reliability of the Results of the Phage Assay with Decontaminated Bovine Faecal Samples

4.3.1 Assessment for Potential of False Negative Results

In the event that the faecal residues remaining in the decontaminated suspension inhibited the downstream phage assay, no plaques will be observed in the phage indicator plate following incubation, even if the test faeces is mycobacteria-positive (i.e. false negative result). The potential for this inhibition was investigated with decontaminated faecal suspensions in which *M. smegmatis* (model for MAP in this project) cells were added.

M. smegmatis culture suspension (approximately 100 to 200 CFU/mL) was transferred to nine 1.5-mL Eppendorf tubes (eight samples and one positive control), 1 mL each. The tubes were centrifuged at 16100 x g for two minutes and the resulting supernatants were discarded. A total of eight BP1 samples (2 for each concentration of NaOH) were divided into two sets, namely A and B, and were decontaminated by DM2 to produce test suspensions for further processing. After decontamination, set A was subjected to the phage assay; 1 mL of each test suspension in set B was used to resuspend the *M. smegmatis* pellet prepared previously and the resulting cell suspension was subjected to the phage assay. A positive control was performed with the suspension of the *M. smegmatis* cell pellet in M7H9-OGC broth to determine the number of PFUs that could be recovered from the *M. smegmatis* pellet alone.

The positive control produced 12 PFUs. Surprisingly, for samples decontaminated with 1% and 2% of NaOH, plaques formed in sets A and B differed by around 32 plaques (data not shown). The results for the samples decontaminated with 4% and 8% NaOH, respectively, were not able to be compared as each exhibited either confluent or complete lysis of the *M. smegmatis* lawn. Hence, it is shown that no significant false negativity was present for faecal samples decontaminated with 1% and 2% of NaOH.

4.3.2 Assessment for Potential of False Positive Results

In the event that the faecal or chemical residues remaining in the decontaminated suspension interfered with FAS inactivation during the phage assay, breakthrough plaques will be observed in the phage indicator plate following incubation (i.e. false positive result). The potential for the occurrence of these background PFUs was investigated with autoclaved faecal suspensions (BP1 and EG2) and no-sample controls. The faecal samples were autoclaved at the suspension stage rather than at the initial stage to ensure better killing of mycobacteria, if present, since faecal solids might shield some of them from being killed for the latter. Complete absence of living microbes in the autoclaved faecal suspensions was assumed in this experiment.

The faecal samples were processed as described in Section 3.6.2 until after they were rocked on the horizontal shaker; the resulting suspensions were harvested and autoclaved at 121ºC for 15 minutes. They were then

decontaminated using the optimised method (2% NaOH + NOA). The no-sample controls were subjected to DM2. Following the phage assay, no plaques were observed in the phage indicator plates for both the autoclaved faecal suspensions and the no-sample controls. This clearly shows that both the optimised method (2% NaOH + NOA) and DM2 do not generate false positive results.

If the plaques observed in the indicator plates were true positives, the removal of the overnight resuscitation step might result in underestimated plaque numbers, since chemical stress in the target mycobacterial cells post-decontamination might render them invulnerable to phage infection. This was also investigated; samples TM1, BP1, and EG1 were used in this test. The samples were decontaminated with 2% NaOH via DM2 and then divided into two sets (A and B) of duplicate reactions for each sample. The decontaminated suspensions in set A were subjected immediately to the phage assay while those in set B were incubated at 37°C for about 18 hours ('resuscitation') prior to the phage assay. As shown in Table 4.4, prior overnight resuscitation of decontaminated faecal samples resulted in significantly higher PFU counts in the phage indicator plates than those without resuscitation. This result further supports the fact that plaques detected from bovine faecal samples decontaminated with NaOH, were genuinely from mycobacterial cells.

Table 4.4: Effect of overnight resuscitation of decontaminated bovine faecal samples in M7H9-OGC-NOA broth at 37°C on PFU recovery in the phage assay

Sample	Replicate	Number of PFUs[†]	
		No resuscitation	With resuscitation
TM1	1	None	Unsure
	2	None	Unsure
BP1	1	18	Confluent lysis
	2	150	Confluent lysis
EG1	1	92	Confluent lysis
	2	242	Confluent lysis

[†]Unsure – The appearance of the medium was not indicative of whether the *M. smegmatis* lawn was completely lysed or no plaques were formed; Confluent lysis – lysis of 80-90% of the *M. smegmatis* lawn, indicating the detection of 10^4 to 10^5 mycobacterial cells

4.4 Evaluation of the Sensitivity of the Phage Assay in Detection of Mycobacterial Cells in Decontaminated Bovine Faecal Samples

Autoclaved faecal sample (EG1) was used in this experiment and initial absence of mycobacteria due to autoclave killing was assumed. The autoclaved faecal sample was distributed into four 4-g aliquots; two were spiked with 10^7 *M. bovis* BCG CFUs each and the other two were spiked with 10^3 *M. bovis* BCG CFUs each. The spiked faecal samples were then decontaminated via the 'optimised method' (2% NaOH + NOA) and then subjected to the phage assay. None of the resulting phage indicator plates exhibited any plaque formation. However, the phage assay with *M. smegmatis* culture was demonstrated to be considerably sensitive, with the detection limit of 100-1000 CFU/mL (Table 4.5).

Table 4.5: Sensitivity of the phage assay with *M. smegmatis* culture.

Number of CFU/mL tested	Number of PFU(s)/mL recovered[†]
10^8	Complete lysis
10^7	Complete lysis
10^6	Complete lysis
10^5	Complete lysis
10^4	Confluent lysis
10^3	264
10^2	28
10	0
Negative control	0

[†]Complete lysis – lysis of 100% of the *M. smegmatis* lawn, indicating the detection of more than 10^5 mycobacterial cells; Confluent lysis – lysis of 80-90% of the *M. smegmatis* lawn, indicating the detection of 10^4 to 10^5 mycobacterial cells

4.5 Analysis of Decontaminated Bovine Faecal Samples for the Potential Presence of Mycobacteria by the Phage Assay

Ten bovine faecal samples from four sampling sites, namely the cattle farm in Kampung Batu Tersusun Batu Putih (BP), the road beside the UTAR East Gate (EG), the road in front of the KTM station (KK), and around Sekolah Kebangsaan Sentosa (SS), were screened in duplicate each. They were decontaminated with the 'optimised method' (2% NaOH + NOA) and then subjected to the phage assay. A 0% contamination rate was achieved for all samples. Out of the ten samples tested, four (40%) were positive for mycobacteria, as evident from the plaques observed in their phage indicator plates (Table 4.6). They were from BP, EG, and KK, respectively. The levels of positivity were in the range of 10^3 to 10^5 mycobacterial cells. However, the PFU positivity for one of the replicates for both the KK samples could not be confirmed as the appearance of the indicator plate was not indicative of whether the *M. smegmatis* lawn was completely lysed (complete lysis) or no

plaques were formed. The faecal samples from SS were consistently negative for mycobacteria, as indicated by no plaque formation in their indicator plates; the yellow colouration of the plates by faecal residues might have obscured the observation of the plaques possibly formed (Table 4.6). Figure 4.8 illustrates the appearance of the indicator plates for the faecal samples that were mycobacteria-positive.

Table 4.6: Number of plaques observed in the phage indicator plates of various bovine faecal samples decontaminated with the 'optimised method'.

Number	Sample	Number of plaques[†]
1	BP2	CF, TNTC
2	EG3	CF, CF
3	KK1	Unsure, CF
4	KK2	Unsure, CF
5	SS1	0, 0
6	SS2	0, 0
7	SS3	0, 0
8	SS4	0, 0
9	SS5	0, 0
10	SS6	0, 0

[†]CF – Confluent lysis (lysis of 80-90% of the *M. smegmatis* lawn, indicating the detection of 10^4 to 10^5 mycobacterial cells); TNTC – Too numerous to count (10^3-10^4); Unsure – The appearance of the medium was not indicative of whether the *M. smegmatis* lawn was completely lysed or no plaques were formed.

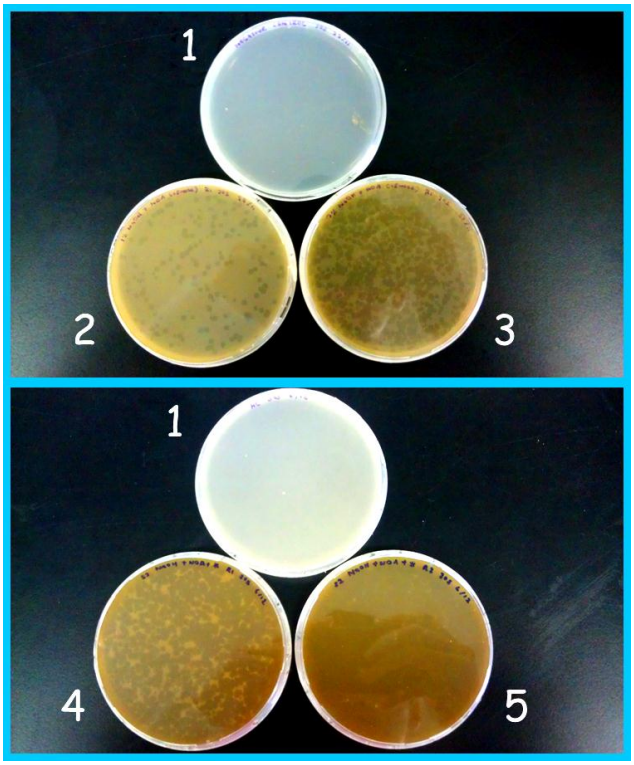

Figure 4.8: Phage indicator plates for different bovine faecal samples showing different levels of PFU positivity. 1) Phage assay negative control; 2) Countable plaques; 3) TNTC; 4) Confluent lysis; 5) Complete lysis[†].

[†]Complete lysis – lysis of all of the *M. smegmatis* lawn, indicating the detection of more than 10^5 mycobacterial cells.

4.6 Investigation of the Feasibility of the Duplex IS*900*/IS*1081* PCR Assay in Detecting Target Mycobacterial DNA in Phage D29 Plaques

Due to the nature of the mycobacteriophage (D29) used, the phage assay is not specific to a particular mycobacterial species. In order to add specificity to the phage assay, a molecular assay is to be performed downstream for specific detection of target mycobacterial DNA in phage D29 plaques. Theoretically, there should only be one genome from a lysed target cell in a positive plaque, and by targeting a multicopy gene such as the IS, detection by a molecular assay is possible. The initial intention was to use a LAMP-based assay but the frequent occurrence of non-specific amplification and false positive fluorescent

signals led to it being abandoned (H. K. Tee, personal communication). The duplex IS*900*/IS*1081* PCR assay, which targets MAP and the MTC complex, respectively, was evaluated instead.

The PCR reaction with the culture DNA extract of *M. bovis* BCG, the positive control for the IS*1081* PCR, yielded a 306-bp band on the gel as expected (Figure 4.9). No amplification was detected for the *M. smegmatis* reaction (negative control) for both IS*900* and IS*1081*, again as expected. However, another negative control, that with *E. coli*, yielded a band of approximately 200-bp in size.

For PCR with plaque DNA extracts from mycobacteria-positive faecal samples, the number of plaques tested for each sample (at least three per sample if possible) was as follow: 12 plaques for BP1, 9 plaques for TM1, 8 plaques for EG1, and 3 plaques for BP2. Out of the 32 plaques examined, none were positive for either IS*900* or IS*1081*; three of the 12 reactions for BP1 are shown in Figure 4.9. Three plaques from the phage assay with *M. bovis* BCG culture were also tested for IS*1081* positivity, but no 306-bp bands were observed upon gel analysis (Figure 4.9).

The potential of PCR inhibition by interfering substances that were co-extracted with the plaque DNA through the extraction procedure was investigated. Instead of excising the plaques formed, areas of the phage

indicator plate that had no plaques were excised and extracted via the same method used for excised plaques. The resulting suspensions (non-plaque plate extracts) were spiked with 2 μL of *M. bovis* BCG DNA each and then subjected to the duplex PCR assay. Upon gel analysis, bands that were 306 bp in size were observed in lanes 14, 15, and 16 (Figure 4.9). This finding indicates that the negative reactions for positive plaques from faecal samples were very likely due to the absence of target DNA, rather than inhibition by medium residues in the plaque DNA extracts.

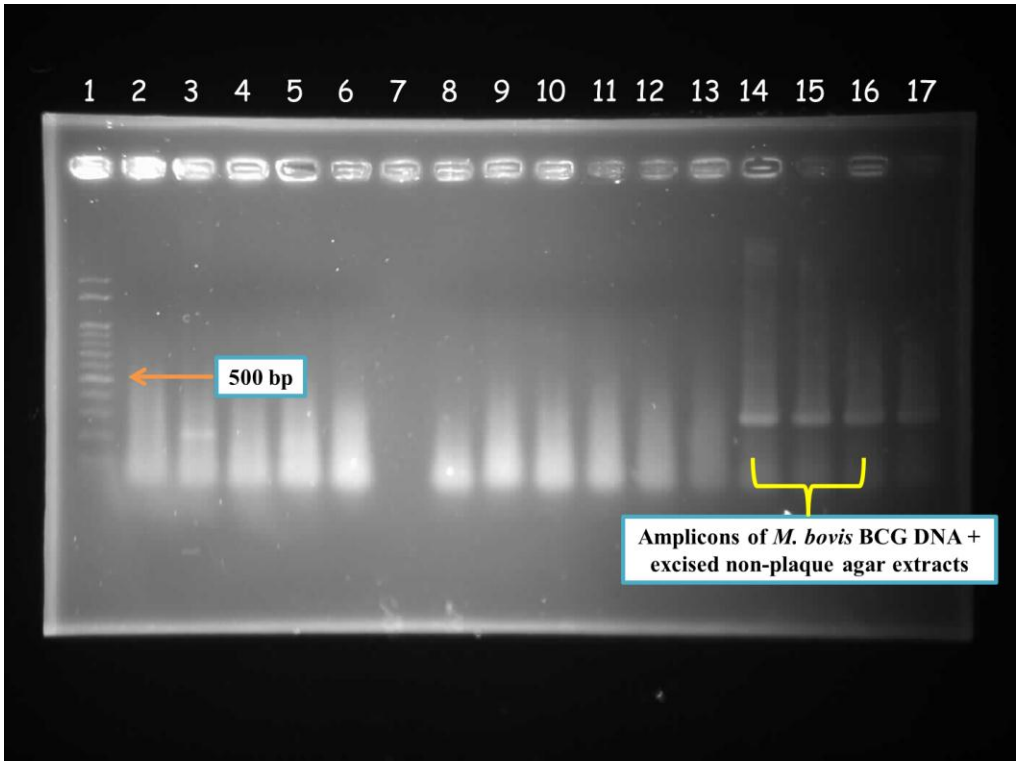

Figure 4.9: Gel analysis of duplex IS*900*/IS*1081* PCR reaction. Lane 1, 100-bp DNA ladder; Lane 2, NTC; Lane 3, E. *Coli* DNA (negative control); Lanes 4, 5, and 6, *M. smegmatis* DNA (negative control); Lanes 8, 9, and 10, Plaques from BP1; Lanes 11, 12, and 13, plaques DNA extracts from *M. bovis* BCG culture; Lanes 14, 15, and 16, *M. bovis* BCG DNA + excised non-plaque agar extracts; Lane 17, *M. bovis* BCG DNA (IS*1081* positive control).

*The orange and yellow arrows show the 500 bp of the DNA ladder and 306 bp amplicons of *M. bovis* BCG DNA respectively.

CHAPTER 5

DISCUSSION

5.1 Physical Appearance and Storage of Bovine Faecal Samples

All the bovine faecal samples tested in this study appeared brown, damp, and firm except for BP1 which appeared rather liquefied and dark brown in colour. Judging from its appearance, there was a high possibility that BP1 was diarrhoeal faeces. Since diarrhoea is one of the symptoms of JD, BP1 was expected to be the most possible one among all the samples to be a MAP-infected sample. As can be seen in Sections 4.2.1 and 4.2.2, the combination of results of both the phage assay and LJ culture for BP1 had expectedly proven the presence of mycobacteria in this faecal sample; the latter showed that the isolated was of a slow-growing species which took more than seven days to form observable colonies, consistent with the growth rate of MAP (Brooks, et al., 2013).

Storage of faecal samples for MAP detection can only be considered effective if it could fulfil two criteria: 1) the survival of MAP in the samples during storage and 2) storage conditions must prevent MAP or the microbiota in them from multiplying. The former, if not fulfilled, will result in false negative detection due to storage-induced death of MAP cells. For the latter, the slow growth of MAP renders its multiplication during storage not of such a concern,

as long as the storage duration is reasonable. More concern is for the rapid-growing microbiota in them that will multiply rapidly, even over a short storage period, and renders the decontamination method used ineffective.

The survival of MAP and *M. bovis* which is evolutionarily related to MAP (Stahl and Urbance, 1990) in cattle slurry has been investigated by a number of researchers due to their pathogenicity to cattle. MAP could survive in cattle slurry for 252 days at 5ºC while *M. bovis* was reported to be able to survive in liquid manure for 176 days at 5ºC (Olsen, et al., 1985; Scanlon and Quinn, 2000). Whittington and Sergeant (2001) on the other hand have proven that MAP was not able of replication when they are outside of its host. These studies had served to justify storing faecal samples at a temperature of 4ºC during this project.

5.2 Evaluation of Various Decontamination Methods for the Phage Assay

As described before and reemphasised here, an effective decontamination method for the detection of MAP by the phage assay needs to ideally fulfil the following criteria: 1) sufficient harshness to eliminate only non-target cells but not the target cells; 2) short turnaround time; and 3) no interference with the formation of confluent *M. smegmatis* lawn.

5.2.1 Hexyldecylcetylpyridinium Chloride

The absence of confluent *M. smegmatis* lawn in the phage indicator plates was the critical factor which confirmed the incompatibility of HPC as a decontaminating reagent, when coupled to the downstream phage assay. The role of the HPC in causing this phenomenon may be controversial because the suspension was centrifuged after the treatment to confine cells to the pellet and HPC to the supernatant. As the supernatant was discarded, the speculation that no HPC residues were left in the pellet served as a factor for the disputation. However, when confluent *M. smegmatis* lawn was observed as NaOH substituted HPC, it was evident; that carry-over HPC residues in some ways had hindered the formation of *M. smegmatis* lawn in the phage assay.

The transparency of the phage indicator plate as shown in Figure 4.4 may be the consequence of either the elimination of the *M. smegmatis* cells by the HPC residues or the complete lysis of the lawn as a result of disrupted D29 inactivation by FAS. Taking notice at Table 4.3, the successful growth of contaminants in the LJ slants would make the former reason an unlikely one to cause the absence of the *M. smegmatis* lawn. Presence of contaminants in the LJ slants implied one thing – be it 0.75%, 0.85%, or even 0.95% HPC, it was not harsh enough to eliminate the non-target cells. If this is the case, the HPC residues were definitely insufficient to kill *M. smegmatis*, a species of bacterium with remarkable chemical resistance compared to other bacterial species (Brooks, et al., 2013). Additionally, according to the processing method (Section 3.7.4), the residues of HPC in the test suspension, if present,

would have been diluted over ten folds when the suspension was mixed with the phage assay reagents (1 mL + 10.8 mL, respectively).

Therefore, it could be confidently assumed that HPC had in some ways interrupted the function of FAS during the inactivation of exogenous phages, causing the complete lysis of the *M. smegmatis* lawn. Despite the study by Park, et al. (2003) which validated the role of citrate in the sequestration of ferrous ions which then prevents the destabilisation of exogenous phage D29 in the phage assay, none is known of the mechanism of HPC in deactivating FAS. HPC has only been previously validated to be used in conjunction with culture detection of MAP in faecal samples (Ridge, 1993; Whipple, et al., 1991) and its evaluation for the phage assay in this study is novel.

5.2.2 Sodium Hydroxide

A trend of increment seemed to dominate in the number of plaques formed with increasing NaOH concentration for those decontaminated with 1% and 2% NaOH (Table 4.2). Those decontaminated with 4% and 8% NaOH on the other hand showed fluctuation in the number of plaques formed. The use of 2% NaOH might have eradicated the non-target cells to a greater degree compared to 1% NaOH. With lesser viable non-target cells in the sample decontaminated with the former, lesser contaminants would compete with the target mycobacterial cells for the limited nutrients, hence leading to the increase in number of plaques formed.

The concentration of 4% and 8% NaOH might be too harsh to the extent that they might have even killed the target mycobacteria. This could explain the observation of which no plaques formed in the phage indicator plates of TM1 samples, which were decontaminated with 4% and 8% NaOH, respectively. BP1 samples, although decontaminated with similar concentrations of NaOH, could still produce plaques because the number of mycobacterial cells in BP1 was likely many times higher than that in TM1. In several studies where NaOH was used to decontaminate other sample types for mycobacterial detection, none used concentrations of higher than 4% due to their excessive harshness that may be fatal to the target mycobacterial cells. (Kamala, et al., 1994; Reddacliff, et al., 2010; Whipple, et al., 1991).

Comparing between 2% NaOH in DM3 and DM5 which had the same treatment duration but the latter incorporated NOA^L during resuscitation while the former did not, it might be surprising to see DM5, which had an extra step of decontamination, could not control contaminants better than DM3, which had no addition of NOA^L. Again, the reason lies within the nature of the faecal samples tested; BP1 and EG2 were likely to have higher amount of contaminants compared to TM1.

These indicated that the amount of mycobacteria and contaminants in each faecal sample varies. Therefore, a ‘universal’ concentration of NaOH is needed to successfully decontaminate faecal samples regardless of how much

contaminants are present and to retain as much viable mycobacterial cells as possible, especially in samples which have only limited amounts of target cells.

5.2.3 Incorporation of NOA during Overnight Resuscitation and in the Phage Indicator Plate

The NOA antibiotic cocktail was formulated to eliminate a broad range of microbes, except mycobacteria. The use of nystatin, oxacillin, and aztreonam in this cocktail enables the killing of a wide range of fungi, Gram-positive bacteria, and Gram-negative bacteria respectively. In a study done by Albert, et al. (2007), NOA^{L} was validated to be the optimal concentration which could control contamination and at the same time would not affect the viability and phage infectivity of *M. tuberculosis*. The phage indicator plates 9 to 12 in Table 4.1 however, had shown otherwise. The incorporation of NOA^{L} during overnight resuscitation clearly could not contain the proliferation of contaminants that survived (Table 4.1).

Due to the its inability to control contamination, NOA^{H} with concentrations of nystatin and oxacillin that were 4 and 256 times higher than those in NOA^{L}, respectively, was incorporated into the phage indicator plates, as performed by Cheah (2010). Although little is known of the possible effects the NOA^{H} may pose to the viability and phage infectivity of mycobacteria, its incorporation in the phage indicator plates would not affect the target cells. This is due to the fact that during its incorporation, target cells in the sample would have already

been infected by phage D29 and ready to be lysed. Therefore, regardless of its effects on viable target mycobacterial cells, they are irrelevant in this context due to the stage of the phage assay in which it is introduced (Cheah, 2010). It is not crucial to what extent the NOA^H affects the viability of the *M. smegmatis* cells forming the lawn, as all the indicator plates incorporated with it throughout this study yielded lawns that were visually confluent, and for every mycobacteria-positive sample, plaques were clearly observed among the lawn.

5.3 Evaluation of Various Decontamination Methods for Culture on Löwenstein-Jensen Slants

5.3.1 Hexyldecylcetylpyridinium Chloride

DM1 was adapted from the method used by Gwóźdź (2006) to isolate MAP from sewage samples. In his study, however, when an additional decontaminating step which consisted of an antibiotic cocktail was added downstream of the HPC step, the contamination rate was reduced down to 4.4%.

The results shown in Table 4.2 then served to justify the necessity of that additional step in this study, although the sample type tested was different. Nevertheless, it is important to note that the main element of this study was never to develop a decontamination method for the isolation of MAP from bovine faeces. Rather, the LJ cultures only served as supporting evidences to

determine the degree of contamination in decontaminated faecal samples and the genuineness of the plaques detected.

5.3.2 Sodium Hydroxide

NaOH with concentrations of 2%, 4% and 8% had been consistently effective in decontaminating different faecal samples (Table 4.3). By substituting HPC with NaOH, this study had found a decontamination method that could effectively decontaminate faecal samples, even when the extra decontaminating step suggested by Gwóźdź (2006) was omitted. The LJ slants inoculated with faecal samples decontaminated with 4% and 8% NaOH have consistently dried up before the other slants. No other studies known have displayed similar observations.

5.3.3 Incorporation of Penicillin in the Löwenstein-Jensen Slants

The incorporation of penicillin into LJ slants was done by Ting (2012) and documented to be the more favourable method in decontaminating soil samples for mycobacterial isolation. Penicillin, which is highly active against many Gram-positive bacteria, had also clearly improved decontamination in this study compared to slants with no added penicillin (Table 4.3). The successful isolation of mycobacteria in LJ culture is such evidence (Figure 4.6).

On a side note, the fact that all non-pigmented MAP colonies analysed by Stevenson, et al. (2002) were of the cattle strain and coincidentally that they were morphologically rough further improved the potential of the isolated colonies in slant 6 of Table 4.3 to be those of MAP.

However, penicillin which deters the formation of peptidoglycan in Gram-positive bacterial cell wall, could not inhibit the growth of fungi such as the molds. This is apparent when slant 8 in Table 4.3, which had penicillin incorporated into it, showed mold-like growth on its surface in the first two weeks of incubation. Microbes such as the Gram-negative bacteria or the fungi are resistant to penicillin due to the protected peptidoglycan in the former's cell wall and the absence of peptidoglycan in the latter's.

5.4 Assessment for the Reliability of the Results of the Phage Assay with Decontaminated Bovine Faecal Samples

Tests in this section were done on both DM2 and the 'optimised method' because the only difference between the former and the latter is the absence and presence of NOA, respectively. As was discussed in Section 5.2.3, NOA^L had been proven to cause no significant effects in the viability and phage infectivity of *M. tuberculosis* (Albert, et al., 2007; Cheah, 2010). In other words, assessing DM2 for potential false and inaccurate results is equivalent to assessing the 'optimised method' because the incorporation of NOA downstream to NaOH treatment will not lead to any changes in the number of

plaques formed. It is of utmost importance to run these tests because the application of the phage assay on bovine faecal samples is still an under-explored area.

5.4.1 Assessment for Potential of False Negative Results

This test was done to investigate the feasibility of the phage assay to detect viable mycobacteria in decontaminated samples. The presence of any form of inhibition will inevitably lead to the underestimation of the levels of mycobacteria within the samples. Since the control (*M. smegmatis* pellet + M7H9-OGC broth) yielded 12 plaques, the phage assay would be proven to produce no false negative results if the spiked samples (*M. smegmatis* pellet + faecal suspension) produced around 12 extra plaques compared to the non-spiked samples. However, a certain degree of deviation from the theoretical results may be tolerated since living cells which are volatile in nature were being dealt with.

Hence, with a ± 32 plaques between the spiked and non-spiked samples, it could still be safely assumed that the phage assay does not produce false negative results because there will be no plaques if false negativity occurred. This test should be repeated with autoclaved faeces in the future to obtain a more reliable conclusion. As non-spiked autoclaved faeces will indisputably produce no plaques, number of plaques formed from spiked autoclaved faeces will then be indicative of the occurrence of false negative results.

5.4.2 Assessment for Potential of False Positive Results

The resulting products of interaction between faecal materials and the decontaminating reagent or the decontaminating reagent itself might interrupt the FAS inactivation of exogenous phage D29, thus causing the formation of plaques even in the absence of mycobacteria within the samples. Hence, to prove that plaques formed through the phage assay resulted genuinely from mycobacteria in the test samples, the tests described in Section 4.3.2 were run.

Autoclaving the faeces suspensions cause the eradication of all viable microbes that might be present in the samples. No-sample control on the other hand substituted faecal material with dH_2O. By retaining the usage of decontaminating reagent, the potential role of faecal materials in causing possible false positivity could be investigated. The results where no plaques were formed in both tests clearly indicate that neither of the factors hypothesised could contribute to false positivity. It is therefore evidential; the coupling of the decontamination method to the phage assay will not produce false positive results.

For further evidence that the plaques formed originated from actual mycobacterial cells, the resuscitation test was done. As shown by Mukamolova, et al. (2006), mycobacterial cells can enter a state of dormancy when they are nutritionally deprived, exposed to environmental stress, or even when they are in the stationary phase. Cheah (2010) on the other hand have shown significant

increment of detectable plaques from decontaminated samples which had been subjected to resuscitation (37°C in M7H9-OGC broth for 18-24 hours) compared to those which were not. During resuscitation, mycobacterial cells which are chemically stressed due to the decontamination steps will recover from dormancy. This recovery restores the vulnerability of the mycobacterial cells to phage infection. In other words, increased phage infectivity (equivalent to increased PFU) after overnight resuscitation is a phenomenon which implies that plaques formed are from genuine mycobacterial cells in decontaminated faecal samples. The consistent escalation in the numbers of plaques formed after resuscitation had served as a powerful justification that the plaques detected were genuinely from infection of mycobacteria that were present in the faecal samples. If the method is prone to false positive results, there should be no difference in the PFU counts with or without resuscitation. Along with the observations for the test with autoclaved faecal suspensions and no-sample controls above, these findings rule out the potential for the occurrence of false positive results with the method used, at least for the faecal samples tested in this study.

5.5 Evaluation of the Sensitivity of the Phage Assay in Detection of Mycobacterial Cells in Decontaminated Bovine Faecal Samples

This test aimed to determine the lowest level of target mycobacterial cells in the faecal samples detectable through the overall method (i.e. the detection limit). The mycobacterial cells present within faecal samples were speculated to be either adhered to or protected by the faecal materials (E. S. G. Cheah,

personal communication). However, when the *M. bovis* BCG culture was spiked onto the autoclaved faeces, the actual condition could not be simulated, causing the mycobacterial cells to be washed away during processing. Hence, no plaques were observed. Nevertheless, it is crucial to assess the sensitivity of the phage assay in the future as this diagnostic method would be impractical if it could only detect mycobacterial cells from heavy shedders.

The absence of plaques formed could not be due to the defects of the phage assay because when it was performed on milk samples, it was proven to be rather sensitive with a detection limit of 100-1000 CFU/mL (Hoe, 2013; Tan, 2013).

5.6 Analysis of Decontaminated Bovine Faecal Samples for the Potential Presence of Mycobacteria by the Phage Assay

It can be concluded that the 'optimised method' could be routinely used as the decontamination method to decontaminate various faecal samples prior to the phage assay as 0% contamination rate was observed. From Table 4.6, it could be clearly inferred that faecal samples from SS were consistently mycobacteria-free. In fact, this result might indicate that mycobacteria, in general, does not cross-infect between distinct herds. Infection normally starts from one or a few infected animals in a given herd, and disseminated throughout the herd.

5.7 Investigation of the Feasibility of the Duplex IS*900*/IS*1081* PCR Assay in Detecting Target Mycobacterial DNA in Phage D29 Plaques

The absence of amplicons for the plaque DNA extracts could be due to three possible reasons: 1) the target genome might be lost through the extraction process; 2) the mycobacteria detected in the faecal samples were neither MAP nor *M. bovis* BCG and 3) the co-extracted medium residues might have inhibited the PCR reaction, as observed by H.K. Tee (unpublished).

The possible reasons could be narrowed down to the first two because the PCR inhibition test in Section 4.5 had proven that the medium residues were not inhibiting the reaction, contrary to what was observed by H.K. Tee (unpublished). Due to the lack of attention for JD in Malaysia, little is known of the composition of bovine faecal microbiota among the local bovid. Hence, the possibility of the second reason being true is unknown.

However, since there should only be one target mycobacterial genome in each positive plaque, it is highly probable that the target genome was lost through the extraction process. In fact, Cheah (2010) had reported rather low PCR positivity rate for the plaque DNA extracts for his *M. tuberculosis*-positive samples. This reason was therefore suggested in this study to be the most probable explanation for the absence of PCR amplification for the positive plaque extracts. Contrary to popular belief, commercial DNA extraction kits, which require more processing steps than the method used in this study, will

not be the solution to this problem as additional procedures would only translate to higher tendency of target loss.

5.8 Future Works

As the duplex PCR assay plays an essential role in the detection of MAP through this combined assay, future works will be devoted primarily on factors 1 and 2 described in Section 5.7 namely, the loss of target DNA during extraction and the probable presence of other mycobacterial species in bovine faecal samples.

The development of a more efficient extraction method is urgently needed to successfully extract the target mycobacterial genome. Faecal samples which are clinically confirmed to be those from MAP-positive animals could be tested with the combined assay to determine whether the absence of PCR amplification was due to limitations of the plaque DNA extraction method or the presence of non-target mycobacterial species in the faeces.

The use of a multiplex PCR targeting different mycobacterial species like the one used by Stanley, et al., (2007) in their study could also be developed to identify more non-MAP mycobacterial species that might be present in bovine faeces.

Additionally, the Loopamp DNA Amplification Kit from Eiken Chemical could be used to replace the false positive-prone in-house LAMP assay. This commercial LAMP assay kit had been optimised to reduce false positivity and therefore could effectively detect the presence of target mycobacterial genome within the positive D29 plaques.

CHAPTER 6

CONCLUSIONS

This project was set out to develop a combined phage and PCR assay for detection of MAP in decontaminated bovine faecal samples. Feasibility of the combined assay for MAP detection in decontaminated bovine faecal samples was partially demonstrated. The optimised decontamination method developed in this study decontaminates faecal samples with 2% of NaOH for 15 minutes, and incorporates both NOA^{L} and NOA^{H} during resuscitation and in the phage indicator plate, respectively. Being able to produce 0% contamination rate and confluent *M. smegmatis lawn* in the phage indicator plates in all bovine faecal samples tested, this method had shown to be superior among the other decontamination methods evaluated. Tests done to assess for potential false and inaccurate results have proven the genuineness of the plaques recovered from all the faecal samples in this study. Sensitivity of the assay however, could not be determined as it was believed that mycobacteria originally present within the faeces are somehow adhered to the faecal materials. Spiked faecal samples could not completely simulate the actual condition hence the mycobacterial cells were likely to have been washed away during processing. LJ slants containing penicillin (50 U/mL) which were inoculated with faecal samples treated with 2% NaOH for 18 hours had acid-fast bacterial colonies grown on them. This further supports the fact that the plaques recovered from the faecal samples were results of genuinely infected mycobacteria. Out of the

ten faecal samples screened, four of them (40%) were mycobacteria-positive. Yet, when 32 of the total plaques recovered were subjected to DNA extraction and then the duplex PCR assay, none of the plaques produced any amplicons. The absence of amplicons could be due to DNA loss during extraction, the absence of MAP or *M. bovis* BCG within the sample, or even the presence of inhibitors in the agar. With the latter factor being omitted through a PCR inhibition test, the effects of the former two factors in affecting the PCR assay will need to be addressed in the near future. The LAMP assay might be a crucial key in making this assay a complete diagnosis method for the detection of MAP. In conclusion, with no previous works done on the development of the combined assay for detection of MAP from bovine cow faeces, this study would be regarded as a milestone in answering numerous unanswered questions in this field of study. Hence, it is the belief of this study that the combined assay would be in the near future, a widely used diagnosis method for the detection of JD in bovine species.

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APPENDIX

APPARATUS AND CONSUMABLES

The lists 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
Electrophoresis system	Hoefer
Fridge and freezer	Copens Scientific, Pensonic
Gel imaging system	Syngene Bio Imaging
Horizontal shaker	Yihder Technology
Laminar-flow cabinet	Telstar
Light microscope	Leica CME
Mini centrifuge	Labo Gene
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	BD
Agarose powder	Vivantis
Aztreonam	Amresco
Basic fuchsin	Merck
Brain heart infusion broth powder	Conda
Calcium chloride ($CaCl_2$)	R & M Chemicals
Crystal violet	Merck
DNA ladder	Fermentas
dNTP mix	Fermentas
DreamTaq polymerase and PCR buffer	Fermentas
Ethanol	Labmart
Ethidium bromide	Bio Basic
Ethylenediaminetetraacetic acid (EDTA)	Systerm
Glycerol	QRëC
Hexyldecylcetylpyridinium chloride (HPC)	Sigma-Aldrich
Iodine	Merck
Löwenstein medium base powder	BD
Luria-Bertani (LB) agar powder	Conda
M. africanum genomic DNA	University of Leicester**
Magnesium sulphate ($MgSO_4$)	QRëC
Methylene blue	Systerm
Middlebrook 7H10 agar powder	BD
Middlebrook 7H9 broth powder	BD
Mycobacteriophage D29	University of Leicester**
Mycobacterium bovis BCG Glaxo	University of Leicester**
Mycobacterium smegmatis MC^2155	University of Leicester**
Nystatin	Bio Basic Canada
Oxacillin	Bio Basic Canada
PCR primers	1st Base
Penicillin G	Bio Basic
Phenol crystals	R & M Chemicals
Safranin	Merck
Sodium hydroxide (NaOH)	Merck
Syringe filters (0.22 µm)	Pall Life Science
Syringe filters (0.45 µm)	Pall Life Science
Syringes	Cellotron
Tris	MP Biomedicals
Tween 80	Systerm

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