The Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Sheep in the Western Cape, South Africa

by
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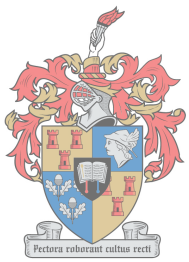
Thesis presented in partial fulfilment of the requirements of the degree of
Master of Science in Food Science



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Declaration

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Summary

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of a gastroenteritis ruminant (cattle, sheep, and goat) disease known as Johne's disease (JD). Subsequently MAP has been speculated as the cause of Crohn's disease (CD) in humans, an infection with similar symptoms, as MAP has been isolated from CD patients. Research has detected MAP in animal derived products such as pasteurised milk, soft cheeses and liver products. It is thought that through these routes, the transmission of MAP to humans has occurred. Statistics in South Africa depict that the Western Cape has the highest ovine JD outbreak, but the true prevalence is unknown. Currently used detection methods in South Africa lack sensitivity and are unreliable, therefore investigation of other more sensitive and reliable methods is required.

The aim of this study was to detect MAP in sheep in the Western Cape, South Africa. The first objective was to optimise a phage assay to specifically detect viable MAP in blood. This followed optimising a polymerase chain reaction (PCR) method. The last objective was to investigate the prevalence of MAP in various sheep sample matrices (blood, faecal and milk), and further compare the results to an enzyme-linked immunosorbent assay (ELISA) test.

Optimisation of the phage assay included establishing a *Mycobacterium smegmatis* (sensor cells) stocks, determine the D29 phage titre and apply these stocks to produce a positive and negative control. However, the optimisation proved to be problematic as the *Mycobacterium smegmatis* (sensor cells) stocks, used to create the lawn for the phage, were contaminated during storage. Therefore the positive and negative controls produced incorrect plaque results and the assay could not be used as a detection method.

As there is currently no published work in South Africa on the investigation of other detection methods besides ELISA, culturing and Ziehl-Neelsen stain; it was decided to investigate PCR as a possible detection method. Optimisation of DNA extraction was conducted on whole blood and buffy coat using two different DNA extraction kits. The DNA extracted from the buffy coat using the *Quick*-DNA Mini Prep kit produced the most and purest DNA compared to the whole blood. Various MAP specific primer sets that target different regions of the MAP genome were tested. The F57 primer sequence was the only primer sequence to detect the presence of MAP. Positive PCR samples were sequenced and aligned using the BLAST website. Results indicated that the PCR samples was 100% identical to MAP ATCC 19698, a bovine strain.

The optimised PCR protocol was used to determine the prevalence of MAP in various sheep sample matrices (faecal, blood and milk). Samples were taken from pregnant Merino and Dohne Merino ewes. Faecal samples did not produce any positive results due to lack of MAP shedding or faecal PCR inhibitors. Blood samples produced the highest positive prevalence at 47.6% from non-vaccinated ewes. The ELISA results indicated positive results for the vaccinated ewes, and also produced three false-positive results between 2017 and 2018, demonstrating the unreliability of the ELISA test. From the 47.6% positive blood PCR results, only two of the ewes produced positive ELISA results. This demonstrated that the PCR method is more sensitive than the ELISA method.

The milk samples produced a prevalence of 26.3%. The low positive prevalence was thought to be as a result of low MAP shedding during sampling. The live weight and body condition score was also taken into consideration, and showed no significant difference between 2017 and 2018.

To conclude, the PCR demonstrated to be more sensitive than the ELISA test, and the blood samples produced the highest prevalence of MAP.

Opsomming

Micobacterium avium subsp. *paratuberculosis* (MAP) is die oorsaak van 'n gastro-enteritis herkouer (bees, skape, bok) siekte bekend as Johne se siekte (JD). Daar word gespekuleer dat MAP moontlik die oorsaak is of ’n rol speel by Chrohn siekte (CD) in mense, 'n infeksie met soortgelyke simptome as Johne se siekte, aangesien MAP ook al geïsoleer is van CD-pasiënte. Navorsing het MAP bespeur in diere afkomstige produkte soos gepasteuriseerde melk, sagte kaas en lewerprodukte. Daar word vermoed dat die oordrag van MAP aan mense deur hierdie roetes plaasgevind het. Statistiek in Suid-Afrika toon dat die Wes-Kaap die hoogste skaap-JD-uitbraak het, maar dat die ware voorkoms daarvan onbekend is. Huidige toetsmetodes in Suid-Afrika is nie sensitief genoeg nie en is onbetroubaar, daarom is die ondersoek na ander meer sensitiewe en betroubare metodes nodig.

Die doel van hierdie studie was dus om die opsporing van MAP in skape in die Wes-Kaap, Suid-Afrika na te vors. Die eerste doel was om 'n fage-toets te optimaliseer, om sodoende lewensvatbare MAP in bloed te kan bepaal. Tweedens, die optimalisering van 'n polimerase ketting reaksie (PKR) metode. Die laaste doelwit was om die voorkoms/teenwoordigheid van MAP in verskillende skaapmonster matrikse (bloed, fekale en melk) te ondersoek en die resultate verder te vergelyk met 'n ensiem-gekoppelde immunosorbens-toets (ELISA).

Optimisering van die fage-toets behels die groei van 'n *Mycobacterium smegmatis* (sensor selle) laag, bepaling van die D29 fage titer, en die gebruik hiervan as ’n positiewe en negatiewe kontrole. Die optimisering was problematies, aangesien die *Mycobacterium smegmatis* (sensor selle) wat gebruik is om die groei-laag vir die fage te skep, tydens die berging daarvan besmet geraak het. Die positiewe en negatiewe kontrole het dus foutiewe plaakresultate opgelewer, en die toets kon nie as 'n opsporingmetode gebruik word nie.

Aangesien daar tans geen gepubliseerde werk in Suid-Afrika is nie oor die ondersoek van ander opsporingsmetodes behalwe ELISA, kultivering en die Ziehl-Neelsen kleuring; is daar besluit om PKR as moontlike opsporingmetode te ondersoek. Optimalisering van DNA-ekstraksie is uitgevoer op die bloed en witbloedselle laag (buffy coat layer) met behulp van twee verskillende DNA-ekstraksie toets-stelle. Die DNA wat uit die witbloedselle geëkstraheer is met behulp van die *Quick*-DNA Mini Prep toets-stel, het die meeste en suiwerste DNA in vergelyking met die bloed geproduseer. Verskeie MAP spesifieke aanvangskodon stelle wat verskillende streke van die MAP genoom teiken, is getoets. Die F57-primerreeks was die enigste aanvangskodon volgorde om die teenwoordigheid van MAP op te spoor. Positiewe PKR monsters is op volgorde van die BLAST-webblad geordend en in lyn gebring. Resultate het aangedui dat die PKR monsters 100% identies was aan MAP ATCC 19698, 'n bees stam.

Die geoptimaliseerde PKR protokol is gebruik om die voorkoms van MAP in verskillende skaapmonster matrikse (bloed, fekale en melk) te bepaal. Monsters is van dragtige Merino- en Dohne Merino-ooie geneem. Fekale monsters het nie enige positiewe resultate opgelewer nie as gevolg van 'n gebrek aan MAP-skuur of fekale PKR-inhibeerders. Bloedmonsters het die hoogste

positiewe voorkoms van 46.7% opgelewer. Die melkmonsters het 'n voorkoms van 26.3% opgelewer. Die lae positiewe voorkoms was vermoedelik die gevolg van lae MAP-vergieting tydens steekproefneming. Die ELISA-resultate het positiewe resultate vir die ingeënte ooie aangedui, en het ook drie vals positiewe resultate tussen 2017 en 2018 opgelewer, wat die onbetroubaarheid van die ELISA-toets getoon het. Die lewendige gewig en liggaamskondisie telling is ook in ag geneem en het geen beduidende verskil tussen 2017 en 2018 getoon nie.

Wat dus hieruit afgelei kan word, is dat die PKR blyk om meer sensitief te wees as die ELISA-toets, en dat die bloedmonsters die hoogste voorkoms van MAP opgelewer het.

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Contents

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The language, style and referencing format used are in accordance with the requirements of *the International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

Abbreviations

Abs	Absorbance
AFLP	Amplified Fragment Length Polymorphism
AGID	Agar Immunodiffusion
ATCC	American Type Culture Collection
BACTEC	Bactenecin
BCS	Body Condition Score
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
bTB	Bovine *tuberculosis*
CAF	Central Analytical Facility
CD	Crohn's Disease
CFU	Colony Forming Units
CMR	Calf Milk Replacer
DAFF	Department of Agriculture, Forestry and Fisheries
dH_2O	Distilled Water
dNTPs	Deoxynucleotide (G, A, T and C)
DTH	Delayed-type Hypersensitivity
EC	Eastern Cape
ELISA	Enzyme-linked Immunosorbent Assay
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAS	Ferrous Ammonium Sulphate
GP	Gauteng Province
HEYM	Herrold's egg-yolk medium
HTST	High Temperature, Short Time
IAP	International Association of Paratuberculosis
IFN-y	Interferon Gamma
JD	Johne's Disease
KZN	KwaZulu-Natal
LB agar	Luria Bertani agar
LB broth	Luria Bertani broth
LW	Live Weight
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MGIT	Mycobacteria growth indicator tubes
MIC	Minimum Inhibitory Concentration
MIRU	Mycobacterial Interspersed Repetitive Units

MP	Media Plus
MP	Mpumalanga
$m.s^{-1}$	Meters per Second
MTC	*Mycobacterium tuberculosis* complex
ng/µL	Nanogram per Microliter
NTB	Nontuberculosis
NTM	Nontuberculosis mycobacteria
NW	North West
OADC	Oleic Albumin Dextrose Catalase
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PFU/mL	Plaque Forming Units per millilitre
PMMS-phage	Peptide-mediated Magnetic Separation phage
qPCR	Quantitative Polymerase Chain Reaction
PCR-REA	Polymerase Chain Reaction-restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
RO	Reverse Osmosis
rpm	Revolutions per minute
RT-PCR	Real-time Polymerase Chain Reaction
SA	South Africa
SSR	Short sequence repeats
TAE	Tris-Acetate-EDTA
TB	Tuberculosis
T_m	Annealing Temperature
UV	Ultraviolet
VNTR	Variable Number Tandem Repeats
WC	Western Cape
ZN	Ziehl-Neelsen

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CHAPTER 1

Introduction

The world population is currently estimated to be 7.4 billion and is predicted to reach over 9 billion by 2050. The population in South Africa has drastically increased from 23 million (1980) to roughly 55 million (2015) and is rapidly growing (DAFF, 2016). Subsequently, the growing population places pressure on the agriculture sector to research innovative ways to meet increasing food demands (Ford *et al*., 2016). As a result, food safety measures are burdened to keep up with food production demands.

Food safety plays a vital role in ensuring that foodborne pathogens are reduced or destroyed, in order to decrease consumer's illnesses. The intensification and increase of food production in the agriculture industry has been linked to an increase in emerging food-borne pathogens (Jones *et al*., 2013). Food-borne pathogens produce a wide range of human illnesses due to the ingestion of contaminated foods with pathogenic microorganisms such as: viruses, parasites, fungi and bacteria (WHO, 2015). Contamination can occur at any stage during food production. From 2013-2017, there were 327 reported food-borne illnesses in South Africa. From 2017 to mid-2018, South Africa had experienced the largest *Listeria monocytogenes* global outbreak, with 928 laboratory-confirmed cases, which was caused as a result of contaminated processed meat (WHO, 2018). The National Institute for Communicable Diseases (NICD) has expressed concerns that many outbreaks are underreported in South Africa due to the lack of data (Shonhiwa *et al*., 2017). In terms of the rise in emerging pathogens, one that has been constantly debated is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Waddell *et al*., 2016; Roberston *et al*., 2017).

This *Mycobacterium* is the causative agent of Johne's disease (JD), a contagious and chronic infection found in the small intestine of ruminants. It has been isolated from livestock ruminants (cattle, sheep and goats) and is intermittently shed, through the various stages of the disease, into their faeces and milk (Grant & Rees, 2010; Chaubey *et al*., 2016). The thick waxy wall of the bacteria, which is comprised of 60% lipids, allows MAP to withstand physical and chemical processing steps in the food production, such as pasteurisation and chlorination (Rowe & Grant, 2006; Foddai *et al*., 2009; Salgado *et al*., 2015; Gerrard *et al*., 2018). Subsequently, MAP has been detected in pasteurised milk, soft cheeses and liver products (Gill *et al*., 2011; Smith *et al*., 2017). The debate about MAP's zoonotic potential is due to research indicating that MAP has been isolated from patients with Crohn's disease (CD); a chronic human gastroenteritis infection with similar symptoms to JD (Robertson *et al*., 2017). It is thought that humans are ingesting foods that are contaminated with MAP, as a result of infected ruminants shedding MAP into animal products such as milk (Chamberlin *et al*., 2001; Eltholth *et al*., 2009).

Johne's disease has a major impact on the agriculture industry as it causes economic losses for farmers due to: decreased milk production, decrease in wool quantity and quality, drastic ruminant weight loss and poor body conformation (Chaubey *et al*., 2016; Smith *et al*., 2017; Gerrard *et al*.,

2018). Although the dairy sector contributes a substantial amount to the agriculture sector in South Africa, there are also other sources; such as red meat, mutton and veal meat and dairy by-products such as cheese, that also act as a potential MAP source of contamination (Gill *et al*., 2011; DAFF, 2017). *Mycobacterium avium* subsp*. paratuberculosis* can be controlled on a farm level using vaccination, culling and quarantine; however, studies have shown these methods are unsuccessful as MAP was still detected after implication of these (Sweeney *et al*., 2012; Wolf *et al*., 2015; Gautam *et al*., 2018).

The first reported cause of Johne's disease in South Africa was in 1923. Between 1976 and 1990, there was an increase in the disease population to 42 counts. With regards to the cases of sheep JD, it was first recorded in 1967 (Michel and Bastianello, 2000). Since 1996, there have been 52 reported infected sheep identified in the Western Cape alone. The Western Cape had produced the highest prevalence of the disease in both dairy cattle and sheep in South Africa (Buergelt *et al*., 2005). Recent statistics (2018), obtained from the Department of Agriculture, Forestry and Fisheries, depicted that the Western Cape had the highest outbreak of ovine JD but Kwa-Zulu Natal produced the highest bovine JD outbreaks (DAFF, undated).

Current identification and detection methods used in South Africa include culturing, Ziehl-Neelsen (ZN) stain and enzyme linked immunosorbent assay (ELISA) (Michel & Bastianello, 2000). However, culturing takes up to 16 weeks and the risk of contamination from background microbes is high. Although rapid, the ZN stain lacks sensitivity and specificity. The ELISA test is used to detect the presence of antibodies in blood and milk samples. However, as the shedding of MAP is intermittent, these antibodies are not always present which can result in false-positive ELISA results. Furthermore, most methods lack the ability to distinguish between viable and dead cells, leading to further inconclusive results. The methods are used in combination to make a final decision on the status of a suspected ruminant. This is because the state veterinarian has to take in to consideration many factors before deciding to slaughter the animal. However, an immediate decision needs to be made and most of the methods are time-consuming. Subsequently, a method that it reliable, sensitive and can give a rapid response needs to be investigated. The phage method is a modern and innovative diagnostic tool that utilizes bacteriophages, in combination with polymerase chain reaction (PCR), to specifically and rapidly detect viable MAP cells within 48 h (Foddai *et al*., 2009; Botsaris *et al*., 2013; Botsaris *et al*., 2016; Swift *et al*., 2016; Waddell *et al*., 2016).

Due to a lack of sensitive and specific detection methods, there is a knowledge gap in understanding MAP. In South Africa, JD is known to be present on many farms but the exact scale is uncertain. Statistics dated from 1995-2017, indicated 1 820 cases, 152 outbreaks, 204 deaths and 861 sheep killed due to ovine JD. Reported bovine JD showed 1 280 cases, 39 outbreaks, 38 deaths and 448 cattle killed. However, not all cases are reported (DAFF, undated). The Department of Agriculture, Forestry and Fisheries released a statement in 2017 that expressed the need to improve current diagnostic methods to detect the presence of MAP at an earlier stage to implement the correct control strategy. Thus, it is important to investigate and implement advanced detection

methods, such as the phage and PCR method, to determine the true prevalence of MAP in the South African context (DAFF, 2017).

Therefore, the overall aim of this research project was to detect the presence of MAP in sheep in the Western Cape, by investigating and optimising the D29 bacteriophage and PCR detection methods. Once optimised, the methods were applied to various sheep sample matrices (blood, faecal and milk) on a research farm, Langgewens, which was known to have a history of Johne's disease. The results were compared to ELISA results to determine which method was more sensitive and reliable. The body condition score (BCS) and live weight was also recorded and taken into consideration. It will also be determined as to which sample matrix produces the highest MAP prevalence.

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CHAPTER 2

Literature Review

2.1 INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) was first described by Johne and Frithingham in 1985 and is currently one of the most common and important diseases, in terms of food and human safety, in the ruminant industry to date (Dreier *et al*., 2006). This mycobacterium was first isolated and grown as a pure culture in 1902, where it was known as *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne*. In 1906, this mycobacterium was first recognised as an infection dissimilar to *Mycobacterium tuberculosis* and received the name *Mycobacterium avium* subsp. *paratuberculosis* (Buergelt *et al*., 2005; Rowe and Grant, 2006). *Mycobacterium avium* subsp. *paratuberculosis* is known to be the aetiology of a ruminant disease, known as Johne's disease (JD) or paratuberculosis. With the final stage of the 'silent' JD leading to death, there are massive economic losses for farmers due to ruminant infertility, decreased productivity, weakened ruminant immune system leading to increased susceptibility to other diseases, resulting in death as the final stage (Collins, 2009; Smith *et al*., 2017). However, MAP can be controlled on a farm level using various control methods such as vaccination, culling or quarantine to prevent the further spread of MAP. However, controlling the spread of the disease has not been very successful as it has continued to spread throughout the livestock industry. This mycobacterium has been isolated from various ruminants such as goats, sheep, cattle and even a domestic dog (Alajmi *et al*., 2016; Bauman *et al*., 2016; Miller *et al*., 2017). Increasing evidence has suggests that MAP could be a possible cause to Crohn's disease (CD), a human's inflammatory disease with similar clinical symptoms to JD (McFadden *et al*., 1987; International Food Information Service, 2009; Krishnan *et al*., 2009).

Mycobacterium avium subsp. *paratuberculosis* has been detected in food products, such as pasteurised milk, cheese, meat products and these are thought to be the possible routes of contamination to humans. This mycobacterium has also been detected in water, which has run-off from infected cattle on farms, in soil, faeces and blood. However, current identification methods lack sensitivity and specificity to identify and detect the presence of MAP in samples. This can lead to inaccurate or false positive or negative results. Reasons such as stage of the disease, type of sample tested, lack of understanding of MAP bacterium characteristics, could all lead to these false results. Currently in South Africa (SA), there are few identification methods used. The prevalence of MAP in ruminants as well as food products from developing countries such as South Africa is also unknown. Therefore, further understanding of the disease is required to determine the correct method that can identify MAP at earlier stages in the disease as well as its presence in various organs.

2.2 *Mycobacterium* GENUS

The *Mycobacterium* genus is comprised of more than 100 species of which all produce a high G+C Gram-positive characteristic (Dalton & Hill, 2011). The genus is part of the *Actinobacteriae* phylum and has been classified, based on its taxonomy, as a relative to *Streptomyces, Rhodococcus* and *Corynebacterium* (Chacon *et al.*, 2004). The *Mycobacterium* genus is segregated into two groups based on their growth: fast and slow growing mycobacteria. Fast growing mycobacteria form colonies within seven days and tend to be non-pathogenic e.g. *Mycobacterium smegmatis*. The slow-growing species are considered the pathogenic mycobacteria and take more than seven days to produce a colony e.g. *Mycobacterium tuberculosis* and MAP. Human and animal diseases such as tuberculosis (TB), bovine tuberculosis (bTB), chronic respiratory diseases and Crohn's disease (CD) have originated from the fast growing mycobacteria genus (Warren *et al*., 2006; Botha *et al*., 2013; Muller *et al*., 2013). The pathogenic mycobacteria are further segregated, on a clinical level, into the *M. tuberculosis* complex (MTC) (e.g. *M. tuberculosis* and *Mycobacterium bovis*), and nontuberculous mycobacteria (NTM) (e.g. *Mycobacterium avium* complex and MAP) (Dalton & Hill, 2011). In general, mycobacteria are aerobic, non-motile and non-spore forming bacteria that are found in environmental niches (including water, soil, biofilms), food, milk, wild and domestic animals (Botha *et al*., 2013).

2.2.1 *Mycobacterium tuberculosis* complex

Members of the MTC, such as *M. tuberculosis* and *M. bovis*, are pathogenic as they cause deadly diseases that affect the pulmonary system in various hosts (Dalton & Hill, 2011). Strains found in the MTC have a 99.9% similarity on a genetic level, however they differ phenotypically with regards to the pathogenicity (Botha *et al*., 2013). Differentiation of the MTC is important to accurately diagnose the disease for health surveillance (Warren *et al*., 2006). It is estimated that a third of the worldwide population has been infected with mycobacteria that has the potential to cause tuberculosis. However, some of these mycobacteria are asymptomatic, meaning they will not develop into tuberculosis (Dalton & Hill, 2011). Although the development of antimycobacterial antibiotics were thought to have controlled the spread of this infectious disease, there seems to be a revival of tuberculosis in immunosuppressed individuals (Müller *et al*., 2013).

2.2.1.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis is the causative agent of TB in human. It was estimated that in 2015, 10.4 million new TB cases were reported worldwide, of which 56% were among men, 34% among woman and 10% among children. South Africa has one of the largest TB populations in the world. In 2015, out of the total 54 million people in South Africa, there were 294 603 TB cases notified (WHO, 2016). Typical symptoms include a chronic cough with blood-tinged sputum (mixture of mucous and saliva), fever with night sweats and weight loss (Müller *et al*., 2013). Clinical diagnosis can only be established once the disease has reached a progressive stage (Botha *et al*., 2013).

Current diagnostic methods that are recommended by WHO include: sputum smear microscopy, rapid molecular tests (Xper® MTB/RIF assay) and culture methods (WHO, 2016). *Mycobacterium tuberculosis* can be spread, from human to human, orally through coughing and is able to be dormant for years in a host's tissue, but is activated when the hosts' immune system is weakened. While the bacteria are dormant in the host, they also become resistant to drug treatment or any host defence mechanisms (Botha *et al.,* 2013). It has been thought that *M. tuberculosis* has evolved from *M. bovis* (Dalton & Hill, 2011).

2.2.1.2 Mycobacterium bovis

Mycobacterium bovis is similar to *M. tuberculosis* as a pathogen and is also a zoonotic disease known as bovine tuberculosis (bTB) (Swift *et al*., 2016b). Symptoms include fever, weakness and weight loss once *M. bovis* has infected the gut and caused the pulmonary disease (Dalton & Hill, 2011). Although *M. bovis* is commonly found in cattle it can also cause tuberculosis in wildlife animals (Dalton & Hill, 2011; Hlokwe *et al*., 2014; Miller *et al*., 2016). Transmission by respiratory routes not only leads to the infection of other animals, but can also be indirectly transmitted through food such as unpasteurised milk to humans (Botha *et al.,* 2013). There is an increase in weakened immune systems in South Africa's population, which causes a grave concern as the possibility of *M. bovis* infecting humans also increases through contaminated foods (Müller *et al*., 2013). In South Africa, TB is a controlled disease and the Director of Animal Health of the Department of Agriculture, Forestry and Fisheries controls the testing of TB according to the Animal Diseases Act 1984 (Act No 35 of 1984) (DAFF, 2013). The tuberlin test, is a diagnostic biological test that can be used to determine if an animal is infected with bTB. Tuberlin is a purified protein derivative that causes a delayed hypersensitive reaction. Positive animals will produce an inflammatory reaction where injected (DAFF, 2013). Control strategies such as vaccination have been advised for dairy and beef cattle farmers to try to manage the spread; however, it is difficult to detect the bTB infection. Immunological testing methods also cannot differentiate between an infected and a vaccinated animal, making control strategies challenging (Gill *et al*., 2011; McAloon *et al*., 2017; Smith *et al*., 2017). If a herd is regarded as infected with bTB, the herd is placed under official supervision (by a state veterinarian) and necessary steps (such as immediate or postponed slaughtering) will be taken to eradicate and prevent the spread of the bTB infection (DAFF, 2013). This leads to a financial loss to farmers and invariably leads to a reluctance to report to the authorities any possible infections.

2.2.2 Nontuberculous mycobacteria (NTM)

This group of pathogenic mycobacteria consist of members, such as *M. avium* complex (MAC) and MAP*,* which do not form part of the MTC (Botha *et al*., 2013). Mycobacteria that form part of the NTM, are saprophytic and cause deadly diseases in susceptible hosts, such as humans and ruminants (Dalton & Hill, 2011). Another characteristic that differentiates NTM from MTC, is the route of infection. Unlike MTC, NTM infect their hosts through environmental samples contaminated with NTM by means of inhaling, ingesting or through exposed open wounds (Gill *et al*., 2011).

Nontuberculous mycobacteria cause diseases from pulmonary to intestinal infections (Dalton & Hill, 2011).

2.2.2.1 Mycobacterium avium complex

This complex consists of similarly related strains, due to their related clinical relationship in the human medical field, which makes them difficult to identify or differentiate and eradicate with antibiotics (Wayne and Kubica, 1986; Dalton & Hill, 2011; Timms *et al*., 2015). Members include *Mycobacterium avium* subsp. *avium*, *Mycobacterium intracellulare* and MAP (Dalton & Hill, 2011). *Mycobacterium intracellulare* has been isolated from fish and black wildebeest in South Africa (Botha *et al*., 2013; Kabongo-Kayoka *et al*., 2015), while *M. avium* has been isolated from household plumbing (Falkinham, 2011). Mycobacteria found in this complex also have the ability to survive under different environmental stresses (low pH, variety of temperatures and even in a chlorine environment) (Dalton & Hill, 2011). The MAC was originally known to be opportunistic human pathogens and caused chronic respiratory infections. MAC has since been associated with immunosuppressed humans (Falkinham, 2011). Most of the reported MAC cases are from human infections; few MAC isolates have been reported from animals, except for MAP (Botha *et al*., 2013).

2.2.2.2 Mycobacterium avium subsp. *paratuberculosis*

Mycobacterium avium subsp. *paratuberculosis* differs from the other members in the *M. avium* complex as it is an obligate parasite that cannot replicate in the environment. However, MAP has the ability to remain dormant and survive outside a host in environmental niches (water, faeces, cattle, slurry and soil) because it is mycobactin dependent. Mycobactin is an iron chelate that allows MAP to respire and remain dormant (Buergelt *et al*., 2005). Genotypically, MAP is rather similar to *M. bovis* and *M. tuberculosis* strains (Rowe & Grant, 2006). Transmission of MAP occurs vertically and horizontally. Vertical transmission includes contamination of milk, semen, colostrum and in pregnancy. Horizontal transmission is through the environment such as water, soil and faeces (Chaubey *et al*., 2016). The main transmission route is faecal-oral, where an uninfected animal consumes feed or water that has been contaminated with faeces containing MAP (Botha *et al*., 2013). In terms of MAP transmission to human, animals or biofilms act as a source of contamination as MAP is unable to replicate in the environment (Collins, 1997). *Mycobacterium avium* subsp. *paratuberculosis* contains a thick and waxy cell wall comprised of 60% lipids. This property attributes to the acid-fast characteristic that allows MAP to resist decolourization through the acidified alcohol mechanism. The lipid content also contributes to the hydrophobicity effect in MAP, which allows MAP to easily form clumps and resist chemical and physical de-clumping processes. These characteristics allow the organism to survive pasteurisation of milk, which is thought to be the possible source of contamination for humans (Timms *et al*., 2015). Therefore, other potential sources of contamination for humans include pasteurised milk, dairy by-products and meat (Gill *et al*., 2011; Bostaris *et al*., 2013; Waddell *et al*., 2016). Isolates from three Crohn's patients, isolated in 1984, were reported to be genetically identical to MAP isolates that were isolated from cattle

(Chiodini *et al*., 1984). Collins (1997) suggests that genetically susceptible humans exposed at childhood, are likely to develop the disease if they encounter unknown microbial or chemical factor(s) in the environment. Buergelt *et al.* (2005) suggests that other exposure triggers such as infection or stress can also lead to MAP activation.

2.3 JOHNE'S DISEASE

Johne's disease (JD) is a severe gastroenteritis with significant impact on the world economy in terms of animal and human health as well as food safety (Chacon *et al*., 2004). The disease is classified into a subclinical (stages one and two) and clinical stages (final stage) based on the progression of MAP. In stage one; the infected ruminant does not shed the mycobacteria and this stage can last for years where the infected ruminant is asymptomatic. In other words, the animal can be infected with dormant MAP. The length of the incubation period can be affected by the age of exposure, dosage, the host susceptibility and lastly the managerial practises (Sergeant, 2001). Stage two is when shedding becomes more progressive as MAP is shed into the faeces as well as bodily fluids. This is where transmission to non-infected ruminants occur, as the cattle or sheep will shed the bacterium in their faeces (Gill *et al*., 2011; Waddell *et al*., 2016). The final phase within this stage is clinical, termed the 'terminal' stage, resulting in continuous shedding of MAP, progressive weight loss, untreatable diarrhoea and decrease in milk production for cattle while shedding of wool is a symptom in sheep (Smith *et al.*, 2017). Clinical signs are more visible in adult animals, typically older than two years. A herd with a positive result in JD can be categorized as follows: clinical cases, asymptomatic silent shedder's, subclinical carriers (non-shedders) and uninfected animals (Collins, 2009). Secondary clinical symptoms can lead to susceptibility towards mastitis and infertility (Waddell *et al*., 2016). Due to the latency period, JD is not classified as an epidemic disease (Davis & Madsen-Bouterse, 2012).

The age of an animal can also affect its susceptibility to MAP infection. Calves that are suckling tend to be more susceptible to infection of MAP. Calves naturally have a higher intestinal acidity, which enhances the survival of this mycobacterium, as it is an acid-fast bacterium. Suckling ruminants also consume lactoferrin and transferrin in the milk from their mothers. These components enhance the presence of MAP as the lactoferrin is a protein present in the milk, which has iron-binding properties, while the transferrin is an iron transporting protein found in the serum. Therefore, due to the iron-chelating properties found in MAP, it is further able to survive in suckling calves. Lastly, opsonin, a natural antibody present in the milk from infected mothers, aids in the uptake of MAP through the use of M-cells (epithelial cells that transport antigens from the lumen of the immune system as an immune response) in the large intestine as well as enterocytes found in the intestinal lining (Buergelt *et al*., 2005).

Johne's disease is further classified by strains. The S strain is isolated from JD infected ovine, while strain C is isolated from JD infected bovine (Whittington & Sergeant, 2001). However, a previous investigation indicated that sheep were more susceptible to either the S or C strain, while

cattle were not as susceptible to the S strain (Moloney & Whittington, 2008). This demonstrates that cross-species transmission of strains exists due to a host preference (EFSA Panel on Animal Health and Welfare (AHAW) *et al*., 2017). The long incubation period of JD also makes it more difficult to notice JD at the clinical stage in sheep than in cattle. Reasons for the difficulty in detecting JD in sheep is due to the fact that diarrhoea is a common symptom in cattle, while only 20% of sheep infected with MAP have diarrhoea 2 years after infection. Ovine JD also mimic symptoms found in other diseases such as caseous lymphadenitis abscesses, scrapie or produce chronic infections (lung, liver and/or kidney). Also, similar symptoms could also be produced as a result of a weakened immune-system (Collins, 2009). One of the more common symptoms of ovine JD is sudden weight loss; however, this symptom is uncertain as numerous other diseases/problems can also cause weight loss (Sergeant, 2001). Botha *et al*. (2013), suggested that because of the complexity involved in identifying and specifying MAP, there are very few reports of MAP isolated from animals in Africa.

2.4 CROHN'S DISEASE

Although the link between JD and CD is a constant debatable subject (Davis & Madsen-Bouterse, 2012; Waddell *et al*., 2016), there has been a suggestion that possible routes of human exposure to MAP should be investigated (Eltholth *et al*., 2009; Robertson *et al*., 2017). Crohn's disease is a recurring systematic inflammatory disease resulting in extraintestinal infection in the gastrointestinal tract, but is also detectable in the large intestine, liver and joints (McFadden *et al*., 1987). In other words, the disease seems to be caused due to a damaged interaction in the immune system- the symbiotic mutualism in the host has been disrupted (Baumgart & Sadborn, 2012). Clinical signs vary in infected patients, depending on the area that is inflamed. For example, illeal lesions produce symptoms of acute appendicitis, while colon lesions lead to abdominal pain and diarrhoea (Buergelt *et al*., 2005). There is no direct cure for CD, therefore, immunosuppressive drugs and surgical removal of infected regions in the intestine helps to control the symptoms. A seminar paper published by Baumgart and Sandborn (2012) noted that 50% of adults infected with CD require surgery within ten years after diagnosis and this disease reduces the patient's life expectancy. According to Behzadi *et al*. (2015), scientific research has shown a strong correlation between environmental factors and the occurrence of CD amongst populations. Environmental factors such as smoking, lack of vitamin D, long time consumption of antibiotics and consuming fast foods has an effect on the occurrence of CD in a population.

In terms of the global incidence and prevalence per 10^5 cases per country, CD has a high incidence rate in Canada (20.2), Australia (17.4), New Zealand (15.2), Brazil (14.6) and Scotland (11.7), and in terms of CD prevalence it is ranked highest in Canada (319), USA (241.3), Sweden (213), Denmark (151) and New Zealand (145). Reports from South Africa are segregated by race (white, black and coloured) and demonstrate a low ranking in the incidence (1.57) and prevalence of CD (Behzadi *et al*., 2015). Basson *et al*. (2014) specifically looked at the association between

race and CD in the Western Cape of SA. They established from their 194 CD patients' data that 18% were white, 78% were Cape coloured and 4% were black (Basson *et al*., 2014).

Wayne *et al*. (2011) compared the homogeneity of MAP strains from human and cattle infections, and saw there was hardly any diversity between the strains. There has been some concern that mothers affected with CD can possibly pass the disease onto new born infants through breast feeding (Naser *et al*., 2000). Naser *et al*. (2000) found that testing breast milk from CD mothers resulted in a positive culture, while control mothers resulted in a negative culture. This provides evidence that excretion of MAP into CD infected mothers' breast milk is possible.

2.5 FOOD AND WATER SAFETY CONCERNS

While the cause of CD is still unknown, there is epidemiological evidence supporting a relationship between MAP and CD (Uzoigwe *et al*., 2007; Waddell *et al*., 2016). Possible routes of transmission include sources such as the consumption of milk and milk based products, consuming meat or organ tissue (liver, spleen and kidney), drinking water from contaminated MAP water systems (Fig. 2.1) (Gill *et al*., 2011; Smith *et al*., 2017). According to Collins (2009), MAP is resistant to heat, ultraviolet (UV) light, freezing and some disinfectants. Although it is still uncertain as to what role MAP plays in CD, it is important to minimize the exposure of MAP to humans (Gill *et al*., 2011).

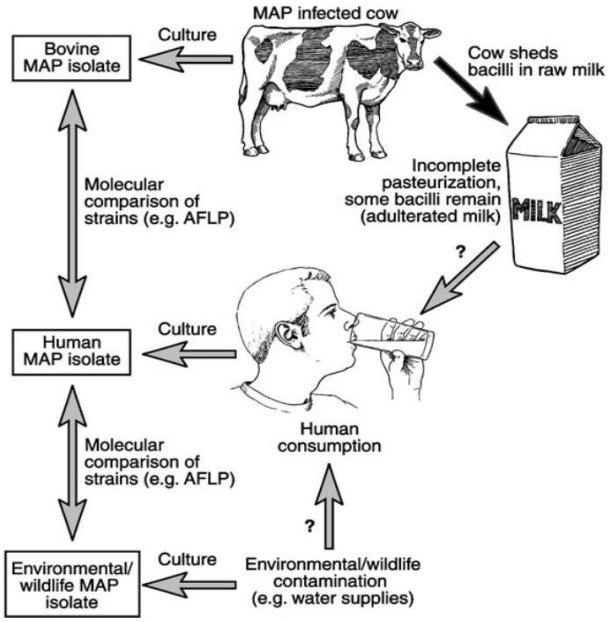

Figure 2.1 A schematic diagram illustrating the possible sources of MAP infection to humans (adapted from Chacon et al., 2016).

2.5.1 Dairy products

Studies have shown, although intermittent, that MAP can be detected in milk whether it is raw, bulk tank milk or even pasteurised milk (Lund *et al*., 2002; Donaghy *et al*., 2009; Waddell *et al*., 2016). This contributes to the spread and infection, acting as a possible source of exposure to humans as well as infant ruminants (Collins, 2009). Robertson *et al*. (2017) claims that raw milk can still be

contaminated from the udder of an infected dairy cow, no matter how good the hygiene practice is. However, Gill *et al*. (2011) suggest that although raw milk can be contaminated with MAP, there is a possibility that other mycobacteria isolates can produce false-negative PCR results. PCR detects the DNA of MAP in milk, from both inactivated and viable cells. Therefore, according to Gill *et al*. (2011), there would be no difference between the pasteurised or bulk raw milk preparations, as PCR testing procedures do not affect the prevalence of MAP DNA.

The current pasteurisation method used in the United Kingdom to pasteurise milk uses the HTST (high temperature, short time) method of 71.7°C for 15 s, while the United States of America uses the batch method with a temperature of 63°C for 30 min (Lund *et al*., 2002). In South Africa, the regulations relating to dairy products and imitation dairy products follow Section 15 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972). The regulation states that either pasteurisation of milk shall use the batch method or using the HTST method, where every particle of milk is treated (Foodstuffs, Cosmetics and Disinfectants Act and Regulations, 1997). Pasteurisation, using various times and temperatures, can determine the survival of microorganisms by plotting log survivors against time (D value). In return, the z-value is determined: the degrees C required to give a 10-fold change in the D value (Klijn *et al*., 2001). Methods that have been tested as alternative sterilising methods to inactive viable MAP cells in raw milk include: UV-C treatment (Donaghy *et al*., 2009), high pressure (Donaghy *et al*., 2007), microfiltration and bactofugation (Grant *et al*., 2005) and homogenization with HTST pasteurisation (Hammer *et al*., 2014). Although the mentioned methods achieved a log reduction, they were inadequate in completely inactivating any MAP present. When testing these methods, it is important to keep in mind the organoleptic properties of the milk during sterilising, as it could lead to rancidity due to the disruption of the fat globule membrane (Donaghy *et al*., 2009). There have been some reports where MAP has been detected in processed cheese and infant formula (Botsaris *et al*., 2010; Botsaris *et al*., 2016; Ricci *et al*., 2016). Although it has been suggested that milk and dairy products are the main routes of MAP exposure to humans, not many other dairy products in retail stores have been tested for the presence of MAP. No reports on testing the prevalence of MAP in milk and dairy products in South Africa and other African countries have been established (Donaghy *et al*., 2009; Ricci *et al*., 2016).

2.5.2 Meat products

Animals such as sheep, goat, cattle and some game meat that have been reared for human consumption, are slaughtered and dressed. During the dressing process, skinned carcasses are inevitably exposed to contaminated faeces, which overflow either from the intestine or from the hide (Eltholth *et al*., 2009; Gill *et al*., 2011; Van Der Merwe *et al*., 2014). Animals that have been reared for human consumption, are generally young animals when slaughtered (Gill *et al*., 2011, Van Der Merwe *et al*., 2014). *Mycobacterium avium* subsp. *paratuberculosis* is more prevalent in older ruminants than young animals, which means most meat should theoretically not contain MAP.

However, if an animal is born from an infected JD mother, the possibility of the young animal developing JD by the age of two years is high.

Waddell *et al*. (2016) reviewed that the likelihood of meat, which has been removed from clinically infected ruminants will produce a low MAP prevalence. A study in Canada tested muscle and organ tissue of 40 known infected cattle for MAP. Results demonstrated that no MAP was detected in any of the organ tissue; however, the MAP was detected in liver samples from 17 of the 40 culled cows (Antognoli *et al*., 2008). Available information is limited on which tissues are most likely to be populated with MAP and therefore which meat products would be contaminated with MAP (Antognoli *et al*., 2008). Mato *et al*. (2017) noted a reduction in the slaughter value as a result of lower carcass weight, bad conformation and fat cover scores due to JD. Therefore, the decrease in weight of an animal as a result of JD can lead to potential economic losses, as a carcass value is dependent on those three characteristics (Mato *et al*., 2017). Further investigation on the population of MAP in meat and organ tissue as well as the preservative treatments on the survival of MAP needs to be conducted. One of the greatest concerns is that MAP has not been recognised as a human pathogen by certain authorities, therefore, there is no limitation on the culling of infected JD ruminants for human meat consumption (Gill *et al*., 2011). No work has been conducted on the MAP in meat prevalence in South Africa and its neighbouring countries.

2.5.3 Environmental samples

Mycobacterium avium subsp. *paratuberculosis,* through evolutionary selection, has been able to survive within the environment using protozoa to form a biofilm (Steed & Falkinham, 2006). Protozoa are able to survive in diverse natural habitats, especially in an environment containing cattle slurry (Brown and Barker, 1999). These unicellular eukaryotic organisms are bacterivores, controlling the bacterial population in the soil allowing *Mycobacterium* and *Listeria* to survive. Their survival is dependent through these organisms by interfering with the fusion of the lysosomal and parasitophoric vacuoles in the host cell (Rowe & Grant, 2006). Limited research has resulted in reports stating that *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* ingested MAP within 180 min at 25°C and found that MAP was able to survive in the amoeba for more than 10 weeks (Rowe & Grant, 2006; Salgado *et al*., 2015). Mura *et al*. (2006) also noted that MAP is able to survive for long periods within the amoeba; for up to 24 weeks.

Human activity has played a role in the evolution of the ecology and epidemiology of MAP; as there is interaction in the environment thereby leading to more sources of exposure (Botha et *al*., 2013). For example, biofilms of MAP found in a plumbing water supply belonging to patients with NTB mycobacterial disease were impossible to remove using cleaning detergents (Falkinham, 2011). Another study found that MAP biofilms were detected in water entering a treatment plant, in potable water systems used for drinking water and in solids extracted from water while under treatment (Pickup *et al*., 2006). The survival of MAP in such systems can be due to the

mycobacterium's resistance to chlorine, which is used to treat and inactivate microorganisms in water (Whan *et al.*, 2001; Steed & Falkinham, 2006).

In terms of MAP survival in water, MAP can survive for 16-20 weeks, and 28 to 90 weeks in sediment, which has led to speculation that water can be a sufficient source of exposure to both humans and animals (Waddell *et al*., 2016). Buergelt *et al*. (2005) found a direct correlation between the amount of MAP present and the soil types. A low pH enhances the spread of Johne's disease as it is at optimum conditions for the mycobacterium to survive and respire by iron uptake. The Western Cape, South Africa, has been reported to have acidic and silcrete-type soils (Michel & Bastianello, 2000). Reports indicated that 47 out of 1718 farms in the Western Cape tested positive for JD in sheep and 46 out of the 47 farms contained acidic soils (Michel & Bastianello, 2000). According to our knowledge, no reports have been published in southern Africa that state the current prevalence of MAP in soil, water and faeces.

2.6 CONTROL STRATEGIES

Understanding the routes of MAP transmission is required before determining how to manage the mycobacterium in flocks/herds (Nielsen *et al*., 2016). Worldwide, MAP is difficult to control and eradicate due to its long incubation periods, stealthy nature, undetected shedders in the flocks/herds, wide range of hosts, a lack of accurate and rapid diagnostic tests and lastly, the lack of knowledge of the genetic variation and disease prevalence amongst the MAP isolates (Chaubey *et al*., 2016). Although Moloney and Whittington (2008) suggest that cross-species transmission of the S and C MAP strains has a low prevalence, they also recommend minimising contact between infected ovine JD sheep or manure with young cattle for their first 12 months of life (Moloney & Whittington, 2008).

In terms of sheep farms' economic losses, they can lose up to 10% of their ewes due to loss of body conditioning from being unable to absorb nutrients (Collins, 2009). Studies in the United States have shown that dairy herds, infected with JD, have caused major economic losses to the dairy industry of more than $200 million (R2.5 billion at $1=R12.54) per annum, at 2002 prices (Chi *et al.*, 2002). Current international management practices include test-and-cull methods, hygiene, vaccination and lastly restriction of animal movements through correct management practices (Chaubey *et al.*, 2016). Testing-and-cull is a difficult control strategy to follow, as most diagnostic tests have various efficacies depending on commercially available kits in South Africa such as the ParaChek™ Johne's absorbed EIA or the HerdChek™ *Mycobacterium paratuberculosis* antibody ELISA, which can lead to a reduced response in positive cattle (Michel & Bastianello, 2000; Chaubey *et al.*, 2016).

Trading of livestock can contribute to spreading of MAP between farms and regions. Therefore, a trace-forward investigation needs to take place to identify the herds' movements and whether there is a possibility of any cross-contamination or infection of JD ruminants. A veterinary examination of the introduced sheep or cattle needs to occur, to test whether they are infected. In Australia, a market assurance program exists, where testing of adult animals every two years for

MAP- ensures a 95% confidence that only 2% of the herd/flock are affected (Whittington & Sergeant, 2001). Internationally, there are no existing official documents with regards to the movement of livestock (EFSA Panel on Animal Health and Welfare (AHAW) *et al*., 2017). However, there have been guidelines proposed by the International Association of Paratuberculosis (IAP) for the certification on the movement of MAP infected livestock (IAP, 2016).

Management practices focus on circumventing healthy sheep or cattle coming into contact with infected herds and reducing the direct MAP load into the environment. It can roughly take up to seven years to have a complete clean-up of JD, due to the chronic nature of the bacterium as well as difficulty in diagnosing the disease (Collins, 2009). Due to calves and lambs being more susceptible to infection from their mothers, it is imperative that good management practices are applied, such as: separation of calves from mothers after birth, keeping non-infected heifers used to replace infected mothers in separate enclosures and lastly, a MAP-free colostrum supplement for feeding. For example: calf milk replacers (CMR) or even reducing lambs and calves exposure to adult's manure that is possibility infected with MAP (Collins, 2009). However, Grant *et al*. (2017) raised concerns on the potential ability of MAP to survive the manufacturing of dried milk-based products, as their research detected the presence of viable MAP from CMR. Improving hygiene and avoiding manure, which is contaminated with MAP, should also be implemented to avoid further spreading of the infection.

In South Africa, a draft by industry and the Department of Agriculture, Forestry and Fisheries (DAFF) was proposed to manage the further spread of JD by implementing a vendor declaration. It stated that reliable diagnostic tests, which are supported by private veterinary certification, should become available to determine the JD stats of an animal (DAFF, 2017). It also noted that the Onderstepoort Veterinary Research Institute is currently validating a diagnostic and reliable method that will be implemented in the future. However, it was noted that due to current diagnostic challenges, the wide-spread occurrence of JD in SA is not known. The draft aims to increase knowledge about the occurrence and spread of JD, once the diagnostic tests have been implemented, so that regular JD testing with vendor declarations can occur. Currently no vaccinations are supported by DAFF in South Africa, only infected farms that have been identified as MAP positive will receive permission from a state veterinarian to use vaccines, such as Gudair®. However, the draft stated that once new motivating information has been found, DAFF will reconsider the possibility of vaccines for wide spread use. Finally, requests were sent to deregulate JD in the Animal Disease Act, 1984 (Act 35 of 1984) from controlled to notifiable disease. However, the draft stated that deregulation can only occur once vendor declarations and results obtained from improved and reliable diagnostic tests have been obtained. This will also enable DAFF to consider a suitable route that can be used to control the further spread of JD (DAFF, 2017). Namibia, unlike Botswana, South Africa and Zimbabwe, has included Johne's disease as a notifiable disease where confirmation of the disease is either regionally or internationally confirmed in reference laboratories (Magwedere *et al*., 2012).

2.7 DETECTION AND IDENTIFICATION METHODOLOGY

Detection and diagnosis of ruminants infected with MAP is a laborious process due to their hydrophobic tendency to form clumps and slow-growing characteristic (Chaubey *et al*., 2016). In order to determine whether a flock has a JD problem, a quarter of the flock requires testing. In this sense it is necessary to begin with the animals that have the lowest body conditioning score to get a better idea of the presence of MAP within the flock as these animals are more likely to have MAP (Collins, 2009). Various diagnostic tests can be utilised to test for the presence of MAP.

Testing can be conducted either directly or indirectly. Direct detection includes the utilization of an agent, while indirect methods include specific immunological responses of the host to test for the bacterium's presence. Methods such as culturing, microscopy such as Ziehl-Neelsen (ZN) staining form part of the direct methods. Indirect detection is comprised of altered interferon-gamma (IFN-y) level, enzyme-linked immunosorbent assay (ELISA) and delayed-type hypersensitivity (DTH). Polymerase chain reaction (PCR), *in situ* hybridization and lastly immunofluorescence are direct pathological detection methods. Each detection method has its own sensitivity and specificity variables, but the most sensitive and specific method needs to be utilised to obtain optimal and quantifiable results (Chaubey *et al.*, 2016). It is noteworthy that shedding of MAP into faecal samples is intermittant, which is why inconclusive data is a possibility -depending on the stage of the infection (Nielsen & Toft, 2008). That is why it has been recommended for a veterinarian to use more than one method to determine the presence of MAP, before deciding to cull the animal (S. Davey, 2017, State Veterinarian, Department of Agriculture, Forestry and Fisheries, Malmesbury, SA personal communication, June 2017).

A summary of noteworthy research on detection methods has been presented in Table 2.1. The methods will be discussed in more detail at a later stage. It should be noted that most of the tests are conducted on faeces, blood and milk, as they are commonly known to contain the MAP, which has been shed during the various stages of the disease.

Table 2.1 Significant international articles demonstrating the detection methods utilised on specific samples dating from 1996-2016

Author(s)	Year	Country	Detection method	Ruminant	Faeces	Blood	Milk	Tissue	Soil	Other
Bauerfeind *et al*.	1996	Germany	IS900 PCR	Sheep, Goats and Cattle	x					
Michel & Bastianello	2000	South Africa	AGID, ELISA and ZN stain	Sheep				x		
Stabel *et al*.	2002	United States	Culture, ELISA and nested PCR	Cattle	x		x			
Corti & Stephan.	2002	Switzerland	IS900 PCR	Cattle			x			
Reddacliff *et al*.	2003	Australia	Radiometric Culture	Sheep	x			x	x	
Bhide *et al*.	2006	India	Nested IS900 PCR	Cattle and Sheep		x				
Stanley *et al*.	2007	United Kingdom	Phage-PCR	Cattle			x			
Kawaji *et al*.	2007	Australia	Radiometric culture and qPCR	Sheep	x					
Antognoli *et al*.	2008	Canada	ELISA, ZN stain and IS900 PCR	Cattle	x					Organ and muscles
Slana *et al*.	2008	Czech Republic	IS900 and F57 real time qPCR	Cattle	x		x			
Foddai *et al*.	2009	Northern Ireland	Phage assay	Cattle			x			
Botsaris *et al*.	2010	Cyprus	Phage-PCR	Cattle, Sheep and Goat			x			Cheese
Fathi *et al*.	2011	Iran	Culture and PCR	Cattle			x			
Botsaris *et al*.	2013	Cyprus	Phage-PCR	Cattle			x			
Swift *et al*.	2013	United Kingdom	ZN stain and Phage-PCR	Cattle		x	x			
Park *et al*.	2014	United States	Real time PCR	Cattle				x		
Galiero *et al*.	2015	Italy	Culture and qPCR	Sheep and Goat						Cheese
Salgado *et al*.	2015	Chile	ZN stain and Real time qPCR	N/A					x	
Bauman *et al*.	2016	Canada	Culture and Real time PCR	Dairy Goat and Sheep	x					
Swift *et al*.	2016	Australia	ELISA and Phage-PCR	Cattle	x	x				
Botsaris *et al*.	2016	Cyprus	Phage-PCR	N/A						Infant formula
Alajmi *et al*.	2016	Germany	Real time PCR	Cattle			x			
Dealbuquerque *et al*.	2017	Brazil	Real time and IS900 PCR	Cattle			x			

PCR: polymerase chain reaction, AGID: agar immunodiffusion, ELISA: enzyme-linked immunosorbent assay, ZN: Ziehl-Neelsen and qPCR: quantitative polymerase chain reaction

2.7.1 Strain differentiation of *Mycobacterium avium* subsp. *paratuberculosis*

South Africa is knowingly lacking data and research in the MAP field. A strain diversity investigation needs to be conducted to identify which strains are present in South Africa and whether a novel strain exists. In Germany, as many as 143 *Mycobacterium avium* subsp. *paratuberculosis* strains have been isolated and detected from dairy cattle faeces (Salem *et al*., 2013). There are various genotyping methods utilised such as multiplex polymerase chain reaction (PCR), pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), short sequence repeats (SSR), mycobacterial interspersed repetitive units (MIRU) and variable number tandem repeats (VNTR) to help identify the various strains (Fernandez-Silva *et al*., 2012; Salem *et al*., 2013). Although high costs are involved, the SSR method was more efficient in the characterization of MAP isolates than the MIRU and VNTR methods (Salem *et al*., 2013). There is a strain difference between JD infected cattle (C) and sheep (S); however, there have been studies that provide evidence of MAP transmission between sheep and cattle (Whittington *et al*., 2001; Moloney & Whittington, 2008). On the same farm plot in Australia, the S type strain was first diagnosed from ovine JD in the 1990s, but was also discovered in a bull in 1999, which was previously considered ELISA negative (Whittington *et al*., 2001). However, when an experimental design with a similar setup was conducted, none of the cattle were infected with the ovine JD strain. This leads to the evidence that MAP does not a have complete specific host (Moloney & Whittington, 2008). Using a PCR-REA method, one can visually distinguish between the S and C type strains using the IS1311 Sequence M56 and M94 primers (Marsh *et al*., 2006; Botsaris *et al*., 2016).

2.7.2 Culture methods

Culturing is considered the “golden standard”, which includes the cultural isolation of MAP from samples by means of Herrold’s egg-yolk medium (HEYM) or Middlebrook 7H10 agar with OADC supplement at 37°C for 12 weeks (Swift *et al*., 2016a). However, the golden standard, namely culturing, is very laborious and time-consuming (Redmond & Ward, 1966; Warren *et al*., 2006; Jasson *et al*., 2010; Chaubey *et al*., 2016). Samples that are best suited for culturing include faecal and tissue specimens (intestinal mucosa and lymph nodes). In order for the slow-growing mycobacterium to grow over the lengthy period, iron is required. Iron is supplemented to MAP through a siderophore known as mycobactin J. Mycobactin helps transport the iron in MAP to ensure the organism survives in the solid medium (Timms *et al*., 2016). Other culturing techniques include the automated culture system known as BACTEC MGIT 960 and the radioisotopic BACTEC 12B or 460. Automated systems shorten the detection time and can detect MAP in samples as low as 10 CFU/mL. The radioisotopic method utilises radioisotopes to radiolabel the MAP culture (Chaubey *et al*., 2016). Although modern culturing techniques are more rapid, these systems mix various acid-fast and non-acid-fast bacteria cultures that can lead to contamination of samples. To overcome such issues, antibiotics (vancomycin, amphotericin B, and nalidixic acid (VAN) or polymyxin B,

amphotericin B, nalidixic acid, trimethoprim, and azlocillin known as PANTA) can be used in both conventional and automated systems (Chang *et al*., 2002; Grant & Rees, 2010).

2.7.3 Antimicrobial susceptibility of *Mycobacterium avium* subspecies *paratuberculosis*

Currently, there is no effective treatment for JD (Collins, 2009). To determine the minimum inhibitory concentration (MIC) of antimicrobial drugs against MAP, increasing concentrations of drugs are incubated with MAP using the EUCAST (European committee on antimicrobial susceptibility testing) guidelines (Williams *et al*., 1999). Williams *et al*. (1999) used the firefly luciferase-based assay to test MAPs antimicrobial susceptibility and found that a combination of amikacin, clarrithromycon and rifuabutin were the most effective treatment towards JD. However, Timms *et al*. (2016) tested clarithromycin, rifampin, clofazimine, ciprofloxacin, ethambutol and streptomycin using Middlebrook 7H10 agar with mycobactin J and isolate 43525 MAP. This study found that ciprofloxacin, streptomycin, clofazimine and rifampin had higher MICs than the other antibiotics (Timms *et al*., 2016). Krishnan *et al*. (2009) tested and compared the BACTEC™ MGIT (960 and ParaTB)™ and conventional agar dilution methods with ciprofloxacin, levofloxacin, azithromycin, clarithromycin, amikacin, ethambutol, clofazimine, isoniazid, dapsone, rifampicin and rifabutin. These are specific drugs usually used to suppress the normal activity of the immune system. It was concluded the MGIT™ media produce rapid and reliable drug susceptibility of MAP (Krishann *et al*., 2009).

2.7.4 Ziehl-Neelsen (ZN) staining

Due to the thick waxy outer layer containing high mycolic acid in their cell wall, MAP are resistant to Gram staining techniques. Therefore, because of their acid-fast characteristic, the ZN (Ziehl-Neelsen) staining method (Fig. 2.2) is utilised to identify the bacteria. Positive results will indicate a bright red cell with a blue background (Swift *et al*., 2014). Ziehl-Neelsen staining is specifically used to test for the presence of MAP cells in faecal, blood, milk and tissue (intestinal lymph nodes and gross lesions) samples under a microscope (Chacon *et al*., 2016). Although this diagnostic test is fast, inexpensive and relatively easy to use, there are some limitations. The staining is not highly specific or sensitive as all *Mycobacterium* display an acid-fast characteristic. Due to ruminants shedding lower amounts of MAP cells during the sub-clinical phase, the ZN stain does not easily detect small concentrations under the microscope. Knowing this, it is recommended to not utilise the ZN staining as a single diagnostic tool, but to rather use it in conjunction with another tool to increase the specificity and sensitivity to detect MAP in sub-clinically and clinically infected ruminants (Wynne *et al*., 2011).

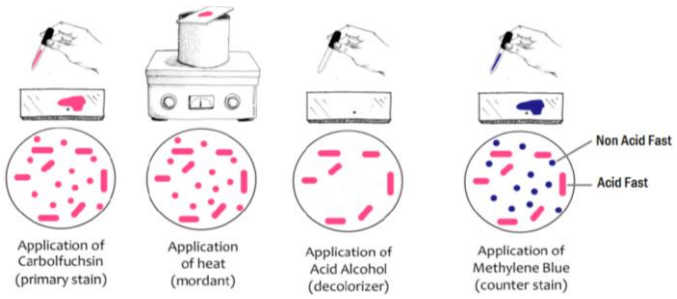

Figure 2.2 Ziehl-Neelsen Staining principle (adapted from Giri, 2016).

2.7.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA utilises the serology characteristics of MAP to diagnose a sample as positive or negative. It is one of the most commonly used assays to determine a ruminants' infection stage, as it has a high specificity ranging from 97.4-98.8% (Chaubey *et al*., 2016). However, in general, ELISA tests lack the ability to detect the bacterium in earlier stages of infection due to low levels of antibodies present. The later the stage of infection, the higher the antibody population, which allows for easier diagnostic quantification. Therefore, false results are most likely to occur in the initial stage of JD (Mato *et al*., 2017). Currently in SA, the state veterinarians routinely utilise the ELISA method when determining the presence of MAP in suspected infected ruminants (S. Davey, 2017, State Veterinarian, Department of Agriculture, Forestry and Fisheries, Malmesbury, SA, personal communication, January 2017). Internationally it has been recommended to combine the ELISA test with a confirmation method such as a polymerase chain reaction technique (Pinedo *et al*., 2008; Smith *et al*., 2017), however this requires skill, time and is expensive to run; particularly in developing countries. Although the test itself is rapid, cheap and easy to use, the sensitivity of the test is low due to the wide range of strains and specific antibodies produced (Nielsen & Toft, 2008). The sensitivity of the ELISA test also increases when combined with PCR when testing faecal samples (Waddell *et al*., 2016).

2.7.6 Polymerase chain reaction (PCR)

Polymerase chain reaction is the rapid identification and detection of a target nucleic acid sequence by means of enzymatic amplification. The PCR amplification process is separated into three stages. The first stage is denaturation, followed by annealing and the last polymerase extension. Each step has specific conditions that need to be monitored using a thermocycler to correctly amplify the specific DNA (Chacon *et al*., 2016; Waddell *et al*., 2016). The PCR can be used to directly detect MAP in food and environmental samples (Grant & Rees, 2010). Insertion sequence's (IS) identify specific sequences from isolated DNA from these samples. There are a number of MAP-specific genomic sequences that have been identified, mainly: IS900 (Green *et al*., 1989), F57 element (Poupart *et al*., 1993), *hsp X* gene (Ellingson *et al*., 1998) and IS*Mav*2 (Strommenger *et al*., 2001). A summary of the target sequences and their respective primers can be seen in Table 2.2. The IS900 sequence was discovered in the 1980s. It is a 1451 base pair element that has been defined

as definitive for the identification of MAP, as there are 14-20 copies of IS900 in the MAP sequence (Grant & Rees, 2010). The F57, *hsp X* gene and IS*Mav*2 sequences have low copy numbers in the MAP genome. Initially the IS900 sequence was routinely used as the standard marker for MAP PCR detection. However, some studies reported environmental mycobacteria and mycobacteria, other than MAP, that contain IS900-like sequences (Cousins *et al*., 1999, Englund *et al*., 2002; Grant & Rees, 2010; Chaubey *et al*., 2016). The F57 sequence is not as sensitive as IS900, but has only been detected in MAP samples and has not been detected in any of the *Mycobacterium avium* complex or any other mycobacterial species (Vansnick *et al*., 2004). Therefore, the F57 single copy sequence holds great potential.

2.7.6.1 Nested PCR method

Nested PCR can be used to avoid any deficient amplification reactions that are caused by inhibitors. This is established by aliquoting the first round of amplification PCR product with a new set of primers. Primers that were used to detect the presence of MAP in cattle milk and tissue (lymph nodes and intestine) samples, that are specific to the IS900 sequence, are TJ1 and TJ2 and are used for the first round and TJ3 and TJ4 for the second amplification set (Pierre *et al*., 1991, Bull *et al*., 2003). Although the method has a relatively high sensitivity, there is a possibility for cross-contamination from aerosol spores, which can result in false-positive and false-negative results (Gill *et al*., 2011). Cross-contamination can be avoided through use of dedicated equipment to analyse PCR products (B.M.C. Swift, 2017, University of Nottingham, Personal communication, April 2017). Buergelt and Williams (2004) tested blood and raw milk for MAP using nested PCR and concluded that the nested PCR was able to detect MAP DNA in subclinical infected cattle, where the ELISA test produced negative results. ELISA assay produced the highest sensitivity values, complementary to faecal culture and PCR, and it was recommended to combined the ELISA and nested PCR methods to increase the overall sensitivity for MAP detection. Little work has been conducted on detected MAP from sheep samples using the nested PCR technique.

2.7.6.2 Quantitative PCR method

Quantitative PCR (qPCR), also known as real-time (RT)-PCR, provides a more sensitive identification for the quantification of MAP. Quantitative PCR utilises fluorescent probes and primers, allowing continuous detection of amplicons during amplification. The quantitative reading is relative to the amount of fluorescence emitted and is directly proportional to the amount of product amplified during analysis (Chaubey *et al.*, 2016). Dealbuquerque *et al*. (2017) used a Qiagen rotor-gene Q thermocycler to detect the presence of MAP and to compare the conventional PCR to qPCR. Although the qPCR is more sensitive to detecting MAP than the conventional PCR, qPCR is also sensitive to inhibitors and requires an increase in the purity of DNA as well as decrease of inhibitors in the samples. Therefore extraction methods to provide good quality DNA samples needs to occur. Nonetheless, this IS900 qPCR method provided the most sensitivity to detecting low CFU present

Table 2.2 Summarised table indicating some published primers with respective target sequence

PCR	Target	Primer	Sequence (5'-3')	Size (bp)	Reference
Single	IS900	IS900/150	CCG CTA ATT GAG AGA TGC GAT TGG	229	Vary *et al*. 1990
		IS900/921	AAT CAA CTC CAG CAG CGC GGC CTC G		
		P11	CGT CGT TAA TAA CCA TGC AG	278	Moss *et al*. 1991
		P36	GGC CGT CGC TTA GGC TTC GA		
		P90	TCG GGG CCG TCG CTT AGG	400	Moss *et al*. 1992
		P91	GAG GTC GAT CGC CCA CGT GA		
		MK5	TTC TTG AAG GGT GTT CGG GGC C	560	Doran *et al*. 1994
		MK6	GCG ATG ATC GCA GCG TCT TTG G		
		MP3	CTG GCT ACC AAA CTC CCG A	314	Bauerfeind *et al*. 1996
		MP4	GAA CTC AGC GCC CAG GAT		
		P90+	GAA GGG TGT TCG GGG CCG TCG CTT AGG	413	Millar *et al*. 1996
		P91+	CGC GTT GAG GTC GAT CGC CCA CGT GAC		
		S204	TGA TCT GGA CAA TGA CGG TTA CGG A	563	Englund *et al*. 2002
		S749	CGC GGC ACG GCT CTT GTT		
	IS*Mav2*	II120	GAT CAT TCC CGG ATG TGT G	269	Strommenger *et al*. 2001
		II121	AGA CTG CGG TGA AAC TGC T		
		ISMav1	GTA TCA GGC CGT GAT GGC GG	312	Stratmann *et al*. 2002
		ISMav2	CCG CAC CAG CGC TCG ATA CA		
		ISMav2-F	GTG AGT TGT CCG CAT CAG AT	494	Shin *et al*. 2004
		ISMavB2	GCA TCA AAG AGC ACC TCG AC		
	F57	F57	CCT GTC TAA TTC GAT CAC GGA CTA GA	432	Vansnick *et al*. 2004
		R57	TCA GCT ATT GGT GTA CCG AAT GT		
		f57p1	TTG GAC GAT CCG AAT ATG T	254	Tasara *et al*. 2005
		f57p2	AGT GGG AGG CGT ACC A		
	hsp X	Left	GAC CGG CTA TCT GTG GAA C	211	Ellingson *et al*. 2000
		Right	CTC GTC GGC TTG CAC CTG		
Nested	IS900	*S204/S749*	*Same as above (Single IS900)*	563	Englund *et al*. 2002
		S346	GCC GCG CTG CTG GAG TTG A		
		S535	AGC GTC TTT GGC GTC GGT CTT G	210	
		TJ1	GCT GAT CGC CTT GCT CAT	356	Bull *et al*. 2003
		TJ2	CGG GAG TTT GGT AGC CAG TA		
		TJ3	CAG CGG CTG CTT TAT CTT CC	294	
		TJ4	GGC ACG GCT CTT GTT GTA GT		
	F57	F57/R57	*Same as above (Single F57)*	432	Vansnick *et al*. 2004
		F57Rn	TGG TGT ACC GAA TGT TGT TGT CAG		
		R57	TCA GCT ATT GGT GTA CCG AAT GT	424	
Quantitative	IS900	forward	CGG GCG GCC AAT CTC		Khare *et al*. 2003
		reverse	CCA GGG ACG TCG GGT ATG		
		probe	FAM-TTC GGC CAT CCA ACA CAG CAA CC-TAMRA		
		F2	AAT GAC GGT TAC GGA GGT GGT		Kim *et al*. 2002
		R2	GCA GTA ATG GTC GGC CTT ACC		
		Probe P2	FAM-TCC ACG CCC GCC CAG ACA GG-TAMRA		
	IS*Mav2*	F	CGC CAA AAT CGA GCA GTT TC		Schönenbrücher *et al*. 2008
		R	TGA GCC GGT GTG ATC ATC TTT		
		Taqman	NED-CGA GTT ACA TGA TCC C-MGB		
		LNA	FAM-CGC TGA GTT CCT TAG-BHQ1		
		F	GAT GAG TGG GTC GAG GAC TAC AA		Wells *et al*. 2006
		R	CCG TTG AGC CGG TGT GAT		
		Probe	FAM-CCA AGC CCT AAA GAT-MGB		
	F57	MAPf57p1	TTG GAC GAT CCG AAT ATG T		Tasara *et al*. 2005
		MAPf57p2	AGT GGG AGG CGT ACC A		
		3iFluo	CAC GCA GGC ATT CCA AGT		
		5iRed705	TGA CCA CCC TTC CCG TCG		
		F57-F	TAC GAG CAG GCA GGC ATT C		Schönenbrücher *et al*. 2008
		F57-R	CGG TCC AGT TCG CTG TCA T		
		F57Taqman	VIC- CCT GAC CAC CCT TC-MGB		
		F57LNA	YY-CCT GAC CAC CCT T-BHQ1		

FAM: 5-carboxyfluorescein, TAMRA: 6-carboxy-tetramethyl-rhodamine, MGC: minorgroovebinder, BHQ1: Black Hole Quencher 1 and YY: Yakima yellow.

in samples such as faeces, milk, tissues (lymph nodes and intestinal mucosa) and blood (Collins, 2009). Park *et al*. (2014) developed a novel DNA pre-treatment extraction method to increase the DNA purity and decrease the amount of qPCR inhibitors. Results indicated that qPCR produced more positive results in identifying and quantifying MAP than culturing after examining unknown field samples. Due to a lack of sensitivity and specificity between the species in the *Mycobacterium* genus, it has been recommended to combine various methods to optimise detection.

2.7.7 Phage-based assay

Polymerase chain reaction amplifies a copy of a specific DNA sequence that aids in easier detection; although PCR does not distinguish between viable and dead organisms. However, a bacteriophage methodology enables you to detect the viable bacterium, by acting as a parasite and infecting the bacterium to reproduce inside the viable cell. Bacteriophage cannot replicate in a dead bacterium cell, which in return allows for more sensitive detection of MAP (Rees & Bostaris, 2012). Mycobacteriophage studies have contributed to the molecular biology and veterinary field tremendously; for example, beneficial in the recombinant production of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG vaccine strains, they are able to identify virulence genes (Ford *et al*., 1998). They also serve a wide range of other purposes such as drug susceptibility, but most importantly, they can determine the presence of viable mycobacteria (McNerney *et al*., 1998).

2.7.7.1 Phage and the life cycle

Bacteriophages are grouped into either the *Siphovirus* or the *Myovirus* group, based on distinctive characteristics such as the capsid structure and nucleic acid type. D29 is categorized into the *Siphoviridae* morphological group. *Siphoviridae* are comprised of a long and simple structure that contains a non-contractile tail. Both families contain the double-stranded DNA genomes, which are located in their capsid. Forman *et al*. (1954) were the first to isolate the D29 bacteriophage, where it was discovered that it was active against *M. tuberculosis*. The D29 bacteriophage, utilised to detect the presence of MAP, affects members of the *Mycobacterium* genus and infects fast and slow-growing mycobacterial species, as it is an obligate lytic phage (Ford *et al.*, 1998). Phages are also segregated, based on their life cycles, into two groups: lytic and lysogenic bacteriophage (Fig. 2.3). The D29 follows the lytic cycle. The term ‘lytic’ is based on the action of the bacteriophage, as it induces a cell lysis on their host cell once they have replicated numerous times, leading to the rupture of the host cell to release the offspring phages (Rees and Bostaris, 2012). Enzymes that attach themselves to the membranes of host cells, and create holes in the membranes, are known as holins. These holins allow for another enzyme, known as lysins, to attack the peptidoglycan of the host cell. The lysin cell wall is comprised of a binding and cutting domain. The binding domain leads the cutting domain to the site of action. This is where the cell wall will rapidly rupture (Hagens & Loessner, 2007).

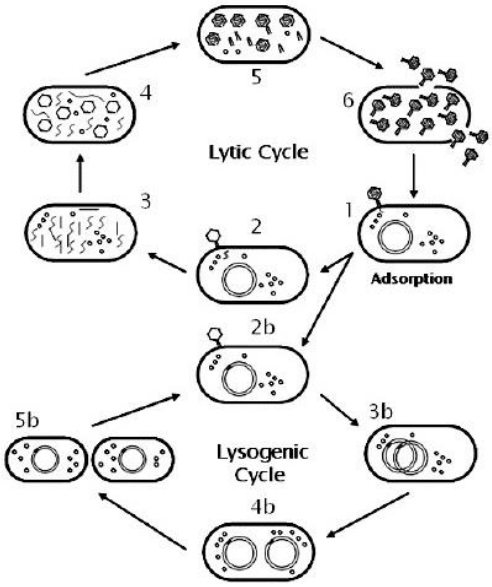

Figure 2.3 Schematic representation of a bacteriophage replication life cycle (adapted from Rees and Bostaris, 2012).

In terms of genomics, D29 is closely related to the L5 mycobacteriophage; however, the D29 cannot produce lysogens, which allows the L5 phage to remain dormant in the host cell until conditions are feasible for lysis (Ford *et al*., 1998).

2.7.7.2 Detection methods

The detection method utilises amplification to detect specific hosts, in other words, the bacteriophage infects the host cell and increases its population once the target cell has been infected (Swift *et al*., 2013). The first phage assay that was developed for the mycobacteria, was known as the *FASTPlaqueTB* (FPTB) assay. An overview of the phage amplification assay can be seen in Figure 2.4. Although it was originally developed to detect the presence of viable *M. tuberculosis*, it has been modified to rapidly detect the presence of viable mycobacteria in 48 h. The D29 bacteriophage is utilised in this particular assay, where it is able to infect a wide range of the species from the *Mycobacterium* genus. As mentioned earlier, the D29 is able to infect the fast and slow-growing mycobacterial species. This is particularly useful as it can infect *M. smegmatis*, which can be utilised to form the lawns on agar plates, to help detect the infected mycobacterial cells within 48 h and also supports the growth of the phage-plaque bacterium cells. The assay is achieved by combining a sputum sample of a supposed infected animal, with the D29 mycobacteriophage and is incubated to initiate the infection of the viable bacteria. Once completed, any remaining and unused phages are eradicated by utilizing a virucide, such as ferrous ammonium sulphate (FAS) known as the virusol (Stanley *et al*., 2007). The virusol ensures that the exogenous mycobacteriophage are inactivated, without affecting the phage replication within the viable host bacteria (McNerney *et al*., 1998). A dilution series from the suspected infected MAP cells is conducted and to reduce the concentration of the virusol. Then the pour plate method, containing the *Mycobacterium smegmatis* sensor cells, commences. A positive result is usually indicated when a plaque has formed. Each plaque

represents each viable bacterium target cell that was infected by the bacteriophage. Swift *et al*. (2013) and Stanley *et al*. (2007) both applied a DNA extraction process to the plaques formed, followed by a RT-PCR, to increase the sensitivity and specificity of MAP detection.

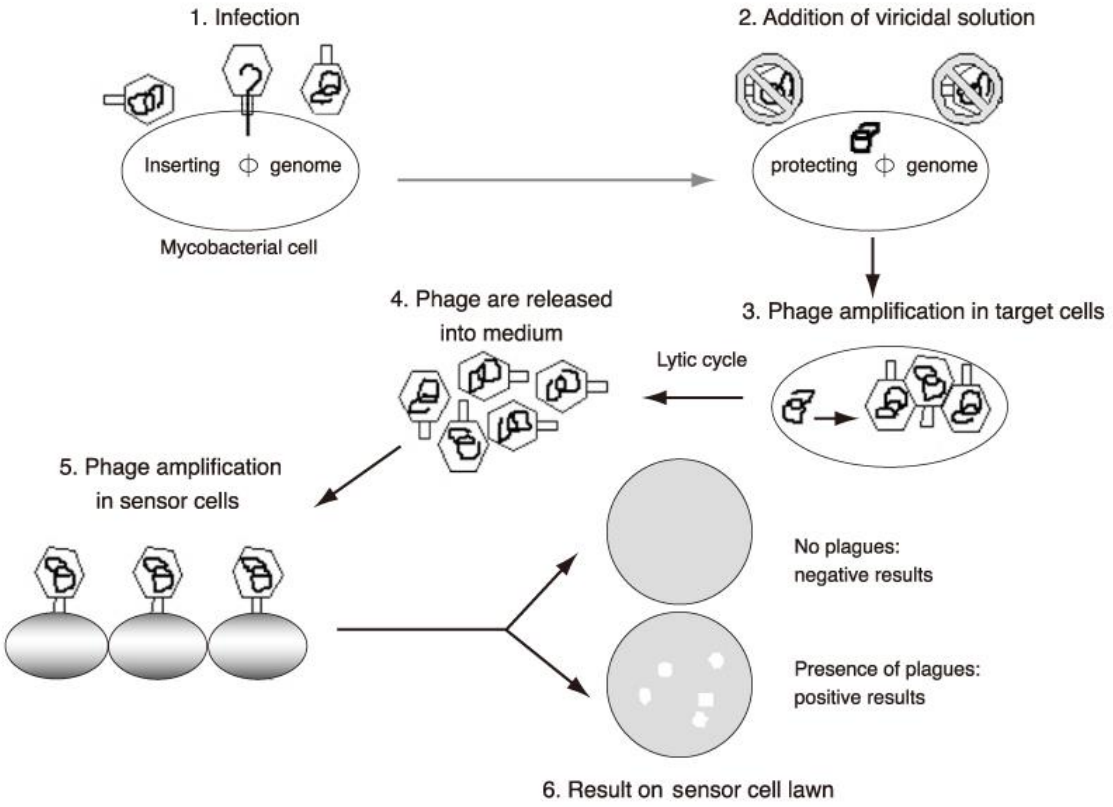

Figure 2.4 A visual overview of the phage amplification assay depicting the infection, inactivation of unattached phage, amplification of target cells and the final results (adapted from Dong Ho Shin, 2006).

There have been very few methods utilizing the phage-assay as a MAP determining tool, as these have not been successful due to the sensitive characteristic of the mycobacterium. Swift *et al*. (2013) initiated the peptide-mediated magnetic separation (PMMS)-phage method with nested PCR amplification to identify MAP cells with the IS900 sequence. Their aim was to combine methodologies and develop a novel protocol for rapid detection of MAP cells in clinical blood samples (Swift *et al*., 2013). However, a 2016 study by Swift *et al*. tested the detection of MAP in the blood of experimentally infected cattle, utilizing a different method. This optimised phage method, in peripheral blood mononuclear cells (PBMC), was more reproducible than isolating MAP cells using PMMS. It was also emphasised to combine the phage method with a PCR-identification step, as the phage method also detected other plaques with no MAP DNA (Swift *et al.,* 2016). Another study by Grant *et al*. (2017) tested the presence of viable MAP utilizing the PMS-culture and PMS-phage assay in CMR and detected MAP in more samples using the PMS-phage assay.

Current detection methods lack sensitivity as they only detect MAP in an infected animal at a later stage of the disease. Application of the phage tool can help aid in a greater understanding of the disease. For example, how MAP progresses, testing determines the occurrence of the systemic disease and lastly determines how MAP influences the serum and milk ELISA's test, the faecal

culture or PCR assay results. Swift *et al*. (2013) showed that although an animal tested negative for ELISA's milk samples, the animal tested positive using the phage-PCR method. Reasoning could be that the test deals directly with the viable organism and results can differ based on the immune response of an animal to the infection (Swift *et al*., 2013). The phage-PCR assay is currently not being used in South Africa, but due to the increase in research interest and results, it has been recommended as a detection method to determine the prevalence in South Africa.

2.8 PREVALENCE AND LEGISLATION IN SOUTH AFRICA

The definition of JD, according to the DAFF, states the following under section B059: "a chronic, contagious enteritis characterized by progressive diarrhoea, weight loss, debilitation, and eventually, death. It affects cattle, sheep, goats, llamas, camels, farmed deer and other domestic and wild ruminants. Distribution is worldwide. The zoonotic risk has been considered minimal, but the isolation of similar organisms from some people with Crohn's enteritis makes this less certain. Aetiology: *Mycobacterium paratuberculosis (johnei)*" (DAFF, 2016). However, DAFF recently released a statement about the current status and the way forward with JD in SA. The statement mentioned that the biggest challenge in the control of JD is the difficulty in identifying infected animals with currently utilized diagnostic testing methods that are not reliable or efficient (DAFF, 2017). To date, there is very little data with regards to the prevalence of MAP in SA. Many of the farmers do not report to DAFF if any of their animals' are infected with JD. The reason being that there are consequences if a farm has been diagnosed with JD; the first step would be the quarantine of their animals and land (Sergeant, 2001). During this process, none of the farmers' animals can be utilised to sell as meat, dairy or any other by-products until the farm has been cleared of MAP. This can lead to major money losses as food-processing companies could ultimately be selling infected products to retail outlets (Smith *et al*., 2017). Therefore, farmers do not report these cases as they know it could create a huge economic loss on their part.

Current data obtained from DAFF (Fig. 2.5) demonstrates the top three provinces with ovine farms that reported the most outbreaks, cases, deaths and kills from January 1993 - May 2017 (DAFF, undated). Reported farms with bovine JD throughout SA can be can be seen in Figure 2.6. 'Outbreaks' represents the diagnosis of JD for the first time on a farm. The outbreak could be a suspicion based on clinical history or findings that is not confirmed, but is believed to be present on the farm. 'Cases' is defined as the number of animals showing the disease that were slaughtered by a state veterinarian for post mortem investigation. The 'deaths' demonstrates the farms that reported the death of an infected animal due to JD. Lastly, the 'kills' category illustrates the total number of farms that slaughtered an animal infected with JD at an abattoir due to the individual/group being tested positive for JD post mortem (S. Davey, 2017, State Veterinarian, Department of Agriculture, Forestry and Fisheries, Malmesbury, SA personal communication, June 2017). The top three provinces for ovine JD reported farms were the Western Cape (WC), Eastern Cape (EC) and Mpumalanga (MP). Overall the WC demonstrates a larger problem as it was the province with the

most outbreaks, deaths and kills; although it should be remembered that the efficiency of the state veterinarians in diagnosing JD in the various Provinces may also influence this data. None-the-less, the EC demonstrated almost double the amount of ovine JD cases than the WC.

When comparing reports between ovine and bovine JD, there are more ovine (Fig. 2.5) than bovine reports (Fig. 2.6). The difference in reported numbers can be explained by the fact that dairy and beef farmers would make a larger economic loss if quarantined, compared to ovine farmers as there are fewer ovine products produced in SA (S. Davey, 2017, State Veterinarian, Department of Agriculture, Forestry and Fisheries, Malmesbury, SA, personal communication, January 2017).

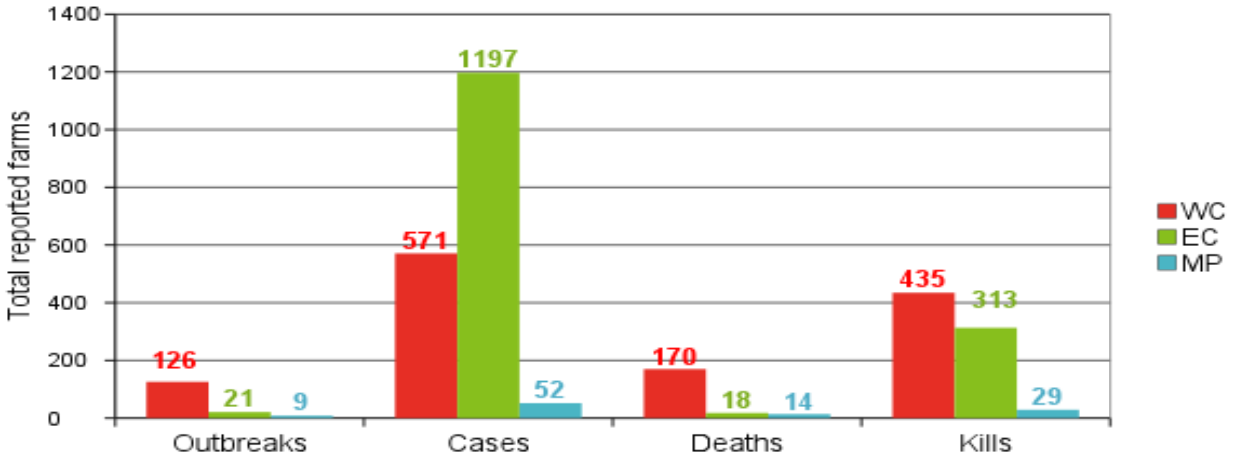

Figure 2.5 The top three provinces with the most reported ovine JD farms in SA dating from January 1993 to May 2017 (data adapted from DAFF, undated).

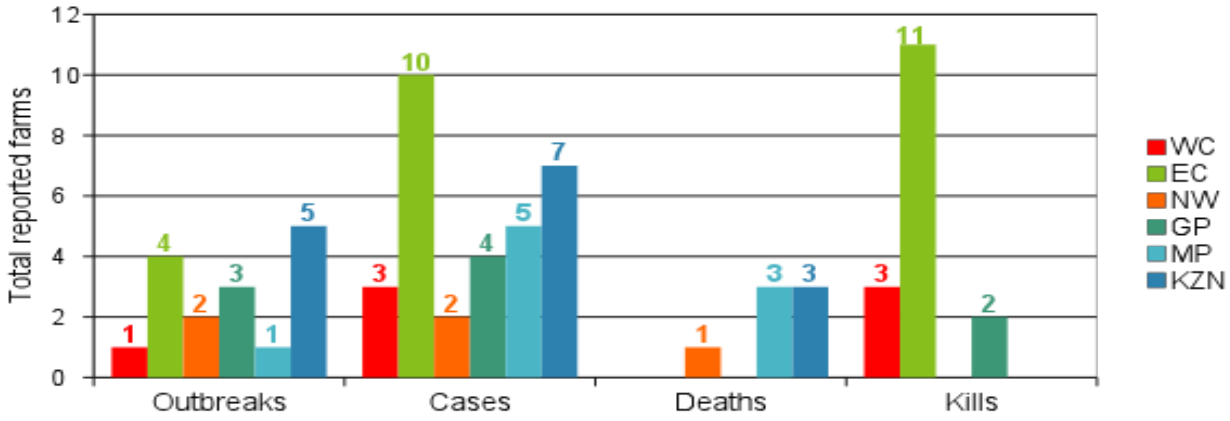

Figure 2.6 The provinces in SA with the most amount of reported bovine JD farms dating from January 1993 to May 2017 (data adapted from DAFF, undated).

As seen in Figure 2.6, the top reported provinces for outbreaks, cases and deaths from bovine farms was the EC. The most death reports were from MP and KZN. According to Sergeant (2001), ovine JD has a different geographic distribution compared to bovine JD. Miller *et al*. (2017) reported a 20% increase in ovine JD reports since 2007 with MAP (Fig. 2.7), where the Swartland municipality had the highest density of reported ovine JD in the Western Cape.

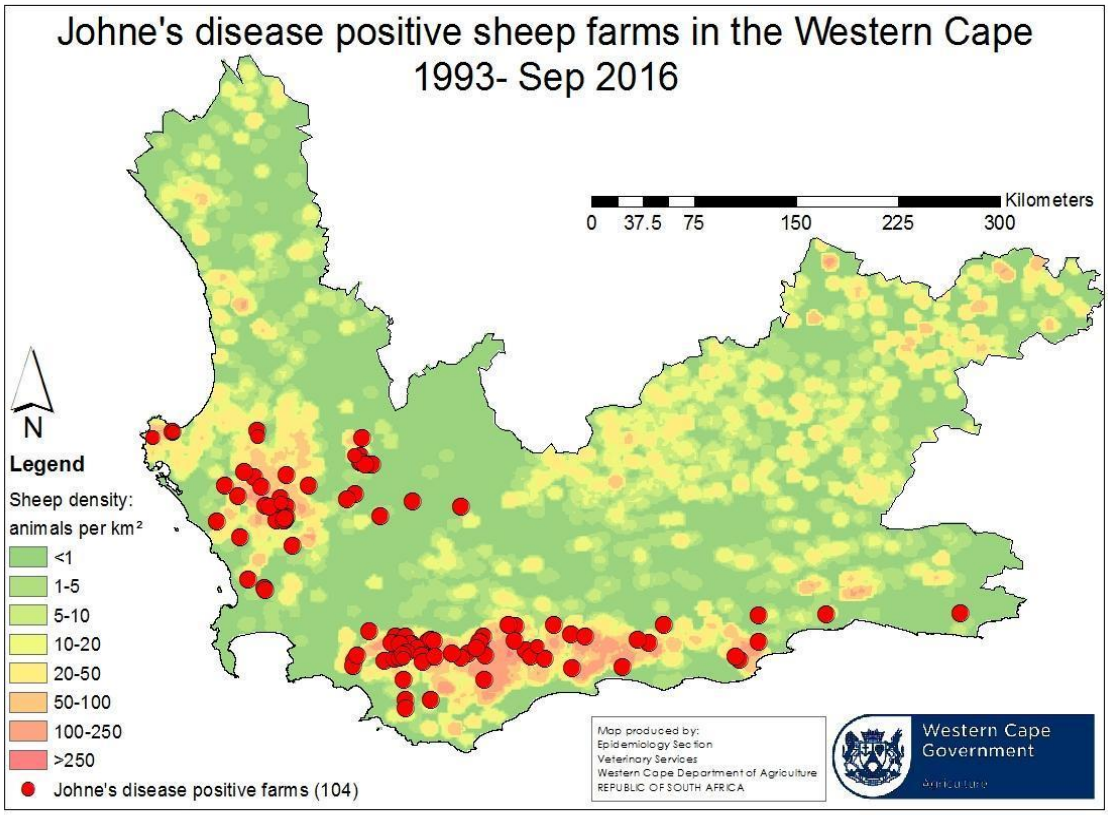

Figure 2.7 A Global Positioning System displaying all the confirmed positive Johne's disease ovine farms throughout the Western Cape (image adapted from the Western Cape Department of Agriculture)

Therefore, based on the above data, it would be beneficial to test and compare the ovine and bovine farms in the WC that have reported JD, in order to identify whether this area is a problematic province with JD.

2.9 AIM OF THIS STUDY

Mycobacterium avium subsp. *paratuberculosis* is a challenging mycobacterium to detect. It is slow-growing and can take up to 16 weeks or more to produce a culture. Johne's disease has a long incubation period before an infected ruminant produces symptoms. During this period, the mycobacterium is intermittently shed into the environment, which leads to the further spread to non-infected ruminants in the flock or herd. This diverts back to the problem where detection of MAP at an earlier stage is problematic, as the mycobacteria are not constantly shed. *Mycobacterium avium* subsp. *paratuberculosis* has been detected in food products, such as pasteurised milk, cheese, meat products and these are thought to be the possible routes of contamination to humans. This mycobacterium has also been detected in water, which has run-off from infected cattle on farms, in soil, faeces and blood. There has been an observation of MAP, in terms of JD, in sheep and dairy herds in the Western Cape, which requires research to assess the occurrence of JD. However, current identification methods lack sensitivity and specificity to identify and detect the presence of MAP in samples. In South Africa, the ELISA, culturing and ZN stain are routinely used to confirm if a ruminant is infected with MAP. However, the lower amounts of MAP cells shed during the sub-

clinical phase limits the ZN stain, as the acid-fast staining cannot easily detect small concentrations under the microscope. The ELISA is unreliable as it is dependent on the presence of antibodies in the infected ruminant. As a result, early stage detection in suspected infected ruminants is limited as MAP is intermittently shed. This increases the possibility of false-negative results as sampling and testing could have occurred during a stage where no MAP shedding occurred. This usually requires the state veterinarian to make a decision whether to slaughter the suspected ruminant and perform a histology examination on the intestine and lymph nodes. However, sometimes a positive ELISA results has been produced, the animal is slaughtered and the histology examination concludes no MAP infection in the intestine or lesions on the lymph nodes. Hence, research into a more rapid and sensitive method needs to be conducted to improve current detection methods in South Africa.

Therefore, the aim of this study was to investigate the D29 phage assay and PCR methods to apply them in South Africa, particularly the Western Cape. This will be completed by optimising the phage assay, followed by the PCR methods established by Rees and Bostaris (2007), Stanley *et al*. (2007), Swift *et al*. (2013) and Swift *et al*. (2016) and applying the methods to various sheep sample matrices (faecal, blood and milk). These results were compared to ELISA results to determine which method is more sensitive and reliable. Only sheep samples were utilised in this study, as it was difficult to source and locate farms that were willing to provide blood, faecal and dairy samples from dairy cows. This masters study is unique to South Africa, as there has been no published work on investigating internationally used methods (such as the rapid phage assay or PCR) to detect MAP.

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CHAPTER 3

Investigating a phage assay to detect *Mycobacterium avium subsp.* paratuberculosis

3.1 ABSTRACT

A phage assay optimisation for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was performed. First, various stock cultures of *Mycobacterium smegmatis* mc^2 155 were grown on Luria Bertani (LB) and Middlebrook 7H10 agar. The Middlebrook 7H10 agar produced the described culture colony of *M. smegmatis*. Various incubation times were tested on the inoculated *M. smegmatis* broths that were isolated from LB and Middlebrook 7H10 agar. The optimal time of incubation was 48 h, as it produced a suspension of cells closest to an OD_{600nm} of 1. A titre was established for two mycobacteriophage D29 stock cultures (S1 and S2) by utilising the suspended *M. smegmatis* broths. The Middlebrook 7H10 culture, which was originally isolated from a broth stock culture, produced the plaques that were used to make stock cultures of D29. The phage assay required optimisation at various stages, however the assay could not be optimised as the *M. smegmatis* stock cultures were contaminated during storage by another bacteria, which hindered the production of any plaques.

3.2 INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a pathogenic and slow growing nontuberculosis mycobacterium (NTM) that is the causative agent of a chronic enteritis infection found in ruminants (sheep, cattle and goats) known as Johne's disease (JD) (Alajmi *et al*., 2016; Bauman *et al*., 2016). Symptoms include inflammation of the small intestine and intermittent shedding, causing the infected ruminant unable to absorb nutrients, which is why farmers have termed it the 'wasting disease' (Nielsen & Toft, 2008; Collins, 2009; Smith *et al*., 2017). As a result, there is a decrease in milk and meat production, which can generate financial losses for farmers. Although the link between Crohn's disease (CD), a human inflammatory disease, and JD has not yet been established, it has been suggested that MAP is also the causative agent of CD in humans. The theory was established due to the similar clinical symptoms that JD and CD produce (McFadden *et al*., 1987; International Food Information Service, 2009; Krishnan *et al*., 2009).

This mycobacterium is spread vertically (milk, semen, colostrum) and horizontally (contaminated water, soil and faeces) to other non-infected ruminants, which also suggests the possible route of exposure of MAP to humans (Chaubey *et al*., 2016). *Mycobacterium avium* subsp. *paratuberculosis* has been detected in: pasteurised milk, infant milk powder, some cheeses, liver and in water systems (Steed & Falkinham, 2006; Botsaris *et al*., 2010; Antognoli *et al*., 2008; Gill *et al*., 2011; Botsaris *et al*., 2013; Botsaris *et al*., 2016; Waddell *et al*., 2016, Gerrard *et al*., 2018). Due to the detection in these samples, it has become an international public health and food safety

concern, urging researchers to determine the prevalence of MAP in ruminants and in the food chain. However, the biggest challenge to determine the true prevalence of MAP is that the currently used diagnostic methods lack sensitivity, specificity and are time consuming (Chaubey *et al*., 2016).

Mycobacterium avium subsp. *paratuberculosis* was first discovered in South Africa in 1967 when an infected Merino ram was imported. After the widespread occurence of MAP in the Western Cape (WC) in the 1990s, enzyme-linked immunosorbent assay (ELISA)'s and Ziehl-Neelsen (ZN) stains were recommended as less labour-intensive diagnostic tests in South Africa (SA) (Michel & Bastianello, 2000). However, the ELISA, which utilises the serological characteristics of MAP to diagnose a sample as positive or negative, cannot detect MAP at an earlier stage of the disease and therefore yields false negative or positive results (Chaubey *et al*., 2016). Although the ZN stain is rapid it is not specific, as it stains on acid-fast mycobacterium (Wynne *et al*., 2011). Another recommended diagnostic method is culturing, although internationally recognised as the golden standard and the only available method that determines viable MAP cells, it is labour intensive and can take up to 16 weeks or longer to produce a single colony of MAP (Jasson *et al*., 2010; Chaubey *et al*., 2016). Recent developments have led to a combined phage and polymerase chain reaction (PCR) rapid detection method known as the *FASTPlaqueTB*™ (Biotec Laboratories, Ipswich, UK) assay. This assay was originally used to detect the presence of *Mycobacterium tuberculosis*, but research has shown it can also detect the presence of MAP in blood, milk and infant milk powder (Stanley *et al*., 2007; Swift *et al*., 2013; Swift *et al*., 2016, Botsaris *et al*., 2016; Gerrard *et al*., 2018). The phage-PCR assay detects viable MAP cells within 48 h. The assay works by using a bacteriophage (D29) that specifically infects viable mycobacterium. Samples are incubated with the bacteriophage for 1 h at 36°C where the viable mycobacteria cells are infected with D29 (Rees & Botsaris, 2012). The phage replicate within the MAP cells to increase the D29 population. Any free D29 that has not attached to a viable cell is killed off with a virucide, such as ferrous ammonium sulphate (FAS) (Stanley *et al*., 2007). These infected cells are then mixed with fast-growing *Mycobacterium smegmatis* with soft agar. As *M. smegmatis* is a fast-growing mycobacterium, it is widely used a host cell for the growth of slow growing mycobacterium. The host cells create a lawn that enables the further replication of the phage, which were initially released from the viable cell, to form a clear zone known as a plaque. To identify whether the plaques formed are MAP, they are picked off of the soft agar followed by a gel DNA extraction. Once the DNA has been obtained from the gel, PCR is conducted using MAP specific primers.

Before samples can be processed, it is important to first optimise and then validate the method in the country of testing. Optimisation should take place by first running the phage assay with *M. smegmatis* to establish a phage titre, followed by validation of the assay with MAP cells. Once the optimisation of the phage assay has been conducted, the PCR method has to be optimised to confirm the MAP DNA. Validation of the phage-PCR assay will be required before samples that require investigation are processed, and hopefully identified rapidly.

This type of research has not yet been implemented in South Africa and therefore sequencing will have to occur to identify if a novel MAP strain is present. The aim of this study was to therefore establish the phage assay by optimising *M. smegmatis*, followed by validation with known MAP positive and negative samples.

3.3 METHODOLOGY

As this was the first time, to our knowledge, that the phage assay had been initiated for the detection of MAP in South Africa, various optimisation trials were required. Firstly, the *M. smegmatis* mc^2 155 needed to be grown on various agars to determine which produced the best growth. Once this had been established, a single colony of the *M. smegmatis* culture needed to be placed in a broth for its use as sensor cells in the phage assay. Various time trials were conducted to determine which would produce a sufficient cell suspension. Once the broth had been incubated for sufficient time, the titre of the D29 bacteriophage needed to be established in order to make stock cultures. Stock cultures were then harvested, and the titre was run again to ensure the correct bacteriophage PFU/mL was obtained. The phage assay was then tested at the various steps to ensure all the correct concentrations were determined.

3.3.1 *Mycobacterium smegmatis*

In order to make up stock cultures of the *M. smegmatis,* to be used as sensor cells in the phage titre and phage assay, various optimising steps were required to take place (Fig. 3.1).

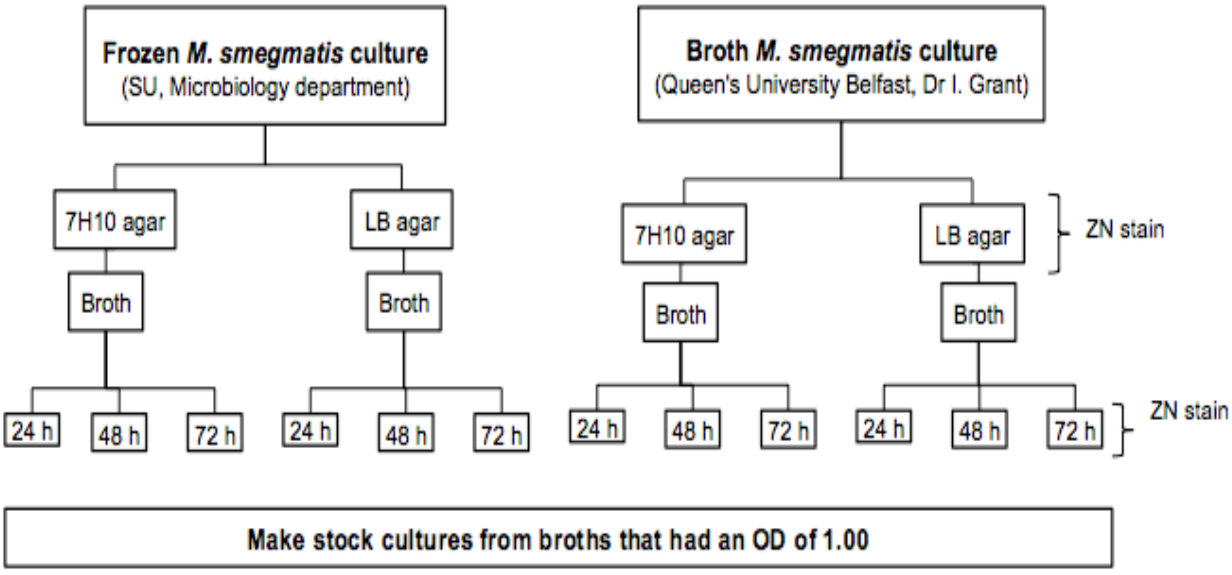

Figure 3.1 Flow diagram exhibiting the steps taken to establish a stock culture of *M. smegmatis.*

3.3.1.1 Determining agar growth medium for Mycobacterium smegmatis

Stock cultures of *M. smegmatis* mc^2 155 were received from various locations and grown on two types of agar (Luria Bertani and Middlebrook 7H10 agar) (Table 3.1).

Table 3.1 *M. smegmatis* mc2 155 stock culture tubes tested during trials

Sample name	Sample composition	Location and reference
Frozen stock culture	Glycerol stock culture	SU*, Microbiology department
Broth culture	Middlebrook 7H9 and 10 %OADC	Queen's University Belfast, Dr I. Grant

*SU - Stellenbosch University

The stock cultures (Table 3.1) obtained were consequently grown on Luria Bertani (LB) agar and Middlebrook 7H10. For the LB agar, 25 g of LB broth (Biolab, Merck, South Africa) and 15 g of Bacteriological agar (BA) (Biolab, Merck, South Africa) was weighed out and added to 1000 mL of distilled water to make 1 L. For the Middlebrook 7H10 (Becton Dickinson, South Africa), 19 g was weighed out and added to distilled water to make 900 mL. Both agars were autoclaved at 121°C for 20 min. The agars were then poured in petri dishes and left to set at room temperature. The streak method was adopted to transfer the various stock cultures received and were streaked out in duplicate. The LB and Middlebrook 7H10 agar require 3-5 days to produce visible colonies of *M. smegmatis* growth. Colonies were visually compared, based on their morphology, size and time taken to grow; and the best-suited agar was determined. Cultures that were creamy white, finely wrinkled, small and smooth were characterized as *M. smegmatis* growth. Cultures were also confirmed as acid-fast Gram-positive bacteria by utilising the Ziehl-Neelsen (ZN) stain (see 3.3.1.3).

3.3.1.2 Preparation of Mycobacterium smegmatis for phage assay

A single colony of *M. smegmatis* mc^2 155 was selected from each LB and Middlebrook 7H10 agar plate and inoculated in 20 mL of Middlebrook 7H9/10% OADC broth (Becton Dickinson, South Africa) and incubated in a shaking incubator at 200 rpm, 37°C (Zhicheng Culturing, ZHWY-100D, South Africa). Trials of incubation periods (24 h, 48 h and 72 h) were tested to determine which period would achieve an OD_{600nm} +/- 1.0 (Fig. 3.1). The OD was measured using a spectrophotometer (Spectroquant® Prove 600, Merck, South Africa). This broth of suspended cells was used as the sensor cells in the amplification of the D29 mycobacteriophage. The broth cell suspension was also confirmed as acid-fast Gram-positive bacteria using the ZN stain (see 3.3.1.3).

3.3.1.3 Confirmation of acid-fast mycobacterium using Ziehl-Neelsen stain

Cultures grown on LB agar, Middlebrook 7H10 agar and in the Middlebrook7H9/10%OADC broth were confirmed as acid-fast Gram-positive mycobacterium using the acid-fast ZN stain. The ZN stain was carried out as described by Gupta *et al*. (2010). Culture smears were performed, air-dried and heat fixed onto a glass slide. The slides were then flooded with 1% of the primary stain, carbolfuchsin, and heated until steam rose for roughly 5 min. Tap (chlorinated/potable) water was used to wash the smears. Thereafter 10% acid alcohol was used to decolourize the stain for 4 min. Slides were rinsed again with tap water. A counter stain, methylene blue, was then applied for 30 sec. Slides were washed with tap water and left to air dry. Slides were examined using the 100x lens with immersion oil under a microscope. Smears were confirmed as acid-fast Gram-positive

bacteria by red, straight or slightly curved rods on a blue background. Bacilli are either found in small groups or alone (Timoney *et al*., 1988).

3.3.1.4 Maintaining Mycobacterium smegmatis stock cultures for the phage assay

Stock cultures of *M. smegmatis* mc^2 155 were maintained according to Microbank™ cryobeads' manufacturing instructions (Pro-lab Diagnostics, South Africa). Briefly, a few single colonies were aseptically isolated from the culture agar plate and placed in the Microbank™ vials with cryopreservative. The vial was inverted four to five times and left to stand for two minutes. A Pasteur pipette was used to aseptically remove the cryopreservative and the tubes containing the beads were placed in the -80°C freezer until required. When *M. smegmatis* was required, a single bead was inoculated in 20 mL Middlebrook 7H9/10% OADC broth and incubated at 37°C.

3.3.2 Mycobacteriophage D29

The mycobacteriophage, D29, was received as gifts from two sources (Table 3.2), as the bacteriophage could not be locally sourced. Before the phage could be used in the phage assay, the titre had to be established to ensure the correct concentration of phage was used in the assay (Fig. 3.2).

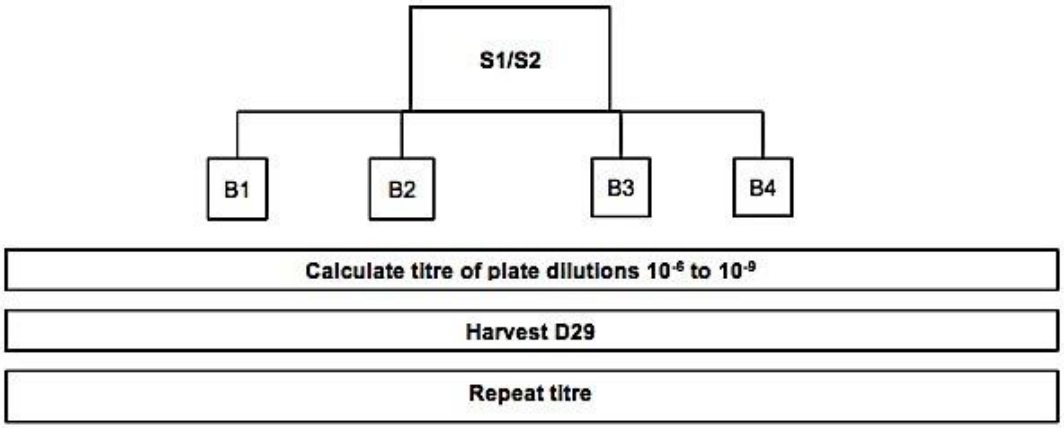

Figure 3.2 Flow diagram indicating the steps taken to produce a phage $1x10^9$ and 10^{10} PFU/mL stock. B1: frozen culture from LB agar, B2: frozen culture from Middlebrook 7H10 agar, B3: broth culture from LB agar and B4: broth culture from Middlebrook 7H10 agar.

Table 3.2 Bacteriophage D29 samples received and tested

Sample name	Sample type	Location and reference
Stock 1 (S1)	Reconstituted D29	Nottingham University, Dr C. Rees
Stock 2 (S2)	Stock culture suspension	Queen's University Belfast, Dr I. Grant

3.3.2.1 Determining the D29 titre of suspension

A 10-fold dilution series was prepared by aliquoting 900 µL Middlebrook 7H9/10% OADC into 2 mL Eppendorf's. The D29 mycobacteriophage was diluted by transferring 100 µL of phage suspension supplied to achieve 10^{-6} to 10^{-9} dilutions. Thereafter, 100 µL of the 10^{-6} to 10^{-9} dilutions were transferred into four duplicated labelled 9 cm Petri dishes. Into each Petri dish 1 mL of the various *M. smegmatis* mc^2 155 broth cultures (B1-B4) made from the various stock cultures (Table 3.1) and 5 mL of Media Plus (MP)(Middlebrook 7H9/10% OADC/1 mM $CaCl_2$) were added. The broth was checked to make sure it was at room temperature before adding the reagents to the Petri dishes. Lastly, 5 mL of Middlebrook 7H10 agar was added and the dishes were swirled in each direction five times to evenly distribute the contents. The agar was left to solidify for roughly 10 min and then incubated at 37°C for 24 h. Plates were examined for plaques (clear circular zones). The titre was established by counting the number of plaques for each dilution up to 300 plaques. The PFU/ml was calculated using equation 1:

Equation 1: $\#\ of\ plaques\ at\ countable\ dilution\ \times diltution\ factor\ \times inoculum\ factor = PFU/mL$

For example: $225\ \times 10^{7}\ \times 10 = 2.25\ \times 10^{10}\ PFU/mL$

The D29 plates were labelled with the result and used as the stock phage suspension. The plates were stored at 4°C until required. Further dilution of the suspension, using Middlebrook 7H9/10% OADC/1 mM $CaCl_2$, was required to achieve the working D29 suspension of 1 x 10^9 PFU/mL for the assay.

3.3.2.2 Harvesting of D29 to make stock solutions

When the D29 was required for the assay, the plates (not the plates with the complete bacterial lawn 'lace effect') were used to harvest the mycobacteriophage (Fig. 3.3).

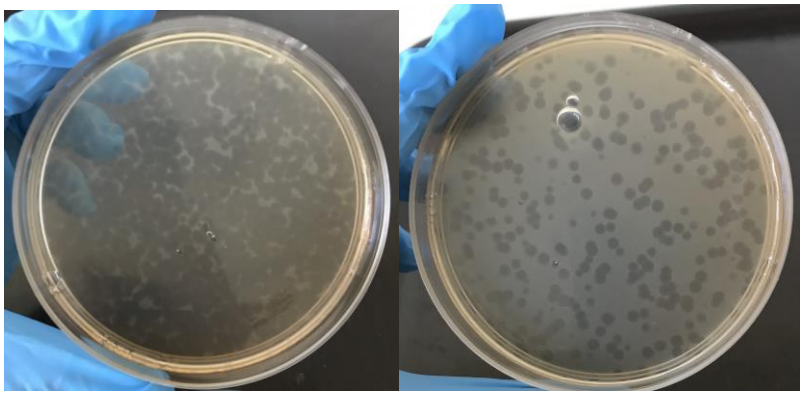

Figure 3.3 The D29 agar plates used to harvest the mycobacteriophage. Plates that were completely lysed were not harvested (left) while the plates that were used to harvest D29 can be seen on the right.

This was completed by adding 10 mL Middlebrook 7H9/10% OADC/1 mM $CaCl_2$ broth in the plate and were incubated at 37°C for 24 h. The broth was then removed using a sterile 10 mL syringe and filtered through a 0.2 µm filter, to remove any debris and bacteria. The D29 mycobacteriophage was then aliquoted and stored at 4°C. The titering procedure was then repeated to quantify the suspension. The phage stock needed to contain at least a $1x10^9$ - 10^{10} PFU/mL. The titre was repeated to ensure the correct PFU/mL was established.

3.3.3 Phage assay optimisation

Once the D29 titre was established, various steps in the assay required optimisation and validation to ensure all concentrations and temperatures were correct. The usual process flow (Fig. 3.4) was adapted from the *FASTPlaqueTB*™ assay to test for the presence of MAP in various samples (blood, milk and sputum). It also indicates the various steps that required optimisation (indicated with green stars). An essential consumable required was the type of reaction vessel that was used.

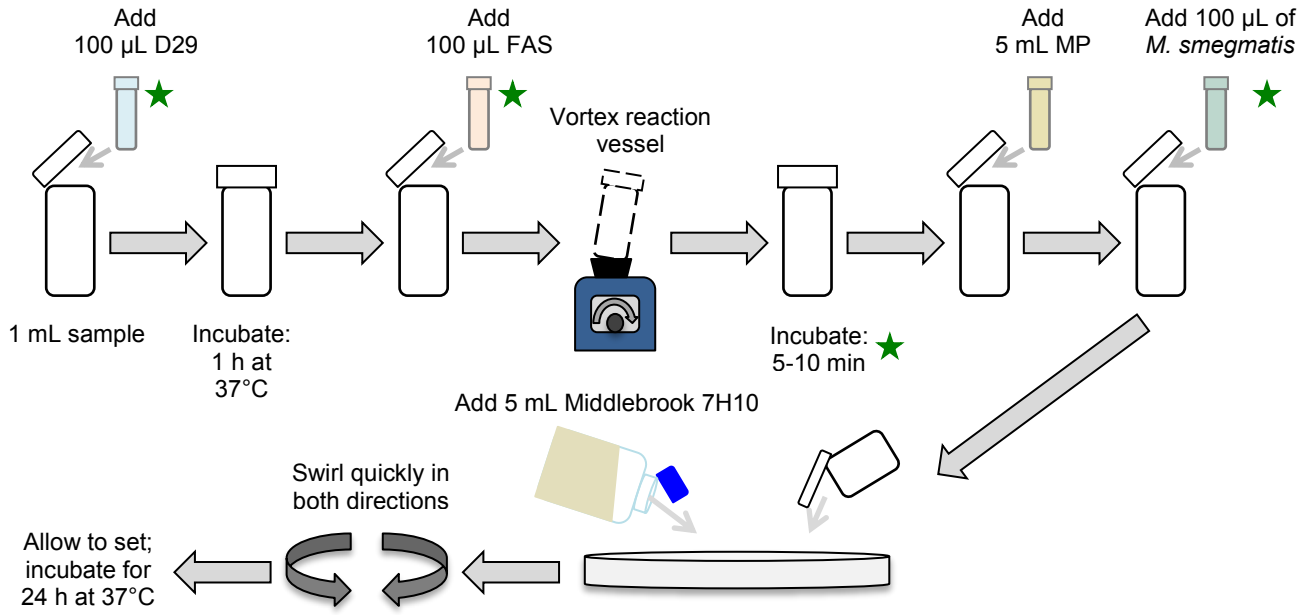

Figure 3.4 *Mycobacterium avium* subsp. *paratuberculosis* phage assay adapted from the FASTPlaqueTB™ assay kit. The green stars illustrate the stages that required optimising.

The reaction vessel needed to have a lid that fitted over the container and not a screw cap. This would ensure that the phage or any other reagents did not get stuck in the lid, to essentially reduce the final concentration of any reagents. The first trial, in the optimisation stages, was to establish the titre (see 3.3.2.1) followed by various concentrations of ferrous ammonium sulphate (FAS), incubation periods for the virucide (FAS) treatment and lastly the use of various *M. smegmatis* grown from various stock cultures (Table 3.1) (see 3.3.1.4). The assay was optimised using the positive (1 mL of *M. smegmatis* 1 x 10^8 CFU/mL serially diluted in MP to 10^{-6}) and negative (1 mL of MP) controls. The positive control was expected to produce more than 20 plaques, while the negative should produce anywhere between zero to ten plaques.

3.3.3.1 Ferrous ammonium sulphate (FAS)

It was important to ensure the correct concentration of virucide (FAS) (Merck, South Africa) is utilised to sufficiently inactivate any unattached D29. A 100 mM solution was made, where various amounts were added to the positive and negative control samples. This included volumes of 90 µL, 100 µL, 110 µL, 115 µL, 120 µL and 125 µL at an incubation time of 5 min. Positive and negative samples were presented on agar plates in duplicate. The positive control was expected to produce more than 20 plaques, while the negative should have produced anywhere between zero to ten plaques.

3.3.3.2 Incubation time

Another critical part of the assay was to ensure that the virucide had sufficient time to inactivate any unattached D29. This was achieved by incubating the virucide with the control samples at time intervals of 5, 7.5 and 10 min with 100 µL of 100mM FAS. It was also essential that the virucide had touched all surfaces of the reaction vessel (the tube that contained the sample). Samples were rotated in 180-degree circles in all directions and left to rest on the workbench until half the time interval was reached. The samples were then vortexed to ensure the entire inside of the reaction vessel had been covered with FAS. The positive and negative samples were plated on agar in duplicate. The positive control was expected to produce more than 20 plaques and zero to 10 plaques for the negative control.

3.4 RESULTS AND DISCUSSION

3.4.1 *Mycobacterium smegmatis*

Various challenges were faced with the enumeration and cultivation of *M. smegmatis* mc^2 155 from the various stock cultures (Table 3.1) for their use as sensor cells. Agars, such as Luria Bertani (LB) and Middlebrook 7H10 agar, were utilised to determine which would correctly grow the mycobacteria sensor cells (Table 3.2). According to Singh and Reyrat (2009), the colony morphology of *M. smegmatis* is finely wrinkled and creamy white. On the other hand, a colony will produce different textures on various media, however this variability is not understood. It is therefore important to know the purpose of the experiment to determine which medium to grow *M. smegmatis*.

Table 3.3 Summarised results comparing LB to Middlebrook 7H10 agar for the growth of *M. smegmatis* from various stock cultures

Stock culture	Agar	Incubation conditions	Appearance
Frozen stock culture	LB*	5 days, 37°C	Yellow, shiny, mucous appearance
	Middlebrook 7H10	3 days, 37°C	Creamy white, small, wrinkle shape
Broth culture	LB*	5 days, 37°C	Yellow, shiny, mucous appearance
	Middlebrook 7H10	3 days, 37°C	Creamy white, small, wrinkle shape

LB* Luria Bertani

As seen (Table 3.3), the various agars produced different culture appearances. When the sensor cells were grown on LB agar, the *M. smegmatis* did not produce the typical bacterial colony as

described by Singh and Reyrat (2009). However, when the *M. smegmatis* was grown on Middlebrook 7H10, sensor cells grew as explained by Singh and Reyrat (2009).

A single colony of each of the above stock cultures from the various agars was incubated in broth. Table 3.4 indicates the OD_{600nm} values from the incubation periods (24 h, 48 h and 72 h) in the broth.

Table 3.4 The OD_{600nm} values obtained from the various stock cultures incubated in broth

Stock culture	**Agar**	**OD value**		
		24 h	48 h	72 h
Frozen stock culture	LB*	0.254	0.756	1.10
	Middlebrook 7H10	0.342	0.814	1.25
Broth culture	LB*	0.459	1.08	1.36
	Middlebrook 7H10	0.512	1.10	1.33

LB* Luria Bertani

As seen in Table 3.4, the cultures that were isolated from the broth culture produced the expected OD values closest to 1 at 48 h. The shortest incubation time of 24 h did not produce a sufficient suspension to be used in the phage assay. The longest incubation period, 72 h, produced a cell suspension greater than 1. It is therefore not necessary to incubate the culture for such an extended time.

Not only did the agars produce different morphology between Middlebrook 7H10 and LB agar, but the suspensions of cells incubated in broth also differed visually (Fig. 3.5).

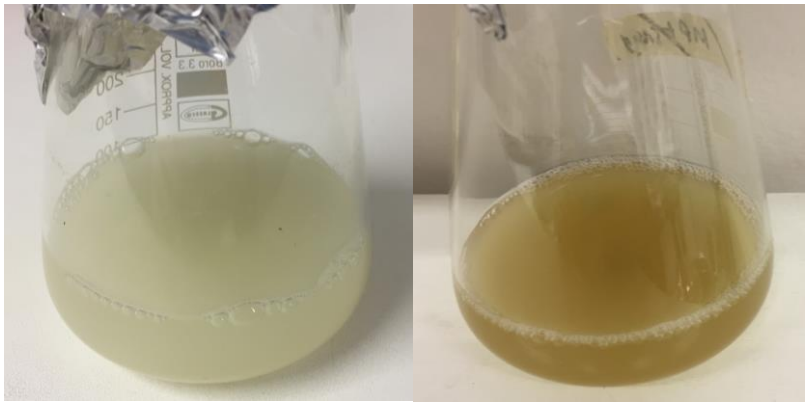

Figure 3.5 The visual difference between suspensions of *M. smegmatis* in Middlebrook 7H9/10%OADC broth from culture isolated from Middlebrook 7H10 (left) and LB agar (right).

It was also seen that *M. smegmatis* grew better in a broth than on a solid media plate. Even if the *M. smegmatis* was grown on Middlebrook 7H10, there were times when the colony that grew on the media was not *M. smegmatis* and turned out to be a Gram-negative bacterium. Ziehl-Neelsen smears were compared to each other and it was seen that the broth (cells isolated from Middlebrook 7H10 agar) produced an acid-fast mycobacterium (Fig. 3.6), while the other broth (culture isolated from LB agar) did not produce acid-fast bacteria.

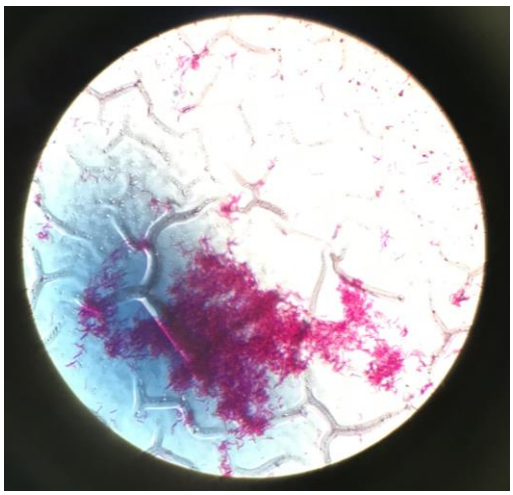

Figure 3.6 The acid-fast bacteria stain from the Middlebrook 7H10 broth suspension.

It was therefore decided to rather use freshly inoculated *M. smegmatis* straight from the cryobeads stock cultures (see 3.3.1.4).

3.4.2 Mycobacteriophage D29

The titre of the D29 was determined by also utilising the various stock cultures of *M. smegmatis* (Table 3.1) incubated in broth and the D29 stock cultures, S1 and S2 (Table 3.2). Only the 10^{-6} to 10^{-9} dilutions were plated, as dilutions 10^{-1} to 10^{-5} would have lysed excessively, making it impossible to calculate the PFU/mL. No plaques were seen from any of the LB agar stock cultures grown in broth (Table 3.5). The reconstituted S1 had produced colonies as well as plaques on the plates, however the plate was contaminated (Fig. 3.7). This led to believe that the S1 stock was contaminated as after mnay attempts, the same conaminated results were produced.

Table 3.5 The number of plaques and PFU/mL calculated from the various stock cultures incubated in broth

Stock culture	**Agar**	**Number of plaques and PFU/mL**							
		10^{-6}		10^{-7}		10^{-8}		10^{-9}	
		S1	S2	S1	S2	S1	S2	S1	S2
Frozen stock culture broth	LB* (B1)	A	A	A	A	A	A	A	A
	Middlebrook 7H10 (B2)	A	A	A	A	A	A	A	A
Broth culture broth	LB* (B3)	A	A	A	A	A	A	A	A
	Middlebrook 7H10 (B4)	C	250 (2.5×10^{9})	C	110 (1.1×10^{10})	C	25 (2.5×10^{10})	C	3 (3×10^{10})

LB* Luria Bertani, A- absent, C- contaminated

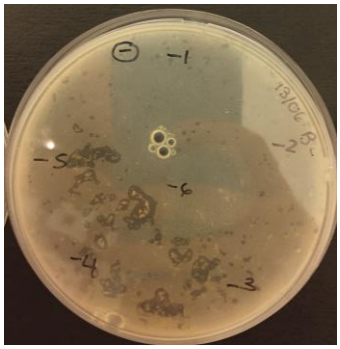

Figure 3.7 A contaminated S1 Middlebrook 7H10 agar plate

No PFU/mL could be calculated for the frozen stock culture either. This can be explained through the understanding that *M. smegmatis* is sensitive to the media, concentrations and temperatures (Foddai *et al*., 2009; Swift *et al*., 2014). Glycerol is known to be the stock standard media used when making stock cultures of microorganisms (Singh & Reyrat, 2009). However, it was also recommended by Dr Irene Grant, that glycerol stocks should not be used as glycerol can interfere with the phage assay, which could also explain why enumeration and cultivation of the phage was problematic (I. Grant, 2018, Queen's University Belfast, IRL, personal communication, March 2018). *Mycobacterium smegmatis* also have the ability to form clumps, which is why Tween80 is recommended when making up stock cultures (Singh & Reyrat, 2009). However, the Tween80 also interferes with the phage assay (I. Grant, 2018, Queen's University Belfast, IRL, personal communication, March 2018). Stock culture (S1) could have been contaminated during transport or between transferring S1 from sample tube to the microcentrifuge tube for the dilution. Another reason could be that the S1 stock culture was not correctly filtered when making stock cultures, which allowed for the growth of bacteria.

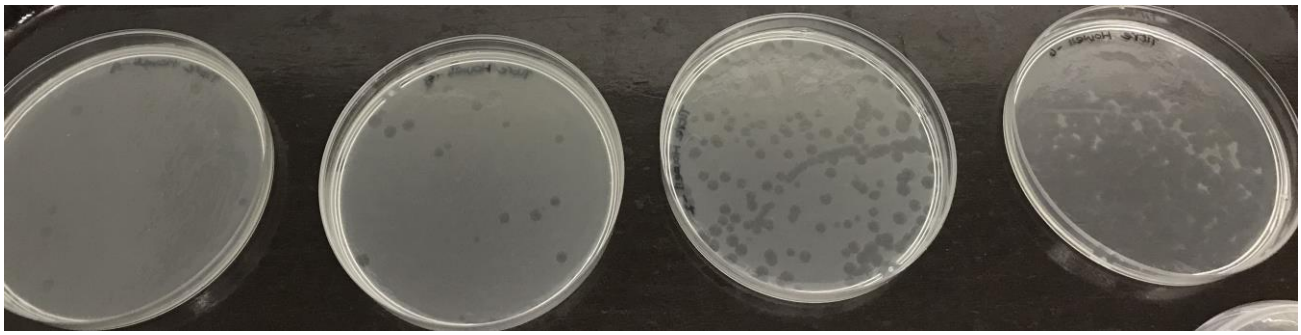

Figure 3.8 The expected outcome of a D29 phage titre (as depicted by S2 Middlebrook 7H10). Plates demonstrate the dilution of the phage from most diluted (far left) at 10^{-9} to concentrated and lysed (far right) at 10^{-6}.

3.4.3 Phage assay

As the titre was established before the phage assay was initiated (Table 3.5), the ferrous ammonium sulphate (FAS) virucide concentration (Table 3.6) and incubation trials (Table 3.7) were just tested on the D29 isolated from the Middlebrook 7H10 broth culture batch.

Table 3.6 PFU/mL determined for the FAS added at various volumes

Sample	90 µL	100 µL	110 µL	115 µL	120 µL	125 µL
Positive	A	A	A	A	A	A
Negative	Lysed	Lysed	Lysed	Lysed	Lysed	Lysed

A- Absent

Table 3.7 PFU/mL determined for the FAS at various incubation times

Sample	5 min	7.5 min	10 min
Positive	A	A	A
Negative	Lysed	Lysed	Lysed

A- Absent

Plaques were absent from all the positive plates while the negative plates had produced lysed or a lacy effect on the plate. Therefore, the plates were repeated using the same parameters as above. The same results were produced. It was decided to dilute the D29 1 x 10^{10} PFU/mL twice. However, this produced no plaques whatsoever on either positive or negative plates. The D29 titre was run again but this too produced no plaques. As a last resort, broth suspensions were made from a single bead from every frozen stock culture received from Dr I. Grant. The titre and the normal phage assay were run, yet still no plaques were produced on the positive or negative control plates. According to Carlson (2004), phage's are fairly fragile and should not be exposed to osmotic shock, strong daylight or fluorescent light as it can lead to a damaged DNA in the phage. Physical interactions such as vortexing or vigorous mixing with a pipette should also be avoided to prevent damage to the phage. It is recommended that due to the viability of the phage, a titre should be established as the phages are prepared and just before they are used in an assay (Carlson, 2004). The phage's that were initially used in the assay were taken straight from cold temperatures of ~4°C, but it is recommended to warm the phage stock to 37°C 5-10 minutes before the assay is performed (Carlson, 2004).

Another possible reason as to why the phage assay and titre stopped working, is that the *M. smegmatis* stock become over-populated with another bacterium. This could have happened when stock cultures were made (see 3.3.1.4). This was confirmed when all stock cultures were grown on Middlebrook 7H10 agar, and yellow, creamy and mucous like cultures grew. The ZN stain was performed, and no acid-fast Gram-positive bacteria were seen.

3.5 RECOMMENDATIONS

The utilisation of distilled water could have had an impact on the preparation of media and other reagents, therefore further research into using reverse osmosis (RO) water would be recommended. Also, it should be ensured that the stock culture of *M. smegmatis* does not contain glycerol or Tween80, and has not been frozen for too long. Care should also be taken that the stock culture can repetitively make a working sensor cell stock culture for the assay before optimising the various

assay stages. A good background of phage laboratory work is also required before optimising is conducted.

3.6 CONCLUSIONS

Although the phage method can rapidly produce sensitive and specific results within 48 h, it was very sensitive to concentration and temperatures. Various trials were conducted to optimise the phage method. However, due to time constraints, difficulty in sourcing local suppliers and a lack of experience- the phage assay could not be optimised nor validated. Recommendations on where improvement can be made were suggested. It was also decided that the PCR method required attention, as currently in South Africa the ELISA, culturing and ZN stain are the only methods routinely used.

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CHAPTER 4

Investigating PCR as a detection tool for *Mycobacterium avium* subsp. *paratuberculosis*

4.1 ABSTRACT

The optimisation of DNA extraction, using two commercial kits, was performed on whole blood and white buffy coat obtained from sheep's blood. DNA was extracted from whole blood and the buffy coat layer using the ZymoBIOMICS DNA Miniprep kit and *Quick*-DNA™ Miniprep Plus kit. The DNA concentration (ng/µL) and quality (260/280 ratio) was measured using a spectrophotometer. The DNA extraction from the buffy coat layer using the *Quick*-DNA™ Miniprep Plus kit produced the highest quality and concentration. All DNA that was extracted underwent a PCR reaction using *Mycobacterium avium* subsp. *paratuberculosis* (MAP) specific primers that targeted different regions of the MAP genome (IS900 and F57). A primer, specific for the *Mycobacterium* genus, was also examined. Completed PCR reactions were run on a 1.2% agarose gel using electrophoresis at 85 V. The DNA fragments were visualised using a UV light. The only MAP specific primer that produced positive bands was the F57 primer. Various temperatures were tested for F57 primer to determine the optimal Annealing temperature(T_m) as the PCR product produced more than one band. Thereafter, the PCR product was sequenced. The sample was aligned to published MAP sequences using the NCBI BLAST tool. Samples were confirmed as 100% identical to *Mycobacterium avium* subsp. *paratuberculosis* (ATCC 19698).

4.2 INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) forms part of the slow-growing and pathogenic *Mycobacterium avium* complex (MAC) and is the causative agent to Johne's disease (JD) in ruminants such as cattle, sheep and goats. During the first two years of infection, the ruminant is asymptomatic but can intermittently shed MAP in their faeces, semen and milk. Therefore, this can lead to the spread of MAP to non-infected ruminants in the herd. Due to the intermittent shedding of MAP, currently used diagnostic methods lack the ability to detect MAP at an earlier stage in the disease (Stevenson, 2015). Another issue is that MAP takes up to 16 weeks to produce a single colony in culturing (Sidoti *et al*., 2011). Johne's disease is further classified by strains. The S strain is isolated from JD infected ovine, while strain C is isolated from JD infected bovine (Whittington & Sergeant, 2001). However, a previous investigation indicated that sheep were susceptible to either the S or C strain, while cattle are not as susceptible to the S strain (Moloney & Whittington, 2008). This demonstrates that cross-species transmission of strains exists due to a host preference (EFSA Panel on Animal Health and Welfare [AHAW] *et al*., 2017).

Since the spread of MAP in the Western Cape (South Africa), little research work has been done on the prevalence of JD in South Africa. The long incubation period of JD makes it more

difficult to notice JD at the clinical stage in sheep than in cattle. This is because diarrhoea is a more common symptom in cattle, while only 20% of sheep infected with MAP will have diarrhoea up to 2 years after infection. What also makes it more difficult is that ovine infected with JD, mimic symptoms of other diseases such as caseous lymphadenitis abscesses and scrapie or produce chronic infections (lung, liver and/or kidney) (Collins, 2009). One of the more common symptoms of ovine JD is the sudden weight loss (Sergeant, 2001). Botha *et al*. (2013) suggested that because of the complexity involved in identifying and specifying MAP, there are very few reports published on research of infected animals in Africa.

In order to detect MAP, the polymerase chain reaction (PCR) method can be utilised. Currently there has been no published work on PCR MAP detection in South Africa. It is therefore necessary to trial various MAP specific primers, as a novel strain could be present in infected ruminants in South Africa. There are a number of MAP-specific genomic sequences that have been identified, mainly: IS900 (Green *et al*., 1989), F57 element (Poupart *et al*., 1993), *hsp X* gene (Ellingson *et al*., 1998) and IS*Mav*2 (Strommenger *et al*., 2001). The IS900 sequence was discovered in the 1980s. It is a 1 451 base pair (bp) element that has been defined as definitive for the identification of MAP, as there are 14-20 copies of IS900 in the MAP sequence (Grant & Rees, 2010). The F57, *hsp X* gene and IS*Mav*2 sequences have low copy numbers in the MAP genome. Initially the IS900 sequence was routinely used as the standard marker for MAP PCR detection. However, some studies reported environmental mycobacteria and mycobacteria, other than MAP, that contain IS900-like sequences (Cousins *et al*., 1999, Englund *et al*., 2002; Grant & Rees, 2010; Chaubey *et al*., 2016). The F57 sequence is not as sensitive as IS900 but has only been detected in MAP samples and has not been detected in any of the *Mycobacterium avium* complex or any other mycobacterial species (Vansnick *et al*., 2004). Therefore, the F57 single copy sequence holds great potential. Although the F57 sequence is 620 bp, which is shorter than the IS900 1 451 bp, it is more specific for MAP detection than the IS900 sequence.

Thus, the aim for this study was to optimise a PCR method to detect MAP. This included testing two different DNA extraction kits to determine which would efficiently extract MAP DNA from sheep's blood. Thereafter, various MAP specific PCR primer sets: P90, F57, TJ1-4 and MYCOGEN, were experimented with.

4.3 METHODOLOGY

All testing was conducted at the Department of Food Science (Stellenbosch University) biosafety level two microbiological laboratory. Various DNA extraction kits were tested to determine which kit would produce a higher quality and quantity of MAP DNA (Fig. 4.1). It was also recommended to test different forms of the sheep blood sample to determine whether it would increase or decrease the DNA quantity and quality. Once this had been established, various MAP specific primers required testing to determine which would produce a positive result. The amplified DNA products that produced a positive result would be sequenced and compared to published DNA sequences by

blasting them on the International Nucleotide Sequence Database, Basic Local Alignment Search tool (BLAST) (National Institutes of Health, International Nucleotide Sequence Database Collaboration, http://www.ncbi.nlm.nih.gov).

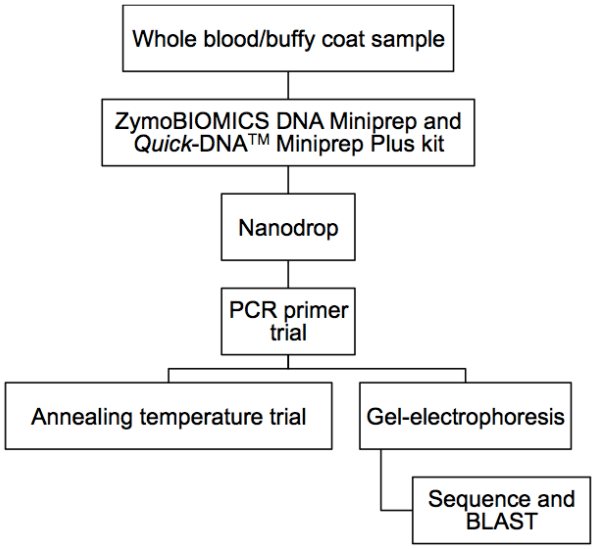

Figure 4.1 Flow chart demonstrating how the optimisation of the PCR method, which was used for the detection of MAP, was carried out.

4.3.1 Blood samples

Ethical clearance, from the Animal Ethics Clearance Committee at Stellenbosch University, and a permit from the Department of Agriculture, Forestry and Fisheries (DAFF) was obtained for the extraction and handling of blood from sheep (SU-AEC project number 2860 and Section 20 under Animal Diseases Act 1984 (Act No 35 of 84). Part of the ethical clearance application and permit from section 20 ensured that only a state veterinarian would be allowed to take blood samples. Dohne Merino sheep blood samples (n=3) were received from veterinarian Dr S. Kotzé (Nuwerus, Moorreesburg, Western Cape, South Africa). The veterinarian had confirmed the samples were MAP positive using ELISA results (Addendum A). For whole blood analysis, blood was removed from the tubes before the rest of the whole blood was centrifuged to obtain the buffy coat layer.

4.3.1.1 Whole blood

Samples were drawn from the sheep using a sterile needle and BD Vacutainer® tubes (lithium preservative) (Becton Dickinson, South Africa) (Whittington & Moloney, 2008). Before the buffy coat separation could occur, 250 and 200 µL of whole blood was removed with a micropipette as required for the two different DNA extraction kits.

4.3.1.2 Buffy coat

Whole blood samples were then centrifuged in the same vacutainer tubes for 30 min at 400 x *g* (1578 rpm) (Sigma 2-16K, Serial # 112234, Germany) at 20°C. The whole blood separated into various sections (Fig. 4.2). The top plasma layer was carefully removed using a sterile Pasteur pipette (VWR Life Sciences, South Africa). The buffy coat layer was removed, using a micropipette, and placed

into a sterile 15 mL Falcon centrifuge tube where 6 mL of sterile phosphate buffered saline (PBS) (VWR Life Sciences, South Africa) was added and centrifuged at 100 x *g* (788 rpm) for 10 min. The PBS was removed, and the buffy coat pellet was re-suspended in sterile 2 mL microcentrifuge tube with 1 mL PBS (Swift *et al*., 2013).

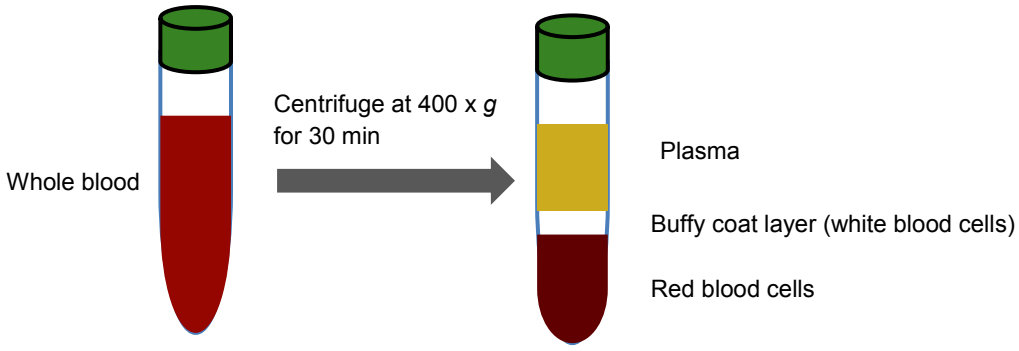

Figure 4.2 Schematic diagram illustrating the separation of whole blood in lithium heparin vacutainer tubes into the plasma, white blood cells (buffy coat layer) and red blood cells.

4.3.2 DNA extraction kits

Two commercially available kits (Table 4.1) were used to determine which would produce a better DNA concentration yield: ZymoBIOMICS DNA Miniprep Kit and *Quick*-DNA™ Miniprep Plus Kit (Zymo Research, Inqaba Biotec, South Africa). Whole blood and the buffy coat layers were tested using both kits. Samples were centrifuged using the Neofuge 13R/13 centrifuge (Heal Force, China).

Table 4.1 The components of the ZymoBIOMICS and Quick-DNA Miniprep kits

ZymoBIOMICS DNA Miniprep Kit	**Quick-DNA™ Miniprep Plus Kit**
ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)	Proteinase K & Storage Buffer
ZymoBIOMICS™ Lysis Solution	BioFluid & Cell Buffer (Red)
Zymo-Spin™ III-F Filter	Zymo-Spin™ IIC-XL
Collection Tubes (2 mL)	Collection Tubes (2 mL)
ZymoBIOMICS™ DNA Binding Buffer	Genomic Binding Buffer
Zymo-Spin™ IIC-Z Column	Wash Buffer
ZymoBIOMICS™ DNA Wash Buffer 1	DNA Wash Buffer
ZymoBIOMICS™ DNA Wash Buffer 2	
ZymoBIOMICS DNAse/RNAse Free Water	DNA Elution Buffer
ZymoBIOMICS™ HRC Prep Solution	
Zymo-Spin™ III-HRC	

4.3.2.1 ZymoBIOMICS DNA Miniprep Kit

Protocol was followed according to manufacturing instructions. Briefly, 250 µL of sample was added to a ZR BashingBead™ Lysis Tube, which contained a mixture of 0.1 and 0.5 mm beads. Thereafter, 750 µL of ZymoBIOMICS™ Lysis Solution was added to the lysis tube and the cap was sealed tightly

to the tube. Lysis tubes were secured into the Omni Bead Rupter 24 (Omni International, United States), and processed for 5 min at a speed of 4.70 $m.s^{-1}$. These tubes were then centrifuged at 10 400 rpm for 1 min. Once completed, 400 µL of the supernatant was transferred to the Zymo-Spin™ III-F Filter in a collection tube, and was centrifuged at 9 500 rpm for 1 min. The filter was then discarded and 1 200 µL of ZymoBIOMICS™ DNA Binding Buffer was added to the filtrate. A quantity of 800 µL of the filtrate mixture was added to the Zymo-Spin™ IIC-Z Column in a collection tube. These tubes were centrifuged at 10 400 rpm for 1 min. The flow-through was discarded and the step was repeated. Approximately 400 µL of ZymoBIOMICS™ DNA Wash Buffer 1 was added to the Zymo-Spin™ IIC-Z Column that contained a new collection tube, and was centrifuged at 10 400 rpm for 1 min. The flow-through was discarded and 700 µL of ZymoBIOMICS™ DNA Wash Buffer 2 was added to the Zymo-Spin™ IIC-Z Column and centrifuged at 10 400 rpm for 1 min. The flow-through was again discarded and 200 µL of the ZymoBIOMICS™ DNA Wash Buffer 2 was added to the Zymo-Spin™ IIC-Z Column and centrifuged at 10 400 rpm for 1 min. The Zymo-Spin™ IIC-Z Column was then transferred to a clean 1.5 mL microcentrifuge tube where 100 µL of ZymoBIOMICS™ DNase/RNase Free Water was added to the tubes and left to incubate at room temperature for 1 min. These tubes were then centrifuged at 10 400 rpm for 1 min. These tubes contained the eluted DNA. The Zymo-Spin™ II-HRC Filter was placed in a new collection tube and 600 µL of ZymoBIOMICS™ HRC Prep Solution was added to the filter. The filter tubes were centrifuged at 9 500 rpm for 3 min. The eluted DNA from the blood samples were transferred to the Zymo-Spin™ II-HRC Filter in a clean 1.5 mL microcentrifuge tube and centrifuged at 13 500 rpm for 3 min. The filtered DNA was then transferred to 0.5 mL microcentrifuge tubes and placed in the -20°C freezer until samples were required.

4.3.2.2 Quick-DNA™ Miniprep Plus Kit

Approximately 200 µL of the blood sample, 200 µL BioFluid & Cell Buffer and 20 µL Proteinase K was added to a microcentrifuge tube. The tube was thoroughly mixed and incubated at 55°C for 10 min. Thereafter, 420 µL of Genomic Binding Buffer was added and rigorously mixed. The mixture was transferred to a Zymo-Spin™ IIC-XL Column in a collection tube. The sample was centrifuged at 12 100 rpm for 1 min. The flow-through was disposed. Afterwards, 400 µL DNA Pre-Wash Buffer was added to the column in a new collection tube and centrifuged for 1 min. The collection tube was emptied and 700 µL of g-DNA Wash Buffer was added. The sample was centrifuged again at 12 100 rpm for 1 min. Subsequently 200 µL g-DNA Wash Buffer was added and the samples was centrifuged for 1 min at 12 100 rpm. The collection tube was emptied. The DNA was eluted by adding 100 µL DNA Elution Buffer to the Zymo-Spin™ IIC-XL Column in a clean microcentrifuge tube. The sample was first incubated for 5 min and centrifuged for 1 min at 12 100 rpm. DNA samples were transferred to smaller 0.5 mL microcentrifuge tubes and placed in the -20°C freezer until required.

4.3.3 DNA concentration and quality

In order to determine if the DNA extraction process had successfully extracted sufficient amounts of DNA, a nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, United States) was used to measure the protein concentration and purity (Leite *et al*., 2013). The spectrophotometer measured the sample using the full UV-VIS spectrum (220-750 nm). A 2 µL control was used to blank the spectrophotometer. The DNA sample was pipetted onto the small receiver holder. The spectrophotometer was then closed by placing a lever (containing the second fibre optic cable) onto the sample. A pulsed xenon flash lamp created the light source for the spectrophotometer to analyse the light that passed through the sample. The data was then recorded on the computer software where the DNA concentration was presented as ng/µL. To ensure the quality of DNA obtained from the sample was good; the nanodrop software calculated the absorbance 260/280 ratio. A value between 1.8 and 2 demonstrated good quality (Leite *et al*., 2013).

4.3.4 PCR primers

Various primer set's (Table 4.2) targeting various genes were tested with the DNA isolated from the sheep whole blood and buffy coat samples.

Table 4.2 Various primers that were utilised in the optimisation of PCR to detect MAP

Primer name	Sequence (5'-3')	T_m (°C)	Product (bp)	PCR conditions
P90[1]	GAAGGGTGTTCGGGGCCGTCGCTTAG	71.1		94°C 5 min, 37 x (94°C
P91	GGCGTTGAGGTCGATCGCCCACGTGAC	72.6	400	30s, 62°C 30s, 72°C 60s) and 72°C 4 min.
P90[2]	GTTCGGGGCCGTCGCTTAGG	68.6		94°C 5 min, 35 x (94°C 2
P91	GAGGTCGATCGCCCACGTGA	66.6	400	min, 55°C 2 min, 72°C 3 min) and 72°C 10 min.
F57f[3]	GGTCGCGTCATTCAGAATC	56.7		94°C 5 min, 37 x (94°C
F57r	TCTCAGACAGTGGCAGGTG	58.8	439	30s, 58°C 30s, 72°C 60s) and 72°C 7 min.
TJ1[4]	GCTGATGCGCTTGCTCAT	56.0		94°C 5 min, 10 x (94°C 1
TJ2	CGGGAGTTTGGTAGCCAGTA	59.4	356	min, 59°C 1 min, 72°C 3 min) and 72°C 7 min.
TJ3[4]	CAGCGGCTGCTTTATATTCC	57.3		94°C 5 min, 30 x (94°C 1
TJ4	GGCACGGCTCTTGTTGTAGT	59.4	294	min, 60°C 1 min, 72°C 3 min) and 72°C 7 min.
MYCOGEN(f)[5]	AGAGTTTGATCCTGGCTCAG	60.4	1030 and 180	95°C 10 min, 35 x (95°C 1 min, 61°C 30 s, 72°C 1
MYCOGEN(r)	TGCACACAGGCCACAAGGGA	64.5		min) and 72°C 10 min.

F: forward, r: reverse. [1]Whittington *et al*. (1998), [2]Schwartz *et al*. (2000), [3]Coetsier *et al*. (2000), [4]Bull *et al*. (2003) and [5]Katale *et al*. (2015)

Each DNA sample was run in duplicate with each primer sequence. Samples were processed by the T100™ Thermocycler (Bio-Rad, South Africa). The Whittington *et al*. (1998) P90/P91 primers detect MAP in the IS900 gene sequence in the region of 15-421 bp. The other P90/P91 primers

detect MAP in the region of 22-421 bp also found in the IS900 gene sequence (Schwartz *et al*., 2000). The F57 primer detects MAP in the f57 sequence in the region 176-197 and 599-617 (Coetsier *et al*., 2000). The TJ 1-4 primers, based on the IS900 insertion sequence, were used to demonstrate a nested PCR reaction, where 10 µL of the PCR product from TJ1 and TJ2 was used as the DNA sample for the PCR reaction of TJ3 and TJ4 (Bull *et al*., 2003). The MYCOGEN primer set was used to detect the presence of the *Mycobacterium* genus and as an internal positive control, to ensure that no false-positive PCR products were produced.

4.3.5 Annealing temperature variations

The annealing temperature (T_m) is the temperature at which the primers attach to the template DNA. The different primers that were used during the PCR optimisation, varied in annealing temperatures (Table 4.2). Therefore, primers that produced more than one band, unless otherwise stated, underwent a PCR annealing temperature variation trial. This would ensure that the DNA product would produce one instead of many bands, due to background noise.

4.3.6 PCR Mixture

In order to process a sample using the PCR method, various reagents were added from the HotStarTaq® *Plus* Master Mix Kit (Qiagen, Whitehead Scientific, South Africa) to create a sample reaction mixture (Table 4.3). Sterile water was used as the negative control for the PCR.

Table 4.3 Sample reaction mixture formula

Component	Volume	Composition
HotStarTaq *Plus* Master Mix (2x)	10 µL	HotStarTaq Plus DNA polymerase, QIAGEN PCR buffer and dNTPs
Primer A	1 µL	Forward primer
Primer B	1 µL	Reverse primer
RNase-free water	3 µL	Water
CoralLoad Concentrate (10x)	2 µL	Two gel-tracking dye
Template DNA	3 µL	Sample DNA
Total reaction volume	**20 µL**	

The T100™ Thermo Cycler (BioRad, South Africa) was used to carry out the PCR reactions for each sample mixture. The PCR conditions required for each primer (Table 4.2) was preloaded onto the machine. Samples were placed in the holder of the thermos cycler, the lid was closed and the pre-saved protocol was selected.

4.3.7 Gel-electrophoresis

Before DNA samples fragments could be separated and analysed, the gel needed to be prepared and set with the wells. A 1.2% agarose gel was used to separate and analyse samples. This was prepared by weighing out 1.2 g in 100 mL of 1x Tris-Acetate-EDTA (TAE) buffer. The mixture was heated in the microwave until the salts had dissolved in the buffer. Subsequently, 10 µL of EZ vision

loading dye x10 000 solution (VWR Life Science, Inqaba Biotec, South Africa) was added to the gel, to ensure that the DNA fragments could be illuminated under the UV light. The gel mixture was poured into a tray, where a comb that contained a certain number of wells was placed in the gel and allowed to set. Once the gel had set, the comb was removed, and the gel was placed in the electrophoresis container. The 1x TAE buffer was poured into the tray and it was imperative to ensure that the gel was completely suspended in the buffer. In the first well, 2 µL of a 100 bp DNA ladder (New England BioLabs, Inqaba Biotech, South Africa) with 2 µL of 6x Gel loading dye (New England Biolabs, Inqaba Biotec, South Africa) was pipetted. The DNA ladder was used as a marker to measure the DNA fragments from the samples after electrophoresis. In the succeeding lanes, the PCR DNA samples, positive and negative controls were aliquoted in volumes of 10 µL into the wells. Once all the samples and controls were loaded, the electrophoresis container was closed. It was important to ensure that the wells were on the negative side of the container, as DNA is negative and the current flows from negative to positive. The electrophoresis machine was set to 85 V for 90 min. Once the gel had finished running, the gel visualisation was performed using the Bio-Rad Gel Doc XR+ System and Imaging Software (Bio-Rad, South Africa). The DNA fragments were analysed while using the DNA ladder as a reference (Fig. 4.3).

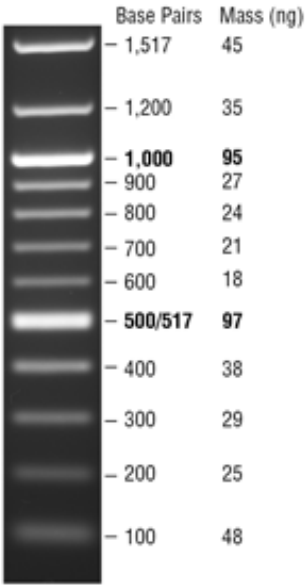

Figure 4.3 The 100 bp DNA ladder that was used as a reference to determine the sample DNA bands. The ladder has a size range from 100 to 1 517 bp (New England Biolabs).

4.3.8 Sequencing and blasting

To verify and identify if the PCR products were MAP positive that were illuminated in the gel after electrophoresis, at the corresponding bp sizes (Table 4.2), PCR products were sent to the Central Analytic Sequencing (CAF) laboratory (Stellenbosch University) (https://www.sun.ac.za/english/faculty/science/CAF). The CAF laboratory cleaned the PCR products, using a post-PCR clean up kit (to remove any dNTP's, primer and mastermix components), followed by a dye terminating sequencing known as Sanger sequencing. The sample sequence

data was converted to a FASTA format and compared to published MAP sequences using the International Nucleotide Sequence Database, Basic Local Alignment Search tool (BLAST) (National Institutes of Health, International Nucleotide Sequence Database Collaboration, http://www.ncbi.nlm.nih.gov). The BLAST website identifies species that are identical to a sample by aligning the DNA sequences.

4.3.9 Statistical analysis

Statistical analysis on data was conducted using XLSTAT to determine the mean, standard deviation and conduct a t-test to determine if values were significantly different.

4.4 RESULTS AND DISCUSSION

Blood samples were specifically taken from Dohne Merino sheep species, as Merino sheep have previously been proven to be more susceptible to MAP than other sheep species (Begg *et al*., 2017). It was also reported that the first infected sheep in South Africa was an imported Merino ram (Van Niekerk & Van der Walt, 1967; Michel & Bastianello, 2000).

Mycobacterium avium subsp. *paratuberculosis* has proved to be a tedious and laborious *Mycobacterium* to detect (Waddell *et al*., 2016). This is due to its slow-growing incubation period, dependence on iron, persistence to survive in the environment and intermittent shedding from an infected ruminant (Stevenson, 2015). In order to eradicate and control the spread of MAP within a herd, sensitive and specific detection methods need to be implemented (Sidoti *et al*., 2011). Not only would the method achieve the mentioned advantages, but it can also contribute towards understanding the potential role MAP plays in Crohn's disease (CD) through the transmission of contaminated food (Möbius *et al*., 2008; Grant & Rees, 2010; Robertson *et al*., 2017).

Currently in South Africa the ELISA test, which is dependent on the presence of antibodies against MAP, is routinely used to test the presence of MAP in a flock/herd. However, this method is unreliable as it has a moderate to low sensitivity that is compromised by the inconsistency of the immune systems' response to MAP, which is also dependent on the stage of the disease (Pinedo *et al*., 2008; Sidoti *et al*., 2011). An ELISA test will also only rarely give results in an animal younger than two years old, thus preventing the determination of early stage infection (Grant & Rees, 2010; Zarei *et al*., 2017). Internationally over the years, the PCR method is said to be more reliable, sensitive and specific than the ELISA test (Waddell *et al*., 2016). Previous research has indicated that the input material, sample DNA, needs to be of high quality. Thus, making the DNA extraction step crucial in ensuring that sufficient amounts of target DNA has been isolated from the particular matrices (blood, faecal, milk and tissue samples) (Sidoti *et al*., 2011; Radomski *et al*., 2013; Park *et al*., 2014; Swift *et al*., 2016).

4.4.1 DNA extraction, quantification and quality

To determine the kit and sample type that would produce the best quantity and quality of DNA, DNA was extracted from the whole blood and white buffy coat layer using the *Quick*-DNA™ Miniprep Plus and ZymoBIOMICS DNA Miniprep. The average and standard deviation (SD) of the DNA concentration (ng/µL) and quality ratio (260/280) was calculated and recorded in Table 4.4. The significant results were determined by using a t-test on a 95% confidence interval, in other words a 5% significance level ($p<0.05$).

Table 4.4 The calculated average concentration and quality of DNA extracted from whole blood and buffy coat layer using two extraction kits

Sample	Extraction Kit	Concentration (ng/µL)		Quality (Abs 260/280)	
		Mean	SD	Mean	SD
Whole blood	*Quick*-DNA[1]	40.68	13.25	1.08	0.14
	ZymoBIOMICS[2]	5.53	3.27	0.66	0.15
Buffy coat	*Quick*-DNA[1]	106.07	81.39	1.47	0.71
	ZymoBIOMICS[2]	20.82	5.40	1.20	0.43

[1]*Quick*-DNA™ Miniprep Plus, [2]ZymoBIOMICS DNA Miniprep, Mean: average and SD: standard deviation

After analysing the sample types (whole blood and buffy coat layer) using the null hypothesis test, no significant difference between the quantity ($p=0.204$) and quality ($p=0.0892$) of DNA extracted was produced. Although there was so significant difference statistically between the sample types, the buffy coat layer would be recommended for MAP DNA extraction as it produced the highest concentration and quality (Table 4.4). Therefore the high ceoncentration and purity of DNA from the buffy coat layer would be beneficial for optimising the PCR detection method (Table 4.4). In terms of the DNA extraction kits (ZymoBIOMICS DNA Miniprep and *Quick*-DNA™ Miniprep Plus), there was a significant difference ($p<0.05$) on the quantity, but there was no significant difference between the kits' quality ($p=0.224$). Although there was not a statistical significant difference between the extracted DNA quantities between the kits, the *Quick*-DNA™ Miniprep Plus extracted a higher concentration of DNA. Based on the statistical observation, it would be recommended to use the *Quick*-DNA™ Miniprep Plus as this kit extracted a better quality DNA than the ZymoBIOMICS DNA Miniprep kit.

In terms of SD, the buffy coat ZymoBIOMICS DNA Miniprep had the greatest deviation from the mean, while the whole blood ZymoBIOMICS DNA Miniprep produced the least. The buffy coat DNA extraction using the *Quick*-DNA™ Miniprep Plus produced the highest quantity and quality DNA. Thereafter, the whole blood DNA extraction using the ZymoBIOMICS DNA Miniprep produced a good quantity, but the buffy coat using the ZymoBIOMICS DNA Miniprep produced a better quality at a lower quantity. Although DNA was obtained from both blood matrices using the two kits, the results need to be interpreted with caution as only three blood samples were obtained from the veterinarian. The reason for the small number of samples is due to the problem of obtaining samples from farms where farmers are willing to allow sample collection from their flock/herd.

The DNA extraction kit components differed slightly. The ZymoBIOMICS DNA Miniprep kit utilised beads with a lysis buffer, while the *Quick*-DNA™ Miniprep Plus did not use beads but used proteinase K at the start of the DNA extraction. Proteinase K is an enzyme (serine protease) used to degrade proteins in their natural structure. This enzyme is useful as it digests unwanted proteins from DNA/RNA originating from microorganisms. The lysis buffer, which is used with the beads, breaks open the cells for DNA extraction of genomic DNA. *Mycobacterium avium* subsp. *paratuberculosis* has a thick and fatty cell wall comprised of 60% lipids. Thus, making general or crude DNA extraction counterproductive. Commercial kits have proved beneficial in the DNA extraction of MAP from various matrices (blood, milk and faeces) (Radomski *et al.*, 2013; Park *et al*., 2014). Although in this study only two extraction kits were tested, there are various commercially available kits that could be tested. However, kits that are MAP-specific are very limited. Other research has demonstrated the use of magnetic capture of MAP during DNA extraction to ensure an increase in DNA extracted (Botsaris *et al*., 2013; Ricchi *et al*., 2016; Swift *et al*., 2016; Robertson *et al*., 2017). According to Husakova *et al*. (2017), when combining the peptide-mediated magnetic separation (PMS)-phage method with IS900 PCR on sheep blood samples, inoculated with 3.5 x 10^1 PFU/mL and diluted 10X Middlebrook 7H9 broth and dynabeads (coated with biotinylated peptides), a limit detection of 10 MAP cells/mL was achieved. Radomski *et al*. (2013) research had indicated that commercial kits produced a lower DNA turnout than the manual DNA extraction method. Park *et al*. (2014) conducted DNA extraction from tissue samples (intestinal tissue from cattle) using two commercial kits and one manual DNA extraction method. The study showed that the use of mechanical breakdown on environmental samples before DNA extraction increased the genomic DNA output. The research also demonstrated that pre-treatment of samples prior to DNA extraction yields 40 times more genomic DNA. These previous studies demonstrate that DNA extraction is complicated, and it is important to test various factors of extraction to ensure the optimal method is applied to samples. Research also showed that pre-treatment is dependent on the sample type. In other words, only environmental, milk and faecal samples require bead bashing. After analysing the results, it was decided that the *Quick*-DNA™ Miniprep Plus kit would be used to isolate DNA from the buffy coat layer.

4.4.2 Primer determination

Molecular detection can be conducted on insertion sequences or single copy genes that are present within the genome of MAP (Grant & Rees, 2010). Primers were selected based on previous research as well as commonly used primers for the detection of MAP. Due to the length of the IS900 gene sequence, two primers were tested to determine which would produce brighter bands. One primer that targets the single copy F57 gene sequence was used in this study. A nested primer set, targeting the IS900 insertion sequence, was utilised as nested PCR is known to be more sensitive and specific than a single PCR. In order to utilise an external control that ensured that the PCR reaction worked, a ‘general’ primer that detects the DNA from mycobacterial species was used.

Results from the PCR products from the various primers, which were fragmented with using gel-electrophoresis, are recorded in Table 4.5.

PCR detection using the IS900 insertion sequence was originally considered to be specific for MAP species as there are 15-20 copies that match the MAP genome. Thus, making the IS900 the diagnostic target gene for testing MAP (Sidoti *et al*., 2011). However, Englund *et al*. (2002) had conducted an IS900 PCR and detected *Mycobacterium cookie*. It was suggested to research alternative genes to IS900 to confirm MAP in samples. Gill *et al*. (2011), also mentioned that PCR systems can produce false-positive results if there is DNA present from other mycobacteria other than MAP. Nonetheless, PCR identification using the phage-PCR assay utilised the IS900 insertion sequence and detection was achieved (Swift *et al*., 2013, Swift *et al*., 2016). The P90 and P91 primers (Whittington *et al.*, 1998; Schwartz *et al*., 2000) did not however produce any bands from any of the DNA extracted. The nested TJ1-4 primers (Bull *et al*., 2003) were also IS900-specific, and also did not produce any bands. Although nested PCR is more sensitive and specific than single PCR, there is possible cause for contamination during the handling of test material and DNA, which could lead to false positive or false-negative results (Gill *et al*., 2011). Therefore, it is generally accepted that the IS900 insertion sequence cannot be used alone as a molecular detection method for food samples (milk, liver and dairy products), and these results can also not be used to determine whether to cull a suspected ruminant (Grant & Rees, 2010; Chaubey *et al*., 2016).

Table 4.5 Primer results after gel-electrophoresis

Primers	**Whole blood**		**Buffy coat**	
	Quick-DNA[a]	ZymoBIOMICS[b]	*Quick*-DNA[a]	ZymoBIOMICS[b]
P90 and P91[1]	-	-	-	-
P90 and P91[2]	-	-	-	-
F57f and F57r[3]	+	+	+	+
TJ1, TJ2, TJ3 and TJ4[4]	-	-	-	-
MYCOGEN(f) and MYCOGEN(r)[5]	+	+	+	+

F: forward, r: reverse. [1]Whittington *et al*. (1998), [2]Schwartz *et al*. (2000), [3]Coetsier *et al*. (2000), [4]Bull *et al*. (2003) and [5]Katale *et al*. (2015). [a]*Quick*-DNA™ Miniprep Plus and [b]ZymoBIOMICS DNA Miniprep. -: no band +: band produced.

The F57 genome has been described to specifically detect only MAP based on a single sequence (Poupart *et al*., 1993; Park *et al*., 2014; Waddell *et al*. 2016). According to Möbius *et al*. (2008), the F57 Map-specific primer also detected *Mycobacterium obuense.* This further demonstrates the complexity in molecular detection of MAP. The problem with utilising single copy gene sequences is that if the DNA sample is of a low concentration, the probability of detecting the DNA is low. Thus, reducing the detection sensitivity of the PCR and increasing the probability of false-negative results (Grant & Rees, 2010). However, the DNA extracted samples in this study all produced bands at 439 bp with the F57 primers. The DNA extracted from the buffy coat using the *Quick*-DNA™ Miniprep Plus produced the brightest bands.

Miller *et al*. (2017) demonstrated the use of a general *Mycobacterium* primer, which detects the presence of the *Mycobacterium* genus. This study detected MAP in an infected dog by using the primer, sequencing the PCR product and then using the BLAST tool on the NCBI website. Although this is one way of detecting MAP in samples, it would seem that further trials and optimisation are required before depending on just this primer alone. This is mainly due to the complexity in various genomes of mycobacteria and previous research has only demonstrated detection of some of the isolates of the *Mycobacterium* genus (Wilton & Cousins, 1992; Katale *et al*., 2015). The primers did detect the presence of mycobacteria in the DNA samples (Table 4.5), however the final confirmation of MAP cannot rely on this primer alone.

As has previously been discussed, the detection of MAP is not easy and even though PCR is sensitive and some-what specific, previous studies have shown that is not always reliable (Gill et al, 2011). Waddell *et al*. (2016) conducted a meta-analysis on research where PCR was tested on various samples (food, blood, faecal), and it could be seen that there was hardly any research conducted on the presence of MAP in Africa, let alone South Africa. As there is currently no published work on primers tested in South Africa, it was difficult to prove if there is only one strain of MAP present. More research should be conducted on more type of samples from various areas of South Africa. It is also unsure whether a novel strain of MAP exists in South Africa. This might explain why none of the IS900 insertion sequence primers detected any of the DNA samples.

4.4.3 Annealing temperature trial

The F57 primer produces DNA products in 500 and 439 bp regions, however according to the original article (Coetsier *et al.,* 2000), only one single band at 439 bp was suppose to be present. According to the suppliers (Table 4.2), the highest annealing temperature for the F57 primers was 58.8°C. Therefore, the following annealing temperatures were experimented on to determine whether a single band could be produced: 53°C, 55°C, 57°C and 60°C. However, no bands were produced at any of these temperatures. Thereafter the F57 PCR products, that produced a 400 and 500 bp at the annealing temperature of 58°C, were sent to the Stellenbosch University Central Analytical Facility (CAF) to obtain a sequence that could be compared to published MAP sequences, using the BLAST tool, on the NCBI website. The 500 bp fragment turned out to be an artefact, which means there was no DNA at that fragment that belonged to MAP. The 400 bp produced 100% identification to *Mycobacterium avium* subsp. *paratuberculosis* (ATCC 19698).

4.5 RECOMMENDATIONS

As only three blood samples were received and tested as positive using the ELISA method, it would be recommended to increase the number of samples and conduct another DNA extraction trial. Not only would it produce more reliable data, but also the standard deviation would hopefully decrease and demonstrate an accurate capturing of data. It would also be beneficial to see if the crude DNA extraction method would produce as high a quantity and quality as the two tested commercial kits in

this study. It would also be recommended to trial the ZymoBIOMICS DNA Miniprep kit with proteinase K to see whether it would produce an increase in the quantity and quality of DNA extracted. An optimisation that include all the other sample matrices (faecal, milk, water and soil) should be incorporated in the PCR optimisation step. It would be beneficial to utilise various F57 primers and determine which would produce a higher sensitivity and specificity. The same recommendation can be applied to the IS900 primers. It would also be beneficial to culture isolates from ELISA positive sheep, by isolating MAP from faecal samples, and using these isolates to perform PCR optimisation, as it is easier to culture from faecal samples than from blood samples (Moloney & Whittington, 2008).

4.6 CONCLUSIONS

During this study, DNA extraction from blood samples was investigated. Two different forms (whole blood and buffy coat layer) of blood, from MAP positive sheep, were tested to determine which would produce a higher concentration and quality of DNA. Two commercially available DNA extraction kits were tested. One kit, ZymoBIOMICS DNA Miniprep, contained bashing beads while the other, *Quick*-DNA™ Miniprep Plus, utilised proteinase K to extract genomic DNA. The DNA that was extracted from both the whole blood and buffy coat were measured using a nanodrop. The DNA that was extracted from the buffy coat using the *Quick*-DNA™ Miniprep Plus kit produced the highest quality and quantity of DNA.

All DNA that was extracted was tested against various primers that targeted different MAP genes (IS900, F57 and *Mycobacteriaceae* genus). The F57 was the only primer that is specific to MAP that produced the correct fragment size after gel-electrophoresis. Annealing temperatures were tested to ensure the correct temperature was utilised in the PCR reaction. Results demonstrated that the correct T_m is in fact 58°C. Thereafter PCR products were sequenced and compared to published MAP sequences using the BLAST tool on the NCBI website. Samples were confirmed as 100% identical to *Mycobacterium avium* subsp. *paratuberculosis* (ATCC 19698).

To conclude, DNA that was extracted from the white buffy coat layer using the *Quick*-DNA™ Miniprep Plus produced the purest DNA. The F57 primer was the only primer to MAP specific fragments after gel-electrophoresis. These conditions and DNA will be used in the following study.

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CHAPTER 5

Investigating the application of PCR to detect *Mycobacterium avium subsp. paratuberculosis* in various sheep sample matrices

5.1 ABSTRACT

Various sample matrices (faecal, blood and milk) were acquired from a known Johne's disease (JD) positive sheep flock from Langgewens research farm in Malmesbury. Pregnant Merino and Dohne Merino ewes were used as the test subjects. Blood, milk and faecal samples were obtained from these sheep to be analysed. The buffy coat layer method was used to extract DNA from the blood samples, and the milk was centrifuged to obtain a milk pellet from where DNA was extracted. Samples underwent DNA extraction using two different extraction kits; *Quick*-DNA™ Miniprep Plus kit for blood samples and ZymoBIOMICS™ DNA Miniprep kit for the faecal and milk samples. Polymerase chain reaction (PCR) was applied to the various samples using the F57 primer sequence sets. Completed PCR reactions were run on a 1.2% agarose gel using electrophoresis at 85 V for 90 min. Positive results produced bands at 439 bp. The buffy coat layer produced the highest prevalence (47.6%) of MAP, followed by the milk pellet samples (26.3%). The faecal samples produced negative results, which could be due to the absence of *Mycobacterium avium* subsp. *paratuberculosis* as it is intermittently shed during the earlier stages in infection. Blood samples were also sent to the Onderstepoort laboratory to conduct a serological test known as the enzyme-linked immunosorbent assay (ELISA) test. The head researcher at Langgewens also provided body condition score (BCS), live weight and ELISA results from 2017. The ELISA results from 2018 were compared with 2017 results, and a decrease was noted between 2017 (52.4%) and 2018 (38.1%) positive ELISA results. False-positive and false-negative ELISA results were produced between the years. There was no significant difference between the mean live weight ($p=0.0747$) and mean BCS ($p=0.361$) between 2017 and 2018. Comparisons between the various PCR sample results, ELISA, BCS and live weight were conducted on vaccinated and unvaccinated ewes. It was determined that the blood PCR test produced the best results, but that BCS and live weight should also be considered when making a final JD diagnosis.

5.2 INTRODUCTION

The purpose of food safety in the food industry is to ensure that foodborne pathogens are reduced, thus decreasing the possibility of emerging pathogens, by implementing regulations and processing procedures. However, with the rise in consumer population, there is an increase in mass produced food of animal origin. This is problematic as it has led to the increase in zoonotic emerging pathogens (Vouga & Greub, 2015). One such a pathogen is *Mycobacterium avium* subsp.

paratuberculosis (MAP) (Greenstein & Collins, 2004). This *Mycobacterium* produces a chronic and severe gastroenteritis disease in ruminants (such as goats, sheep and cattle) known as Johne's disease (JD) (Robertson *et al*., 2017).

Johne's disease is classified into asymptomatic, symptomatic and clinical stages. Initially the infected ruminant does not produce symptoms but sheds the *Mycobacterium* into blood, faeces, milk and semen- which can last for years after initial infection (Collins, 2009). Thereafter, symptoms such as rapid weight loss, profuse diarrhoea, decrease in milk production, shedding of wool and reduction in body conformation occur and these are termed the clinical stages (Whittington *et al*., 2017). Massive economic losses for farmers are caused as a result of the clinical stage due to ruminant infertility, decreased productivity, weakened ruminant immune system leading to increased susceptibility to other diseases (Sergeant, 2001; Gautam *et al*., 2018). Increasing evidence suggests that MAP could be a possible cause for Crohn's disease (CD), a human inflammatory disease with similar clinical symptoms to JD (Chaubey *et al*., *2016*; Chaubey *et al*., 2017).

Mycobacterium avium subsp. *paratuberculosis* has been detected in food products such as: pasteurised milk, soft cheeses, meat products (derived from animal liver) and is thought to be the possible source of contamination to humans (Grant & Rees, 2010; Gill *et al*., 2011; Botsaris *et al*., 2013; Gerrard *et al*., 2018). Not only has MAP been detected in food products, but it has been detected in run-off water from farms with infected ruminants, soil, faeces and blood from abattoirs (Foddai *et al*., 2009; Möbius *et al*., 2015; Hahn *et al*., 2017). Control methods such as vaccination, culling or quarantine have been used to prevent the further spread of MAP (Wolf *et al*., 2015; Chaubey *et al*., 2016). In spite of this, these methods have been unsuccessful in prevention, as it has been demonstrated that MAP has spread through the livestock trade (Windsor & Masters, 2010). Windsor & Masters' (2010) study on the effect of vaccination against JD demonstrated that at least six years after initial vaccination, MAP was still being shed into the environment. The prevalence of MAP in ruminants as well as food products from developing countries such as South Africa is also unknown. The first case of ovine JD was discovered in the 1960s in South Africa from an imported infected Merino ram (Michel & Bastianello, 2000). According to the Department of Agriculture, Forestry and Fisheries (DAFF), the Western Cape (South Africa) has the highest reported cases of ovine JD; higher than bovine JD (DAFF, 2017).

Further understanding of the disease is required to determine the correct diagnostic method that can identify MAP at earlier stages in the disease. Detection methods have been improved over the years. Methods such as culture, enzyme-linked immunosorbent assay (ELISA), Ziehl-Neelsen (ZN) stain and polymerase chain reaction (PCR) are internationally used (Grant & Rees, 2010; Chaubey *et al*., 2016). At present, in developing countries such as South Africa, the ELISA and ZN stain are routinely used by state veterinarians (Michel & Bastianello, 2000). World-wide, these currently used detection methods lack sensitivity and specificity to identify and detect the presence of MAP in various samples (Park *et al*., 2014), subsequently leading to inaccurate or false positive or negative results (Robertson *et al*., 2017). Reasons for the lack in sensitivity and specificity of

these methods are due to the stage of the disease at which the samples are taken, the type of sample and the lack of understanding MAP's characteristics by farmers, consumers and the food industry (Whittington *et al*., 2017). State veterinarians are required to make a decision on the status of an examined ruminant based on results obtained from these methods, however with the possibility of false-negative results the only way to give a definitive answer, is to slaughter the animal and perform a histology examination on the intestines and lymph nodes (DAFF, 2017).

The aim of this study was to test for the presence of MAP, from suspected Merino and Dohne Merino sheep, in various sample matrices (blood, faeces and milk) using PCR. The PCR results were compared to ELISA results to determine whether the ELISA or PCR method was more sensitive and reliable. The live weights and body condition score (BCS) of the suspected sampled sheep were also taken into consideration.

5.3 METHODOLOGY

Sheep samples were obtained from Langgewens Research farm, Malmesbury (Fig. 5.1). Roughly a quarter (n=21) of the flock were selected based on their history of poor body condition score, suspected infection of MAP and confirmed history of JD. The sheep were Merino and Dohne Merino ewes and were mostly all pregnant subjects- which increases the stress level in the ruminant. The flocks contained sheep that were obtained from different regions of the Western Cape, mainly Langgewens and Elsenburg. Some of the sheep had been vaccinated against ovine JD (n=9), with the Gudair® vaccine: a heat inactivated MAP F316 strain with mineral oil (Park & Yoo, 2016; Hautam *et al*., 2018).

Figure 5.1 The Merino and Dohne Merino sheep at Langgewens research farm, Malmesbury (taken May 2018).

5.3.1 Sample acquisition

Samples were acquired in compliance with the ethic's report and permit submitted for this project (SU-AEC project number 2860 and Section 20 under Animal Diseases Act 1984 (Act No 35 of 84)). Basically, blood, faecal and milk samples were taken from sheep that were suspected to be infected with JD (Table 5.3). The state veterinarian, Dr S. Davey, aseptically took blood samples from the

jugular vein using a sterile needle and BD vacutainer tube (Becton Dickinson, Inqaba Biotec, South Africa) (Fig. 5.2). Blood samples were kept cool in a polystyrene container until further analysis later that day. Dr S. Davey had also sampled blood into separate tubes to conduct an ELISA test, which was completed by the Agriculture Research Council veterinary research laboratory in Onderstepoort (100 Soutpan Road, Onderstepoort, Pretoria, 0110, South Africa). Faecal samples were aseptically obtained (by removing from the anus) using sterile latex gloves (Fig. 5.2). Before faecal and blood samples were taken from a specific sheep, a professionally trained sheep examiner (Dr J. Cloete) performed a body condition score (BCS) analysis (Fig. 5.3) and recorded the live weight (kg). The BCS is used to determine the body fatness to muscle ratio in sheep. Each sheep was examined around the backbone and short ribs using a 1-5 scale, where 1 indicated emaciated body condition and 5 indicated an extremely fat sheep (Whittington *et al*., 2017). The head researcher at Langgewens, Professor S. Cloete, also provided the ELISA and BCS results from 2017, in order to compare results between the years.

Figure 5.2 The state veterinarian Dr Sewellyn Davey took blood samples for MAP DNA extraction (left). Faecal samples taken from the same sheep (right) at Langgewens research farm, Malmesbury (taken May 2018).

Figure 5.3 The body condition score (BCS) being conducted by Dr J Cloete on a suspected infected Merino sheep at Langgewens research farm, Malmesbury (taken May 2018).

Samples were then transported to the Stellenbosch University Food Science department (as stated in the permit obtained under Section 20 of the Animal Diseases Act 1984 (Act No 35 of 84)), where the faecal samples were placed in the freezer (-20ºC) until further analysis. Blood samples were immediately processed by centrifuging the blood samples to obtain the white buffy coat layer (Chapter 4, section 4.3.1.2). Subsequently, DNA extraction was performed on the buffy coat layer and faecal samples.

Milk samples were obtained at a later stage, as the ewes were still pregnant when blood and faecal samples were taken. Milk samples were placed in sterile 50 mL Falcon centrifuge tubes (VWR Life Science, Inqaba Biotech, South Africa) (Fig. 5.4) and placed in a cooled polystyrene container until further processing and analysis. Milk samples were centrifuged to separate the milk into the cream, liquid layer and pellet. Thereafter, DNA extraction was performed on the pellet.

Figure 5.4 Sampling milk from the same Merino and Dohne Merino sheep at Langgewens research farm, Malmesbury (taken July 2018).

5.3.2 DNA Extraction

5.3.2.1 Buffy coat layer

Once the blood was centrifuged to obtain the buffy coat, the *Quick*-DNA™ Miniprep Plus kit was used to extract DNA from the samples (Chapter 4, section 4.3.2.2). The DNA samples were stored in 1 mL microcentrifuge tubes in the freezer (-18°C) until further analysis.

5.3.2.2 Faecal and milk

The DNA was extracted from the faecal and milk samples using the ZymoBIOMICS™ DNA Miniprep kit (Chapter 4, section 4.3.2.1).

Faecal samples were removed from the freezer (-18°C) and left to defrost at room temperature. Thereafter, 200 mg of faeces was weighed and placed into the bead beating tubes with 750 µL of ZymoBIOMICS™ Lysis Solution. Tubes were placed in the Omni Bead Rupter 24 (Omni International, United States), and processed for 5 min at a speed of 4.70 $m.s^{-1}$. The

methodology proceeded as previously described (Chapter 4, section 4.3.2.1). The DNA samples were stored in 1 mL microcentrifuge tubes in the freezer (-18°C) until further analysis.

Milk samples were processed on the day of collection. Briefly, 50 mL of milk was centrifuged at 2 500 *x g* for 15 min (Fig. 5.5). The cream layer was removed using a sterile spatula and the liquid layer was removed using sterile Pasteur pipettes. Thereafter, 3 mL of sterile phosphate buffer saline (PBS) was added to the milk pellet, and the sample was centrifuged at 2 500 *x g* for 10 min. The PBS was removed from the tube and the pellet was resuspended in a 1 mL microcentrifuge tube with new PBS. Approximately 200 µL of the milk pellet was added to the ZR BashingBead™ Lysis Tube with 750 µL of ZymoBIOMICS™ Lysis Solution. It was recommended by manufacturers to add 2% (v/v) proteinase K to the lysis tube. Once added, the samples were incubated for 30 min at 55°C. The tubes were then placed in the Omni Bead Rupter 24 (Omni International, United States), and processed for 5 min at a speed of 4.70 $m.s^{-1}$. Thereafter, the methodology proceeded as previously described (Chapter 4, section 4.3.2.1). The DNA samples were stored in 1 mL microcentrifuge tubes in the freezer (-18°C) until further analysis.

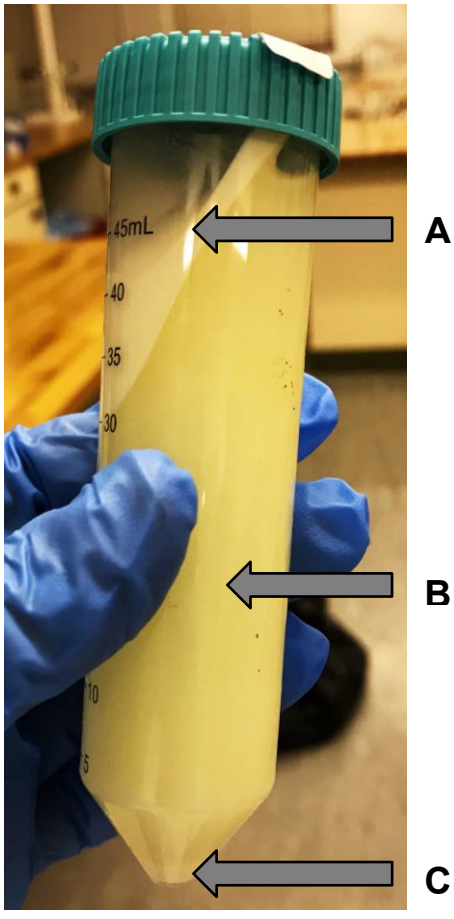

Figure 5.5 An ewes' milk sample after centrifuging for 15 min (taken at Department of Food Science, July 2018). A: cream layer, B: liquid milk layer and C: milk pellet with high microbial population.

Before samples were subjected to PCR, the DNA extracted from the various samples was measured using a spectrophotometer (Fig. 5.6), to determine the quantity (ng/µL) and purity (Abs 260/280). A good purity ratio reads between 1.8 and 2.0 (Whittington *et al*., 2017; Botaro *et al*., 2017). Thereafter, the F57 primer set sequence was used for the PCR MAP detection, as the method had previously been optimised (Chapter 4, section 4.3.4) (Coetsier *et al*., 2000). The PCR reaction mixture was comprised of: 10 µL HotStarTaq *Plus* Master Mix (2x), 1 µL forward primer, 1 µL reverse primer, 2 µL CoralLoad Concentrate (10x), 3 µL RNase-free water and 3 µL DNA sample. The PCR reaction followed previously optimised conditions (Chapter 4, section, 4.3.4), as seen in Table 5.1.

The PCR products were subjected to gel-electrophoresis to determine if MAP DNA was detected. A 1.2% agarose gel was utilised. Briefly 1.2 g was weighed and added to 100 mL of 1X TAE buffer solution. The media was heated until the crystals were dissolved. Thereafter, 10 µL of EZ vision loading dye x 10 000 solution (VWR Life Science, Inqaba Biotec, South Africa) was added to the gel. The agar was added into a PCR tray, with a comb, and left to set. Once set, the gel was placed in the electrophoresis bath, which contained 1X TAE buffer solution. The DNA samples and a 100 bp DNA ladder (New England BioLabs, Inqaba Biotech, South Africa) was added into the respective well (Chapter 4, section 4.3.7 for specific steps). The gel ran for 90 min at 85 V. The gels were then visualised using the Bio-Rad Gel Doc XR+ System and Imaging Software (Bio-Rad, South Africa). Positive PCR results would indicate bands at 439 bp (Coetsier *et al*., 2000)

Figure 5.6 The spectrophotometer used to measure the quantity and purity of the DNA samples (taken at the Central Analytical Facility, Stellenbosch University, May 2018).

Table 5.1 The PCR primer set and conditions that were used for the detection of MAP

Primer	Sequence (5'-3')	PCR conditions
F57f	GGTCGCGTCATTCAGAATC	94°C 5 min, 37 x (94°C 30 s, 58°C 30 s, 72°C 60 s) and 72°C 7 min.
F57r	TCTCAGACAGTGGCAGGTG	

f: forward and r: reverse

To ensure no false-positive PCR results were produced, a couple of PCR positive samples were randomly selected and sent to the Central Analytical Facility (CAF) Stellenbosch University) (https://www.sun.ac.za/english/faculty/science/CAF) for sequencing (Chapter 4, section 4.3.8).

5.4 RESULTS AND DISCUSSION

Internationally used detection methods used to detect the presence of MAP lack sensitivity, specificity and are laborious (Park *et al*., 2014). Due to the increase in demand on the food industry to mass produce foods, emerging pathogens are also increasing (Greenstein & Collins, 2004; Chaubey *et al*., 2016). Although there is currently no recent and relevant work published on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) prevalence in South Africa, MAP is a world-wide food safety problem. Increasing evidence has shown that MAP has been detected in various food types that have been derived from infected ruminants (Eltholth *et al*., 2009; Sweeney *et al*., 2012; Robertson *et al*., 2017). Studies have demonstrated that, although the disease is intermittent in the shedding of the *Mycobacterium*, detection methods cannot detect MAP at an earlier stage. This is problematic as the correct control methods cannot be implemented at an earlier stage to prevent the further spreading of MAP, thereby reducing the effectiveness of implemented biosecurity on the farm (Wolf *et al*., 2015; Botaro *et al*., 2017).

Currently in South Africa, the enzyme-linked immunosorbent assay (ELISA), Ziehl-Neelsen (ZN) stain and histology of slaughtered animals suspected of JD, are diagnostic tests that are routinely used to determine the prevalence of MAP in a flock or herd (DAFF, 2017). Whereas internationally, different PCR methods (conventional, nested-PCR and RT-PCR), targeting various genes of MAP, are optimised and implemented to detect MAP in diverse sample matrices (Grant & Rees, 2010; Chaubey *et al*., 2016). In South Africa, when a state veterinarian is determining the JD status of a flock or herd, they are pressured into making immediate decisions. When sampling blood for an ELISA test, the body condition score (BCS) is taken into consideration. If a ruminant produces a low BCS and has a history illustrating a drastic decrease in live weight, a state veterinarian has to consider past ELISA results, if any are available. A decision then needs to be made to determine if it is necessary to slaughter a suspected infected ruminant at an abattoir and perform a histology examination for lesions on the lymph nodes and intestines (DAFF, 2017). However, sometimes if the ruminant has a low BCS, a decrease in live weight and has a history of positive ELISA results, the ruminant can produce negative results during the histology examination (S. Davey, 2018, State Veterinarian, Western Cape Agriculture Department, Malmesbury, South Africa, personal communication, May 2018). Depending on the farmer and where the animal is slaughtered, the carcass can be de-boned, and meat could be sold to either the abattoir or a distributor (Gill *et al*., 2011). It is therefore important to investigate a more sensitive and specific method that can be used to determine the status of a suspected ruminant. By doing this, the correct control method can be used on the farm to prevent spreading to the rest of the flock, which in turn increases the biosecurity of the farm and prevents the bacteria spreading into the food chain.

5.4.1 Body condition score and live weight

Research has shown that most ruminants tend to produce symptoms and signs of JD when they are older than 3 years (Fernández-Silva *et al*., 2014). In this study, most of the samples were taken from ewes that were at least 3 years of age or slightly older. As the majority of the ewes were pregnant when the ELISA, blood and faecal samples were taken, their bodies were under additional physiological stress. Stress tends to increase the prevalence of bacteria in infected ruminants, as their immune systems are weakened (Windsor, 2015). Unlike other livestock ruminants (cows or goats), which produce symptoms such as severe weight loss, diarrhoea, continuous shedding of MAP and decrease in milk production; sheep only produce chronic weight loss as a sign of clinical JD (Fernández-Silva *et al*., 2014). The mean live weight (Table 5.2) from the sampled ewes at lambing was recorded and compared with the previous year's records. Results indicated that there was an increase in the ewes mean live weight between 2017 and 2018 (Table 5.2). In terms of visual signs of ovine JD based on the live weight, there was no significant difference between 2017 and 2018 values (p=0.0747). There were, however, three ewes (4290, 4149 and 3060) that underwent a noticeable amount of weight loss (Table 5.3). Nonetheless, these results cannot be used alone to assess the JD status of a sheep. The body condition score (BCS) was also used as an indicator of the ewes condition in terms of muscle and fat ratio. The lower the BCS, the thinner the animal, which is more prominent in infected ruminants at a later stage of JD (Whittington *et al*., 2017).

Table 5.2 Comparing the average live weight's (kg) and body condition score (BCS) of pregnant ewes between 2017 and 2018

Measurement	Live Weight (kg)		BCS	
	2017	2018	2017	2018
n	21	21	21	21
Mean	62.7	70.3	3.17	3.35
Median	61.0	68.0	3.00	3.50
Maximum	87.5	88.0	4.00	4.00
Minimum	36.0	52.5	2.00	1.50
Standard deviation	15.3	11.0	0.555	0.709

n: sample size, mean: average and median: midpoint value

The mean BCS for 2017 and 2018 was calculated, and it can be seen that the average BCS between the two years was similar (3.17 in 2017 and 3.35 in 2018) (Table 5.2). There was no difference in the maximum BCS between the years, but there was a slight difference between the minimum BCS scores. Overall, there was no significant difference in the mean BCS between 2017 and 2018 (p=0.361). However, it must be noted that ewe numbers 4226, 4149, 4162, 3060 and 3217 dropped in their BCS between 2017 and 2018 (Table 5.3). Sheep number 4149 had the largest decrease in BCS and live weight from 2017 to 2018, which is one of the reasons that the head researcher decided to slaughter the ewe- however no histology was performed on the ewe (as she had been slaughtered

Table 5.3 The live weight, body condition score, PCR and ELISA results obtained from the Merino and Dohne Merino ewe samples (blood, faeces and milk)

Origin	Sheep	Breed	Gudair®	Live weight (kg)		BCS		Faeces PCR	Blood PCR	Milk PCR	ELISA	
				2017	2018	2017	2018				2017	2018
E	4289	M	V	64.0	71.0	3.0	4.0	-	+	-	+	+
E	4079	M	V	56.0	68.0	3.0	4.0	-	+	-	+	-
E	5023	M	V	42.5	52.5	2.0	2.5	-	+	-	+	+
E	5105	M	V	50.0	70.5	3.0	3.5	-	+	-	+	+
E	5137	M	V	52.0	67.5	3.0	3.5	-	+	-	+	+
E	4226	M	V	61.0	61.5	**3.5**	**2.5**	-	+	-	+	+
E	4290	M	V	**76.5**	**69.5**	4.0	4.0	-	+	-	+	+
E	4149	M	V	**57.5**	**55.5**	**3.0**	**1.5**	-	+	**S**	+	+
E	5178	M	V	46.0	59.0	2.5	3.0	-	+	-	+	+
E	4162	M		60.0	65.5	**3.5**	**3.0**	-	+	-	-	-
L	6046	M		36.0	56.0	2.5	2.5	-	+	+	+	-
L	6102	M		43.0	67.5	2.5	3.0	-	+	+	-	-
L	3203	M		56.0	63.0	3.0	3.0	-	+	-	-	-
L	4156	M		63.5	70.0	3.5	3.5	-	+	+	-	-
L	3060	M		**72.0**	**67.5**	**3.5**	**2.5**	-	+	+	-	-
L	3214	DM		78.5	84.5	3.5	4.0	-	+	**S**	-	-
L	3046	M		74.5	80.0	4.0	4.0	-	+	+	-	-
L	4143	M		75.0	86.5	3.5	4.0	-	-	-	-	-
L	4170	M		84.0	87.0	3.5	4.0	-	+	-	-	-
L	3217	M		86.5	88.0	**4.0**	**3.5**	-	+	-	-	-
L	6003	DM		53.0	85.5	3.0	4.0	-	-	-	+	-

L: Langgewens, E: Elsenburg, M: merino, DM: Dohne Merino, V: vaccinated, BCS: body condition score, PCR: polymerase chain reaction, S: slaughtered and ELISA: enzyme-linked immunosorbent assay. Highlighted blocks under 'live weight' and 'BCS' indicated a decreased value between 2017 and 2018. Highlighted blocks under 'PCR' and 'ELISA' headings indicate a positive MAP result.

for humane reasons before the experimental sampling occurred). Based on the BCS and live weight scores, it would appear that the flock is healthy. Nonetheless, the ELISA and PCR results need to be taken into consideration before making a final conclusion on the JD status of the flock.

5.4.2 PCR results from various matrices

In this study, various samples (blood, faecal and milk) were obtained from suspected infected Merino and Dohne Merino sheep at Langgewens research farm in Malmesbury. The aim of the study was to determine which matrix sample (blood, faecal or milk) would produce a positive result, and furthermore compare it to ELISA results to determine if the PCR or ELISA method would be more beneficial to use as a detection method.

Polymerase chain reaction (PCR) was conducted using the F57 primer set sequence on the various sample matrices (Coetsier *et al*., 2000). In terms of positive PCR results, the blood samples produced the highest MAP prevalence (90.5%) followed by milk (26.3%) (Table 5.3). A couple of positive PCR samples taken from non-vaccinated ewes were sequenced and the NCBI website confirmed a 100% identification to the ATCC 19698 MAP strain, which was previously isolated from a bovine (Stevenson, 2015). This demonstrates that the infection must have originated from an infected cow and was spread through contaminated faeces.

The vaccination of the ewes needed to be taken into consideration. Stevenson (2015) has emphasised that it is difficult to differentiate between a vaccinated or infected ruminant as detection methods cannot differentiate between the two. The ewes were vaccinated with the Gudair® vaccine, which is comprised of a heat-inactivated strain of MAP. Specifically analysing the ewes that were vaccinated and produced a positive PCR result, only the blood PCR produced a positive result. These ewes did not produce a positive result for the faecal or milk samples. Therefore, out of the 19 positive blood samples (90.5%), 42.9% were vaccinated ewes. This in turn means that only 47.6% (10/21) of the ewes were positive and had not been vaccinated. The positive PCR results from the vaccinated animals does however indicate that the PCR is effectively working.

The faecal samples produced no positive results (Table 5.3); this could be as a result of the numerous PCR inhibitors, such as phytic acid and polysaccharides, found in faeces (Leite *et al*., 2013; Acharya *et al.,* 2017). Inhibitors in samples reduce the accuracy and sensitivity of PCR reactions, which can lead to an under estimation of the amount of target DNA (Park *et al*., 2014). Hahn *et al*. (2017) emphasised the important aspects of good quality DNA for a PCR reaction, which requires removing DNA amplification inhibitors, sufficiently disrupting the MAP cell wall and extracting DNA from a homogenous sample. Leite *et al*. (2013) conducted a comparison of various commercial DNA extraction kits to determine which kit would sufficiently extract DNA, and remove PCR inhibitors, from spiked MAP faecal samples. Results depicted that the most suitable DNA extraction kit for faecal samples, was the ZR Faecal DNA Miniprep kit. A similar kit was used in this study, the ZymoBIOMICS™ DNA Miniprep kit, as it can be applied to both faecal and milk samples. The kit followed the same steps as the kit recommended by Leite *et al*. (2013), the only difference is

the kit can be altered to extract DNA from more than one sample matrix type. The DNA that was extracted from the various matrices, was measured using a spectrophotometer to determine the quantity and purity (Table 5.4). According to the Qiagen HotStarTaq *Plus* Master Mix protocol, a minimum of 5-10 ng genomic DNA is required for an optimal PCR reaction, but no more than 1 µg per 50 µL PCR reaction should be used (Qiagen, 2016). The results indicated that the DNA in the faecal samples contained a high purity of 1.92 and a high DNA quantity of 113.6 ng/µL. Therefore, the negative PCR results cannot be completely due to inhibitors or DNA quantity; as the DNA extraction kit had sufficiently extracted a high purity and quantity of MAP DNA. Another possible reason for the negative faecal results, could be that MAP is intermittently shed in the faeces, and the samples could have been taken during the stage where no shedding occurred (Park *et al*., 2014; Slater *et al*., 2016).

Table 5.4 The average quantity (ng/µL) and purity (Abs 260/280) of DNA extracted from the various sample matrices

Sample	Quantity (ng/µL)	Purity (Abs 260/280)
Blood	50.8 ± 25.8	1.82 ± 0.0856
Faecal	113.6 ± 50.5	1.92 ± 0.152
Milk	4.2 ± 5.40	2.01 ± 1.69

Abs: absorbance and ± indicates standard deviation

Blood samples can be used for a range of diagnostic tests, such as ZN stain, ELISA, PCR and the phage assay (Chaubey *et al*., 2016; Swift *et al*., 2016). The concentration of MAP cells, by centrifuging the samples, is higher in blood and there are fewer PCR inhibitors (Grant & Rees, 2010). In this study, the white blood cells, or the buffy coat layer, was used to detect for the presence of MAP. The DNA isolated from the buffy coat produced a purity of 1.82 and quantity of 50.8 ng/µL (Table 5.4). Most studies recommend that the buffy coat layer be used when sampling from blood, as it contains a higher microbe concentration than in the whole blood state (Gill *et al*., 2011; Swift *et al*., 2013; Swift *et al*., 2016). The prevalence of MAP in blood samples from the non-vaccinated ewes produced the highest positive PCR results at 47.6% (Table 5.3). Only ewe numbers 4143 and 6003 produced negative results. As there are fewer inhibitors in blood, a standard DNA extraction kit was used that did not require bead bashing.

Chaubey *et al*. (2017) has agreed that MAP is a troublesome bacterium to detect due to its slow growing characteristics, waxy cell wall and clumping nature. Fernández-Silva *et al*. (2014) expressed concern that ovine JD is a serious threat to sheep production, as the symptoms remain hidden and tend to show indirect production effects. *Mycobacterium avium* subsp. *paratuberculosis* has also been detected in milk from infected ruminants, which has increased the interest of MAP as it has been linked as a possible cause to Crohn's disease (CD) in humans (Park *et al*., 2014). The milk samples that were drawn from the suspected ewes produced a low prevalence of positive PCR results (26.3%) (Table 5.3). The DNA extraction was performed on the milk pellet, as it has been

proven to contain a higher microbial population than in the whole milk (Gerrard *et al*., 2018). Proteinase K enzyme was used, in addition with the ZymoBIOMICS™ DNA Miniprep kit, to extract the DNA and decrease any inhibitors from the milk pellet. The DNA extracted from the milk pellet produced the highest purity at 2.01 but the lowest quantity 4.2 ng/µL. Milk is known to contain a few PCR inhibitors such as proteins, fats and calcium ion (Schrader *et al*., 2012). However, Tasara and Stephan (2005) stated that the level of direct MAP shedding is higher in faeces than in milk. This statement contradicts the results that were found in this study (Table 5.3), as the milk samples produced a higher MAP prevalence than the faecal samples in the PCR results. However, the faecal and milk samples were taken at different times, which could explain the differences in results. Newly born lambs are prone to infection as their immune systems are weak (Gill *et al*., 2011). Colostrum, from JD infected ruminants, has been tested to contain MAP as it produced a positive ELISA and PCR result (Elolth *et al*., 2009). Therefore, it would be beneficial to separate the lambs from infected ewes to prevent the further spread of Johne's. Although calf milk replacers (CMR) have been suggested as an alternative to prevent infection in lambs, real time PCR has detected the presence of MAP in powdered CMR (Grant *et al*., 2017). Therefore, constant surveillance of a flock needs to be implemented to ensure that the further spread of the infection from ewes to lambs does not occur (Whittington *et al*., 2017).

5.4.3 ELISA results from blood samples

The ELISA test was applied to blood collected in different tubes to that where upon DNA extraction was performed for the PCR test. Results between 2017 and 2018 were recorded (Table 5.3). Ewe number's 4289, 5105, 5023, 5137, 4226, 4290, 4149 and 5178 produced positive ELISA results in 2017 and 2018. It is interesting to note that all these ewes came from Elsenburg and have been vaccinated against JD. A decrease was noted between 2017 (52.4%) and 2018 (38.1%) in positive ELISA results. Three ewes (6046, 4079, and 6003) had produced a positive ELISA result in 2017 but a negative result in 2018. These results demonstrate the concern of using the ELISA test as a detection method, as results can vary between sampling times. The ELISA test is used to detect the presence of a ruminants' serological response to MAP, in other words the test is dependent on the presence of antibodies used to fight the MAP infection (Gill *et al*., 2011; Park & Yoo., 2016). Variation is mainly due to the ruminants' intermittent immune response to MAP infection (Leite *et al*., 2013). Botaro *et al*. (2017) conducted a study to compare ELISA milk results on various test days over a period of two years in cows. There was a significant difference in test day milk product records between the cows and this also demonstrated the intermittent immune response to MAP. It has been recommended that a post mortem examination as well as culturing of tissue samples be used in conjunction with ELISA, to make a complete diagnosis (de Silva *et al*., 2018). However, these measures could result in a negative diagnosis and culturing takes up to 16 weeks to produce a culture, if no contamination has occurred (Chaubey *et al*., 2016). Faecal culture and ELISA have been recommended to be used together when determining the JD status of a ruminant (de Silva *et*

al., 2018). It must be noted that the ELISA test is used as a flock or herd diagnostic test, while PCR can be used to determine the JD status of an individual ruminant or the flock (Park & Yoo., 2016; Whittington *et al*., 2017).

5.4.4 Overall analysis of diagnostic tests

Evaluating the results obtained from the various diagnostic tests and matrices as a whole (Table 5.3), a further analysis can be made on the JD status of the individual ruminants. According to Acharya *et al*. (2017), there is a poor correlation between a ruminants' immunological response to ELISA and faecal shedding of MAP. This can also be seen in the results obtained for this study (Table 5.3), as there were no positive faecal results, while there were positive ELISA results. As some of the ewes were vaccinated against JD using Gudair®, 42.9% produced a positive PCR result from blood that was taken from vaccinated animals and 47.6% from non-vaccinated ewes (Table 5.3). However, the positive blood PCR results from the non-vaccinated ewes did not agree with the ELISA results, as only ewe numbers 6046 and 6003 produced positive PCR and ELISA results while the rest of the non-vaccinated ewes produced negative ELISA results. These results further demonstrate the unreliability of the ELISA test and that PCR is a more sensitive method. Stevenson (2015) has concluded that a disadvantage of vaccination, is that diagnostic tests will not be able to differentiate between an infected and a vaccinated ruminant. This study agrees with this statement, based on the positive ELISA results between 2017 and 2018 of ewes 4289, 5105, 5023, 5137, 4226, 4290, 4149 and 5178. However, the same cannot be said about ewe 4079, as the ELISA produced a positive result in 2017 and a false-negative result in 2018. This ewe had produced a positive PCR result for the blood sample but had an increase in BCS from 2017 to 2018. It would also seem that there was a false-positive ELISA result produced for ewe 6003 in 2017, as all the samples came out negative for PCR and the 2018 ELISA was also negative. Ewe 6003 also had a good BCS of 3.0 in 2017, which had increased in 2018 to 4.0, and a good live weight between the years. Ewe numbers 6046, 6102, 4156, 3060 and 3046 all produced PCR positive results for the milk samples. All ewe numbers mentioned had produced an average BCS of 2.5 in 2017, which had increased slightly to 3.0 in 2018. These ewes had also had an increase in their live weight between 2017 and 2018. None of the vaccinated ewes produced positive PCR results from the milk or faecal samples. Which demonstrates that the vaccine has improved the ruminants' immunity towards MAP. Although this statement is contradicted by ewe numbers 4226, 4290 and 4149, as they had a decrease in either BCS or live weight and has produced a positive ELISA and blood PCR result. These results demonstrate the difficulty in determining the true JD status of a vaccinated animal. Overall, according to the blood and milk PCR results (Table 5.3), the tested group would be classified as Johne's disease positive as 47.6% (10/21) of the ewes tested positive for MAP from blood samples, 26.3% (5/19) had MAP positive milk samples and 38.1% were positive on the ELISA in 2018 (8/21).

5.5 RECOMMENDATIONS

Only 21 sheep were sampled from the flock to evaluate the detection tests and sample matrices. An increase in sample size would demonstrate a clearer picture in regard to the definitive sample matrix and diagnostic test that should be used. There was no pre-treatment used on the faecal samples before DNA extraction. Acharya *et al*. (2017) has recommended that although inhibitors in faecal samples are unavoidable, due to the complexity of the matrix, a dilution treatment before DNA extraction would reduce the inhibitors in the sample; this pre-treatment could be evaluated in future studies. Although a low prevalence of MAP DNA extracted from the milk pellet was detected; it is recommended to test the various milk layers (cream, liquid and pellet) to determine which could produce the highest MAP prevalence. There was a variation between the blood, milk PCR and ELISA results; therefore, ELISA results should have been taken at both pregnancy and post lambing stages to determine if there was a difference in the immune response to MAP before and after lambing. Another interesting angle for future projects, would be to take blood, faecal and ELISA results from the lambs and compare them to their mothers' JD status.

5.6 CONCLUSIONS

The aim of this research chapter was to determine which sample matrix would produce a positive MAP result and to compare PCR test results with that from the ELISA test, so as to determine which diagnostic method/test should be recommended for flock diagnosis. It is rather difficult to conduct a Johne's disease diagnosis on ruminants, as there are many factors that need to be taken into consideration, such as stage of infection when samples were taken, vaccination status and the sensitivity of the diagnostic test. The study demonstrated that the PCR method is more sensitive and specific than the ELISA method, as PCR detected a higher positive prevalence of MAP in the non-vaccinated ewes blood samples than the ELISA test. It was shown that the BCS and live weight changes should also be taken into consideration when making a final decision. This study also revealed that each sample matrix has its own factors to consider, such as PCR inhibitors and pre-treatment requirements prior to DNA extraction. None the less, these results would seem to indicate that the blood is the best sample matrix to use when determining the JD status of a flock or individual ruminant.

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CHAPTER 6
General Discussion and Conclusion

Food safety plays a critical role in the food industry by implementing processing protocols that are used to reduce or eliminate pathogenic food-borne microorganisms to a level that is safe for consumers. However, with the rise in consumer population, there is an increase in emerging pathogens (Jones *et al*., 2013; van Doorn, 2014). In order to improve food safety control regulations, it is imperative to understand the type of microorganisms that are emerging from various food matrices. It is also important to investigate the possible transmission routes of these microorganism along the food production process, from sampling of raw materials to retail products (Vouga & Greub, 2016).

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of Johne's disease (JD), a chronic gastro-enteritis found in ruminants such as cattle, sheep and goats. The bacterium is intermittently shed into faeces and milk through the various stages of the disease, even though JD symptoms vary amongst ruminants (Gill *et al*., 2011; Chaubey *et al*., 2016). It has been proven that MAP has the ability to survive physical or chemical sterilising processes, such as pasteurisation and chlorination, due the bacterium's thick waxy wall comprised of 60% lipids (Rowe & Grant, 2006; Foddai *et al*., 2009; Salgado *et al*., 2015; Gerrard *et al*., 2018). Subsequently the zoonotic potential of MAP has been debated as the possible cause of Crohn's disease (CD), a human infection similar to JD (Robertson *et al*., 2017). This is mainly because MAP has been detected in CD patients as well as in pasteurised milk, soft cheeses and animal liver products (Gill *et al*., 2011; Waddell *et al*., 2016; Smith *et al*., 2017). In order to improve control programs (used on farms to prevent the further spread of MAP) and improve food safety measures, it is essential to improve detection methods for early stage detection (DAFF, 2017). This is currently being achieved globally, except in developing countries such as South Africa. In August 2017, the Department of Agriculture, Forestry and Fisheries (DAFF) released a statement that expressed their concerns in that currently used diagnostic testing methods, such as the Ziehl-Neelsen (ZN) stain, enzyme-linked immunosorbent assay (ELISA) and culturing, are contributing to the challenge of controlling JD (Michel & Bastianello, 2000; DAFF, 2017). The ZN stain only confirms if an acid-fast bacterium is present but cannot specify the specific microorganism (Chaubey *et al*., 2016). The ELISA is dependent on the serological properties of an animal, and with the intermittent shedding and low prevalence of MAP during the early stage, the test can produce false-negative results. Lastly, culturing can take up to 16 weeks to produce a single colony if no contamination has occurred. Ultimately, these detection methods are unreliable and not sensitive when identifying infected ruminants that are not demonstrating symptoms (DAFF, 2017). This statement typically correlates to ovine JD, as sheep only produce signs at the clinical stage of JD, and by the time detection methods produce a positive confirmation the disease has spread to the rest of the flock and control measures are ineffective. Therefore, it is

essential that diagnostic methods are improved in South Africa to determine the true prevalence of MAP in various ruminants so as to improve on control methods and in turn improve food safety measures.

The overall aim of this study was to detect the presence of MAP in sheep in the Western Cape. In order to achieve this, various objectives had to be completed. The first objective (chapter 3) was to optimise the D29 phage assay, which is a virus that specifically detects viable *Mycobacterium* cells in blood, milk, and sputum samples (Foddai *et al*., 2009; Grant & Rees, 2010; Botsaris *et al*., 2013; Swift *et al*., 2016). However, the assay could not correctly be optimised as there were many factors that hindered the phage from effectively working. Phage have been known to be very sensitive to concentration and temperature (Grant & Rees, 2010) and this proved to be the main contributing problem during optimisation of the assay. Various trials were conducted to optimise the phage method. However, due to time constraints and difficulty in sourcing reagents and consumables from local suppliers, the phage assay could not be optimised nor validated. It was also decided that the polymerase chain reaction (PCR) method required further investigation, as currently in South Africa the ELISA, culturing and ZN stain are the only methods routinely used by state veterinarians to determine the status of a flock.

This led to the second objective in this research study; to optimise a PCR method to detect MAP (chapter 4). Three sheep blood samples were received and confirmed as positive by a veterinarian using the ELISA method. Before DNA extraction was performed, the blood was centrifuged into different physiological states, whole blood and the white blood cells (buffy coat layer) (Swift *et al*., 2016). Thereafter, two commercial DNA extraction kits were evaluated. The DNA that was extracted from the white buffy coat layer using the *Quick*-DNA™ Miniprep Plus produced the purest DNA. The genetics of MAP is rather complicated as there are different MAP strains, S and C, which tend to be species specific (Whittington & Sergeant, 2001). In other words, it is more common for a strain S to be found in ovine samples and strain C from bovine. However, research has shown that ovine are susceptible to S and C strains while cattle are less susceptible to the S strain (Moloney & Whittington, 2008). This demonstrates that cross-species transmission of strains exists due to a host preference (EFSA Panel on Animal Health and Welfare (AHAW) *et al*., 2017). As there has been no published work on which primers have been evaluated in South Africa, it was essential to trial primer sets that are commonly used internationally. The different MAP specific PCR primer set sequences were tested and the only positive sequence, was that of the F57 primer (Coetsier *et al*., 2000). The F57 sequence is a single copy identical to the MAP genome with a small target region of 620 bp, compared to the 1 456 bp IS900 sequence that has 14-20 copies identical to the MAP genome (Vansnick *et al*., 2004). It would be beneficial to utilise various F57 target regions and determine which would produce a positive result. Sequencing and blasting the positive PCR products depicted that strain ATCC 19689 was present, which happened to be originally isolated from a bovine (Stevenson, 2015). The F57 primer set, PCR reaction and MAP positive DNA was used for the next research objective.

According to the South African Animal Diseases Regulations (Animal Disease Act, No 35 of 1984), it is the responsibility of a farm manager or owner to prevent further spreading of a disease by implementing control steps if they suspect their animal(s) are infected with a disease. It is therefore the manager or owner's responsibility to report their suspicion to a state veterinarian, whom is then required to test the suspected animal(s). In terms of JD, a state veterinarian uses ELISA, ZN stain and culturing to determine the status of the animal(s) (DAFF, 2017). Other measurements, such as body condition score (BCS) and live weight, are also taken into consideration before making a decision (Windsor, 2015; Gautam *et al*., 2018). However, a state veterinarian is usually required to make an immediate decision on the status of an animal and all the currently used diagnostic methods take time to produce results. Subsequently, the suspected animal is usually slaughtered for a histology examination, where the intestine and lymph nodes are examined for swelling and lesions (DAFF, 2017). Occasionally, the state veterinarian will only use the ELISA method to make a decision. This can be problematic as sometimes a positive ELISA result is produced; the animal is slaughtered, and the histology examination reports no lesions or swelling (Husakova *et al*., 2017). This demonstrates the unreliability of the ELISA detection method and emphasises the need to improve detection methods to not only determine the true prevalence of MAP, but to also aid state veterinarians in their decision making.

Therefore, the third objective (chapter 5) was to test various sheep sample matrices (blood, faecal or milk) using the optimised F57 PCR, and determine which sample matrix would produce a positive result. The PCR results were also compared with ELISA results, to determine which diagnostic method should be recommended for JD diagnosis. It was rather difficult to conduct a Johne's disease diagnosis on ruminants, as there were many factors that need to be taken into consideration, such as the body condition score (BCS), live weight, infection stage when samples are taken and the vaccination status of suspected ruminant. Only 21 sheep were sampled from the flock to evaluate the detection tests and sample matrices. An increase in sample size would have demonstrated a clearer picture in regard to the definitive sample matrix and diagnostic test that should be used. Samples were specifically taken from pregnant ewes, as the ewe was under more stress. This was beneficial to the study as the possibility of MAP shedding was increased. Some of the ewes were vaccinated, which was taken into consideration when discussing the JD status of the ewes. The study demonstrated a difference in positive results between the diagnostic methods. Specifically looking at the non-vaccinated ewes, 47.6% produced a positive PCR blood result but only two of these ewes produced a positive ELISA result. Therefore, the PCR method was more sensitive and specific than the ELISA method. This could be as a result that PCR is not dependent on the immune response of a ruminant. The study also revealed that each sample matrix has its own factors to consider, such as PCR inhibitors and pre-treatment requirements prior to DNA extraction. No pre-treatment was used on the faecal samples before DNA extraction, which could explain why no positive results were detected. Acharya *et al*. (2017) has recommended that a dilution treatment before DNA extraction would reduce the inhibitors in the faecal sample. A low

prevalence of DNA was extracted from the milk pellet, therefore a future trial on the various milk layer's (cream, liquid and pellet) to determine which would produce the highest MAP prevalence would be beneficial in optimising a milk PCR detection (Chamberlain *et al*., 2001; Gerrard *et al*., 2018). Overall results indicated that the blood sample was the best sample matrix to use when determining the JD status of a flock or individual ruminant. As samples were taken from the ewes while they were pregnant, it would be interesting to take blood, faecal and ELISA results from the lambs and compare them to their mothers' JD status.

Currently, the South African Animal Diseases Act, 1984 (Act No 35 of 1984), has Johne's disease as a controlled disease. However, DAFF has proposed a draft, with the help from industry, to prevent the further spread of JD and have requested to change the status of JD in the Animals Disease Act from controlled to notifiable. But the draft also emphasised that results from improved diagnostic methods must first be obtained to demonstrate the true prevalence of the JD situation in South Africa before regulation can happen (DAFF, 2017). This study has demonstrated that the PCR method is a reliable and rapid tool that can be used to screen various sample matrices to determine the presence of a pathogenic microorganism. It would benefit the state veterinarians to incorporate the PCR as part of their JD testing routine, as it can help make a definitive answer about the status of a suspected individual or flock. Although a conventional PCR cannot differentiate between viable and dead cells, a quantitative or real-time PCR method can. It is recommended to evaluate various MAP specific primers and probes in South Africa. This study demonstrated that the F57 primer set was successfully used to detect the presence of MAP in a sheep flock and that various sample matrices can be used. It also demonstrated to be more reliable than the ELISA test but the BCS and live weight should also be taken into consideration. Although the phage assay could not successfully be optimised and validated, it is recommended to continue investigating possible variations to get the assay working. The phage assay combined with the optimised PCR detection method can contribute in assisting state veterinarians in determining the true scale of JD in South Africa, as the combined diagnostic method can detect MAP during the early stage of JD and produce a result in 48 h. This can ultimately help improve control methods as they can be implemented earlier to not only prevent the further spread, but also improve the biosecurity of the farm and in turn preventing the bacterium entering the food chain and improving the food safety aspect.

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Addendum A

To whom it may concern

Date: 14/3/18

I, Dr Sewellyn Davey (Malmesbury State Veterinarian), give consent that S. Kotzé (Veterinarian's full name) could take blood samples on my behalf at Nuwerus (location of farm). The following additional information was also provided:

- Name of farmer: André Kirsten
- Name of farm: Kirsten boerdery (Specifically Nuwerus.)
- Number of samples: 3x
- Animal species (e.g. merino): Dohne Merino.
- Date of sampling: 27/2/18
- Test used to confirm that samples were MAP positive: ELISA

Sampling procedure was that follows standard protocol procedure and was correctly transported and stored- following the DAFF section 20 application form of Shannon Howell (student at Stellenbosch University) and Dr Maricel Krügel (Supervisor of Shannon Howell).

REPUBLIC OF SOUTH AFRIC.
DEPARTMENT OF AGRICULTURE
OFFICIAL VETERINARIAN
15 MAR 2018
DR SC DAVEY Bsc B.V.Sc.
REG NO: 80/1486
WESTERN CAPE PROVINCE

Dr Sewellyn Davey
State Veterinarian Malmesbury

Date signed 15 March 2018

S. Kotzé

Veterinarian
Name: S. Kotzé
Reg number: 014/1113

Date signed 14/3/18

Shannon Howell
Student at Stellenbosch University

Date signed 15/03/2018

M Krügel

Dr Maricel Krügel
(Supervisor of Shannon Howell
at Stellenbosch University)

Date signed 15/03/2018