Longitudinal monitoring of *Mycobacterium bovis* and non-tuberculous mycobacteria in cow's milk from a rural community

By

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Declaration

I declare that I am the sole author of this thesis and that it has not previously been submitted as an exercise for a degree at the University of Pretoria, or any other university.

_____ _____

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Table of Contents

Declarations……..2

Acknowledgements……..3

List of figures and tables…….7

Abbreviations…….9

Thesis summary……..11

Chapter 1: General information……13

Introduction……13

Problem statement……15

Justification……15

Objectives……16

Chapter 2: Literature review……..17

Genus *Mycobacterium*……..17

Tuberculosis in humans……18

Nontuberculous mycobacteria in animals and humans……...20

Bovine tuberculosis……21

Epidemiology of mycobacteria……25

Diagnosis of mycobacterial infection……..27

Control and treatment of bovine tuberculosis……33

Chapter 3: Materials and methods……...35

Bacterial suspensions……...36

Spiking of milk preparation……...36

Decontamination of spiked milk……...37

Culture of spiked milk samples……39

Preparing a smear from LJ slopes with visible isolates……...39

Collection of milk samples from cattle infected with *M. bovis*…….40

Study area……..40

Introduction of study to stakeholders……41

Selected dip tanks……41

Comparative intradermal tuberculin test……...41

Milk collection……43

Laboratory analyses……44

Decontamination with hydrochloric acid……44

Inoculation on LJ pyruvate slopes and monitoring…….....44

Polymerase chain reaction (PCR) for detection of

Mycobacteria spp……45

Limit of detection of *M. bovis* using conventional

PCR (cPCR)…….....46

Phenotypic characterisation of nontuberculous mycobacteria……..47

Animal ethics and consent forms……47

Chapter 4: Results…….....48

Comparison of decontamination methods……48

Limit of detection for *M. fortuitum* in pasteurised milk……48

Efficacy of decontamination methods on contaminated milk……50

Monitoring of *M. bovis* and NTM from cow's milk……51

Study area and study animals……51

Limit of detection for *Mycobacterium bovis* on cPCR ……...52

Collection of milk samples……..53

Mycobacterium species culture on LJ pyruvate slopes…….....53

Mycobacterium species identification (cPCR)…….. ..53

Culture of milk samples using MGIT pyrazinamide……57

NTM identification……57

Chapter 5: Discussion……59

Comparison of decontamination methods for isolation

of *Mycobacterium fortuitum*……..59

Detection of *Mycobacterium bovis* and non-tuberculous mycobacteria……61

Limitations of the study……65

Conclusion……..66

References……..67

Appendices……..80

List of figures and tables

Figure 2.1. Transmission cycle of *Mycobacterium tuberculosis*……19

Figure 2.2. A. Bovine lung infected with *M.bovis* B. Dissected…….. 23
lymph node from an infected cow.

Figure 2.3. Measurement of the skin thickness for diagnosis of……32
bovine TB

Figure 3.1: Preparation of spiked milk suspension……..35

Figure 3.2: Serial dilutions of bacteria *(M. fortuitum*, *E. coli* and *S. aureus*)……36

Figure 3.3: Study area: Northern KZN, uMkhanyakude district, Big 5 False Bay……40

Figure 3.4: A. cattle being packed in a crush pen at a dip tank in Hluhluwe, KZN…..41
B. Image of cattle tied during preparation of performing the comparative
intra-dermal tuberculin test.

Figure 3. 5 Sampling time frame indicating the days on which……..43
milk collection was performed.

Figure 4.1 Limit of detection for *M. fortuitum* using 2% hydrochloric…….49
acid and 5% oxalic acid

Figure 4.2 Limit of detection for *M. fortuitum* using 4% sodium……..49
hydroxide, Novel hypertonic-saline hydroxide and cetylpyridinium chloride

Figure 4.3 Distribution of bovine TB positive cattle among four dip tanks…….51

Figure 4.4 Gel image showing *M. bovis* limit of detection on cPCR with……..53
a band size of 268bp. The dilution factor ranged
from 10^0 CFU/ml to 10^6CFU/ml.

Figure 4.5 Phylogenetic relationship amongst species of mycobacteria…….58
identified in raw cattle milk

Table 2.1: Modified mycobacteria that are pathogenic for animals and man……..…17

Table 2.2. Mycobacterium identification by PCR ……..30

Table 4.1 Efficacy of decontamination methods on milk contaminated…..……...50

with *E. coli* and *S. aureus*

Table 4.2 Results of Comparative Intradermal Tuberculin testing (CITT)..……...51

of cattle at four dip tanks

Table 4.3 Summary of detection limit of *M. bovis* cells on conventional PCR…..……52

Table 4.4 Summary table of results of milk samples collected and tested ……..……..56

by cPCR and culture

Table 4.5 Summary of NTM and *M. bovis* growth on LJ pyruvate and.....……57

MGIT Pyrazinamide

Appendices

Table 3.1 Preparation of 5% oxalic acid (OA)……...80

Table 3.2 Preparation of Novel hypertonic-saline hydroxide (HS-SH)……..…80

Table 3.3 Preparation of 1% cetylpyridinium Chloride (CPC).....80

Table 3.4 Preparation of 2% hydrochloric acid (HCl)……80

Table 3.5 Preparation of 4% NaOH (sodium hydroxide).....…...80

University of Pretoria animal ethics……81

Department of Agriculture, Forestry and Fisheries approval……..82

Farmers consent form……...84

Raw data……86

Abbreviations

AFB	Acid-Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
Bovine TB	Bovine Tuberculosis
CFTT	Caudal Fold Tuberculin Test
CPC	Cetylpyridinium chloride
cPCR	Conventional Polymerase Chain Reaction
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPC	Hexadecylpyridinium Chloride Monohydrate
HS-SH	Hypertonic-saline hydroxide
IFN-y	Interferon gamma
KNP	Kruger National Park
KZN	KwaZulu Natal
LJ	Löwenstein–Jensen
MAC	*Mycobacterium avium complex*
M. bovis	*Mycobacterium bovis*
M.TB	*Mycobacterium tuberculosis*
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacterium Growth Indicator Tube
MRI	Magnetic Resonance Imaging
MTBC	*Mycobacterium tuberculosis complex*
NaOH	Sodium hydroxide
OA	Oxalic acid

OIE	World Organisation for Animal Health
PCR	Polymerase Chain Reaction
SCITT	Single Comparative Intradermal Tuberculin Test
TB	Tuberculosis
WAHID	World Animal Health Information Database
WHO	World Health Organisation

Thesis summary

Title: Longitudinal monitoring of *Mycobacterium bovis* and non-tuberculous mycobacteria in cow's milk from a rural community.

By: Koketso D Mazwi

Promoter: Anita L Michel

Degree MSc (Veterinary Science Tropical Diseases)

Bovine tuberculosis (Bovine TB) is a chronic respiratory disease of cattle caused by *Mycobacterium bovis* (*M. bovis*) which also affects other domestic animals, wildlife and humans (zoonotic TB). Raw milk from bovine TB infected animals can serve as a vehicle for the transmission of *M. bovis* to humans and the disease is therefore a potential threat to human health and livelihoods.

The purpose of the study was to 1. Compare the efficacy of decontamination methods and sensitivity of *Mycobacteria* spp. for the primary isolation of *Mycobacterium* spp. from milk using *M. fortuitum* as a model. 2. To investigate the frequency of *M. bovis* shedding in milk from bovine TB infected cows at regular intervals.

For the first objective, a known concentration (10^9 cells/mL) of *M. fortuitum*, *E. coli* and *S. aureus* were inoculated into pasteurised milk. *E. coli* and *S. aureus* were included as contaminants in the experimental samples as these organisms have previously been detected in milk from communal cattle. The spiked milk aliquots were decontaminated with one of the following methods: 1. 5% Oxalic acid (OA) for 15 min 2. 7% NaCl and 4% NaOH (5 mL each) for 15-20 min and 5 mL phosphate buffer 3. 1% CPC for 5 hours 4. 2% HCl for 10 min and distilled sterile water 5. 4% NaOH for 10 min and washed with distilled sterile water. The frequency of *M. bovis* and NTM shed in milk was investigated by collecting milk samples from skin test positive cattle between March 2017 to March 2018. A total of 41 TB skin test positive cattle were identified. The collection of milk was done 10 times at different time intervals with a total of 131 milk samples targeting preferentially but not exclusively the same cows.

This study showed that *M. fortuitum* survived and grew confluent on culture, incubated at 37°C for 2 months when 2% HCl and 5% OA is used for decontamination and inoculated on LJ pyruvate resulting in a method of isolating the mycobacteria. The frequency of *M. bovis* shed in milk could not be determined. This is because samples collected in 2017 were found positive

for NTM on culture and cPCR, and only 2 samples were positive for *M. bovis* on cPCR. Samples collected in March 2018 were positive for *M. bovis* on cPCR yet negative on culture. 14/121 milk DNA samples were positive for *M. bovis* whereby 12 were collected in 2018. Two slopes were contaminated on culture hence 77/119 slopes were positive for NTM. Isolates were sequenced and resulted in mycobacteria closely related to *Mycobacterium brasiliensis*, *Mycobacterium* sp. py137, *Mycobacterium* sp. strain EPG1. It is speculated that due to poor nutrition, which resulted in reduced milk production caused by drought experienced in the study area, the concentration of *M. bovis* within the samples was very low to be detected using cPCR. In addition, NTM as a fast growing organism may have inhibited growth of *M. bovis* on culture.

CHAPTER 1

INTRODUCTION

1.1 Background information

Mycobacterium bovis

Mycobacterium bovis and nontuberculous mycobacteria (NTM) all belong to the Mycobacteriaceae family. *M. bovis* is the infectious cause of bovine tuberculosis (Bovine TB) (Grange et al., 1996). Bovine TB is a world-wide chronic infectious disease which affects a range of mammalian hosts such as: humans, domestic and wild animals (Delahay et al., 2002). *M. bovis* is mostly transmitted from cattle to cattle and cattle to wildlife or vice versa by inhalation, saliva, urine or droppings, discharge lesions and most importantly from lactating cows to calves (DAFF, 2012) (https://www.nda.agric.za > docs > infopaks). The infection within the animal may disseminate in the body further progressing to a chronic infection which may result in animals becoming thin, with a dull hair coat and poor appetite, further presenting with severe lung lesions, moist cough and difficult breathing (DAFF, 2012) (https://www.nda.agric.za > docs > infopaks). Drought in most areas results in malnourished animals which increases the possibility of infection in these animals (Griffin et al., 1996).

The possibility of shedding the mycobacteria and the concentration of bacteria shed by a single animal relies on the infectious dose, site and stage of infection (Phillips et al., 2003). Discharge of *M. bovis* in faeces, urine or milk probably happens in instances of generalised or progressive TB, ordinarily described by disseminated infection and lesions mostly observed in the liver, kidneys and udder, or in the meninges (Neill et al., 1992).

In South Africa, bovine TB affects domestic cattle which was first reported in 1880 by Hutcheon. *M. bovis* was first reported in the 1920s in African free-ranging wildlife (Paine and Martinaglia, 1929) and years later in the 1990s, it was found in African buffalo (*Syncerus caffer*) in the Kruger National Park (KNP), South Africa (Kriek et al., 1996).

The rate of infection in both humans and livestock in developing countries is increasing annually, this is due to the lack of ability to control bovine TB (Cosivi et al., 1998a). The inability to control bovine TB in developing countries is mainly due to insufficient funding to enforce the test and slaughter scheme (Grange, 2001, Michel et al., 2008). The transmission of *M. bovis* from cattle to humans occurs through eating or drinking contaminated unpasteurised dairy products. It is therefore important to ensure the food safety of milk as a significant source of protein and other nutrients, since contamination with *M. bovis* may lead to high rates of infections from animals to humans (Sweeney et al., 1992).

Nontuberculous mycobacteria (NTM)

An emerging number of NTM has recently been identified which poses harm to human-kind. NTM include both opportunistic pathogens such as *M. fortuitum*, *M. chelonae, M. abscessus* group and *M. smegmatis*, as well as non-pathogenic pathogens such as *M. brasiliensis* and *M. gilvum* (Khaledi et al., 2017). NTM were neglected mycobacteria and regarded as insignificant organisms, however, recently more than 160 NTM species have been isolated and characterised from different samples. At most one-third of the mycobacteria have been associated with diseases infecting humans (Liu et al., 2016).

The frequency of pulmonary infections caused by NTM is reported to be increasing in Europe, North America and Southern Africa (Aliyu et al., 2013). Pulmonary infections are most commonly due to *Mycobacterium avium complex* (MAC), *Mycobacterium kansasii* and *Mycobacterium abscessus* (Johnson and Odell, 2014). NTM have recently been reported to cause skin and soft tissue infection (Atkins and Gottlieb, 2014). This is caused by *Mycobacterium ulcerans*, a slow growing mycobacteria which mainly affects the skin and subcutaneous tissues resulting in non-ulcerated and ulcerated lesions.

Genitourinary infections caused by NTM are infrequently reported. Infection in humans is due to MAC, *M. fortuitum* and *M. abscessus* (Huang et al., 2010). NTM can also lead to infection of the sinuses, lymph nodes, joints and central nervous system (Huang et al., 2010). Environmental mycobacteria are found in the environment, soil and water. Though associated with other mycobacteria that can cause infection, NTM do not cause TB or leprosy (Huang et al., 2010).

1.2 Problem statement

Mycobacteria can be transmitted from cattle to humans via milk but the actual risk to human health is not known. Consumption of unpasteurised milk increases the risk of transmission from cows to humans. The frequency of *M. bovis* and NTM shedding in milk as well as the most important species of NTM found in milk is unknown.

To assess the impact of zoonotic *M. bovis* on human health, it is essential to know the frequency of *M. bovis* and NTM shedding in milk from naturally infected cows. This information is crucial for selecting appropriate measures to control the spread of infection from cows to humans.

1.3 Justification

Mycobacterium genus consists of *Mycobacterium leprae*, NTM and *Mycobacterium tuberculosis* complex (MTBC). Bovine TB caused by *M. bovis* remains a threat to animals and humans in many countries, more particularly in developing countries due to lack of *M. bovis* control in animals. Increased numbers of NTM are isolated from the environment, animal and human samples. Environmental mycobacteria are developing pathogens resulting in opportunistic infections in humans and animals. The health effects of human-mycobacterial interactions are unpredictable and likely considerably more extensive than presently perceived. The spread of infection from infected animals to humans (zoonotic TB) is primarily through consumption of contaminated milk and inhalation of the bacteria; hence it poses an occupational risk for farmers, hunters, butchers and even the community itself.

In Africa, 85% of cattle and 82% of the human population live in rural areas where bovine TB has been diagnosed (Cosivi et al., 1998a). *M. bovis* cases in humans are believed to be mainly due to consumption of unpasteurised milk. However, it has remained unknown how frequently *M. bovis* is shed in milk of infected cows in a communal farming system where humans are most likely to be exposed to milk-borne pathogens. This information is crucially important when assessing the risk and impact of zoonotic TB exposure in humans.

1.4 Objectives

1.4.1 Aim

The aim of the study was to monitor the frequency at which *M. bovis* and NTM are shed by infected cows.

1.4.2 Specific objectives

- Identify an optimised method for isolation of *Mycobacterium* spp. from milk in communal cows with a high level of bacterial contamination.
- To monitor the frequency of NTM and *M. bovis* shedding in milk from 100 bovine TB skin test positive cows.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus Mycobacterium

Mycobacteria are slow growing and acid-fast organisms. Due to their unique cell wall, the bacteria resist decolourisation with acid-alcohol. Mycobacteria consist of MTBC, *Mycobacterium leprae* and NTM. MTBC evolved from the Horn of Africa (Sweeney et al., 1992, Blouin Y, 2012). MTBC consists of species such as *M. tuberculosis, M. africanum*, *M. canettii*, *M. bovis*, *M. microti*, *M. caprae*, *M. pinnipedii*, *M. mungi*, *M. orygis*, *M. suricattii*, *Dassie bacillus* and *M. bovis* BCG (Ingen et al., 2012). In the previous years, human TB was exclusively known to be caused by *M. tuberculosis*. An emerging number of other *Mycobacterium* species are recently identified which also poses harm to human-kind. *M. leprae* also known as Hansen's bacillus is commonly found in warm tropical countries and causes leprosy in humans. NTMs are a heterogeneous group of mycobacterium pathogens which inhabits the soil and water also known as the environmental Mycobacteria (Griffith et al., 2007b). Currently, more than 160 NTM species have been identified although most are not life-threatening to humans with healthy immune systems while other species await classification (Euzeby, 2013). Unlike gram staining organisms, mycobacteria has increased concentration of lipids in their cell wall associated with several properties. These properties include the ability to resist staining, resistance to several antibiotics, ability to survive treatment with acidic and alkaline compounds (Leslie, 2004).

Table 2.1: Mycobacteria that are pathogenic for animals and man. Modified from (Quinn et al., 2002).

***Mycobacterium* species**	**Main hosts**	**Species occasionally infected**	**Disease**
M. tuberculosis	Man, captive primates	Dogs, cattle, psittacine birds, canaries	Tuberculosis (worldwide)
M. bovis	Cattle	Deer, badgers, man, other mammalian species	Tuberculosis

M. africanum	Man		Tuberculosis (regions in Africa)
M. avium complex	Most avian species except psittacines	Pigs, cattle, humans	Tuberculosis
M. marinum	Fish	Man, aquatic mammals, amphibians	Tuberculosis
M. leprae	Man	Armadillos, chimpanzees	Leprosy
M. avium ssp. paratuberculosis	Cattle, sheep, goats, deer	Other ruminants	Paratuberculosis (Johne's disease)

2.2 Tuberculosis in humans

M. tuberculosis is an obligate (Martínez A, 1999), infective bacterial species in the family *Mycobacteriaceae* and a causative agent of TB in humans (Cudahy and Shenoi, 2016). The tubercle bacillus was discovered in 1882 by Robert Koch. The infection is one of the oldest world known affecting humans. The term "tuberculosis" is originally from the Latin word '*tubercular*' (meaning a small lump) (Dubos and Dubos, 1952).

Patients presented to the hospital for diagnosis complain of symptoms such as a persistent cough for 2-3 weeks or more, chest pain, blood tinged sputum, breathlessness, weight loss, loss of appetite, fever and night sweating (Lawn & Zumla, 2011). Non-communicable diseases such as diabetes mellitus may increase susceptibility to TB in both poor and affluent populations, and consequently raise new concerns/challenges regarding prevention and early diagnosis (Creswell et al., 2010, Ferrara et al., 2012).

In spite of its significant global impact, TB is preventable and treatable therefore the disease can be controlled (Marais and Zumla, 2013). Nevertheless, the development and spread of multidrug resistance may lead to major health implications, as the detection and treatment of multi- and extensively drug-resistant TB (M/XDR-TB) are still the main concern (Chiang et al., 2013, Falzon et al., 2014). The epidemiology of transmission of the malady has additionally been impacted with the incidence of co-infection with HIV and occurrence of MDR-TB (Coovadia et al., 1998, Allix-Béguec et al., 2008).

The infection remains asymptomatic in most people, thus not leading to medical conditions (Sulis et al., 2016). As reported by Sulis and co-workers, approximately 5-15% of the latently infected patients may later develop the disease, consequently becoming TB cases with transmission potential (Sulis et al., 2016). The transmission cycle of *M. tuberculosis* consists of phases which include, (1) transmission of bacteria, (2) immune response, (3) latency and (4) activation of the disease (figure 1) (Paulson, 2013).

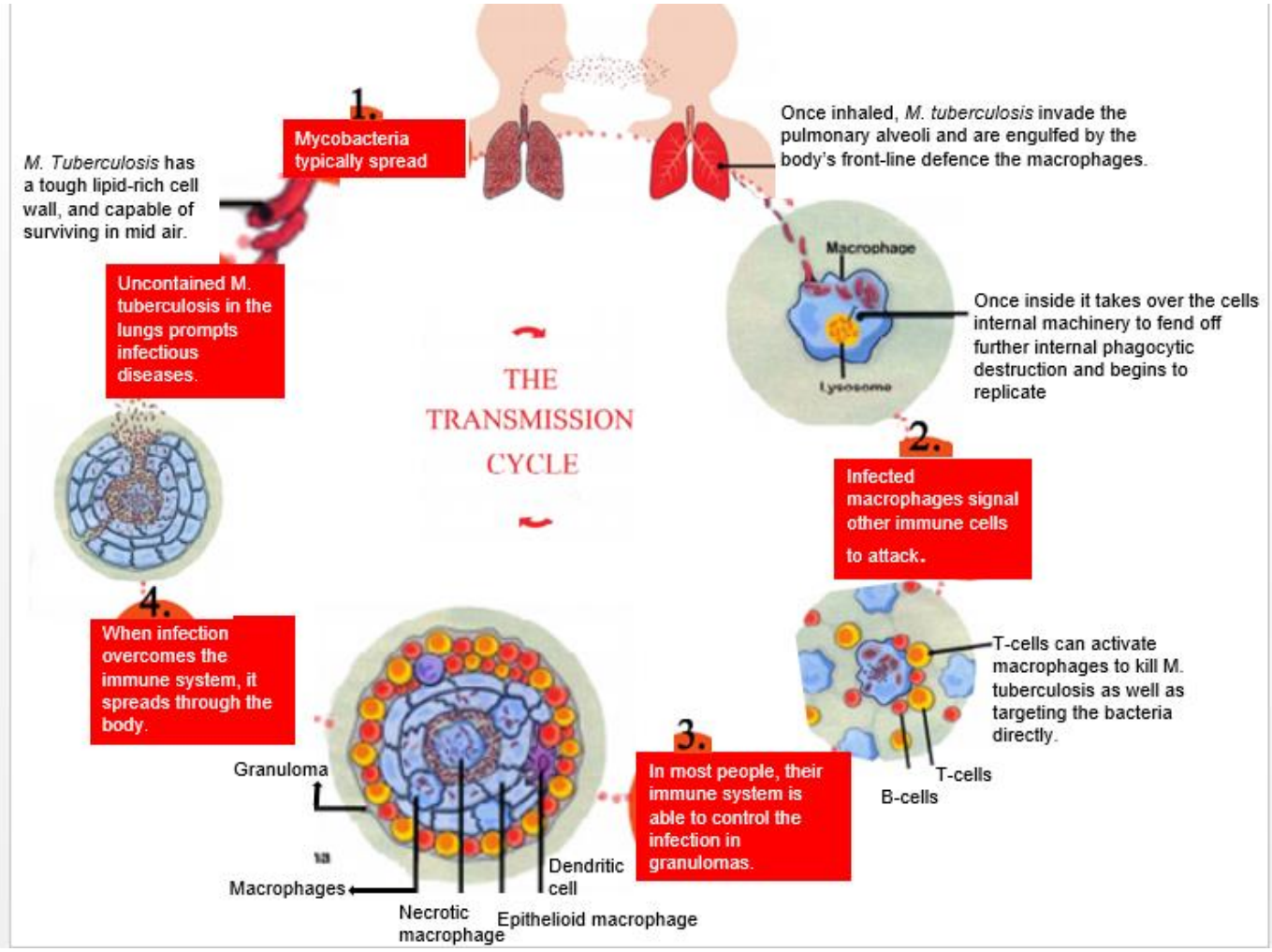


Figure 2.1. Transmission cycle of *M. tuberculosis* in humans (Paulson, 2013).

2.3 NTM in animals and humans

The improvement of molecular methods and phylogenetic studies in the 1990's, brought about an increase in the discovery of new NTM species (Biet and Boschiroli, 2014). NTM represents an expansive and diverse population of mycobacteria which has been broadly undervalued and unrecognised regardless of their important effect on animal health (Biet and Boschiroli, 2014). The atypical mycobacteria are not part of the MTBC. NTM include both slow growing (*M. absecussus*, *M. chelonae*, *M. fortuitum*) and rapidly growing mycobacteria (*M. lentiflavum*, *M. intermedium*, *M. branden*) (Falkinham, 2002). NTM are recovered from natural and human-influenced environments causing illness in human, animals, and birds (Falkinham, 2002).

Ruminants acquire infection mainly through consumption, this was observed by Kankya after isolating the organism from the intestinal content and mesenteric lymph nodes of cattle and pigs (Kankya et al., 2011). Transmission of NTM also occurs through aerosol (respiratory) and cutaneous route, due to inhalation of dust in the kraal or when grazing in the field (Courtenay et al., 2006). The conditions in which cattle get sensitised with NTM are not well known (Biet and Boschiroli, 2014). Identification of NTM as the reason for non-specific response in bovine TB diagnosis should originate from clinical examination and be supplemented by experimental investigations (Biet and Boschiroli, 2014), for better treatment and control practices.

NTM in humans

It is significant to acknowledge that, individuals who are both immunocompetent and/or immunocompromised may suffer from disseminated disease resulting from NTM infection (Griffith et al., 2007). This infection could affect organs such as the skin and tissues (Griffith et al., 2007). In both human and animals, NTM infections cause presentation of clinical signs that are similar to those in patients with TB (Nasiri et al., 2018). This poses a diagnostic and therapeutic challenge particularly in developing countries (Nasiri et al., 2018) since most (except *M. kansasii*) are resistant or partially susceptible to the second anti-tubercular drugs (Gopinath and Singh, 2010). Due to the lack of information on NTM, misdiagnosis and incorrect treatment is administered to patients infected with NTM.

Most cases reported of pulmonary infections due to NTM are caused by pathogens such as *M avium*, *M. intracellulare*, *M. kansasii*, *M. chelonae*, *M. abscessus* and *M. fortuitum* (Gopinath and Singh, 2010). Their strains and prevalence vary from one location to another (Gopinath and Singh, 2010). Many of this organisms are resistant to high temperature and low pH (Kirschner Jr et al., 1992, Bodmer et al., 2000). In western countries, the prevalence of NTM isolated from most laboratories is greater than of *M. tuberculosis* (Cassidy et al., 2009). Clinical symptoms vary from one individual to another but a chronic cough usually with purulent

sputum is often seen (Johnson and Odell, 2014). Systemic symptoms of patients with NTM including fatigue, weight loss, and malaise are common with advancing disease (Johnson and Odell, 2014).

2.4 Bovine tuberculosis

Bovine TB is primarily a disease of cattle (reservoir) caused by *M. bovis*. The symptoms of bovine TB take time to manifest in cattle (Une and Mori, 2007). Animals with pulmonary infection usually present with a moist cough at the beginning of the day, in cool climate days and may have dyspnea or tachypnoea (Une and Mori, 2007). In animals with a progressed infection, organs such as retropharyngeal or other lymph nodes enlarge and result in rupturing (Ramos et al., 2015a). Enlarged lymph nodes may result in obstruction of blood vessels, airways as well as the digestive tract (Ramos et al., 2015a). Animals may experience respiratory distress from blocked airways, constipation, and intermittent diarrhoea when the digestive tract is involved (Une and Mori, 2007). It is a challenge to make a diagnosis based on the clinical signs presented by the animal (Cousins, 2001).

2.4.1 Bovine tuberculosis in wildlife

Wildlife species have a significant role to play in the dynamics of bovine TB transmission (Anderson, 1981, Organization, 2003,). All mammals are susceptible to bovine TB yet the degree of susceptibility varies greatly. Sporadic disease reports have indicated that bovine TB is a growing concern in wild animals from game parks, reserves as well as farms within South Africa (Michel et al., 2006a, Hlokwe et al., 2016). Bovine TB can threaten conservation efforts for endangered species (Michel et al., 2010) and that has an economic impact on animals and universal trade (Medeiros et al., 2010).

The significance of bovine TB infection in wild animals relates to four aspects: i) conservation issues, as the disease may lead to an increased mortality rate of livestock, wild animals, including endangered species, ii) influence on livestock productions, due to spillback of infection from wildlife to cattle, iii) influence on transmission to human from wildlife as a reservoir of infection iv) Economic effects on private game farmers (De Garine-Wichatitsky et al., 2013).

It was reported in 1998 that buffalo herds in Kruger National Park (KNP) had a mean bovine TB prevalence of 38.2%, 16.0%, and 1.5% in the southern, central and northern region of the park, respectively (Rodwell, 1999). As suggested by Kloeck, the disease was introduced into

KNP between the years 1950 and 1960, by buffalo which are believed to have contracted the bacilli from livestock in the southeast region of KNP (Kloeck, 1998). Recently, Hlokwe and colleagues diagnosed *M. bovis* in buffalo from the Madikwe game reserve in the North West province, which had the same genotype found in wildlife from KwaZulu Natal (KZN) (Hlokwe et al., 2016). The buffalo contracted the infection from other game animals which were introduced into the reserve. This is an indication that through transportation of animals, the disease is also being moved across places. Bovine TB has also been diagnosed in the Hluhluwe-iMfolozi Park in the northern region of KZN which is believed to have been spilled over from cattle (Michel et al., 2006b).

African buffalo are the maintenance hosts of *M. bovis* in southern Africa and circulate *M. bovis* in large environments (De Vos et al., 2001). Cross described how drought conditions may favour the spatial spread of the disease, by prompting herds to explore new areas and mix with previously unrelated herds (Cross et al., 2005). The first report of *M. bovis* in free-ranging African lions was in 1996 in two lionesses in the KNP (Keet et al., 1996). The frequent exposure of lions to large amounts of infectious buffalo tissues, led to a spatial spread of bovine TB within lion prides in areas where the prevalence of bovine TB is high in buffalo (Keet, unpublished data).

Since 1929, other African wildlife species reported to have been infected in South Africa with *M. bovis* include African buffalo (*Syncerus caffer*), bush pig (*Potamochoerus porcus*), chacma baboon (*Papio cynocephalus*), cheetah (*Acinonyx jubatuseland*, eland (*Taurotragus oryx*), honey badger (*Mellivora capensis*), Impala (*Aepyceros melampus*), large spotted genet (*Genetta tigrina*), leopard (*Panthera pardus*), lion (*Panthera leo*), spotted hyaena (*Crocuta crocuta*), and warthog (*Phacochoerus aethiopicus*), black rhino (*Diceros bicornis*), white rhino (*Ceratotherium*), wild dogs (*Lycaon pictus*), giraffe (*Giraffa camelopardalis*), blue wildebeest (*Comochaetess taurinus*), nyala (*Tragelapus angasii*), horse (*Equus ferus caballus*) (Michel et al., 2006c, Hlokwe, 2014, Miller et al., 2017a, Miller et al., 2017b)

Thus far an increased number of 60 wild vertebrate species worldwide have been identified to be infected with *M. bovis*, however, only a limited number were confirmed to act as maintenance hosts (De Lisle et al., 2001, Thoen et al., 2009). A study conducted in the Queen Elizabeth National Park in Uganda and in Kafue lechwe National Park situated in Zambia demonstrated that, African buffalo and lechwe (*Kobus leche kafuensis*) respectively, act as maintenance hosts of *M. bovis* (Woodford, 1972, Krauss et al., 1984). *M. bovis* was isolated in Spain from red deer (*Cervus elaphus*), fallow deer (*Dama dama*), wild boars (*Sus scrofa*) and Iberian lynx (*Lynx pardina*) (Aranaz et al., 2004).

Domesticated animals and wildlife have co-existed for the previous 6500 years in Africa regularly having similar space and resources (Bradley et al., 1998, Osofsky, 2005). Wildlife-livestock interfaces in Africa have been significantly transformed during the past years, due to the increasing movement of livestock into game parks for better pasture. In most countries where bovine TB is primarily a disease of domesticated cattle, it can be controlled effectively through a test and slaughter programme (Michel, 2002). Once spillover of the infection occurs from cattle to wildlife species as maintenance host, traditional measures are not effective for control and eradication of the disease in cattle nor in wildlife. (Michel, 2002).

2.4.2 Bovine TB in cattle

Bovine TB is characterised by the systematic formation of granulomatous lesions or tubercles that affect the lungs, lymph nodes and other organs (Ayele, 2004). Lesions may also develop within the lymph nodes of the udder and cows with infected udders can excrete *M. bovis* in the milk (Department of agriculture, 2012). These granulomas are typically yellowish and either caseous or calcified, and frequently embodied. A few tubercles may be quite small in size and can be missed by the naked eye, unless the tissue is sectioned (Shown on Figure 2).

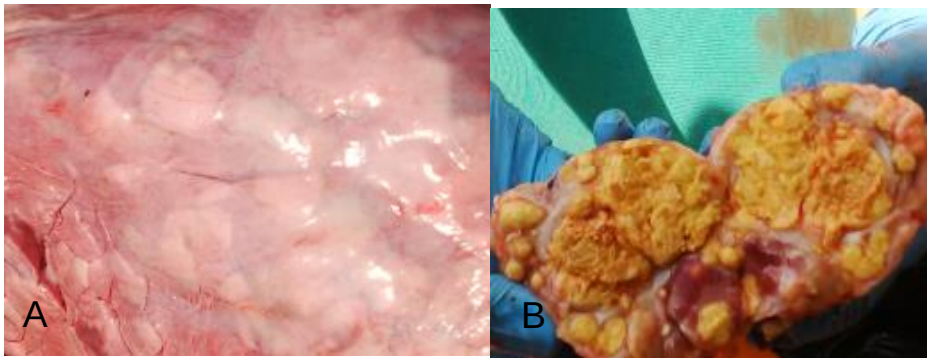


Figure 2.2. A. Bovine lung infected with *M. bovis.*

B. Dissected lymph node from an infected cow.

Despite successful eradication of bovine TB mostly in some developed countries, some developing countries continue to encounter persistence of *M. bovis* in their cattle population. Cattle shed *M. bovis* in its aerosol, nasal secretions, and faecal excretions and in milk. The pattern of how the bacteria are shed as well as the quantity of bacteria in milk that is considered harmful is not well understood. Research suggests that the shedding of the bacteria occurs immediately after the cow is infected (Neill et al., 1992). The bacilli are mostly transmitted through aerosol and contact in cattle, this usually happens between cattle that

graze in the same area and those housed together. Spread from dairy cattle to calves is often due to consumption of the milk or colostrum from infected cows.

The research conducted in Tanzania and South Africa to evaluate the frequency of people consuming milk from their cattle, showed that respectively over 90% and 97% of farmers owning cattle consumed milk daily (Michel et al., 2015). Essential variances, nonetheless, were witnessed in the treatment prior to ingestion (Michel et al., 2015).

2.4.3 Bovine tuberculosis in humans (Zoonotic TB)

According to Machado, humans are susceptible to *M. bovis,* however, more cases are reported on infection with *M. tuberculosis* (Machado, 2015). They become infected through close interaction with infected animals, consumption of raw infected milk, and undercooked meat. Researchers report a global estimation of 9 million TB cases in humans annually which accounts for both *M. bovis* and *M. tuberculosis* (WHO, 2014, Olea-Popelka et al., 2017). The infection of *M. bovis* in humans differs from one country to another depending on the percentage of infected cattle, consumer habits and food hygiene practises (Tschopp et al., 2010). According to WHO, by the year 2010, an estimated number of 121 263 new cases of zoonotic TB were reported with an estimated 10 545 deaths due to *M. bovis* (WHO, 2014).

From the year 1993 to 2007, it was reported in the Netherlands that *M. bovis* infection represents about 1.4 percent of all TB cases (Majoor et al., 2011). In San Diego, California, infection of *M. bovis* was observed in 45% children while adults accounted for 6% (Rodwell et al., 2010, Olea-Popelka et al., 2017). In countries where the infection is endemic in cows, and the owners consume milk without pasteurisation, the rate of infection is likely to be elevated (Tschopp et al., 2010). In developing countries where there is lack of resources and inadequate veterinary control measures, there are high possibilities of undetected milk-borne transmission to humans (Cosivi et al., 1998b, Ashford et al., 2001). The infection leads to extra-pulmonary TB in humans (Ndambi et al., 2007, Oloya et al., 2008).

Cow's milk has always been an essential diet in most countries, both developed and developing. In most developed countries, the pasteurisation of milk has been implemented however the opposite could be said about certain areas in developing countries. Health alarms about consumption of milk originated as early as the mid-19th century, because the public were having queries about the unhygienic conditions of cattle and dairy processing plants (Davis et al., 2014). Human TB infection due to consumption of milk was common at this time and was reported as a result of bacterial contamination (Gillespie et al., 2003, Garber, 2008). Due to

such observations, regulators and hygienists enhanced better methods of milking cows as well as how milk was distributed to consumers (Leedom, 2006, Gould et al., 2014).

Individuals such as dairy labourers who spend long periods of time in close contact with animals susceptible to *M. bovis*, are advised to seek therapy for any sickness displaying manifestations of TB (Control, 2012). Clinical signs caused by *M. bovis* in humans are indistinguishable to those caused by *M. tuberculosis.* Signs include weakness, loss of appetite, weight loss, diarrhoea, large prominent lymph nodes and intermittent hacking cough (Claeys et al., 2013). Patients with HIV/AIDS eventually have an immunosuppressed system hence being susceptible to the infection. Reports show that the risk of *M. bovis* infection in humans is likely to increase in patients with HIV/AIDS (Cosivi et al., 1998).

2.5 Epidemiology of mycobacteria

2.5.1 Epidemiology of zoonotic TB

The lack of routine screening for *M. bovis* in humans results in the burden of *M. bovis* being underestimated as a leading cause of TB in humans. In addition to this fact, laboratory procedures used to analyse human TB don't separate *M. bovis* from *M. tuberculosis* (Olea-Popelka et al., 2017). As indicated by WHO, it is evaluated that *M. bovis* caused an expected 147 000 new cases and 12 500 deaths in 2016 (WHO, 2017).

In countries where dairy products are pasteurised before consumption as well as having the infection control in cattle, the prevalence established is usually found to be lower than in countries where there is a lack of control. For instance, *M. bovis* caused roughly 0.5% of culture-confined human TB in 2007. This has been decreasing from the years 2005 to 2008 as, the yearly rate of *M. bovis* diminished from 0.065 to 0.047 for every 100 000 populace (Mandal et al., 2011). Iran has a rapidly growing livestock industry, the spread and lack of identification of circulating *M. bovis* strains are possibly the most essential contributor to increasing economic losses from bovine TB as opposed to the importation of infected dairy cattle (Tadayon et al., 2008).

According to O'Reilly and Daborn, in Africa, approximately 85% of cattle and 82% of the human population reside in areas where the infection had been identified (O'Reilly and Daborn, 1995). Animals that share grazing and water facilities are more likely to spread bovine TB among each other. This was seen in a study conducted in Zambia, where cattle and Kafue lechwe (*Kobus leche kafuensis*) graze together and a prevalence rate of up to 50% at herd level was reported (Oloya et al., 2007, Munyeme et al., 2009). In Cameroon, the infection has

been reported to be endemic and *M. bovis* has been isolated from meat bound for human utilisation (Egbe et al., 2016).

The herd prevalence rates of bovine TB has been reported to be 51% for Tanzania (Kazwala et al., 2001, Munyeme et al., 2008). In South Africa the rate has dropped in domesticated cattle to 0.04% in 1995 but recently, increased sporadic outbreaks have been reported due to lack of control (Michel et al., 2008). There is a concern that livestock keepers, abattoir workers, and consumers are at risk of zoonotic transmission of *M. bovis* through consumption of unpasteurised dairy products and close interaction with infected animals (Egbe et al., 2017).

According to OIE reports, most African countries detected bovine TB in livestock during the period 1996-2011, 4 countries suspected *M. bovis* infection in livestock whereas 4 countries reported the absence of bovine TB due to implementation of effective bovine TB control (Olea-Popelka et al., 2017). The World Animal Health Information Database (WAHID) of the World Organisation for Animal Health (OIE) reported that 70 nations announced cases of bovine tuberculosis in their cattle populaces in 2010, and 49 nations in 2011 (Olea-Popelka et al., 2017).

2.5.2 Epidemiology of NTM

NTM are found in soil and water, however factors affecting transmission to humans are not well known (Halstrom et al., 2015). Throughout the past decade, it has been proposed by several researchers that the occurrence of NTM is increasing in both laboratory isolation and disease prevalence (Johnson and John, 2014). The increase in prevalence may be due to a maturing populace as the incidence of symptomatic disease is higher in post-menopausal women and older men (Halstrom et al., 2015). It is challenging to precisely portray the incidence and prevalence of NTM pulmonary infections since the isolation of the organism does not demonstrate clinical infection (Johnson and John, 2014). Most NTM infections, unlike potentially health-threatening infections, do not require public health reporting which obstructs an exact comprehension of the epidemiology (Johnson and John, 2014).

To understand the ecological repositories and transmission routes of pathogenic NTM, different environments, countries, and atmosphere must be explored (Halstrom et al., 2015). NTM have been isolated from specimens such as faeces, clinical isolates (Haican et al., 2016), livestock and wildlife (Woodring and Vandiviere, 1990, Tschopp et al., 2010), water, soil, milk, fish and birds (Miller and Miller, 1993). In a study from Oregon, NTM pneumonic infections were related with more densely populated territories proposing that urban civil water supply introduction predisposed individuals to disease (Winthrop et al., 2011). However in Japan,

NTM were more ordinarily found in agriculturists and plant specialists than in urban patients with bronchiectasis, suggesting a more important etiologic role for exposure to soil than water sources (Maekawa et al., 2011). An increased risk of NTM infection is likewise present in patients with rheumatoid joint pain, diabetes mellitus, alcoholism or non-pneumonic malignancies (Heitzman, 1978, Halstrom et al., 2015).

For reasons that are not totally understood, some pathogenic NTM tend to cluster in particular land distribution (Griffith et al., 2007). A study led by Khaledi et al (2016) in Iran, had *M. fortuitum* as the most predominant fast-growing mycobacteria and the most common slow-growing mycobacteria were *M. simiae* with the prevalence of 44.2% and 14.3% respectively. In China, a test showed that the prevalence of NTM infections among tuberculosis suspects was 6.3% in Mainland, while the southern region had the highest NTM prevalence of 8.6% by the year 2016 (Yu et al., 2016). In Nigeria, 444 (28%) culture positive cases of pneumonic tuberculosis were recognised, of these, 375 (85%) were due to MTBC (354 cases of M. tuberculosis, *20 M. africanum* and 1 *M. bovis*) and 69 (15%) were due to infection with NTM (Yu et al., 2016) . A prevalence of 56% which was higher than expected was obtained among culture-positive sputum specimens collected from people living with HIV with TB symptoms in Botswana (Agizew et al., 2017).

2.6 Diagnosis of mycobacterial infection

2.6.1. Direct detection

Laboratory detection of mycobacterial infection

I. Direct smear microscopy

Two kinds of acid fast bacilli (AFB) stains are generally used: the carbol fuchsin stain and the fluorochrome stain (Pfyffer, 2015, Somoskövi et al., 2001). The most widely recognized technique for analysis of TB in humans is sputum smear microscopy developed over 100 years back. Routine screening for mycobacterial disease is typically performed just for *M. tuberculosis* and the outcomes will be negative in 85% of patients with NTM infections (Erasmus et al., 1999, Woodring and Vandiviere, 1990). Sputum smear microscopy requires 10,000 to 100 000 organisms/ml to visualise pathogenic or saprophytic mycobacteria (Katoch, 2004). NTMs are not clearly seen on routine gram stain hence the fluorochrome technique for staining is considered (Ryan and Ray, 2004). Analysis of TB disease from tissues, milk, faeces or other biological material with microscopy is poor because of low quantities of mycobacteria and increased concentration of other pathogens (Madigan, 2012). For this reason, collection

of high quality specimens is necessary to avoid contamination. Direct staining is very rapid but lacks specificity and does not identify the species of mycobacteria causing the infection.

II. Culture

Mycobacteria are classified as either slow growing or fast-growing organisms. *M. bovis* takes 3 to 8 weeks to grow on culture before any colonies can be observed whereas fast growing NTM grow in 3 days. NTM are environmental mycobacteria but are often cultured from several specimens collected from both human and animals. Both broth and solid media are recommended for the isolation of NTM (Johnson and Odell, 2014). Culturing specimens on solid media allows the opportunity to visualize characteristics of colony growth (Woods, 2011). The culture of mycobacteria can give more data on the mycobacterial species than a direct smear and histopathology and thus it is regarded as a gold standard method.

On culture, the identification of mycobacteria is based on the phenotypic (pigmentation, growth rate, and colony morphologies) and biochemical (pyrazinamidase test, amidase test, niacin test) characteristics of cultures isolated in vitro (Vitale et al., 1998). Historically, NTM were classified according to their rate of growth, morphology and pigment production, this is known as Runyon classification (ROGALL et al., 1990). They include slow growing mycobacteria such as 1. Runyon I: photochromogens (*M. kansasii*, *M. marinum*, *M. asiaticum,* and *M. simiae*) which are slow growing and produce a yellow-orange pigment when exposed to light 2. Runyon II: scotochromogens (*M. gordonae* and *M. scrofulaceum*), slow growing and produce a yellow-orange pigment regardless of exposure to light or grown in the dark 3. Runyon III: non-chromogens (MAI complex), slow growing and produce no pigment regardless of culture conditions and rapid growing mycobacteria which includes 4. Runyon IV (*M. fortuitum*, *M. peregrinum*, *M. abcessus* and *M. chelonae*), visible colonies in 5 days with no pigmentation (Brown-Elliott and Wallace, 2002).

Fast growing bacteria tends to overgrow before *M. bovis* on culture (Madigan, 2012). Infected tissues from the respiratory tract, lymph nodes as well as other infected organs with lesions are used for culture. All specimens require decontamination but those collected from non-sterile areas such as sputum, nasal swabs, and milk require more stringent decontamination methods to reduce/inhibit the growth of other micro-organisms. Samples must be collected with care to reduce cross-contamination between samples. The decision and technique for decontamination is critical since the procedure affects the viability of the mycobacteria and has the capability to destroy other micro-organisms (Madigan, 2012).

Specimens such as bronchoalveolar lavage fluid and sputum can be false positive on culture obtained from patients with chronic lung disease because isolation of NTM may be a signal of

airway colonisation and not infection while patients with non-cavitary pulmonary infections may lead to false negative results for NTM (Albelda et al., 1985, Erasmus et al., 1999). In patients diagnosed with HIV/AIDS, positive cultures of sputum or bronchoalveolar lavage fluid indicate pulmonary non-tuberculous mycobacterial (NTMB) infection even if the chest radiograph is normal (Marinelli et al., 1986, Woodring and Vandiviere, 1990) .

Culture media

a) Solid media for growth of *M. bovis*

Löwenstein–Jensen medium (LJ) is mostly used for culturing *Mycobacterium* species. LJ supplemented with sodium pyruvate is used to support the growth of *M. bovis* but reduce the growth of the micro-organisms present in the sample whereas glycerol supports the growth of *M. tuberculosis* (Che-Engku-Chik et al., 2016). Higher bacterial contamination are often found in communal milk samples hence decontamination of the sample is necessary, this has a direct importance on the outcome obtained from the slopes. MTBC grows only when incubated at 37°C +/- 1°C, whereas other mycobacteria can grow at various temperature ranges.

b) Liquid media for detection of mycobacterial species

Mycobacteria Growth Indicator Tube (MGIT) is another technique created by Becton Dickinson. The tube is aimed for the detection and recovery of *Mycobacterium* species. Growth is recognised by a nonradioactive detection system using fluorochromes for detection and drug screening (Tortoli et al., 1999). The recovery of mycobacteria within the tube is accelerated with the MGIT system, but this is only of partial benefit if it cannot be accompanied by rapid species identification. This system helps in early detection of mycobacterial growth in 7 to 12 days and has also been of importance for drug susceptibility testing (Tortoli et al., 1999, Bemer et al., 2002).

iii. Molecular techniques

Molecular techniques such as polymerase chain reaction (PCR), real-time PCR, multiplex real-time PCR and restriction fragment length polymorphism (RFLP) have been applied for the identification and differentiation of mycobacterial species (Esfahani et al., 2012). The identification is made by comparing the nucleotide sequence of the organism with a known amplicon sequence (Johnson and Odell, 2014).

Table 2.2. Mycobacterium identification by PCR.

Region targeted	Identification	Reference
32 KDa protein	Mycobacteria spp.	(Soini et al., 1994)
65KDa heat shock protein	Mycobacteria at genus level	(Telenti et al., 1993)
MPB70	MTBC	(Cousins et al., 1992, Cousins et al., 1991)
16S-23S rRNA	All bacteria	(Hashemi-Shahraki et al., 2013)
RD1, RD4 and RD9 gene	*M. bovis BCG*, *M. bovis* and *M. tuberculosis*	(Warren et al., 2006)
IS6110	MTBC	Thierry et al., 1990

Weaknesses associated with the use of PCR-based technologies in diagnoses include issues of sampling such as: low concentrations of the organisms often found in the sample, samples comprising inhibitors which prevent effective PCR reactions and difficulties in extracting DNA from mycobacteria due to the organism's robust cell wall (Strain et al., 2011). The molecular biological technique such as spoligotyping is used to characterise mycobacterial DNA samples and definitively identify them. It is based on the polymorphism identified in spacer sequences present within direct repeat regions of the *M. tuberculosis* genome (Heyderman et al., 1998). Spoligotyping has advantages since in principle, it can be used simultaneously for the detection and typing of MTBC in one assay and performs typing on non-viable cultures (Heyderman et al., 1998,Driscoll et al., 1999).

iv. Necropsy and Histopathological diagnosis

A conditional diagnosis of mycobacterial infection can be made after visual detection of lesions during necropsy (Comer, 1994). In most countries, bacteriology and histopathology are the most utilised procedures for conclusive diagnosis of mycobacteria in both human and cattle (Courcoul et al., 2014). In cattle, the necropsy technique is initiated to inspect cattle that are reactors to tuberculin test for the diagnosis of bovine TB (Comer, 1994). An examination of tissues for macroscopic lesions is done when culling of the infected cattle is performed and collection of intact tissues for laboratory examination is done (Comer, 1994). The tissues are cut in situ and examined for lesions. In humans and other animals, formalin-fixed, paraffin embedded tissue blocks are used for histological diagnosis. TB can be diagnosed only as

chronic granulomatous inflammation suggestive of TB on a routine surgical pathological report. Nonetheless, the presence of prolonged granulomatous inflammation does not indicate a conclusive diagnosis of mycobacteria. This is because the histopathological characteristics are found in several diseases including foreign body reaction, fungal infection, sarcoidosis, cat scratch disease, leprosy and brucellosis (Park et al., 2003). According to a study conducted by Varello, the specificity of histopathology compared to that of bacterial culture demonstrated the average of 92.3% with a low sensitivity (Varello et al., 2008). As a result, for a definite diagnosis, AFB stain, culture and TB-PCR should be implemented (Park et al., 2003).

2.6.2 Indirect diagnosis

i. Comparative Intradermal Tuberculin Test

Several methods have been used for the indirect diagnosis of TB in infected animals, however the Single Comparative Intradermal Tuberculin Test (SCITT) is most widely used (Machado, 2015). The procedure is primarily used in cattle to screen for *M. bovis* infection (El Idrissi and Parker, 2012). There are two different tests, the single intradermal tuberculin test which only uses bovine tuberculin PPD, whereas the comparative intradermal tuberculin test uses both the bovine tuberculin PPD and avian tuberculin PPD (El Idrissi and Parker, 2012). Both intradermal tests are based on the delayed type hypersensitivity reaction to the injected tuberculin (OIE Manual, 2009) (https://www.oie.int > doc > ged). This reaction is a complex cell-mediated immune response comprising of specific and nonspecific components. The dominating response is caused by previously sensitised T cells (i.e. cells previously exposed to components of the mycobacteria) which recruit and coordinate the infiltration of other cell types into the injected area thereby leading to the development of a measurable transient swelling (OIE Manual, 2009) (https://www.oie.int > doc > ged).

The comparative intradermal tuberculin test is used to distinguish among animals infected with *M. bovis* and those sensitised to tuberculin as a result of previous exposure to other mycobacteria (OIE Manual, 2009) (https://www.oie.int > doc > ged). In most cases, NTM will cause a strong reaction to avian tuberculin PPD (PPDa) whereas cattle which have been exposed to *M. bovis* will result in a strong reaction to bovine tuberculin PPD (PPDb). The response of the tuberculin is observed by measuring the swelling of the injected region. Swelling is observed after 72 hours following injection (figure 8) (Lepper et al., 1977, Francis et al., 1978, Strain et al., 2011).

The tuberculin skin test is commonly used in most countries for screening infection in animals as part of the test-and-slaughter scheme to control bovine TB. However, like many other tests,

the tuberculin test has limitations and should be carried out by an experienced veterinarian or animal technician for accuracy (El Idrissi and Parker, 2012). False positive results in cattle are mainly due to temporary sensitisation with NTM (Department of Agriculture, Forestry and Fisheries, Bovine TB manual, 2013). It is advisable to re-test animals after a period of 3 months and collect blood for the Bovigam®TM (Prionics) assay for conclusive outcomes. In chronically infected animals, the test results may be false negative due to an unresponsive reaction of the infected animals (Department of Agriculture, Forestry and Fisheries, Bovine TB manual, 2013). The tuberculin test remains not validated in most non-bovid and non-cervid species. In instances whereby infected animals are not identified, progress in herd sanitation programme can be impeded. In addition, removal of false positive reactors may also have serious implications on cattle management (Department of Agriculture, Forestry and Fisheries, Bovine TB manual, 2013).

Figure 2.3. Measurement of the skin thickness for diagnosis of bovine TB (Department of Agriculture, Forestry and Fisheries, Bovine TB manual, 2013).

ii. Bovigam

In order to achieve the success rate in diagnosis for control of bovine TB in cattle, an assay for IFN-γ has been created to identify and evaluate the release of cytokine (immunological messenger or effector molecule) when whole blood is cultured with bovine and avian tuberculin (Rothel et al., 1990, Gormley et al., 2006). The Bovigam is an in vitro assay developed in late 1990 in Australia based on the detection of cytokine interferon-gamma (IFN-γ). This assay was developed for the diagnosis of bovine TB in cattle (Wood and Jones, 2001, De la Rua-Domenech et al., 2006). IFN-γ is primarily released by the T-cells after antigenic stimulation (De la Rua-Domenech et al., 2006). It has a significant role in the immune responses to tuberculous mycobacteria as the main macrophage activation factor (De la Rua-Domenech et al., 2006).

The principle behind the IFN-γ assay is the detection of the host's cell-mediated immune (CMI) response against *M. bovis* infection. As reported by Gormley and colleagues, using both the tuberculin test and Bovigam assay will result in an early detection of infection (Neill et al., 1994). This will reduce the chances of cattle to cattle transmission within the herd. Like any other assay, the Bovigam assay has shortcomings that negatively affects its effectiveness such as such as: 1. Delay in the processing of the blood sample collected. Blood samples need to be transported to the laboratory on the same day of collection for processing, as a delay of 24 hours may result in a 30% decrease in the ELISA OD value. 2. Effects of tuberculins and corticosteroids changes the sensitivity of the IFN-γ assay. This is attributed to the influence of tuberculins used and produced in different countries with dissimilar conditions and the combinations of antigens (whole proteins and derived peptides) also have an effect on the sensitivity of the assay (Rothel et al., 1992, Gormley et al., 2006). The Bovigam assay has an advantage compared to the SCITT because it has the ability to detect *M. bovis* in cattle as early as 14 days post infection, thus ensuring an early removal of infected cattle from the herd (Buddle et al., 1995, Gormley et al., 2006). Unlike the intradermal tuberculin test, the Bovigam assay does not compromise the immune status of the cattle (Gormley et al., 2006). The use of the intradermal tuberculin test involves two visits to the farm, however, only one farm visit for once off blood collection is necessary when using the Bovigam assay.

2.7 Control of bovine tuberculosis

Bovine TB is a chronic debilitating disease in South Africa and several other countries. Providentially it has been controlled by the animal health legislation due to its long-term disadvantageous effect (DAFF, 2012) (https://www.nda.agric.za > docs > infopaks). The disease has negative impact on both human and animal health leading to a substantial economic burden on the agricultural industry (Ramos et al., 2015b). The animal health legislation has implemented the slaughtering out policy of bovine TB positive animals under the TB Eradication Scheme (Muller et al., 2013) . The aim of this scheme is to eradicate the disease from cattle in South Africa. Müller and colleagues argued that the control of *M. bovis* in developed countries has been improved such that it is insignificant, this is because infected cattle were eliminated and pasteurisation of milk was introduced and well-practised (Muller et al., 2013). However, in developing nations a dissimilar situation is observed. The present arrangement of testing high-risk cattle brought into herds has decreased the number of episodes caused by bought cattle (Goodchild and Clifton-Hadley, 2001)

Control of bovine tuberculosis in wildlife

There are various reasons that could lead to validating control measures in wildlife populations such as effect of bovine TB on endangered species, tourism, exportation of wildlife to other countries and the risk of transmission to humans and livestock (De Garine-Wichatitsky et al., 2013). This is because once the infection is introduced in wildlife, it then becomes difficult to eradicate (Munyeme et al., 2009). Although the intradermal tuberculin test is only validated for cattle, it may be used for monitoring of wildlife and livestock by frequent testing (Hlokwe et al., 2016). Regardless of its numerous confinements in wildlife, the diagnostic test is currently used for buffalo (Jolles et al., 2005). The choice of appropriate control measures may differ from place to place (Michel et al., 2006c).

As a result of the cost measures and trouble of diagnosing TB in wildlife (non-approved tests and cost of immobilizing), it becomes difficult to practise the test-and-slaughter method in most of the countries (De Garine-Wichatitsky et al., 2013). The use of fences across the parks which separates wildlife from cattle reduces the chances of spillover (De Garine-Wichatitsky M, et al., 2013). The rate of bovine TB in Hluhluwe-Umfolozi has significantly declined compared to previous years (Cooper D, 2005). The decrease is due to a control programme which limits intervention of buffalo followed by tuberculin testing and removal of positive animals from the herds (Michel et al., 2006). Another known case of progress in maintaining bovine TB from Minnesota was when more than 9700 deer were tested and in 4 years the deer population had decreased by an estimated 55% (Tessaro, 1986, Carstensen and DonCarlos, 2011).

The best and valuable risk investigations for *M. bovis* ought to consider the bi-directional nature of transmission (Tessaro, 1986). This then permits the implementation of risk mitigations to inhibit the spread between cattle and wildlife (Tessaro, 1986). The eradication of bovine TB in wildlife through culling of the animals is expensive and not practical hence according to Meyer and colleague, preventing the spread of infection in wildlife populace should be implemented (Meyer and Meagher, 1995). The primary discovery of an outbreak is important in controlling the infection within a population, nevertheless, this requires frequent testing of wildlife (Michel et al., 2006c). Moreover, investigation of infected wildlife is important to recognize *M. bovis* susceptible wildlife population (Michel et al., 2006c). Building up a complete national monitoring system for *M. bovis* in wildlife that is strategically and financially practical could yield economic advantages for animals health by inhibiting transmission from domesticated animals to wildlife through primary detection (Miller and Sweeney, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Methods used for the decontamination of milk

Store bought pasteurised milk was used for this experiment.

3.1.1 Bacterial suspensions

The milk was experimentally spiked with *M. fortuitum* ATCC® 6841™, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). These cultures were obtained from the DVTD bacteriology laboratory. Confluent colony growth of the organisms were inoculated into 10 ml of phosphate buffered saline (PBS) solution supplemented with Tween 20. Teflon homogenisers (Thermo Fisher Scientific, South Africa) were used to homogenise the bacterial suspensions. The concentration of the bacteria (*M. fortuitum*, *E. coli* and *S. aureus*) was measured using an automated cell counter (Biorad.TC20™).

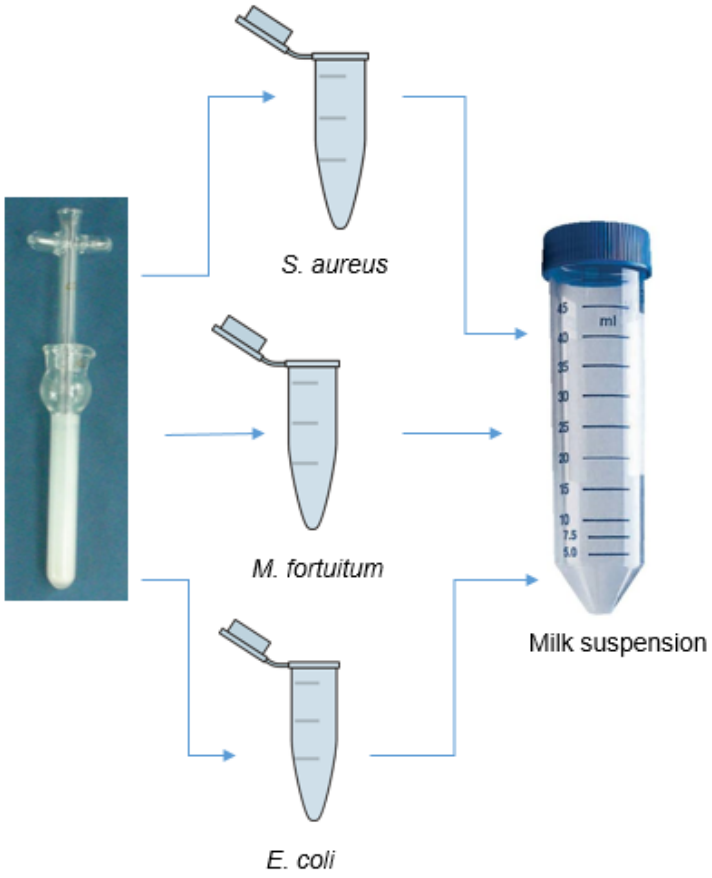


Figure 3.1: Preparation of spiked milk suspension.

3.1.2 Preparations of serial dilutions

To identify the limit of detection of *M. fortuitum* in milk and to determine the efficacy of decontamination methods on contaminated milk following a given decontamination method, serial dilutions of *M. fortuitum*, *E. coli* and *S. aureus* were performed (Figure 3.2). The minimum number of detectable *M. fortuitum* and the ability of the methods to effectively decontaminate the milk were determined by inoculating samples with known concentrations of viable contaminants. This was confirmed by inoculation on LJ (Löwenstein-Jensen) pyruvate slopes.

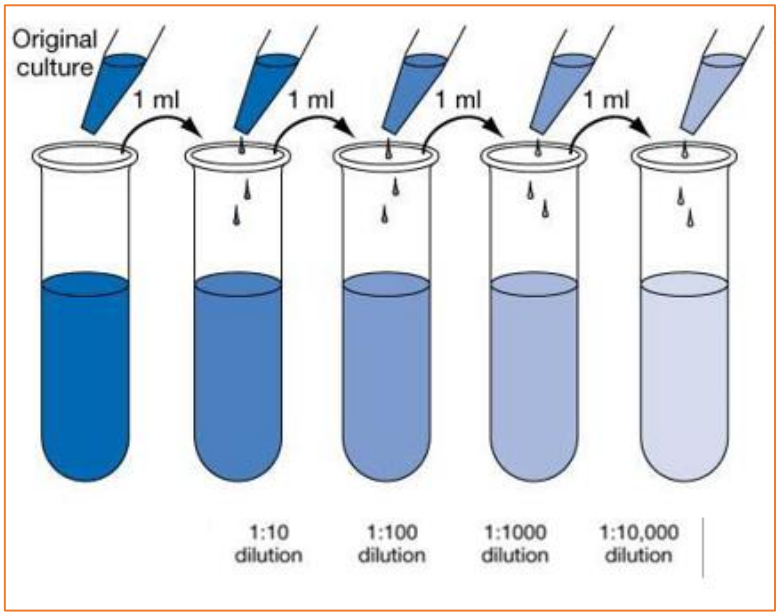


Figure 3.2: Serial dilution of bacteria *(M. fortuitum*, *E. coli* and *S. aureus*).

The following procedure was used to prepare the dilution series:

Sterile 50 ml tubes were labelled 10^8, 10^7, 10^6…10^0 (representing 1 x 10^x CFU/ml). Using an automatic pipet-aid and a sterile disposable pipette, 9 ml of pasteurised milk was aliquotted into each tube. 1 ml solution of 1 x10^9 CFU/ml *M. fortuitum* was pipetted into the first tube (labelled 10^8). When contaminants where included, 1 ml solution of 1 x 10^9 CFU/ml *M. fortuitum*, 0.5 ml of 1 x 10^9 CFU/ml *E. coli* and 0.5 ml of 1 x 10^9 CFU/ml *S. aureus* were pipetted into the first (10^8) marked tube containing 8 ml of pasteurised milk. The suspension was pipetted up and down to adequately disperse the bacteria evenly throughout the tube. Using a new sterile disposable pipette, 1 ml from the first tube (10^8) was added into the tube labelled 10^7 containing 9 ml of pasteurised milk. The suspension mixing procedure was repeated using a dilution factor of 10 until 1 colony forming unit in a millilitre of milk was achieved (1 x 10^0 CFU/mL).

3.1.3 Decontamination of spiked milk

Preparations for the decontamination methods mentioned below are described in Annex 1. Following centrifugation, a loop full of the pellet from each decontamination procedure was used to inoculate the LJ pyruvate media and slopes were incubated at 37°C for 2 months.

Decontamination of spiked milk samples with 5% oxalic acid (OA).

The 10 ml serially diluted suspensions were treated with an equal volume (10 ml) of 5% OA. The acid mixture was incubated at room temperature for 15 minutes while occasionally shaking the tubes for homogenisation and decontamination. The tubes were centrifuged (eppendorf centrifuge 5810 R) at 956 g for 10 minutes at 4°C. The supernatants were carefully discarded into a container containing disinfectant (10% Corox). (www.tbcare1.org>participant_manual, 2018).

Decontamination of spiked milk samples with Novel hypertonic-saline hydroxide (HS-SH).

The 10 ml serially diluted suspensions were treated with an equal volume (10 ml) of 5% HS-SH (5 ml of 7% NaCl and 5 ml of 4% NaOH). The suspensions were mixed frequently for 15-20 minutes at room temperature using a vortex mixture. Tubes were then incubated at 37°C for 20 minutes. Phosphate buffer (5 ml) was added into the tubes and centrifuged (eppendorf centrifuge 5810 R) at 956 g for 15 minutes at 4°C. (www.tbcare1.org>participant_manual, 2018).

Decontamination of spiked milk samples with 1% cetylpyridinium Chloride (CPC).

The 10 ml serially diluted suspensions were treated with an equal volume (10 ml) of 1% CPC. Milk suspensions were incubated at room temperature for 5 hours. Following incubation, samples were centrifuged (eppendorf centrifuge 5810 R) at 1258 g for 15 minutes at 4°C. Pellets were suspended into PBS, centrifuged at 1258 g for 15 minutes at 4°C. Supernatants were discarded into a container containing disinfectant (10% Corox). (Dundee et al., 2001).

Decontamination of spiked milk samples with 2% hydrochloric acid (HCl).

The 10 ml serially diluted suspensions were treated with an equal volume (10 ml) of 2% HCl. Milk suspensions were incubated for 10 minutes at room temperature and centrifuged (eppendorf centrifuge 5810 R) at 1301 g for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded into a container containing disinfectant (10% Corox). Pellets were washed with 10 ml distilled sterile water and the suspensions were shaken vigorously. The

washed suspensions were centrifuged at 1301 g for 10 minutes at 4°C. The supernatants were discarded into a container containing disinfectant. (Manual, 2009).

Decontamination of spiked milk samples with 4% NaOH (sodium hydroxide).

The 10 ml serially diluted suspensions were treated with an equal volume (10 ml) of 4% NaOH. Samples were incubated for 10 minutes at room temperature and centrifuged (eppendorf centrifuge 5810 R) at 1301 g for 10 minutes at 4°C. The supernatants were discarded, pellets were washed with 10 ml of distilled water and suspensions were shaken vigorously. The washed suspensions were centrifuged for 10 minutes at 4°C. (Manual, 2009).

3.1.4 Culture of spiked milk samples

For each experiment, two slopes were inoculated from each tube of spiked milk on LJ pyruvate media. A negative control was included in the form of pasteurised milk for all the different decontamination protocols. Slopes were incubated at 37°C for a period of 2 months. Growth of the cultures was monitored from day to day in the first week of incubation and later monitored on a weekly basis. Exposure of slopes to other microorganism present within the laboratory was avoided at all times by not opening the slopes when monitoring, this was to reduce the possibility of introducing contaminants into the slopes.

3.1.5 Preparing a smear from LJ slopes with visible isolates using Ziehl-Neelsen staining (ZN-staining)

Following incubation, slopes which had growth at week 6-7 were selected for Ziehl-Neelsen staining. A sterile inoculating loop was used to pick individual colonies from the slopes and streak them onto a microscope slide.

The slide with the smear was heat fixed by passing the slide 3-4 times through the flames of a Bunsen burner. The slide was flooded with carbol fuchsin and heated gently. The smear was rinsed with water until no colour appeared on the effluent. An acid-alcohol solution was poured on the slide and allowed to decolourise for 5 min. The slide was washed with water and counterstained with malachite green solution for 2 min. The slide was washed with water and allowed to air dry. The slide was examined with a compound microscope using the 100x oil immersion objective.

3.2 Collection of milk samples from cattle infected with *M. bovis*

3.2.1 Study area

Hluhluwe is a residential town in northern KZN, South Africa located in uMkhanyakude District Municipality. It is situated between several game reserves including the iSimangaliso Wetland Park and Hluhluwe –iMfolozi Park on the banks of the Hluhluwe River. It is well known for its natural diversity, game reserves, and cultural heritage.

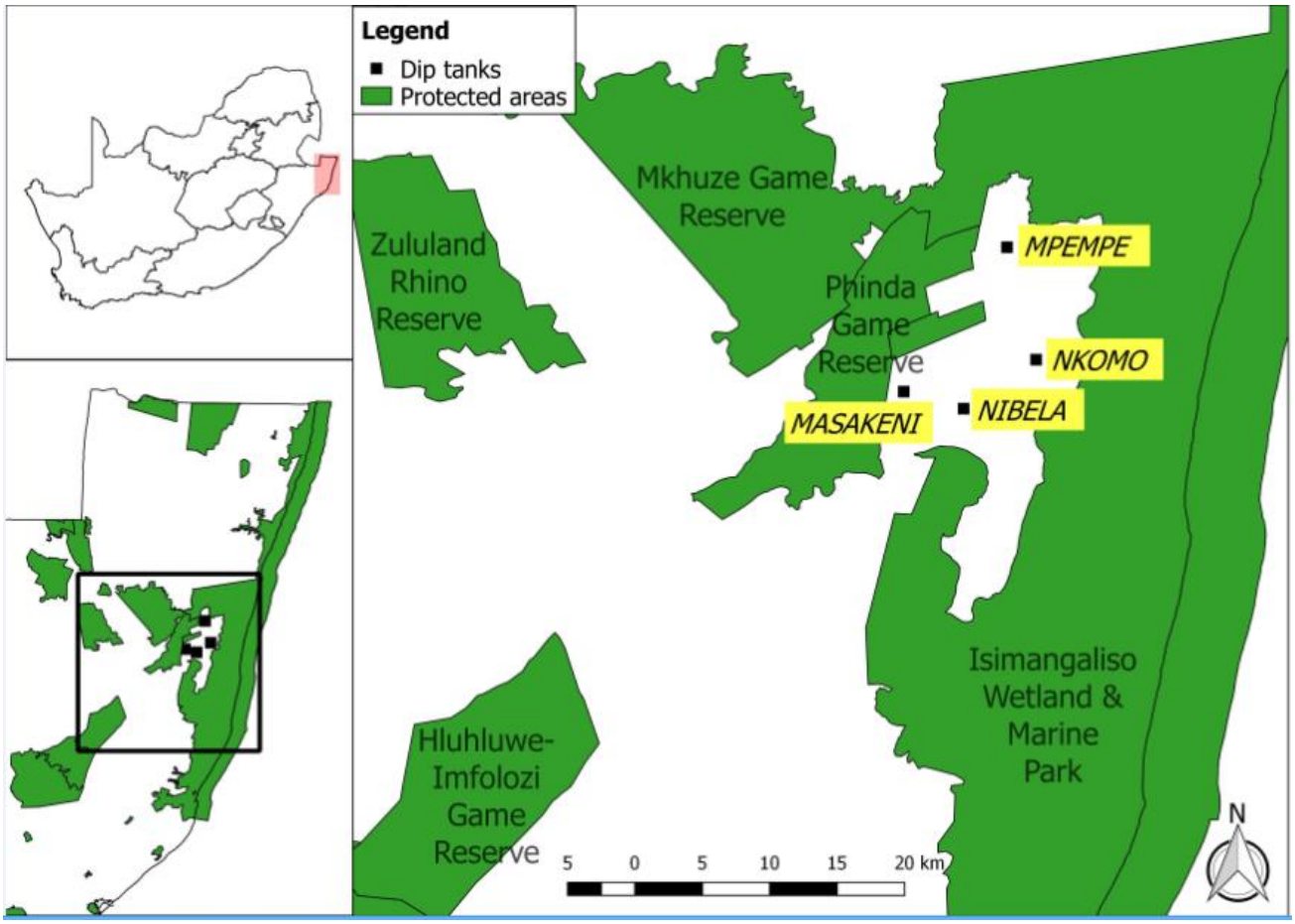


Figure 3.3: The figure represents the study area in northern KZN, uMkhanyakude district, Big 5 False Bay Municipality. The green shaded regions indicate protected areas. The dip tanks are indicated by a black square with the appropriate label highlighted in yellow.

3.2.2 Introduction of study to stakeholders

The Department of Agriculture and Rural Development (KZN) which is the custodian of livestock and wildlife health in the area was approached and informed of the study. Veterinary officials responsible for the dip tanks of the study were officially informed and the study was explained to them. Each of the dip tanks of the study was visited. Farmers and animal health technicians were told about the study prior to the commencement. During these meeting, farmers and technicians had an opportunity to ask questions and seek clarification on any aspect of the study that they did not understand.

3.2.3 Selected dip tanks

Four dip tanks were selected in the study area: Masakeni, Nibela, Nkomo and Mpempe. The dip tanks are located a few kilometres apart from each other in different villages. Previous studies had been conducted in the area and bovine TB had been found to be present in cattle (Munjere, 2017). All farmers were notified about the research project and asked to bring their cattle to their allocated dip tank for TB testing using the CITT. Farmers were given consent forms to sign indicating that the participation was voluntary.

3.2.4 Comparative intradermal tuberculin test

Restraining of animals

The cattle were directed into the cattle crush pen. A random selection of pregnant and lactating cattle was performed at all the dip tanks. Selected cattle were tied on either the horns or around the neck by rope and excessive force was avoided.

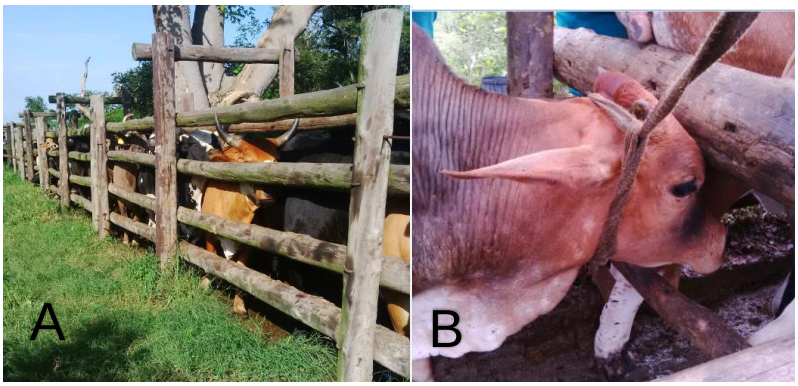


Figure 3.4: A. cattle being packed in a crush pen at a dip tank in Hluhluwe, KZN.B. Image of a heifer tied during preparation of performing the comparative intra-dermal tuberculin test.

Shaving of the animals and measurement of the skin thickness

The randomly selected cattle were restrained in a crush pen and two areas of almost 5cm x 4cm were shaved several cm's apart on the left side of the neck. Before shaving, the areas were palpated to ensure no existing body lumps or any sort of skin damage which would affect the interpretation of the results was present. The selected cattle were ear tagged for identification by an animal health technician from the Department of Agriculture and Rural Development in Hluhluwe.

The skin thickness of the selected regions where bovine tuberculin PPD and avian tuberculin PPD was to be injected were measured using a Hauptner caliper and the measurements were recorded.

Injection of bovine tuberculin PPD and avian tuberculin PPD

Bovine tuberculin PPD and avian tuberculin PPD were kept at 4-8°C in a cooler box containing frozen ice packs. Separate McLintock syringes were used for the application of bovine and avian tuberculin intradermally into the distal and cranial shaved sites, respectively.

Reading and interpretation of skin test results

After a period of 72 hours, the injected cattle were brought back to the cattle crush for the reading of the tuberculin test. The regions of injection were again palpated for swelling on both the injected sites by the same animal technician who injected the tuberculin. The swelling was measured using a calliper by the same animal technician and thereafter positive cattle where T- branded on the neck. Ear tags were removed from negative and avian reactors.

Negative reactions were characterised by adifference of the skin thickness between bovine and avian injection sites of 0 mm or less and a positive difference of the skin thickness in the increase between bovine and avian injection sites of 1 mm to 2 mm. Suspect reactions were characterised by a positive difference of the skin thickness in increase between bovine and avian injection sites of 3 to 4 mm. Positive reactions were characterised by a positive difference of the skin thickness in the increase between bovine and avian injection sites of 5 mm or more.

Identification of skin test positive cattle

Animals which were test positive and lactating or pregnant were recruited into the project. An ear tag which was labelled using a tag marker was inserted on all the cattle recruited. TB test positive cattle which were not lactating and not pregnant, were not included in the study.

3.2.5 Milk collection

Milk collection was done at dip tanks and homesteads while cattle were in the cattle pen. The collection was done longitudinally. An aseptic procedure was followed whereby 70% of ethanol was used to rinse the hands of the person responsible for milking as well as rinsing the udder to minimise the possibility of contamination. Sterile 50 ml tubes were used for collection of the milk. For each animal, one sterile tube was used. The tubes were identified by labels and immediately placed in a cooler box, which contained frozen ice packs.

The collection was done in March 2017, followed by a collection in June and October 2017. The collection of samples in October was done from as many available cows as possible on a daily basis followed by weekly basis as indicated in Figure 3.5. The collection in October was different because it served to determine if the cattle were shedding on a monthly basis. The following round of collections was done in December 2017 and finally the last collection in March 2018. Milk samples were placed into a portable freezer. The samples were transported to the University of Pretoria, Faculty of Veterinary Sciences and kept in a -20 freezer within the BSL2+ laboratory until they were processed for further analysis.

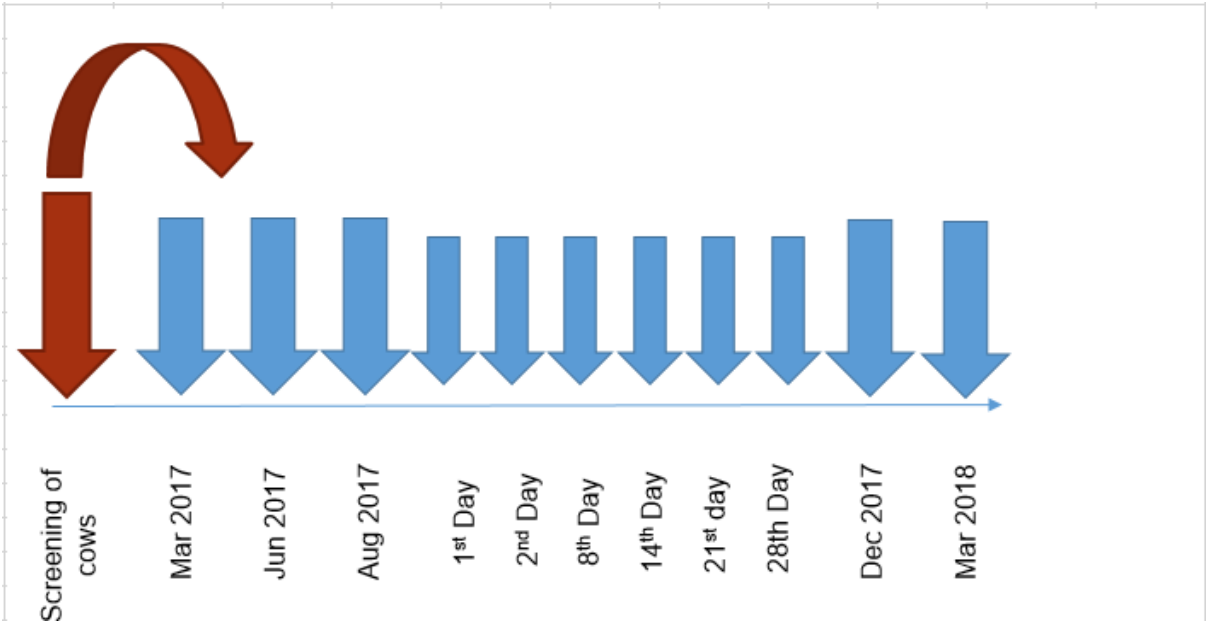


Figure 3.5 Sampling time frame indicating the months and days on which milk collection was performed. The arrow in the diagram illustrates that following screening of the cows, milk samples were collected on the specific dates as explained above on 3.2.5.

3.2.7 Laboratory analyses

Preparations of milk before processing

Milk tubes were removed from the -20 freezer in BSL2+ laboratory and the milk samples were allowed to defrost at room temperature. The tubes were mixed vigorously to allow even distribution of microorganisms in the samples. Using the biosafety level 2 laboratory, 10 ml of milk was removed from each milk sample tube and added into a sterile 50 ml tube. The tubes were centrifuged at 1816 g for 10 minutes at 4°C.

DNA extraction from milk samples

DNA was extracted using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, South Africa). The extraction and purification was performed following the manufacturer's protocol.

3.2.8 Decontamination with hydrochloric acid

The milk samples (10 ml) previously used for DNA extraction were treated with 10 ml of 2% HCl and incubated for 10 minutes at room temperature. Samples were centrifuged at 2465x g for 10 minutes at 4°C. Following centrifugation, the supernatants were discarded and pellets were washed with 10 ml distilled sterile water and the suspensions were shaken vigorously. The neutralised suspensions were centrifuged at 2465x g for 10 minutes at 4°C. Supernatants were discarded into a container containing disinfectant (10% Corox). Pellets were homogenised with an inoculation loop before inoculating on LJ pyruvate slopes.

3.2.9 Inoculation on LJ pyruvate slopes and monitoring

Löwenstein–Jensen medium with pyruvate

For each milk sample, two slopes of LJ pyruvate were used for inoculation using a sterile inoculating loop. The samples were incubated at 37°C for a period of 10 weeks. Monitoring of the samples was done on weekly basis for observation of colony growth of the target organism and contamination. Sub-culturing of the isolates was performed for propagating the isolate for further analysis when slopes were partially contaminated or partially liquefied and it was possible to salvage the isolate.

Mycobacteria Growth Indicator Tube (MGIT) with pyrazinamide

Due to the fast growth of NTM observed on LJ pyruvate inhibiting the growth of slow mycobacteria such as *M. bovis,* the MGIT supplemented with 100 gm/ml pyrazinamide was used to test whether it would inhibit the growth of the specific NTM species isolated from milk. For each milk sample, two tubes of the MGIT were used for inoculation using a sterile inoculating loop. The samples were incubated at 37°C in the BD Bactec MGIT 320. The machine was monitored on daily basis for observation of growth within the tube.

3.2.10 Polymerase chain reaction (PCR) for detection of mycobacteria

DNA amplification for heat shock protein using primers TB 11 and TB 12

A PCR master mix of 19 µl was prepared as follows: 12.5 µl Dream Taq polymerase, 0.5 µl of each primer (20 µm) [(Primer TB11 F (5'-ACC AAC GAT GGT GTG TCC AT-3'), Primer TB12 R (5'-CTT GTC GAA CCG CAT ACC CT -3')] and 5.5 µl of nuclease-free water (Thermo Fisher Scientific, South Africa). From each sample, 6 µl of DNA was added to a separate PCR tube containing 19µl master mixture in the PCR laboratory. Each sample was tested in duplicate. The mix was amplified on a thermal cycler (Veriti 96 well) with a heated lid, preheated to 105°C. The thermal cycling program used was: 45 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, final extension at 72°C for 10 minutes. The amplified DNA was held at 4°C (Telenti et al., 1993). The target DNA has a product size of 439bp.

DNA amplification for detection of MPB70 gene using primers TB 1 and TB 2

A PCR master mix of 19 µl, was prepared in the clean laboratory as follows: 12.5 µl Dream Taq polymerase, 0.5 µl of each primer (20 µm) [(Primer TB1 F (5'- GAA CAA TCC GGA GTT GAC AA-3'), Primer TB 2 R (5'- AGC ACG CTG TCA ATC ATG TA-3')] and 5.5 µl of nuclease-free water (Thermo Fisher Scientific, South Africa). From each sample, 6 µl of DNA was added to a separate PCR tube containing 19 µl master mixture in the PCR laboratory. Each sample was tested in duplicate. The mix was amplified on a thermal cycler (Veriti 96 well) with a heated lid, preheated to 105°C. The thermal cycling program was used following a protocol by Cousins: 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 30 seconds, 62°C for 3 minutes and 75°C for 3 minutes, no final extension. The amplified DNA was held at 4°C (Cousins et al., 1991, Cousins et al., 1992). The target DNA has a product size of 372bp.

DNA amplification to detect the regions of difference (RD4)

A PCR master mix of 19 µl, was prepared in the clean laboratory as follows: 12.5 µl Dream Taq polymerase, 0.4 µl of each primer (20 µm) [(5'- ATG TGC GAG CTG AGC GAT G- 3'), (5'- TGT ACT ATG CTG ACC CAT GCG-3'), (5'- AAA GGA GCA CCA TCG TCC AC-3')] and 5.3 µl of nuclease free water (Thermo Fisher Scientific, South Africa). From each sample, 6 µl of DNA was added to a separate PCR tube containing 19 µl master mixture in the PCR laboratory, with each sample tested in duplicate. The mix was amplified on a thermal cycler (Applied Biosystems Veriti 96 well) with a heated lid, preheated to 105°C. The thermal cycling program was used at: 94°C for 15 minutes, 45 cycles of denaturation at 94°C for 30 seconds, 62°C for 3 minutes, 75°C for 3 minutes, with no final extension. The amplified DNA was held at 4°C (Warren et al., 2006). The target DNA has a product size of 268bp.

Analysis of PCR products

An agarose gel was prepared by dissolving 2x g of agarose (agarose LE, Lasec) per 100 ml of 1 x TAE Buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3). A microwave oven was used to dissolve the solution. Ethidium bromide (3 µl) was used as the staining dye. The amplified samples were prepared for loading onto the gel by mixing 10 µl of the amplified samples with 2 µl of loading dye (name? Inqaba Biotechnical Industries, SA). A 100 bp DNA ladder (Quick-Load® ladder) (Inqaba Biotechnical Industries, SA) was prepared by mixing 1 µl of DNA ladder with 9 µl of water and 2 µl of gel loading buffer and loaded into the wells. Each well on the gel was loaded with 10 µl of the sample mixture. The gel ran at 120 volts for 1 hour. The gel was photographed under UV light by a molecular imager (ChemiDOC^{TM}XRS+).

3.11 Limit of detection of *M. bovis* using conventional PCR (cPCR)

M. bovis was used for serial dilutions. The concentration of the organism was detected using the automated cell counter (1 x 10^8 CFU/ml). 1 ml of the solution was added into 9 ml of pasteurised milk. A serial dilution of the solution was performed to the dilution factor of 10^0 cells/ ml. The PureLink Genomic DNA Mini Kit was used to extract DNA from the samples. The DNA samples were screened for Mycobacteria using cPCR. This was of importance in a context of determining the limit of the detecting *M. bovis* in milk using cPCR.

3.12 Phenotypic characterisation of nontuberculous mycobacteria

Raw sequences were assembled and edited using CLC Genomics Workbench version 8.0 (http://www.clcbio.com). The assembled and edited sequences were aligned using MAFFT version 7 online version. Aligned sequences were viewed and checked manually using Mega version 6.0. Phylogenetic tree was constructed using Mega version 7 applying the default parameters with 1000 bootstraps.

3.13 Animal ethics and consent forms

An animal ethics application form was submitted to the Animal Ethics Committee (AEC) of the University of Pretoria to obtain approval to use animals for research or testing purposes. An approval from July 2017 to July 2018 was granted to work on 100 cattle (Project number V074-17). Permission from the National Director of Animal Health, South Africa, to do research under Section 20 of the Animal Diseases Act (Act 35 of 1984) was also obtained (Shown in the appendix).

A consent form was prepared for the farmers with bovine TB herds. Consent was obtained from the farmers to use their animals in the study. Before they agreed, the project was fully explained to them to understand the study and their role in the study. Farmers were allowed to ask more questions. The study area was in northern KZN where most residents communicate in Zulu. Thus, the consent form was translated to Zulu.

CHAPTER 4

RESULTS

Introduction

This chapter reports the results from experiments that were carried out to compare decontamination methods for the primary isolation of *Mycobacterium spp*. from milk and to monitor shedding of *Mycobacterium bovis* and nontuberculous mycobacteria from bovine TB positive cattle.

Optimisation of the method for isolating *M. bovis* from milk is of particular importance in the context of higher bacterial contamination often found in milk (Bradner, L.K., 2013), that is more different bacteria are found within the milk. On culture, *M. bovis* takes over 4-6 weeks to grow hence regarded as slow growing mycobacteria while most contaminating bacteria from the environment or pathogens potentially colonising the udder generate faster. An organism such as *E. coli* has a generation time of 20 minutes. With slower growth rates, *M. bovis* would be out-competed by such bacteria. For this reason, an optimised decontamination method for milk is of use as to process the communal unpasteurised milk before culture.

4.1. Comparison of decontamination methods for the primary isolation of *Mycobacterium spp*. from milk

.4.1.1 Limit of detection for *M. fortuitum* in pasteurised milk

Five decontamination methods were performed on pasteurised milk inoculated with *M. fortuitum.* This Mycobacterium species was preferably used since it is considered to be a fast growing mycobacterium (4-5 days). The decontamination was performed on pasteurised milk inoculated with *M. fortuitum* to evaluate the sensitivity of mycobacteria in the decontaminants (Figure 4.1-4.3).

The limit of detection was described as the lowest number of *M. fortuitum* observed on the LJ pyruvate slopes following each decontamination protocol. As shown in Figure 4.1 below: on the third day of incubation, the limit of the detection of *M. fortuitum* following decontamination with 2% HCl and 5% OA was $1x10^3$ CFU/ml and $1x10^4$ CFU/ml respectively. After 2 months of incubation, the limit of detection was reduced to $1x10^1$ CFU/ml and $1x10^2$ CFU/ml respectively.

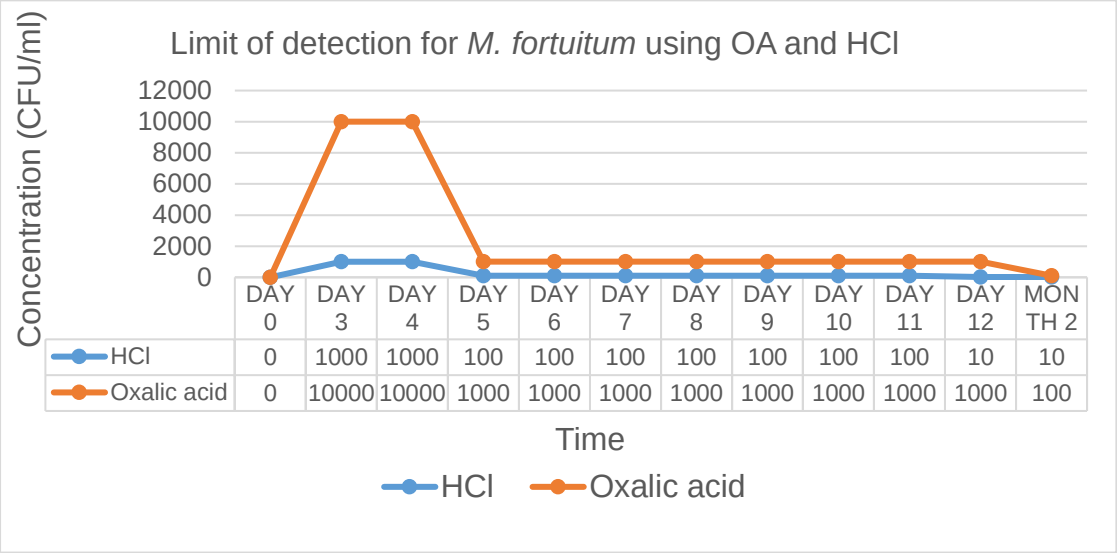


Figure 4.1 Limit of detection for *M. fortuitum* using 2% hydrochloric acid and 5% oxalic acid. The y-axis and x-axis represents concentration and time respectively. LJ slopes were monitored from day 0 to month 2 following inoculation.

The limit of detection for *M. fortuitum* following decontamination with 1% CPC and 4% NaOH on the third day of incubation was $1x10^5$ CFU/ml and $1x10^6$ CFU/ml, respectively. After 2 months of incubation, the limit of detection remained $1x10^5$ CFU/ml for CPC and reduced to $1x10^4$ CFU/ml for NaOH. The limit of detection of *M. fortuitum* following decontamination with HS-SH was $1x10^6$ CFU/ ml and $1x10^5$ CFU/ml on the third day and last day of incubation respectively (Figure 4.2).

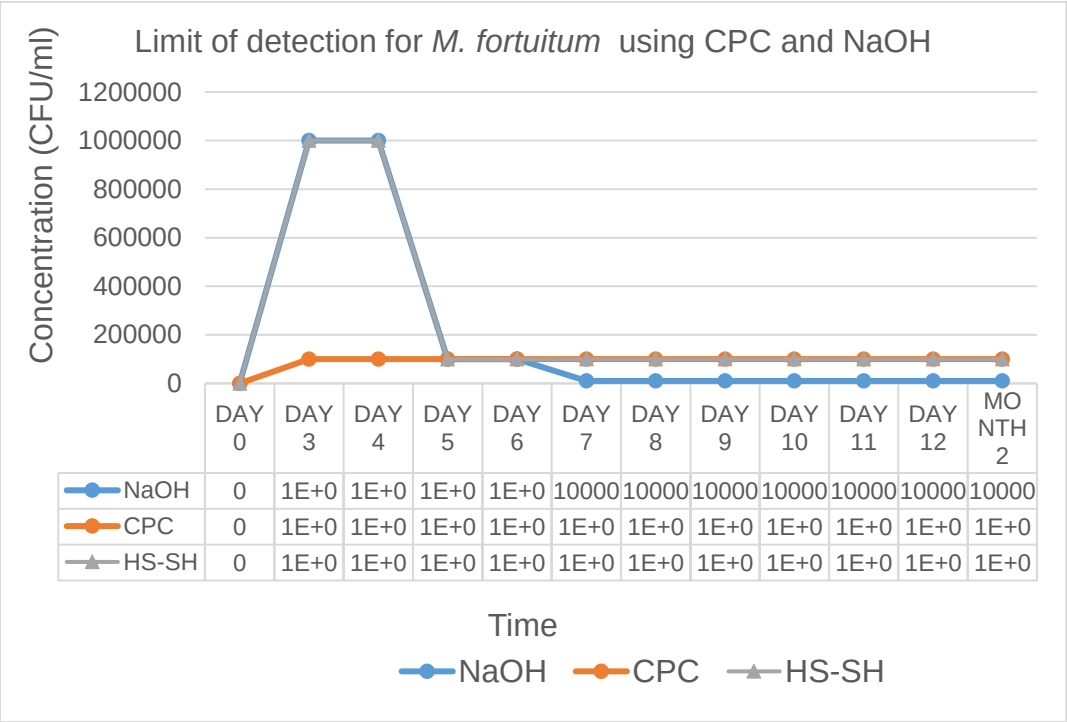


Figure 4.2 Limit of detection for *M. fortuitum* using 4% sodium hydroxide, Novel hypertonic-saline hydroxide and 1% cetylpyridinium chloride. The y-axis and x-axis represents concentration and time respectively. LJ slopes were monitored from day 0 to month 2 following inoculation.

4.1.2 Efficacy of decontamination methods on contaminated milk

No growth of contaminants was observed on slopes inoculated using milk samples decontaminated with 2% HCl, 5% OA, 4% NaOH and HS-SH (7% NACl and 4% NaOH). However, there was growth on slopes from milk samples decontaminated using 1% CPC, (18/32 (56%)). The details of the results are presented below in Table 4.1.

Table 4.1 Efficacy of decontamination methods on milk contaminated with *E. coli* and *S. aureus*

Decontamination Methods	No. of milk samples	No. of slopes inoculated	No. of slopes contaminated	Positive control	Negative control
2% HCL	16	32	0	*M. fortuitum* confluent growth	No growth
5% OA	16	32	0	*M. fortuitum* confluent growth	No growth
4% NaoH	16	32	0	*M. fortuitum* confluent growth	No growth
7% NACl and 4% NaOH	16	32	0	*M. fortuitum* confluent growth	No growth
1% CPC	16	32	18	Contaminated	No growth

4.2 Monitoring of *Mycobacterium bovis and nontuberculous mycobacteria* from cow's milk

A total number of 176 cattle from all the dip tanks were injected with tuberculin for the CITT. The number of cattle depended on the farmers who were willing to participate in the project. However, only 149 cattle were brought back to the dip tanks for reading of the CITT on day 3 of the test (Table 4.2).

Table 4.2 Results of Comparative Intradermal Tuberculin testing (CITT) of cattle at four dip tanks

CITT TESTING	NUMBER OF CATTLE
DAY 1- TUBERCULIN INJECTION	176
DAY 3- READING OF CITT	149
BOVINE TB POSITIVE CATTLE	36
BOVINE TB NEGATIVE CATTLE	98
BOVINE TB SUSPECT CATTLE	15

A total of 36 cattle tested positive on CITT. The distribution of positivity was as follows: Nibela 14, Mpempe 13, Nkomo 8 and Masakeni 1. The distribution is represented as percentages in the pie chart below in figure 4.3. All TB positive lactating and pregnant cattle were included in the study. The cattle produced different amount of milk during sampling, ranging from 5 ml to at least 50 ml.

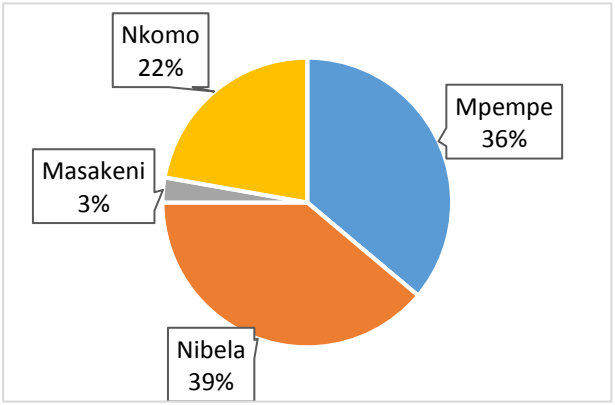


Figure 4.3 Distribution of bovine TB positive cattle (n=36) among four dip tanks

Limit of detection for *Mycobacterium bovis* on cPCR

The primary aim of this work (objective 1) was to optimise the detection of *M. bovis.* The limit of detection for *M. bovis* on cPCR was part of the optimisation of decontamination methods. The procedure set up as described in section 3.11 above. Table 4.3 summarises the limit of detection of *M. bovis* on cPCR. Of the 7 serially diluted samples, cPCR detected *M. bovis* cells from DNA with concentration of $1x10^2$ CFU/ml to $1x10^6$ CFU/ml. Samples below $1x10^2$ CFU/ml could not be detected using the cPCR technique. As illustrated in figure 4.4, lane 10^0 and 10^1 shows no amplification of *M. bovis* while 10^2-10^6 showed amplification of the organism.

Table 4.3 Detection limit of *M. bovis* cells on conventional PCR from the different dilutions

Sample ID	Cells concentration per 6 ul	Results
7	1 000 000	Detected
6	100 000	Detected
5	10 000	Detected
4	1 000	Detected
3	100	Detected
2	10	Not detected
1	1	Not detected

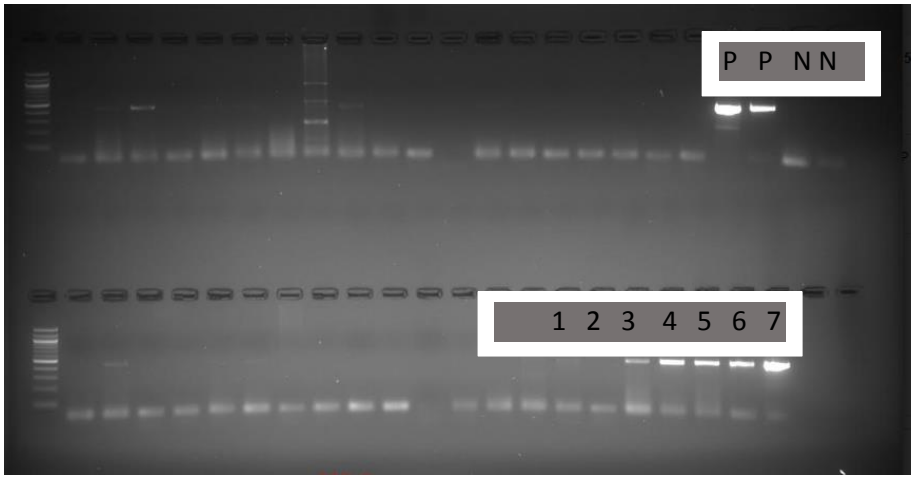


Figure 4.4. Gel electrophoresis picture of a 2% agarose gel. Lane 1 (10^1 CFU/ml) and 2 (10^2 CFU/ml) failed to show amplification product of 439 bp. Lane 3-7 (10^3 CFU/ml -10^7 CFU/ml) showed amplification product of 439 bp for mycobacteria. P and N represents the positive and negative control. The other samples are part of the field milk samples, which were analysed for detection of mycobacteria, however very faint bands were observed.

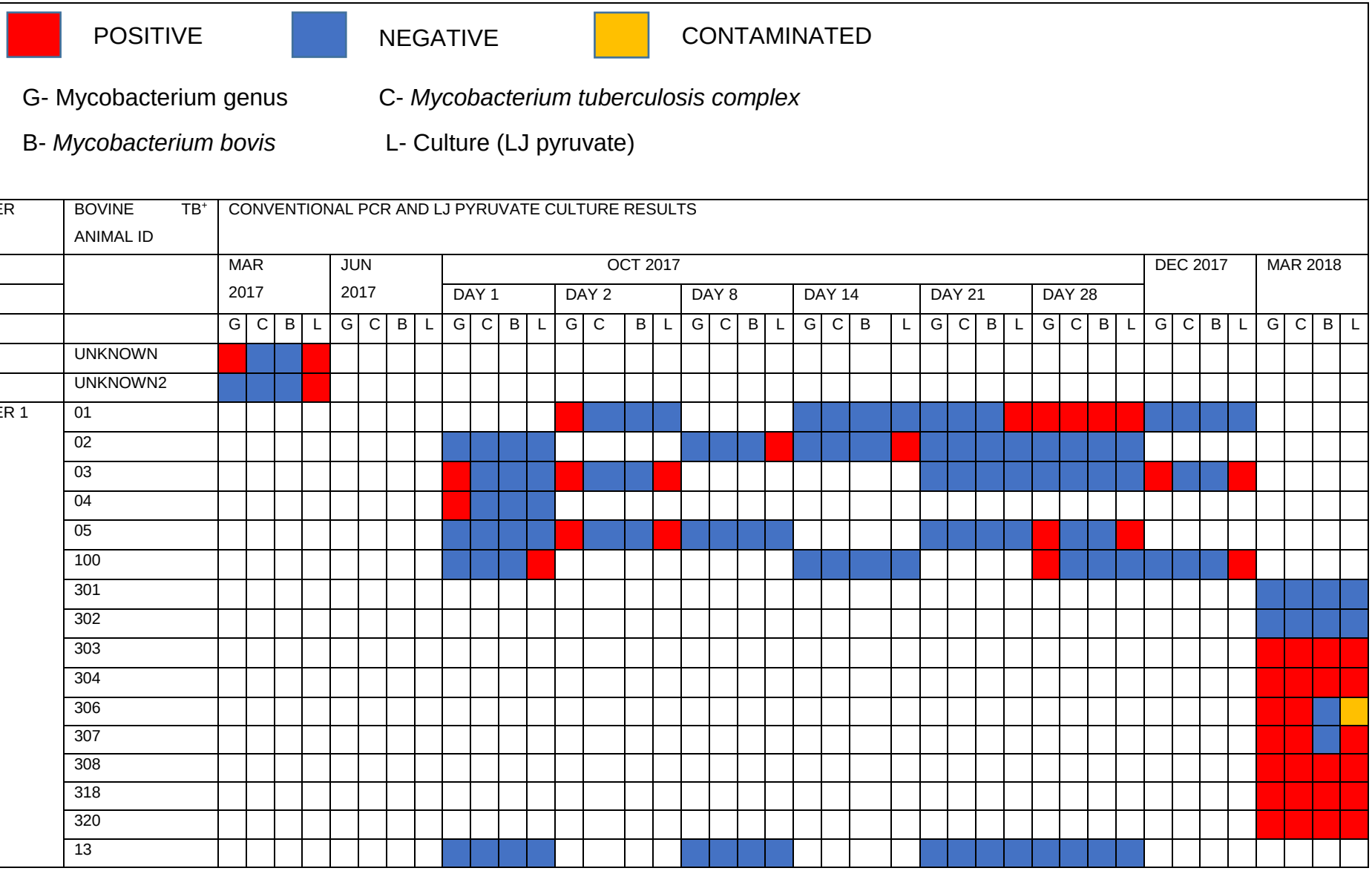
Mycobacterium species culture of milk samples on LJ pyruvate medium

Table 4.4 represents the results from culture of the field milk samples. Milk samples showed predominance rapid growth of NTM. Between the first and second week of incubation, scanty isolates were observed on LJ pyruvate slopes. Of these 131 slopes cultured mycobacterium isolates were demonstrated on 79 slopes. These were seen as overlapping small convex yellow, orange mucoid and hard light yellow colonies. Co-infection with different *Mycobacterium* species was confirmed in 12 LJ pyruvate slopes of which 2 different colonies were observed.

Mycobacterium species identification (cPCR)

DNA was directly extracted from raw milk samples for identification of *Mycobacterium* species. Of 131 samples tested on cPCR, 60 samples were identified as *Mycobacterium genus* by detecting the 65KDa which yields a 439bp fragment. Of these 60, MTBC were identified from 19 samples by detecting MPB70 gene yielding a 372bp fragment and 13 samples were positive for *M. bovis* which detects the region of difference through 268bp fragment. As shown on table 4.4, 12 milk samples from 2018 were positive for *M. bovis*. However, only one milk sample from 2017 collection was *M. bovis* positive on cPCR.

Table 4.4 Milk samples collected and tested by cPCR and culture from March 2017 to March 2018. The white blocks on the table indicates the absence of the cattle at the day of collection.


POSITIVE
NEGATIVE
CONTAMINATED
G- Mycobacterium genus
C- Mycobacterium tuberculosis complex
B- Mycobacterium bovis
L- Culture (LJ pyruvate)
FARMER
BOVINE TB+ ANIMAL ID
CONVENTIONAL PCR AND LJ PYRUVATE CULTURE RESULTS
MAR 2017
JUN 2017
OCT 2017
DAY 1
DAY 2
DAY 8
DAY 14
DAY 21
DAY 28
DEC 2017
MAR 2018
G C B L
UNKNOWN
UNKNOWN2
FARMER 1
01
02
03
04
05
100
301
302
303
304
306
307
308
318
320
13

		MAR 2017				JUN 2017				OCT 2017																								DEC 2017				MAR 2018			
										DAY 1				DAY 2				DAY 8				DAY 14				DAY 21				DAY 28											
		G	C	B	L	G	G	C	B	L	G	G	C	B	L	G	G	C	B	L	G	G	C	B	L	G	G	C	B	L	G	G	C	B	L	G	G	C	B	L	G
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Culture of milk samples using MGIT pyrazinamide

Table 4.5 represents NTM and *M. bovis* growth on the MGIT pyrazinamide slopes and LJ pyruvate slopes. NTM growth was observed on both LJ pyruvate and MGIT pyrazinamide. However, no growth of *M. bovis* was observed. Of the 43 samples inoculated in the MGIT pyrazinamide, 39 tubes yielded positive results and 4 tubes were negative. These results demonstrated that the growth of rapid growing organisms was not inhibited by pyrazinamide.

Table 4.5 Summary of NTM and *M. bovis* growth on LJ pyruvate and MGIT Pyrazinamide

Culture method	No. of slopes/tubes cultured	No. of positive slopes/tubes	No. of negative slopes/tubes	No. of contaminated slopes/tubes
LJ pyruvate solid medium	131	79	50	2
MGIT pyrazinamide	43	39	4	0

Nontuberculous mycobacterium identification

The four isolates that were sequenced were representatives of all the mycobacterium cultures observed on LJ pyruvate slopes. Sequences were analysed and the phylogenetic tree was constructed using Mega version 7 (Molecular Evolutionary Genetics Analysis software version 7.0).

Sequenced analyses of the 16S rRNA gene sequences illustrated that isolates F01 and F03 shared 100 % sequence similarity with *Mycobacterium brasiliensis*, isolate F02 shared 100 % sequence similarity with *Mycobacterium sp. py137* and isolates F04 shared 100 % sequence similarity with *Mycobacterium sp. strain EPG1* (Figure 4.5). Sample F01 and F03 had different morphological characteristics; however they were both identified as *Mycobacterium brasiliensis.* This was due to samples incubated for a longer period of time hence changing colour and shape compared to the initial morphology observed.

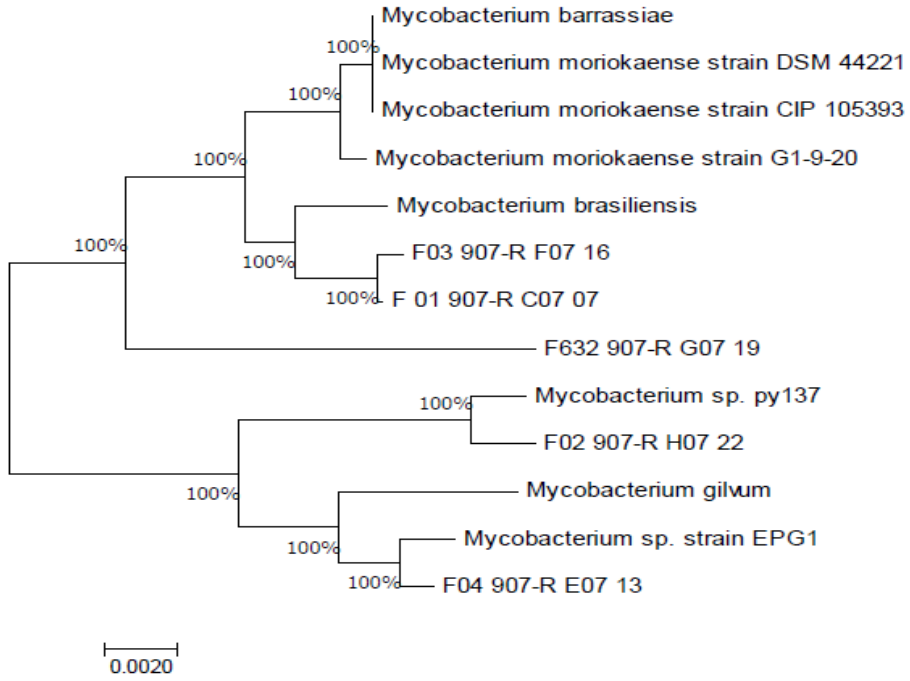

Figure 4.5 Phylogenetic relationships amongst species of mycobacteria identified in raw cattle milk. The assembled and edited samples were aligned using MAFF Version 7 online version.

Chapter 5

Discussion

5.1 Comparison of decontamination methods for isolation of *Mycobacterium fortuitum*

The present study was to compare decontamination methods for isolation of mycobacterial species and to monitor the shedding of *M. bovis* and NTM in cow's milk from a rural area. To obtain this, five decontamination methods where compared in both pasteurised milk and contaminated milk. Pasteurised milk was used to identify the limit of detection for *M. fortuitum* as a preferable mycobacterial organism due to its fast growing ability. Contaminated milk was used to detect the efficacy of the decontamination methods on the other organisms present in the milk. For the second objective, culture and conventional PCR were used for the detection of *M. bovis* and NTM within the milk samples.

Mycobacterial species include both fast and slow growing organisms, infecting both humans and animals. Slow growing mycobacteria include species such as *M. bovis* which grows on culture after 6-8 weeks. Whereas, fast growing mycobacteria such as *M. fortuitum* take 3-5 days to show growth on Löwenstein–Jensen medium following culture. A limited number of bacteriological studies have addressed the primary isolation of mycobacterial species from communal cow's milk. Most research studies are conducted on commercial milk samples rather than communal milk samples (Leite et al., 2003, Munjere, 2017). An increased difference in the microbiological quantity of communal and commercial milk samples has been observed in previous studies (Munjere, 2017).

Isolation of mycobacterial species from clinical and environmental samples has always been reported to be difficult due to different microorganisms being present in the samples. Protocols for culturing of mycobacterial species ought to be adjusted to incorporate conditions that will inhibit the growth of contaminating microorganisms but remain minimally lethal to mycobacterial species. The use of decontamination methods affects the number of mycobacterial organisms present within the milk samples. Therefore, this implies that samples which have a low concentration of mycobacterial species may result in failure to yield an isolate on culture. This may be due to the harsh treatment applied to the samples.

The microbiological quality of milk as a sample for mycobacterium culture can differ greatly depending whether it is collected from commercial or communal cattle. For this reason, it is easier to process commercial milk samples on culture since the risk of contaminated slopes is reduced. In contrast, during a pilot investigation, milk samples were collected from one of

the selected dip tanks in this project (Munjere, 2017). The study involved assessment of the microbiological quality of the milk samples from known bovine TB positive cattle herds. An overwhelming increase of organisms such as *E. coli* and *S. aureus* was detected in the milk samples. It was reported that 59% of the milk samples yielded 100 CFU/ml *E. coli* whereas others were reported as too numerous to count (Munjere, 2017). The decontamination method used in the standard SOP from tissues to prevent contamination and the failure to isolate *M. bovis* despite its detection by PCR. These findings prompted the optimisation of the method reported in the current study.

As shown from the spiking experiment, 2% HCl and 5% OA were the most suitable methods for isolation of mycobacterial species in our study. The period at which the slopes were incubated free from contamination proved that slow growing mycobacteria will grow unhindered when milk samples are treated with 2% HCl and 5% OA. The use of 2% HCl as decontamination method of choice on communal milk samples resulted in adequate results, whereby an insignificant number of 2 of 131 samples resulted in contaminated cultures.

This study highlighted significant differences between the five decontamination methods in milk in terms of minimum detection limit and efficacy. Treatment of milk samples using either 2% HCl for 10 minutes or 5% OA for 15 minutes at room temperature and incubation at 37°C, were shown to be superior to the other methods for decontaminating milk prior to culture for *M. fortuitum*. Oxalic acid is a strong dicarboxylic acid (organic compound). It is a colourless crystalline solid which dissolves completely in water. The 5% oxalic acid decontamination method is well known to be appropriate for sputum or respiratory samples from patients who are likely to be colonized with *Pseudomonas aeruginosa* (www.tbcare1.org>participant_manual, 2018). This method was modified and used for decontaminating milk samples in this study. A study in Brazil previously decontaminated milk samples using 5% OA and *M. bovis* and NTM were isolated from 23 of 128 milk samples (Leite et al., 2003).

Comparison of the decontamination method revealed that the limit of detection was highest when using HCl, followed by oxalic acid, NaOH and HS-SH. CPC had the lowest limit of detection and a cloudy layer was formed on the slopes which made it difficult to count the colonies of *M. fortuitum.* Isolates on LJ pyruvate slopes were observed at a subsequent time when milk samples were decontaminated with CPC compared to the other decontamination procedures. In contrast with our study, an increased recovery of *M. bovis* was observed by Medeiros when samples were decontaminated with 0.75% CPC, whereas the use of sodium hydroxide (NaOH) resulted in a reduced recovery of the organism (Medeiros et al., 2012). This

contrast may be due to the increased concentration of CPC used in this study which inhibits the growth.

The efficacy of the decontamination methods was evaluated on contaminated milk samples inoculated with *S. aureus*, *E. coli* and *M. fortuitum*. When samples were decontaminated with either 2% HCl, 4% NaOH, 5% OA and HS-SH, the contamination was totally inhibited on the LJ slopes incubated at 37ºC. Most chemical reagents used for sample decontamination have adverse effect on the isolation of mycobacterial species cultured, increasing the possibility of isolation however reducing the number of recovered colonies. Our results differs from other reports on the application of 1% CPC as a preferred technique for effective inhibitions of milk borne pathogens. According to literature, CPC is effective against many pathogens including *E. coli* (Dundee et al., 2001). However, in this study contamination was observed on the LJ slopes inoculated with milk samples following decontamination with 1% CPC.

A recent study in India compared the use of hypertonic-saline-sodium hydroxide method with modified Petroff's method (highly concentrated NaOH (4%)) (Srivastava et al., 2008). This study was conducted to recover *M. tuberculosis*. As reported by the researchers, modified Petroff's method is a feasible method for decontamination and concentration, however, it kills 60-70% of the mycobacteria (Chaudhary and Mishra, 2013). This may be a result of the highly concentrated NaOH used. The study reported an increased recovery of *M. tuberculosis* with HS-SH as a decontamination method compared to modified Petroff's method.

5.2 Detection of *Mycobacterium bovis* and non-tuberculous mycobacteria

Löwenstein–Jensen (LJ) pyruvate medium was used to culture mycobacteria from milk samples following decontamination. This culture medium was developed by Lowenstein who integrated Congo red and malachite green to inhibit growth of contaminants. LJ with pyruvate integrated pyruvic acid into LJ basal medium to stimulate the growth of *M. bovis* and *Mycobacteria* spp. except for *M. tuberculosis*. For this reason, the use of LJ pyruvate medium was carried out for isolation of *M. bovis* and NTM from bovine TB positive milk samples.

In this study, communal milk samples were centrifuged at 1816x g for 10 minutes at 4°C. Milk samples collected from bovine TB positive cattle were treated with 2% HCl and washed with distilled sterile water. No *M. bovis* were observed but abundant growth of a variety of NTM species was observed on the LJ pyruvate slopes.

LJ pyruvate slopes were incubated at 37°C. *M. bovis* are microaerophilic organisms that grow at ±37°C however NTM grow at different temperature. Low concentration of *M. bovis* were present in the milk samples however, its growth on medium was inhibited. A decrease in mycobacterium concentration may be a result of the low volumes of milk obtained from the cattle. Lack of proper diet due to drought may result in a reduced milk production and hence lower excretion rate.

Intermittent release during a short period post infection may have resulted in the low excretion of organism within the milk samples. A study conducted in Brazil evaluated the pattern of mycobacteria during the 15 days of milk collection. Their study indicated an intermittent and irregular character of *Mycobacterium* spp isolation. The growth of NTM on culture was more rapid compared to *M. bovis* (Pardo et al., 2001). In this study, a rapid growth of NTM was observed on the LJ slopes. A confluent (lawn-like) growth of NTM was seen at week three following inoculation. The growth of *M. bovis* at week four was then inhibited due to reduced nutrients and availability of space to generate.

Polymerase chain reaction (PCR) is a popular molecular biology technique in which DNA is replicated enzymatically without necessarily using living organisms. As reported by Sumiyah and colleagues, one specific technique cannot solely be used as a tool for identification of novel species of *Mycobacteria spp* (Sumiyah et al., 2017). The diversity of techniques and tests that are essential and the time required for a proper identification are the two main limitations to species level differentiation of mycobacteria.

By cPCR, we could identify NTM, MTBC and *M. bovis* DNA from milk samples. A limit of detection for identification of *M. bovis* on cPCR was performed. DNA present at a concentration of $1x10^2$ CFU/ml and $1x10^1$ CFU/ml failed to amplify. This may also be due to a limited efficacy of the extraction kit. This indicated that milk with low concentration of *M. bovis* will result as negative when amplified using cPCR. The use of real-time PCR may be more sensitive on samples with low concentration compared to cPCR.

Due to inability of isolating *M. bovis* on culture from all samples collected in 2017 and 2018, conventional polymerase chain reaction (cPCR) was used to directly detect the organism with increased sensitivity. The increase of sensitivity showed a low concentration of MTBC within the DNA when amplified on cPCR. A low concentration of DNA was observed in samples collected during the year 2017. On the agarose gel, very indistinct bands yielding 372bp fragment were observed when cPCR was amplified for identification of MTBC. However, definite bands yielding 372bp fragment were observed when samples were tested for *Mycobacterium* genus. The *Mycobacterium* genus includes both NTM and MTBC. Only 1 of 107 milk samples had specific band for *M. bovis* from the 2017 milk samples.

Milk samples collected in March 2018 yielded results different from those observed from the 2017 samples when subjected to cPCR. An increased number of milk DNA were strongly positive for *Mycobacterium* genus and MTBC. The detection rate of *M. bovis* on cPCR had also improved, more samples tested positive for *M. bovis* as compared to samples collected in 2017. This molecular method should be particularly useful for dysgonic slow growing mycobacteria, since diagnosis through culture is not assured due to growth data being difficult to obtain. The advantage of PCR compared to culture is the ability to detect non-living organisms whereas in culture non-living organisms will not generate. This was evidenced in this study when *M. bovis* was not isolated on culture, however it was identified on cPCR.

The inability to isolate *M. bovis* in 2017 was hypothesized to be due to the drought that was experienced in the study area at the time of collection. It had been reported in 2016 by the KZN cabinet that the uMkhanyakude district municipality was experiencing a crisis as the dry season kept attacking the nation (https:www.white elephant.co.za/blog/post/drought, 2016). Territories such as Mtubatuba, Hluhluwe, Jozini, Umhlabuyalingana and Hlabisa had particularly been affected by the severe drought. Particularly in Hluhluwe, the water level in dams were low at 22 percent.

In Brazil, isolation and identification of mycobacteria from livestock was performed in 128 milk samples (Leite et al., 2003). Only one milk sample was positive for *M. bovis*. NTM were identified in 9 of the pasteurised milk samples and 14 of the raw milk samples. Another study in Brazil sampled 802 milk, these were analysed for direct detection of *M. bovis* using real time PCR (da Silva Cezar et al., 2016). Only one milk sample tested positive for *M. bovis* and the identity was confirmed by sequencing. In the year 1998, Kazwala cultured 805 milk samples. 3.9% (31) were positive for *Mycobacteria* spp (Kazwala et al., 1998). An increased percentage of NTM (87%) was isolated whereas only two isolates were confirmed as *M. bovis.* These results correspond with our findings in terms of low identification of *M. bovis* in milk samples. This reflected a low detection of *M. bovis* within the milk samples with an increased detection of NTM. Our study showed that the isolation of NTM in communal milk is more common than that of *M. bovis,* especially in dry seasons. This may be due to a higher concentration of NTM in the milk samples compared to *M. bovis*. As a result, fast growing NTM outgrow the slow growing *M. bovis* on culture.

The 79 culture positive slopes were identified with growth of only NTM. Several reasons may have resulted in this observation. The use of 2% HCl on the field samples was successful in inhibiting the growth of contaminants present within the milk samples. The culture isolates on LJ pyruvate slopes had different colony morphology. Co-infection was observed in other milk samples, whereby two different isolates were identified in one slope. Co-infection with different

members of *Mycobacterium* spp is common in animals. This contribute more difficulties to the diagnostic process. The isolates were sequenced and found to be closely related to *Mycobacterium sp.* strain EPG1, *Mycobacterium sp*. py137 and *Mycobacterium brasiliensis*. These are known as non-pathogenic mycobacteria to humans. The isolation of non-pathogenic mycobacteria in cattle milk do not appear to pose a threat for human health but they merely complicate the isolation of slow growing mycobacteria.

The isolation of several other NTM in milk may, however, be of public health significance because others are found to be pathogenic to man, particularly in immunocompromised people. Infection of NTM in cattle may be transmitted through several routes including environmental transmission and consumption of milk. Drinking of raw and fermented milk is still practiced, particularly in developing countries. Although fermentation results in formation of lactic acid when the milk is soured and thus brings down the pH which inhibits numerous pathogenic microorganisms, mycobacterial species are known to survive for 14 days in fermented milk (Michel et al., 2015).

NTM can survive and replicate in the environment. Communal kraals are hardly ever cleaned to remove the faeces excreted by the cattle on a daily basis. Possible infection of cattle with NTM gaining entry to the udder through faeces is considered a reason for isolation of NTM from milk samples. During the milking process, sterile tubes were used for collection hence contamination of collecting tubes is not considered the cause. However contamination through the hands of individual's responsible for milking is possible because rinsing of hands with 70% alcohol may not have effectively cleaned the milker's hands.

The study showed that the shedding of NTM in milk is not affected by drought as compared to that of *M. bovis*. The low levels of milk production under drought conditions in the area which may have resulted in bad health condition of the cattle had no effect on the isolation of NTM. This was observed when samples collected from the same animals at different times of the year were found positive for NTM on culture. Samples monitored in 2017 showed growth of NTM from 62 of 107 samples, however, an increased isolation was observed in samples collected in the rainy season in March 2018, when 17 of 24 samples were reported positive for NTM growth on LJ pyruvate slopes.

In this study, all samples that were found positive for *M. bovis* on cPCR were positive for only NTM on culture. The inhibition of *M. bovis* growth may be a result of fast growing mycobacteria observed on the slopes or non-living *M. bovis* in the milk samples. For this study, the use of liquid media (MGIT) was used for culturing milk samples for growth of *M. bovis*. The MGIT tubes were supplemented with pyrazinamide antibiotic for inhibition of NTM growth. A total of 43 milk samples following decontamination were subjected to culture using the MGIT

pyrazinamide tubes. However, the use of pyrazinamide for inhibition of NTM growth was not successful. Even though NTM were not isolated from the MGIT tubes, detection of growth by the MGIT machine within the first week of culture suggested the presence of fast growing mycobacteria. This study showed that the NTM isolated from the communal milk samples are resistant to pyrazinamide, a drug used in the treatment of human TB.

Despite the low levels of *M. bovis* in the milk samples, the presence of the organism results in an increased risk of transmission to humans. This circumstantial evidence suggests low or intermittent shedding of the mycobacteria in cow's milk, and that repeated sampling of milk from the same positive may increase the rate of isolation. To achieve this, animals should be kept/ closed in an isolated place where pasture and water are available, and milk samples can be collected on daily basis for a period of time. A situation whereby researchers depend on farmers to bring their cattle to dip tanks for collection of milk samples has proved to be unreliable. This was because not all cattle recruited into the study were returned to the dip tanks when collection of milk was to be done, making the longitudinal collection from the same animal difficult.

Limitations of the study

The target of this study was to identify 100 bovine TB positive cattle from owners who gave consent to participate in the study. This number was expected to yield a sufficient number of lactating cows available for longitudinal sampling. In this study, a total number of 176 cattle were skin tested using the CITT, this was done at the dip tank. This included 57 from Mpempe dip tank, 45 from Nibela dip tank, 43 from Masakeni dip tank and 31 from Nkomo dip tank. A reduced number of 149 returned back to the dip tanks after 3 days to read the skin test. The reduced availability of cattle was due to other farmers not willing to participate in the project thus their cattle could not be recruited. The area was experiencing a severe and prolonged drought between the years 2015-2017. Most of the farmers were moving their cattle from their homesteads to areas with better access to pastures and availability of water. Due to this reason, several cattle could not return to the dip tanks for the skin test reading.

Animals could not be followed to their respective homes or kraals to read the test because the cattle did not return to their kraals for weeks or months and it would not have been possible to read the results of the skin test in the absence of a crush pen. Furthermore, farmers could not indicate where their cattle could be due to the movement from one place to the other.

The total number of 36 bovine TB positive cattle from all four dip tanks was identified. This included non-lactating, lactating and pregnant cattle. An increased number of bovine TB positive cattle were not lactating at that period. Cattle found to be lactating had little milk due

to calves drinking most of the milk in the morning before they were brought to the dip tanks. Pregnant bovine TB positive cattle were recruited into the study for collection of milk as soon as they gave birth which was almost towards the end of the milk collection period in December 2017.

Conclusion

Despite advances in control programs, bovine TB remains a major threat in animals and humans. The present study evaluated 5 decontamination methods for recovery of *M. fortuitum* and monitored the shedding of *M. bovis* and NTM in known infected cattle. The study showed that 2% HCl and 5% oxalic acid were preferred decontamination methods in terms of limit of detection and efficacy. NTM were isolated from milk samples, however no *M. bovis* was observed from culture. Inhibition of *M. bovis* growth on culture may be due to fast growing mycobacteria observed on LJ pyruvate slopes. In addition, this study provided significant information that proper sampling techniques are necessary to assure reduced contaminating bacteria prior to culture.

References

AGIZEW, T., BASOTLI, J., ALEXANDER, H., BOYD, R., LETSIBOGO, G., AULD, A., NYIRENDA, S., TEDLA, Z., MATHOMA, A. & MATHEBULA, U. 2017. Higher-than-expected prevalence of non-tuberculous mycobacteria in HIV setting in Botswana: Implications for diagnostic algorithms using Xpert MTB/RIF assay. *PloS one,* 12**,** e0189981.

ALBELDA, S. M., KERN, J. A., MARINELLI, D. L. & MILLER, W. T. 1985. Expanding spectrum of pulmonary disease caused by nontuberculous mycobacteria. *Radiology,* 157**,** 289-296.

ALIYU, G., EL-KAMARY, S. S., ABIMIKU, A. L., BROWN, C., TRACY, K., HUNGERFORD, L. & BLATTNER, W. 2013. Prevalence of non-tuberculous mycobacterial infections among tuberculosis suspects in Nigeria. *PloS one,* 8**,** e63170.

ALLIX-BÉGUEC, C., HARMSEN, D., WENIGER, T., SUPPLY, P. & NIEMANN, S. 2008. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. *Journal of Clinical Microbiology,* 46**,** 2692-2699.

ANDERSON, R. 1981. Population ecology of infectious diseases agents. *Theoretical Ecology***,** 318-355.

ANONYMOUS 2009. OIE Manual,Bovine tuberculosis. *World assembly of delegates of the OIE in***,** 7.

ANONYMOUS 2013. Department of Agriculture, Forestry and Fisheries, Bovine scheme manual 6. Https://www.nda.agric.za > vetweb > policy.

ANONYMOUS 2015. Review of Literature: Mycobacterium tuberculosis Genome Polymorphisms, Strain Diversity and Association with Diseases.

ANONYMOUS 2016. https:www.white elephanant.co.za/blog/post/drought.

ARANAZ, A., DE JUAN, L., MONTERO, N., SÁNCHEZ, C., GALKA, M., DELSO, C., ALVAREZ, J., ROMERO, B., BEZOS, J. & VELA, A. I. 2004. Bovine tuberculosis (Mycobacterium bovis) in wildlife in Spain. *Journal of Clinical Microbiology,* 42**,** 2602-2608.

ASHFORD, D., WHITNEY, E., RAGHUNATHAN, P. & COSIVI, O. 2001. Epidemiology of selected mycobacteria that infect humans and other animals. *Revue Scientifique et technique (International Office of Epizootics),* 20**,** 325-337.

ATKINS, B. L. & GOTTLIEB, T. 2014. Skin and soft tissue infections caused by nontuberculous mycobacteria. *Current opinion in infectious diseases,* 27**,** 137-145.

AYELE, W. Y. N. S. D. Z. J. W. M. G. P. I. 2004. Bovine tuberculosis:an old disease but a new threat to Africa. 8**,** 924-937.

BEMER, P., PALICOVA, F., RÜSCH-GERDES, S., DRUGEON, H. B. & PFYFFER, G. E. 2002. Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of Mycobacterium tuberculosis. *Journal of Clinical Microbiology,* 40**,** 150-154.

BIET, F. & BOSCHIROLI, M. L. 2014. Non-tuberculous mycobacterial infections of veterinary relevance. *Research in Veterinary Science,* 97**,** S69-S77.

BLOUIN Y, H. Y. S. C. F. M. V. R. D. C. C. G. M. P. L. K. P. 2012. Significance of the identification in the Horn of Africa of an exceptionally deep branching Mycobacterium tuberculosis clade.

BRADLEY, D. G., LOFTUS, R. T., CUNNINGHAM, P. & MACHUGH, D. E. 1998. Genetics and domestic cattle origins. *Evolutionary Anthropology: Issues, News, and Reviews: Issues, News, and Reviews,* 6**,** 79-86.

BROWN-ELLIOTT, B. A. & WALLACE, R. J. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clinical Microbiology Reviews,* 15**,** 716-746.

BUDDLE, B., DE LISLE, G., PFEFFER, A. & ALDWELL, F. 1995. Immunological responses and protection against Mycobacterium bovis in calves vaccinated with a low dose of BCG. *Vaccine,* 13**,** 1123-1130.

CARSTENSEN, M. & DONCARLOS, M. W. 2011. Preventing the establishment of a wildlife disease reservoir: a case study of bovine tuberculosis in wild deer in Minnesota, USA. *Veterinary Medicine International,* 2011.

CHAUDHARY, S. & MISHRA, B. 2013. Comparison of hypertonic saline-sodium hydroxide method with modified Petroff's method for the decontamination and concentration of sputum samples. *International Journal of Infection and Microbiology,* 2**,** 78-81.

CHE-ENGKU-CHIK, C. E. N., YUSOF, N. A., ABDULLAH, J., OTHMAN, S. S., SAID, M. H. M. & WASOH, H. 2016. Detection of tuberculosis (TB) using gold standard method, direct sputum smears microscopy, PCR, qPCR and electrochemical DNA sensor: A mini review. *Journal of Biochemistry, Microbiology and Biotechnology,* 4**,** 16-21.

CHIANG, C. Y., VAN WEEZENBEEK, C., MORI, T. & ENARSON, D. A. 2013. Challenges to the global control of tuberculosis. *Respirology,* 18**,** 596-604.

CLAEYS, W. L., CARDOEN, S., DAUBE, G., DE BLOCK, J., DEWETTINCK, K., DIERICK, K., DE ZUTTER, L., HUYGHEBAERT, A., IMBERECHTS, H. & THIANGE, P. 2013. Raw or heated cow milk consumption: Review of risks and benefits. *Food Control,* 31**,** 251-262.

COMER, L. 1994. Post mortem diagnosis of Mycobacterium bovis infection in cattle. *Veterinary Microbiology,* 40**,** 53-63.

CONTROL, C. F. D. 2012. Mycobacterium bovis (bovine tuberculosis) in humans. *Center for Disease Control and Prevention factsheets*.

COOVADIA, H., JEENA, P. & WILKINSON, D. 1998. Childhood human immunodeficiency virus and tuberculosis co-infections: reconciling conflicting data. *The International Journal of Tuberculosis and Lung Disease,* 2**,** 844-851.

COSIVI, O., GRANGE, J., DABORN, C., RAVIGLIONE, M., FUJIKURA, T., COUSINS, D., ROBINSON, R., HUCHZERMEYER, H., DE KANTOR, I. & MESLIN, F. 1998a. Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. *Emerging Infectious Diseases,* 4**,** 59.

COURCOUL, A., MOYEN, J.-L., BRUGERE, L., FAYE, S., HENAULT, S., GARES, H. & BOSCHIROLI, M.-L. 2014. Estimation of sensitivity and specificity of bacteriology, histopathology and PCR for the confirmatory diagnosis of bovine tuberculosis using latent class analysis. *PloS one,* 9**,** e90334.

COURTENAY, O., REILLY, L., SWEENEY, F., HIBBERD, V., BRYAN, S., UL-HASSAN, A., NEWMAN, C., MACDONALD, D., DELAHAY, R. & WILSON, G. 2006. Is Mycobacterium bovis in the environment important for the persistence of bovine tuberculosis? *Biology Letters,* 2**,** 460-462.

COUSINS, D. 2001. Mycobacterium bovis infection and control in domestic livestock. *Revue Scientifique et Technique-Office International des Epizooties,* 20**,** 71-85.

COUSINS, D., WILTON, S. & FRANCIS, B. 1991. Use of DNA amplification for the rapid identification of Mycobacterium bovis. *Veterinary Microbiology,* 27**,** 187-195.

COUSINS, D. V., WILTON, S., FRANCIS, B. & GOW, B. L. 1992. Use of polymerase chain reaction for rapid diagnosis of tuberculosis. *Journal of Clinical Microbiology,* 30**,** 255-258.

CRESWELL, J., RAVIGLIONE, M., OTTMANI, S., MIGLIORI, G. B., UPLEKAR, M., BLANC, L., SOTGIU, G. & LONNROTH, K. 2010. Tuberculosis and non-communicable diseases: neglected links, missed opportunities. *European Respiratory Journal***,** erj00843-2010.

CROSS, P. C., LLOYD-SMITH, J. O., JOHNSON, P. L. & GETZ, W. M. 2005. Duelling timescales of host movement and disease recovery determine invasion of disease in structured populations. *Ecology Letters,* 8**,** 587-595.

CUDAHY, P. & SHENOI, S. V. 2016. Diagnostics for pulmonary tuberculosis. *Postgraduate Medical Journal,* 92**,** 187-193.

DA SILVA CEZAR, R. D., LUCENA-SILVA, N., BATISTA FILHO, A. F. B., DE MELO BORGES, J., DE OLIVEIRA, P. R. F., LÚCIO, É. C., ARRUDA-LIMA, M., DE ASSIS SANTANA, V. L. & JUNIOR, J. W. P. 2016. Molecular detection of Mycobacterium

bovis in cattle herds of the state of Pernambuco, Brazil. *BMC Veterinary Research,* 12**,** 31.

DAFF 2012. Department of agriculture, forestry and fisheries. Cattle (bovine) TB.

DAVIS, B. J., LI, C. X. & NACHMAN, K. E. 2014. A Literature Review of the Risks and Benefits of Consuming Raw and Pasteurized Cow's Milk. *A response to the request from The Maryland House of Delegates' Health and Government Operations Committee. John Hopkins Report, Maryland, USA*.

DE GARINE-WICHATITSKY, M., CARON, A., KOCK, R., TSCHOPP, R., MUNYEME, M., HOFMEYR, M. & MICHEL, A. 2013. A review of bovine tuberculosis at the wildlife–livestock–human interface in sub-Saharan Africa. *Epidemiology & Infection,* 141**,** 1342-1356.

DE LA RUA-DOMENECH, R., GOODCHILD, A., VORDERMEIER, H., HEWINSON, R., CHRISTIANSEN, K. & CLIFTON-HADLEY, R. 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. *Research in Veterinary Science,* 81**,** 190-210.

DE LISLE, G. W., MACKINTOSH, C. & BENGIS, R. 2001. Mycobacterium bovis in free-living and captive wildlife, including farmed deer. *Revue Scientifique et Technique-Office International des Epizooties,* 20**,** 86-111.

DE VOS, V., BENGIS, R. G., KRIEK, N. & MICHEL, A. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (Syncerus caffer) in the Kruger National Park, South Africa. *The Onderstepoort Journal of Veterinary Research,* 68**,** 119.

DELAHAY, R., DE LEEUW, A., BARLOW, A., CLIFTON-HADLEY, R. & CHEESEMAN, C. 2002. The status of Mycobacterium bovis infection in UK wild mammals: a review. *The Veterinary Journal,* 164**,** 90-105.

DEPARTMENT OF AGRICULTURE, F. A. F. 2012. Cattle (bovine) TB.

DRISCOLL, J. R., MCGARRY, M. A. & TABER, H. W. 1999. DNA typing of a nonviable culture of Mycobacterium tuberculosis in a homeless shelter outbreak. *Journal of Clinical Microbiology,* 37**,** 274-275.

DUBOS, R. J. & DUBOS, J. 1952. *The white plague: tuberculosis, man, and society*, Rutgers University Press.

DUNDEE, L., GRANT, I., BALL, H. & ROWE, M. 2001. Comparative evaluation of four decontamination protocols for the isolation of Mycobacterium avium subsp. paratuberculosis from milk. *Letters in Applied Microbiology,* 33**,** 173-177.

EGBE, N., MUWONGE, A., NDIP, L., KELLY, R., SANDER, M., TANYA, V., NGWA, V. N., HANDEL, I., NOVAK, A. & NGANDALO, R. 2016. Abattoir-based estimates of mycobacterial infections in Cameroon. *Scientific Reports,* 6**,** 24320.

EGBE, N., MUWONGE, A., NDIP, L., KELLY, R., SANDER, M., TANYA, V., NGWA, V. N., HANDEL, I., NOVAK, A. & NGANDALO, R. 2017. Molecular epidemiology of Mycobacterium bovis in Cameroon. *Scientific Reports,* 7**,** 4652.

EL IDRISSI, A. & PARKER, E. 2012. Bovine tuberculosis at the animal-human-ecosystem interface. *EMPRES Transboundary Animal Diseases Bulletin,* 40**,** 2-11.

ERASMUS, J. J., MCADAMS, H. P., FARRELL, M. A. & PATZ JR, E. F. 1999. Pulmonary nontuberculous mycobacterial infection: radiologic manifestations. *Radiographics,* 19**,** 1487-1503.

ESFAHANI, B. N., YAZDI, H. R., MOGHIM, S., SAFAEI, H. G. & ESFAHANI, H. Z. 2012. Rapid and accurate identification of Mycobacterium tuberculosis complex and common non-tuberculous mycobacteria by multiplex real-time PCR targeting different housekeeping genes. *Current Microbiology,* 65**,** 493-499.

FALKINHAM, J. O. 2002. Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine,* 23**,** 529-551.

FALZON, D., MIRZAYEV, F., WARES, F., BAENA, I. G., ZIGNOL, M., LINH, N., WEYER, K., JARAMILLO, E., FLOYD, K. & RAVIGLIONE, M. 2014. Multidrug-resistant tuberculosis around the world: what progress has been made? *European Respiratory Journal***,** erj01018-2014.

FERRARA, G., MURRAY, M., WINTHROP, K., CENTIS, R., SOTGIU, G., MIGLIORI, G. B., MAEURER, M. & ZUMLA, A. 2012. Risk factors associated with pulmonary tuberculosis: smoking, diabetes and anti-TNFα drugs. *Current Opinion in Pulmonary Medicine,* 18**,** 233-240.

FRANCIS, J., SEILER, R., WILKIE, I., O'BOYLE, D., LUMSDEN, M. & FROST, A. 1978. Sensitivity and specificity of various tuberculin tests using bovine PPD and other tuberculins. *Veterinary Record*.

GARBER, K. 2008. Drinking it raw-or not. Health officials face off with farmers over unpasteurized milk.

GILLESPIE, I., ADAK, G., O'BRIEN, S. & BOLTON, F. 2003. Milkborne general outbreaks of infectious intestinal disease, England and Wales, 1992–2000. *Epidemiology & Infection,* 130**,** 461-468.

GOODCHILD, A. & CLIFTON-HADLEY, R. 2001. Cattle-to-cattle transmission of Mycobacterium bovis. *Tuberculosis,* 81**,** 23-41.

GOPINATH, K. & SINGH, S. 2010. Non-tuberculous mycobacteria in TB-endemic countries: are we neglecting the danger? *PLoS neglected tropical diseases,* 4**,** e615.

GORMLEY, E., DOYLE, M., FITZSIMONS, T., MCGILL, K. & COLLINS, J. 2006. Diagnosis of Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam®) assay. *Veterinary Microbiology,* 112**,** 171-179.

GOULD, L. H., MUNGAI, E. & BARTON BEHRAVESH, C. 2014. Outbreaks attributed to cheese: differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. *Foodborne Pathogens and Disease,* 11**,** 545-551.

GRANGE, J. 2001. Mycobacterium bovis infection in human beings. *Tuberculosis,* 81**,** 71-77.

GRANGE, J. M., YATES, M. D., KANTOR, I. N. D. & ORGANIZATION, W. H. 1996. Guidelines for speciation within the Mycobacterium tuberculosis complex.

GRIFFIN, J. M., MARTIN, S. W., THORBURN, M. A., EVES, J. A. & HAMMOND, R. F. 1996. A case-control study on the association of selected risk factors with the occurrence of bovine tuberculosis in the Republic of Ireland. *Preventive Veterinary Medicine,* 27**,** 217-229.

GRIFFITH, D. E., AKSAMIT, T., BROWN-ELLIOTT, B., CATANZARO, A., DALEY, C., GORDIN, F., HOLLAND, S. M., HORSBURGH, R., HUITT, G., IADEMARCO, M. F., ISEMAN, M., OLIVIER, K., RUOSS, S., VON REYN, C. F., WALLACE, R. J. & WINTHROP, K. 2007. An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. *Am J Respir Crit Care Med,* 175**,** 367-416.

HAICAN, L., LULU, L. & YI, J. 2016. Identification of Species of Nontuberculous Mycobacteria Clinical Isolates from 8 Provinces of China," BioMed Research International.

HALSTROM, S., PRICE, P. & THOMSON, R. 2015. Review: Environmental mycobacteria as a cause of human infection.

HASHEMI-SHAHRAKI, A., BOSTANABAD, S. Z., HEIDARIEH, P., TITOV, L. P., KHOSRAVI, A. D., SHEIKHI, N., GHALAMI, M. & NOJOUMI, S. A. 2013. Species spectrum of nontuberculous mycobacteria isolated from suspected tuberculosis patients, identification by multi locus sequence analysis. *Infection, Genetics and Evolution,* 20**,** 312-324.

HEITZMAN, E. R. 1978. Diagnosis of Diseases of The Chest. *Radiology,* 128**,** 300-300.

HEYDERMAN, R., GOYAL, M., ROBERTS, P., USHEWOKUNZE, S., ZIZHOU, S., MARSHALL, B., MAKOMBE, R., VAN EMBDEN, J., MASON, P. & SHAW, R. 1998. Pulmonary tuberculosis in Harare, Zimbabwe: analysis by spoligotyping. *Thorax,* 53**,** 346-350.

HLOKWE, M. T. 2014. *Molecular characterization of Mycobacterium bovis from livestock and wildlife in South Africa: genetic marker optimization and identification using whole genome sequence data.* University of Pretoria.

HLOKWE, T. M., DE KLERK-LORIST, L.-M. & MICHEL, A. L. 2016. Wildlife on the move: a hidden tuberculosis threat to conservation areas and game farms through introduction of untested animals. *Journal of Wildlife Diseases,* 52**,** 837-843.

HUANG, C. T., CHEN, C. Y., CHEN, H. Y., CHOU, C. H., RUAN, S. Y., LAI, C. C. & HSUEH, P. R. 2010. Genitourinary infections caused by nontuberculous mycobacteria at a university hospital in Taiwan, 1996–2008. *Clinical Microbiology and Infection,* 16**,** 1585-1590.

INGEN, J. V., RAHIM, Z., MULDER, A., BOEREE, M. J., SIMEONE, R., BROSCH, R. & SOOLINGEN, D. V. 2012. Characterization of Mycobacterium orygis as *M. tuberculosis* complex subspecies.

JOHNSON, M. M. & JOHN, A. O. 2014. "Nontuberculous Mycobacterial Pulmonary Infections." Journal of Thoracic Disease. 6**,** 210-220.

JOHNSON, M. M. & ODELL, J. A. 2014. Nontuberculous mycobacterial pulmonary infections. *Journal of Thoracic Disease,* 6**,** 210.

JOLLES, A. E., COOPER, D. V. & LEVIN, S. A. 2005. Hidden effects of chronic tuberculosis in African buffalo. *Ecology,* 86**,** 2358-2364.

KANKYA, C., MUWONGE, A., DJØNNE, B., MUNYEME, M., OPUDA-ASIBO, J., SKJERVE, E., OLOYA, J., EDVARDSEN, V. & JOHANSEN, T. B. 2011. Isolation of non-tuberculous mycobacteria from pastoral ecosystems of Uganda: public health significance. *BMC Public Health,* 11**,** 320.

KAZWALA, R., DABORN, C., KUSILUKA, L., JIWA, S., SHARP, J. & KAMBARAGE, D. 1998. Isolation of Mycobacterium species from raw milk of pastoral cattle of the Southern Highlands of Tanzania. *Tropical Animal Health and Production,* 30**,** 233-239.

KAZWALA, R., KAMBARAGE, D., DABORN, C., NYANGE, J., JIWA, S. & SHARP, J. 2001. Risk factors associated with the occurrence of bovine tuberculosis in cattle in the Southern Highlands of Tanzania. *Veterinary Research Communications,* 25**,** 609-614.

KHALEDI, A., BAHADOR, A., ESMAEILI, D. & GHAZVINI, K. 2016. Prevalence of Nontuberculous Mycobacteria (NTM) in Iranian Clinical Specimens: Systematic Review and Meta-Analysis. *J Med Bacterial,* 5**,** 29-30.

KHALEDI, A., BAHADOR, A., ESMAEILI, D. & GHAZVINI, K. 2017. Prevalence of nontuberculous mycobacteria (NTM) in Iranian clinical specimens: Systematic review and meta-analysis. *Journal of Medical Bacteriology,* 5**,** 29-40.

KLOECK, P. 1998. Tuberculosis of domestic animals in areas surrounding the Kruger National Park. *Proceedings of the Challenges of Managing Tuberculosis in Wildlife in Southern Africa, Zunkel, July***,** 30-31.

KRAUSS, H., ROETTCHER, D., WEISS, R., DANNER, K. & HUBSCHLE, O. 1984. Wild animals as a source of infection for domestic animals: investigations in Zambia.[Wildtiere als Infektions potential fur Nutztiere: Untersuchungen an Wild in Sambia.]. *Giessener Beitrage zur Entwicklungsforschung, Reihe I,* 10**,** 133-149.

KRIEK, N., KEET, D., RAATH, J., DE VOS, V., HUCHZERMEYER, H. F. & BENGIS, R. G. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (Syncerus caffer-Sparrman) population in the Kruger National Park: a preliminary report.

LEEDOM, J. M. 2006. Milk of nonhuman origin and infectious diseases in humans. *Clinical Infectious Diseases,* 43**,** 610-615.

LEITE, C. Q. F., ANNO, I. S., LEITE, S. R., ROXO, E., MORLOCK, G. P. & COOKSEY, R. C. 2003. Isolation and identification of mycobacteria from livestock specimens and milk obtained in Brazil. *Memorias do Instituto Oswaldo Cruz,* 98**,** 319-323.

LEPPER, A., NEWTON-TABRETT, D., CORNER, L., CARPENTER, M., SCANLAN, W., WILLIAMS, O. & HELWIG, D. 1977. The use of bovine PPD tuberculin in the single caudal fold test to detect tuberculosis in beef cattle. *Australian veterinary journal,* 53**,** 208-213.

LESLIE, M. 2004. The Good, the Bad, and the Deadly. *Science,* 304**,** 1421-1421.

LIU, H., LIAN, L., JIANG, Y., HUANG, M., TAN, Y., ZHAO, X., ZHANG, J., YU, Q., LIU, J. & DONG, H. 2016. Identification of Species of Nontuberculous Mycobacteria Clinical Isolates from 8 Provinces of China. *BioMed Research International,* 2016.

MACHADO, A. D. C. 2015. *Mapping of the distribution of Mycobacterium bovis strains involved in bovine tuberculosis in Mozambique*.

MADIGAN, G. 2012. *Evaluation of Different Methods for the Detection of Mycobacterium bovis in Lymph Node Tissue.* National University of Ireland Maynooth.

MAEKAWA, K., ITO, Y., HIRAI, T., KUBO, T., IMAI, S., TATSUMI, S., FUJITA, K., TAKAKURA, S., NIIMI, A., IINUMA, Y., ICHIYAMA, S., TOGASHI, K. & MISHIMA, M. 2011. Environmental Risk Factors for Pulmonary Mycobacterium avium-intracellulare Complex Disease.

MAJOOR, C. J., MAGIS-ESCURRA, C., VAN INGEN, J., BOEREE, M. J. & VAN SOOLINGEN, D. 2011. Epidemiology of Mycobacterium bovis disease in humans, The Netherlands, 1993–2007. *Emerging Infectious Diseases,* 17**,** 457.

MANDAL, S., BRADSHAW, L., ANDERSON, L. F., BROWN, T., EVANS, J. T., DROBNIEWSKI, F., SMITH, G., MAGEE, J. G., BARRETT, A. & BLATCHFORD, O. 2011. Investigating transmission of Mycobacterium bovis in the United Kingdom in 2005 to 2008. *Journal of Clinical Microbiology,* 49**,** 1943-1950.

MANUAL, O. T. 2009. Bovine tuberculosis. *World assembly of delegates of the OIE in***,** 7.

MARAIS, B. J. & ZUMLA, A. 2013. History of tuberculosis and drug resistance. *New Engl J Med,* 368**,** 88-90.

MARINELLI, D. L., ALBELDA, S. M., WILLIAMS, T. M., KERN, J. A., IOZZO, R. V. & MILLER, W. T. 1986. Nontuberculous mycobacterial infection in AIDS: clinical, pathologic, and radiographic features. *Radiology,* 160**,** 77-82.

MARTÍNEZ A, T. S. K. R. 1999. Sliding Motility in Mycobacteria. Journal of Bacteriology. ;181**,** 7331-7338.

MEDEIROS, L., MARASSI, C., DUARTE, R., DA SILVA, M. & LILENBAUM, W. 2012. Comparison of decontamination methods for primary isolation of Mycobacterium bovis in paucibacillary bovine tissues. *Letters in applied Microbiology,* 54**,** 182-186.

MEDEIROS, L. D. S., MARASSI, C. D., FIGUEIREDO, E. E. S. & LILENBAUM, W. 2010. Potential application of new diagnostic methods for controlling bovine tuberculosis in Brazil. *brazilian Journal of Microbiology,* 41**,** 531-541.

MEYER, M. E. & MEAGHER, M. 1995. Brucellosis in free-ranging bison (Bison bison) in Yellowstone, Grand Teton, and Wood Buffalo National Parks: a review. *Journal of Wildlife Diseases,* 31**,** 579-598.

MICHEL, A., BENGIS, R. G., KEET, D., HOFMEYR, M., DE KLERK, L., CROSS, P., JOLLES, A. E., COOPER, D., WHYTE, I. & BUSS, P. 2006a. Wildlife tuberculosis in South African conservation areas: implications and challenges. *Veterinary Microbiology,* 112**,** 91-100.

MICHEL, A., HLOKWE, T., COETZEE, M., MARE, L., CONNOWAY, L., RUTTEN, V. & KREMER, K. 2008. High Mycobacterium bovis genetic diversity in a low prevalence setting. *Veterinary Microbiology,* 126**,** 151-159.

MICHEL, A. L. 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences,* 969**,** 251-255.

MICHEL, A. L., BENGIS, R. G., KEET, D., HOFMEYR, M., DE KLERK, L., CROSS, P., JOLLES, A. E., COOPER, D., WHYTE, I. & BUSS, P. 2006b. Wildlife tuberculosis in South African conservation areas: implications and challenges. *Veterinary Microbiology,* 112**,** 91-100.

MICHEL, A. L., BENGIS, R. G., KEET, D. F., HOFMEYR, M., KLERK, L. M. D., CROSS, P. C., JOLLES, A. E., COOPER, D., WHYTE, I. J., BUSS, P. & GODFROID, J. 2006c. Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Veterinary Microbiology,* 112**,** 91-100.

MICHEL, A. L., GEOGHEGAN, C., HLOKWE, T., RASELEKA, K., GETZ, W. M. & MARCOTTY, T. 2015. Longevity of Mycobacterium bovis in raw and traditional souring milk as a function of storage temperature and dose. *PloS one,* 10**,** e0129926.

MICHEL, A. L., MÜLLER, B. & VAN HELDEN, P. D. 2010. Mycobacterium bovis at the animal–human interface: A problem, or not? *Veterinary Microbiology,* 140**,** 371-381.

MILLER, M., MICHEL, A., VAN HELDEN, P. & BUSS, P. 2017a. Tuberculosis in rhinoceros: an underrecognized threat? *Transboundary and emerging diseases,* 64**,** 1071-1078.

MILLER, M. A., BUSS, P. E., VAN HELDEN, P. D. & PARSONS, S. D. 2017b. Mycobacterium bovis in a free-ranging black rhinoceros, Kruger National Park, South Africa, 2016. *Emerging Infectious Diseases,* 23**,** 557.

MILLER, R. S. & SWEENEY, S. J. 2013. Mycobacterium bovis (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. *Epidemiology & Infection,* 141**,** 1357-1370.

MILLER, W. T. & MILLER, W. T. 1993. Pulmonary infections with atypical mycobacteria in the normal host. *Mycobacterial Diseases in the Normal and Compromised Host.*

MULLER, B., DURR, S., ALONSO, S., HATTENDORF, J., LAISSE, C. J. M., PARSONS, S. D. C., VAN HELDEN, P. D. & ZINSSTAG, J. 2013. Zoonotic Mycobacterium bovis: induced tuberculosis in humans. 19**,** 899-908.

MUNJERE, J. 2017. *Pilot investigation of selected milk-borne pathogens in communal cattle in the uMkhanyakude district of Kwa-Zulu-Natal South Africa.* University of Pretoria.

MUNYEME, M., MUMA, J., SAMUI, K., SKJERVE, E., NAMBOTA, A., PHIRI, I., RIGOUTS, L. & TRYLAND, M. 2009. Prevalence of bovine tuberculosis and animal level risk factors for indigenous cattle under different grazing strategies in the livestock/wildlife interface areas of Zambia. *Tropical Animal Health and Production,* 41**,** 345.

MUNYEME, M., MUMA, J., SKJERVE, E., NAMBOTA, A., PHIRI, I., SAMUI, K., DORNY, P. & TRYLAND, M. 2008. Risk factors associated with bovine tuberculosis in traditional cattle of the livestock/wildlife interface areas in the Kafue basin of Zambia. *Preventive Veterinary Medicine,* 85**,** 317-328.

NDAMBI, O. A., HEMME, T. & LATACZ-LOHMANN, U. 2007. Dairying in Africa-Status and recent developments. *Livestock Research for Rural Development,* 19**,** 111.

NEILL, S., CASSIDY, J., HANNA, J., MACKIE, D., POLLOCK, J., CLEMENTS, A., WALTON, E. & BRYSON, D. 1994. Detection of Mycobacterium bovis infection in skin test-negative cattle with an assay for bovine interferon-gamma. *The Veterinary Record,* 135**,** 134-135.

NEILL, S., HANNA, J., MACKIE, D. & BRYSON, T. 1992. Isolation of Mycobacterium bovis from the respiratory tracts of skin test-negative cattle. *The Veterinary Record,* 131**,** 45-47.

O'REILLY, L. M. & DABORN, C. 1995. The epidemiology of Mycobacterium bovis infections in animals and man: a review. *Tubercle and Lung disease,* 76**,** 1-46.

OLEA-POPELKA, F., MUWONGE, A., PERERA, A., DEAN, A. S., MUMFORD, E., ERLACHER-VINDEL, E., FORCELLA, S., SILK, B. J., DITIU, L. & EL IDRISSI, A. 2017. Zoonotic tuberculosis in human beings caused by Mycobacterium bovis—a call for action. *The Lancet Infectious Diseases,* 17**,** e21-e25.

OLOYA, J., MUMA, J., OPUDA-ASIBO, J., DJØNNE, B., KAZWALA, R. & SKJERVE, E. 2007. Risk factors for herd-level bovine-tuberculosis seropositivity in transhumant cattle in Uganda. *Preventive Veterinary Medicine,* 80**,** 318-329.

OLOYA, J., OPUDA-ASIBO, J., KAZWALA, R., DEMELASH, A. B., SKJERVE, E., LUND, A., JOHANSEN, T. B. & DJONNE, B. 2008. Mycobacteria causing human cervical lymphadenitis in pastoral communities in the Karamoja region of Uganda. *Epidemiology and Infection,* 136**,** 636-643.

ORGANIZATION, W. H. 2003. *The world health report 2003: shaping the future*, World Health Organization.

OSOFSKY, S. A. 2005. *Conservation and Development Interventions at the Wildlife/livestock Interface: Implications for Wildlife, Livestock and Human Health: Proceedings of the Southern and East African Experts Panel on Designing Successful Conservation and Development Interventions at the Wildlife/Livestock Interface: Implications for Wildlife, Livestock and Human Health, AHEAD (Animal Health for the Environment And Development) Forum, IUCN Vth World Parks Congress, Durban, South Africa, 14th and 15th September 2003*, IUCN.

PAINE, R. & MARTINAGLIA, G. 1929. Tuberculosis in wild buck living under natural conditions. *Journal of Comparative Pathology and Therapeutics,* 42**,** 1-8.

PARDO, R. B., LANGONI, H., MENDONÇA, L. J. P. & CHI, K. D. 2001. Isolation of Mycobacterium spp. in milk from cows suspected or positive to tuberculosis. *Brazilian Journal of Veterinary Research and Animal Science,* 38**,** 284-287.

PARK, D. Y., KIM, J. Y., CHOI, K. U., LEE, J. S., LEE, C. H., SOL, M. Y. & SUH, K. S. 2003. Comparison of polymerase chain reaction with histopathologic features for diagnosis of tuberculosis in formalin-fixed, paraffin-embedded histologic specimens. *Archives of Pathology & Laboratory Medicine,* 127**,** 326-330.

PAULSON, T. 2013. A mortal foe. *Nature,* 502**,** S2-S2.

PHILLIPS, C., FOSTER, C., MORRIS, P. & TEVERSON, R. 2003. The transmission of Mycobacterium bovis infection to cattle. *Research in Veterinary Science,* 74**,** 1-15.

QUINN, P., MARKEY, B., CARTER, M., DONNELLY, W. & LEONARD, F. 2002. Veterinary Microbiology and Microbial Disease. First Published Blackwell Science Company, Iowa. State University Press.

RAMOS, D., SILVA, P. & DELLAGOSTIN, O. 2015a. Diagnosis of bovine tuberculosis: review of main techniques. *Brazilian Journal of Biology,* 75**,** 830-837.

RAMOS, D. F., SILVA, P. E. A. & DELLAGOSTIN, O. A. 2015b. Diagnosis of bovine tuberculosis: review of main techniques. *Brazilian Journal of Biology,* 75**,** 830-837.

RODWELL, T. C. 1999. *The epidemiology of bovine tuberculosis in African buffalo (Syncerus caffer)*, University of California, Davis.

RODWELL, T. C., KAPASI, A. J., MOORE, M., MILIAN-SUAZO, F., HARRIS, B., GUERRERO, L., MOSER, K., STRATHDEE, S. A. & GARFEIN, R. S. 2010. Tracing the origins of Mycobacterium bovis tuberculosis in humans in the USA to cattle in Mexico using spoligotyping. *International Journal of Infectious Diseases,* 14**,** e129-e135.

ROGALL, T., WOLTERS, J., FLOHR, T. & BÖTTGER, E. C. 1990. Towards a phylogeny and definition of species at the molecular level within the genus Mycobacterium. *International Journal of Systematic and Evolutionary Microbiology,* 40**,** 323-330.

ROTHEL, J., JONES, S., CORNER, L., COX, J. & WOOD, P. 1990. A sandwich enzyme immunoassay for bovine interferon-γ and its use for the detection of tuberculosis in cattle. *Australian Veterinary Journal,* 67**,** 134-137.

ROTHEL, J., JONES, S., CORNER, L., COX, J. & WOOD, P. 1992. The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle: conditions affecting the production of gamma-interferon in whole blood culture. *Australian Veterinary Journal,* 69**,** 1-4.

RYAN, K. & RAY, C. 2004. Staphylococci. *Sherris Medical Microbiology: An introduction to infectious disease. 4th ed. New York: McGraw-Hill, Medical Publishing Division*.

SOINI, H., BÖTTGER, E. C. & VILJANEN, M. K. 1994. Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. *Journal of Clinical Microbiology,* 32**,** 2944-2947.

SRIVASTAVA, K., CHAUHAN, D., GUPTA, P., SINGH, H., SHARMA, V., YADAV, V., THAKRAL, S., DHARAMDHEERAN, J., NIGAM, P. & PRASAD, H. 2008. Isolation of Mycobacterium bovis & M. tuberculosis from cattle of some farms in north India--possible relevance in human health. *Indian Journal of Medical Research,* 128**,** 26-32.

STRAIN, S. A., MCNAIR, J., MCDOWELL, S. W. & BRANCH, B. 2011. Bovine tuberculosis: a review of diagnostic tests for M. bovis infection in badgers. *Report: Agri-Food and Biosciences Institute. Available online: http://www. dardni. gov. uk/afbi-literature-review-tb-reviewdiagnostic-tests-badgers. pdf (accessed 22 March 2016)*.

SULIS, G., CENTIS, R., SOTGIU, G., D'AMBROSIO, L., PONTALI, E., SPANEVELLO, A., MATTEELLI, A., ZUMLA, A. & MIGLIORI, G. B. 2016. Recent developments in the diagnosis and management of tuberculosis. *NPJ primary care respiratory medicine,* 26**,** 16078.

SUMIYAH, R., DEEPTI, N., MUDIT, C. & SHARMA, N. 2017. Identification of non-tuberculous mycobacteria in faeces of cattle and buffaloes using polymerase chain reaction-restriction fragment length polymorphism analysis. *Haryana Veterinarian,* 56**,** 87-91.

SWEENEY, R. W., WHITLOCK, R. H. & ROSENBERGER, A. E. 1992. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *Journal of Clinical Microbiology,* 30**,** 166-171.

TADAYON, K., MOSAVARI, N., SADEGHI, F. & FORBES, K. J. 2008. Mycobacterium bovis infection in Holstein Friesian cattle, Iran. *Emerging Infectious Diseases,* 14**,** 1919.

TELENTI, A., MARCHESI, F., BALZ, M., BALLY, F., BÖTTGER, E. & BODMER, T. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *Journal of Clinical Microbiology,* 31**,** 175-178.

TESSARO, S. V. 1986. The existing and potential importance of brucellosis and tuberculosis in Canadian wildlife: a review. *The Canadian Veterinary Journal,* 27**,** 119.

THIERRY, D., CAVE, MD, EISENACH, K. D., CRAWFORD, J. T., BATES, J. H, GICQUEL, B. & GUESDDON, J, L. 1990. IS6110, an IS-like element of *Mycobacterium tuberculosis complex*. Nucleic acids research, 18 (1), 188.

THOEN, C. O., LOBUE, P. A., ENARSON, D. A., KANEENE, J. B. & DE KANTOR, I. N. 2009. Tuberculosis: a re-emerging disease in animals and humans.

TORTOLI, E., CICHERO, P., PIERSIMONI, C., SIMONETTI, M. T., GESU, G. & NISTA, D. 1999. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. *Journal of Clinical Microbiology,* 37**,** 3578-3582.

TSCHOPP, R., ASEFFA, A., SCHELLING, E., BERG, S., HAILU, E. & ET AL. 2010. Bovine Tuberculosis at the Wildlife-Livestock-Human Interface in Hamer Woreda, South Omo, Southern Ethiopia. . *PLoS ONE,* 5.

UNE, Y. & MORI, T. 2007. Tuberculosis as a zoonosis from a veterinary perspective. *Comparative Immunology, Microbiology and Infectious Diseases,* 30**,** 415-425.

VARELLO, K., PEZZOLATO, M., MASCARINO, D., INGRAVALLE, F., CARAMELLI, M. & BOZZETTA, E. 2008. Comparison of histologic techniques for the diagnosis of bovine tuberculosis in the framework of eradication programs. *Journal of Veterinary Diagnostic investigation,* 20**,** 164-169.

VITALE, F., CAPRA, G., MAXIA, L., REALE, S., VESCO, G. & CARACAPPA, S. 1998. Detection of Mycobacterium tuberculosiscomplex in cattle by PCR using milk, lymph node aspirates, and nasal swabs. *Journal of Clinical Microbiology,* 36**,** 1050-1055.

WARREN, R., GEY VAN PITTIUS, N., BARNARD, M., HESSELING, A., ENGELKE, E., DE KOCK, M., GUTIERREZ, M., CHEGE, G., VICTOR, T. & HOAL, E. 2006. Differentiation of Mycobacterium tuberculosis complex by PCR amplification of genomic regions of difference. *The International Journal of Tuberculosis and Lung Disease,* 10**,** 818-822.

WHO 2014. Global Tuberculosis Report. *http://www.who.int/tb/publications/global_report/en/ (accessed*

267 March 17, 2015).

WINTHROP, K. L., VARLEY, C. D., ORY, J., CASSIDY, P. M. & HEDBERG, K. 2011. Pulmonary disease associated with nontuberculous mycobacteria, Oregon, USA. *Emerging Infectious Diseases,* 17**,** 1760.

WOOD, P. & JONES, S. 2001. BOVIGAMTM: an in vitro cellular diagnostic test for *bovine tuberculosis*. *Tuberculosis,* 81**,** 147-155.

WOODFORD, M. H. 1972. *Tuberculosis in the African buffalo (Syncerus caffer), in the Queen Elizabeth National Park, Uganda*, Juris.

WOODRING, J. H. & VANDIVIERE, H. M. 1990. Pulmonary disease caused by nontuberculous mycobacteria. *J. Thorac Imaging,* 5**,** 64-76.

WOODS, G. L. 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. *Approved Standard M24-A2,* 31.

YU, X., LIU, P., LIU, G., ZHAO, L., HU, Y., WEI, G., LUO, J. & HUANG, H. 2016. The prevalence of non-tuberculous mycobacterial infections in mainland China: Systematic review and meta-analysis. *Journal of Infection,* 73**,** 558-567.

https://www.bd.com/en-uk/products/diagnostics-systems/identification-and-susceptibility-systems/mgit-(mycobacteria-growth-indicator-tube)-system

http://n0gp49ausy2iunoe2ldace1b.wpengine.netdna-cdn.com/wp-content/uploads/2016/02/BM0210.jpg

Annex 1

Table 3.1 Preparation of 5% OA

Preparation of 5% OA (sigma-Aldrich, South Africa)
Oxalic acid 5 g
Distilled water 100 ml

Table 3.2 Preparation of HS-SH

Preparation of HS-SH (sigma-Aldrich, South Africa)	
NaCl 7 g	NaOH 4 g
Distilled water 100 ml	Distilled water 100 ml

Table 3.3 Preparation of 1% CPC

Preparation of 1% CPC (sigma-Aldrich, South Africa)
Cetylpyridinium chloride 1 g
Distilled water 100 ml

Table 3.4 Preparation of 2% HCl

Preparation of 2% HCl (sigma-Aldrich, South Africa)
HCl 2 g
Distilled water 100 ml

Table 3.5 Preparation of 4 % NaOH

Preparation of 4 % NaOH (sigma-Aldrich, South Africa)
NaOH 4 g
Distilled water 100 ml

Annex 2: University of Pretoria Animal Ethics

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Patterns of longitudinal *Mycobacterium Bovis* shedding in cow's milk from communal cattle**
PROJECT NUMBER	**V074-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**KD Mazwi**

STUDENT NUMBER (where applicable)	**U_12225305**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

CONDITIONS:

1. *DAFF approval is required for the study. Only the National Office can grant approval in terms of the Animal Diseases Act – please submit a copy*
2. *The education material must include information on the dangers of uncooled/unpasteurised milk being consumed*

ANIMAL SPECIES	**Cattle**	
NUMBER OF SAMPLES	**100**	
Approval period to use animals for research/testing purposes		**July 2017- July 2018**
SUPERVISOR	**Prof. A Michel**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 3 October 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S*4285-15*

Annex 3: Department of Agriculture, Forestry and Fisheries approval

agriculture, forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries Private Bag Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za Reference: 12/11/1/1/611

Petronillah Sichewo
Department of Tropical Diseases
Onderstepoort

Dear Petronillah Sichewo

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)
Your fax / memo / letter/ Email dated 2 June 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;

3. Samples must be packaged and transported in accordance with International Air Transport
   Association (IATA) requirements and/or National Road. Traffic Act, 1996 (Act No. 93 of 1996);

4. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982(Act No. 19 of 82);

5. No animal to be used in the study may be purchased or originate from an area under any veterinary restriction;

6. All growing pigs used in the study must be destroyed humanely and carcasses incinerated at the completion of the study.

Title of research/study: The epidemiology of bovine tuberculosis at the wildlife-livestock-human interface in northern Kwa-Zulu Natal, South Africa

Researcher (s): Petronillah Sichewo

Institution: Department of Tropical Diseases, Onderstepoort

Your Ref./ Project Number:

Our ref Number: 12/11/111/6/1

Kind regards,

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2016 -06- 2 1

Annex 4: Farmer's consent form

FARMER'S INFORMATION LEAFLET & INFORMED CONSENT FORM

Researcher's name: Mazwi Desiree

Student Number: 12225305

Department of Veterinary Tropical Diseases,

University of Pretoria

Dear Farmers

I am a masters student (1st year) in the study of TB, in the Department Veterinary Tropical Diseases, University of Pretoria. You are invited to volunteer for your animals to take part in our research project on: **Longitudinal monitoring of *Mycobacterium bovis* and *non-tuberculous mycobacteria* from cow's milk in a rural community**

This letter gives information to help you to decide if you want your animals to take part in this study. Before you agree you should understand the study and your role in the study. . If you do not understand the information or have any other questions, you are free to ask us. You should not agree to take part unless you are completely happy about what we expect.

The aims of this study is to determine the frequency of *M. bovis* shedding and quantity of *M. bovis* excreted in milk from Bovine TB infected cows and to optimize the detection method for *M. bovis* in fresh milk with a high level of bacterial contamination.

We would like you to give us permission to collect milk samples from your cattle and these will be collected in a time interval for 6 months. The milk will be used to test for bovine TB. The animal is not harmed in anyway when these samples are collected.

The Animal Ethics Committee of the University of Pretoria, Faculty of Veterinary Sciences, telephone numbers 012 529 8349 have given permission for this study. Your taking part in this study is voluntary. You can refuse to participate or stop at any time without giving any reason and your animals will still receive the same standard services from State veterinarians.

Note: Filling in the form means that you have understood the study and agreed that your animals become part of the study. . As a result information obtained from your form (without your name) may be used for e.g. publication, by the researchers.

We thank you for your help and time.

Yours truly,
Desiree Mazwi.

FARMER'S INFORMATION LEAFLET & INFORMED CONSENT FORM

VERBAL FARMER'S INFORMED CONSENT (applicable when farmers cannot read or write)

I, the undersigned, ……..…, have read and have explained fully to the farmer, named …….. the information leaflet, which has indicated the nature and purpose of the study in which I have asked the farmer and his animals to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The farmer indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing the treatment of his animals.

I hereby certify that the farmer has agreed to participate in this study.

Farmer's Name	Farmer's Signature	Date
Investigator's Name	Investigator's Signature	Date
Witness's Name	Witness's Signature	Date

Annex 5: Raw data

MARCH 2017

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 12	RD 4	CULTURE
1	112	Positive	Negative	Negative	Positive
2	120	Positive	Negative	Negative	Negative
3	76	Positive	Negative	Negative	Positive
4	74	Positive	Negative	Negative	Positive
5	UNKNOWN	Positive	Negative	Negative	Positive
6	75	Positive	Negative	Negative	Positive
7	95	Positive	Negative	Negative	Negative
8	33	Negative	Negative	Negative	Positive
9	36	Negative	Negative	Negative	Positive
10	127	Negative	Negative	Negative	Negative
11	96	Positive	Positive	Negative	Positive
12	18	Negative	Negative	Negative	Positive
13	14	Positive	Negative	Negative	Positive
14	UNKNOWN 2	Negative	Negative	Negative	Positive
15	110	Negative	Negative	Negative	Positive
16	65	Positive	Negative	Negative	Negative
17	45	Positive	Negative	Negative	Positive
18	*M. bovis* BCG	Positive	Positive	Positive	
19	MTB ATCC	Positive	Positive	Positive	
21	Negative	Negative	Negative	Negative	

JUNE 2017

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	99	Positive	Negative	Negative	Positive
2	34	Positive	Negative	Negative	Positive
3	21	Negative	Negative	Negative	Negative
4	115	Negative	Negative	Negative	Positive
5	87	Negative	Negative	Negative	Positive
6	28	Positive	Negative	Negative	Negative
7	32	Positive	Positive	Negative	Positive
8	72	Positive	Negative	Negative	Positive
9	111	Positive	Negative	Negative	Positive
10	163	Negative	Negative	Negative	Negative
11	90	Negative	Negative	Negative	Negative
12	109	Positive	Negative	Negative	Positive
13	66	Positive	Negative	Negative	Negative
14	38	Positive	Negative	Negative	Positive
15	41	Positive	Positive	Negative	Negative
16	63	Positive	Negative	Negative	Positive
17	159	Positive	Negative	Negative	Negative
18	92	Positive	Positive	Negative	Positive
19	39	Negative	Negative	Negative	Positive
20	*M. bovis* BCG	Positive	Positive	Positive	
21	MTB	Positive	Positive	Positive	
23	Negative	Negative	Negative	Negative	

DAY 1

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	109	Negative	Negative	Negative	Positive
2	100	Negative	Negative	Negative	Positive
3	03	Positive	Negative	Negative	Negative
4	127	Negative	Negative	Negative	Negative
5	04	Positive	Negative	Negative	Negative
6	112B	Negative	Negative	Negative	Negative
7	28	Negative	Negative	Negative	Negative
8	13	Negative	Negative	Negative	Negative
9	02	Negative	Negative	Negative	Negative
10	72	Negative	Negative	Negative	Negative
11	127	Negative	Negative	Negative	Negative
12	05	Negative	Negative	Negative	Negative
13	115	Negative	Negative	Negative	Positive
14	122	Negative	Negative	Negative	Positive
15	112A	Negative	Negative	Negative	Negative
16	Unknown	Negative	Negative	Negative	Negative
17	76	Negative	Negative	Negative	Negative
18	121	Negative	Negative	Negative	Negative
19	27	Negative	Negative	Negative	Positive
20	35	Positive	Positive	Negative	Positive
21	*M. bovis* BCG	Positive	Positive	Negative	
22	MTB ATCC	Positive	Positive	Negative	
23	Negative	Negative	Negative	Negative	

DAY 2

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	121	Negative	Negative	Negative	Positive
2	05	Positive	Negative	Negative	Positive
3	120	Positive	Negative	Negative	Positive
4	122	Negative	Negative	Negative	Positive
5	03	Positive	Negative	Negative	Positive
6	01	Positive	Negative	Negative	Negative
7	120	Negative	Negative	Negative	Positive
8	44	Positive	Negative	Negative	Negative
9	112	Positive	Negative	Negative	Positive
10	76	Negative	Negative	Negative	Negative
11	*M. bovis* BCG	Positive	Positive	Positive	
12	MTB ATCC	Positive	Positive		
13	Negative	Negative	Negative	Negative	
14	Negative	Negative	Negative		

DAY 8

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	02	Negative	Negative	Negative	Positive
2	05	Negative	Negative	Negative	Negative
3	13	Negative	Negative	Negative	Negative
4	115	Negative	Negative	Negative	Positive
5	121	Negative	Negative	Negative	Positive
6	122	Negative	Negative	Negative	Positive
7	*M. bovis* BCG	Positive	Positive	Positive	
8	MTB ATCC	Positive	Positive	Positive	
9	Negative	Negative	Negative	Negative	
10	Negative	Negative	Negative	Negative	

DAY 14

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	44	Negative	Negative	Negative	Negative
2	127	Negative	Negative	Negative	Negative
3	120	Negative	Negative	Negative	Positive
4	122	Negative	Negative	Negative	Positive
5	110	Negative	Negative	Negative	Positive
6	02	Negative	Negative	Negative	Positive
7	28	Negative	Negative	Negative	Negative
8	01	Negative	Negative	Negative	Negative
9	100	Negative	Negative	Negative	Negative
10	*M. bovis* BCG	Positive	Positive	Positive	
11	MTB ATCC	Positive	Positive	Positive	
12	Negative	Negative	Negative	Negative	
13	Negative	Negative	Negative	Negative	

DAY 21

PCR ID		ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1		03	Negative	Negative	Negative	Negative
2		05	Negative	Negative	Negative	Negative
3		122	Positive	Negative	Negative	Positive
4		13	Negative	Negative	Negative	Negative
5		27	Negative	Negative	Negative	Negative
6		121	Positive	Negative	Negative	Positive
7		28	Negative	Negative	Negative	Negative
8		35	Negative	Negative	Negative	Negative
9		01	Negative	Negative	Negative	Positive
10		02	Negative	Negative	Negative	Negative
11		110	Negative	Negative	Negative	Positive
12		120	Positive	Negative	Negative	Positive
13		*M. bovis* BCG	Positive		Positive	
14		MTB ATCC	Positive	Positive	Positive	
15		Negative	Negative	Negative	Negative	

DAY 28

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	01	Positive	Positive	Positive	Positive
2	02	Negative	Negative	Negative	Negative
3	03	Negative	Negative	Negative	Negative
4	05	Positive	Negative	Negative	Positive
5	100	Positive	Negative	Negative	Negative
6	13	Negative	Negative	Negative	Negative
7	27	Negative	Negative	Negative	Negative
8	35	Negative	Negative	Negative	Negative
9	44	Negative	Negative	Negative	Negative
10	*M. bovis* BCG	Positive	Positive	Positive	
11	MTB ATCC	Positive	Positive	Positive	
12	Negative	Negative	Negative	Negative	
13	Negative	Negative	Negative	Negative	

DECEMBER 2017

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	100	Negative	Negative	Negative	Positive
2	109	Negative	Negative	Negative	Positive
3	111	Negative	Negative	Negative	Positive
4	27	Negative	Negative	Negative	Positive
5	127	Positive	Negative	Negative	Negative
6	110	Positive	Negative	Negative	Positive
7	03	Positive	Negative	Negative	Positive
8	01	Negative	Negative	Negative	Negative
9	112	Negative	Negative	Negative	Positive
10	*M. bovis* BCG	Positive	Positive	Positive	
11	MTB ATCC	Positive	Positive		
12	Negative	Negative	Negative	Negative	

MARCH 2018

PCR ID	ANIMAL ID	TB 11 & 12	TB 1 & 2	RD4	CULTURE
1	301	Negative	Negative	Negative	Negative
2	302	Negative	Negative	Negative	Negative
3	303	Positive	Positive	Positive	Positive
4	304	Positive	Positive	Positive	Positive
5	306	Positive	Positive	Negative	Contaminated
6	307	Positive	Positive	Negative	Positive
7	308	Positive	Positive	Positive	Positive
8	320	Positive	Positive	Positive	Positive
9	401	Positive	Negative	Negative	Negative
10	404	Positive	Negative	Negative	Positive
11	407	Positive	Negative	Positive	Positive
12	413	Positive	Positive	Positive	Positive
13	415	Positive	Positive	Negative	Positive
14	417	Positive	Positive	Positive	Positive
15	419	Positive	Negative	Negative	Positive
16	420	Negative	Negative	Negative	Contaminated
17	421	Positive	Negative	Positive	Positive
18	422	Positive	Positive	Positive	Positive
19	423	Positive	Positive	Negative	Positive
20	424	Negative	Negative	Negative	Negative
21	426	Positive	Positive	Positive	Positive
22	427	Positive	Negative	Positive	Positive
23	*M. bovis* BCG	Positive	Positive	Positive	
24	MTB ATCC	Positive	Positive	Positive	
25	Negative	Negative	Negative	Negative	
26	Negative	Negative	Negative	Negative	