THE EPIDEMIOLOGY OF TUBERCULOSIS IN CATTLE AND HUMANS LIVING IN THE WILDLIFE-LIVESTOCK-HUMAN INTERFACE IN THE RURAL MNISI COMMUNITY, MPUMALANGA PROVINCE, SOUTH AFRICA

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DECLARATION

I declare that this thesis, which I hereby submit for the degree **Philosophiae Doctor** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any tertiary institution.

Jolly Musoke

November 2015

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DEDICATION

Jeremiah 29:11: “I alone know the plans I have for you, plans to bring you prosperity and not disaster, plans to bring about the future you hope for.”

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AFLP	Amplified Fragment Length Polymorphism
AIDS	Acquired Immune Deficiency Syndrome
BTB	Bovine Tuberculosis
CIDT	Comparative Intradermal Tuberculin Skin Test
CFU	Colony Forming Unit
CPC	Cetylpyridinium Chloride
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
EAI	East African Indian
ELISA	Enzyme-Linked Immunosorbent Assay
GKNPC	Greater Kruger National Park Complex
GNP	Gonarezhou National Park
HiP	Hluhluwe iMofolozi Game Reserve
HIV	Human Immunodeficiency Virus
IFN-ɣ	Gamma Interferon
INH	Isoniazid
KNP	Kruger National Park
LAM	Latin American and Mediterranean
LSP	Large Sequence Polymorphism
MDR-TB	Multidrug-Resistant Tuberculosis
MDG	Millennium Development Goal
MGIT	Mycobacterial Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit
MOTT	Mycobacterium Other Than Tuberculosis

MTBC	*Mycobacterium Tuberculosis* Complex
NTM	Non-Tuberculous Mycobacteria
NTP	National Tuberculosis Control Programme
OD	Optical Density
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampin
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
TB	Tuberculosis
TST	Tuberculin Skin Test
UK	United Kingdom
VNTR	Variable Number Tandem Repeat
WHO	World Health Organisation

THESIS SUMMARY

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The aim of this study was to investigate the prevalence and epidemiological significance of tuberculosis (TB) in bovine and humans living at a wildlife/livestock/human interface, as well as the risk factors associated with TB transmission at that interface. The Mnisi community was chosen as it is located at the western border of the Kruger National park (KNP) and enables research at the wildlife/livestock/human interface. The first objective of the study entailed investigating the presence of bovine tuberculosis (BTB) in 10% of Mnisi's livestock, using the comparative intradermal skin test. A low individual prevalence of 0.33 % (95% CI.0.14 - -0.79) was detected. Further investigations into the causative agent in livestock, using genotyping techniques identified the KNP parental strain, *M. bovis* KNP – VNTR -1 strain.[1] Supporting records from the provisional Mpumalanga Veterinary Services and the physical location of dip-tanks where BTB was detected, it was established the infection was a result of spillback infection from wildlife in the neighbouring KNP. The epidemiological significance of BTB in human TB was investigated through the isolation and genetic characterisation of the *Mycobacterium tuberculosis* complex (MTBC) strain population in the Mnisi community. *Mycobacterium bovis* was not detected in the human population. However, a high genetic diversity of *M. tuberculosis* was observed among the 13 isolates obtained.The *M. tuberculosis* isolates were identified as the following eight families: T; Beijing; LAM 11_ZWE; EAI5; MANU1; X1; X2; and S families. The predominant lineage was as T family, sub-lineage ST53. Based on the high diversity (8 clusters/13isolates)

and the predominance of the T family, it was concluded that the TB population structure within the Mnisi community was largely impacted by human migration from urban towns and neighbouring Mozambique. A questionnaire was administered to investigate BTB transmission risk factors at the livestock/human interface. It was established that there were low risk levels of BTB transmission at the human/livestock interface mainly based on the fact that the majority of the households in the community obtained pasteurised milk commercially, and although undercooked/raw meat and organs were preferred, the majority of respondents reported that they discarded the meat if changes in meat quality were observed.

Chapter 1: Literature review

1.1 General Introduction

Tuberculosis (TB) is one of the leading human infectious diseases in the 21th century, with approximately 9 million cases reported and above 1.5 million deaths in the year 2013.[2] *Mycobacterium tuberculosis* is primarily known as the causative agent of human TB. However, three other members of the *Mycobacterium tuberculosis* complex, namely *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium canettii* can cause TB in humans.[3] In cattle, *Mycobacterium bovis* is primarily known as the causative agent of bovine tuberculosis (BTB), however research has shown that other members of the *Mycobacterium* complex, particularly *M. tuberculosis*, can cause TB in livestock.[4]

In 1882, Koch and Behring were under the impression that *M. tuberculosis* was attenuated in bovines and could be used as a vaccine against *M. bovis* in cattle.[5] Conversely, *Mycobacterium bovis* was thought to be less virulent in humans and could be used as a vaccine in man against *M. tuberculosis*. With further research, virulent human tubercle bacilli were isolated from milk of a vaccinated cow and more supporting literature stated that BTB has zoonotic (infectious disease that can be transmitted between animals and humans) potential and can be detrimental to human health.[6,7] *Mycobacterium tuberculosis* has also been shown to be virulent in cattle. For instance, Berg *et al*., (2009), reported the isolation of *M. tuberculosis* from TB suspect lesions in cattle in Ethiopia, suggesting mycobacteria transmission from human to animals.[4] Similarly *M. tuberculosis* was isolated from cattle showing typical tuberculous lesions at slaughter in South Africa (Michel, unpublished data). However, there is limited literature on the virulence of *M. tuberculosis* in bovines within southern Africa and little research has been done on the socio-economic impact *M. tuberculosis* has on livestock farming.[8] In addition, there is an inadequate understanding of the *Mycobacterium tuberculosis* complex epidemiology at the interface between humans and animals (both wildlife and livestock). Investigating this interface is essential in the fight against the TB epidemic overall, as better understanding of the disease epidemiology will assist in designing suitable interventions to prevent disease spread and containing reservoir hosts.

Due to cultural traditions and poverty, many households in developing countries rely on obtaining dairy and other livestock products from subsistence cattle farming or communal

small scale farmers. In some households these communal products are the main affordable source of animal protein, particularly for the very young and the old. Additionally, there is a common perception that raw milk and its products are more tasty and nutritious. However, the main limitation of livestock products derived from communal as compared to commercial farming, is the limited access to veterinary inspection before human consumption, raising questions with regard to food safety. A study in the Southern Highlands of Tanzania, isolated *M. bovis* from raw milk; thus, selling of unpasteurised dairy products such as raw milk, sour milk, and unpasteurized cheeses, which is very common in these areas could be of potential public health concern.[9] Undetected pathogens including those causing zoonotic BTB may be prevalent within communities, with little or no adequate control measures. Under-resourced health centre services and infrastructure do contribute to low detection and under-diagnosis of zoonotic diseases. Collaboration of these health centres with research institutes would not only assist in the fight against zoonotic diseases but would aid in establishing if there is the presence of these diseases, their significance and population group at risk.

1.2 Mycobacteria

1.2.1 Taxonomy

Mycobacteria are characterised as aerobic, acid-fast, non-motile, non-spore forming, slow growing bacilli.[10] The genus can be sub-divided into different clusters; however the broad classification is in two groups, namely; *Mycobacterium tuberculosis* complex (MTBC) and Non-tuberculous mycobacteria (NTM) also referred to as mycobacterium other than tuberculosis (MOTT).[11] *Mycobacterium tuberculosis* complex organisms are obligate pathogens that cause the infectious disease tuberculosis (TB), whereas NTM are mycobacteria that do not cause TB. However, in certain conditions NTMs can cause TB like symptoms.[12] Currently, *Mycobacterium tuberculosis* complex has twelve closely related members, which include: *Mycobacterium tuberculosis*, *Mycobacterium canetti*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium bovis BCG*, *Mycobacterium pinnipedii*, *Mycobacterium mungi*, *Mycobacterium caprae*, *Mycobacterium suricattae*, *Mycobacterium orygis*, *dassie bacillus* and *Mycobacterium microti*.[13-15] *Mycobacterium tuberculosis* and *M. bovis* are the two most commonly reported complex species that cause TB in humans and animals.[16]

Members of the MTBC are characterized by 99.9% or more similarity at the nucleotide level and identical 16S rRNA sequences. The MTBC is thought to have evolved from a single

ancestral *M. tuberculosis* that has diversified through spontaneous mutations and progressive loss of genetic material.[16] The two common members, *M. tuberculosis* and *M. bovis* have less than 0.05% average sequence difference.[17] Smith and co-workers described how population bottlenecks and selective sweeps could have shaped the phylogeny of the MTBC and the lack of genetic diversity within *M. bovis* particularly in Great Britain.[18] Despite the high genomic similarity between MTBC species and strains, they have different epidemiological significance (i.e. pathogenicity, host susceptibility), thus it is essential to speciate members and identify the different strains.

1.2.2 *Mycobacterium bovis*

Mycobacterium bovis has the widest host range of the MTBC and can infect a diverse range of free-living wildlife, captive wildlife, domestic livestock, and humans.

1.2.2.1 *M. bovis* in animals

Mycobacterium bovis was first isolated from domestic cattle and it is considered the key maintenance host of *M. bovis*, hence the name bovine tuberculosis (BTB).[19]. Until 1920, BTB was a major disease in cattle, with endemic levels reported in the UK (40% of cattle in the UK). The disease caused significant economic loss as a result of reduced animal productivity and trade restrictions.[20] Many developed countries have successful eradicated or limited the disease, mainly through 'testing and culling' of cattle. However, in many developing countries, particularly in Africa, the status of BTB in cattle remains unknown due to the sporadic or lack of BTB control strategies.[21] According to the World Organization for Animal Health (OIE), 70 countries reported BTB in their cattle population in the year 2011, thus BTB remains epidemiologically significant.

Due to the broad host spectrum of *M. bovis*, it has established itself in a number of maintenance host species in a range of countries within the wildlife populations.[22] Maintenance or reservoir hosts are species where the pathogen, in this scenario *M. bovis*, can persist through horizontal transmission in the absence of an external source of infection. Maintenance hosts serve as continuous source of infection to other species which share the same ecosystem.[23] Studies in the United Kingdom (UK) have identified the European badger (*Meles meles*); in New Zealand the brush-tail possum (*Trichosurus vulpecula*); in Spain, European wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) as the wildlife maintenance hosts. [24,25] In Zambia, Kafue lechwe (*Kobus leche*) has been reported as the maintenance host of *M. bovis* in the wildlife population.[26] In the case of South Africa, African buffalo

(*Synceros caffer*) are considered the main *M. bovis* wildlife reservoir, whereas the warthog (*Phacochoerus aethiopicus*) and kudu (*Tragelaphus strepsiceros*) have shown potential as maintenance hosts.[26-28]

Spill-over or dead-end hosts are defined by sporadic *M. bovis* infections or infections that only persist in the particular host population if a true maintenance host shares the ecosystem (serves as an external source).[23] However, spill-over hosts can serve as maintenance in cases of high density populations.[29] Examples of spill-over hosts of *M. bovis* are goats (*Capra hircus*), sheep (*Ovis aries*), cats (*Felis ula*), chacma baboon (*Papio ursinus*) and warthog (*Phacochoerus aethiopicus*).[19,28,30,31]

1.2.2.1.1 *M. bovis* in South African wildlife

Bovine tuberculosis in free-ranging wildlife was first diagnosed in 1928 in the Eastern Cape region of South Africa.[32] Since then *M. bovis* infections in wildlife has been reported across South Africa including national game reserves such as Hluhluwe iMfolozi Park and Kruger National Park (KNP), where the disease is endemic.[33,34]

The introduction of BTB in the KNP is thought to have been in the late 1950's to early 1960's. The disease is believed to have been transmitted from an adjacent farm with BTB infected cattle to African buffalo (*Syncerus caffer*) in the southern border of the park, across the Crocodile River in the Komatipoort, Malelane region.[26] Bovine tuberculosis remained undetected until the year 1990 where a young buffalo bull was confirmed to be infected with BTB.[32] This prompted an investigation into the BTB status of buffalo in the KNP. In the year 1991, BTB prevalence of 0%, 4.4% and 27.1% were reported in the north, central and south of the KNP, respectively.[35] In the following survey (1998), a significant increase of 1.5%, 10% and 38.2% in the north, central and south of the park was recorded.[35] The disease had spread northwards at an estimated rate of 6 km per year.[36] The African buffalo (*Syncerus caffer*) is considered the maintenance host; in addition, warthog (*Phacochoerus aethiopicus*) and kudu (*Tragelaphus strepsiceros*) have potential as maintenance hosts.[26,27] As the disease spread, it had spilled over into other species including, among others, lions (*Panthera leo*), leopard (*Panthera pardus*), spotted hyena (*Crocuta crocuta*), giraffe (*Giraffa camelopardalis*) and baboons (*Papio ursinus*).[26,32]

The implications of BTB are not restricted to wildlife in the KNP, but also affect shared ecosystems in other parts of the Greater Limpopo Transfrontier Conservation area. The

isolation of the paternal KNP strain in buffalos in the neighbouring Gonarezhou National Park (GNP), Zimbabwe, provide evidence of an epidemiological link of BTB infections between the KNP, South Africa and GNP, Zimbabwe.[37] Not only do the implications of BTB affect wildlife but also the health of livestock and humans that live in the periphery to these parks. A few studies have investigated the spillback of BTB from wildlife to livestock and implications to humans and the risk factors.[38] However, no studies have provided evidence of BTB spillback transmission from wildlife to livestock and the potential zoonotic implications for humans.

1.2.2.2 *M. bovis* in humans

Human TB caused by *M. bovis* is referred to as zoonotic tuberculosis.[20] Though *M. tuberculosis* is clinically indistinguishable from *M. bovis*, the latter is commonly associated with extra-pulmonary TB. Kazwala *et al*., 2001 reported significantly higher association of *M. bovis* with extra-pulmonary TB (26.8%) compared to pulmonary TB (4.3%) in Tanzania due to the ingestion of the tubercle bacilli.[39] Zoonotic TB can present as cervical lymphadenitis (scrofula), gastrointestinal lesions, lupus vulgaris (chronic skin TB) or a combination of lymphadenopathy and lupus vulgaris termed scrofuloderma.[40,41] *Mycobacterium bovis* can also present itself as pulmonary TB, as reported by Gumi *et al*., 2012 and Malama *et al*., 2013, who isolated *M. bovis* from sputum samples in Ethiopia and Zambia, respectively.[42,43]

Between 1954 and 1970, human TB due to *M. bovis* was estimated at a global prevalence of 3.1% (Gervois *et al*., 1972 cited by Cosivi *et al*., 1998).[44] A more recent comprehensive review conducted by Muller *et al*., 2013, estimated 1.4% zoonotic TB incidence rates outside Africa, whereas in Africa an estimated 2.8% of zoonotic TB cases were reported, at a crude estimated rate of 7/100 000 population/year.[45] Despite these estimates, the review highlighted the current presence of zoonotic TB particularly in developing countries and a great data shortage on the incidences. The limited data is largely due to the difficulty in distinguishing *M. tuberculosis* and *M. bovis* as they are clinically, radiological and pathologically indistinguishable.[40] It requires specialised techniques (e.g. genotyping or biochemical tests on culture) and resources to distinguish the MTBC members. Very few developing countries have these resources, trained personnel and laboratory facilities to differentiate, hence the gap in knowledge and the underestimation of the zoonotic TB impact.

In Africa, the investigations into the importance of *M. bovis* as a zoonotic disease are mainly focused on the north-eastern and eastern regions of Africa, predominately in Ethiopia.[45-48] To the best of our knowledge there is no information on the incidence and significance of *M. bovis* in humans TB, in South Africa. As a result, the epidemiological significance of *M. bovis* at the wildlife/livestock/human interface in communities at close periphery to KNP is unknown. This is despite reports that have shown *M. bovis* in South African livestock, and acknowledgement of endemic levels in South African game reserves.[33,34,38]

1.2.3 *Mycobacterium tuberculosis*

Humans are considered the only maintenance host of *M. tuberculosis*. However, *M. tuberculosis* is not restricted to humans as it has been shown to cause disease in domestic animals as well as wildlife species.[49]

1.2.3.1 *M. tuberculosis* in animals

The true prevalence and epidemiological significance of *M. tuberculosis* in animals worldwide is currently unknown. There are sporadic reports on *M. tuberculosis* in cattle. In India, *M. tuberculosis* was isolated in 37% of the sampled cattle.[50] In Ethiopia, *M. tuberculosis* prevalence of 27% was detected in grazing cattle.[51] Of great concern, Romero *et al*., (2011), reported the isolation of multi-drug resistant *M. tuberculosis* strain from cattle sampled in Spain.[52] Though, these reports highlight the sporadic isolation of *M. tuberculosis* from cattle, the reports provide circumstantial evidence to justify public health concerns but the zoonotic risk of *M. tuberculosis* spillback from cattle to humans has not been established.[22] Further research into the potential of cattle as *M. tuberculosis* reservoirs for human spillback infection is required.

Infections due to *M. tuberculosis* in livestock are not restricted to cattle. Camels, goats and pigs are among other livestock species *M. tuberculosis* have been isolated from.[30,53-55]

There are reports on the incidence of *M. tuberculosis* in wildlife, both free-ranging and captive. Angkawanish *et al*., (2010) reported the isolation of *M. tuberculosis* in domesticated Asian elephants (*Elephas maximus*), Thailand.[56] Similarly, Michel *et al*., (2003) and (2013) reported the isolation of *M. tuberculosis* in eight different species in a National Zoological Garden of South Africa.[57,58] In free-ranging wildlife, species that *M. tuberculosis* has been isolated include vervet monkeys and Chacma baboons in South Africa.[58]

These studies highlighted the importance of humans as source of TB infection to animals.

1.2.3.2 *M. tuberculosis* in humans

Human TB is an ancient disease that can be traced back as far as 3200 - 3100BC.[59] The disease has re-emerged with devastating consequences and is currently among the top leading causes of death by a single infectious disease.[2] In the year 2013, 9 million new cases of TB and 1.5 million deaths due to TB were reported worldwide. Africa had a quarter of the new cases and the highest continental incidence of 280 per 100 000 population.[2] However, South Africa had a much higher prevalence compared to the continental average. South Africa had a TB incidence of 860 per 100 000 population and was ranked third in new cases per 100 000 population.[2] The high incidence of TB observed in Africa is attributed to human immuno-deficiency virus (HIV), with an estimated four of every five HIV-positive TB related case in Africa.[2]

1.2.4 Transmission of *Mycobacterium tuberculosis* complex at the wildlife/livestock/human interface

The route in which MTBC bacilli are transmitted to a host affects the pathogenicity and the infectious dose required to establish infection.[60] The transmission can occur in several ways, however, the principal vehicles of transmission are the respiratory and oral routes.[19]

The respiratory route entails the inhalation of aerosol droplets containing tubercle bacilli and requires very low infectious dose, as low as 1 colony forming unit (CFU).[61] The aerosol droplet from a host with open pulmonary TB can contain 1 to 400 bacilli.[10] Hence, one droplet can potentially transmit infection but depends on a variety of other factors such as the immune status of the host and the virulence of the MTBC strain.[62] The oral route entails the consumption of mycobacteria contaminated food (e.g. milk, feed, etc), water and other material (e.g. faeces) and requires high infection doses. Several million bacilli are reportedly required for the gastrointestinal infection.[63]

1.2.4.1 Transmission of *M. bovis*

Mycobacterium bovis has a wide host range and the ability to establish itself in multiple species.

1.2.4.1.1 Transmission of *M. bovis* in livestock

A wide spectrum of factors influences *M. bovis* transmission between cattle.

Husbandry practices play a key role in the transmission and prevalence of *M. bovis* within livestock.[64] An intensive husbandry system promotes close contact between animals in an

enclosed environment, thus increasing the probability of *M. bovis* transmission from an infected animal via the aerosol route. Whereas in extensive livestock farming, livestock have an open environment, however livestock interact indirectly through sharing of water sources and grazing land. Additionally, livestock in extensive farming do closely interact in kraals, at dip-tanks, auctions and market points.[19] Ameni *et al*., (2006) reported a difference in lesion distribution between the two farming practises.[65] In livestock kept indoors, typical of intensive farming, lesions were localized in the respiratory tract. With animals grazing on pastures, commonly observed in extensive farming, lesions were predominant in the digestive tract. Some researchers report the prevalence of *M. bovis* is higher in intensive as compared to pastoral systems.[66] Whereas, in other studies, the reverse is reported whereby higher prevalence of BTB was observed in extensive compared to intensive systems.[67]

Within cattle herds, vertical and pseudovertical transmission between calves and dams has been reported. The transmission may occur through the consumption of infected colostrum/milk and/or the close contact during grooming.[68] Congenital infections of the calf in utero do occur but are rare.[69]

The introduction of animals has been shown to increase the risk of BTB in the herd.[70,71] In communal farming, it is often observed that breeding bulls are exchanged between herds with limited veterinary assistance. Similarly, illegal/uncontrolled movement of livestock for trade is often observed in rural communities. These practices do increase the risk of introducing BTB into different herds.

Other risk factors reported to affect the transmission of BTB within livestock include livestock breeds, herd size and age.[39,72]

1.2.4.1.2 Transmission of *M. bovis* in wildlife

Among the African buffalo, the respiratory route is the primary transmission vehicle within herds and between herds. This theory is supported by necropsy reports of TB lesions largely associated with lungs and the upper respiratory tract.[36,73] The African buffalos are gregarious animals found in large herds of an estimated average of 250 and they spend large parts of the day in close proximity with each other, which facilitates aerosol transmission.[74]

Within a single wildlife species, for example the herbivorous kudu, transmission may occur by more than one route. These include: respiratory, oral and percutaneous (cut through the skin) transmissions. BTB infection in kudu is generally characterised by severe abscesses

seen as swollen head and neck (retropharyngeal, parotid, submandibular and cervical) lymph nodes.[75] These infected lymph nodes have draining tracts which lead to contamination of the environment, thus providing opportunity for *M. bovis* transmission to other susceptible herbivores.[26] Kudus are known as super-shedders of *M. bovis*.[27]

In large carnivores, aggressive interaction between prey and predator facilitates several routes of BTB infection. For instance in lions, transmission may occur through direct ingestion of infected material (i.e. lesions, organs, lymph nodes). Percutaneous infection may also occur through bite wounds, due to the intra-species aggression. Additionally, infection could occur through the respiratory route, during suffocation of the prey. TB lesions in lions can be found in the general lymphatic system, particularly mesenteric, peripheral and head lymph nodes.[26] Restriction fragment length polymorphism (RFLP) analysis has confirmed *M. bovis* strains isolated from buffalo and lions as identical, a finding which provides scientific evidence of transmission between prey and predator.[76]

Scavenging omnivores (baboons, warthogs) and carnivores (spotted hyenas, leopards) are too at risk of infection through consumption of infected scavenged material or contaminated environment.

1.2.4.1.3 Transmission of *M. bovis* between wildlife and livestock

The on-going transmission of *M. bovis* between wildlife and livestock is well documented in literature and is a significant obstacle in the eradication of BTB worldwide.[60]

In New Zealand, brush-tail possum are known as the wildlife reservoirs of BTB infection for cattle as well as deer.[77] Possums terminally infected with BTB have been seen to exhibit erratic behaviour that attracts the attention of inquisitive cattle and deer.[78] It is reported that the inquisitive animals would sniff, touch the noises and in many cases extensively lick the terminally infected possums.[79,80] This increased encounter results in a high risk of *M. bovis* spillback infection to cattle. In the UK, badgers are identified as wildlife reservoirs for BTB. The transmission of *M. bovis* from badgers to cattle is reported to occur through two possible routes, direct or indirect transmission.[81] Direct contact between badger and cattle through close encounters facilitates aerosol transmission. Whereas, indirect transmission entails consumption of pasture contaminated with urine, faeces and/or sputum by infectious badgers.[82] In South Africa, it is reported that the transmission of BTB from the wildlife maintenance host (i.e. African buffalo) could occur through direct (aerosol route) or indirect

(sharing contaminated grazing and/or water sources).[26] However, no ecological studies in southern Africa have confirmed the transmission route of BTB spillback infection from wildlife to cattle.

As a result of the risk of disease transmission at the wildlife/livestock interface and the threat of BTB maintenance in wildlife, numerous control policies have been implemented worldwide. In Ireland, the controversial policy to cull badgers and vaccination trials are the main components of BTB eradication.[83,84] In New Zealand, the BTB control policy entails reducing the population of possums as well as oral vaccination of possums .[85]

In South Africa, particularly the KNP, a great amount of man-power and capital has been invested to create a physical barrier (game fences), separating livestock and communal land around the KNP from wildlife. However, these fences do not guarantee complete separation. Natural disasters such as the great water flood of the year 2000 and elephant stampedes have caused great damage to the fences, leaving the fences permeable. Kudus, impalas (*Aepyceros melampus*) and buffaloes have been seen to escape through the damaged fences.[86,87] Hence, direct or indirect contact between wildlife and livestock is observed. Bovine TB remains a threat for transmission through possible contact.

1.2.4.1.4 Transmission of *M. bovis* to humans

It is widely reported that the transmission of *M. bovis* from livestock to humans is mainly through the consumption of raw unpasteurised milk, raw/undercooked meat and/or aerosol transmission.[13,23,44] Raw milk is considered the main vehicle for transmission.[88] Tuberculosis of the udder (tuberculous mastitis) and/or *M. bovis* bacteraemia in early infections are the main source of *M. bovis* contamination in milk.[12]

Milk is an essential commodity especially in developing countries as it is very nutritious. It is often an affordable, cheap source of protein, animal fat and calcium, particularly consumed by children for their growth and development. The introduction of pasteurised milk in developed countries resulted in a rapid drop, to near disappearance of zoonotic TB.[89] However, communities still practise the consumption of raw unpasteurised milk, particularly rural communities in Africa.[12] This food consumption practise is of public health concern as the isolation of *M. bovis* in communities that consume raw unpasteurised milk is being reported over the past few years.[9,50,90,91] Hence, the risk of zoonotic TB is still prevalent despite advances in health research.

Human-to-human transmission of *Mycobacterium bovis* is rare.[41] However, sporadic studies have been reported and were mainly attributed to co-infection with human immuno-deficiency virus (HIV).[45] There is a vast knowledge gap in the significance of human-to-human transmission in the prevalence of zoonotic TB, particularly in communities with a high burden of HIV.

The transmission of *M. bovis* infection from animals to humans is not limited to livestock. Wildlife can directly transmit *M. bovis* to humans through the consumption of undercooked/raw bush-meat and/or the respiratory route.[92] In developing countries, bush meat hunting (poached wildlife) is considered a cheap source of protein and it is common for the whole carcass (muscle, organs with lymph nodes as well as offal) to be consumed. Hence, there is a potential risk of *M. bovis* infections in these communities.[23] The risk of *M. bovis* transmission from wildlife is not limited to hunters and their families, but also veterinarians, veterinary support system, care-givers, and abattoirs workers as they too are at risk through occupational exposure to aerosols from infected animals.[93]

1.2.4.2 Transmission of *M. tuberculosis* to animals

The common route of *M. tuberculosis* transmission from humans to animals is through the aerosol route, whereby humans cough up tubercle bacilli from open pulmonary lesions. Close proximity with animals either through occupation duties (e.g. animal handlers and caretakers) or sharing shelter with livestock, facilitates aerosol transmission.[44,47] Regassa *et al*., (2008) reported the prevalence of BTB was threefold higher in cattle owned by farmers with active TB compared to farmers with no active TB.[48] Similarly, Ameni *et al*., (2013) reported cattle owned by TB positive farmers had a statistically higher ($p < 0.01$) increase in comparative intradermal tuberculin skin test (CIDT) reaction compared to TB-free households.[94]

Other routes of transmission include the oral route, through consumption of contaminated feed, water and other materials. Humans can contaminate the animal feed and environment through sputum, faeces or urine. In a study conducted in the Germany state of Hessen between 1968 and 1972, 114 cattle were infected. Nine of 12 farmers' were diagnosed with genitourinary TB; of interest one farmer had infected 48 cattle in four herds (Schliesser, 1974 cited by Yates and Grange, 1988).[95] Other unusual possible routes of transmission include cultural practises where farmers spit chewed tobacco into the livestock mouth, as means of anti-parasitic treatment.[51]

Despite reports of *M. tuberculosis* in animals, questions still remain concerning the relative virulence and factors that influence the progression from infection to disease of *M. tuberculosis* in animals.[40] As mentioned above, Ameni *et al*., (2013) reported cattle owned by TB positive farmers had higher chances of reacting to CIDT test; however, no TB transmission was established.[94] It was concluded that TB transmission from the farmers to cattle by the aerosol route sensitizes the cattle but rarely leads to disease or infection. This study is among other literature that highlights the incomplete understanding of the pathogenesis of *M. tuberculosis* in animals.

1.3 Clinical signs

Tuberculosis is a chronic debilitating disease in both animals and humans. In early stages of infection, no distinct clinical signs are observed. However, during the late stages, clinical signs may manifest but will depend largely on the organs and organ system affected.[40]

1.3.1 Animals

In advanced stages of BTB, animals are observed to have difficulty in breathing, as a result animals stand with their elbows adducted; neck extended and lowered head to facilitate breathing.[22] Additional clinical signs include coughing, emaciation, anorexia, enlargement of lymph nodes, dull hair coat and sunken eyes.[96]

1.3.2 Humans

The clinical symptoms of TB in humans are chronic coughing for more than 2 weeks, unexplained weight-loss, fever and/or night sweats.[97]

As mentioned above, the clinical signs of zoonotic TB are indistinguishable from TB caused by *M. tuberculosis*. However, zoonotic TB is often associated with extra-pulmonary TB and can present itself in various forms, including cervical lymphadenitis, and/or lupus vulgaris.[40,41]

1.4 Diagnosis of tuberculosis in cattle

There are numerous techniques used to diagnose TB. These include tuberculin skin tests, gamma interferon (IFN-ɣ) assay, post-mortem examination, necropsy, histopathology, biochemical tests, enzyme-linked immunosorbent assay (ELISA) and bacteriological examination.[98,99]

1.4.1 Tuberculin skin testing

Tuberculin skin tests (TST) are the international standard for ante mortem diagnosis of BTB in cattle but are also used in human medicine.[100]

The TST is based on the delayed type hypersensitivity to mycobacterial tuberculo-protein (purified protein derivative (PPD)).[101] The PPD is injected intradermally and the dermal swelling, as a result of the cell-mediated immune response, is measured after 72 h.[102] There are different TST methods which include single intradermal, comparative intradermal (CIDT), short thermal and Stormont tests.[98] The type of TST used depends on the prevalence of TB and exposure to other sensitising mycobacteria from the environment.[103]

The CIDT test is more commonly used as it can differentiate between infections from *M. bovis* and sensitization from other environmental mycobacteria. The CIDT test has the sensitivity range of 55.1-93.5% and specificity range of 88.8 – 100%.[98] The CIDT test is read by measuring the increase in skin thickness at the site injected with bovine tuberculin as compared to the site of the avian tuberculin injection. The recommended cut-off for classifying positive cattle by OIE standards is greater than or equal to 4 mm more on the bovine injection site. However, there have been reports that a lower cut-off of 2 mm in countries such as Southern Chad and Ethiopia increases the sensitivity of detecting positive animals.[104,105] Though these lower cut-offs do suggest improved sensitivity there is still a need to optimise these modified test interpretation for targeted population (i.e. cattle breeds) and the different husbandry systems in various countries.

Advantages of TST are that it is convenient, cost-effective, and highly available. The limitations are the lack of standardization in methodology and interpretation of results, low sensitivity and specificity in areas with low TB prevalence.[103] Skin tests are influenced by the stage and severity of the disease.[106]

1.4.2 Gamma Interferon assay

This in vitro assay is a laboratory based technique detecting specific cell mediated immune response by circulating lymphocytes.[100] The assay consists of two stages. First, whole blood collected in heparin is incubated with antigens (i.e. bovine PPD tuberculin, avian PPD tuberculin and specific antigens) for 18 – 24 h. During this incubation phase, production and release of IFN-ɣ by predominantly T lymphocytes is induced. In the second stage, the plasma

supernatant is harvested in order to quantify the IFN-ɣ present using a sandwich enzyme-linked immunosorbent assay (ELISA).[107]

The advantages of IFN-ɣ assay is the increased sensitivity and the early detection of infections. The IFN-ɣ assay has an estimated sensitivity range of 73.0 to 100% and specificity range of 85.0 to 99.6%.[100] In addition, IFN-ɣ is used as a confirmatory test to reactors of TST or as an ancillary test to increase diagnostic sensitivity (parallel testing).[101]

The limitations of the IFN-ɣ assay include the reduced specificity. The introduction of defined mycobacterium antigens such as ESAT-6 and CFP-10 has improved the specificity.[108] Other limitations of IFN-ɣ are the high costs and the logistical demand of processing collected blood within 24 h after collection.

1.4.3 Post-mortem diagnosis

A tentative diagnosis of TB can be made through the detection of typical granuloma lesions during post mortem examination.[109] Granuloma or tubercle lesion formation is a result of the host immune system attempting to localize the pathogen and allowing inflammatory (i.e. macrophages) and immune mechanisms to destroy the bacilli.[110] The development of lesions depends on the stage of infection and the immune response of the host. These lesions can occur anywhere in the body. In humans, the lesions are commonly found in the lungs.[111] These lesions begin as an exudative bronchopneumonia and progress to classical caseous granuloma formation, followed by massive necrosis (tissue destruction) and cavity formation in the lung.[10] Similarly, in cattle, lesions usually appear in the lung as well as pharynx and thoracic cavity.[112] The lung lesions appear as encapsulated yellowish foci of caseous necrosis which may spread to the visceral and parietal pleura.[110]

As part of BTB control strategies, the detection of gross lesions in abattoir surveillance is used as a cost-effective method to trace back the herd of origin to establish herd level infection.[113] In high BTB incidences, macroscopic post-mortem inspection is used as diagnosis in animals, however, the final diagnosis can only be reached using other confirmatory methods such as culture and molecular methods.[101]

The limitations of post-mortem is that the sensitivity depends on necropsy techniques employed and anatomical sites examined.[109]

1.4.4 Isolation, bacterial culture and biochemistry tests

Bacteriological isolation of MTBC from clinical samples (e.g. sputum, tissue, lesions, milk) remains the gold standard for definitive diagnosis of TB infection in both animals and humans.[109] The isolation of MTBC entails sample collection, homogenization (depending on the sample type), decontamination and culture on media.

There are a wide variety of media available for both solid and liquid culture. The two commonly used are egg-based Lowenstein-Jensen (with or without pyruvate) agar or Middle brook $7H_{10}$ or $7H_{11}$ medium.[98] Though there is a wide range of media to enrich the growth of mycobacteria, the challenge remains that the colony growth and characteristics are not sufficient to definitively identify the species of mycobacteria. Conventional biochemical tests such as niacin production, nitrate reduction and urease test were in the past commonly used to definitively identify mycobacteria species.[114] The limitations of these biochemical techniques are the long periods of incubation. In addition they are labour intensive and cannot distinguish at strain level. Hence, the introduction of molecular genotyping techniques with potentially improved sensitivity, flexibility and speed.

1.4.5 Molecular diagnostic techniques

Early methods of diagnosis and characterization, particularly the phenotypic techniques have limited ability to distinguish MTBC due to the high genomic similarity. More recent molecular diagnostic tests have increased sensitivity, reduced turnaround time and are reproducible.[115] Hence, they are increasingly used in parallel with phenotypic techniques.

These molecular techniques are mainly based on the amplification of nucleic acids (DNA or RNA) using techniques such as polymerase chain reaction (PCR) and the detection of mutations in the genes by sequencing or nucleic acid hybridization.[116-118] Examples of molecular diagnostics techniques include, restriction fragment length polymorphism (RFLP), Real-time PCR and Line Probe Assay.[119]

The disadvantages of molecular techniques are that the sensitivity can be significantly compromised by PCR inhibitors in the specimens, the expensive equipment required and the techniques are more prone to contamination, thus lead to false positives.[120]

1.5 Genotyping techniques

With improved technology, there has been a great advancement in molecular genotyping techniques, which have proved useful in epidemiological studies. Over the recent decades, molecular epidemiology has been a great tool in answering questions such as the source of infection and the risk factors for the spread of infection.[17]

The common genotyping techniques include polymerase chain reaction (PCR) of genomic regions of difference (deletion mapping), spoligotyping, variable number tandem repeat (VNTR), IS*6110*- based restriction fragment length polymorphism (RFLP) and a range of other genotyping methods.

1.5.1 Deletion mapping

Comparative genomic analysis of the whole DNA sequence of certain MTBC strains (e.g. *M. tuberculosis* H_{37}Rv, CDC 1551, *M. bovis*, and *M. bovis* BCG) has demonstrated the loss of genetic material among members of the MTBC, referred to as regions of difference (RD) or large sequence polymorphism (LSP).[121,122] These deletions can result in differences in the genome content, hence possible variations in phenotypic traits (e.g. resistance to antibiotics) and enhancement or loss of virulence.[123,124] These polymorphisms are reported as important in generating genetic diversity within the MTBC complex and can be used as a tool to differentiate members of the MTBC and the study of phylogeny and evolution.[125-127]

The deletion analysis can either be performed by a PCR-based method or automated microarray techniques.[121] The advantages of the method are that it is sensitive, rapid and less laborious.[127] The disadvantage is that it has limited discriminatory power in closely related strains.

1.5.2 Restriction Fragment Length Polymorphism with IS6110 hybridization (IS6110-RFLP)

The IS*6110*-RFLP typing technique is based on the detection of difference in the number of IS*6110* copies (ranging from zero to 25) and the molecular weight of DNA fragments in which the insertion are found after enzymatic restriction of the DNA.[17,128] IS*6110* belongs to the enterobacterial IS3 family of mobile elements, with 1361 base pairs (bp) and the sequence is exclusive to MTBC.[129]

IS*6110*-FLP is considered the traditional gold standard of genotyping *M. tuberculosis*, as it has very high discriminatory power.[130] However, there are a number of limitations which include the need for large amounts (2 - 3µg) of high quality DNA, a challenge to obtain in slowing growing mycobacteria.[121] Additionally, the low discriminatory power of the technique in strains with five or fewer IS*6110* copies number and the technique is technically demanding.[131] Based on these limitations, alternative techniques have been sought after. These include commonly spoligotyping and Mycobacterial Interspersed Repetitive Unit - Variable Number Tandem Repeat (MIRU-VNTR).

1.5.3 Spoligotyping

This genomic technique is based on detecting the presence or absence of non-repetitive sequence of spacers found between the Direct Repeat (DR) elements of the mycobacterial genome.[132] The DR regions are 36 bp, which are interspersed with non-repetitive DNA spacer sequence of 35-41 bp.[17] The combination of DR region and spacer is referred to as direct variable repeat unit. There are 94 spacer sequences; however 43 spacer units are typically selected for spoligotyping strains of the MTBC.[132]

Spoligotyping entails the amplification of the DR locus and subsequent differential hybridization of the amplicons with membrane bound oligonucleotide complementary to the variable spacer regions.[129] Loss of hybridization signal of any of the 43 spacer signals polymorphism. The presence or absence of each of the 43 direct variant repeat spacer units is commonly interpreted with corresponding ones and zeros, respectively, producing spoligo patterns which appear barcode like for each isolate.

Spoligotyping is the most commonly used molecular typing to differentiate strains of *M. bovis* due to low IS*6110* copies; however this technique is widely used for characterizing *M. tuberculosis* isolates as well.[17,133,134]

One of the main advantages of spoligotyping is the standardized interpretation of results across international laboratories, which is facilitated by the website www.Mbovis.org or spoligotyping database SpolDB4 that assigns an international recognized octal number.[135] Additional advantages of spoligotyping are the technique is simple, highly reproducible and requires low DNA. However, the main disadvantage is the limited discriminatory power to distinguish closely related strains.[130]

Gori *et al*., (2005) recommended the use of spoligotyping for simultaneous detection and typing of TB in health clinical settings.[136] Authors reported sensitivity and specificity of 98% and 96% respectively in the clinical samples obtained in Milan. However, the recommendation is not feasible in developing countries as the resources to purchase spoligotyping kits and equipment are high and the technical support is limited. Currently, spoligotyping is mainly restricted to research centers in developing countries.

1.5.4 Mycobacterial Interspersed Repetitive Unit - Variable Number Tandem Repeat (MIRU-VNTR)

The MIRU-VNTR technique is based on determining the number of repetitive mini-satellite elements located as tandem repeats at various loci of the mycobacterial chromosome.[137] The repetitive units are 40-100 bp and are located in 41 loci throughout the *M. tuberculosis* H37Rv.[138] Shortly after the establishment of the MIRU-VNTR technique, 12 MIRU loci were recommended in combination with spoligotyping.[128] For improved discriminatory power of the technique, the recommendations increased to 15 MIRU loci for molecular epidemiological discrimination studies and 24 MIRU loci for high-resolution phylogenetic studies, particularly for *M. tuberculosis*.[139] VNTR-typing is not restricted to *M. tuberculosis*, it has been shown to have high discriminative potential among *M. bovis* strains.[140,141] The loci panel reported in *M. bovis* epidemiological studies includes 16 to 29 loci panel.[142,143] However, there is no specific set of loci that has been standardized and the allelic diversity of loci varies from country to country and between MTBC members.

The MIRU-VNTR technique is a PCR based method that uses primers specific to each repeat locus. Because the length of the repeat unit is known, the size of the amplicons would reflect the number of copies of the repeat unit.[128] Results of the typing are presented as a string of numbers (multi-digital code) indicating the number of repeats at each locus.[137] The original protocol included visualization of amplicons on agarose gel and sizes were determined with the use of molecular markers. MIRU-VNTR typing could also be performed using capillary or denaturing high-performance liquid chromatography.[139] With advancement of technology, MIRU-VNTR has been automated and has improved the technique by increasing the throughput, reproducibility, less time consuming and computerized analysis.[128] Disadvantages of the automated MIRU-VNTR are the need for specialized expensive equipment and software for analysis.

MIRU-VNTR other advantages include better discriminatory power compared to spoligotyping and IS*6110* RFLP, particularly strains with low copies of *IS6110.* In addition, MIRU-VNTR results can be compared across labs and there is a global database (www.miru-vntrplus.org) available.[130]

MIRU-VNTR is considered by some authors as the new gold standard for *M. tuberculosis* genotyping.[128]

1.5.5 Other methods

A wide variety of other methods for genotyping MTBC and other mycobacteria are available.[121,129] These include amplified fragment length polymorphism (AFLP), large sequence polymorphism (LSP), the emerging approach of single nucleotide polymorphism (SNP) typing and whole genome sequencing and deletion typing.[17,130] These emerging techniques are likely to replace aforementioned techniques in the future. Each of the typing methods has their own benefits and limitations. Choice of technique will depend on sample condition, objective of investigation as well as equipment and technical expertise.

1.6 Cost and socio-economic impact of bovine tuberculosis

Bovine tuberculosis is a chronic debilitating disease for the infected animal (clinical symptoms mentioned above). However, the impact of BTB is not limited to the individual animal but also affects agriculture economics and livelihoods of farmers, through losses in livestock production; changes in herd demographic composition; restrictions in markets and trade; as well as costs implementing BTB surveillance and control programmes. It has been shown that BTB positive cows can have up to a 5% loss in fertility of the annual number of calves born; 10% reduction in milk production and 5% loss of meat product through condemned/confiscated organs or whole carcass. [144,145]

The literature on the cost and impact of BTB is based on old European and Canadian studies, in Africa investigations into the cost of the disease are rare. A cost estimate study of BTB in Ethiopia by Tschopp *et al.*, (2013) reported no measurable loss in asset value or cost of disease in rural and urban production systems.[145] The study had a number of limitations but did highlight that the economic impact of BTB is extremely difficult to accurately calculate as there is a huge lack of data, including information on livestock demographic and productivity in African settings.

1.7 Control of zoonotic TB

Great strides in investigating the epidemiological significance and transmission routes of BTB have assisted in designing control measures (e.g. test-slaughter of TB infected cattle and pasteurization of milk) which have drastically reduced the prevalence of BTB both in livestock and humans.[146] This success in eradicating BTB is predominately observed in Western European countries where there are well-functioning veterinary services, with cooperating farmers and finances (public funds) to compensate farmers. Very few African countries, including sub-Saharan Africa have such infrastructure and resources. Many of the African countries have unregulated animal movement, lack animal identification which in turn limits trace back of infected animals; strained veterinary services and lack of financial resources. It was estimated that the annual testing cost per animal in African settings (i.e. Ethiopia) would be approximately 2 US$ without the cost of compensation, many African countries cannot afford these high prices.[145] Hence, effective control measures are compromised. Some African countries (i.e. South Africa) however, have taken a step further to include the test and slaughter policy as part of the governments BTB control programme, however the success of the policy largely depends on political support and economic resources.

Alternative strategies for the control of BTB particularly in resource poor countries include post-mortem inspection. Currently many African countries have few abattoirs as communities largely dependent on informal slaughter houses. However, invest in post-mortem inspection facilities and training of meat inspectors could provide a cost effective method for surveillance and control of BTB. In addition, awareness with communities particularly in rural areas could provide more cooperation from farmers.

However, since the WHO highlighted the importance of zoonoses to public health and ‘One Health’. As well as the declaration of BTB as a ‘Neglected zoonoses’, increased attention is focused on research into the epidemiology of zoonotic diseases in developing countries.[92]

1.8 Problem statement

Bovine tuberculosis is endemic in buffaloes in the Kruger National Park (KNP) and has spilled over into other wildlife species and adjacent game farmers.[33,37] Apart from sporadic TB surveillance in the cattle population adjacent to the KNP, there has been no study to investigate the potential spillback of BTB from the endemic KNP into livestock in

neighbouring communities.[38] Due to the potentially negative implications on livestock and human health arising from interaction observed between livestock and wildlife, it is of importance to investigate the prevalence of BTB in these communities at the interface.[147]

Over the past decades, increased human contact with livestock is being observed in response to a rise in demand for food production from animal origin, to sustain the demand for animal protein by an increasing human population.[148] There are potential risks in the close interaction between livestock and humans, as infectious diseases have been shown to spread from animals to humans and vice versa.[6] However, the true prevalence of zoonotic diseases in livestock is unknown, particularly in southern Africa. There is limited understanding of factors which promote or inhibit the spread of diseases at the human-animal interface. In addition, the lack of understanding of the human-animal interface compromises initiatives in controlling the transmission and impact of zoonotic diseases such as TB.

1.9 Aims and objectives

The aims and objectives of this study were to investigate the presence and if applicable, the prevalence of tuberculosis in livestock and genetically characterise the causative agent. In addition, investigate the incidence and significance of *M. bovis* in humans through the isolation and the genetic characterisation of the MTBC strain population in TB patients. The potential risk factors for TB transmission between livestock and humans were investigated through a questionnaire based interview.

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Chapter 2: Spillback transmission of *Mycobacterium bovis* from wildlife in the Greater Kruger National Park Complex to neighbouring livestock

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For the purpose of this thesis, a more detailed account of the study is presented below.

2.1 Abstract

The objective of this study was to investigate the presence and if applicable the prevalence and molecular profile of bovine tuberculosis (BTB) in cattle bordering the Greater Kruger National Park Complex (GKNPC). A total of 1166 cattle were randomly selected at 15 dip-tanks within the study area. Comparative intradermal tuberculin skin testing was performed to determine the BTB status and the interferon gamma assay was performed as an ancillary test. A total of four BTB cases were detected with an overall prevalence of 0.33 % (95% CI. 0.14 - -0.79). A single *M. bovis* genotype was identified as the causative agent in all infected cattle, the dominant Kruger National Park (KNP) strain designated KNP – VNTR -1. Based on the genotype of the causative agent and circumstantial evidence of wildlife/livestock interaction, the finding is strongly indicative of BTB spillback from GKNPC wildlife to livestock outside the park.

2.2 Introduction

Bovine tuberculosis (BTB) is an infectious disease caused by *Mycobacterium bovis* (*M. bovis*). The pathogen has a wide host range inclusive of humans, domestic and wild animals. Great strides in controlling BTB (e.g. test-slaughter of TB infected animals and pasteurization of milk) have drastically reduced the prevalence of the disease in livestock and humans, particularly in developed countries. However, in southern Africa, similar to a number of other developing countries, BTB remains a challenge in livestock and humans due to uncontrolled BTB status or inactive schemes lacking funds to support control efforts.[1,2] An additional factor that compromises the effective eradication of the disease both in developed and developing countries remains the wildlife reservoirs which pose a threat of re-infection in livestock.[3] In Sub-Saharan Africa, particularly South Africa and Uganda, African buffaloes (*Syncerus caffer*) serve as wildlife maintenance hosts whereas in Zambia, lechwe antelopes (*Kobus leche Kafuensis*) have been identified.[4,5] New reports have suggested greater kudu (*Tragelaphus strepsiceros*) and common warthog (*Phacochoerus africanus*) as potential maintenance hosts that should be considered in BTB control strategies.[4]

Humans and animals, located at the wildlife/livestock/human interface are susceptible to influence dynamics of diseases at this interface, among other factors the direct contact or sharing of resources (e.g. water and land).[6] These interactions are limited where wildlife conservation areas are fenced, i.e. in the Kruger National Park (KNP) in South Africa, while open ecosystems provide more frequent opportunities for pathogen(s) exchange.[7] Bovine tuberculosis is endemic in buffaloes and has spilled into other wildlife species particularly in the KNP and adjacent game reserves which have become part of the conservation area of KNP to form the Greater Kruger National Park Complex (GKNPC) by dropping neighbouring game fences.[8] However, apart from sporadic TB surveillance in the cattle population adjacent to the GKNPC, there was no study to investigate a potential spillback of BTB from the endemic GKNPC into livestock in neighbouring communities.[3] Due to the potentially negative implications on livestock and human health arising from interaction observed between livestock and wildlife, it is of importance to investigate the prevalence of zoonotic diseases in these communities.[5]

This study aimed to investigate the status and origin of BTB in livestock in a rural community situated adjacent to the GKNPC where BTB infection has been confirmed in multiple wildlife species.[4]

2.3 Materials and Methods

2.3.1 Study area and cattle population

The Greater Kruger National Park complex (GKNPC) is a joint conservation area between Kruger National Park and nine neighbouring private game reserves. Within this conservation area, wildlife are allowed to move freely in an area of 33 948 km.[2 9] This study was conducted in a rural community situated west of the GKNPC, in Mpumalanga Province, South Africa, 24°33'-45'S, 31°15'-30'E (Figure 2.1). The study area forms part of the Mnisi Tribal Authority and shares boundaries with three of the private game reserves. Although efforts are made to minimize livestock/wildlife interaction through fences and buffer zones established by doubling fencing, interaction between wildlife and livestock is observed when fences are compromised.[10]

2.3.2 Ethical Statement

Ethical approval for this study was obtained from the institutional ethical committee at the University of Pretoria Animal Use and Care committee (Ref: V043-12); and The Department of Agriculture, Forestry and Fisheries, Republic of South Africa (12/11/1/1/6/1). As BTB is a controlled disease, thus routine testing was performed under the supervision of the Mpumalanga Veterinary Services.

2.3.3 Study animals

A cross-sectional study was conducted between August 2012 and February 2013. Testing was performed at the 15 dip-tanks in the study area.

All cattle in the study area are required by law to register and attend dip-tank inspection as the community falls under the Foot and Mouth disease control zone. Farmers are assigned and registered to a particular dip-tank by the agricultural authorities and given a unique number to a particular dip-tank, referred to as stock card number. In the latter, the numbers of cattle, births and deaths, as well as animal movement within a herd are recorded. Animals selected for testing in the study were randomly selected from a list of stock cards presented during visits to particular

dip-tanks. Ten percent of registered animals under a particular stock card were randomly tested. However, a minimum of 2 to 3 animals per stock card were tested. All stock card owners willing to participate were included to reach a target of 10% of the cattle assigned to each dip-tank. Animals under the age of 2 years were not included in the study.

2.3.4 Sample size

The estimated population size of cattle in the study area is 15 000. A hypergeometric function was used to estimate the number of samples required to obtain, with 95% confidence, at least 3 test positive animals if the prevalence was 0.5%. A total of 1222 animals were to be tested but 1682 cattle were recruited at the 15 dip-tanks to compensate for an anticipated lack of farmers' compliance to return animals for reading of the skin test after 72hrs.

2.3.5 Intradermal tuberculin test (skin test)

The comparative intradermal tuberculin test (CIDT) was applied in the determination of the BTB status in cattle. The procedure and interpretation of test results were according to the guidelines of the World Organization for Animal Health (OIE).[11] Briefly, animals were classified as inconclusive reactors if their increase in skin thickness at the site injected with bovine tuberculin was between 2 mm and 4 mm greater than the reaction shown at the site of avian tuberculin injection. A positive BTB reactor showed an increase of more than 4 mm greater than the increase at the avian injection site. The net increase with regard to the bovine site was referred to as bovine bias.

2.3.6 Interferon gamma assay (Bovigam)

The interferon gamma assay (IFN-ɣ) was performed as an ancillary test to the CIDT, to increase the specificity of detecting BTB infected cattle. Whole blood in heparin was collected immediately after reading of the skin reaction (72 hours post PPD injection) from all CIDT positive and inconclusive animal reactors with a bovine bias of greater than 3 mm. The assay was performed according to a modified protocol for the interferon gamma assay as reported previously.[12] Briefly, whole blood in heparin was stimulated with bovine PPD, avian PPD and fortuitum PPD and incubated at 37°C in an incubator with 5% CO_2 for 12 hours. Thereafter, plasma was harvested from each tube and stored at -20°C till further processing. In the BSL 2+ laboratory, the plasma was thawed to room temperature and assayed for the presence as well as

the concentration of IFN-ɣ using the Bovigam 2G TB kit (Thermo Fischer Scientific) as per manufacturer's instructions. The optical densities (OD) of the processed plasma were measured using an ELISA reader at 450nm. Positive and negative controls were included (pokeweed mitogen and unstimulated blood, respectively). Interpretation of results was according to Michel *et al*., 2011.[12] In summary, optical densities measured for plasma stimulated with bovine, avian, fortuitum PPDs were recorded as OD_{bov}, OD_{av}, OD_{fort}, respectively. OD_{pwh} represented the optical density measured for the positive control, pokeweed, whereas OD_{nil} represented optical density measured for negative control, unstimulated plasma. Animals were classified as BTB positive if ($OD_{bov} - OD_{av} > 0.2$) and $OD_{fort} - OD_{nil} \leq 0.15$). Avian reactors were classified if animals had $OD_{av} > (OD_{bov} + 0.1 \times OD_{bov})$. Animals were classified as multiple reactor (immune response to bovine and fortuitum PPDs) if ($OD_{bov} - OD_{av} > 0.2$) and ($OD_{fort} - OD_{nil} > 0.15$). Animals were classified as equal reactor (equal immune response to bovine and avian PPDs) if $(OD_{bov} + 0.1 \times OD_{bov}) > OD_{av} > (OD_{bov} - 0.1 \times OD_{bov})$. To check the validity of the test, OD_{pwh} should be > 0.5.

2.3.7 Sample collection, culturing

Test animals classified as positive based on CIDT and IFN-ɣ including recent offspring of test positive cattle were purchased and culled. Standard sets of tissue samples from the lymph nodes of the head and thorax were collected. In addition, affected tissue was collected where visible lesions were observed. The tissue samples were stored at -20°C. Tissue samples were processed and cultured as described.[13] Samples were defrosted in the biohazard cabinet in the BSL2+ laboratory thereafter sectioned using sterile scissors. Samples were homogenized with a motor and pestle with sterilized sand to assist in grinding. The homogenate was decontaminated by adding equal volume of 4% NaOH and incubated at room temperature for 10 mins. Samples were then centrifuged at 3500 rpm for 10 mins and the supernatant was discarded. The sediment was neutralized with distilled water. The suspension of each sample was inoculated onto two sets of Löwenstein-Jensen media, one media set was supplemented with glycerol and the second set supplemented with glycerol and pyruvate. Media sets were inoculated in duplicates. Cultures were incubated at 37°C for 12 weeks with weekly inspections of growth. Non-pigmented colonies were stained using the Ziehl-Neelsen staining method to test for Acid Fast Bacilli (AFB).

2.3.8 Collection of milk

Approximately 10 ml of milk was drawn from lactating cows that were CIDT positive. The milk samples were stored at -20°C until processed in the BSL 2+ laboratory. Processing of each milk sample entailed thawing to room temperature and adding equal volumes of 1% Cetylpyridinium Chloride (CPC) solution. Samples were incubated at 20°C in the dark for a week. Thereafter, samples were centrifuged at 3500 rpm for 30 mins. The supernatant was discarded, whereas the pellet and the cream were neutralized with 25ml of sterilized water. The sample was centrifuged again at 3500 rpm for 10 mins and the supernatant was discarded. The pellet was inoculated on two sets of Löwenstein-Jensen media. The medium slopes were inoculated as described above and incubated at 37°C for 12 weeks, with weekly inspection of growth. Non-pigmented colonies were stained using the Ziehl-Neelsen staining method to test for AFB.

2.3.9 Template DNA preparation

One loop full of AFB cells were suspended in 100µl of sterile distilled water and heated at 95°C for 25 mins. Samples were then cooled on ice then centrifuged briefly. The supernatant was used as crude DNA template for genotyping techniques, i.e. spoligotyping and variable number tandem repeat (VNTR) genotyping.

2.3.10 Molecular characterization

Heat killed AFB isolates were characterized using spacer oligonucleotide typing (spoligotyping) with a commercial kit (Ocimum Biosolutions, Indianapolis, IN, USA).[14] Spoligopatterns obtained were referenced according to the *M. bovis* spoligotype database (www.mbovis.arg).

Variable number tandem repeat (VNTR) typing was performed as described [15]. Briefly, a panel of 13 loci (ETR A, B, C, and E; Qub 11a, b, 18 and 26; MIRU 16, 23 and 26; M. tub 12 and 21) were individually amplified and the PCR products separated on 2% agarose gels. DNA fragment sizes were also estimated by using a 100 base pair ladder PLUS as well as the Quantity One 1-D analysis software installed in the Gel Doc system. These were converted into tandem repeat copy numbers and the resulting VNTR profiles were saved in an Excel spreadsheet. Dendogram was created using an unweighted pair group mean average (UPGMA) tree, using the Bionumerics software package version 7 (Applied Maths, Sint-Martens-Latem).

2.3.11 Data analysis

The prevalence and its 95% confidence interval of positive and inconclusive test results within dip-tanks were calculated in logistic regressions using dip-tanks as explanatory variables. Positive and inconclusive (including positive results) results were analyzed separately. In the event that a dip-tank had no positives, the upper bound of the confidence interval was calculated by the exact method. The overall prevalences were calculated using robust logistic regressions. In the analysis, dip-tanks were used as primary sampling units and the data was weighted according to the cattle population and the number of cattle tested in each dip-tank. Finally, the distance to the park (shortest distance between the dip-tank and the park's fence) was tested as continuous explanatory variable.

2.4 Results

2.4.1 Intradermal tuberculin skin test

A total of 1682 head of cattle were tested in the 15 dip-tanks located within the study area. Only 1166 of the 1682 head of cattle were presented for measuring the skin reactivity after 72 hours, resulting in a 67 % farmers' compliance with the BTB testing scheme (Table 2.1).

Table 2.1: Results of comparative intradermal tuberculin testing at 15 dip-tanks in the study area

Dip tank	No. of cattle	No. of cattle tested (%)	No. of inconclusive test animals (% and 95% CI)	No. of test positive animals (% and 95% CI)
Welverdiend A (A)	1648	178 (10.8)	6 (3.4; 1.5 – 7.3)	1 (0.6; 0.1 – 3.9)
Thlavekisa (B)	556	55 (9.9)	3 (5.5; 1.8 – 15.6)	0(0; 0 – 5.3)
Gotternburg (C)	963	104 (10.8)	2 (1.9; 0.5 – 7.4)	0(0;0 – 2.8)
Clare A (D)	706	72 (10.2)	3 (4.2;1.4 – 12.1)	0(0;0 – 4.1)
Utah Scheme (E)	585	82 (14.0)	1(1.2; 0.2 – 8.1)	0(0; 0 – 3.6)
Welverdiend B (F)	786	75 (9.5)	0(0; 0 - 3.9)	0 (0; 0 – 3.9)
Eglington (G)	1092	86 (7.9)	3 (3.5; 1.1 – 10.3)	0(0;0 - 3.4)
Share (H)	709	70 (9.9)	3 (4.3; 1.4 – 12.5)	0(0; 0- 4.2)
Seville B (I)	850	75 (8.8)	1 (1.3; 0.2 – 8.9)	0(0; 0 – 3.9)
Shorty (J)	545	48 (8.8)	1(2.1; 0.3 – 13.4)	1(2.1; 0.3 – 13.4)
Hlalakahle (K)	436	49 (11.2)	3 (6.1; 2 – 17.3)	1 (2; 0.3 – 13.1)
Utah A (L)	812	79 (9.7)	2 (2.5; 0.6 – 9.6)	0 (0; 0 -3.7)
Seville A (M)	903	50 (5.5)	1 (2; 0.3 – 12.9)	0(0; 0 – 5.8)
Athol (N)	1298	83(6.4)	2(2.4; 0.6 – 9.1)	1 (1.2; 0.2 – 8.1)
Clare B (O)	943	60 (6.4)	1 (1.7; 0.2 – 10.9)	0(0;0 – 4.9)
Total	**12832**	**1166 (9.1)**	**32**	**4**

Letters in brackets represents the coding for the particular dip-tank

The overall individual BTB prevalence detected was 0.33 % (95% Conf. 0.14 –7.9). Positive reactors were detected in four of the 15 dip-tanks (Figure 2.1). In each of the dip tanks a maximum of one positive animal was detected. The distance of the dip tank to the park had no significant effect on the number of infected animals (P > 0.2).

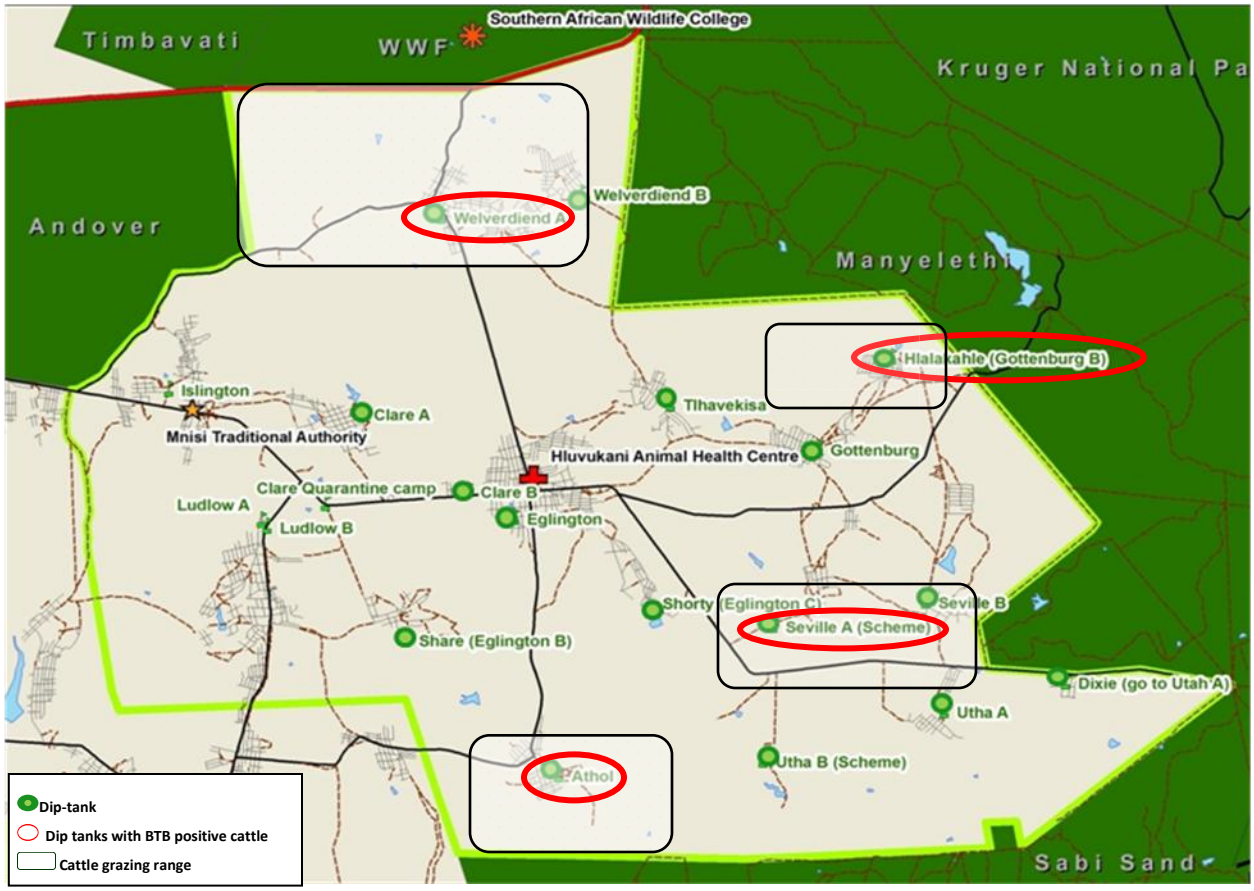

Figure 2.1: Map of study area including dip tanks. Dip tanks at which BTB positive cattle were detected are indicated by red circles. Shortest distance between individual dip tanks and the game fence represented in brackets as follows; Dip-tank: Welverdiend A (3.1 km), Thlavekisa (3 km), Gotternburg (4.2 km), Clare A (7.3 km), Utah Scheme (2.3 km), Welverdiend B (1 km), Eglington (6.1 km), Share (5.8 km), Seville B (0.5 km), Shorty (6 km), Hlalakahle (1.2 km), Utha A (1 km), Seville A (4.3 km), Athol (2 km), Clare B (6.4 km). Dip tanks sampled are indicated by green circles.

2.4.2 Interferon gamma assay

The interferon gamma assay was performed on all CIDT positive reactors (n = 4). Animal J1 was diagnosed as an avian reactor by the IFN-ɣ assay and was therefore not culled (Table 2.2). The remaining three animals (Animal ID: A1, K1, and N1) were culled based on positive reaction on CIDT testing and IFN-ɣ assay. On macro-pathological examination, animals A1 and K1 had lesions mainly in the bronchial and thoracic lymph nodes. Animal A1 had an additional caseous lesion in the right lung. All three culled animals were culture positive and yielded *M. bovis* (Table 2.2).

Test animals classified as inconclusive CIDT reactors, with a bovine bias greater than 3 mm but lower than 4mm, were further tested using the IFN-ɣ gamma assay. One animal, MI, yielded a positive test result and was culled (Table 2.2). No visible lesions were found during the post mortem examination but *M. bovis* was isolated on culture of the prescapular lymph node.

The young calf (3 months old) born to the BTB positive cow K1 had no visible lesions in the lymph nodes but a small caseous lesion was detected in the right lung from which *M. bovis* was isolated.

Table 2.2: Results of comparative intradermal tuberculin testing in comparison to whole blood interferon gamma assay, pathological examination and culture results

Animal ID	Bovine bias	CIDT	IFN-ɣ assay	Macro-pathology	Culture
N1	8.2	Pos	ND	NVL	*M. bovis*
A1	5.5	Pos	Pos	Lesions in mediastinal (focal, < 1.5 cm), bronchial (confluent, < 8 cm) lymph nodes and one lesion (focal, < 2 cm) in the lung lobe	*M. bovis*
J1	5.4	Pos	Neg	NA	NA
K1	4.8	Pos	Pos	Lesions in bronchial (focal, < 1.5 cm) and lumbar (focal, < 2 cm) and renal (focal, < 1 cm) lymph nodes	*M. bovis*
K1 calf	-	ND	- ND	One lesion in the lung (< 10mm)	*M. bovis*
MI	3.8	Inconclusive	Pos	NVL	*M. bovis*
OI	3.8	Inconclusive	Neg	NA	NA
HI	3.5	Inconclusive	Neg	NA	NA
AI	3.5	Inconclusive	Neg	NA	NA
GI	3.1	Inconclusive	Neg	NA	NA

ND: Not done due to poor sample quality

NA: Not applicable. Animals were not culled

NVL: Non-visible lesions

CIDT: Comparative intradermal tuberculin skin test results

No AFB were isolated from the milk samples obtained from the two lactating cows, K1 and MI (Table 2.2)

2.4.3 Molecular characterization

Genetic characterisation of AFB isolates obtained in this study was identified as SB 0121 spoligotype. A complete match of the 13 loci in the VNTR analysis was obtained with the majority of isolates from KNP and identified as *M. bovis* VNTR 1 strain (Figure 2.2).

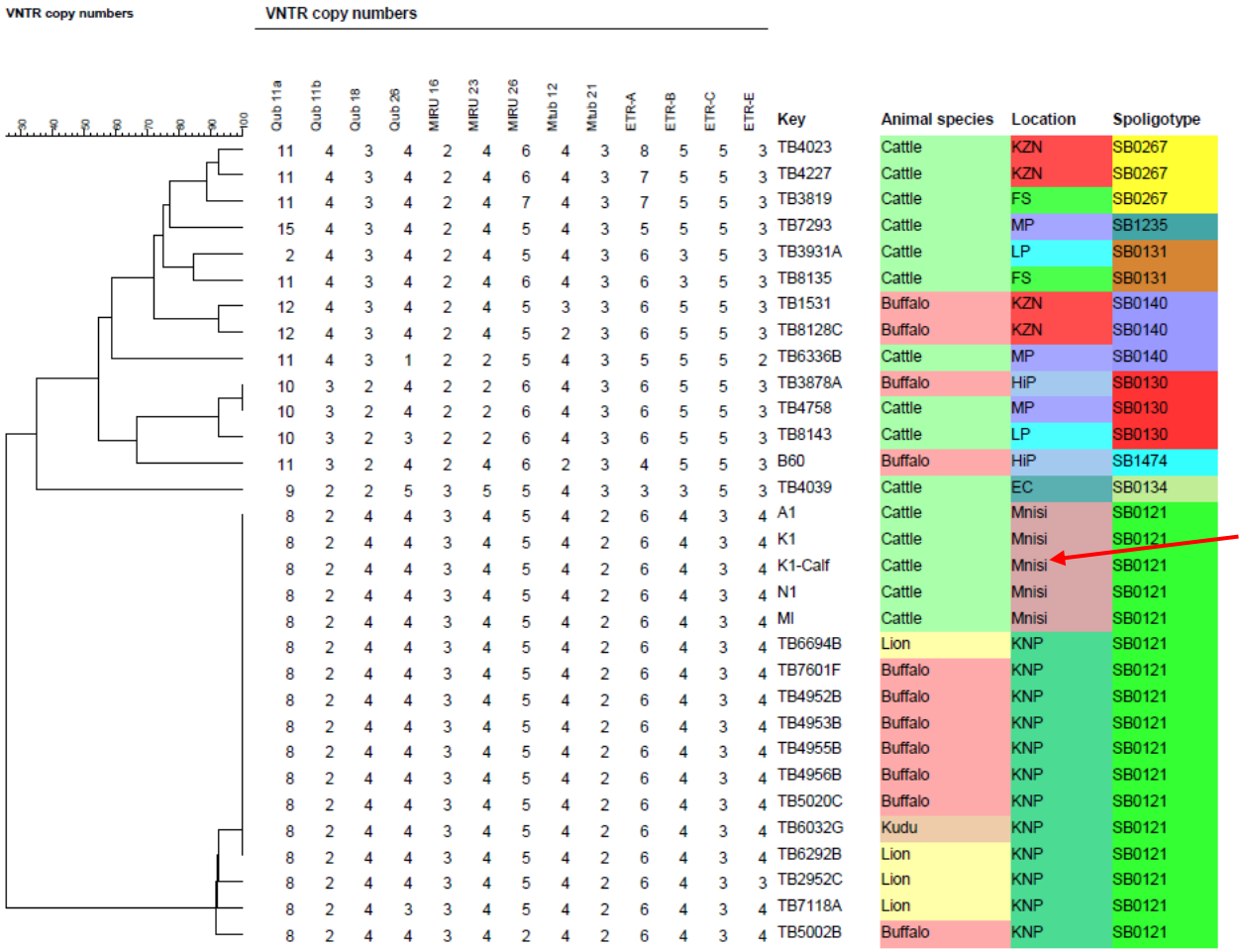

Figure 2.2: Dendogram depicting the genetic homology between *M. bovis* isolates obtained from BTB positive cattle in the Mnisi community (pointed out with the red arrow) in comparison with selected *M. bovis* strains isolated in South Africa. Colours differentiate the isolates. EC, Eastern Cape; FS, Free State; HiP, Hluhluwe iMofolozi Game Reserve; KNP, Kruger National Park; KZN, Kwa-Zulu Natal; LP, Limpopo; MP, Mpumalanga and current study area, Mnisi; VNTR, variable number tandem repeat typing.

2.5 Discussion

Previous field and molecular epidemiological investigations suggested that BTB was introduced into the Greater Kruger National Park Complex (GKNPC) through spill-over of *M. bovis* from infected cattle on a commercial farm located on the southern boundary of the Kruger National Park (KNP), most probably prior to 1960.[8] Over the past 50 years the disease has been seen to spread northwards affecting buffalo herds in all regions of the park including the neighbouring Gonarezhou National Park in Zimbabwe.[16] Great concerns have emphasised the potential risk of BTB spillback to livestock in neighbouring communities but evidence proved that spillback was lacking.[3]

In this study, BTB was detected in communal cattle directly bordering three private game reserves which form part of the GKNPC and hence share the KNP ecosystem. A single causative agent was isolated from all infected cattle and genetically identified as *M. bovis* spoligotype SB0121, VNTR type 1 strain. This *M. bovis* genotype has been reported to be the parent and dominant *M. bovis* strain responsible for the BTB outbreak in GKNPC.[15] The complete match observed between the four *M. bovis* isolates detected in the cattle and the outbreak strain in the GKNPC strongly suggests a spillback infection from wildlife as the source of *M. bovis* infection in the neighbouring cattle population.

Bovine tuberculosis infected cattle were detected in four dip-tanks at close proximity to game fences separating the communal area from the GKNPC. There was no statistically significant association between the distance from the game fence and the presence of infection at dip-tanks, possibly because of the overall low number of infected cattle. However, it was observed that the home range of cattle increased substantially during the dry months to meet their nutritional needs.[10,17] It was common practice that cattle in neighbouring dip tanks would share grazing and come into close proximity of the game fence during the day. When the grazing range of cattle in the study area was investigated it was observed that animals registered with the infected Seville A dip tank, although located the furthest from the game fence (4.3 km), grazed along the nearest fence shared with a BTB infected game reserve. For infected dip tanks Welverdiend A, Athol and Hlalakahle, the home range of registered animals extended to areas close to two other infected reserves (Musoke, unpublished data). This led to the conclusion that the minimum

distance between wildlife and livestock in the study area was much smaller than anticipated by the location of the dip tanks or the homesteads. Furthermore, interaction between cattle and wildlife was observed in the study area when livestock escaped into the park, or, *vice versa*, wildlife escaped into the communal area.[10] These interactions are observed despite efforts to minimize such interactions by implementation of game fences and buffer zones i.e. double game fencing.[18] Natural events like floods and destruction by large wildlife are able to damage these fences resulting in interactions between wildlife and livestock.[19] Although we could not determine the mode of infection in the diseased animals, it may either have occurred through indirect contact such as sharing water sources or grazing or, to a lesser extent, by direct contact between livestock and wildlife.[10] Post mortem examination showed that lesions were associated with the lung and thoracic lymph nodes, suggesting a respiratory route of infection.[20] During this study, a farmer reported that one of the herds found with a BTB infected cow had escaped into the GKNPC several months prior to CIDT testing. Additionally, the presence of wildlife on communal land where cattle were grazing was reported by 63% (94/149) of farmers interviewed in a recent questionnaire survey conducted in the same study area (C Molefe, 2013 pers.comm). Wildlife observed to have escaped into the community within the study area included mainly buffalos, greater kudu and warthogs, which all play an essential role in the transmission of BTB.[3] Greater kudu, warthogs are capable of crossing intact game fences with ease.[3,10,19]

On the other hand, the question arose whether the original KNP – VNTR -1 outbreak strain could have persisted and spread outside the KNP since the original spill-over event to buffaloes. The study area is approximately 180 km from the area where the infection was originally transmitted from cattle to buffalo across the KNP's southern boundary. Between 1996 and 2012, provincial State Veterinary Services of Mpumalanga tested a total of 96806 head of cattle in the relevant three veterinary districts of Bushbuckridge (the district where the study area is located), Nsikazi (south of Bushbuckridge, bordering the GKNPC in the west) and Nkomazi (south of KNP) using the CIDT as part of their annual BTB surveillance. No BTB reactors were detected in Bushbuckridge nor in Nsikazi districts. Between one and three bovine reactor animals were detected in the Nkomazi district in three unrelated outbreaks in 2009, 2010 and 2011 (du Plessis, per. comm.). All outbreak strains were genotyped and their spoligotypes as well as their VNTR profiles were found to be different from each other as well as from the KNP dominant strain

VNTR-1 (Results not shown). This information serves as an additional body of evidence that supports the hypothesis that BTB infected cattle in this study contracted *M. bovis* infection from neighbouring wildlife in the GKNPC.

The young calf (3 months old) born to one of the BTB infected cows (Hlalakahle, K1) was found to have a small lesion in the right lung lobe and was identified as the same *M. bovis* strain as the dam. Bovine tuberculosis transmission to neo-natal calves occurs predominantly intra-uterine via the haematogenous route.[21] Alternatively, it is likely that the calf contracted *M. bovis* via aerosol transmission based on the localization of a single granulomatous lesion in the lung. This finding warrants further research into BTB transmission within the herd.

Despite BTB being detected in this study, the BTB prevalence at individual animal level was low at 0.33 % (95% CI. 0.14 - -0.79). This is similar to findings in extensively farmed cattle in Ethiopia, where the overall individual BTB prevalence was 0.8% [22], while a prevalence of 30% was reported at intensive husbandry systems in dairy farms in urban and peri-urban areas in the same country.[20] In the present study, only one positive animal per BTB infected dip-tanks was detected, suggesting a low transmission of BTB among the animals of one dip-tank. The low BTB prevalence detected may, on the one hand, be attributed to the low animal density generally found in extensive farming systems.[23] On the other hand, the communal herd composition in the study area, comprising 70.9% indigenous cattle breeds [24] may have played a contributing role, as indigenous cattle appear to be more resistant to BTB compared to introduced cattle breeds.[25]

No BTB was detected in the milk samples collected from the positive CIDT cows (K1 and MI). The number of milk samples collected was very low due to the overall low number of infected cattle. Thus, no conclusive hypothesis on the potential shedding of *M. bovis* into the milk and the potential risk to public health could be concluded. There is a need to further investigate the presence and potential risk of zoonotic diseases within the study community by widening the milk sampling range as the detection of BTB in the cattle herds is of public health concern.

2.6 Conclusion

In conclusion, the detection of the dominant *M. bovis* GKNPC strain in livestock at the wildlife/livestock interface in combination with skin testing information demonstrating the absence of this *M. bovis* strain from neighbouring farming districts prior to this study and the observed wildlife/livestock contact in the study area provide sufficient evidence to conclude that the BTB infection diagnosed in cattle in the study area was the result of *M. bovis* spillback transmission from GKNPC wildlife. These findings are of great concern not only to livestock health and production in communities bordering the GKNPC but more importantly to public health and human livelihoods due to the zoonotic potential of BTB and the economic impact in affected areas.

2.7 Acknowledgments

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Chapter 3: Lack of evidence for tuberculosis transmission at the livestock/human interface in a rural Mnisi community, Mpumalanga province, South Africa

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This chapter is a working paper and has not yet been submitted for publication.

3.1 Abstract

The aim of this study was to investigate the risk factors for bovine tuberculosis (BTB) (*Mycobacterium bovis*) and human tuberculosis (*Mycobacterium tuberculosis*) transmission at the livestock/human interface in a rural community in the Mpumalanga province. A total of 174 questionnaires were administered to cattle owners (n= 52) and patients with clinical symptoms of tuberculosis (TB) (n= 122) to determine the food consumption and livestock management practices that may place them at risk of TB transmission at that interface. It was observed that although milk formed part of the community's diet, milk was not consumed daily but rather when affordable. The majority (92.1%) of respondents regardless of whether they owned cattle or not, sourced milk commercially (pasteurised). The consumption of undercooked/raw meat as well as organs was common. However, if visible abnormalities in the meat quality were observed, the meat/organs were discarded. A large number (92.3%) of patients with confirmed TB did not own cattle nor did they consider themselves to have close interactions with livestock. Based on these findings we concluded that in this particular community, the risk of acquiring zoonotic diseases transmitted by cattle (i.e. bovine tuberculosis) was low. Similarly, livestock had a low risk of acquiring human TB due to the few TB patients among livestock owners. This study provides evidence that the common assumption that all rural communities in developing countries consume milk primarily raw (unpasteurised) should not be generalised and hence the risk of contracting zoonotic diseases varies depending on the communities' practises.

3.2 Introduction

Tuberculosis (TB) is a wide-spread, potentially fatal infectious disease caused by mycobacteria classified under the *Mycobacterium tuberculosis* complex (MTBC). *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the two commonly reported MTBC members that cause TB in humans and animals, respectively.[1]

Shortly after the discovery of the causative agent of TB in 1882, Robert Koch reported that *M. bovis* was pathogenic only in livestock and attenuated in humans.[2] However, with further investigations by the British Royal Commission in 1911, it was established that *M. bovis* can be virulent in humans.[3,4] Human TB caused by *M. bovis* is commonly referred to as zoonotic TB.[5] In the 1930's and 1940's more than 50% of cervical lymphadenitis (form of extra-pulmonary TB) in children was attributed to *M. bovis* infection in Great Britain.[6] Approximately, 6% of human TB deaths in 1930's were due to zoonotic TB,.[5] The introduction of bovine tuberculosis (BTB) control strategies in livestock led to a drastic decline in reports of *M. bovis* infections in humans as well as cattle. The control strategies included pasteurisation of milk and test and slaughter of infected cattle. Currently, zoonotic TB due to *M. bovis* in developed countries is at very low levels, approximately 1%.[7,8] In contrast, in developing countries the true prevalence of *M. bovis* infections in humans is currently unknown and is potentially a serious public health concern.

Muller *et al*., (2013) conducted an extensive literature review to estimate the global occurrence of zoonotic TB in humans.[9] A substantial lack of information on zoonotic TB was highlighted from many parts of the world. Despite the limited data, it was clear that *M. bovis* infections in humans remain prevalent and Africa had the highest prevalence estimates compared to other continents. The higher rates in Africa are attributed to a lack or partial implementation of BTB control strategies. Sporadic reports from Africa on zoonotic TB cases are mainly derived from work conducted in the north-eastern and eastern parts of the continent (Ethiopia and Tanzania). There is currently no published literature on the occurrence of zoonotic TB in humans in South Africa. This knowledge gap is despite numerous reports on the prevalence of *M. bovis* in South African livestock and endemic levels in free-ranging wildlife.[10,11]

In the current study population, BTB was detected in livestock. Had this diagnosis been made in a developed country, the outbreak would have been eradicated with effective BTB control measures and the public health concern would be negligible. However, because limited resources hinder effective BTB control in a developing country, the human health consequences of the current BTB outbreak remain unknown and warrant further investigation. What makes the public health concerns more alarming is that the study population is in a region of high reports of immunocompromised individuals, thus increasing the susceptibility of the study population to zoonotic diseases. It is therefore of importance to investigate the risk factors for BTB transmission to humans in the particular study community.

Countries in southern Africa have the highest TB incidences in the human population, with South Africa at an estimated TB incidence of 840/100 000 population in the year 2013.[12] *Mycobacterium tuberculosis* is the main causative agent of TB in humans, but it has been shown that it can be virulent in livestock, particularly cattle.[13] In South Africa, two reports of *M. tuberculosis* isolation in cattle have been confirmed (T Hlokwe, 2013 pers. comm)). Despite this, no known studies in southern Africa, including South Africa have investigated *M. tuberculosis* transmission from humans to livestock and the associated risk factors.

Hence, this study aimed to investigate the risk factors for the transmission of *M. bovis* infections from livestock to humans in the study community and vice versa, the transmission of *M. tuberculosis* from humans to livestock.

3.3 Materials and methods

3.3.1 Study area

This study was conducted in a small rural community that forms part of the Mnisi Tribal chieftainship, located in the north-eastern corner of Bushbuckridge Municipal area, Mpumalanga Province, South Africa. The community directly borders the west of the Greater Kruger National Park Complex (GKNPC) conservation area with a comprehensive wildlife/livestock/human interface.

3.3.2 Study population

The community is home to approximately 40, 000, mainly Shangaan speaking people.

This study to assess the potential risk factors for TB transmission at the livestock/human interface was conducted between June 2013 and February 2015. The study included four different sub-groups. Group 1 and 2 entailed patients registered at the local clinics, whereas group 3 and 4 was farmers registered at the local dip-tanks. No respondents belonged to more than one group.

Group 1: Comprised of TB positive patients enrolled at the local healthcare clinics (Hluvukani, Welverdiend, Utah and Gotternburg clinics) in the community. These patients were diagnosed as TB positive by the local health clinics. A total of 78 TB patients were enrolled.

Group 2: included patients enrolled at the four local clinics with unconfirmed TB status but presented with clinical signs consistent with TB (coughing for more than 2 weeks, unexplained weight-loss and/or night sweats). No confirmed laboratory results were available due to the patient confidentiality clause. Forty-four patients were recruited as part of this study group.

Group 3: included farmers who owned cattle which had previously (Chapter 2) been tested and found negative for BTB.[14] These participants were recruited at the local livestock dip-tanks during their weekly animal inspections. Participants were informed of the study and farmers who offered their consent were enrolled. The TB status of the farmers was unknown at the time of recruitment. A total of 42 farmers were enrolled as part of this study group.

Group 4: entailed all farmers whose cattle herds had previously been confirmed as BTB infected, based on the isolation and genetic characterisation of the causative *M. bovis* strains. Farmers whose cattle herds had been classified as inconclusive BTB status were also recruited as part of this study group. These cattle herds with inconclusive BTB status refers to reactors in the previous study (Chapter 2) that had an increase of between 2 mm and 4 mm skin thickness greater in the site injected with bovine tuberculin than the reaction shown at the site of avian tuberculin injection. Participants were informed of the study and farmers who offered their consent were recruited. Ten farmers were enrolled as part of this study group.

3.3.3 Questionnaire design

A personal interview in the local language, Shangaan, was administered by a translator recruited from the community, with the assistance of local clinic health service providers. A questionnaire survey form comprising the following variables was used: individual and household demographic; socioeconomic characteristics; household consumption practices with regard to meat and milk; as well as livestock management practices (questionnaire attached as Appendix 1). The individual characteristics assessed included the TB status of the respondent within the past 12 months from the time of the interview, the frequency of contact with livestock, and gender. The TB diagnosis and history of members in the same household was recorded. Socioeconomic characteristics included employment status and education level of the respondent. Livestock management variables assessed included the BTB infection status of cattle in the household. Milk consumption practices evaluated included the source of milk (e.g. own cows, local farmer or retail shops) and the frequency of consumption. Meat consumption variables evaluated included the type of meat component consumed (meat, head or organs) and preparation methods. The respondent's zoonotic awareness was also reviewed in closed ended questions.

3.3.4 Ethics Statement

The study was carried out with ethical approval from the University of Pretoria, Faculty of Health Sciences Research Ethics Committee and from the Mpumalanga Provincial Government, Department of Health Provincial Research and Ethics Committee.

3.3.5 Data Analysis

Questionnaire responses were coded and entered in Microsoft Excel workbooks. To check for errors, the captured data were compared with the questionnaire responses and data corrections were made with the assistance of the project statistician. Some respondents made invalid responses which were excluded from the analyses; some respondents did not provide responses to all questions. Analyses were performed using SAS version 9.3 of the SAS system for Windows. Frequency counts and percentages were used to summarise and describe the responses. Comparisons were made between selected groups of respondents using 2-way tables and the chi-square test for association test was applied where practical. Fisher's exact test was used for tables where the data were too sparse to meet the assumptions of the chi-square test.

Many of the tables were considered too sparse for any statistical analysis and descriptive analysis was done instead.

3.4 Results

3.4.1 Socioeconomic characteristics of cattle owners versus non-cattle owners

A socioeconomic comparison between cattle owners (groups 3 and 4, n = 52) and non-cattle owners (from groups 1 and 2, n = 111) was performed. A statistically significant difference (p-value = 0.04) in unemployment/employment status was observed between the two groups. Among cattle owners, higher unemployment rate of 92.3% (n = 48) was observed, as compared to 79% (n = 64) unemployment rate in non-cattle owners. In the analysis of the employment status of both cattle owners and non-cattle owners, 27% (n = 30) of the non-cattle owners did not provide their employment status and were thus excluded from the chi-square test. There was also a statistically significant difference (p-value = 0.006) in the education levels of the two groups. Formal education was highest among non-cattle owners (75.4%) as compared to cattle owners at 53.8% (Figure 3.1).

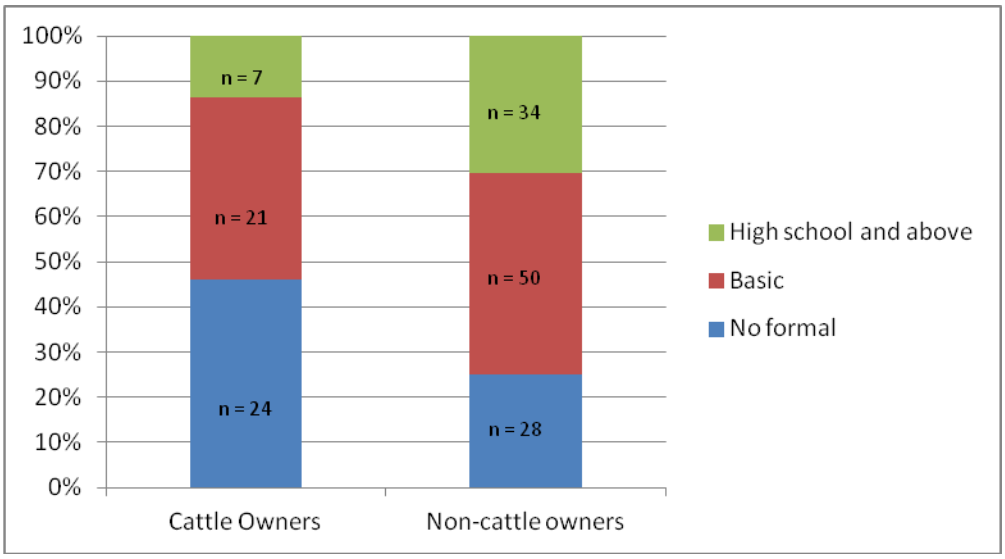

Figure 3.1: Education levels in cattle owners (groups 3 and 4) and non-cattle owners (groups 1 and 2). Basic education defines formal education from Grade 0 to 9 (General Education and Training) according to the Department of Basic Education, South Africa, whereas high school and above defines Grade 10 to 12 (Further Education and Training) including all levels of tertiary education.

3.4.2 Demographic and socioeconomic characteristics of TB patients

The majority (92.3%) of TB positive patients (group 1) registered at the four local clinics did not own cattle, nor did they consider themselves to have close interactions with livestock, while the remaining proportion of 0.076 (n = 6) (95% CI: 0.0350 – 0.1608) were farming with cattle. Sixty-five percent of the TB positive patients were unemployed (Table 3.1), 15.4% (n=12) did not indicate their employment status. Of the respondents employed, professional positions that required specialised training accounted for 5.1%. The majority (41%) of TB positive patients responded that their highest education level was basic education. A total of 19.2% of TB positive patients reported a history of TB cases in other members of their household.

Table 3.1: Socio-demographic data of confirmed TB patients enrolled in this study (n = 78)

	Frequency (n)	Percent (%)
Occupation		
Industrial	11	14.1
Professional	4	5.1
Unemployed	51	65.4
No response	*12*	*15.3*
Education		
No formal	18	23.1
Basic (Grade 0 to 9)	32	41.0
High school and above (Grade 10 and above)	27	34.6
No response	*1*	*1.3*
History of TB in *other* members		
Yes	15	19.2
No	63	80.8

3.4.3 Food consumption practises

3.4.3.1 Milk consumption

In groups 1 and 2 the 11 TB patients who owned cattle were excluded from these analyses. The 11 respondents were excluded as they were not mutually exclusive to either cattle-owners or TB patients, thus to avoid duplication of responses the patients were excluded. Almost all respondents in the three study groups consumed milk as part of their diet (Table 3.2). However, milk was not consumed daily but rather when affordable. Ninety percent or more of the respondents in each of the three groups consumed pasteurised milk, obtained commercially at retail stores. Despite farmers owning livestock, the majority did not consume milk from their cows. In group 3, 95.2% and in group 4, 90% obtained milk commercially.

Table 3.2: Milk consumption practises in the study population

Practices	**Group1 and 2** (Patient's with clinical symptoms of TB, without cattle) n=111	**Group 3** (Farmer's with BTB negative herd) n=42	**Group 4** (Farmer's with BTB infected/inconclusive herd) n=10
Milk consumption			
○ Yes	81 (73)	42 (100)	9 (90)
○ No	7 (6.3)	0	0
○ *No response*	*23(20.7)*	0	*1(10)*
Source of milk**			
• Own cows/local farmer			
○ Yes	0	2 (4.8)	5 (50)
○ Not selected (interpreted as No)	111 (100)	40 (93.2)	5 (50)
• Commercial (pasteurised)			
○ Yes	101 (91.0)	40 (95.2)	9 (90)
○ Not selected (interpreted as No)	10 (9.0)	2 (4.8)	1 (10)
Consumption frequency			
○ Daily	3 (2.7)	0	5 (50)
○ Weekly	5 (4.5)	0	0
○ When affordable	68 (61.3)	41 (97.6)	4 (40)
○ When sick or for family function	5 (4.5)	1 (2.4)	0
○ *No response*	*30(27.0)*		*1 (10)*

Numbers in brackets represents percentage of respondents within each group
**Multiple responses were allowed for this question.

3.4.3.2 Meat consumption

The 11 TB patients in groups 1 and 2 who owned cattle were also excluded from these analyses. Undercooked meat/organs was defined as medium-rare food product whereas well-cooked referred to thoroughly cooked food product. Undercooked meat or organs were preferred by the majority of respondents in the study (Table 3.3). Within study groups 3 and 4 (both of which comprised cattle owners), none of the participants indicated that they ignored any visual abnormalities in the meat or organs, whereas, in study groups 1 and 2, a small percentage (6.3%) reported ignoring the visual abnormalities on the meat or organs and proceeded with preparation

of the meal. The majority of respondents discarded the meat or organs by either feeding it to the dogs or throwing it away into dug pits.

3.4.4 Awareness of zoonotic diseases

As compared to patients at the clinics (groups 1 and 2), the farmers (group 3 and 4) were more aware of zoonotic disease transmission. More farmers knew that BTB could be transmitted from animals to humans, rather than the alternative route that human TB can also be transmitted from humans to animals.

Table 3.3: Meat consumption practises in the Mnisi community

Practices	**Group 1 and 2** (Patient's with clinical symptoms of TB, without cattle) n=111	**Group 3** (Farmer's with BTB negative herd) n=42	**Group 4** (Farmer's with BTB infected/inconclusive herd) n=10
Meat consumption behaviour**			
• Well-cooked meat/organs			
○ Yes	21 (18.9)	2 (4.8)	7 (70)
○ Not selected (interpreted as No)	90 (81.1)	40 (95.2)	3 (30)
• Raw/undercooked meat and organs			
○ Yes	78 (70.3)	40 (95.2)	8 (80)
○ Not selected (interpreted as No)	33 (29.7)	2 (4.8)	2 (20)
Response to visual changes in meat quality			
○ Abnormalities on any meat/organs are ignored	7 (6.3)	0	0
○ Additional treatment of the meat (boil for longer, add lots of salt, overcook)	14 (12.6)	8 (19)	1 (10)
○ Product is discarded	76 (68.5)	31 (73.8)	8 (80)
○ *Other* or no response*	*14(12.6)*	*3(7.1)*	*1 (10)*
Awareness			
• TB can spread from animals to humans	31 (27.9)	27 (64.3)	7 (70)
• TB can spread from humans to animals	23 (20.7)	17 (40.4)	5 (50)

Numbers in brackets represent percentages of respondents within each group
**Multiple responses were allowed for this question.
* Frying or non-meat eater

3.5 Discussion

The aim of this study was to investigate the risk factors for BTB (*M. bovis*) transmission from cattle to humans based on the food consumption patterns and livestock management practices. Additionally, the risk factor for human TB (*M. tuberculosis*) transmission from humans to livestock was investigated by evaluating the contact between TB patients and livestock. These risk factors were investigated through a questionnaire based interview.

During the interviews it was observed that the majority (92, 3%) of cattle owners referred to themselves as unemployed. Suggesting that they perceived themselves as livestock owners/keepers and their farming activities were not regarded as a source of business or dependable income. [15,16]

Tuberculosis of the udder (tuberculous mastitis) is the main cause of *M. bovis* contamination of raw milk and dairy products.[5,17] In the current study population, the majority (92.1%) of respondents consumed pasteurised milk obtained commercially. The almost absolute lack of utilisation of milk from own cows and/or local farmers is in disagreement with the commonly held general view that rural communities largely depend on and consume milk from their own livestock and/or that of local farmers. One possible explanation of the shift to commercially obtained milk is the low milk production observed in subsistence farming. The main constraint for subsistence farming is the erratic and limited rainfall, which has a direct negative impact on livestock nutrition and the milk yield.[18] Given the opportunity rural communities prefer to buy milk from formal sources despite their low socioeconomic status (high unemployment rates). However, sourcing of milk commercially in the low socio-economic environment appeared to impact the milk consumption pattern and it was observed that the community did not consider milk as a priority to the daily diet. In conclusion, based on the milk consumption practises observed in this study, milk was a low risk factor for BTB transmission from livestock to humans.

The majority of respondents in the present study population, both cattle owners and non-cattle owners reported consumption of medium cooked to rare meat/organs on a regular basis. In developing countries, the consumption of the carcass includes muscle, organs, offal and their associated lymph nodes (where most TB bacilli reside). While in developed countries the consumption of meat refers only to large skeletal muscles. *Mycobacterium bovis* occurs rarely in

the muscle but it is possible during bacteraemia, which means it would be limited to the early phase of infection, thus the view in developed countries that meat poses a low risk for zoonotic TB.[5,19] However, settings in developing countries differ as there is evidence that undercooked/raw meat/organ is an important BTB risk factor. Hambolu *et al*., 2013 reported on a highly risky behaviour of consuming *M. bovis* infected parts of the lung in Nigeria, among meat traders.[20] Twenty-eight percent of the meat traders sold the lungs despite visible contamination, whereas 14 % consumed raw meat. Similarly, a study in northern Tanzania reported that 17.8% of respondents consumed raw meat.[21] Based on the highly strained veterinary services observed in developing countries, with little to no meat inspection, undercooked meat and organs remain a BTB risk factor. An additional risk is that local farmers are unskilled in hygienically preparing the carcass for consumption, potentially missing TB lesions or contaminating the muscle. However, when respondents in this study were questioned on their behaviour if the meat showed visible abnormalities, the majority reported to discard the meat/organs. Based on the findings in this study in terms of consumption behaviour of raw/undercooked meat/organ, this community can be classified of being at risk of *M. bovis* and other meat-borne pathogens. However, the observation that meat/organs with visual abnormalities were discarded, together with the high infectious dose required for oral infection (requires about ten times higher dose than aerosol transmission) mitigates the risk factor for BTB transmission. These findings highlight the importance of improved meat inspections, awareness within the community and better veterinary public health services, particularly in households that own and slaughter cattle at their homes.

In literature, there are reports that humans can serve as a source for sensitization of cattle to TB.[13,22] Tamiru *et al*., 2013 reported that in Ethiopia, TB in cattle was more prevalent in animals owned by TB positive farmers as compared to negative farmers.[23] Hence, part of the objectives of this study was to investigate the risk of TB transmission from humans to livestock through assessing the level of contact. The majority (92.3%) of confirmed TB patients (group 1) did not own cattle nor did they consider themselves to have close interaction with livestock. Compared to the general population, the level of cattle ownership observed in this study was lower than the reported 25%.[24] Secondly, no high risk behaviour seemed to exist (e.g. sharing of the same houses) among individuals that owned cattle. These findings are contradictory to the direct contact between livestock and farmers reported in other developing countries, such as sleeping in the same houses with poor ventilation.[1] Or a local practise in Ethiopia of spitting chewed tobacco

juice directly into the mouth of livestock, as means of anti-parasitic treatment.[22] Thus, due to the minimal contact observed in this study there was a low probability of human TB (*M. tuberculosis*) transmission from humans to livestock through the aerosol route, despite the TB incidence of 630 per 100 000 population within the study district. However, there are two reports on the isolation of *M. tuberculosis* from the respiratory lymph nodes of livestock in South Africa (T Hlokwe, 2013 pers. comm). These findings highlight the need for further investigations into how *M. tuberculosis* is transferred to livestock within South African husbandry practices, both commercial and communal settings.

Farmers in this study (groups 3 and 4) generally had a high level of awareness on BTB transmission. These findings could be attributed to the extension services rendered to farmers during BTB testing of their cattle herds. However, despite this awareness, farmers still consumed meat/organs undercooked/raw (group 3, 95.2%; group 4, 80%). These findings are similar to reports by Hambolu *et al*., 2013, stating that despite the fact that 84% of meat handlers in an abattoir claimed they knew of BTB, 22% ate infected lungs and 14% ate raw meat.[20] The knowledge of farmers in this study was incomplete as the majority knew of only one mode of pathogen transmission. This limited knowledge highlights the importance of not only relying on awareness as a mode of control, but also improved BTB control measures by the veterinary services to reduce the risk of zoonotic TB transmission.

The main limitation of this study was the design of an open ended questionnaire. As a result respondents could provide more than one answer. This became a challenge in the analysis as some responses could not be validated.

3.6 Conclusion

In summary, we concluded from the findings of this study that the general Mnisi community and farmers are at low risk of BTB transmission from livestock, based on their milk consumption practises, as the majority of the population obtained pasteurised milk commercially. There is a risk of BTB transmission to the community due to the consumption of all commodities of the carcass uncooked/raw, however this risk appears to be mitigated by the fact that visible abnormalities in the meat/organs are discarded, reducing the risk of high infective doses required for oral infection. Similarly, livestock were at low risk of acquiring TB from humans as low levels of contact with TB patients were observed. This study is in contrast with the common

assumption that rural communities in developing countries have no access to pasteurised milk, have close contact with livestock and are hence all at high risk of contracting zoonotic diseases. This rather depends on the socio-economic context, infrastructure and prevailing cultural community practises.

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Chapter 4: Characteristics of tuberculosis patients and the evaluation of compliance to the national TB management guidelines at clinics in a rural community in Mpumalanga province, South Africa

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This chapter has been accepted for publication as a short report in the Southern African Journal of Infectious Diseases.

For purposes of this thesis a more detailed account of the study is presented.

4.1 Abstract

The objective was to provide detailed description of age and gender characteristics of tuberculosis (TB) patients as well as to report on the compliance of the clinics in the rural settings to the national TB guidelines in terms of diagnosing the disease. A total of 62 TB positive patients' files were reviewed. It was found that TB cases were equally distributed between males and females (n = 31). In males, the age categories of 35 – 39 yrs and 45 -49 yrs were impacted most by the disease whereas in females a slightly younger age category of 30 - 34 yrs was primarily affected. Patients were diagnosed using: smear microscopy (41.9%); chest radiography (37.1%); Xpert MTB/RIF (9.7%); symptoms (3.2%); abdomen sonar (1.6%); and no record (6.5%). This study reports on similar epidemiological trends as for TB patients elsewhere in South Africa. On the other hand it does serve as a baseline study used to investigate TB patient population characteristics in a rural community setting. Lack of complete compliance was identified, including large dependencies on chest x-ray as first line of diagnosis and inadequate diagnosis of extra-pulmonary TB. These findings could assist in identifying health systems gaps for provincial and national control programs.

4.2 Introduction

Tuberculosis (TB) remains a global health crisis claiming 1.3 million deaths and 8.6 million new cases reported in the year 2012.[1] The African continent has an estimated 27% of these TB cases. According to the World Health Organisation (WHO), South Africa is in the top three countries on the African continent bearing the highest TB burden, with TB incidence of 1003 per 100 000 population in the year 2012. The Department of Health reported a lower TB incidence (all TB) of 687 per 100 000 population (2012). Although the two reports provide different values for the year 2012, they both highlight the alarming rates of TB in South Africa. There have been notable achievements in TB disease management. This is evidenced by the latest WHO Global Tuberculosis Report 2014, which shows a decline in TB incidence to 860 per 100 000 people.[2] Hence the country is on track in achieving the Millennium Development Goal 2015 (MDG) for the reduction in TB disease burden by 2015.

The South African national tuberculosis programs (e.g. Health System's Trust) and international health organisations (e.g. WHO) rely on obtaining critical data from TB registers compiled at provincial or national levels. Often these TB registers are incomplete and do not reflect the individual communities bearing the brunt of the TB disease.[3] It is of importance to conduct community-level studies, profiling people infected with TB and comparing with international and national reports. In addition, it is of importance to investigate local risk factors for contracting TB. Community-level studies reflect the TB situation with specific reference to local drivers of the disease and are essential for targeted, appropriate and tailor-made control measures and interventions, particularly in rural, resource-poor settings.

The aim of this study was to retrospectively investigate the trends in the characteristics of TB patients in a small rural community and investigating local risk factors for contracting TB. In addition, we wanted to assess if the diagnostic techniques used are in line with current national TB control policies.

4.3 Materials and methods

4.3.1 Study area and study population

This study was conducted in a small rural community that forms part of the Mnisi Tribal chieftainship, located in the north-eastern corner of the Bushbuckridge Municipal area, Mpumalanga Province, South Africa. This study community is entered in the Mnisi Community Engagement Program in partnership with the University of Pretoria. The community was selected based on the geographical location, at the western border of the Greater Kruger National Park Complex (GKNPC) conservation area (Figure 4.1). Part of the research platform focuses on studying the epidemiological significance of zoonotic diseases at the wildlife/livestock/human/environment interface, under the 'One Health' theme. The community is home to over 40, 000, mainly Shangaan speaking people. The community has high levels of unemployment and a low family income is the main socio-economic indicator.

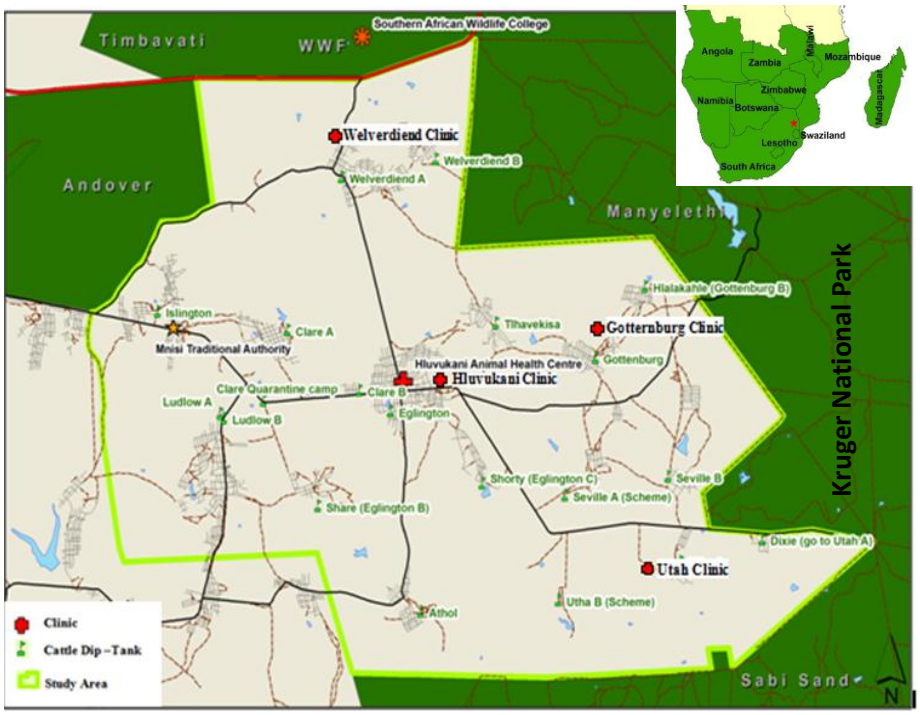

Figure 4.1: Geographical location of the health clinics in the Mnisi study site

4.3.2 Health Services

The study community's main health service providers are four government primary health care clinics (Hluvukani, Welverdiend, Utah and Gotternburg clinics) and one referral hospital, Tintswalo. The referral hospital is a 423 bedded acute government hospital located 48 km from the study community. All sputum testing, including Xpert MTB/RIF, a cartridge based automated diagnostic technique that detects DNA sequences specific for *Mycobacterium tuberculosis* complex and resistance to rifampicin antibiotic using nucleic acid amplification, are performed by the National Health Laboratory Services in Bushbuckridge.[4] The X- Ray facilities are available at Tintswalo hospital.

The community is under the administration of Ehlanzeni district, the largest of the three municipalities in Mpumalanga province, with a population of 1,688,616 (2011).[5] According to the Ehlanzeni Report 2012/13-2015/16 IDP, in 2010, the district had the highest number of reported TB cases (all TB cases) in Mpumalanga province, 12, 459 in the district.[5] The latest (2012) health survey reported a TB incidence of 630 per 100 000 population.[6] The local TB control program is aligned with the World Health Organization's Directly Observed Treatment Short-Course (DOTS) strategy.

4.3.3 Data collection

Between June 2013 and June 2014, a retrospective study was conducted at the four primary healthcare clinics. Patient demographic data and disease classification were extracted from the TB patient files actively enrolled at the four clinics during the study period (one year). Data from the patient files was directly entered into Microsoft Excel. The demographic data collected included: patient age, gender; TB patient category (i.e. new case, defaulter, treatment failure, multi-drug resistant); whereas disease classification included site of TB disease and diagnostic method. In this study, patient category and site of disease were defined as stated in the National Tuberculosis Control Programme (NTP) (2014).[7] Briefly, new TB cases referred to patients who had never had anti- tuberculosis medication or had taken TB treatment for less than four weeks. A defaulter TB case referred to a patient who completed at least one month of TB treatment and returns after interrupting treatment for two or more months. Re-treatment after failure referred to patients with positive smear or culture at the end of treatment. Multi-Drug Resistant TB (MDR-TB) defined cases were TB isolates reported as resistant to at least isoniazid (INH) and rifampin

(RIF). Extra pulmonary TB is the spread/dissemination of TB in organs other than the lungs, including pleura, lymph nodes or abdomen.

4.3.4 Data analysis

The statistical correlation between age and gender was assessed using a linear regression model.

4.3.5 Ethics Statement

The study was carried out with ethical approval from the University of Pretoria, Faculty of Health Sciences Research Ethics Committee and from the Mpumalanga Provincial Government, Department of Health Provincial Research and Ethics Committee.

4.4 Results

4.4.1 Health Services

A total of 62 TB positive patient files were reviewed. Patients were diagnosed as positive using the following techniques: smear microscopy, 41.9% (n = 26); chest radiography, 37.1% (n = 23); Xpert MTB/RIF assay, 9.7% (n = 6); symptoms, 3.2% (n = 2); abdomen sonar, 1.6% (n = 1); and no record, 6.5% (n = 4).

4.4.2 Gender and age distribution

The TB cases were equally distributed between males and females (n = 31 each).

There was no significant difference between the age distribution among males and females. However, the highest incidence of TB was observed in patients aged 30 – 34 yrs. Specific to males, the age categories of 35 – 39 and 45 – 49 years were mainly impacted, whereas in females, the highest impact of TB was observed in the age category 30 – 34 yrs (Figure 4.2). The mean age of patients was 42.

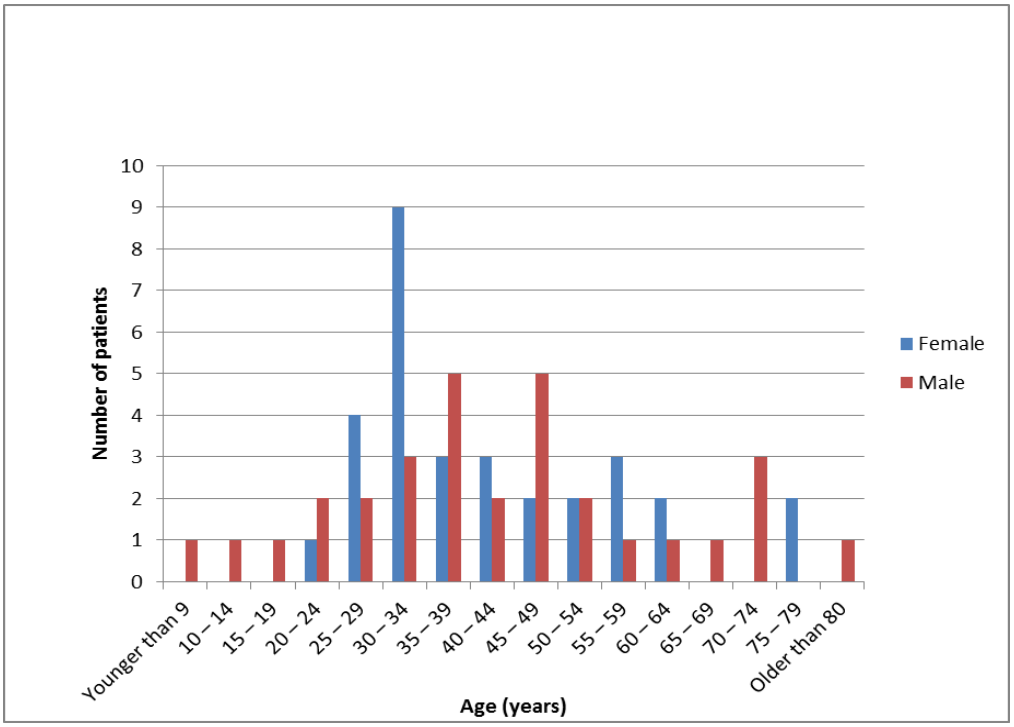

Figure 4.2: The age distribution of TB positive patients in the Mnisi community

A total of 87.1% (n = 54) of the reviewed TB cases were classified as new cases; 6.5% (n = 4) were classified as re-treatment after default; 4.8 % (n = 3) were re-treatment after failure cases. One case (1.6%) was identified as a Multi-Drug Resistant TB (MDR-TB) case.

Nine cases (14.5%) were classified as extra-pulmonary TB, of this, two cases were adenitis, and two other cases were TB of the pleura.

4.5 Discussion

A total of 62 patient files actively enrolled in the four primary healthcare clinics for TB treatment were reviewed. This retrospective study was conducted to serve as a preliminary investigation into the TB patient population characteristics and the compliance of the local clinics to the national TB guidelines in terms of diagnosing the disease.

It was observed that TB cases were equally distributed between males and females and the age category most impacted by TB was patients aged 30 – 34 years. These findings follow the trends reported in recent years with TB disease being commonly observed in younger generations, particularly males in their 30's and females in their 20's.[8] There is a clear shift in age trends from the 19th century when TB was a disease of the elderly in South Africa, mainly affecting persons

>60 years of age, particularly men.[9] The drivers of TB during that time (l9th century) were attributed to the migration of people from the rural communities to cities for employment in the then emerging gold mines. The migrants were mainly older males that had not been previously exposed to TB and had poor working conditions, poor nutrition, exposure to silica dust and sharing overcrowded hostels.[8] Hence, older males were vulnerable and mostly affected by TB. The current age shift can be largely attributed to the high rise of human immunodeficiency virus (HIV). Estimates by the WHO, indicate one-third of the world's population has latent TB (infected with TB bacilli but has not progressed to disease) and in the event of co-infection with HIV, the risk of progression from latent TB into active disease is 20-31 fold.[10, 11, 12, 13]

The TB patients were diagnosed using smear microscopy (41.9%), chest radiography (37.1%), Xpert MTB/RIF (9.7%) and no record (11.3%). According to the NTP guidelines, diagnosis of TB depends, among other factors, on the presentation of patients with TB symptoms, screening practices at the health facilities, and turnaround time for delivery of results. However, the tests used are to follow provided guidelines. Part of the South African TB control campaign was the introduction and rolling out of new diagnostic tools. One of the innovative techniques is Xpert MTB/RIF. South Africa was the first high TB burden country to implement Xpert MTB/RIF in its government polices as the first diagnostic tool for all suspect TB cases.[14] In the Mnisi study population, it was observed that in more recent cases, the use of Xpert MTB/RIF had replaced smear microscopy as first line of diagnosis. It can therefore be concluded that the NTP components were in the process of being implemented, and the national roll-out implementation of the new diagnostic tool is beneficial to communities including those in rural areas.

It was, however, observed that there was a great dependence on using chest x-rays (37.1%) as first line diagnosis, especially in patients transferred from the referral Tintswalo hospital to the clinics. This is contradictory to the NTP policies, as the report states that chest x-rays should not be used as the only diagnostic test. Chest x-rays are to be used in patients who cannot produce sputum or patients with negative Xpert MTB/RIF results and HIV positive or in patients with extra-pulmonary TB. The x-ray findings are to be interpreted with the patient's history and clinical findings.[7] Similar findings on the over-reliance of chest x-rays for diagnosis of TB has been reported in a case study in Durban, were 45% TB cases were diagnosed on the basis of a

chest x-ray.[15] This finding highlights the urgent need for the complete implementation of all components of the NTP within the study community and supporting health systems.

In the study community, the majority (87.1%) of TB cases were classified as new. These results are in accordance with trends reported nationally; classifying the majority of TB cases as new (85.0%); 7.6% relapse; 0.9% treatment failure; 2.2% treatment after default and 4.3% other.[1] The high number of new TB cases reflects high TB transmission rates.[16] High contact rates, low case finding, delays in diagnosis, resource-limited health systems and delays in chemotherapy are among the factors known to contribute to the high TB transmission rates. To achieve long term control of TB, reduction of the transmission rates should be one of the main priorities.

In Mpumalanga province, in the year 2010, 10% (2, 331) of reported TB cases were extra-pulmonary.[5] A slightly higher percentage of extra-pulmonary TB cases were observed in the study population, at 14.5% (n= 9). Compared to other African countries with a high human TB burden (Zimbabwe, Uganda, Mozambique, Kenya) the observed 14.5% in this study was within the reported range, of 10 – 16% of extra-pulmonary TB cases.[17] However, compared to Ethiopia, one of the countries with the highest incidence rate (33%) of extra-pulmonary TB, the observed cases in this study was lower.[18] Complete details of these cases and site of infection were not available from the TB patient files and TB register reviewed in this study. However, the few files which were classified as extra-pulmonary TB were adenitis (n = 2), and TB of the pleura (n = 2). Extra-pulmonary TB is highly elusive in detection and requires clear and complete communication in the health system in order to administer timely and appropriate chemotherapy. In addition, the NTP (2014) guidelines state that patients suspected of extra-pulmonary TB are to have specimens taken from the site and Xpert MTB/RIF, culture and drug susceptibility testing performed. However, in the cases of the Mnisi patient files, this protocol had not been followed. The lack of a complete set of information flags the severe constraint in diagnosing extra-pulmonary TB patients and the urgency to raise awareness among health workers and diagnostic services on the importance of complete investigation and reporting according to the NTP guidelines.

Tuberculosis, both extra-pulmonary and pulmonary can be caused by different members of the *Mycobacterium tuberculosis* complex (MTBC). Although *Mycobacterium tuberculosis* is the most common causative agent in humans, *Mycobacterium bovis,* known as the cause of TB in

bovines, can cause TB in humans. The main transmission route of *M. bovis* from livestock to humans (zoonotic disease) is through the consumption of contaminated, raw unpasteurised milk and/or meat/organs. Zoonotic TB is often associated with extra-pulmonary TB (e.g. retropharyngeal adenitis).[19] In the study population , *M. bovis* was confirmed in livestock (Chapter 2).[20] It is therefore of importance to investigate the epidemiological significance of zoonotic *M. bovis* in resource limited community settings such as Mnisi. The MTBC causative agent of TB, particularly extra-pulmonary TB was rarely investigated in the Mnisi local clinics. The under diagnosis of extra-pulmonary TB and the lack of investigating the causative agent of TB has been reported as common in other developing countries.[21,22] The reasons provided were the difficulties in obtaining samples from the extra-pulmonary site, the specialized diagnosing equipment required and trained laboratory personnel. In developing countries, epidemiological studies of extra-pulmonary TB are mainly conducted for research purposes and are rarely part of the routine diagnostic process.[22] It can therefore be concluded that in the current study, *M. bovis* cannot be ruled out as a causative agent for a fraction or all of the reported 14.5% cases of extra-pulmonary TB. This finding warrants further investigation into the significance of *M. bovis* or *M. tuberculosis* in the extra-pulmonary TB cases reported in Mnisi area. *Mycobacterium bovis* is resistant to one of the first line antimicrobial treatment, pyrazinamide; therefore accurate identification of the causative MTBC member can render efficient chemotherapy.

The main limitation of this study was the lack of complete TB population data for the Mnisi community particularly data for patients referred from the local Mnisi clinics to Tintswalo hospital. Tintswalo hospital was not included in this study as it did not form part of the study area and associated research approval, hence no access to hospital data was possible. The limited data prohibited analysis of TB prevalence within the Mnisi area. The TB register file and patient files were partially incomplete and paper based which compromised tracing of patients.

An additional limitation of this study was the inability to link TB and HIV status due to restricted ethical approvals which only allowed access to the TB data and not the HIV data. It can, however, be said that with regard to HIV prevalence, Mpumalanga province is ranked in the top three of the nine provinces in South Africa.[23] Therefore the high HIV prevalence in the province could infer the same level high level of HIV in the Mnisi study population.

4.6 Conclusion

From this study it was observed that TB equally impacted both males and females at the age category of 30-34 years. Extra-pulmonary TB accounted for 14.5% of all TB cases; this warrants further investigation into the MTBC causative agent in order to provide a comprehensive control of TB, inclusive for both *M. tuberculosis* and *M. bovis*. The four primary health clinics in the study area had the basic components of the NTP guidelines implemented. However, there is a need for implementation and awareness of all the components. This includes less dependence on chest x-ray for diagnosis and improved diagnosis of extra-pulmonary TB.

This study highlights the importance of community based studies in providing preliminary data on the TB population characteristics which will be of use in informing policy-makers towards targeted TB control policies. In addition, this preliminary data can help to identify health systems gaps for provincial and national tuberculosis control programs.

4.7 Acknowledgements

We would like to thank the Mpumalanga Health Services as well as the Mnisi Health Clinics nurses and all staff for their collaboration, assistance and communication with patients for the purpose of this study. We also thank Jaqui Somerville and Rene Ehlers for their assistance with the statical analysis and Thuli Anastasia Maabane for her assistance in the field. We are thankful to Ongole Joven Jebio and Ravi Raveendranathan for assistance in editing the manuscript.

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Chapter 5: Investigation into *Mycobacterium tuberculosis* and *Mycobacterium bovis* in patients at the wildlife/livestock/human interface, Mpumalanga Province, South Africa

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This chapter is a working paper and has not yet been submitted for publication.

5.1 Abstract

The aim of this study was to investigate the presence of *Mycobacterium bovis* in a small rural community located at the wildlife/livestock/human interface and genetically characterise TB isolates obtained within the community. A total of 191 sputum samples was collected from patients with TB symptoms as well as farmers within the community. No *M. bovis* was isolated and all 13 isolates were identified as *Mycobacterium tuberculosis* by deletion typing PCR (RD 4 and RD 9 PCR). Eight of the spoligo-patterns obtained from the 13 isolates were compared on the international spoligotyping database (SITVIT2) and a high level of diversity was detected. The *M. tuberculosis* isolates were identified as belonging to the T; EAI5; MANU 1; X1; Beijing; LAM 11_ZWE; X2; and S families. The predominant lineage was identified as T family, sub-lineage ST53. MIRU-VNTR genotyping further sub-categorised spoligotyping clusters into unique patterns. Based on the diversity of TB strains from the small group of isolates studied it appeared that human migration between the study community and urban towns as well as neighbouring Mozambique had a great impact on TB strain diversity detected. These findings indicate that improved TB control policies are required that take into account, human migrations. It is concluded that more effective TB control programs implemented by urban employers (e.g. mines) can effectively contribute to a reduction of the TB burden in the rural community settings. In addition, bovine tuberculosis did not appear to have any epidemiological significance in this study population as supported by the lack of *M. bovis* detection in the human population.

5.2 Introduction

Tuberculosis (TB) is a fatal infectious disease that claimed 1.3 million deaths in the year 2012, worldwide.[1] The main causative agent of TB is *Mycobacterium tuberculosis*. However, *Mycobacterium bovis* commonly identified as the causative agent of bovine tuberculosis (BTB) can cause TB in humans and often referred to as zoonotic TB.[2] The main transmission route of BTB from livestock to humans is through the consumption of *M. bovis* contaminated raw unpasteurised milk and/or meat/organs.[3] In the current study population, BTB was detected within the community's livestock, and this finding is of public health concern and warrant further investigation into the prevalence of *M. bovis* within the community.[4]

The pathogenicity of TB infection is attributed to a complex interplay of host, environmental and pathogen factors.[5] One of the essential pathogen factors is the genetic makeup of the infecting mycobacteria. It has been well established through reports in literature that different *Mycobacterium tuberculosis complex* (MTBC) lineages and strains induce different immune responses and pathogenesis in animal models.[5-7] Similarly in human studies, different *M. tuberculosis* lineages manifest variably between individuals and have different epidemiological significance.[8,9]

The global TB health crisis has been attributed to a diverse range of *M. tuberculosis* genotypes that are reported at different frequencies and vary widely between communities, countries and continents.[10,11] In South Africa, numerous studies have investigated the population structure of *M. tuberculosis* genotypes with special emphasis on drug resistant strains. High levels of diverse genotypes were reported, with some reports suggesting a link between certain *M. tuberculosis* genotypes (e.g. Beijing lineage) and drug resistance, whereas others reported no association.[12-14] The lack of consistency between these studies highlights the incomplete knowledge of TB transmission within South Africa and shows the importance of continued investigation. There is a need for on-going surveillance of TB population structure within different settings in an attempt to better understand TB transmission. Additionally, investigation into population structure provides insight into whether specific strains are over-represented in certain communities or specific group(s) and have significant epidemiological relevance in particular settings.[15] These studies are an essential part to TB control programs.

Advances in molecular techniques have facilitated the insight into MTBC genetic diversity and epidemiological links between particular TB genotypes and specific outbreaks. These

techniques are based on detecting specific variations, if any, in well characterised repeats sequences, such as the *IS6110*, and/or direct repeat (DR) region.[16] The "gold standard" for genotyping *M. tuberculosis* is currently *IS6110* - based restriction fragment length polymorphism (RFLP).[17] Though *IS6110*-RFLP has high discriminatory power among *M. tuberculosis* strains, the method is requires high technical expertise, needs large amount of DNA and has limited discriminatory power for strains with less than six *IS6110* copy numbers.[17,18] To compensate for *IS6110*-RFLP limitations, alternative genotyping techniques such as spoligotyping and mycobacterial interspersed repetitive units of variable-number tandem repeats (MIRU-VNTR) are commonly used and have acceptably high discriminatory power.[19]

Spoligotyping is based on detecting the presence or absence of 43 unique spacer regions found between direct repeat elements of the *M. tuberculosis* strain.[8] Whereas, MIRU-VNTR is based on the determination of the number of repetitive mini-satellite elements found in tandem repeats at various loci throughout the *M. tuberculosis* chromosome.[16] Each of these two techniques, MIRU-VNTR and spoligotyping, on their own has lower discriminatory power but in combination the discriminatory power is comparable to *IS6110*-RFLP. [20,21]

This study aimed to investigate the genetic characteristics of *Mycobacterium tuberculosis complex* in TB patients registered with one of four primary health clinics as well as farmers recruited from the dip-tanks in the community. This in order to investigate the presence of *M. bovis* in the study population; assesses the transmission patterns; whether specific TB strains were more prevalent; and more successfully transmitted within the community. This objective was investigated through the isolation and genetic characterisation of the causative agent of TB, using spoligotyping and MIRU-VNTR typing.

5.3 Materials and methods

5.3.1 Study area

This study was conducted in a small rural community that forms part of the Mnisi Tribal chieftainship, located in the north-eastern corner of the Bushbuckridge Municipal area, Mpumalanga Province, South Africa. The community is located at the western border of the Greater Kruger National Park Complex (GKNPC) conservation area, with a comprehensive wildlife/livestock/human/environment interface. The community has over 40, 000 people and has high levels of unemployment.

5.3.2 Clinics

Participants in this study included patients actively enrolled at the four local clinics (Hluvukani, Welverdiend, Utah and Gotternburg) (chapter 4). The study was carried out from June 2013 through to Feb 2015 (20 months). Participants incorporated TB positive patients diagnosed by the health clinics (retrospective study). Patients with clinical symptoms (coughing for more than 2 weeks, unexplained weight-loss and/or night sweats), with no confirmed laboratory results were also enrolled as TB suspects (prospective study). From each patient, one sputum sample was collected at the clinics as TB patients/suspects attended follow-up treatment and diagnosis. In some instances, sputum was collected at the patient's home when insufficient sample was obtained from the clinics or as follow-up cases.

5.3.3 Dip-tanks

In this prospective part of the study, farmers were incorporated as a subset of participants enrolled (Figure 5.1). Recruitment was conducted by a local research assistant, with the help of the Animal Health Technicians, at the 15 livestock dip-tanks in the study community. The farmers were classified into two categories; farmers whose cattle tested positive or owners of herds with an inconclusive bovine tuberculosis (BTB) status (6 farmers and members of their households (total n = 19); and 20 farmers whose animals tested negative for BTB previously (chapter 2).[4] From each participant farmer, a sputum sample was collected at the dip-tanks during the routine livestock inspection. In some instances sputum samples were collected by the local research assistant at the farmer's home.

5.3.4 Sputum collection, processing and mycobacterial culture

A total of 191 sputum samples were collected, each in one 50 ml sterile falcon tube. A set of 117 samples were stored in cetyl-pyridinium chloride (CPC) solution. Cetyl-pyridinium chloride (CPC) is used as a transport and storage medium for sputum specimens for the isolation of mycobacteria.[22] Equal volumes of CPC solution (1 % CPC) were added to each sputum sample. The remaining samples (n = 74) samples were collected without additions, according to recommendations of the local clinics, due to logistical difficulties experienced, resulting in prolonged storage and increased risk of leakage. All sputa were kept frozen at -4°C until processed at the BSL 2+ laboratory, University of Pretoria, Department of Veterinary Tropical Diseases, Onderstepoort, South Africa.

At the laboratory (biosafety level BSL2+), CPC-treated sputa were thawed to room temperature. Thereafter, samples were slightly heated in a warm bath at 28°C, to insure CPC

was at optimum temperature (no crystals had formed). Samples were then centrifuged at 3,000 x g for 30 mins. The supernatant was discarded. One hundred µl of each pellet was inoculated in duplicate onto each of two sets of Lowenstein –Jensen (LJ) media supplemented with glycerol, the other set of LJ media supplemented with pyruvate. Inoculated media were incubated at 37°C for 8 weeks and monitored weekly.

The non-treated CPC samples were decontaminated by the n-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) method, using a commercial kit (MycoPrep; Becton Dickinson (BD), Dickinson, Sparks, MD), according to the manufactures instructions. Thereafter, sediments were re-suspended in phosphate –buffered saline (PBS) and inoculated on two sets of solid media (LJ with glycerol and pyruvate) in duplicate and liquid media (Bactec mycobacteria growth indicator tube 320 (MGIT 320, BD Diagnostics)) system. One hundred µl of sample was inoculated onto each media slope, whereas 0.5 ml of sample was inoculated in MGIT tubes as described by the manufacturer. The inoculated solid media slopes were incubated at 37°C and examined weekly for 8 weeks. The inoculated MGIT tubes were scanned into BD machine and monitored by the machine at 37°C for 42 days.

On LJ media, negative cultures were classified if no visible growth was detected after the 8 week incubation period. Mycobacteria positive cultures were provisional characterized if non-pigmented, waxy, wrinkled colony morphology were observed after 1 - 12weeks incubation of LJ slants. MGIT tubes that flagged positive on the MGIT 320 machine were checked for visual morphology, clear liquid with flaky growth detected after 1 – 12 weeks incubation. Microscopic examination of positive cultures both from LJ and MGIT was conducted using the Ziehl-Neelsen (ZN) staining method to verify the presence of acid-fast bacilli (AFB). *Mycobacterium tuberculosis* H37 and *Mycobacterium bovis* BCG were used as control.

5.3.5 Genotyping techniques

5.3.5.1 DNA Extraction

Acid fast bacilli (AFB) and positive culture controls (*M. tuberculosis* H37 and *M. bovis* BCG) were heat killed by mixing two loop-full of colonies in 1 ml PBS in 1.5 ml Eppendorf tubes, followed by heat inactivation at 95°C for 20 mins. The heat killed suspensions were briefly centrifuged (1 min) at 3000 x g. Thereafter, the supernatant was transferred to clean Eppendorf tubes and the pellet was discarded. The supernatant was used as crude DNA templates in polymerase chain reactions (PCR) for both spoligotyping and MIRU-VNTR.

5.3.5.2 Polymerase Chain Reaction (PCR)

The region of difference RD4 and RD9 deletion typing was used to distinguish the MTBC as previously described (Warren *et al*., 2006), with slight modifications.[23] In brief, each PCR reaction contained 1 µl crude DNA template, 13.6 µl DreamTaq PCR Master Mix (2x) (Thermo Scientific), 0.5 µl of each primer (50pmol/µl) (Table 5.1) and was made up to 25 µl with nuclease-free water. Amplification was initiated by first incubating at 95°C for 15 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min and an extension step at 72°C for 1 min. An elongation step at 72°C for 10 min followed after the final amplification cycle. .

PCR amplicons were electrophoretically fractioned in 2 % agarose in 1x Tris-borate-EDTA, at 3.5V/cm for 90 min and visualized by staining with ethidium bromide. A Gel Doc XR (TM) Imaging system (Bio-Rad) was used to visualize and capture gels.

Table 5.1: PCR primer sequence and corresponding regions of difference

RD region	Primer sequence
4	5’ ATGTGCGAGCTGAGCGATG 3’
4	5’ TGTACTATGCTGACCCATGCG 3’
4	5’ AAAGGAGCACCATCGTCCAC 3’
9	5’ CAAGTTGCCGTTTCGAGCC 3’
9	5’ CAATGTTTGTTGCGCTGC 3’
9	5’ GCTACCCTCGACCAAGTGTT 3’

Primer sequences according to Warren *et al*., 2006

5.3.5.3 Spoligotyping

Spoligotyping was performed for all AFB using a commercial kit (Ocimum Biosolutions Ltd, Hyderabad, India) as described by manufacture’s guidelines with slight modifications. In brief, PCR amplification was preformed, and each PCR reaction contained 2 µl of each primer, DRa and DRb; 12.5 µl of Qiagen master mix; 5 µl crude DNA sample and PCR reactions was made up to 25 µl with sterilized water (3.5 µl). The PCR cycles entailed 15 mins denaturation at 96°C, followed by 30 cycles of 1 min at 96°C, 1 min at 55°C and 30 sec at 72°C. The elongation step followed and was at 72°C for 10 mins.

Thereafter, hybridization of PCR amplicons was performed. In brief, 25 µl of PCR product was added to 150 µl 2xSSPE/0.1% SDS and heated at 100°C for 10 mins and cooled on ice

immediately after. Loaded the heat denatured product on pre-washed membrane and hybridized at 60°C for 60 mins. Membranes were processed as recommended and exposed to light sensitive film for approximately 15 mins.

An electronic database was created by entering the spoligotyping results in Microsoft Excel sheet as a binary code representing positive (n) or negative (o) hybridization result. Obtained binary format were entered in the SITVIT2 database (Pasteur Institute of Guadeloupe; http://www.pasteur-guadeloupe.fr/tb/bd_myco.html).

A cluster was defined as two or more isolates with identical patterns.

5.3.5.4 Mycobacterial interspersed repetitive-unit-variable number tandem repeat (MIRU-VNTR)

The MIRU-VNTR typing was performed on isolates grouped into spoligotyping clusters obtained above, using the 24 MIRU-VNTR typing kit supplied by Quardruplex versions (GenoScreen, France), according to the manufacture's guidelines. In brief, MIRU-VNTR genotyping entailed PCR amplification, whereby each PCR reaction contained 8 µl of MIRU-VNTR quardruplex mix and 2 µl of sample crude DNA. The PCR cycle included 15 mins denaturation at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 59°C and 90 sec at 72°C. The elongation step followed the last cycle and was at 72°C for 10 mins. PCR amplicons were electrophoretically fractioned using ABI 3130 genetic analyzer (Applied Biosynthesis, USA). Thereafter, assignment of the alles was performed using the MIRU-VNTR calibration kit (GenoScreen, France). Analysis of data was done using the GeneMapper software version 4.0 (Applied Biosystems, USA).

The 24-locus MIRU-VNTR panel comprised: MIRU 02, VNTR 42, VNTR 43, MIRU 04, MIRU 40, MIRU 10, IRU 16, VNTR 1955, MIRU 20, QUB 11b, ETR A, VNTR 46, VNTR 47, VNTR 48, MIRU 23, MIRU 24, MIRU 26, MIRU 27, VNTR 49, MIRU 31, VNTR 52, QUB 26, VNTR 53 and MIRU 39. The MIRU-VNTR profiles were reported as a series of numbers that correspond to the number of alles at each of the loci and were entered in an excel sheet. These numerical patterns were then analyzed using the MIRU-VNTRplus database (www.miru-vntrplus.org).

5.3.6 Dendrogram

A dendrogram was constructed for spoligotype clusters and corresponding MIRU-VNTR results. The un-weighted pair group method with arithmetic averages (UPGMA) algorithm was used to construct the dendrogram. The categorical coefficient was used to calculate the distance matrix.

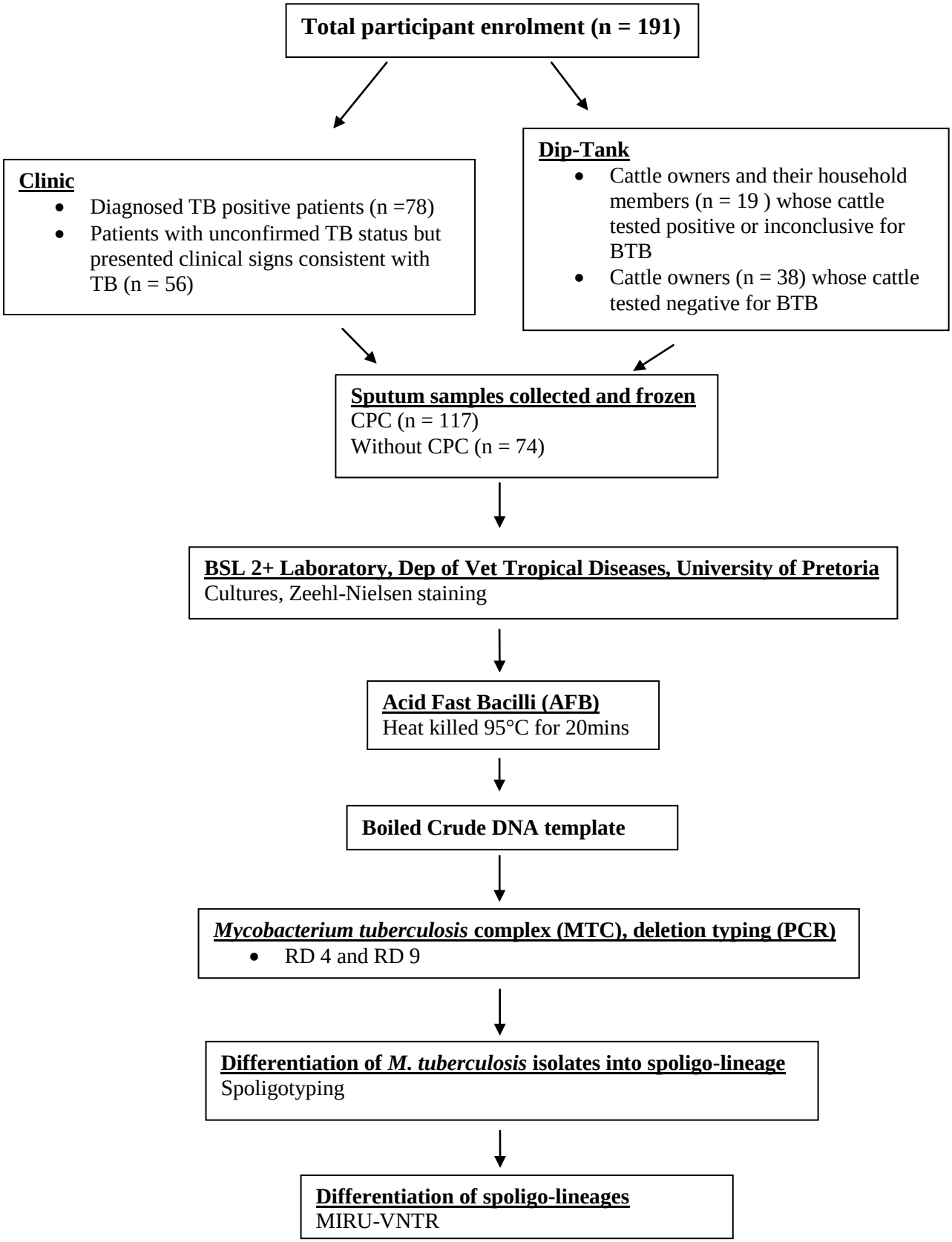

Figure 5.1: Algorithm depicting the isolation and genetic characterisation of Mycobacterium tuberculosis complex from patients as well as farmers in the Mnisi study population

5.3.7 Ethical Statement

The study was carried out with ethical approval from the University of Pretoria, Faculty of Health Sciences Research Ethics Committee and from the Mpumalanga Provincial government, Department of Health Provincial Research and Ethics Committee. Verbal consent was obtained from the patients and farmers to be enrolled in the study under anonymous records.

5.4 Results

From June 2013 to Feb 2015 (20 months) a total of 191 sputum samples were collected and processed from the Mnisi community. Thirteen AFB isolates were identified using Ziehl-Neelsen staining and confirmed as *M. tuberculosis* using region of difference (RD), RD 4 and RD 9 PCR (Figures 5.2 and 5.3). Eleven of the thirteen isolates originated from participants enrolled at the clinics (Appendix 2). The remaining two isolates were collected from livestock farmers; one farmer was among the participants recruited at the dip-tank (cattle herd with BTB negative status) whereas the second farmer owned a cattle herd with inconclusive BTB status.

All AFB isolates were identified and confirmed as *M. tuberculosis*.

5.4.1 PCR

Figures 5.2 and 5.3 show deletion typing results having used primer sets RD 4 and RD 9 (respectively) for the 13 MTBC isolates. Ten of the 13 isolates had PCR products corresponding to reference strain *M. tuberculosis* H37, using primer RD 4 (172bp) and RD 9 (235bp). The three remaining isolates, 366, 367 and 809 were not amplified using RD 4 primer but were amplified using RD 9. Isolate 809 had a band size slightly larger than the expected 235bp, using primer RD 9 (Figure 5.3).

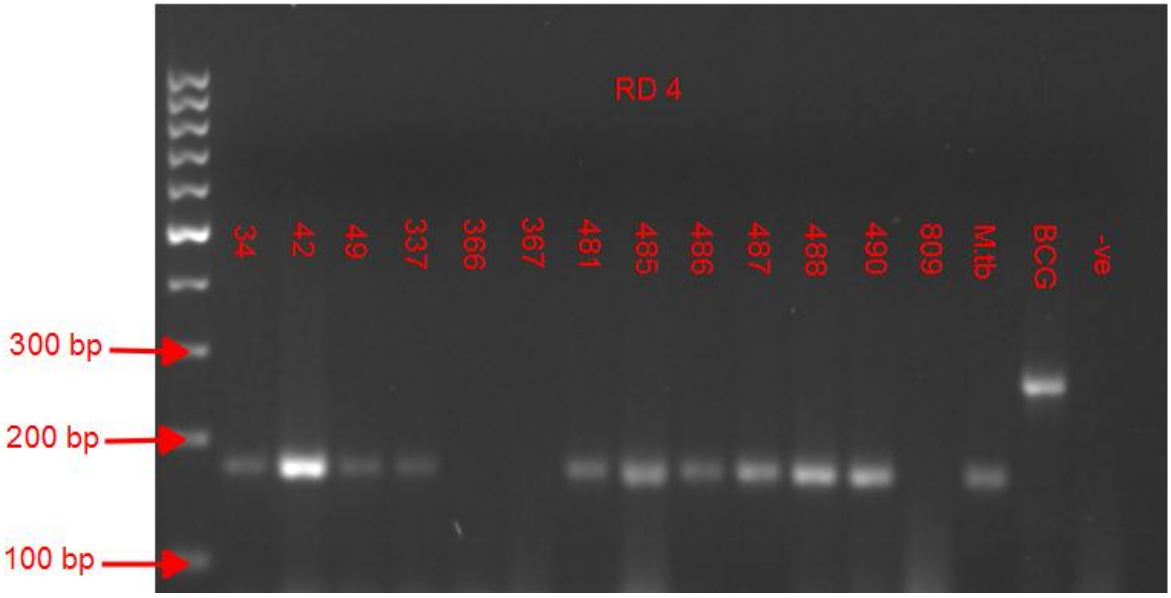

Figure 5.2: 2% agarose gel showing the electrophoretic fractionation of deletion typing PCR products. Crude DNA template from: the 13isolates, 34, 42, 49, 337, 366, 367, 481, 485, 486, 487, 488, 490, 809; positive controls, *M. tuberculosis* H37, and *M. bovis* BCG; as well as negative control (no DNA template but water added), were amplified using RD 4 forward and reverse primers.

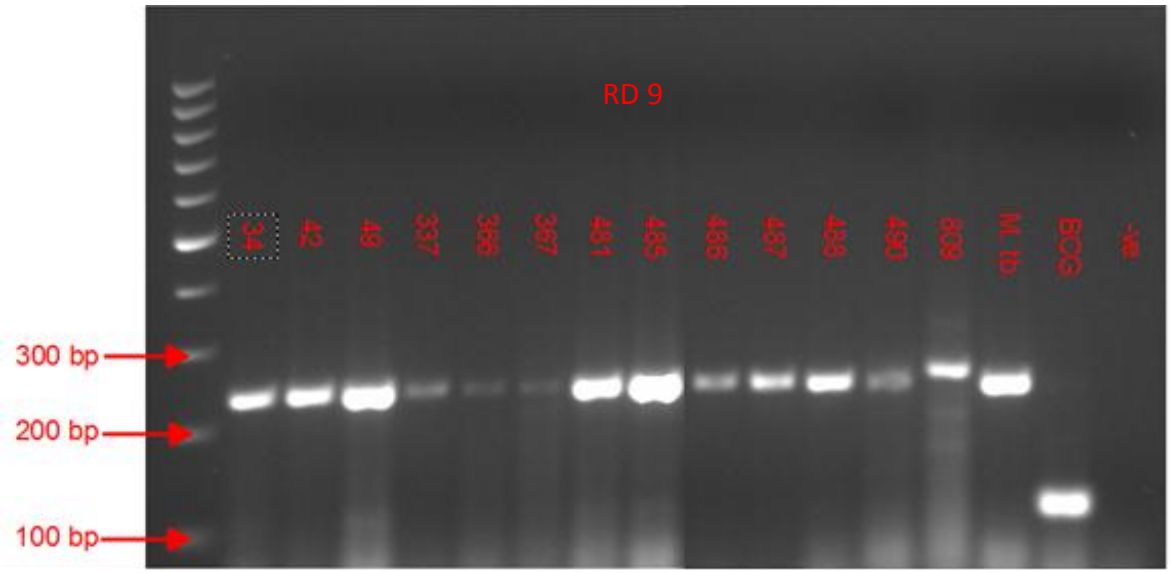

Figure 5.3: 2% agarose gel showing the electrophoretic fractionation of deletion typing PCR amplicons. Crude DNA template from: the 13 isolates, 34, 42, 49, 337, 366, 367, 481, 485, 486, 487, 488, 490, 809; positive controls, *M. tuberculosis* H37, and *M. bovis* BCG; as well the negative control (no DNA template but water added), were amplified using RD 9 forward and reverse primer.

5.4.2 Spoligotyping

Eight distinct spoligotyping patterns were detected among the 13 *M. tuberculosis* isolates, including three clustered patterns and five un-clustered patterns. All eight resulting spoligotyping patterns were compared within the international spoligotyping database (SITVIT2 database). A total of eight different lineages were identified. These included: T1; EAI5; MANU 1; X1; Beijing; LAM 11_ZWE; X2; and S families (Table 5.2). The T1 family sub-lineage SIT53 was the predominant lineage detected in this study population, represented by three isolates. EAI5 and MANU1 clusters, each consisted of two isolates, whereas X1 represented two isolates with different SIT numbers. The remaining four lineages were each represented by one isolate.

Table 5.2: Spoliogtype patterns for the thirteen *M. tuberculosis* isolates detected in this study

Participant no.	Lineage	SIT[a]	Spoligotyping pattern	No of isolates
809c 481 337	T1	53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	3
42 366	EAI5	236	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	2
486 487	MANU1	100	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■	2
34 367	X1	336 119	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■□■■■ ■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	1 1
49	Beijing	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1
485	LAM 11_ZWE	59	■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	1
490	X2	137	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■□□□□■	1
488	S	34	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1

5.4.3 MIRU-VNTR

MIRU-VNTR genotyping analysis sub-categorised the three spoligotyping clusters into seven uniquely distinct patterns (Figure 5.4). No clusters were observed. Highest allelic diversity was observed at the MIRU 40 loci (Figure 5.4 and Appendix 3). Of interest, from the MANU 1 spoligotype cluster, the two isolates only had one mutation at locus QUB 26.

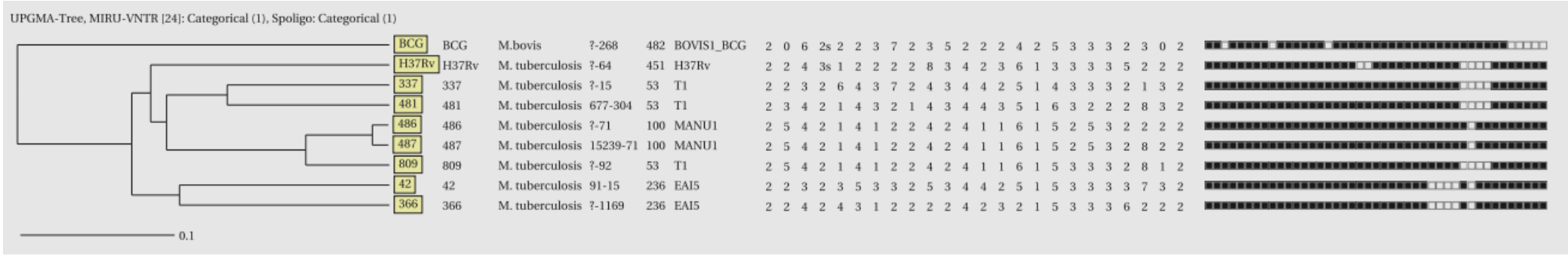

Figure 5.4: Combined dendrogram of seven mycobacterial isolates using spoligotyping and mycobacterial interspersed repetitive-unit-variable number tandem repeat (MIRU-VNTR) typing

5.5 Discussion

The main aim of this study was to investigate the presence of *Mycobacterium tuberculosis* complex in patients with clinical symptoms of TB and recruited farmers from the community. Additionally, genetic diversity of TB isolates within the community was investigated in order to assess possible transmission patterns. This preliminary study was conducted through the isolation and genetic characterisation of the causative agent of TB.

Of the isolates obtained in this study, none were identified as *M. bovis*. All isolates were identified as *M. tuberculosis*. The apparent absence of *M. bovis* in the study population is attributed to the low BTB prevalence (0.33 %) detected in the resident livestock (chapter 2). In addition, previous findings in chapter 3 concluded that there was a low risk of *M. bovis* transmission from livestock to humans based on the community's food consumption practises regarding meat/organs and milk, as well as the limited human/livestock interaction. Hence, there was a low probability of *M. bovis* transmission from livestock to the human population. However, based on this study, it cannot be concluded that BTB is not a public health concern. There are limitations in this study that could have influenced the detection of BTB. One possible reason was that this study only analysed sputum samples. *Mycobacterium bovis* is mainly associated with extra-pulmonary TB and the great difficult in diagnosing the disease and obtaining fine needle aspirates could contribute to the under-diagnoses.[1] Thus, further studies, with larger sample numbers and wider sample range (e.g. fine needle aspirates) are required in order to determine the true extent of the epidemiological significance of BTB in public health.

M. tuberculosis is the main causative agent of TB in humans worldwide and the different *M. tuberculosis* lineages/sub-lineages induces different pathogeneses in hosts.[2,3] Numerous studies in South Africa have characterised the circulating *M. tuberculosis* strains.[4-6] However, these studies were mainly focused on drug resistant strains and were main hospital level based. There is a lack of community based studies in rural settings, investigating the TB population structure. These are of importance in order to determine the local drivers of the disease, as they are essential for targeted, appropriate and tailor-made control measures and interventions. In this preliminary study, *M. tuberculosis* was detected with a high level of spoligotyping diversity. Spoligopatterns were assigned to eight lineages/sub-lineages, namely T; EAI5; MANU 1; X1; Beijing; LAM 11_ZWE; X2; and S. The T family, sub-lineage ST53 was the predominant

genotype detected, represented by three of the thirteen (23.1%) isolates (Table 5.2). Our findings are consistent with investigations by Stavrum *et al*., 2009, who reported the T family as the predominant (25.8%) genotype across eight of the nine provinces in South Africa, with 11.1% of all isolates identified as T lineage, sub-lineage ST53 genotype.[5] Of the nine provinces, predominance of the T lineage was observed in two of South Africa's bigger urban provinces, namely, the Free State and Gauteng.[5] Other studies conducted in South Africa have widely reported on the varying prevalence of the T family sub-lineage ST53; however there is limited information on the epidemiological significance.[4,7,8] The few reports in literature describe the T family as a group of strains clustered in one group as they could not be classified in any of the established genotypic lineages with well-established phylogeographical specificity, such as Haarlem (H); or East-African Indian (EAI).[9] The lack of literature highlights the challenge that still remains to link genetic variability of *M. tuberculosis* genotypes with clinical significance and varied TB pathogenicity.

The three spoligotyping clusters obtained in this study were split into unique patterns by MIRU-VNTR typing. The most polymorphic locus observed was at MIRU 40 (Figure 5.4). This finding is consistent with reports by Sola *et al*., 2003, that consider MIRU 40 among the top five highly discriminating loci of the standard 12 MIRU loci set.[10]

Interestingly, the two isolates (486 and 487) clustered under the MANU1 had a very close epidemiological link, however showed one change at the QUB 26 locus during the MIRU-VNTR analysis (Figure 5.4). This finding suggests a recent ancestor, and possible mutation. Conclusive epidemiological significance cannot be drawn based on a single locus, as loci mutate at different rates.[11] Further, investigations into *M. tuberculosis* strain mutations within the community are warranted.

Epidemiological studies have indicated that a high level of *M. tuberculosis* strain diversity within a closed community does not support the theory that transmission occurred within the community, rather that the transmission source was external.[12-14] In this study, despite it being a preliminary investigation and few isolates obtained, spoligotyping and MIRU-VNTR genotyping results showed a high diversity among the *M. tuberculosis* isolates. It can therefore be hypothesised that the high *M. tuberculosis* strain diversity observed was a result of introduction of external strains into the study population. It was expected that TB lineages reported would be

those prevalent in Mpumalanga province, namely Latin American and Mediterranean (LAM) and in other studies the East African Indian (EAI) genotype.[5,7] However, the predominant lineage detected was the T family; this suggests the predominance of the T family is a result of migration from urban towns into the rural community. In the questionnaire interviews conducted earlier (chapter 3), it was established that migration of people from the study community to urban towns in search for job opportunities and education was common. Supporting evidence to the hypothesis that migration impacted the diversity of *M. tuberculosis* strains observed in this study was findings in chapter 3, where it was reported that the majority of employed TB patients registered at the clinics were industrial employees. This meant that the majority of TB patients were migrant workers, as industrial employment was inferred urban employment. Of interest was the observation that the TB lineage patterns observed in this study were similar to those reported from Mozambique by Viegas *et al*., 2010, who reported LAM (37%), EAI (29.7%), T clade (11.6%) and the Beijing (7%).[9] These findings add to the hypothesis that migration has an impact on TB population structure. The impact of migration on the TB population structure highlights the importance of continued surveillance within South Africa and neighbouring countries for improved TB control policies and better understanding of TB transmission.

A limitation of this study was the long storage of samples before processing and culture. The delay was due to the remote distance of the study community and the difficulty in logistics to transport samples. There is a need for further investigation in improved storage and transportation of samples from remote communities to health and laboratory facilities.

5.6 Conclusion

This study served as a preliminary investigation into the causative agent of MTBC in the community. Based on the small group of isolates obtained, *M. bovis* was not detected. This finding provides further supporting evidence that the risk of BTB from livestock to humans in the Mnisi study population at present is probably negligible. Nonetheless, a wide diversity of *M. tuberculosis* strains was detected, with eight spoligo-patterns observed from the 13 isolates. One would have assumed that in the small rural community, a particular TB lineage would be over-represented. However, based on the diversity and predominate lineage detected in the small group of isolates obtained, it can be concluded that there is an introduction of a variety of *M. tuberculosis* lineages into the community from urban towns and possibly neighbouring

Mozambique. Additionally, migration has an impact on the prevalence of TB within the community. There is a need for improved TB control policies, particularly from urban employers, in order to reduce the impact and burden of TB in rural communities. The improved strategies include better communication with health service providers where patients/migrants work/originate from, and improved awareness within the community on the importance of TB diagnosis as well as treatment.

5.7 Acknowledgements

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Chapter 6: General discussion and conclusion

Tuberculosis is a fatal infectious disease caused by members of the MTBC that can cross boundaries between humans, livestock and wildlife.[1] In the human population, *M. tuberculosis* is the main causative agent; however, the numbers of TB cases attributed to *M. bovis* are unknown and probably underestimated, particularly in developing countries. The reason linked to the underestimation of zoonotic TB is that the status of *M. bovis* in livestock is unknown due to the sporadic and/or lack of BTB control.[2] Additionally, a number of wildlife species have been established as *M. bovis* reservoirs that serve as a source of infection/re-infection into livestock.[3,4] These reports have focused on TB in one or two types of host(s); however, the holistic approach (One Health) of investigating TB at the interface between human/livestock/wildlife and the environment is not well established, compromising the control of this deadly disease. Against this background the overall aim of this study was to investigate the epidemiological significance of TB, including a variety of potential risk factors, at the wildlife/livestock/human interface in the study area of the Mnisi community.

The prevalence of BTB was investigated in the community's livestock through a cross-sectional survey. CIDT testing was performed on approximately 10% of cattle registered with one of the 15 dip-tanks in the study area. It was established that BTB was present at a low prevalence of 0.33% (95% CI.0.14 – -0.79) and the causative agent was identified as *M. bovis* and genotyped as the KNP parental strain, KNP-VNTR-1.[5] The source of infection was identified as the spillback of BTB from wildlife in the endemically infected KNP as the identical *M. bovis* genotype was detected. Other potential sources of infection were excluded as the physical location where BTB positive cattle were observed was directly bordering the GKNP. Additionally, from the retrospective records from Mpumalanga Veterinary Services, no cases of BTB due to the KNP genotype were observed in the neighbouring livestock. The transmission of BTB from wildlife to livestock is attributed to direct or indirect contact between livestock and infected wildlife (e.g. African buffalo, great kudu and/or the common warthog) shedding *M. bovis*.[4] Jori *et al*., (2011) reported an observation rate of 162/year of study for African buffalo in the communal areas in close periphery with KNP, whereas the greater kudu and the common warthog were sighted at a rate of 511 and 621/year of study, respectively.[6] These break-outs were the result of damages on the physical barriers intended to separate wildlife and livestock, commonly due to elephant

damages or human activities.[7] The break-outs could also lead to indirect transmission, whereby environmental contamination of water points and grazing areas by infected wildlife is a possibility.[8] Despite these reports on direct or indirect interactions at the wildlife-livestock interface, the associated risk of disease transmission has been established for infections such as foot-and-mouth disease, while the risk of BTB transmission back to livestock has not been fully quantified in sub-Saharan Africa.[7] It has been theoretical only and this study provided the first evidence of wildlife-to-livestock BTB transmission in Africa.[8,9]

From this study it cannot be deduced when the transmission of BTB from wildlife to Mnisi livestock occurred, or whether the transmission between the infected animals was directly from wildlife or subsequent transmission between the cattle. Despite no information being available on the time frame of transmission, it is evident that wildlife definitely played a key role in BTB infection within the Mnisi livestock.

The concerns therefore extended to include the implications of detecting BTB in the study community's livestock. Bovine tuberculosis is a controlled disease in terms of the Animal Diseases Act, 1984 (Act 35 of 1984).[10] This implies that BTB is monitored, controlled and notifiable to the government extension services. The BTB eradication scheme entails the 'slaughtering out' policy. This policy was implemented in the study area by the veterinary extension services as soon as BTB had been confirmed by culture. The control strategies to be implemented included: the testing of all cattle in the four dip-tanks where BTB was detected; the quarantine of BTB positive herds; and the retesting of BTB positive herds after three month intervals. However, due to lack of resources and limited facilities these control strategies were not sustainable and could not be fully implemented. The compensation of slaughtered BTB positive cattle to owners was among the challenges. It was highly expensive to obtain the tuberculin needed for the CIDT testing. There was limited manpower as well as infrastructure to test the cattle in the communal setting. Hence, the full implementation of the BTB control strategies was unsuccessful. These constraints are similar to reports that the lack of veterinary infrastructure in developing countries remains a great challenge in the surveillance and control of BTB.[11] Despite the consequences of limited resources being observed, the cost of BTB is not known in African settings. There is a need for economic analysis relating to the losses due to BTB infected cattle in the absence of an enforced test-and-slaughter scheme and the cost of serial

follow-up testing as well as removal of positive animals. These estimates are essential in identifying cost effective control programmes in resource poor settings.

The success achieved through the traditional 'test and slaughter' BTB control policy in livestock is reported as limited in eradicating the disease, particularly at the wildlife-livestock interface due to the constant re-infection from wildlife reservoirs.[12] Hence, alternative BTB control strategies entailing the monitoring of infected wildlife reservoirs have been implemented, particularly in developed countries. These include limiting the population size of the wildlife reservoir through culling (lethal control) or vaccination (non-lethal control) programs.[13-15] In New Zealand, BTB has been successfully controlled by maintaining a low density of the wildlife reservoir (i.e. brushtail possum) through culling.[16,17] In the UK there has been a slow shift away from the controversial culling of badgers to the recently licenced injectable vaccine.[18] In South Africa the lethal control through testing and culling is currently focused on BTB positive African buffalo.[19] However, BTB in South African wildlife ecosystems is a multi-host pathogen and additional wildlife reservoirs species, such as the greater kudu and possibly warthog may compromise this BTB control initiative.[4] In addition, the 'testing and culling' of African buffalos has a great ecological impact as they are bulk grazers and top prey species for lions. African buffalo are also under great conservation protection, as part they form part of the beloved 'Big 5' African wildlife species and have a large economic value for tourism. This differs with countries such as New Zealand where the BTB wildlife reservoirs (i.e. possums) are not indigenous and are considered pest, thus, less conservation pressures.[20,21] The most feasible alternative control strategy could potential be vaccination. There have been successful trials on the use of vaccination for BTB control in both wildlife reservoirs and cattle in developed countries (e.g. New Zealand, Spain and Great Britain).[22-24] However, in South Africa, Klerk *et al*., (2005) reported that the vaccine protection was not statistically significant in African buffalo.[25] Further, research is required for an effective vaccine as part of a BTB control strategy, particularly in African settings.

Had the control of BTB been effective in the cattle in the study area, there would be little concern for a zoonotic risk to the community. However, due to the above mentioned limited resources that hinder effective BTB control, the impact of zoonotic TB remained unknown and raised public health concerns. Hence, part of the objectives of this study was to investigate the

presence of *M. bovis* in the study population, through the isolation and genetic characterisation of causative agents of TB in the community over a one year period (June 2013 through to Feb 2015). *Mycobacterium bovis* was not detected in the human population and this was primarily attributed to the low prevalence of BTB in the livestock.

The risk factors of *M. bovis* transmission from livestock to humans were investigated using a questionnaire based interview covering aspects of food consumption practices with regard to meat and milk; as well as livestock management practices. Meat consumption patterns in the community revealed a potential risk as all commodities of the carcass were consumed undercooked/raw. However, this risk appeared to be at least partially mitigated by the fact that visible abnormalities in the meat/organs were generally discarded, reducing the risk of high infective doses required for oral infection. It was also established that the majority of the community's households, including farmers, obtained pasteurised milk commercially and milk was consumed when affordable rather than on a daily basis. These findings are contradictory to reports that often refer to milk as an important part of the daily diet.[26,27] The sporadic consumption of milk observed in this study is possibly attributed to the low family income (i.e. when affordable) within the community that is explained in more detail below. Based on the questionnaire survey, a low percentage (7.7%) of TB patients in the study community owned livestock compared to 25% cattle ownership in the overall community as reported by a recent independent study population.[28] Hence, it was established that the risk of BTB transmission from livestock to humans was low based on the community's food consumption patterns and the few TB patients among cattle owners. In conclusion, BTB had no or little impact on the study population. However, this conclusion warrants further research as due to the limited medical service provision, only sputum was sampled in this study. For future investigations, a wider spectrum of samples (i.e. sputum, fine needle aspirates and samples from extra-pulmonary sites) should be included when investigating the significance of zoonotic TB.

The main socio-economic indicators in this study community are high levels of unemployment and low family income. An independent study conducted within the same study community reported that 88% of interviewed households of which 50% were farmers, had a monthly dispensable income of less than R 4000.[29] High unemployment trends were observed in the study questionnaire survey. Of interest, the vast majority (92.3%) of farmers referred to themselves as

unemployed. It was concluded from this finding that farmers did not regard the purpose of cattle ownership as livestock farming to generate income but rather as livestock keeping for cash reserves and status. This observation is identical to studies in other local or foreign African communities that livestock are largely kept as a form of investment and social status, not necessarily as a source of daily supply of food.[30,31] As a consequence livestock only produce milk for certain parts of the year (calving season) and animals are slaughtered for special occasions (e.g. funerals), which in turn influences the consumer behaviour. These observations support the above mentioned findings that the majority of the study community consumed pasteurised milk sourced commercially and, due to the low family income, milk was consumed only when affordable, resulting in highly sporadic consumption. These reports challenge the school of thought in terms of assessing the risk of zoonotic disease transmission through food consumption and contact with livestock. The characteristics of disease transmission and the level of risk will largely depend on the community setting and cannot be generalised for "rural communities in developing countries" as it is often done.

In literature, there are sporadic reports of *M. tuberculosis* in cattle. These studies highlight humans as a source for sensitization of cattle to *M. tuberculosis*.[32,33] However, the epidemiological significance of *M. tuberculosis* at the human/livestock interface is not well established, particularly in communities with high human TB prevalence. Additionally, the zoonotic risk of *M. tuberculosis* spillback from cattle to humans has not been established.[34] Hence, part of the objectives of this study was to investigate the epidemiological significance and risk of *M. tuberculosis* transmission from humans to livestock. *Mycobacterium tuberculosis* was not detected in the livestock sampled. In addition, it was established that the risk of *M. tuberculosis* transmission from humans to livestock was minimal as there was a low contact between TB patients and livestock, with only 7.7% of confirmed TB patients owning cattle. Ideally, the cattle owned by the TB patients would have been TB tested in this study. However, due to the regulatory constraints imposed by the Department of Agriculture, Forestry and Fisheries (DAFF) as well as the difficulty in tracing back cattle herds, these animals were not tested in this study. Further research is required to understand the impact of human TB at the human/livestock interface, particularly in these communities with high TB incidence (630 per 100 000 population). Additionally, comparative analysis with reports on the isolation of *M.*

tuberculosis in cattle from other developing countries (e.g. Ethiopia and Zambia) would contribute to the better understanding on human tuberculosis infection pressure on livestock.

From the questionnaire survey, a number of respondents mentioned that they fed their dogs meat/organs with visual abnormalities. This finding is of concern as there are reports of TB transmission to companion animals, however these studies were focused on developed countries and none were from resource poor settings in developing countries.[35-37] Hence, the significance of TB in the dog population as potential sentinel hosts should be investigated in the Mnisi study population.

In conclusion, in this study it was established that BTB is a reality at the wildlife/livestock interface and wildlife in the KNP serve as a potential source of infection and reinfection to neighbouring livestock. However, due to the low BTB prevalence in the community's livestock, BTB did not have a significant impact at the livestock/human interface, as *M. bovis* was not detected in the human population. Given a hypothetical increase in the prevalence of BTB, our findings suggest that the risk of BTB transmission at the livestock/human interface would be low based on the reported community's food consumption patterns (i.e. consumption of pasteurised milk). With regard to the risk of *M. tuberculosis* transmission from human to livestock, it was considered low based on the minimal contact TB patients had with livestock.

There is a need for improved inter-sectoral collaboration under the "One Health" theme (medical and veterinary services) in the study area, not only for zoonotic TB but other zoonotic pathogens (e.g. brucellosis). The benefits from the close collaboration include shared resources such as laboratory facilities, thus reducing logistic and time costs for sending samples to different laboratories. In addition, there would be improved awareness of zoonotic diseases in the study area and better information dissemination for implementation of surveillance as well as control programmes.

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Appendices

Appendix 1

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF VETERINARY TROPICAL DISEASES

Good day.

My name is ..... from the ..... clinic. We are in collaboration with a team of researchers from the Department of Veterinary Tropical Diseases of the Faculty of Veterinary Science, at the University of Pretoria. We are conducting a short survey investigating TB at the human-livestock interface.

Your participation in the survey is voluntary. You may refuse to answer any questions you feel uncomfortable with. Refusing to participate will not affect you or your household in any way.

The information you supply will be kept confidential and no attempt will be made to identify your answers in any way as these will be bulked with the answers of other respondents to form a data file to be used by professional statisticians to perform appropriate statistical analyses.

Please respond to the best of your ability and please ask if you find any questions to be unclear.

FORMAL CONSENT TO PARTICIPATE

Are you willing to participate in this study by supplying answers to the questions that follow?

Yes	1
No	2

Address of Respondent

Patient number

Patient mobile number ***(Only if stock card numbers are not available)***

Respondent number / Sputum sample number

If the respondent is willing to take part in the survey, please REMOVE this sheet from the rest of the questionnaire

1

Investigation into the prevalence of Tuberculosis at the human-animal interface

For office use

Respondent number / Sputum sample number V1 [ ][ ][ ][ ] 1

Please answer the questions by circling an appropriate number in a shaded box or by writing your answer in the shaded space provided

1. Where have you been **recruited** from?

Clinic	1
Dip-tank	2
Household	3

V2 [ ] 6

2. Please supply the **name** of the **enumerator**.

V3 [ ][ ] 8

3. Please supply the **name** of the **Clinic**.

V4 [ ][ ] 11

4. **Date** of **visit** (*please use dd/mm/yy*).

V5 [ ][ ][ ][ ][ ][ ] 14

5. On what **date** were you **diagnosed** with **TB** **(Please supply the most recent date as dd/yy/mm. If you have NOT been diagnosed with TB, please leave blank)**

V6 [ ][ ][ ][ ][ ][ ] 21

6. **Occupation** of respondent.

V7 [ ][ ] 28

7. How would you **rate** your **contact** with **cattle**?

Once a month	1
Once a week	2
Once a day	3
All the time	4

V8 [ ] 31

Question 8 ***follows on the next page ..***

For office use

8. What is your **gender**?

Male	1	V9	33
Female	2		

9. Please complete the following table in respect of the number of **members** of your **household** for the **age category** mentioned.

Age category	Number		
Children aged below 12 yrs		V10	35
Persons aged 12 yrs to 18 yrs		V11	38
Persons aged more than 18 yrs to 30 yrs		V12	41
Persons aged more than 30 yrs to 60 yrs		V13	44
Persons older than 60 yrs		V14	47

10. What is your **highest** educational level?

No formal education	1	V15	50
Primary school education	2		
Secondary school education	3		
High school education	4		
Tertiary/College/University education	5		

11. Which of the following do you **receive** as **monthly income**?
(If applicable, you may indicate more than a single answer)

Government grants	1	V16	52
Stable salary (employee)	2	V17	54
Selling of livestock	3	V18	56
Selling of livestock products	4	V19	58
Remittance	5	V20	60
Business other than farming	6	V21	62
Other (specify):		V22	64

12. In the table below indicate the **number** of those members of your **household**, excluding yourself, who have been **diagnosed** with **TB** in the last 6 months per **age category**. ***(If no member has been diagnosed, leave blank).***

Age category	Number		
Children aged below 12 yrs		V23	67
Persons aged 12 yrs to 18 yrs		V24	70
Persons aged more than 18 yrs to 30 yrs		V25	73
Persons aged more than 30 yrs to 60 yrs		V26	76
Persons older than 60 yrs		V27	79

Question 13 ***follows on the next page ...***

For office use

13. How would you describe your **status**? ***(Please mark only what is applicable to you)***

I am a member of the household	1	V28	82
I am head of the household	2	V29	84
I am a cattle owner	3	V30	86
I am employed as a herdsman	4	V31	88
I am employed as a herdswoman	5	V32	90
I work with cattle on a daily basis	6	V33	92

14. What is the **size** of your cattle **herd** or cattle you **look after**?
(If you do not have a herd or look after cattle, leave blank)

V34 94

15. Please supply the **number on each stock card** you are currently in charge of. ***(If you do not have stock card numbers with you, please supply your Mobile number on the cover sheet. If you do not have stock cards, please leave blank)***

Number on Stock card 1:	V35	101
Number on Stock card 2:	V36	110
Number on Stock card 3:	V37	119
Number on Stock card 4:	V38	128
Number on Stock card 5:	V39	137
Number on Stock card 6:	V40	146

16. Where are your cattle or the cattle you look after **dipped**?

V41 155

17. How do you **mainly consume** the **milk** from any cows you may have?
(Please mark a single answer only to indicate the most frequently consumed product).

I do not have any milk cows	1	V42	158
I consume it raw	2		
I consume it after boiling it	3		
I consume it after it has soured	4		

Question 18 follows on the next page ...

For office use

18. Do you **sleep** in an **enclosed space** with your **cattle** or the cattle you **look after**?

Yes	1	V43	160
No	2		

19. Have any of your **cattle** shown signs of **coughing** for **more** than **2 weeks**, over the past 3 months?

Yes	1	V44	162
No	2		

20. Have your cattle been **tested** for **bovine TB** in the past 3 months?

Yes	1	V45	164
No	2		

21. What was the result of the **bovine TB test**?

No test was conducted for bovine TB	1	V46	166
Positive	2		
Negative	3		

22. Have your cattle been **tested** for **Brucellosis** in the past 3 months?

Yes	1	V47	168
No	2		

23. What was the **result** of the **Brucellosis test**?

No test was conducted for Brucellosis	1	V48	170
Positive	2		
Negative	3		

24. Please indicate the **source** of the milk you consume.
(If applicable, you may indicate more than a single answer)

From Own cows	1	V49	172
From Supermarkets	2	V50	174
From Kiosks	3	V51	176
From Taxi ranks	4	V52	178
From Bus stations	5	V53	180
From Pension points	6	V54	182
From another local farmer	7	V55	184
From Road stalls	8	V56	186
Other (specify):		V57	188

For office use

25. Please indicate the **type** and **frequency** of the milk consumed by you **(If applicable, you may indicate more than a single answer)**

Milk type consumed	Never	Once a week	More than once a week		
Fresh milk (commercial)	1	2	3	V58	191
Fresh milk (untreated)	1	2	3	V59	193
Boiled milk	1	2	3	V60	195
Soured milk (commercial / mafi)	1	2	3	V61	197
Soured milk (home-made)	1	2	3	V62	199

26. How frequently is **milk consumed per week** in your household?
(Please supply only a single answer)

Not at all	1	V63	201
Daily	2		
Every second day	3		
Less than 3x a week	4		
Only when we need to	5		
Only when we need to for a sick household member	6		
Only when needed for a large family function	7		

27. What do you **mainly use to clean** the utensils used with milk?
(Please supply only a single answer)

Cold water without detergent	1	V64	203
Hot water without detergent	2		
Cold water with detergent	3		
Hot water with detergent	4		
Other (specify):			

28. After slaughtering cattle at your home, what is your preferred **method** of **meat consumption** for the components mentioned below? ***(If you do not consume a component mentioned, please leave the method blank)***

Component	Method of consumption				
	Raw	Undercooked	Well-cooked		
Blood	1	2	3	V65	205
Meat	1	2	3	V68	211
Organs (kidneys)	1	2	3	V71	217
Head	1	2	3	V74	223

Question 29 follows on the next page ....

For office use

29. If any meat product has any **spots** or **abnormalities**, what do you do?

I ignore the matter	1	V77 [ ][ ] 229
I boil the meat product	2	
I fry the meat product	3	
I overcook the meat product	4	
Add lots of salt	5	
I give it to dogs	6	
Other (specify):		

30. Are you aware that **bovine TB** can **affect humans**?

Yes	1	V78 [ ] 232
No	2	

31. Are you aware that **human TB** can **affect animals**?

Yes	1	V79 [ ] 234
No	2	

Thank you for your time and co-operation

Appendix 2:

Characteristics of *M. tuberculosis* isolates from the Mnisi community between June 2013 and Feb 2015

Participant no.	Recruitment site	Location	Status of TB	Gender
34	Clinic	Welverdiend	New diagnosed patient (16 days)	Male
42	Clinic	Welverdiend	Diagnosed patient (37 days)	Female
49	Clinic	Welverdiend	New diagnosed patient (15 days)	Female
337	Dip-Tank	Eglington	Unknown	Male
366	Clinic	Gotternburg	New diagnosed patient (6 days)	Male
367	Clinic	Gotternburg	New diagnosed patient (8 days)	Female
481	Clinic	Hluvukani	New diagnosed patient (3 days)	Male
485	Clinic	Hluvukani	New diagnosed patient (3 days)	Male
486	Clinic	Hluvukani	New diagnosed patient (1 day)	Male
487	Clinic	Hluvukani	New diagnosed patient (13 days)	Female
488	Clinic	Hluvukani	New diagnosed patient (15 days)	Male
490	Clinic	Hluvukani	New diagnosed patient (8 days)	Female
809	Dip-Tank	Utah	History of TB	Male

Appendix 3: Spoligotyping patterns of defined clusters using un-weighted pair group method and the corresponding mycobacterial interspersed repetitive units (MIRUs)-variable –number tandem repeats (VNTRs) data

Sample no	Spoligotyping pattern	ST no	Family	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348	
BCG	Nnonnnnnonnnnnno nnnnnnnnnnnnnnnnn nnnnnooooo	482	BOVIS 1_BCG	2	0	6	2s	2	2	3	7	2	3	5	2	2	2	4	2	5	3	3	3	2	3	0	2	2062s22372 3522242533 32302
H37Rv	Nnnnnnnnnnnnnnn nnnoonnnnnnnnnnno ooonnnnnnn	451	H37RV	2	2	4	3s	1	2	2	2	2	8	3	4	2	3	6	1	3	3	3	3	5	2	2	2	2243s12222 8342361333 35222
42	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnoooon onnnnnnnnn	236	EAI 5	2	2	3	2	3	5	3	3	2	5	3	4	4	2	5	1	5	3	3	3	3	7	3	2	2232353325 3442515333 3732
337	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnnnno ooonnnnnnn	53	T1	2	2	3	2	6	4	3	7	2	4	3	4	4	2	5	1	4	3	3	3	2	1	3	2	2232643724 3442514333 2132
366	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnoooon onnnnnnnnn	236	EAI 5	2	2	4	2	4	3	1	2	2	2	2	4	2	3	2	1	5	3	3	3	6	2	2	2	2242431222 2423215333 6222
481	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnnnno ooonnnnnnn	53	T1	2	3	4	2	1	4	3	2	1	4	3	4	4	3	5	1	6	3	2	2	2	8	3	2	2342143214 3443516322 2832
486	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnnnnn onnnnnnnnn	100	MANU 1	2	5	4	2	1	4	1	2	2	4	2	4	1	1	6	1	5	2	5	3	2	2	2	2	2542141224 2411615253 2222
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809	Nnnnnnnnnnnnnnn nnnnnnnnnnnnnnnno ooonnnnnnn	53	T1	2	5	4	2	1	4	1	2	2	4	2	4	1	1	6	1	5	3	3	3	2	8	1	2	2542141224 2411615333 2812

UNIVERSITEIT VAN PRETORIA
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ANIMAL USE AND CARE COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: V043-12

25 September 2012

Prof A Michel
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(anita.michel@up.ac.za)

Dear Prof Michel

V043-12 : Investigating the role of cattle in the epidemiology of tuberculosis and bovine brucellosis in humans in the Mnisi community of Mpumalanga, South Africa *(J Musoke)*

The application for ethical approval, dated 25 June 2012 was approved by the Animal Use and Committee at its meeting held on 17 September 2012.

Kind regards

Elmarie Mostert

AUCC Coordinator

Copy *Mr J Musoke*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Investigating the role of cattle in the epidemiology of tuberculosis and bovine brucellosis in humans in the Mnisi community of Mpumalanga, South Africa**
PROJECT NUMBER	**V043-12**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. J Musoke**

STUDENT NUMBER (where applicable)	**UP_27410872**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Cattle (Bovine)**	
NUMBER OF ANIMALS	**1130**	
Approval period to use animals for research/testing purposes		**June 2012-June 2013**
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 May 2012
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

28/03/2013

Approval Notice
New Application

Ethics Reference No.: 100/2013

Title: Investigating the role of cattle in the epidemiology of tuberculosis and bovine brucellosis in humans in the Mnisi community of Mpumalanga, South Africa

Dear Jolly Musoke

The **New Application** for your research received on the 11 March 2013 was approved by the Faculty of Health Sciences Research Ethics Committee on the 27/03/2013

Please note the following about your ethics approval:

- Ethics Approval is valid for 3 years.
- Please remember to use your protocol number (100/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
Standard Conditions:

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

We wish you the best with your research.

Yours sincerely

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee
University of Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

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✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

MPUMALANGA PROVINCIAL GOVERNMENT

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Department of Health

Litiko Letemphilo | Umnyango WezaMaphilo | Departement van Gesondheid

Enquiries: Molefe Machaba (013) 766 3009/3511/3534

15 October 2012

Ms Jolly Musoke
Department of Veterinary Tropical Diseases
Para-clinical Building
Onderspoort
PRETORIA
0110

Dear Ms Jolly Musoke

INVESTIGATING THE ROLE OF CATTLE IN THE EPIDEMIOLOGY OF TUBERCULOSIS AND BOVINE BRUCELLOSIS IN HUMANS IN THE MNISI COMMUNITY OF MPUMALANGA, SOUTH AFRICA

The Provincial Research and Ethics Committee has approved your research proposal in the latest format that you sent. No issues of ethical consideration were identified.

Kindly ensure that you provide us with the report once your research has been completed.

Kind regards,

Dr WRM Mapharga
Chairperson: PHREC

15/10/2012
Date

Machaba
Molefe Machaba
Research and Epidemiology

15/10/2012
Date

Siyanakekela

MPUMALANGA