DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND LAMP ASSAY FOR DETECTION AND QUANTIFICATION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CELLS IN RAW MILK SAMPLES

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By

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ABSTRACT

DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND LAMP ASSAY FOR DETECTION AND QUANTIFICATION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CELLS IN RAW MILK SAMPLES

TAN POH SUAN

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent for Johne's disease, a chronic intestinal disease affecting cattle. This disease has caused severe economic losses to the dairy and poultry industries worldwide. The prolonged subclinical phase of this disease and lack of a reliable laboratory screening method pose a challenge to its diagnosis. To address this challenge, a combined phage amplification and molecular assay was developed in this study for detection of MAP in raw milk from cattle. Assay development was conducted with *M. smegmatis*- and *M. bovis* BCG-spiked milk samples; these mycobacterial species were used to model MAP. Spiked milk samples were processed and then subjected to the phage assay. The resulting plaques were enumerated and a few were subjected to the IS*1081* loop-mediated isothermal amplification (LAMP) assay. The genus-specific phage assay detects viable mycobacterial cells while the downstream LAMP assay identifies the infecting species. During this study, it was shown that direct phage assay on milk samples was not possible as severe

contamination by rapid-growing organisms obscured plaque observation; prior decontamination with 1% (w/v) NaOH was necessary. Milk components were also shown to be inhibitory to the phage assay. The sensitivity of the phage assay with spiked milk samples was 10-100 CFUs/ml, equivalent to those reported for sputum specimens and mycobacterial cultures. It was initially thought that chemical decontamination and milk inhibition would affect the assay sensitivity, but this was not the case. The downstream LAMP assay on plaque DNA extracts was able to detect the IS*1081* of spiked *M. bovis* BCG, suggesting its potential in adding specificity to the phage assay. The combined assay developed can yield results within 48 hours of specimen receipt and can therefore facilitate rapid identification of infected cattle. Further optimization of this assay and its evaluation on MAP-infected milk samples await.

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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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(TAN POH SUAN)

APPROVAL SHEET

This project report entitled "**DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND LAMP ASSAY FOR DETECTION AND QUANTIFICATION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CELLS IN RAW MILK SAMPLES**" was prepared by TAN POH SUAN 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 **TAN POH SUAN** (ID No: 09ADB08503) has completed this final year project entitled "**DEVELOPMENT OF A COMBINED PHAGE AMPLIFICATION AND LAMP ASSAY FOR DETECTION AND QUANTIFICATION OF VIABLE *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CELLS IN RAW MILK SAMPLES**" supervised by Dr. Eddy Cheah Seong Guan (Supervisor) from the Department of Biological Science, Faculty of Science.

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

Yours truly,

_____

(TAN POH SUAN)

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LIST OF ABBREVIATIONS

BCG	Bacille Calmette-Guérin
BLASTn	Nucleotide-nucleotide Basic Local Alignment Search Tool
Bst	*Bacillus stearothermophilus*
$CaCl_2$	calcium chloride
CDC	Center for Disease Control and Prevention
CFU	Colony-forming unit (s)
dNTP	deoxyribonucleoside triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
E-value	expect value
FAS	ferrous ammonium sulphate
IS	insertion sequence
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani
MAA	*Mycobacterium avium* subsp. *avium*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MAS	*Mycobacterium avium* subsp. *sylvaticum*
Mtb	*Mycobacterium tuberculosis*
MTBC	Mtb complex
$MgSO_4$	magnesium sulphate
Mtb	*Mycobacterium tuberculosis*
NaCl	sodium chloride
NaOH	sodium hydroxide

NCBI	National Center of Biology Information
NOA	nystatin-oxacillin-aztreonam
NTC	no-template control
NTM	non-tuberculous mycobacteria
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
OGC	OADC-glycerol-calcium
PCR	polymerase chain reaction
PFU	plaque-forming unit (s)
rDNA	ribosomal deoxyribonucleic acid
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
UV	ultraviolet

x *g*	times gravity
bp	base pair
g	gram
ml	milliliter
µl	microliter
cm	centimeter
M	molar
s	second
µM	micromolar
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

While tuberculosis has been recognized as one of the leading causes of human mortality since the ancient era, paratuberculosis or Johne's disease has been described as one of the most chronic and widespread pathogenic diseases affecting domestic animals primarily ruminants (Haas and Haas 1996; Cocito et al., 1994; Larsen et al., 1963). Nowadays, paratuberculosis is the most predominant and economic exhaustive infectious diseases affecting dairy cattle worldwide (Hasonova and Pavlik, 2006).

Johne's disease was first described by Johne and Frothingham in 1895 upon discovery of the pathological association between the detection of acid-fast bacilli in the granulomatous lesion of the intestinal tract of cattle in Germany (Hermon-Taylor et al., 1990). The disease was initially speculated to be tuberculosis. Since guinea pigs are extremely vulnerable to *Mycobacterium tuberculosis* (Mtb) and *M. bovis* infection, failure of the organism to grow and infect guinea pigs suggested that the disease was non-tuberculous. In 1906, Bang proposed pseudotuberculous enteritis as the alternative term for the disease (Cocito et al., 1994). The causative agent of the disease, *M. avium* subsp. *paratuberculosis* (MAP), was subsequently identified by Twort in 1910 when its cultivation was successful and this allowed for further molecular characterization; the latter revealed a high GC content of 66

to 67% GC in MAP genome, allowing it to be distinguishable from other mycobacterial species (Imaeda et al., 1988). Johne's disease resembles tuberculosis and therefore is also known as paratuberculosis. This was because MAP has caused the intestinal infection of ruminants, and it is in the same genus as the Mtb which has caused tuberculosis in humans, cattle, and other species (Chiodini 1993).

MAP is an acid-fast bacillus that carries a specific signature sequence IS*900* (Sweeney, 1996). It is an extremely slow grower, requiring incubation time ranging from weeks to months to show visible colonies on solid medium (Merkal et al., 1970; Chiodini et al., 1984). Animals susceptible to MAP infection include all domestic, wild, and exotic ruminants as well as other non-ruminant species such as the primates (Chiodini and Van Kruiningen, 1983).

Despite many wildlife ruminants such as deer, antelopes, camels and others are also affected by paratuberculosis, the studies in the veterinary field focus on domestic ruminants that are of economic importance, particularly cattle, sheep and goat (Chandler 1961). Indeed, finding by Worthington (1963) that paratuberculosis, to some extent, has exerted chronic impact on the cattle industry and this implied the significance of the present study which focused on laboratory detection of MAP in milk from cattle, to enable further actions such as culling infected animals and movement restriction to control the disease widespread.

Since the early 19th century, paratuberculosis has gained attention from animal producers besides diagnosticians and researchers, because it accounts for significant economy losses to the livestock, dairy and related industries globally (Kennedy and Benedictus, 2001; Nielsen and Toft 2009). Many epidemics of this disease have been reported in several regions in Europe as well as in the United States and now it is an epizootic disease worldwide. Countries affected are those having an economical supportive livestock industry, which include Canada, the United States and all European countries except Sweden (Stevenson et al., 2009).

In the United States, the incidence of paratuberculosis has increased dramatically over the years and has caused annual losses exceeding USD 1.5 billion (Jones 1989). Severe reduction in milk production, poor healthy circumstances including continuous or intermittent diarrhea and weight loss, followed by premature culling or death are primary causes of economic losses to dairy cattle producers. Other factors include reproduction dysfunction, poor feed conversion, and expenses involved in herd replacement.

In the perspective of consumers, there is a risk of infection with MAP upon consumption of MAP-contaminated water or milk containing MAP cells that survived the pasteurization process. Transmission of MAP to humans, especially those who are immunocompromised, can possibly result in Crohn's disease, an intestinal disease in human. Despite the zoonotic potential of MAP to cause

Crohn's disease remains controversial, there is no evidence to disregard the association between them.

Hence, it is important to have a rapid diagnostic system to detect viable MAP in infected cattle during the early stage of the disease, thereby controlling the transmission to other healthy calves to alleviate the economic loss and minimizing possibly transmission to human. However, the characteristic of paratuberculosis development has placed a serious obstacle to rapid detection of MAP or paratuberculous calves because most of the infected calves appear phenotypically similar as those healthy ones. This is because paratuberculosis has a long subclinical phase during disease development, in which infected animals will remain asymptomatic but may excrete MAP into feces, milk, and colostrum. Therefore, MAP-contaminated milk and colostrum from asymptomatic mothers serve as primary sources of MAP for calves via ingestion (Chiodini et al., 1984; Sweeney et al., 1992; Streeter et al., 1995; Sweeney 1996).

Indeed, infection of newborn calves is the leading cause of paratuberculosis (Cocito et al., 1994). Apart from that, up to 10^8 or more MAP cells per gramme of feces has been reported and this contributed to rapid spread of the pathogens throughout the herds through ingestion of the feces (Chiodini et al., 1984). Due to these oral administration routes, as the disease developed to the clinical phase, MAP might have been transmitted and infected almost 50% of herdmates (Delisle

et al, 1980; Larsen et al., 1963). Whitlock et al., (2000) reported that a minimum of 25 other healthy animals are infected for every clinical animal identified. This also reflected that asymptomatic animals will outnumber symptomatic ones (Brugere-Picous 1987).

Currently available diagnostic tools are able to detect MAP despite its long subclinical phase. An example is acid-fast Ziehl-Neelsen (ZN) staining. This differential staining is based on the ability of mycolic acid in cell wall of mycobacteria to retain carbol fuchsin stain after decoloration using acid alcohol. Resistance to decoloration enables mycobacteria stained pink, differentiable from other microorganisms which are stained blue by the methylene blue counterstain. Besides, culture is the definitive and sensitive method in diagnosing MAP. Modified Löwenstein-Jensen (LJ) medium are preferred media used due to its high specificity and sensitivity in detecting mycobacteria (Shin 1989). Other media used include Herrold's egg yolk medium (HEYM) with mycobactin and Middlebrook 7H9-OADC solid medium. As MAP cells have generation time of about 16 weeks, formation of colonies after incubation for this length of time implied presence of MAP cells. Other ways of detection include conventional PCR amplification of the insertion sequence IS*900*, the unique genetic marker specific to MAP (Collins et al., 1989). PCR methods enable quick detection of MAP in less than three days as this method does not require the cultivation of the cells (Ayele et al., 2001).

However, each of these methods has its limitation. First, acid-fast staining used to identify MAP cells can occasionally generate false-negative result when the cells are cell-wall deficient and are not able to retain the color of carbol fuchsin stain (Juneja and John 2010). It also could not distinguish MAP cells from other mycobacteria. Culture methods, on the other hand, are time-consuming because MAP is a slow grower and it needs long incubation time about 4 months, to show visible colonies on solid culture medium (Shin et al., 2008). This would increase the risk of contamination by other fast growers. Conventional PCR methods have no live/dead differentiation capability and PCR probes are expensive, thus are not affordable to developing countries. Moreover, PCR inhibitors might be present in the clinical samples, affecting accuracy of diagnostic results (Ayele et al., 2001).

Another established tool is the phage assay that can rapidly detect mycobacteria. As it is inexpensive, it is used in developing countries. Phage assay using mycobacteriophage that infects only mycobacterium has been used detect the presence of the MAP in just 24 hours (Foddai et al., 2010). Overnight incubation of phage-infected mycobacterial culture that showed plaque formation indicates there are mycobacteria present in the sample. Mycobacteria that have been infected by the phages and burst on a lawn of *M. smegmatis* indicator cells, producing clear zones known as plaques. Plaque formation thus implies presence of mycobacteria but cannot conclude the types of species that present. Therefore, specificity of the phage assay was enhanced via the loop-mediated isothermal amplification (LAMP) assay performed downstream. With the use of primers

targeting on insertion sequence unique to a specific mycobacterial species, plaque DNA that generates positive signal such as fluorescing green upon the addition of SYBR Green dye confirms that the plaques formation is due to mycobacterial species that carries the signature sequence in which the LAMP primers are targeting on.

There is no one test capable of identifying a single mycobacterium up to the species level, and only after multiple tests are performed on the signature markers and expected results are obtained, the organisms isolated from the specimen can be certainly classified as MAP (Jenkins 1981). Combination of both phage and LAMP assays enables identification of paratuberculous cattle even in early of the disease without any clinical signs displayed upon detection of MAP in specimens obtained from the animal (cited in Foddai et al., 2010).

The aims and objectives of this project were:

I. to develop and evaluate a combined phage amplification and LAMP assays for rapid detection and quantification of MAP in raw milk samples;
II. to determine the sensitivity of the combined assay to detect MAP in milk samples;
III. to investigate the potential inhibition of phage amplification assay by milk components.

CHAPTER 2

LITERATURE REVIEW

2.1The Genus *Mycobacterium*

2.1.1 General Features

The genus *Mycobacterium* belongs to the family *Mycobacteriaceae*, which consists of about 127 species based on the approved list of Bacterial Nomenclature (Skerman et al., 1989). All of these species are generally acid-fast, rod-shaped, have thick cell walls and grow slowly. The cell wall of mycobacteria is highly complex and well organized structure. This is because it comprises of peptidoglycan covalently attached to a linear galactofuran, which is connected to several highly branched arabinofuran molecules that are bound to mycolic acids. The mycolic acids are arranged perpendicular to the membrane plane and this has contributes to the thickness of the cell wall, thus creating a unique lipid-rich barrier important for physiological and possible disease-inducing aspects of mycobacteria (Brennan 2003). Indeed, the low permeability of the mycobacterial cell wall has enabled the cells to have high degree of intrinsic resistance to most chemotherapeutic agents and antibiotics (Jarlier and Nikaido 1994).

Slow growth rate is amongst one of the remarkable features of mycobacteria. Based on Falkinham III (cited in Kazda et al., 2009), the growth rate of mycobacteria might be reduced due to the channeling of a substantial proportion

of energy and carbon into the synthesis of the long fatty acids, lipids and waxes in the outer membrane. As the rRNA genes are responsible for the rate of protein synthesis, the low copy numbers of ribosomal RNA genes has contributed to the slow growth rate of mycobacteria (Maaloe and Kjeldgaard 1966). Nevertheless, the slow growth rate was strongly varied in different mycobacterial species (Lewin and Sharbati-Tehrani 2005). Highly pathogenic species such as Mtb belong to the group of slow-growing mycobacteria with the doubling time of 16 hours. Apathogenic and opportunistic species such as *M. smegmatis* are members of the fast growers, with the doubling time of 3 hours.

2.1.2 Classification of Mycobacteria

There are three major classes of pathogenic mycobacteria, namely Mtb complex (MTBC), non-tuberculous mycobacteria (NTM), and *M. leprae,* grouped based on their molecular and biochemical profiles. *M. leprae* is different from MTBC and NTM as it cannot be cultured on synthetic media and can only be grown by infecting its animal host. NTM are distinguishable from MTBC and *M. leprae* because they are either nonpathogenic or are opportunistic pathogens. Examples of opportunistic NTM are *M. avium, M. kansasii*, *M. haemophilum*, and *M. simiae*, which are frequently associated with infection in immunocompromised patients.

Different mycobacteria also vary in their degree of virulence and pathogenesis, they could be environmental saprophytes, opportunistic pathogens and obligate

pathogens (Quinn et al., 2011). The two important obligate pathogens of the genus are Mtb which causes tuberculosis and *M. leprae*, the etiological agent of leprosy. Additionally, the primary pathogenic mycobacteria that infect animals encompass *M. bovis*, which is responsible for bovine tuberculosis and *M. avium* subsp. *paratuberculosis* (MAP), an etiological agent of the widespread Johne's disease in ruminants especially cattle.

2.2 *Mycobacterium avium* subsp. *paratuberculosis*

MAP is an obligate pathogen that causes paratuberculosis. It has all the general features of a mycobacterium with length of about 0.5 to 1.5 µm. Besides, it is a slow grower that forms visible non-pigmentative rough colony on culture medium only after incubation of up to 16 weeks. There were two main strains of MAP, C strains, found in cattle and S strains, found in sheep and cross-species transmission of both strains could occasionally occur between the two hosts (Collins et al., 2002).

Based on Thorel et al. (1990), MAP is an extremely fastidious organism, as most of its strains are mycobactin-dependent. Mycobactin is essential for transportation of high amounts of iron across cell membranes, for the growth of MAP (Barclay and Ratledge, 1983). Genetically, MAP has 66-67% of GC DNA base composition (McFadden et al., 1987). It also has the insertion sequence IS*900* which is present in 15 to 20 copies in its genome (Green et al., 1989). MAP is

closely related to *M. avium* subsp. *avium* (MAA) and *M. avium* subsp. *sylvaticum* (MAS) on the basis of the DNA homology (van der Giessen et al., 1992). Despite this, MAP can be still differentiated from MAA and MAS based on the presence of the multicopy IS*900* and its strong mycobactin-dependence.

2.3 Paratuberculosis/Johne's Disease

2.3.1 General Introduction

Johne's disease was first described by Johne and Frothingham in 1895 upon discovery of the association between granulomatous enteritis with the presence of acid-fast bacilli in the intestinal tract (Hermon-Taylor et al., 1990). The exact identity of the causative agent responsible for this enteritis remained enigmatic until 1912, when Twort succeeded in cultivating and characterizing this mycobacterium. The causative organism had thus been identified as MAP and Johne's disease was renamed paratuberculosis.

Currently, paratuberculosis has been a chronic infectious enteritis naturally affecting both domesticated and exotic ruminants all over the world, including dairy and beef cattle, sheep, goats, red deer, and camelids, without any effective treatment to date (Fecteau and Whitlock 2011; Chiodini et al., 1984). Besides domestic and wild ruminants, non-ruminant wildlife such as foxes, rats, rabbits, wood mice and badgers have been found to harbor MAP.

2.3.2 MAP Infection in Cattle

Feces from diseased cows serve as the original source of MAP. Domestic ruminants such as calves are usually predisposed to MAP infection upon nursing from an udder contaminated with fecal MAP besides exposure to MAP-contaminated manure in infected pens. This was because the infected animals could shed MAP into their feces, which then contaminates milk, nearby pasture and water. Additionally, milk originated from both symptomatic and asymptomatic cows had also been reported to harbor the organisms (Sweeney 1996; Streeter et al., 1995). These findings are important because the latter infers that subclinically infected calves can continuously shed MAP into milk without being able to be detected, thereby silently spreading the disease to other animals.

Sweeney et al. (1992) has revealed the infestation of MAP in the intestinal tract of animals occurred upon oral uptake of MAP-containing milk from paratuberculous cattle. MAP will then cross the intestinal mucosa by penetrating the epithelial M cells in the Peyer's patches, following which macrophages will phagocytise MAP and thus facilitate allow its intracellular multiplication. MAP will then disseminated to a large area of the intestine as well as to neighboring organ systems. As the disease advances with time, protein-losing enteropathy becomes apparent, leading to low levels of proteins in the blood, edema, and progressive thickening of the gut (Patterson and Berrett, 1969). Eventually, MAP will be

sloughed from the intestinal mucosa into the lumen and discharged with stools into the environment.

2.3.3 Development of Paratuberculosis and Clinical Signs

Developmental characteristics of paratuberculosis have compromised the efforts to eradicate the disease from cattle herds, including the difficulty to detect it in the young cattle in the earliest stages (Whitlock et al., 2000). Three clinical stages are involved paratuberculosis becomes apparent for phenotypic visualization. Stage I is described as subclinical and non-excretory at which paratuberculous cows are asymptomatic and non-appreciable MAP shedder. Stage II is characterized as subclinical and excretory, during which MAP in the intestinal mucosa and lumen are constantly increased in numbers with infected cows remain as asymptomatic shedders. Stage III is the chronic terminal stage because during this clinical and excretory phase, infected cows become symptomatic with non-alleviatory diarrhea, emaciation followed by infertility, severe reduction in milk yield, and eventually death in cachectic state.

Absence of clinical signs during the silent stage I and subclinical stage II have set obstacles for interpretation of test results (Dargatz et al., 2001, Whitlock et al., 2000). However, it is possible to screen for early infected calves at stage II through the detection of excretory MAP in stool or in milk. Therefore, milk can be a potential reservoir used for paratuberculosis diagnosis. Heavy chemical

decontamination to get rid normal microbiota that are present does not need to perform on raw milk sample as it is generally sterile unlike fecal sample.

It was hypothesized that infected calves will discharge MAP into the milk and the detection of MAP in it would diagnose the milking calves to be paratuberculous. The diseased calved diagnosed have to be culled prematurely before they progress to the terminal stage during which underrated calves within the herds will be infected through oral ingestion of MAP-contaminated water, milk or feces, the latter by accident.

2.3.4 Incidence of Paratuberculosis

The first paratuberculosis was reported on cattle in several European countries and in the United States. The disease has since spread worldwide and is now a common disease in all countries with a significant dairy industry, except Sweden and certain states in Australia regions that has been officially declared free of the disease (Buergelt et al., 1978).

Epidemiology has revealed that about 70% of bovine herds in northern Italy and about 60% of Sardinian sheep flocks were infected with this pathogen. Incidence of paratuberculosis was also reported to be increasing in other parts of European continents such as Netherlands, Finland and Scotland (Nielsen and Toft 2009).

The disease was also widespread in about 70% of dairy herds in New Zealand, particularly infecting dairy cattle and goats. African continent country such as in Zambia, paratuberculosis has been a greater problem in sheep than in cattle, whereas in Tunisia, MAP is only found in camels. Nevertheless, there was a lack of epidemiological studies regarding the true distribution of paratuberculosis especially in Asian countries (Nielsen and Toft 2009). Therefore, Asian countries such as Malaysia, China, Japan and Korea are still with contemporary, albeit infrequent reports of paratuberculosis.

2.3.5 Impact on the Dairy Industry

Economic losses due to paratuberculosis have been reported to be more adverse than that due to other bovine illness such as enzootic bovine leucosis because most cases of paratuberculosis are subclinical (Chi et al, 2002). The major economic impacts involve worsening productive performance particularly substantial reduction in milk yield and forced culling. This was because subclinical carriers alone have been estimated to cause losses of maximum 2800 pounds of milk per lactation corresponded to 16% lesser milk (Hendrick et al., 2005). Additional loss from the early culling was due to higher calves replacement costs and unrealized future income for the producers.

Both major impacts on Friesian cattle have been reported to occupy about 70% of sum direct economic losses in MAP infection (Groenendaal et al., 2002). Besides,

the reduced productivity in US dairy industry due to paratuberculosis has costed a loss of USD 250 million annually (Ott et al., 1999). Economic losses in Australia in the year 1989 were estimated to be AUD 2.0 million annually. Additionally, the losses on an average infected Dutch dairy herd have been estimated to be EUR 19 per cattle annually average.

2.4 Zoonotic Potentiality for Crohn's Disease in Humans

Crohn's disease (CD) is a rare chronic human intestinal disease first suggested by Dalziel in 1913 (cited in Chiodini 1993). CD is characterized by transmural inflammation capable of affecting any part of the gastrointestinal tract. It is a life-long disease and a major healthcare problem today. CD patients experience deteriorating symptoms such as profuse urgent diarrhea, vomiting and fever (Isselbacher et al., 1994). Despite its chronicity, there is currently no known efficient treatment for the disease and the causative link between MAP and CD is still controversial with an ongoing scientific debate.

The zoonotic potential of MAP was suggested based on the detection of MAP in the blood or mucosal tissues of the CD patients and the similarities between paratuberculosis in ruminants with the CD in humans (Sibartie et al., 2010; Collins 2003). Milk has been proposed to be the most probable route of MAP transmission to human especially those who are immunocompromised, because MAP has been reported to survive high temperature short time pasteurization

(Chiodini and Hermon-Taylor 1993). In fact, viable MAP has been detected in retail pasteurized milk in several countries such United Kingdom and Brazil (Carvalho et al., 2012; Grant et al., 2002). Successful detection of MAP cells in pasteurized milk also indicated that 100 CFU/ml or more MAP cells must have present in raw milk and it is unsafe for human consumption (Grant et al., 1998).

2.5 Diagnostic Techniques to Detect MAP

2.5.1 Detection Based on Phenotypic Visualization

Clinical-based diagnosis of paratuberculous calves is based on the visualization of phenotypes such as intermittent or persistent diarrhea, reduced in milk production, decreased in fertility, loss of appetite, weight loss and increased emaciation leading to eventual death. In fact, diarrhea in calves contributes to high suspicion for paratuberculosis because diarrhea was a response to histamine released as a result of antigen-antibody reaction in the infected intestine (Merkal et al., 1970).

However, phenotypic visualization could not completely eradicate the diseased calves as it is able to detect only symptomatic calves, not those of subclinical form. For a single diseased cattle displayed clinical symptoms and thus visually diagnosable, about 75% of the healthy calves within the herd will be infected by MAP and remain undetectable for many years (Brugere-Picoux 1987). Besides, evidence has shown that most newborn calves infected upon ingestion of milk from diseased mother, only display clinical symptoms after two years old (Tiwari

et al., 2007). The presence of subclinically infected asymptomatic calves thus hampers the detection of MAP infection by phenotypic visualization.

2.5.2 Acid-fast Smear Microscopy

Acid-fast bacilli do not stain well with dyes, including Gram stain dyes. Therefore, more concentrated dyes together with heating are used to stain the mycobacteria. The presence of high mycolic acids content of the mycobacterial cell wall contributes to its ability to retain the carbolfuchsin stain that are more easily removed in other bacterial species after decolorization by acid-alcohol. Under the light microscope, acid-fast bacilli will appear pink in the culture smear due to the carbol fuchsin whereas non-acid-fast bacilli will appear blue due to the methylene blue counterstain.

However, the acid-fast staining is limited in its specificity because it cannot differentiate MAP from other mycobacterial species as well as *Nocardia* species which are also acid-fast. Besides, staining method provides no live/dead differentiation. Moreover, MAP and some other mycobacterial species can sometimes exist as spheroplasts, which are incapable of holding the carbol fuchsin stain, thereby becoming undetectable and generating false-negative result (Thompson 1994; Chiodini 1992).

2.5.3 Culture

Culture is long considered the “gold standard” for diagnosis of paratuberculosis due to its specificity and high sensitivty. Conventional MAP culture requires decontamination of the specimen followed by concentration of the organisms before inoculation on a growth medium. Solid media are usually used such as Herrold’s egg yolk medium (HEYM) supplemented with mycobactin J, Middlebrook 7H10 agar and modified Löwenstein-Jensen (LJ) slant. HEYM is one of the most commonly used culture media in veterinary diagnostic laboratories. On HEYM, the MAP colonies appear small, rough and off-white to yellow in colour (Collins 2003). On Middlebrook agar in the absence of Tween 80, MAP colonies are very rough in appearance and resemble those of Mtb. In the presence of Tween 80, MAP colony morphology becomes smooth and domed and resembles that of *M. avium* (Collins 2003). Based on Tell et al. (2003), LJ slant is more sensitive for detection of acid-fast organisms with shorter incubation time as compared to HEYM.

Despite diagnosing paratuberculosis by culture yields reliable result, it is laborious, time consuming and recovery of organism has been found to vary with ruminant species tested (Ellingson et al., 2005). Long incubation time of more than 16 weeks to yield visible MAP colonies has also caused the culture prone to contamination by other fast-growing contaminants such as *Escherichia coli*. Besides, sensitivity of culture is compromised because the chemicals used for

decontamination of fast-growing microflora will also affect MAP viability (Whittington 2002).

2.5.4 Molecular Techniques

Polymerase chain reaction (PCR) has long been used as to diagnose paratuberculosis by detection of MAP and the most widely used target gene is IS*900* (Green et al., 1989). Presence of 15 to 20 copies of this insertion element provides an increased sensitivity, making IS*900*-based PCR a popular molecular diagnostic method for paratuberculosis (Li et al., 2005). Successful diagnosis of paratuberculosis by amplifying the unique signature sequence of MAP, IS*900*, enables MAP-infected calves to be distinguished from those infected by other mycobacterial species, *M. bovis* for instance (Green et al., 1989). This would be especially valuable to PCR detection of MAP in raw milk.

Nevertheless, the efficiency of PCR amplification can be limited by the presence of PCR inhibitors in real biological samples including food such as milk (Abu Al-Soud and Radstrom 1998). Thus, DNA extraction and purification is very important for successful MAP detection. PCR method is also very expensive and unaffordable by developing countries. More importantly, PCR detection cannot facilitate live/dead differentiation (cited in Stanley et al., 2007).

2.6 Combination of Phage and LAMP Assays for Rapid Detection

2.6.1 Phage Amplification Assay

Mycobacteriophage D29 was first isolated from soil and propagated on *M. smegmatis*, *M. ranae* and *M. butyricum* by Froman et al., (1954). The phage has a head of uniform size which is with diameter of 65 nm and possesses regular shape with a tail of variable length. The optimal pH for the phage stability is between pH 9 and 10. The phage also carries double-stranded DNA and six structural polypeptides (Schafer et al., 1977). Mycobacteriophage D29 is a lytic phage capable of infecting both fast and slow-growing mycobacteria (Ford et al., 1998).

Phage amplification assay using mycobacteriophage D29 is a cost effective diagnostic method that can rapidly detect the presence of viable mycobacterial cells. It is also highly sensitive as it is able to detect as low as 10 CFUs of *M. smegmatis* (McNerney et al., 1998). Diagnosing paratuberculosis is based on the infection of viable MAP cells by mycobacteriophages D29. Subsequent multiplication of phages within the infected MAP cells will give rise to plaque formation upon addition of *M. smegmatis*. *M. smegmatis* cells are used as sensor cells because they grow relatively faster. The internalized phages that survive after virucide treatment will be released and infect these sensor cells, generating opaque plaques.

Each plaque is a representative of a viable mycobacterial cell present in the original sample. In milk, the potential infecting mycobacterial species are *M.*

bovis besides MAP. As the mycobacteriophage D29 used is genus-specific, this implies that plaque formation may be due to the presence of other mycobacteria besides MAP. Therefore, a downstream molecular assay has been used to confirm the presence of MAP to add specificity to the phage assay,

2.6.2 Loop-mediated Isothermal Amplification Assay

Combination of molecular assay with phage assay can enhance the specificity of the phage assay through amplification of the MAP-specific IS*900*. While Stanley et al., (2007) have combined phage assay with IS*900*-based PCR, this project employed the novel combination of phage and IS*900* LAMP assays for specific detection of MAP in raw milk.

The LAMP assay was developed by Notomi et al., (2000). Since then, this assay is well known with its potentially rapid, accurate and cost-effective nucleic acid amplification method. More than 180 reports evaluating the LAMP assay have been published in many publications including journals (Misawa 2007). This technique is highly specific because of the use of four to six primers that respectively recognise six to eight regions within a target DNA. It is also highly efficient because DNA can be amplified 10^9-10^{10} times in less than an hour with high accuracy (Notomi et al., 2000). Besides, it is very rapid because the *Bst* DNA polymerases large fragment used has high strand displacement activity. Thus, single amplification temperature is possible and time delay for thermal cycling as needed in conventional PCR, can be avoided.

LAMP assay is performed under isothermal temperature at 65˚C for 1 hour and generates large number of amplicons which has added sensitivity to the assay (Notomi et al., 2000). The product can be visually inspected by addition of SYBR Green dye, thereby eliminating the need for operations such as gel electrophoresis which is needed in nested PCR and conventional PCR to detect the amplicons (Iwamoto et al., 2003). It is very simple, cost effective and easy to perform because a simple water bath can be used to perform the assay. As no costly specialized equipment is needed, it is more economical and practical than conventional PCR or real-time PCR (Parida 2008). LAMP reaction also shows high tolerance to biological products because its sensitivity is less interfered by the various components in clinical samples than is PCR, thus omitting the need for DNA purification (Kaneko et al., 2007). Thus, LAMP instead of other amplification methods, was used in this project.

CHAPTER 3

METHODOLOGY

3.1 Experimental Design

The overall experimental design for this project is summarized in Figure 3.1 below.

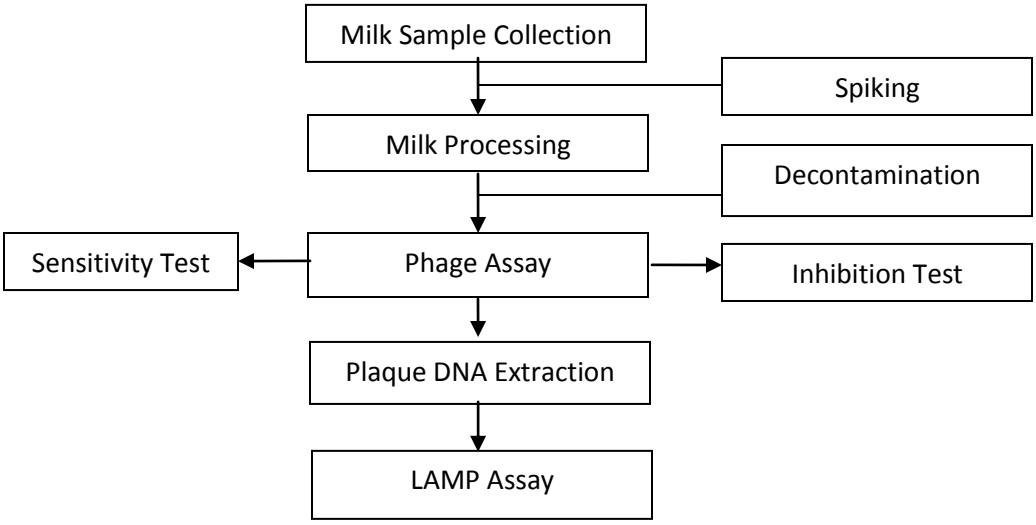

Figure 3.1: Overview of the experimental design for the project

3.2 Apparatus and Consumables

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

3.3 Preparation of Culture Media

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

3.3.1 Luria-Bertani Agar and Broth

An amount of 16 g of LB agar powder was dissolved with dH_2O to a final volume of 400 ml. LB broth was prepared by dissolving 10 g of the broth powder with dH_2O to a final volume of 400 ml.

3.3.2 Middlebrook 7H9 Broth and Middlebrook 7H9-OADC Broth

Middlebrook 7H9 broth was prepared by mixing 0.94 g of Middlebrook 7H9 broth powder and 0.5 g of glycerol with dH_2O to a final volume of 180 ml. The autoclaved broth left to cool to room temperature and was supplemented with 10% (v/v) OADC before use.

3.3.3 Middlebrook 7H9-OADC-Tween Broth

Middlebrook 7H9 broth was prepared as before, with the addition of 10% v/v OADC and 0.05% (w/v) Tween 80 before use.

3.3.4 Middlebrook-7H9-OGC Broth and Agar

Middlebrook 7H9 broth was prepared as before, with the addition of 10% (v/v) OADC and 1 mM $CaCl_2$ before use. Middlebrook 7H9-OGC agar was prepared as described for the broth, with the addition of 1.5% (w/v) agar powder.

3.3.5 Middlebrook 7H10 Agar

An amount of 7.6 g Middlebrook 7H10 agar powder and 2.5 g of glycerol were mixed with dH_2O to a final volume of 360 ml. The agar was boiled for five minutes to dissolve the powder. The agar was left to cool and was supplemented with 10% (v/v) OADC prior to use.

3.4 Preparation of Reagents

All reagents were sterilized by autoclaving at 121˚C for 15 minutes unless otherwise stated.

3.4.1 Acid-alcohol

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

3.4.2 Carbol Fuchsin Stain

An amount of 0.3 g basic fuchsin was dissolved in 10 ml of 95% v/v ethanol and 5 ml of molten phenol crystals was dissolved in 95 ml of dH_2O. Both solutions were mixed and allowed to stand for five days. The resulting carbol fuchsin solution was filtered with a filter paper prior to use.

3.4.3 Ferrous Ammonium Sulphate (FAS), 50 mM

Stock solution of 50 mM FAS was freshly prepared before use. An amount of 0.196 g of FAS powder was dissolved in dH_2O to a final volume of 10 ml. The solution was then filter sterilized using the 0.22-µm syringe filter.

3.4.4 Glycerol Solution, 65% (v/v)

Glycerol solution (65% v/v) was prepared by mixing 162.5 g glycerol, 20 ml of 1 M MgSO4 and 5 ml of 1 M Tris-HCl (pH 8) with dH_2O to a volume of 200 ml.

3.4.5 Methylene Blue Stain

Methylene blue counterstain was prepared by dissolving 0.3 g of methylene blue chloride in dH_2O to a final volume of 100 ml.

3.4.6 Mycobacteriophage Buffer

A volume of 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 $CaCl_2$ were mixed with dH_2O to a volume of 200 ml.

3.4.7 Phosphate Buffer, 67 mM, pH 6.8

Phosphate buffer was prepared by mixing 0.2 M of KH_2PO_4 and 0.2 M of K_2HPO_4 in dH_2O to final concentrations of 34 mM and 33 mM, respectively.

3.4.8 TAE Buffer

Stock solution of 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 liter. The stock solution was diluted 50 fold with dH_2O before use.

3.4.9 Tris-Cl, 1 M, pH 7.6

An amount of 31.53 g Tris-Cl powder was dissolved in dH_2O to a final volume of 200 ml to obtain the 1 M of Tris-Cl solution. The pH was adjusted to 7.6 with NaOH.

3.4.10 Tween 80, 10% (w/v)

Tween 80 solution (10% w/v) was prepared by mixing 10 g of Tween 80 with dH_2O to a final volume of 100 ml and warmed in the water bath at 40˚C for 30 minutes. The solution was then filter sterilized with 0.2-µm syringe filter and stored at 4˚C away from light.

3.5 Culture

3.5.1 Measuring Optical Density of Cultures

The optical density of cultures was measured at the wavelength of 580 nm (OD_{580nm}) using the spectrophotometer. Dense cultures (OD_{580nm} > 1.0) were diluted ten-fold with Middlebrook 7H9 broth prior to measurement.

3.5.2 Ziehl-Neelsen Acid-fast Staining

For smear from solid culture, a loopful of colonies was emulsified in a drop of dH_2O on a microscope slide, whereas for that from broth culture, a loopful of culture suspension was spread into a thin smear on the slide. This was followed by heat fixing of the smear. Subsequently, the smear was flooded with carbol fuchsin solution. Then, the slide was flamed until steam appeared and the smear was left to stain for 5 minutes. Following rising with tap water, the smear was decolorized with acid-alcohol until the stained smear appeared faintly pink. The slide was rinsed again, and this was followed by staining with the methylene blue

counterstain for 1 minute. The slide was then rinsed and blotted dry with the filter paper. The stained smear was examined under oil immersion at 1000x magnification.

3.5.3 Gram Staining

Bacterial smears were prepared and heat fixed as described in Section 3.5.2. The smear was left stained with crystal violet for 1 minute. The slide was then rinsed with tap water, followed by fixation with iodine for 1 minute. The slide was rinsed again and then decolorized with alcohol for not more than 10 seconds. Following rinsing, the smear was stained with the counterstain safranin for 30 seconds. The slide was then rinsed and blotted dry. The stained smear was examined under oil immersion at 1000 x magnification.

3.5.4 Long-term Storage of Culture

Glycerol stocks of *M. smegmatis* and *M. bovis* BCG were prepared by mixing exponential phase cultures with 65% (v/v) glycerol solution in equal proportions. A volume of 1 ml of the mixture was then aliquoted into a 1.5-ml cryovial and stored at -80˚C.

3.5.5 Cultivation of *M. bovis* BCG

Two aliquots of *M. bovis* BCG glycerol stocks were thawed and used to inoculate 5 ml of Middlebrook 7H9 in a 30-ml universal bottle each. Cultures were incubated static at 37˚C; it took about 10 days to reach OD_{580nm} of 1. *M. bovis* BCG suspension was subcultured into 25 ml of Middlebrook-7H9-OADC-Tween broth in a 100-ml conical flask to OD_{580nm} of approximately 0.05. The broth culture was incubated static at 37˚C for 3-4 days. Exponential-phase *M. bovis* BCG has a doubling time of 24 hour (Cheah 2010).

3.5.6 Cultivation of *M. smegmatis*

M. smegmatis was grown by transferring a few colonies from a plate culture into 5 ml of Middlebrook 7H9 broth in a 28-ml universal bottle and colonies were smeared on the wall of the bottle to avoid clumpy suspension. The resulting *M. smegmatis* suspension was inoculated into 25 ml of Middlebrook 7H9-OADC-Tween broth in a 100-ml conical flask to OD_{580nm} of approximately 0.05. The culture was incubated at 37˚C with shaking at 200 rpm for up to 24 hours. Exponential *M. smegmatis* doubles every 3 hours.

3.5.7 Enumeration of Colony-forming Units (CFUs)

Drop plate method was used to enumerate CFUs of liquid cultures (Hoben and Somasegaran 1982). Firstly, ten-fold serial dilutions of a liquid culture were

carried out using Middlebrook 7H9 broth as diluent in 1.5-ml Eppendorf tubes. For each dilution, three 20-µl drops were plated out on Middlebrook 7H10 agar. Culture plates were incubated at 37˚C for 2-3 days for *M. smegmatis* colonies to become apparent. The number of CFUs in the test culture was counted using the dilution that yielded 10-100 CFUs.

3.5.8 Culture Isolation of Raw Milk Contaminants

Ten-fold serial dilution was performed on a raw milk sample using LB broth as the diluent. For each dilution, 100 µl of the sample was spread onto a LB agar plate. All plates were incubated at 37˚C for up to 24 hours. Each different colony obtained was subcultured onto a new LB plate for downstream molecular identification.

3.6 Processing of Milk Samples

3.6.1 Milk Centrifugation

Each milk sample was decanted into a sterile Schott bottle. The milk was thoroughly shaken for even mixing each time before a volume of 50 ml of milk was transferred into a 50-ml centrifuge tube. The milk was then centrifuged at 3000 x *g* for 15 minutes and the resulting creamy top layer was removed using a spatula (sterilized with 70% of ethanol and flaming) (Figure 3.2). The supernatant

was discarded and the pellet was resuspended in 1 ml of Middlebrook 7H9 broth and then transferred to a new centrifuge tube for further processing.

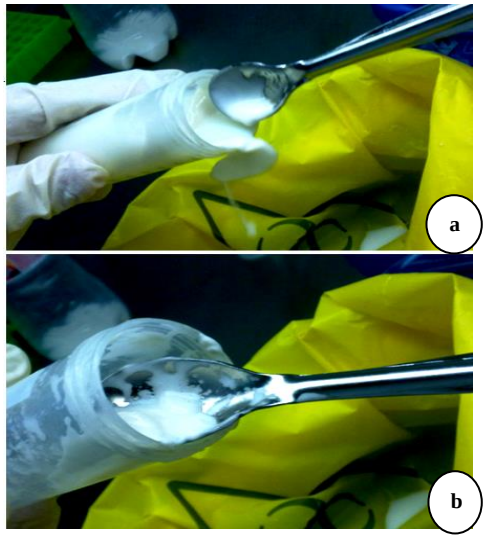

Figure 3.2: Removal of the creamy layer of raw milk sample. a) Cream was removed before whey was be discarded; b) fatty residues removal.

3.6.2 NaOH Decontamination

In a 50-ml centrifuge tube, 1 ml of 2% (w/v) NaOH was added to 1 ml of concentrated milk suspension. The mixture was vortexed for no more than 30 seconds and then left at room temperature for 15 minutes. Following decontamination, 20 ml of 67 mM phospahate buffer was added to neutralize the mixture. Tube contents were mixed by inversion and then centrifuged at 3000 x *g* for 15 minutes. The resulting supernatant was discarded and the pellet was resuspended in 2 ml of Middlebrook 7H9-OGC broth. The suspension was

incubated at 37˚C for 18-24 hours to resuscitate NaOH-stressed mycobacterial cells.

3.7 Mycobacteriophage D29

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

Molten Middlebrook 7H9-OGC agar was distributed into 50-ml centrifuge tubes, each with 9 ml of the agar, and maintained at 55˚C until use. *M. smegmatis* culture was grown in Middlebrook 7H9-OADC broth for 24 hours at 37˚C with shaking at 200 rpm. The resulting *M. smegmatis* culture was left to stand for at least 30 minutes. To 9 ml of molten 7H9-OGC agar, 1 ml of upper homogenous suspension of *M. smegmatis* culture was added and the mixture was mixed by inversion and then poured into a *P*etri dish. The resulting phage indicator plate was left to dry for 10-15 minutes under laminar air flow before use.

3.7.2 Propagation of Phage D29

A volume of 100 µl of D29 phage at approximately 4 x 10^3 PFU/ml was pipetted and spread onto a phage indicator plate. The plate was incubated at 37˚C for up to 24 hours. Large number of plaques were formed on the *M. smegmatis* lawn following incubation. A volume of 10 ml of Middlebrook 7H9-OGC broth was pipetted onto the phage indicator plate, followed by an overnight incubation. About 5 ml of the phage suspension was transferred into a 50-ml centrifuge tube

and then filtered twice with 0.22-µm syringe filters. The resulting phage D29 suspension was distributed in 1-ml aliquots into 1.5-ml Eppendorf tubes and stored at 4˚C away from light.

3.7.3 Enumeration of Phage D29

Ten-fold serial dilution of mycobacteriophage D29 was performed in 450-µl aliquots of MP phage buffer. For each dilution, three 10-µl drops of D29 suspension were spotted onto a phage indicator plate. Agar plates were incubated at 37˚C after the drops had dried.

3.7.4 Phage Amplification Assay

A volume of 500 µl of 10^8 PFU/ml phage D29 was pipetted into 1 ml of sample and 1ml 7H9 broth as control. The tube was gently swirled to mix its contents. The reaction was incubated for 1 hour at 37˚C for phage infection of target cells. Following incubation, 300 µl of 50 mM FAS was added into the reaction vessel to kill exogenous phages. Then, 1 ml of reaction and 1 ml of *M. smegmatis* lawn culture were pipetted into 9 ml of molten Middlebrook 7H9-OADC agar. Tube contents were mixed by inversion and then poured into a *P*etri dish. The plate was incubated at 37˚C for up to 24 hours for plaque formation.

3.8 Genotypic Identification of Contaminant Isolates in Milk Samples

3.8.1 Presto Mini gDNA Bacteria Kit

The gram type of each contaminant isolate was determined prior to DNA isolation with the extraction kit due to slight variation in the protocols for both Gram-positive and Gram-negative bacteria. DNA extraction was performed according to the manufacturer's instructions.

3.8.2 Universal 16S rDNA PCR Assays

Universal 16S rDNA PCR was carried out for contaminant isolates in 25-µl reaction consisting of 5 µl of template DNA, with final concentration of 1x DreamTaq Green Buffer, 250 nm of each forward and reverse primers, 0.2 mM of dNTPs, and 0.05 U of DreamTaq DNA polymerase each. Forward primer was called 16S-338F and reverse primers was called 16S-515R. The sequences for the forward and reverse primer were 5'-ACT CCT ACG GGA GGC AGC-3' and 5'-ACC GCG GCT GCT GGC AC-3' respectively. PCR was performed in the thermocycler, beginning with 95˚C for 3 minutes for *Taq* polymerase activation, followed by 40 cycles of denaturation at 95˚C for 30 s, annealing at 60˚C for 60 s, and extension at 72˚C for 60 s. Non-template control (NTC) was included and *M. smegmatis* DNA was used as positive control in every PCR run.

3.8.3 Gel Analysis of PCR Amplicons

16S PCR amplicons were separated on 2% (w/v) agarose gels. 10 µl of amplicons for each sample, NTC and positive control were analyzed on the gel along with 2.5 µg of GeneRuler 100-bp DNA ladder. Gel electrophoresis was performed at 80 V for 45 minutes and the gel was then stained with ethidium bromide (EtBr) for 10 minutes and destained with dH_2O for 2 minutes before viewing under the ultraviolet (UV) transilluminator.

3.8.4 Gel Purification of PCR Amplicons

PCR amplicons of the expected size of 199 bp were excised from the gel and purified using the Invisorb Spin DNA Extraction Kit according to the manufacturer's instructions. The purified amplicons were assessed for both purity (A_{260}/A_{280}) and concentration using the nanospectrophotometer. Pure DNA should have A_{260}/A_{280} ranging from 1.8-2.0 and their concentrations should be more than 10 ng/µl for DNA sequencing.

3.8.5 DNA Sequencing and Analyses

Purified PCR amplicons were sent to Medigene Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. Analyses of the resulting DNA sequences were performed using the Basic Local Alignment Search Tool (BLAST) at the National Center of

Biology Information (NCBI) website. The identity of each isolate was identified based on the sequence identity with type strains and the expect value (E-value).

3.9 Genotypic Identification of Mycobacterial DNA in D29 Plaques

3.9.1 Plaque DNA extraction

Plaque DNA was prepared according to the method described by Stanley et al., (2007). Plaques were excised from a phage indicator plate using 200 µl pipette tips and transferred into 1.5-ml Eppendorf tube containing 10 µl of deionized distill water each. Tube contents were heated at 95˚C for 15 minutes, pulse centrifuged, and then left at -20˚C until extraction. The tubes were left to thaw and centrifuged at full speed for 2 minutes and the resulting supernatant used for the IS*1081* LAMP assay.

3.9.2 Design of IS*1081* LAMP Primers

Six primers were designed for LAMP reactions, two inner primers, two outer primers and two loop primers. The two inner primers are the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages. The two outer primers are called the forward primer (F3) and the backward primer (B3), whereas the loop primers are called the forward loop primer (FLP) and

backward loop primer (BLP). IS*1081* LAMP was used as a model to demonstrate the potentiality of combined LAMP and phage assay to rapidly detect mycobacteria in milk. The gene sequence of IS*1081* (accession no. Mb1076) was obtained from the BoviList database by the Institut Pasteur (http://genolist.pasteur.fr/BoviList/). The primers for LAMP were designed to amplify a region of *IS*1081 of MTBC using the PrimerExplorer V4 software. The primers sequences were listed in Table 3.1.

3.9.3 IS*1081* LAMP Assay

The IS*1081* LAMP assay was performed in 25-µl reaction containing template DNA, with final concentration of 1.67 x LAMP buffer, 10 mM $MgSO_4$, 1.33 M betaine, 2.33 mM dNTPs, 0.33 µM each of forward (F3) and backward (B3) primers, 2 µM each of forward inner (FIP) and backward inner (BIP) primers, 1.33 µM each of forward loop (FLP) and backward loop (BLP) primers, and 0.53 U of *Bst* DNA polymerase. Amplification was performed in a water bath maintained at 65˚C for 40 minutes and then the reaction was terminated at 80˚C for 5 minutes.

Table 3.1 Sequences of IS*1081* primers

Primer	Sequence
IS *1081*-F3	5'-AGC ACC TCG ACA CCG-3'
IS *1081*-B3	5'-AGG TAG CGC CGT CCT TC-3'
IS *1081*-FIP	5'-TTC CTG GGG GTT GTT GGA CCA GGA CCT GCT GGC GTT CAC-3'
IS *1081*-BIP	5'-TCG TGG GCA TCT TCC CCG ATC CAT TCG TCG TGT TGT TCG-3'
IS *1081*-FLP	5'-CCA GAT CTG CTT GGG GAA GGC-3'
IS *1081*-BLP	5'-GCC TCG ATC ATC CGC CT-3'

CHAPTER 4

RESULTS

4.1 Sampling

4.1.1 Collection of Milk Samples

Two different cattle farms located within the same area, Kampung Tersusun Batu Putih, Kampar, were selected for milk sampling. A total of 26 milk samples were collected as available from these two farms and the volumes sampled varied from 250 ml to 1 liter, depending on the amount needed for a particular processing. The number of milk samples collected do not represent the number of milking cattle available.

One farm (designated Farm A in this project) with three milking cattle is owned by Mdm Suaran Kaur. The other farm (designated Farm B) with also three milking calves is owned by Mr Nadaraja. Milk samples from the three cattle in Farm A were labeled A1, A2, and A3, respectively, while the samples from the three cattle in Farm B were labeled B1, B2, And B3, respectively. Milk sampling was performed by farmers, in which samples from Farm A were sampled in the afternoon between 2-4 pm, whereas samples from Farm B were sampled very early in the morning between 5-6 am. Milk samples from Farm A were always placed in used mineral water bottles (Figure 4.1) and collected a day before

processing and were stored at 4˚C, whereas those from Farm B were placed in plastic bags and delivered early in the morning and were therefore processed fresh.

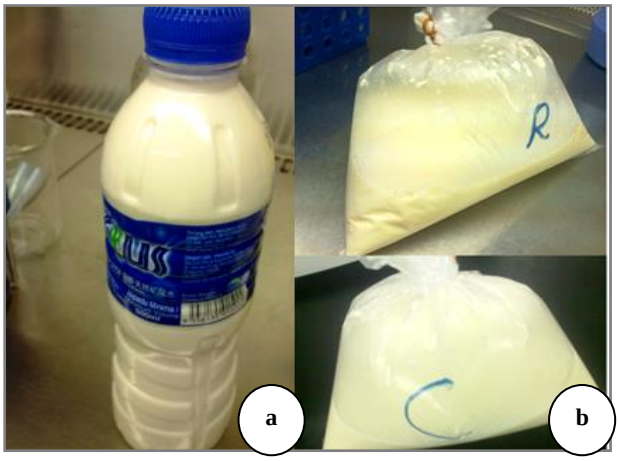

Figure 4.1: Sampling containers for milk samples. a) Used mineral water bottle for Farm A sample; b) disposable plastic bags for Farm B samples.

4.1.2 Appearance and Characteristics of Milk Samples

Generally raw milk samples were visually and physically varied in their color intensity, viscosity, and ease of processing either among the samples collected from the same or different farms. In term of color intensity, all raw milk samples varied from milky white to yellowish white even for milk samples from the same farm (Figure 4.1b) and for samples from the same cattle that were collected on different days. The milk samples from Farm A were more viscous than those from Farm B and this difference determined the ease of subsequent processing. For pasteurized milk samples from Dutch Lady and Magnolia, both were watery and milky white, giving rise to the thinnest and softest fatty layers after centrifugation (Figure 4.2).

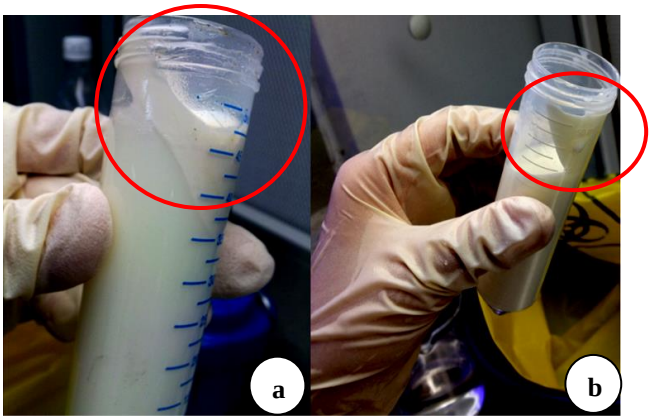

Figure 4.2: Appearance of the creamy layer. a) Raw milk; b) pasteurized milk

Creamy layer was indicated by red circle for clarity.

4.2 Direct Analysis of Raw Milk Samples by the Phage Assay

During the early stage of this project, the raw milk samples were directly processed through the phage amplification assay without prior chemical decontamination. However, the phage assay plates were contaminated following overnight incubation. Generally, different milk samples from the same or different farms have varying degree of contamination. For instance, plates for Farm A milk samples were consistently contaminated upon 24 hours of incubation. Sample A1 was more heavily contaminated as compared with Samples A2 and A3 (Figure 4.3). There was an equal degree of contamination observed among all of the milk samples from Farm B (Figure 4.3), but the contamination was not as heavy as those from Farm A. Therefore, an attempt to minimize the contamination was performed by storing the milks at -20˚C overnight prior to the phage assay, and the resulting phage assay plates indeed showed lesser contamination (data not shown).

From the contaminated phage assay plates, single colonies of dominant contaminants were isolated from the mixed culture for downstream analysis. Gram-staining was performed and the results revealed the Gram-type and morphology of the contaminants. They were Gram-negative cocci (Isolates IKP BC) and cocco-bacilli (Isolate 2KP B), Gram-negative rods (Isolates 1KP SC, 1MN BW, 3KP W and 3KP S), Gram-positive cocci (Isolate 2KP BY) and Gram-positive cocci in cluster (Isolate 1MN SY). Gram types and examples of cocci cocco-bacilli, and rods were shown in Figure 4.4. All contaminant colonies also have different color, morphology, size and surface texture and these are summarized in Table 4.1.

Pasteurized milk was also included for comparison with the raw milk. Samples of pasteurized milk used were from Dutch Lady and Magnolia. They were processed fresh shortafter opening and there was no contamination in the phage assay plates following overnight incubation. Indeed, a smooth *M. smegmatis* lawn was formed on the agar surface without any discrete elevated colonies (data not shown). Nevertheless, contaminating bacteria were found to grow on the phage assay plates during incubation, when the pasteurized milk had been opened and stored for about three to six days (Figure 4.5).

Table 4.1: Description of the contaminants colonies from raw milk samples

Sample	Description
A1	Heaviest contamination. Majority of the colonies were yellow and white, varied in size, had smooth and glistening surface and appeared as discrete entity. Most of the colonies were circular in shape..
A2	Milder contamination. The dominant contaminants have bigger sized colonies and were yellow, whereas smaller sized colonies were milky white in color. They appeared as punctiform and circular colonies that grew close to each other.
A3	Milder contamination. Colonies had greenish wrinkled surface and were milky light white in color whereas smaller colonies had smooth surface.
B1	Mildest contamination. Contaminant colonies varied in size. They were with big milky white colony and small yellowish colony, and both had smooth surface.
B2	Mildest contamination. Colonies were light yellow with soft edge.

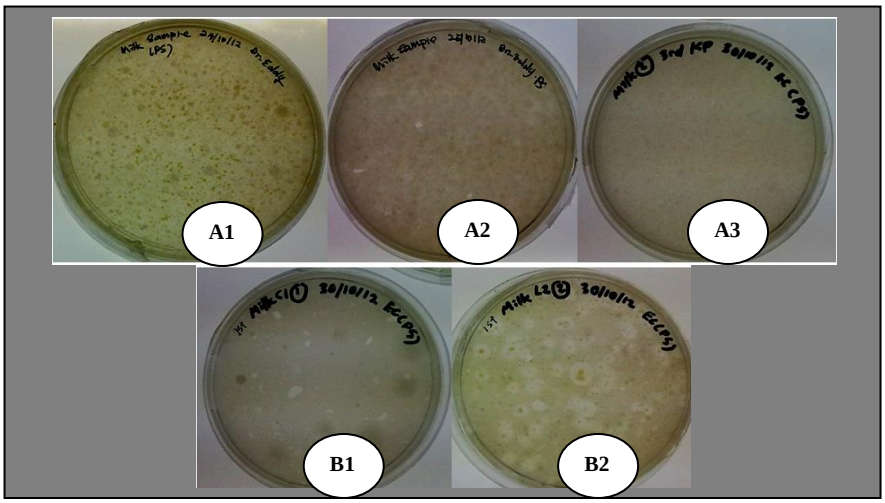

Figure 4.3: Phage assay plates for non-decontaminated raw milk samples.

Agar plates shown represented one of the duplicates prepared for the particular sample; three milking cattle from Farm A, A1, A2 and A3; two milking cattle from Farm B, B1 and B2 were shown.

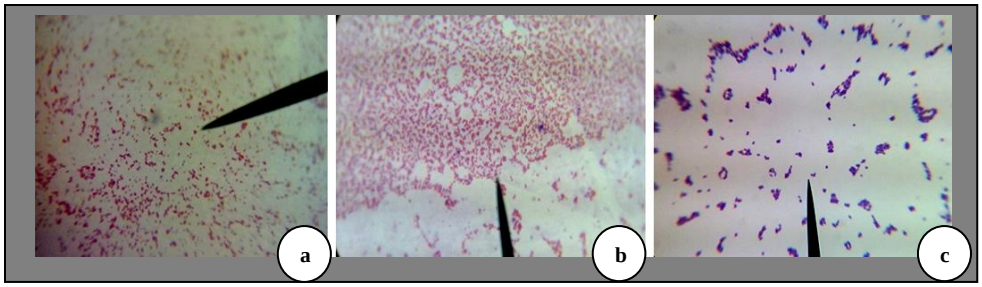

Figure 4.4: Gram-stained contaminant isolates from raw milk observed under oil immersion (1000x). a) Gram-negative cocci (IKP BC); b) Gram-negative rods (1KP SC); c) Gram-positive cocci (2KP BY).

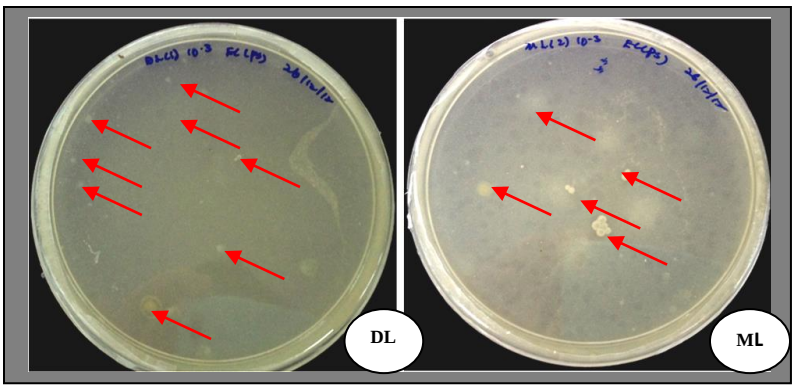

Figure 4.5: *M. smegmatis*-spiked pasteurized milk samples that were processed through the phage assay. DL: Dutch Lady; ML: Magnolia.

Contaminants colonies are indicated by red arrows for clarity.

4.2.1 Identification of Milk Contaminants by 16S rDNA Sequencing

As heavy contamination on phage assay plates would obscure plaque development, the identification of contaminating bacteria present in the milk was thus significant. This was because based on the contaminant profile, a more stringent decontamination protocol could be developed.

Based on the Gram staining results, genomic DNA was extracted from the contaminants using methods specific to their Gram-type as stated in the Presto Mini gDNA Bacteria Kit protocol. Gel electrophoresis of the amplicons following PCR showed bands of the expected size of 199 bp in great intensity and brightness (Figure 4.6). The 16S amplicons were then excised from the gel and subjected to purification using the Invisorb® Spin DNA Extraction Kit. Nanospectrometer was used to measure the concentration and purities of the

purified amplicons. Almost all purified amplicons showed A_{260}/A_{280} values of 1.8-2.0 and their concentrations were more than 10 ng/µl. A simple boiling method (95˚C for 15 minutes) was initially employed to release DNA from bacterials cells; however, it resulted in non-specific amplification (data not shown).

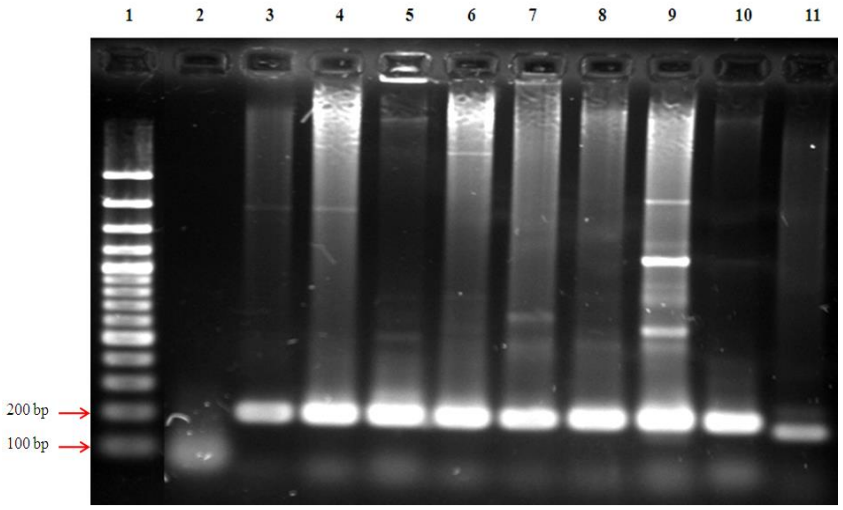

Figure 4.6: Gel analysis of universal 16S amplicons of contaminant isolates from raw milk sample

Lane 1, 100-bp DNA ladder; Lane 2, NTC; Lanes 3 and 4, contaminant isolates from Sample A1 (1KP SC and 1KP BC); Lanes 5 and 6, contaminant isolates from Sample A2 (2KP BY and 2KP B); Lanes 7 and 8, contaminant isolates from Sample A3 (3KP W and 3KP S); Lanes 9 and 10, contaminant isolates from Sample B1 and Sample B2 (1MN BW and 1MN SY); lane 11, *M. smegmatis* positive control.

4.2.2 Potential Sources of Milk Contaminants

All gel-purified amplicons were sent for DNA sequencing. BLASTn result of the DNA sequence with the lowest E-value and the highest percentage of similarity of each contaminant isolate was selected (Table 4.2). The milk contaminants isolated

from raw milks mainly belong to the genera *Enterobacter*, S*taphylococcus* and *Pseudomonas*.

Table 4.2: BLASTn analysis of contaminants in raw milk samples

Contaminant Isolate	BLASTn Identity	Accession	Score (bits)	E-value	Identity (%)
1KPBC	16S ribosomal RNA gene (*Enterobacter* sp. *AJAR-A3*)	KC355282.1	294	$1e^{-76}$	99
1KPSC	16S ribosomal RNA gene (*Enterobacter* sp. *AJAR-A3*)	HM103366.1	283	$2e^{-73}$	99
2KPB	16S ribosomal RNA gene (*Enterobacter* sp. *oral* taxon C98 clone UF045)	GU430681.1	272	$5e^{-70}$	97
2KPBY	16S ribosomal RNA gene (*Staphylococcus sciuri* strain -Y114)	JX134623.1	270	$2e^{-69}$	97
3KPS	16S ribosomal RNA gene (Uncultured *Acinetobacter* sp. isolate DGGE band A13a)	AM711883.1	292	$4e^{-76}$	99
3KPW	16S ribosomal RNA gene (*Pseudomonas aeruginosa* strain NRRL B-14935)	DQ459316.1	279	$3e^{-72}$	99
1MNBW	16S ribosomal RNA gene (*Klebsiella pneumoniae* strain SI-2)	KC211309.1	278	$1e^{-71}$	98
1MNSY	16S ribosomal RNA gene (*Staphylococcus sciuri* strain -Y114)	JX134623.1	281	$9e^{-73}$	99

4.3 Chemical Decontamination of Milk Samples

Direct analysis of raw milk samples through the phage assay gave heavily contaminated phage assay plates (Figure 4.3), which hampered the detection and observation of plaques. Therefore, prior chemical decontamination needed to be carried out. In this study, NaOH decontamination was preferred as it is a common method used to decontaminate specimens containing mycobacteria (Vestal 1975).

A preliminary determination of the NaOH concentration to be used for the decontamination was carried out. Two concentrations were tested, 1% (w/v) and 2% (w/v), on Sample A1. For both concentrations, there was no contamination on following incubation and the lower concentration was chosen because higher concentration of NaOH could affect the downstream phage assay by killing mycobacterial cells.

Even though 1% (w/v) NaOH was able to control contamination, some of the phage assay plates were still contaminated following 24-hour incubation, but this did not obscure plaque development and observation (Figure 4.7). However, further incubation led to severe contamination, which masked the possible development of additional plaques. Although this extended incubation could allow more plaques to form, the numbers were negligible and hence, all phage assay plates were incubated for 24 hours only in further experiments. Due to the varying NaOH-suppressive effect on contaminant growth, the efficiency of 1%

(w/v) NaOH in controlling contamination was quantitatively assessed based on the number of contaminant-free samples following 24-hour of incubation as shown in Table 4.3.

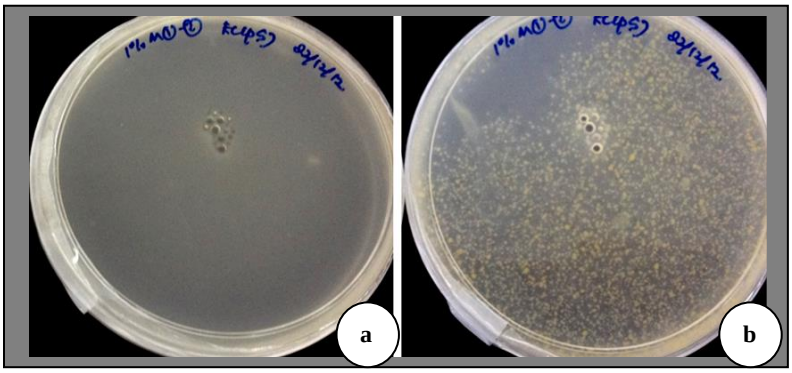

Figure 4.7: Phage assay plates of raw milk Sample B1 treated with 1% (w/v) NaOH. a) 24 hours incubation; b) 48 hours incubation.

Table 4.3: Effectiveness of 1% (w/v) NaOH to keep samples free from contamination after overnight incubation

Sample	A1	A2	B1
No. of decontamination trials	8	6	7
% contaminant-free	62.5	0.0	42.9

Milk samples from the same cattle processed on different days showed varying degree of contamination.

4.4 Inhibitory Effect of Milk Components on Phage Assay

The inhibition test was carried out by spiking *M. smegmatis* cell pellets with NaOH-treated raw milk and NaOH-treated 7H9 broth (control) followed by the phage assay. Pasteurized milk samples were also tested for comparison. Results

showed that the resulting plaques for raw milk were low in number, with an average of 1.44 x 10^5 PFUs/ml, which is a ten-fold reduction as compared to that for the control, with an average of 3.09 x10^6 PFUs/ml. This large difference suggests that there was potential inhibition from milk components on the phage assay. The pasteurized milk samples also recorded low numbers of recovered cells with 2.66 x10^5 PFUs/ml, but it was greater than that of raw milk, implying that it was not as inhibitory as the raw milk. Besides reduction in plaque counts, other inhibition effects included smaller sized plaques formed for both raw and pasteurized milks (Figure 4.8).

Potential inhibition by milk components on the phage assay could cause the actual number of mycobacterial cells present in milk being underestimated. Thus, an additional washing step was incorporated before NaOH decontamination using 20 ml of 7H9 broth with the aim to remove inhibitors from milk, hoping to recover and detect cells via the phage assay. Nevertheless, this extra washing step resulted in the reduction of plaque counts (Table 4.8). For instance, the raw milk Sample A3 generated about three folds reduction in plaque counts when the washing step was incorporated. Both control and pasteurized milk also experienced the reduction in plaque counts.

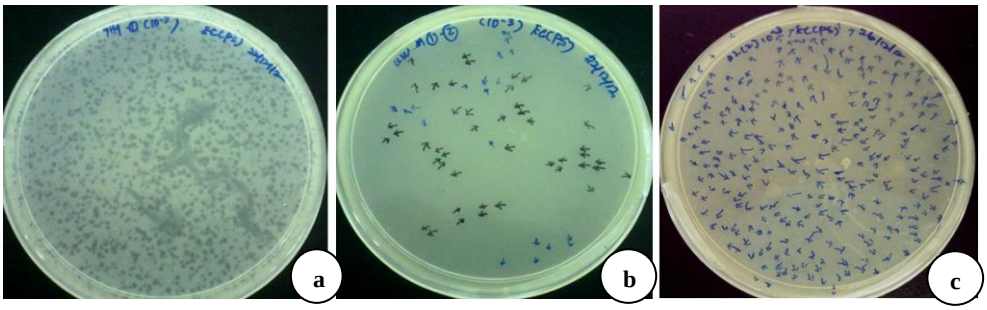

Figure 4.8: Inhibitory effect of milk components on the phage assay. a) Middlebrook 7H9 broth (control); b) raw milk sample (B1); c) pasteurized milk (DL)

Plaques in plates for b) and c) were indicated by black and blue arrow respectively for clarity.

Table 4.4: PFU counts for raw milk and pasteurized milk samples under different treatments

Treatment	7H9 Broth	Sample A3	Pasteurized Milk (DL)
Decontamination Without Washing	Confluent Lysis	305 PFUs/plate	Confluent lysis
Decontamination With Washing	Confluent Lysis (visually lesser than without washing)	114 PFUs/plate	Unclear discrete plaques formation

*The average of duplicate data were shown.

4.5 Sensitivity of the Phage Amplification Assay with *M. smegmatis*-spiked Milk Samples

Besides the potentiality of the phage amplification assay to rapidly detect mycobacterial cells in milk, it was also important to know its detection sensitivity. Theoretically, the lower number of mycobacterial cells that can be detected, the more sensitive the assay is. Despite of the potential inhibition of milk components and the need for decontamination, raw milk Sample B1 processed through phage assay still showed similar sensitivity level as that for non-decontaminated pure

culture; both were capable of detecting mycobacterial cells ranging from 10 to 100 CFUs/ml (Table 4.5).

In order to assess the effect of 1% (w/v) NaOH decontamination on the cells in milk, the spiked milk sample were subjected to processing with and without decontamination. However, due to contamination problem, it was not possible to process the milk without decontamination, and thus the efficiency of decontamination was assessed on pure *M. smegmatis* culture. Based on 1% (w/v) NaOH decontamination on the pure culture, there was a clear reduction in the CFUs and PFUs (Table 4.6). For instance, both CFUs and PFUs were reduced by about ten folds with decontamination treatment. These findings imply that with decontamination, the number of cells would be significantly reduced. However, decontaminated raw milk still retained similar sensitivity as that for non-decontaminated pure culture.

Table 4.5: Sensitivity of the phage assay assessed using raw milk samples

M. smegmatis (CFUs/ml)	Phage D29 (PFUs/ml)	
	Raw Milk Sample (B1)	Culture
10^6	Confluent Lysis	Complete Lysis
10^5	Anomalous	Complete Lysis
10^4	Confluent Lysis	Confluent Lysis
10^3	2628	661
10^2	551	119
10^1	2	9
10^0	0	0

*_M. smegmatis_ at exponential phase was about 10^6 CFUs/ml

Table 4.6: Effect of 1% (w/v) NaOH decontamination on PFU and CFU counts of *M. smegmatis* culture

Treatment	Without Decontamination		With Decontamination	
	Phage D29 (PFUs/ml)	*M. smegmatis* (CFUs/ml)	Phage D29 (PFUs/ml)	*M. smegmatis* (CFUs/ml)
	1.42×10^7	6.20×10^7	2.50×10^6	4.00×10^6
	1.76×10^7	5.90×10^7	2.45×10^6	5.00×10^6
	1.57×10^7	4.10×10^7	5.17×10^6	5.00×10^6
Average	1.58×10^7	5.40×10^7	3.37×10^6	4.67×10^6

Each determination was tested with approximately 10^7 CFU in triplicate

4.6 Limitations of the Combined Assay

Non-reproducibility and inconsistency of results were observed among replicates of the same milk samples. The plaque counts obtained were not consistent among the replicates despite same numbers of mycobacterial cells were spiked into each of them. Inconsistent results among replicates were found either among the

samples from the same farms or among the same samples from the same cattle. For the former, Samples B1 and B2 were both used for the phage assay sensitivity test, but only Sample B1 yielded consistent results among its replicates, whereas Sample B2 did not (data not shown). The ways of the pellet formation were also found to be varied among replicates of the same sample collected from the same cattle but, processed on different days; this could have exerted some impacts on the reproducibility among the replicates. For example, Sample A1 processed on different days yielded pellets that varied in color and depth of formation (Figure 4.9).

Another problem faced during this project was the poor clarity of the plaques of *M. smegmatis* cells spiked into milk samples, which caused the enumeration of plaques to be challenging. Interestingly, clearer plaques were produced when the carry-over milk clumps were allowed to sediment and only the resulting upper homogenous suspension was used for the phage assay (Figure 4.10). However, the sedimentation did not always produce similar clarity of plaque appearance.

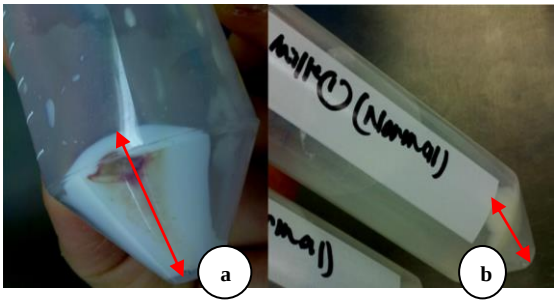

Figure 4.9: Pellets from Sample A1 obtained on different days of processing. a) Opaque pellet was about 2.5 cm long; b) gel-like pellet was about 1 cm long.

Depth was indicated by red double-head arrows.

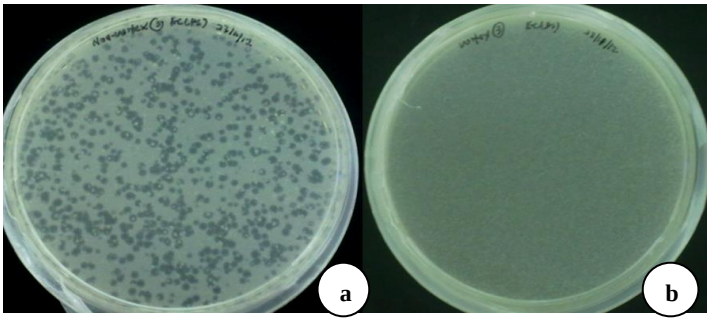

Figure 4.10: Effect of sedimentation of milk clumps prior to the phage assay on the clarity of plaques. a) With sedimentation; b) Without sedimentation

4.7 Development of the IS*1081* LAMP Assay for Identification of Mycobacterial DNA in D29 Plaques

The LAMP assay was optimized for the inner primer concentration. Initial attempt involved the use of 40 µM inner primers as recommended by Eiken Chemical Co., Ltd (Eiken Chemical 2005). Although the use of higher concentrations of primers increased the sensitivity of the assay, its specificity was compromised. *M. smegmatis* DNA (tube 4) which was without the targeted sequence, yielded positive SYGR Green signals (Figure 4.11). Nonetheless, the intensity of the fluorescence emitted was much lower as compared with those for Mtb and *M.*

bovis BCG DNA. Therefore, the concentration was reduced to 30 µM and it produced expected results, in which Mtb DNA was positive by the LAMP assay whereas *M. smegmatis* DNA was negative.

The IS*1081* LAMP assay was specific because results revealed that both *M. bovis* BCG plaque DNA and pure genomic DNA were positive by the LAMP assay, while LAMP assay, while *M. smegmatis* DNA which carries no targeted sequence was negative as expected (Figure 4.12). Surprisingly, another replicate of *M. bovis* plaque DNA yielded false-negative SYBR Green signal, which could be due to the losts of single genome of *M. bovis* BCG, which could be easily lost during DNA extraction. On the other hand, NTC turned green after the addition of SYBR Green dye, indicating false positive signal which could be due to cross-contamination.

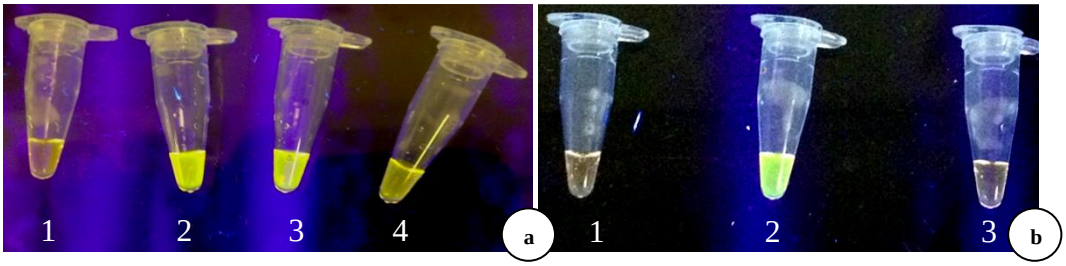

Figure 4.11: LAMP reactions using different inner primer concentrations.

a) 40 µM Inner Primers

: Tube 1, NTC; tube 2, Mtb DNA; tube 3, *M. bovis* BCG DNA; tube 4, *M. smegmatis* DNA

b) 30 µM Inner Primers

: Tube 1, NTC; tube 2, Mtb DNA; tube 3, *M. smegmatis* DNA

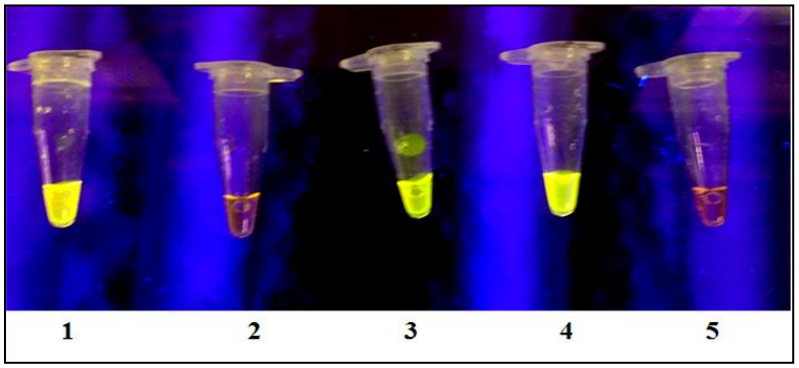

Figure 4.12: LAMP detection of *M. bovis* BCG DNA in D29 plaques

Tube 1, NTC; tubes 2 and 3, *M. bovis* BCG plaque DNA, tube 4, *M. bovis* BCG pure DNA, tube 5, *M. smegmatis* pure DNA.

CHAPTER 5

DISCUSSION

5.1 Sampling

5.1.1 Collection of Milk Samples

Raw milk samples from Farm A were collected and chilled on ice during transportation to the laboratory as recommended (Stanley et al., 2007). However, raw milk samples from Farm B were delivered directly by the farmer without temperature control. Despite of different delivery systems of the milk sample had no observable effect on the milk microbiota, delivery of milk without temperature control has been reported to cause the proteolysis of milk constituents such as fat globules during transportation (Franciosi et al., 2012).

There was an interval of 1-2 hours from the time of sampling to time of milk collection for both Farms A and B. For Farm B samples which were sampled earlier in the morning, they were delivered earlier to the laboratory, thus enabling fresh processing and eliminating the need for storage. However, Farm A performed sampling late in the afternoon, the collected milk samples were thus stored at 4˚C and processedthe next day. According to Standard Methods for the Examination of Dairy Products, cooling and storing milk samples at 4˚C is

recommended until testing. However, low teperature storage could affect the raw milk quality, thereby possibly interfering with the downstream analysis, phage assay in this case (Endrizzi et al., 2012). Indeed, cold storage of raw milk has been shown to favor the growth of psychrotrophic bacteria in milk, in which extensive amount of extracellular spoilage enzymes are produced, leading to spoilage of the milk (Hantsis-Zacharov and Halpern 2007). Nevertheless, as there was no absolute control on the time of sampling, an attempt to minimize the storage effect on raw milk was performed by processing the samples as early as possible following sampling.

5.1.2 Appearance and Characteristics of Milk Samples

In this project, raw milk samples were supplied by two cattle farms located within the same area in Kampar, Perak. It was found that samples from different cattle within the same farm differed in their color intensity and viscosity and these could be contributed by both physiological and physical factors. Examples of physiological states of cattle that might influence these include dehydration due to lack of water uptake, grazing behavior and starvation, whereas physical factors include length of time after the last lactation before sampling, age and fatness or leanness.

In fact, high metabolic rate in milking cows could have caused them to be afflicted with homeostatic disorders (Allison and Laven 2000; Sender 2001). The

lost of balance in metabolism such as high acidity could have contributed to the yellowish white color of Sample B1 and dehydration of cattle from Farm A could have led to production of viscous milk. For the latter, bacterial spoilage that occurred during cold storage could have also turned the milk more viscous. Therefore, Farm B raw milks which were free from storage effect appeared to be more diluted (Pesta et al., 2007). These suggest that variations are most likely present among milk samples either from similar or different cattle, contributing to the differences in the characteristics observed.

5.2 Direct Analysis of Raw Milk Samples by the Phage Assay

Stanley et al. (2007) reported that there was very little contaminant growth when milk samples were directly subjected to the phage assay without decontamination. However, the raw milk samples used in this project were heavily contaminated. This could be due to regional differences such as high temperature and relatively high humidity in tropical rainforest region here, causing frequent condensation which encourages the growth of bacteria (Karel and Lund 2003). This necessitated decontamination of the raw milk to remove non-mycobacterial organisms prior to the phage assay.

5.2.1 Identification of Milk Contaminants by 16S rDNA Sequencing

Gel analyses showed that the 16S amplicons of all contaminant isolates yielded bands with the expected size of 199 bp (Figure 4.6). Using DNA extracted with a commercial kit, desired bands all of the isolates appeared to be more intense and brighter, indicating high numbers of 199-bp amplicons were successfully extracted. Interestingly, using boiling released-DNA, it resulted in non-specific amplicons, even for NTC. Although smearing and non-specific bands were also observed for kit-extracted DNA, they were far lesser as compared to those for the boiling method.

Non-specific amplification could be due to low purity of the crude DNA lysate released from boiling, in which cell debris and medium residues could have interfered with primer annealing, resulting in non-specific amplification (Weighardt et al., 1993). However, with isolation of DNA using kit, there was still a little non-specific amplicons. This necessitated the need to increase PCR stringency, possibly by incorporating dimethyl sulfoxide and formamide into PCR reactions (Cheah, 2010). Nonetheless, non-specific bands would not be a problem as 199-bp amplicons could be selectively excised out for sequencing.

The resulting DNA sequence of purified 16S amplicons were analyzed with the BLASTn program available in the NCBI website. BLASTn results revealed that DNA sequences of contaminant isolates have high similarity to those of the

genera *Enterobacter* and *Staphylococcus*, with percentage of identity of 97-99% (Table 4.1). The potential identities were chosen for the isolates based on the lowest E-value, because it indicates the lowest probability that the sequence similarity occurs by chance but rather it is genetically due to sequence homology.

5.2.2 Potential Sources of Milk Contaminants

16S rDNA sequencing revealed that most of the contaminants belong to the genera *Enterobacter*, *Staphylococcus* and *Pseudomonas*, which are fast grower and thus can easily overgrow the slow growing mycobacteria, outcompeting them in the growth media, leading to culture loss (Falkinham III 2002). Indeed, based on FDA (2012), these contaminants are frequently associated with contamination of raw milk and several routes are possible for introduction of these contaminants into milk.

Contamination could be introduced during the milking process. In fact, *Enterobacter* spp. from fecal materials could have entered through the tract opening and colonized the interior of the udder, thereby introducing them into the milk. This implies that fore milk would harbor these contaminants. Besides, *Staphylococcus* spp. which are part of the skin microbiota, could also be introduced into the milk from the skin of farmers and cattle during the milking process. Milk is normally sterile or contains relatively few bacteria if it is secreted from the lactating glands of healthy animals but it can be endogenously

contaminated with MAP if the lactating cattle has been infected with MAP. However, milk is prone to external contamination following extraction from cattle (Rosenthal 1991) Based on Burton (1986), the contaminant organisms might have arisen from several sources, such as the sampling buckets, disposable plastic bags and surfaces of the containers used to contain the milk (Figure 4.1). Nonsterile bottles and plastic bags could have introduced contaminants into the milk. Furthermore, storage at 4˚C could also increase the risk of contamination (Hantsis-Zacharov and Halpern 2007). This was especially for the raw milk samples from Farm A which were always collected in the evening and could not be processed on the day of collection. Overnight storage at 4˚C could only slow down the growth of contaminants to some extent. Furthermore, psychrotrophic *Pseudonomas* spp. were able to grow and replicate at low temperature.

Apart from this, milking process is normally carried out under not sanitary condition, in which milk could have been contaminated by microbes in the air. Russell (n.d.) from Virginia Tech University reported that high proportion of bacteria could be introduced from the air when milk is collected under non-hygienic situation. *Pseudomonas* spp. from the environment could have contaminated the milk during the milking process. This explains their detection in the raw milk samples during this project, as revealed by the 16S rDNA sequencing. There were several recommendations to minimize the occurrence of contamination, such as washing and disinfection of the mammary before milking which would be effective to prevent introduction of microorganisms into the milk

(Cociuba 2008). Mechanical milking systems equipped with cold and warm water supply important for proper hygiene of the milking devices could also be considered.

For pasteurized milks which have been treated with high temperature in a short time under commercial condition, majority of the endogenous microorganisms would have been killed (Renner 1986). However, substantial growth of microorganisms in phage assay plates were observed when the milk was stored at 4˚C after opening for a few days and air was the most likely source of contamination. This has been previously reported for those which were stored at 4˚C for about five days before expiration (He et al., 2009). Hence, it was recommended to process pasteurized milks stored at 4˚C within 48 hours as practiced by some researchers (Ellingson et al., 2005). Overall, raw milk samples were more severely contaminated as compared to pasteurized milks as the latter have been heat-treated commercially to remove almost all of the contaminating organisms.

5.3 Chemical Decontamination of Milk Samples

Prior decontamination must be performed on raw milk samples in this project to remove non-mycobacterial organisms prior to the phage assay. The decontamination is suitable for mycobacterial culture because it relies on the resistance of mycobacteria to acid, base, or detergents (Peres et al., 2009).

Concentration of 1% (w/v) NaOH was used because it was sufficient to kill those non-mycobacterial contaminants in the raw milk samples, even though some contaminants survived after overnight incubation. However, 1% (w/v) was still chosen for decontamination as is widely used for sputum decontamination (Peres et al., 2009). Concentration of 2% (w/v) NaOH could also inactivate the growth of contaminants, but it could be very harsh to mycobacterial cells (Park et al., 2003). Therefore, 1% (w/v) NaOH was used throughout the project.

Even though the raw milk samples have been decontaminated, the growth of contaminating bacteria could still be observed on the phage assay plates following overnight incubation. Only one of the raw milk samples, B2, showed no contamination; other samples almost always showed contamination. This implies that 1% (w/v) NaOH decontamination is not totally effective to suppress the growth of these fast growers. This has also been reported by McClean et al., (2011). Although the contamination did not obscure the observation of plaques, the contamination worsened upon further incubation and significantly obscured plaque development and observation. Since the extra incubation showed only slight increase in plaque numbers which were negligible, incubation of all of the phage assay plates for 24 hours was sufficient for analysis.

The success of chemical decontamination relies on the amount of contaminant microbiota present in the sample (cited in Ratnam et al., 1987). However, since

the numbers of contaminants associated with different milk samples are not known, which could have caused different contamination rate among different samples, a more effective decontamination could be achieved upon lengthening the exposure time from 15 to 30 minutes using 1-2% NaOH (Burdz et al., 2003). If 2% (w/v) were used, the exposure time should be reduced to compensate for the increased strength of NaOH (Ratnam et al., 1987). However, with milder decontamination, the higher recovery rates of mycobacteria would be obtained although it could lead to higher rates of contamination (Sommers and Good 1985). Therefore, 1% (w/v) NaOH was used to decontaminate raw milk samples in this project after considering the balance between the decontamination power and the need to maintain the viability of mycobacterial cells in them.

Besides, a study on sputum decontamination revealed that decontamination with 2% (w/v) NaOH decontamination would kill about 20-90% of mycobacteria (Weitzman 2007). As sputum is more viscous than milk, there is more protection exerted on the high number of microbiota in the sputum, which necessitates the use of 2% (w/v) NaOH for sputum decontamination (C. E .D. Rees, personal communication). However, 1% (w/v) NaoH is recommended for sputum in which the phage assay is to be performed downstream as 2% (w/v) NaOH could have affected the phage assay possibly by affecting viability of mycobacterial cells (Dundee et al., 2001). All these justify the use of 1% (w/v) NaOH throughout the project.

Chemical decontamination has been reported to possibly kill MAP cells in milk obtained from diseased cattle, suggesting significant reduction of chance to detect MAP is possible (Gao et al., 2009). Thus, alternative method such as freezing of raw milk, which has been shown to significantly lower the bacterial numbers without damaging mycobacterial cells, may ultimately be considered (Murray and Coey 1959).

5.4 Inhibitory Effect of Milk Components on the Phage Assay

Based on the results, the *M. smegmatis* suspension in decontaminated raw milk recorded ten-fold reduction in the plaque count (1.44×10^5 PFUs/ml) as compared to the control (3.09×10^6 PFUs/ml), suggesting that raw milk could possibly inhibit the phage assay. Even though the exact mechanism of inhibition was unclear, however, it was most probably due to the binding of milk residues to the receptors on the target cells, thereby blocking adsorption of D29 phage to the host cells (Cheah 2010). Besides, infectivity of D29 phages on mycobacterial cells could also possibly be interfered by a wide range of substances deposited from cattle body fats into in the milk; these include carcinogens, pesticides, dioxin, and antibiotic residues (Mercola 1997). This would be especially true for cattle injected with growth hormone to increase milk production, which would have reduced their body fats. With lesser body fats, toxic compounds would be secreted into the milk instead (Mercola 1997). In addition, Bickley (1996) reported that different portions of milk, such as the cream and whey carry inhibitory substances

for PCR assay, they might also possibly cause the MAP detection by the phage assay to be challenging, but this is still under investigation.

Interestingly, the phage assay plate with *M. smegmatis* suspension in decontaminated pasteurized milk was subjected to lesser inhibition than that for raw milk, because the former showed two folds higher PFUs/ml as compared to the latter. This difference could be due to the removal of the whey proteins which might be inhibitory to the phage assay from the milk during pasteurization. This is because whey protein are heat susceptible and easily being denatured by pasteurization (Renner 1986). With the removal of whey protein, pasteurized milk could probably be less inhibitory as compared to the raw milk. The removal of whey protein also explains pasteurized milk is more diluted than raw milk.

Due to the presence of inhibitory substances in the milk, the effect of washing the milk pellet prior to NaOH decontamination and subsequent inhibition of phage assay was investigated. This extra washing step might contribute to removal of milk inhibitors in addition to those washing step during decontamination. Nevertheless, with this washing step, both raw and pasteurized milks showed reduction in PFUs. This could possibly be explained by the cell loss as a result of this extra washing step. Millar et al., (1996) reported that MAP cells would be in the cream, whey and pellet fractions of centrifuged milk; this suggests that with extra centrifugation step, more whey and cream layers (if any) would be discarded,

thus more cells would be removed as well. Hence, cell loss due to the extra washing step was suspected affect the detection sensitivity of the phage assay and is therefore not recommended for processing of milk sample.

5.5 Sensitivity of the Phage Amplification Assay on *M. smegmatis*-spiked Milk Samples

Results showed that the sensitivity level of the phage assay on *M. smegmatis*-spiked milk samples was between 10-100 CFUs/ml (Table 4.5). This was same as the typical sensitivity level reported for phage assay on sputum sample (Biswas et al., 2008). Besides, the sensitivity level of that of *M-smegmatis*-spiked milk was the same as that for pure culture without decontamination (Table 4.5). However, there was a reduction of ten folds of CFUs/ml of pure culture after NaOH decontamination (Table 4.6). Indeed, decontamination has been shown to affect the viability of mycobacterial cells even though they are relatively resistant to the NaOH used (Albert et al., 2007). Since the detection sensitivity was similar between the decontaminated raw milk samples and non-decontaminated culture, it could suggest that milk components might protect mycobacterial cells from the harsh effect of NaOH.

5.6 Limitations of the Combined Assay

During the project, the major limitation of the phage assay with raw milk sample is the non-reproducible and inconsistent results observed among replicates of same sample. The major reason could be due to the uneven distribution of milk

components that might have led to inconsistent pellet formation following centrifugation, thus affecting the extent of exposure of mycobacterial cells to NaOH action and also downstream inhibition of the phage assay. Even though the raw milk samples were well mixed by vigorous shaking before aliquoting into centrifuge tubes, the exact degree of homogenous distribution of milk constituents in each tube was unclear.

Apart of this, based on Stabel et al., (1997), Grant et al., (1996) and Hope et al., (1996), laboratorially grown mycobacterial cells might be more protective than *in vivo* mycobacteria against unfavorable condition; thus, this might imply different effect of NaOH decontamination on *in vivo* mycobacterial cells in raw milk. Furthermore, different species of mycobacteria vary in their level of resistance to chemical treatments due to difference in their cell wall compositions (Falkinham III, 2002). Despite all these limitations, the results of this test would give some insights on the sensitivity of the phage assay

As phage D29 only infects viable mycobacterial cells, the organism might be dead and thus become undetected by the phage assay (Stanley et al., 2007). The diagnosis of the milking cattle for paratuberculosis could be challenging when viable mycobacterial cells were killed during milking process. Besides, MAP cells in different physiological states have varied cell wall composition and thickness. These variations have been shown to significantly affect MAP susceptibility to

phage infection (Piuri and Hatfull, 2006; Dusthackeer et al., 2008). Therefore, MAP-negative samples could have harbored dead cells that could not be detected by the phage assay. Moreover, based on Sweeney et al., (1992), *in vivo* MAP cells in raw milk could be as low as 2 CFUs/50 ml and this level is beyond the detection limit of the phage assay observed in this project.

5.7 Development of the IS*1081* LAMP Assay for Identification of Mycobacterial DNA in D29 Plaques

As D29 phage is genus-specific, thus the phage assay is able to detect virtually all of the viable mycobacterial species and it could not distinguish among potential mycobacteria which are always associated with cattle infection, such as *M. bovis* and NTM including *M. fortuitum, M. farcinaogenes* besides MAP (Ford et al., 1998; Barclay and Ratledge 1983). Therefore, a LAMP assay was performed downstream to help in identifying MAP by targeting on MAP-specific insertion element, IS*900*, thereby adding specificity to the phage assay. With LAMP assay, it allows rapid detection of the desired amplicons using SYBR Green dye and negates the need for gel electrophoresis. Insertion element was selected as the target gene of amplification because they are present in multiple copies within a single genome; this would increase the chance of their detection (Stanley et al., 2007). In this project, IS*1081* LAMP assay was used as a test model to demonstrate the potential usefulness of the combined phage and LAMP assay, which targets the IS*1081* that are present exclusively in MTBC, Mtb and *M. bovis* BCG (van Soolingen et al., 1992).

IS*1081* LAMP was performed by designing primers that are specifically targeting on the IS*1081* elements. The reaction was started with 40 µM inner primers as recommended (Eiken Chemical 2005). Results revealed that the reactions with Mtb DNA and *M. bovis* BCG DNA were positive by the LAMP assay as expected. The presence of desired amplicons suggests that the LAMP primers recognized and thus amplified the six distinct regions on target sequence, thereby generating a large amount of amplicons which are stem-loop DNA structure with several inverted repeats of the target (Notomi et al., 2000). Subsequent binding of SYBR Green dye to its double-stranded amplicons caused them to emit green fluorescence with high intensity (Monis et al., 2005; Vitzthum and Bernhagen 2002). However, *M. smegmatis* DNA without target generated false positive signal, which was probably due to primer-dimer formation as well as non-specific binding of inner primers to the non-target template at high concentration (Figure 4.11a). Indeed, it is generally known that use of higher amount of primers maximizes the chance of mispriming, thereby generating nonspecific amplicons (Pestana et al., 2010).

Due to the false positive result, the concentration of the inner primers was reduced to 30 µM, and the results were as expected because Mtb DNA was positive by the LAMP assay whereas *M. smegmatis* DNA was negative (Figure 4.11b). Even though with 40 µM, the sensitivity of the LAMP assay was expected to be reliably

higher, but the test showed that they compromised its specificity. Therefore, the concentration of 30 µM could possibly be the optimal concentration for the inner primers and was used throughout the project.

The successful detection of *M. bovis* BCG plaque DNA from the spiked raw milk samples implies the high potentiality of the combined phage and LAMP assays to (Figure 4.12). This is because *M. bovis* BCG plaque DNA was positive by the LAMP assay. This observation agreed with the finding that IS*1081* elements are present in *M. bovis* BCG genome (Collins and Stephens 1991). Nevertheless, one of the duplicates of *M. bovis* BCG plaque DNA tested was not positive by the LAMP assay. This could probably due to the lost of single copy of genomic DNA which theoretically presents within a plaque during the extraction, which has been previously reporrted by Cheah (2010). Even though multiple copies of IS were targeted throughout the *M. bovis* BCG genome, the need for single-cell detection could stretch the sensitivity of the IS*1081* LAMP assay. Interestingly, the NTC yielded false positive result which could possibly due to the carry-over contamination from previous LAMP reactions (Figure 4.12). As LAMP reaction produces an abundant of amplicons and due to the need to open the reaction tubes to add SYBR Green dye for amplicon detection, which is the nature of post-amplification detection, it runs a high risk of contamination with amplicons deposited from previous reactions (Zhu et al., 2009).

To address this challenge, use of calcein fluorescence which allows closed-tube detection of amplicons, thereby minimizing the risk of cross contamination, could be an ideal choice of amplicon detection for LAMP assay in the future (Boehme et al., 2007). Alternatively, amplicons detection based on turbidity, which relies on precipitation of pyrophosphate, by-products of LAMP reaction, could be considered (Enosawa et al., 2003). Precautions such as opening the reaction tubes in a dedicated area for amplicon analysis, separated from that for preparation of reagents and reaction mixture, as well as the use of filter tips are necessary to minimize the risk of contamination.

5.8 Future Works

The potential usefulness of the developed assay could be demonstrated on cattle diagnosed to have paratuberculosis, by working together with veterinarians. Besides, MAP cells instead of *M. smegmatis* cells could be used as spiking model in the future. Fine tuning of milk processing protocol could also be performed such as by passing all of the milk through a gauge needle to declump the MAP cells which have tendency to form large clump of cells (Keswani and Frank 1998). Effectiveness of 1% (w/v) NaOH decontamination could also improved by incorporating antibiotics such as nystatin-oxacillin-aztreonam (NOA). In addition to this, pellet and cream layers after centrifugation could be combined and decontaminated together or zwitterionic detergent could be incorporated, to increase the recovery rate of mycobacterial cells (Dundee et al., 2001;

Anonymous 1998). The use of lecithin which stabilizes mycobacterial cell walls, thereby counteracting with the toxicity of NaOH, might be necessary (Thornton et al., 1998). With respect to inhibition of milk components, development of a more effective method which considers the balance between successful removal of inhibitory components and maximal impact on the detection sensitivity. The storage method which has been speculated to possibly diminish some natural inhibitory property of milks, could be considered (Keogh 1958). Besides, the sensitivity level of phage assay could be increased to detect as low as one mycobacterial cell by optimizing the phage inoculate and incubation times (McNerney et al., 2004). For the IS*1081* LAMP assay, the resulting amplicons should be analyzed by restriction digestion followed by the gel electrophoresis to further confirm the identity of the products. In addition to this, calcein fluorescence which enables closed-tube detection of amplicons and the use of filter pipette tips, may be necessary to minimize the risk of post-amplification contamination. Furthermore, multiple LAMP assays could be performed to increase its versatility to detect other potential mycobacterial species infecting cattle, such as *M. bovis*.

CHAPTER 6

CONCLUSION

In this study, a combined phage amplification and molecular assay was developed for detection and identification of MAP in raw milk from cattle. *M. smegmatis* and *M. bovis* BCG were used as model organisms in this assay development. Initial analysis of raw milk directly by phage assay without decontamination caused severe contamination after overnight incubation. 16S rDNA sequencing revealed that most of the contaminants belonged to the genera *Enterobacter* and *Staphylococcus* which are correlated to microbiota found in fecal materials and skin respectively. These reiterate that prior decontamination of raw milk with 1% (w/v) NaOH is necessary. Although the growth of contaminating organisms could still be observed following overnight incubation, they did not obscure the observation of plaques. Extended incubation of phage assay plates resulted in negligible numbers of plaque formation and led to severe contamination, suggesting 24-hour incubation is sufficient.

Milk components were also shown to be inhibitory to the phage assay, which caused approximately ten folds reduction in the PFUs detected. This was attributed to the potential binding affinity of milk residues to the receptors on the target cells, thereby preventing the adsorption of the phage D29 to the host cells. To address this challenge, additional washing step was incorporated before

decontamination with the intention to remove these inhibitors. However, it caused cell loss rather than minimizing the inhibition. The sensitivity of the phage assay with spiked milk samples was 10-100 CFUs/ml, equivalent to those reported for sputum specimens and mycobacterial cultures. As chemical decontamination and milk inhibition could possibly affect phage assay sensitivity, in which the former could affect mycobacterial viability, the sensitive detection level thus implies possible protection of milk components on the cells from NaOH action. The phage assay is genus-specific and able to detect viable mycobacterial cells; in order to identify the mycobacterial species, a downstream LAMP assay was performed. Successful amplification of IS*1081* from *M. bovis* BCG plaque DNA extracts by the LAMP assay suggests its potential in adding specificity to the phage assay. Even though post-amplifcation contamination was a problem in this study, it could be overcome such as by employing the use of calcein fluorescence and turbidity methods which permit closed-tube detection of amplicons. The use of filter tips could also be considered. All of these alternatives could minimize the risk of contamination.

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APPENDIX A

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
Laminar air flow cabinet	Telstar
Centrifuge machines	Hettich Zentrifugen, Sigma
Fridge and freezer	Copens Scientific, Pensonic
Gel imaging system	Syngene Bio Imaging
Shaking incubators	Labnet 211DS, N-Biotek
Static incubators	Memmert
PCR thermocycler	Kyratec
Water baths	Memmert
Light microscope	Leica CME
Spectrophotometer	Genesys 10S UV-VIS
Mini centrifuge	Labo Gene
pH meter	Eutech Instruments
Vortex mixer	VELP Scientifica

Table A2: List of consumables and their respective manufacturers

Consumable	Manufacturer
Agar-agar powder	Merck
Agarose powder	SeaKem LE
Basic fuchsin	Merck
Crystal violet	Merck
Deoxynucleoside triphosphate (dNTP) mix	Fermentas
DNA extraction kit	Geneaid
DNA ladder	Fermentas
Ethanol	Labmart
Ethidium bromide	Bio Basic
Gel purification kit	Invitek
Glycerol	QRëC
Iodine	Merck
Luria-Bertani (LB) agar powder	Merck
Magnesium sulfate ($MgSO_4$)	ORëC
Methylene blue	Systerm
Middlebrook 7H9 broth powder	BD
Middlebrook 7H10 agar powder	BD
Mycobacterium smegmatis MC^2155	University of Leicester*
Mycobacterium bovis BCG	University of Leicester*
Mtb CDC 155 genomic DNA	University of Leicester*
PCR primers	1^{st} Base
Phenol red	R & M Chemicals
Potassium dihydrogen phosphate	R & M Chemicals, QRëC
Safranin	Merck
Sodium hydroxide (NaOH)	Merck
SYBR Green dye	Invitrogen
Syringes	Cellotron
Syringe filters (0.2 µm)	Pall Life Science
Taq polymerase and PCR buffer	Fermentas
Tris	MP Biomedicals
Tween 80	Systerm, Sigma-Aldrich

* Kind donation from the University of Leicester, UK.