Molecular characterization of *Mycobacterium bovis* from livestock and wildlife in South Africa: Genetic marker optimization and identification using whole genome sequence data

By

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List of Abbreviations and Symbols

AIDS	Acquired immunodeficiency syndrome
AFB	Acid-fast bacilli
ARC	Agricultural Research Council
BCG	Bacillus Calmette Guérin
bp	base pairs
BTB	Bovine tuberculosis
BWA	Burrows Wheeler transformation algorithm
CA	California
C1	Cattle strain 1
C2	Cattle strain 2
C8	Cattle strain 8
C8v	Variant of cattle strain 8
CFP	Culture filtrate protein
CITT	Comparative intradermal tuberculin test
Cm	Centimetre
CMI	Cell mediated immunity
DEFRA	Department of Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DR	Direct repeat
EC	Eastern Cape Province
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immuno sorbent assay
ESAT-6	Early secretary antigenic targets-6
Etc.	Et cetera
ETR	Exact tandem repeat
ESX-1	Early secretary antigenic target system 1
FS	Free State Province
GKNPC	Greater Kruger National Park Complex
GP	Gauteng Province
ha	Hectare
HCL	Hydrochloric acid
HiP	Hluhluwe-iMfolozi Park
HIV	Human immunodeficiency virus
IDT	Intradermal tuberculin
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IMP	Immunoperoxidase
IN	Indianapolis
IS	Insertion sequence
KNP	Kruger National Park
KZN	KwaZulu-Natal
GLTP	Greater Limpopo Transfrontier Park
GP	Gauteng Province
NaOH	Sodium hydroxide
n/a	not available
L-J	Löwenstein-Jensen
LN	Lymph node
LNP	Limpopo National Park

LP	Limpopo Province
MAC	*Mycobacterium avium* complex
MIRU	Minisatellite interspersed repetitive unit
MLVA	Multiple locus variable analysis
MOTT	Mycobacteria other than tuberculosis
Moz	Mozambique
MP	Mpumalanga Province
MSG	Monosodium Glutamic acid
MST	Minimum spanning tree
MTBC	*Mycobacterium tuberculosis* complex
MPB70	Mycobacterial protein bovis 70
MPB83	Mycobacterial protein bovis 83
ml	millilitre(s)
mm	millimetre
mM	millimolar
nm	nanometre
NZ	New Zealand
NW	North West Province
OIE	Office International des Epizooties
OVI	Onderstepoort Veterinary Institute
pM	Pico mole
PCR	Polymerase Chain Reaction
PGRS	Polymorphic G-C rich sequences
PO SID	Per oral semel in die (once daily dosing)
PPD	Purified protein derivative
PPDA	Purified protein derivative from *M. avium*
PPDB	Purified protein derivative from *M. bovis*
Qub	Queen's University of Belfast
RD	Region of difference
REA	Restriction enzyme analysis
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
SA	South Africa
SITT	Single intradermal tuberculin test
SNP	Single nucleotide polymorphism
SNR	Spioenkop Nature Reserve
TB	Tuberculosis
TCH	Thiophen-2-carboxylic acid hydrazide
TE buffer	Tris-EDTA buffer
TH1	T helper 1
Th2	T helper 2
TR	Tandem repeat
UK	United Kingdom
VNTR	Variable Number of Tandem Repeats
UPGMA	Unweighted pair group method with arithmetic averages
USA	United States of America
UV	Ultra Violet
V	Voltage
WC	Western Cape Province
WHO	World Health Organization
ZN	Ziehl Neelsen
µl	microliter(s)
°C	Degree Celsius

Summary

Molecular characterization of *Mycobacterium bovis* isolated from livestock and wildlife in Southern Africa: Genetic marker optimization and identification using whole genome sequence data

by

Motlatso Tiny Hlokwe

Promoter: **Professor A L Michel**

Co-Promoters: **Professor P D van Helden and Dr C Sabeta**

Degree: **PhD in Veterinary Science (Veterinary Tropical Diseases)**

Bovine tuberculosis (BTB) is a disease caused by *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* complex, which are bacteria that infect both domestic and wildlife host species. The zoonotic nature of the disease is of great concern globally. Over 50 million animals are infected all over the world, resulting in economic losses of approximately three billion United States dollars per year. Molecular typing techniques have greatly improved knowledge regarding the sources and modes of BTB transmission in many countries including South Africa. The current study was aimed at filling the knowledge gap regarding the epidemiology of the disease in livestock and wildlife species in the country for the purpose of effective control and management (in wildlife) as well as ultimate eradication (in livestock). Identification of a suitable typing technique for potential application in South Africa was the core purpose of the study. In an attempt to determine the manner of transmission within and between animal populations and map the geographical spread of this zoonotic disease, samples were collected from a variety of animal species throughout the country. In Chapter 2, molecular techniques (i.e. Restriction Fragment Length Polymorphism, spoligotyping and variable number tandem repeat) were used to identify and characterize *Mycobacterium bovis* infecting buffaloes in the Hluhluwe-iMfolozi Park (HiP) and three epidemiologically related game reserves over a 15-year time frame. The findings demonstrated that the majority of the *M. bovis* infections in buffaloes were as a result of a single strain which has persisted throughout the years with spill-over to other wildlife species. Evidence of further *M. bovis* infections due to strains previously undetected in the park was also shown. With a view to providing a simple and reliable method suitable for studying the epidemiology of BTB in the country, we describe, in Chapter 3,

how we evaluated the utility of known VNTR sequences as epidemiological markers to describe the molecular epidemiology of BTB in South Africa, with special emphasis on the Kruger National Park (KNP) where there is evidence of clonal expansion of the C8 parental cattle strain. A set of VNTR loci that provided sufficient discriminatory power for application in epidemiological studies was identified. Potential use of VNTR typing in exploring evolutionary changes was also highlighted. To further establish the genetic diversity of the isolates and study the population structure of *M. bovis,* the loci were used to study a larger panel of *M. bovis* isolates from different regions (Chapter 4). The findings showed that besides cattle, at least 16 animal species contracted the infection, and highlighted a strong evidence of intra and inter-species transmission of *M. bovis*. Furthermore, the study also showed that despite the fact that a national control programme for BTB is applied in commercial cattle, there is a high diversity of *M. bovis* persisting in the country, highlighting the importance and need for intensified diagnostic testing and consideration of alternative control measures such as vaccination. Together with results obtained in Chapter 5, the study further indicated the presence of *M. bovis* infection in previously uninfected (or unknown status) game parks or reserves. Overall, three novel *M. bovis* strains (i.e. SB1474, SB2199 and SB2200) were identified and their patterns are now available in the international *M. bovis* database. We also describe for the first time in South Africa, *M. bovis* infection in a blue wildebeest (*Comochaetes taurinus*), nyala (*Tragelaphus angasii*) and giraffe (*Giraffa camelopardalis*); and a rare case of *M. bovis* infection causing pulmonary tuberculosis in a horse (*Equus ferus caballus*) is described in Chapter 6. To improve VNTR typing method for clonally linked isolates from the KNP, we sequenced whole genomes of local *M. bovis* isolates in Chapter 7. The tandem repeat loci identified were exploited for polymorphisms in *M. tuberculosis* complex isolates including *M. bovis*, with two of the five polymorphic loci identified showing potential discriminatory capabilities for clonally linked isolates. The epidemiological information gained in this investigation is particularly important in the context of the establishment of the Greater Limpopo Transfrontier Park (GLTFP) where the potential spread of BTB from Kruger National Park to Limpopo National Park (Mozambique) and Gonarezhou National Park (Zimbabwe) (or *vice versa*) is of great concern. The availability of the local *M. bovis* genomes provides an opportunity for future comprehensive sequence comparisons which may assist in further deducing the phylogenetic and evolutionary relationships of *M. bovis* isolates and efforts towards vaccine developments.

Chapter 1
General introduction

1.1 Literature Review

1.1.1 Bovine tuberculosis

Bovine tuberculosis (BTB) is a chronic debilitating disease in cattle and mammalian species including humans (Cosivi *et al*. 1998; Michel *et al.* 2006; Smith *et al.* 2006). The disease can, however, occasionally be acute and rapidly progressive. Early infections are often asymptomatic. Animals with pulmonary tuberculosis such as cattle usually have a cough that is worse in the morning as well as during cold weather, or may have dyspnoea or tachypnea. In the terminal stages, animals may become extremely emaciated and develop acute respiratory distress. In some animals such as greater kudu (*Tragelaphus strepsiceros*), the retropharyngeal or other lymph nodes become enlarged and may rupture and drain (Keet *et al.* 2001). Greatly enlarged lymph nodes can also obstruct blood vessels, airways or the digestive tract. In the case that the digestive tract is involved, intermittent diarrhoea and constipation may be seen (Bojkovski *et al.* 2011). Bovine tuberculosis may also be sub acute in some animal species such as cervids, and the rate of progression is variable. In some animals, the only symptom may be abscesses in isolated lymph nodes, and clinical signs may only develop many years later after the initial infection remains dormant only to be reactivated during periods of stress and old age. In other cases, the disease may be disseminated, with a rapid progression (Cassidy, 2006; Pollock *et al*. 2006).

1.1.2 Aetiological agent of tuberculosis

The genus *Mycobacterium* of the family *Mycobacteriaceae* includes non-motile and non-sporulating acid-fast rods of various lengths, ranging from 0.3 µm to 0.6 µm in diameter and 1.5 µm to 3 µm in length (Koch, 1882; Quinn, 1994). Mycobacteria possess a waxy coat that makes it difficult for the host‘s defence mechanisms to destroy them, and this results in a slow chronic disease (Taylor *et al*. 2007b). Most Mycobacteria of clinical significance are slow growing organisms that usually appear on culture media in 3-6 weeks (Thoen *et al.* 1981). *Mycobacterium bovis* is the major causative agent of BTB

and it is usually isolated from domestic cattle, but also infects a range of mammalian species, including humans. *M. bovis* is one of the species of the *Mycobacterium tuberculosis* complex (MTBC). The mycobacteria grouped in the MTBC are nearly (99 %) identical at the nucleotide level and have identical 16S rRNA (Brosch *et al.* 2002; Smith *et al.* 2006). Traditionally, the MTBC comprises tubercle bacilli of distinct subgroups: *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis*, *M. bovis BCG* (the vaccine strain), *M. caprae*, *M. pinnipedii*, *M. microti*, *M. mungi*, *M. suricattae* as well as *Dassie* and Oryx bacilli which are rare MBTC variants (Brosch *et al.* 2002; Huard *et al*. 2006; Alexander *et al.* 2010; Parsons *et al.* 2013). Recently, *M. orygis* was proposed as a name for isolates previously labelled as Oryx bacillus (Van Ingen *et al.* 2012), however, Dassie bacillus' standing within the MTBC still needs to be defined (Mostowy *et al.* 2004).

Historically, taxonomic segregation of the MTBC has been based upon each species' unique combination of growth, morphology, physiological and biochemical characteristics (Niemann *et al*. 2000). Variation in geographical distribution, host preference, virulence, and relative human virulence also differentiated the tubercle bacilli (Huard *et al.* 2003). Given that members of the MTBC are genetically closely related and pathologically similar, despite widely different host-adaptation, it has been suggested that different host-adapted forms be referred to as "ecotypes" rather than species (Smith *et al.* 2006). Although it has been conventionally established that *M. tuberculosis* and *M. africanum* (divided into subtype I and subtype II) are isolated from humans, *M. microti* from voles, and *M. bovis* predominantly from cattle, reports of MTBC organisms in a variety of other domesticated and wildlife species hosts pose a challenge in this classification scheme. The host spectrum of *M. bovis* is considered to be the broadest of the complex, causing disease across a variety of animals, including cattle, seals, goats, wildlife species and humans (Mostowy *et al.* 2005). *M. caprae* and *M. pinnipedii* are associated with tuberculosis in goats and seals, respectively (Aranaz *et al.* 2003; Cousins *et al.* 2003). *M. canettii* is a rare, smooth variant of *M. tuberculosis* and is usually isolated from patients from or with connection to Africa (Brosch *et al.* 2002).

1.1.3 Evolution of *M. bovis*

Mycobacterium tuberculosis complex organisms have an unusually high degree of conservation in their housekeeping genes and previous reports had suggested that members of the MTBC underwent an evolutionary bottleneck at the time of speciation, estimated to have occurred roughly 15 000 to 20 000 years ago (Sreevatsan *et al*. 1997). However, recent studies have suggested that speciation of MTBC might have occurred at the time of initial human migrations about 40 000 to 45 000 years ago (Hershberg *et al.* 2008). Before the advent of whole genome sequence availability of *M. tuberculosis*, human tuberculosis was thought to have evolved from bovine tuberculosis by adaptation of an animal pathogen to the human host. This hypothesis was based on the fact that *M. tuberculosis* is almost exclusively a human pathogen, whereas *M. bovis* has a broad host spectrum (Stead *et al*. 1995). *M. tuberculosis* complex organisms have several variable genomic regions known as regions of difference (RD). By making use of the nearly complete *M. bovis* AF2122/97 genome sequence and the *M. tuberculosis* H37Rv sequence, researchers have identified regions of difference (RD1-14) which ranged in size from 2 kb to 12.7 kb, in the *M. tuberculosis* H37Rv; however, these regions are absent in *M. bovis* bacillus Calmette-Guérin Pasteur (Behr *et al.* 1999; Gordon *et al*. 1999). In addition, regions which are H37Rv related deletions (RvD1-5) and *M. tuberculosis* specific deletion 1 (TbD1), which were absent from the *M. tuberculosis* H37Rv genome relative to other members of the *M. tuberculosis* complex, were identified (Brosch *et al*. 1999; Gordon *et al.* 1999).

Brosch and co-workers (Brosch *et al*. 2002) have analysed the distribution of these variable regions in the genomes of a representative and diverse panel of strains belonging to the MTBC. From this work, it has been shown that *M. bovis* has undergone numerous deletions events in the RD4-10 and RD12-13 regions relative to *M. tuberculosis*. *M. canettii* and ancestral *M. tuberculosis* strains have neither of these regions deleted, suggesting that the common ancestor of the tubercle bacilli resembled *M. tuberculosis* or *M. canettii* and that it could well be a human pathogen. It was also proposed that an *M. africanum*-like clone diverged from *M. tuberculosis* with the loss of the RD9 locus and that the successive loss of DNA in this lineage resulted in the derivation and clonal expansion of *M. microti*, the dassie bacillus, the Oryx bacillus, *M. pinnipedii*, *M. caprae* and finally *M. bovis* as they adapted in new hosts (Brosch *et al.* 2002; Mostowy *et al*. 2002).

Huard and co-workers highlighted the importance of inspecting the genomes of the MTBC organisms since they may contribute new and unexpected insights in understanding the biology and evolutionary history of MTBC (Huard *et al*. 2006). Indeed, very recently, and correctly so, a newly identified member of the *M. tuberculosis* complex named *M. mungi*, has found a standing in the phylogeny of *M. tuberculosis* complex in a position between *M. africanum* type 1(a) and dassie bacillus (Alexander *et al*. 2002). In addition, *M. orygis* was also characterized as MTBC subspecies and *M. suricattae* which causes tuberculosis in meerkats (Parsons *et al.* 2013). Figure 1 illustrates the evolutionary scenario of MTBC organisms.

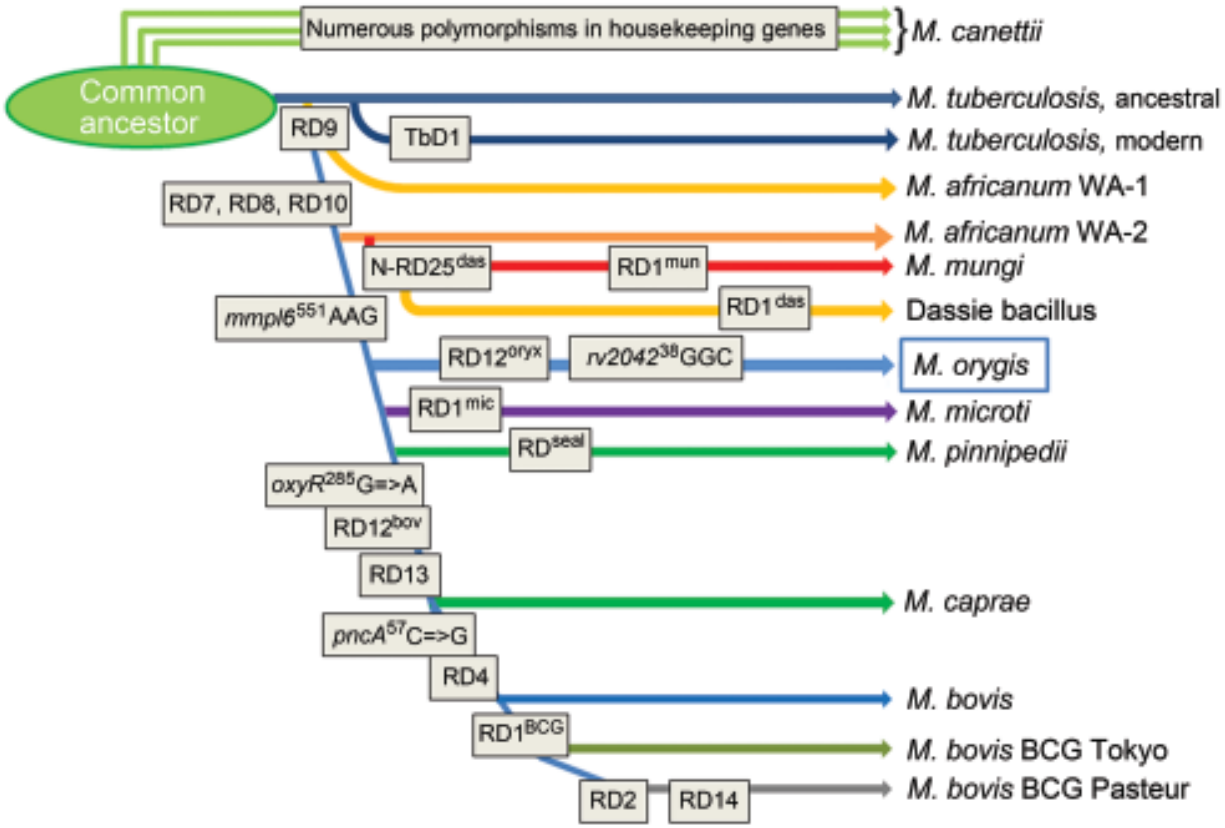

Figure 1 Evolutionary scenario of MTBC organisms. Updated phylogeny based on deleted regions demonstrated through genomic analysis (adapted from Brosch *et al*. 2002; Van Ingen *et al.* 2012).

1.1.4 Immune response to *M. bovis* infection

A better understanding of the dynamics of the event following *M. bovis* exposure and subsequent infection in animals would be of significant benefit in developing new tools appropriate for controlling BTB (Pollock and Neill, 2002). After infection of animals with *M. bovis*, there is an initial interaction between macrophages and mycobacteria, which define subsequent events and the consequences of exposure to tubercle bacilli (Pollock and Neill, 2002). Bacteria can either be killed or eliminated from the host, lie dormant, lead to development of active tuberculosis, or reactivate from dormancy at some stage in the future (Welsh *et al*. 2005).

Mycobacterium bovis infection causes a delayed hypersensitivity type (DTH) reaction which is regarded as an indicator of a cellular immune response that is a result of infection due to *M. bovis* (Thom *et al*. 2004). T-cell recognition of the Mycobacterial antigens may be the major response to tuberculosis (Welsh *et al*. 2005; Neonakis *et al*. 2008). Upon injection of the antigen, the Langerhans's cells process the antigen and present it to local memory cells, whether they are CD4+ or CD8+. These T-cells together with activated Langerhans's cells secrete numerous cytokines that cause early hallmarks of inflammation. Within two hours neutrophils begin to infiltrate the injection site. During the early stages of inflammation, leukocytes migrate exclusively through the post capillary venules and do not follow an obvious gradient of cytokine. Although cytokines such as members of the chemokine family that are secreted by T-cells and macrophages are chemo-attractant for numerous immune cells, it is unlikely that these cytokines are directly regulating the influx of cells from the vasculature. Instead, it is probable that the venular endothelium is recruiting the cells to the local site (Black, 1999). Two types of T-helper cells have been described, i.e. the Th1 and Th2. The Th1 cell secretes interferon gamma, which activates macrophages and induces a cell mediated immune response. Th2 cells secrete cytokines such as IL-4, IL-5 and IL-6, which activate B-cells and induce humoral immunity (Mosmann and More, 1991). Initially, animals develop strong anti-mycobacterial cell-mediated immune (CMI) responses. As the disease progresses, the cellular immune response decreases, whereas an increased humoral response based on IgG1 antibodies can be clearly demonstrated. The humoral response does not control the infection, progression of pathology or increased bacterial load (Medeiros *et al*. 2010). The progression of the disease may explain the anergy of some infected cattle to common CMI based tests. The absence of CMI response in infected animals occurs particularly when the bacterial

load is high (McNair *et al*. 2001). A typical immune response profile in the course of *M. bovis* infection is illustrated in Figure 2.

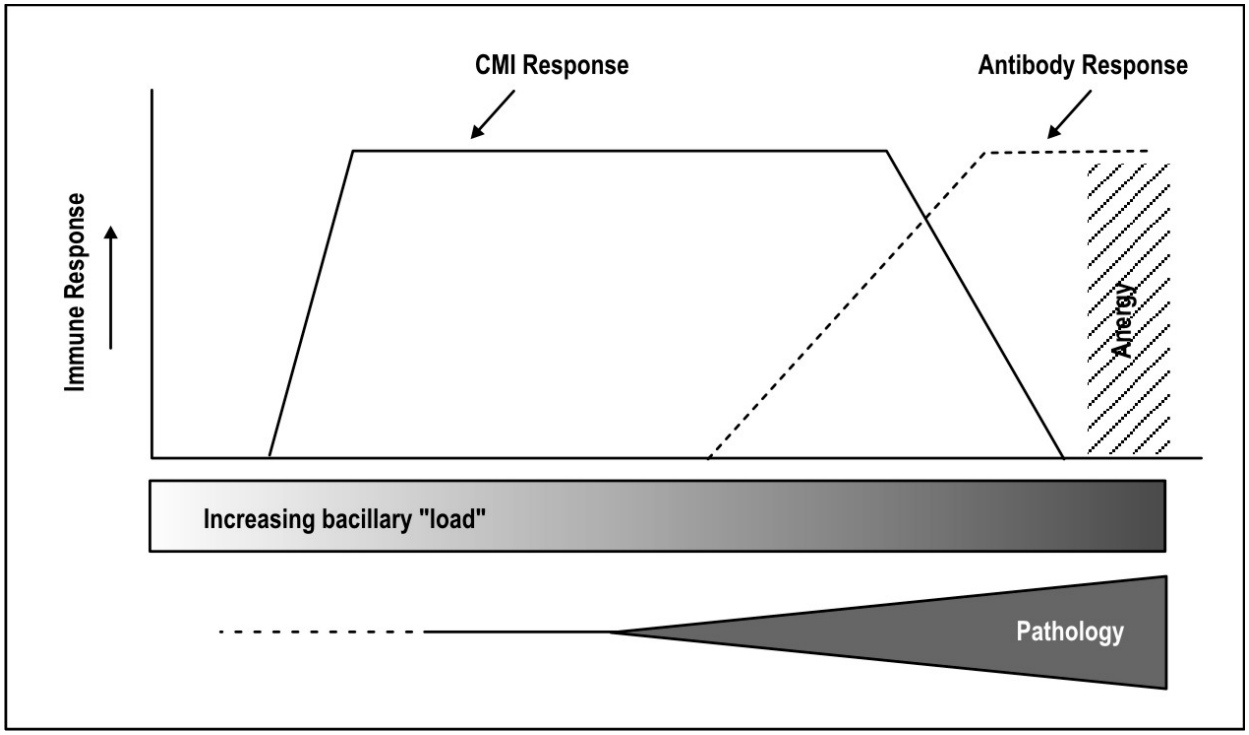

Figure 2 Illustrates a typical immune response profile in the course of *M. bovis.* During the early stages of the disease, the cell mediated immune (CMI) response predominates. As the disease progresses, CMI responses diminishes and the humoral immune response takes over. Finally, in the clinical stages of the disease, reached only by a minority of infected animals, *M. bovis* specific immunity disappears completely, while bacterial shedding occurs (adapted from Pollock and Neill, 2002).

1.1.5 Diagnosis of bovine tuberculosis

Accurate diagnosis of tuberculosis due to *M. bovis* is notoriously difficult in live animals, yet it is equally important to understand the epidemiology of BTB and devise effective strategies to limit its spread (Drewe *et al*. 2010). Intermittent excretion of *M. bovis* appears to occur in many species (Bengis *et al*. 2001; Gavier-Widen *et al.* 2001). None of the currently available diagnostic test can be considered 100 % sensitive and 100 % specific in all animal species including cattle, that is, to be perfect gold standards able to determine accurately the infection status for each animal tested (Drewe *et al*. 2010; Hartnack and Torgerson, 2012). There is also an increasing recognition that individual diagnostic tests do not detect all infected cattle. In addition, routine meat inspection

may fail to detect this notifiable disease in many animal species (Van de Burgt *et al.* 2013). Therefore, a comparative strategy for the diagnosis of bovine tuberculosis should include test results for the detection of both cellular and humoral immune responses where there may be animals infected at different stages of infection (Marassi *et al*. 2013). Different diagnostic tests largely applied for bovine tuberculosis are discussed below.

1.1.5.1 Intradermal tuberculin test

The primary diagnostic test used for the diagnosis of bovine tuberculosis is the intradermal tuberculin test (tuberculin skin test), which has remained in the forefront of tuberculosis (TB) diagnosis in both cattle and humans for over a century (Whelan *et al*. 2010). The intradermal tuberculin (IDT) test involves the intradermal injection of tuberculins, which are purified protein derivatives (PPDs) and the subsequent detection of swelling and induration at the site of injection 72 hours later. The test was developed by Robert Koch in 1890. The tuberculin used by Koch had, however, failed to live up to its initial claims of having curative properties, but its diagnostic potential was realised and was first demonstrated in cattle before its widespread use in humans (Snider, 1982). The test is based on a measurable cellular immune response against *M. bovis* that is elicited during the first stages of the disease. It is often done as a single intradermal tuberculin test (SITT), based on the intradermal inoculation of *M. bovis* purified protein derivative (PPDB). Different mycobacteria are known to share some proteins amongst themselves, or even with other bacteria. For this reason, not only individuals infected with MTBC mycobacteria may respond immunologically to tuberculin prepared from *M. bovis*. As a result, a comparative tuberculin test known as comparative intradermal tuberculin test (CITT) in which skin responses to tuberculin prepared from *M. bovis* (PPDB) are interpreted with reference to responses to tuberculin prepared from *Mycobacterium avium* (PPDA) have been developed (Monaghan *et al*. 1994). The sensitivity of the CITT in different countries and settings has neen reported to range from 75-95.5 % (De la Rua Domenech *et al.* 2006) and from 77-95 % (Monaghan *et al.* 1994). This practice has been widely used for the diagnosis of BTB in regions where environmental mycobacteria are known to have a major influence on the animal's response (Monaghan *et al*. 1994). The CITT has a higher specificity by recognizing cross reaction against environmental mycobacteria (Monaghan *et al*. 1994; Jolley, 2007). This use of comparative tuberculin test has

resulted in improvements in specificity, but many attempts have been made to identify reagents for use in better defined tests (Pollock *et al.* 2000). For example, the use of ESAT-6 (early secretary antigenic targets-6) resulted in an increased specificity in the skin test when compared to PPD based tests. However, it also led to a decrease in sensitivity. In addition, the best results could only be achieved using high concentrations of the recombinant protein making the test very expensive (Pollock *et al.* 2003).

The specificity of tuberculin skin test is also compromised by vaccination with the *Mycobacterium bovis*-derived vaccine strain bacilli Calmette-Guérin (BCG). In an attempt to overcome this constraint, Whelan and co-workers developed a skin test for bovine tuberculosis capable of differentiating infected from vaccinated animals. In this study, a cocktail of the MTBC recombinant protein antigens ESAT-6, CFP-10 (culture filtrate protein-10), MPB70 and MPB83 elicited delayed type hypersensitivity skin test response in 78 % of naturally infected tuberculin positive cattle (Whelan *et al*. 2010). Other limitations linked to tuberculin skin tests include the logistical drawbacks with regard to repeated handling of animals to read test results after 72 hours; and the minimum testing intervals (Green *et al*. 2009) as well as the source of the tuberculin PPD (Cagiola *et al*. 2004). In a recent study, significant variations were found with regard to the relative potencies of PPD-B and PPD-A tuberculins from different manufacturers (Bakker *et al.* 2009). Improvements in the production and quality control of tuberculins have the ability to enhance sensitivity and specificity of the intradermal tuberculin test (Schiller *et al.* 2010).

1.1.5.2 Culture

Bacteriological isolation and identification of *M. bovis* is considered the gold standard for bovine tuberculosis diagnosis. Clinical samples such as tissues, sputum, bronchial and tracheal washes etc. collected from suspect cases are cultured on a specific media (Medeiros *et al.* 2010). Milk samples can also be used for culture; however, microbial spread to the mammary glands does not necessarily occur rendering this sample type less reliable (Pollock *et al.* 2005). In addition, negative test results do not exclude the possibility of infection, especially in areas with a low disease incidence and low bacteriological load, a complicating factor for accurate diagnosis (Jolley *et al.* 2007). The egg-based Löwenstein Jensen (L-J) media are most commonly used in veterinary

mycobacteriology. Agar-based media such as Middlebrook 7H10 and 7H11 may also be used (OIE, 2009). Glycerol supplement is inhibitory to *M. bovis*, while pyruvate (0.4 %) enhances growth. *M. bovis* grow well in airtight containers (Quinn *et al*. 2003). Culture, although highly specific, is mostly performed *post-mortem* and culture slopes/plates may require an incubation period of four (Pollock *et al*. 2005) to ten weeks (Alexander *et al*. 2002). In general, the outcome of mycobacterial culture is time consuming, expensive and delays disease confirmation (Stewart *et al*. 2013). However, it is generally known that mycobacteria including *M. bovis* grow well in liquid media. Several studies have evaluated both the liquid culture systems and L-J solid media for the recovery of mycobacteria. For example, Garcia-Elorriaga and co-workers isolated and identified mycobacteria in a liquid culture system-MB/BacT in 8-46 days, and 21-63 days in Löwenstein-Jensen media (García-Elorriaga *et al*. 2006). In another study, the time to detection for mycobacteria was 18.2 and 27.9 days for the Versa TREK liquid culture system and L-J medium respectively. In this study, a contamination rate of 4.2 % in liquid media and 7.4 % on solid media was found (Falconi *et al.* 2008). Although a high recovery rate and shorter time to detection were found, some studies have however reported higher contamination rates of culture in liquid system compared to L-J medium, a concern that should be carefully evaluated to reduce the costs of the test (Chihota *et al.* 2010).

1.1.5.3 Colony morphology

Mycobacterium bovis shows a dysgonic colony shape on L-J medium (Patterson and Grooms, 2000), and a microaerophilic growth on Lebek medium (Niemann *et al.* 2000). It is a slow growing and aerobic *Mycobacterium*, which can take up to ten weeks to grow at 37 °C (Alexander *et al.* 2002). Colonies are small, moist, rounded with irregular edges, smooth and off-white in colour (Pfeiffer, 2007; OIE, 2009). Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*; however, every isolate needs to be confirmed using further tests (Medeiros *et al*. 2010).

1.1.5.4 Biochemical and susceptibility testing

Species level identification of *M. bovis* can be done by using the following biochemical tests: niacin production, nitrate reduction, catalase activity at 68 °C and room temperature, tween hydrolysis, aryl sulphate and thiophen-2 carboxylic acid hydrazide

(TCH) sensitivity. A negative activity for niacin, nitrate reduction, catalase at 68 °C, tween hydrolysis, aryl sulphate and TCH are considered as typical for *M. bovis* (Srivastava *et al*. 2008). *M. bovis* isolates are also resistant to pyrazinamide (Niemann *et al*. 2002), a first line antituberculosis drug. The biochemical tests, however, need sufficient bacterial growth, are time consuming and do not allow unambiguous species identification in every case (Richter *et al*. 2003). They were the only tools for mycobacterial species identification before the advent of DNA methods and are rarely used nowadays (Warren *et al*. 2006).

1.1.5.5 Microscopic and histopathological examination

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials as well as on cultured isolates. Important staining characteristic of mycobacteria is their resistance to acid decolourization. The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl Neelsen stain, but fluorescence acid-fast stain may also be used (Thoen, 1981). A rapid fluorescent staining method employing a rapid modified auramine O fluorescent stain was recently found to outperform a standard auramine O stain according to a number of measurable parameters (Hendry *et al.* 2009). Microscopic examination of smears is faster and cheaper than any other method; however, visualization of acid-fast bacilli (AFB) is not able to discriminate among members of the *Mycobacteriaceae* family, or between members of the genus *Mycobacterium* and other organism which are acid-fast, including certain species of Legionella, Norcadia, Rhodococcus, Cryptosporidium and Cyclospora (Eisenstadt and Hall, 1995). In addition, the method lacks sensitivity and can only reveal AFB in the concentration above 10^4 bacteria per millilitre (Ajobiewe and Tiri, 2013). The presumptive diagnosis of tuberculosis can be made if the tissue has characteristic histopathological lesions typical for the affected tissues (caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid-fast organism in histopathological sections may not be detected. Histopathological examinations are practical and inexpensive, and useful to make decisions on grossly suspect carcasses. Another advantage is the increased diagnostic sensitivity when performed in conjunction with culture (Liebana *et al*. 2008).

1.1.5.6 DNA based tests

1.1.5.6.1. Identification of *Mycobacterium* species

Several techniques have been developed to target genes which are present in all mycobacteria such as 16S ribosomal RNA gene (Avaniss-Aghajani *et al.* 1996), *hsp65* (Devallois *et al.* 1997), *dna*J (Takewaki *et al.* 1993), *rec*A (Blackwood *et al.* 2000), *dna*J *(*Takewaki *et al.* 1993), *rpo*B encoding for the B subunit of RNA polymerase (Kim *et al*. 1999), the gene of the 32-kDa protein (Soini *et al.* 1994) and the *gyr*B encoding the B subunit of DNA gyrase (Kasai *et al.* 2000). However, none of these mycobacteri species-specific gene sequence difference can presently differentiate all the mycobacterial species commonly isolated in the clinical laboratories (Dauendorffer *et al.* 2003). However, some of them (i.e. 16S ribosomal DNA sequencing) work well to differentiate mycobacteria other than tuberculosis (MOTTs) from each other and from the MTBC species (Avaniss-Aghajani *et al.* 1996). Members of the MTBC species can also be differentiated from the MOTTs by PCR amplification using primers that target a sequence encoding the MPB70 antigen (Alexander *et al*. 2002).

1.1.5.6.2. Speciation of the *M. tuberculosis* complex

Members of the *M. tuberculosis* complex exhibit remarkable sequence identity at nucleotide level (Sreevatsan *et al.* 1997). Differentiation of these species is necessary for epidemiological purposes and for the treatment of patients especially in areas where tuberculosis has reached epidemic proportions or wherever the transmission of *M. bovis* between animals or animal products and human is a concern. *M. bovis* is naturally resistant to pyrazinamide, a first-line anti-tuberculosis drug (Neimann *et al.* 2000). In addition, differentiation can be important to rapidly identify isolates of *M. bovis* BCG recovered from immunocompromised patients (Parsons *et al.* 2002). To specify certain MTBC groupings, a well-defined and subspecies restricted single nucleotide polymorphism (SNPs) in the *gyr*A*, kat*G, *pnc*A; *oxy*R, *hsp*65 and *gyr*B have been used. These involve the sequence analysis and/or digestion of PCR products followed by restriction fragment length polymorphism (PCR-RFLP) analysis (Haas *et al.* 1997; Frothingham *et al.* 1997; Goh *et al*. 2001). The loci are however, unable to differentiate all of the MTBC subspecies (Goh *et al*. 2001).

Comparative genome analysis studies employing several different genetic hybridization strategies revealed regions of difference (RDs) representing loss of genetic materials in *M. bovis* BCG compared to *M. tuberculosis* H37Rv. PCR analysis of the regions of difference sequences established that some of the regions are restricted to one MTBC strain or subspecies, while other appeared to be differentially distributed among the MTBC grouping (Brosch *et al.* 2002). Several studies have demonstrated that PCR analysis of these regions accurately differentiated several MTBC bacteria when used to evaluate large collections of isolates (Parsons *et al*. 2002; Huard *et al*. 2003; Warren *et al*. 2006; Nakajima *et al.* 2010). Alternatively, some molecular typing techniques such as spoligotyping can identify *M. bovis* isolates and provide some molecular typing information on the isolate that is of epidemiological value (Kamerbreek *et al*. 1997). Detecting the presence of *M. bovis* DNA in tissues prepared for culture may provide a simple and rapid diagnostic test. However, it should be noted that negative test results do not exclude the possibility of infection, especially in an area with low disease incidence and low bacteriological load (Jolly *et al.* 2007). Mycobacteria other that *M. bovis* are routinely isolated from tissues submitted for diagnostic culture. These non *M. bovis* mycobacteria may interfere with PCR diagnostic tests performed on these tissues if the test does not have sufficient specificity. However, this problem can be overcome by application of PCR using specific primers for MTBC organisms including *M. bovis* for example, IS*6110* PCR (Thacker *et al*. 2011). IS*6110* specific PCR can also be applied to detect MTBC organisms in sections prepared from formalin fixed tissues (Miller *et al*. 1996; Sethusa, 2006; Govender, 2013). Although direct PCR can produce rapid results, it is recommended that culture be used in parallel for confirmation of disease diagnosis (OIE, 2009).

Techniques such as gene probes for identification of Mycobacteria are commercially available. Example is the DNA strip assay-GenoType MTBC system (HAIN Lifescience, Germany), intended for the differentiation of members of the MTBC. The assay is based on *gyr*B DNA sequence polymorphisms (Chimara *et al*. 2004) and the RD1 deletion of *M. bovis* BCG (Behr *et al*. 1999). Specific oligonucleotides targeting these polymorphisms are immobilised on membrane strips. Amplicons from the multiplex PCR react with these probes during hybridization. This technique is rapid, easy to perform and is suited for routing diagnostic laboratory use (Richter *et al*. 2003).

1.1.5.7 Gamma Interferon assay

The gamma interferon test (IFN-γ) is an assay commonly applied as an ancillary test for the *ante-mortem* diagnosis of BTB in cattle, goats and sheep. It is an *in vitro* assay based on the cellular immune response and measures IFN gamma released into whole blood culture (Wood and Jones, 2001; Michel *et al*. 2011), in response to specific antigen stimulation. For this test, blood samples are incubated with mycobacterial antigens, typically bovine and avian tuberculins for 20-24 hours at 37 °C. The T-lymphocytes present in the blood respond to the antigens and release a cascade of biological products, most notably gamma interferon. Gamma interferon is regarded as a pivotal cytokine released during the immune response to tuberculosis. The gamma interferon present in the plasma supernatant is quantified in a sandwich ELISA. A measurable gamma interferon can be detected as early as 3-5 weeks following infection even when the level of exposure is very low (Dean *et al*. 2005). This assay has been evaluated and incorporated into BTB control programmes and used as an ancillary diagnostic method to the tuberculin skin testing (Anon, 2008a) in many countries (Wood and Jones, 2001) including South Africa (Michel *et al*. 2011). Since lymphocytes stimulation with antigens is done *in vitro*, and does not alter the immune status of an animal, it is therefore not necessary to wait 60-90 days to repeat the test when the initial test is found inconclusive, a distinct limitation of the tuberculin skin test (Marassi *et al*. 2013). Cattle can be re-tested using gamma interferon test seven days following tuberculin skin testing (Palmer *et al.* 2006). In addition, the test offers an opportunity to employ a range of *M. bovis* specific antigens in addition to the standard avian and bovine tuberculins to increase specificity of the tests and also to differentiate between infected and vaccinated animals (Vordemeier *et al.* 2008). Animals are frequently exposed to or infected with various non-tuberculous mycobacteria. Cross-reactive responses to PPD-B may occur as many antigens contained within the PPD-B are shared between non-tuberculous and tuberculous mycobacteria. Indeed, different non-tuberculous mycobacteria are known to induce cross-reactive immune response in livestock and wildlife species to which they are exposed. These immune responses interfere with both tuberculin skin test and IFN-γ assay (Schiller *et al.* 2010; Michel *et al.* 2011). The most significant improvement in specificity of the test comes from the use of more defined antigen like the early secretory antigenic target (ESAT-6) and culture filtrate protein (CFP-10). Both antigens are regarded as largely specific to MTBC organisms including *M. bovis* (Veyrier *et al*. 2011). The application of diagnostic tests for bovine tuberculosis in wildlife poses technical difficulties and the use of gamma

interferon assay offer a simplified approach to testing wildlife species. In South Africa, Michel and co-workers conducted a study whereby the gamma interferon test was optimised for use in African buffaloes (*Syncerus caffer*). In this study, researchers also found that inclusion of an additional tuberculin, Fortuitum PPD (prepared from *M. fortuitum*) increased specificity for gamma interferon test without compromising its sensitivity (Michel *et al*. 2011).

A wider range of antigen in addition to ESAT-6 and CFP-10 is needed for improving the sensitivity of antigen based IFN-γ assays (Schiller *et al.* 2010). Schiller and co-workers had evaluated the Rv0899 (outer membrane protein A of *Mycobacterium tuberculosis*) as a stimulation antigen in IFN-γ assay. This antigen was found to induce IFN-γ responses in cattle experimentally infected with *M. bovis* as early and as persistently as ESAT-6 and CFP-10, which are the current leading diagnostic antigens. In addition, Rv0899 detected five of six of *M. bovis* infected cattle which did not respond to ESAT-6 and CFP-10 stimulation (Schiller *et al.* 2010).

1.1.5.8 Serological tests

Serological tests are relatively easy to perform and can be used to rapidly test a large number of samples (Serujballi *et al*. 2002; Medeiro *et al*. 2010). They are considered to be useful tools for the diagnosis of BTB; however, they are limited to those periods when antibodies are a feature of the immune response. There is a switch in the nature of the immune response against *M. bovis* as the infection progresses (see Figure 2). During the later stages of infection, there is a strong humoral response and assays based on cell immune response IDT test and IFN-γ assay can either be negative or inconclusive (Serujballi *et al.* 2002; Medeiros *et al.* 2010). Antibody responses to *M. bovis* positively correlate with *M. bovis* elicited pathology and *M. bovis* antigen burden and therefore data from these assays are supportive for immunopathogenesis and vaccine efficacy studies (Waters *et al.* 2010; Waters *et al.* 2011).

New antigens are being investigated with variable results in order to identify infected animals in their first stages of disease and to differentiate between infection and vaccination (Whelan *et al*. 2010). MPB70 and MPB83 have been used independently or in association with other antigens such as ESAT-6 and CFP-10, for diagnostic purposes (Aagard *et al*. 2006). MPB70 and MPB83 proteins are identified as early B-cell targets

in *M. bovis* infection and could detect antibodies in experimentally infected cattle about 7 (MPB70) to 18 (MPB83) weeks before a positive tuberculin response could be measured (McNair *et al.* 2001; Lin *et al.* 2007). A selection of highly specific antigens could help eliminate cross reactions between other species of mycobacteria such as *M. avium* subsp. *paratuberculosis* and *M. kansasii* (Olsen *et al*. 2001; Waters *et al.* 2006; Marassi *et al.* 2009). Although serological tests are easy to perform and relatively inexpensive, it should be borne in mind that in their various forms, ELISA tests have a range of sensitivity and specificity indexes, especially when there are animals in various stages of infection. ELISA tests require further improvements with regard to efficiency (Marassi *et al*. 2013).

The newly available serological test, the IDEXX *M. bovis* antibody test was recently released and added to the registry list by the World Organisation for Animal Health (OIE, 2011). The test makes use of a cocktail blend of mycobacterial antigen MPB70 and MPB83 coated on the microtiter plates (Waters *et al.* 2011). Addition of this test to the BTB control programmes can increase detection by identifying infections other tests missed. For example, in a study done on the Irish cattle herds, addition of the IDEXX *M. bovis* ELISA increased overall sensitivity to 91 %. The test was validated for cattle from nine countries and validation for South African cattle is in progress (http://www.idex.com/pubwebresources/pdf/en/us/livestock-poultry/09-71473-00).

1.1.6 Bovine tuberculosis in domesticated animals

1.1.6.1 Cattle and domesticated or farmed buffalo

Mycobacterium bovis is the primary source of BTB in cattle, swamp or Asiatic water buffalo (Cousins, 2001). *M. bovis* causes BTB in cattle and was previously reported to be present in almost every country of the world (Smith *et al.* 2006). All species of cattle are affected, however, some cattle breeds are more resistant to *M. bovis* infections (*Bos indicus*, i.e. Zebu and Brahman) than others (*Bos taurus*, i.e. European cattle breeds) (O'Reilly and Daborn, 1995). In Western Azerbaijan, Iran, *M. bovis* was isolated in one farmed buffalo specimen out of 140 specimens. In this region, buffaloes are bred in close contact with cattle, however, before this report; no incidences of BTB in buffaloes were reported in 26 years (Tadayon *et al.* 2006). In a study done in Pakistan, Khan and co-workers have noted that domesticated buffaloes which are high milk producers tend to suffer more from bovine tuberculosis (Khan *et al*. 2008).

M. bovis infection in cattle rarely presents clinical disease. More commonly it appears in healthy animals responding to immunological tests based on tuberculin (Collins, 2006). In cattle, as well as in many other animal hosts, the route of transmission can be deduced by the pattern of lesions observed in slaughtered animals. Animal with lesions restricted to the thoracic cavity are presumed to have been infected by the inhalation of aerosols, while those with lesions in the mesenteric lymph nodes are thought to have acquired the infection by ingestion (Pollock and Neill, 2002). However, tuberculosis lesions in cattle are most often found in the lungs and associated lymph nodes, and liver (Neill *et al.* 1994). In some cases, lesions are most commonly observed in the lower respiratory tract. The upper respiratory tract and associated tissues also displayed the disease in a significant number of cases (De la Rua Domenech *et al*. 2006). Respiratory excretion and inhalation of *M. bovis* is considered to be the main route through which cattle-to-cattle transmission occurs in bovine (Biet *et al.* 2005).

1.1.6.2 Pigs

Pigs are susceptible to *M. bovis* infection and *M. bovis* was a common cause of tuberculosis in these animal species in the early to mid-1900s (Cousins, 2001). Tuberculosis in domestic pigs is rare in countries that have successfully implemented tuberculosis control programmes. For example, in South Africa, *M. bovis* accounted for 2.5 % of the isolated mycobacteria from pigs with pathological lesions (Cousins *et al*. 2004). A recent study in Uganda has found that about one in fifty slaughter pigs with suspect lesions in the mesenteric lymph nodes were infected with *M. bovis* (Muwonge *et al.* 2012). This prevalence is higher considering that convenience sampling was used, which might have missed infected lymph nodes that had not yet developed lesions (Muwonge *et al.* 2012). *M. bovis* infection in pigs was also reported in Nigeria (Jenkins *et al*. 2011) and Argentina (Barandiaran *et al*. 2011). Tuberculosis is, however, not considered to be particularly contagious amongst pigs or to spread easily from pigs to other animals (Cousins, 2001).

1.1.6.3 Goats

A goat adapted strain (ecotype) of the *M. tuberculosis* complex known as *M. caprae* has been recognized mainly in central and southern Europe, where it has been occasionally isolated from tuberculous lesions in goats, cattle, red deer, pigs and wild boars

(Ikonomopoulos *et al*. 2006; Boniotti *et al.* 2009; Rodriguez *et al.* 2011). This member of the *M. tuberculosis* complex was first described in Spain in goats with disseminated lesions by Aranaz and co-workers (Aranaz *et al.* 1999). Spain is the second country in the European Union with the largest goat population. It also has the biggest problem of tuberculosis in these animal species (Napp *et al.* 2013). *M. caprae* spreads fast in goats (Rodriguez *et al.* 2011). In Spain, goat infected with *M. caprae* act as reservoirs of BTB and may pose a threat to neighbouring cattle herds (Napp *et al.* 2013). *M. caprae* infection in Spain represents 7.4 % of all MTBC isolates from domestic and wild animals, with the majority of the outbreak involving goats (Rodriguez *et al.* 2011). In countries that are virtually free of animal tuberculosis such as Germany and, Austria and the Czech Republic, a large number of cases in cattle and red deer are caused by *M. caprae* (Rodriguez *et al.* 2011). According to Rodriguez and co-workers, *M. caprae* has never been isolated outside continental Europe, except from a European patient in Australia (Sintchenko *et al.* 2006).

Mycobacterium bovis is also known to cause tuberculosis in goats, although the importance of the disease varies depending on countries. In Nigeria, Cadmus and co-workers had isolated *M. bovis* from goats from a slaughterhouse in Ibadan (Cadmus *et al*. 2009). An outbreak of goat tuberculosis caused by *M. bovis* infection that affected several linked goats herds was disclosed in south-west Wales and the west of England in 2008. Retrospective movement tracings identified a goat herd in south-west Wales as the probable common source of infection (Daniel *et al*. 2009). Caprine TB is extremely rare in Great Britain following the introduction of bovine tuberculosis eradication scheme in the 1950s (O'Reilly and Daborn, 1995), with *M. bovis* infections recorded in only four goat premises between 1971 and 2008 (Daniels *et al*. 2009). *M. bovis* infections in goats have also been reported in domestic goats in France, Germany, Italy, USA, India, Taiwan and Australia (O'Reilly and Daborn, 1995) as well as in Portugal (Duarte *et al*. 2008).

1.1.6.4 Sheep

Tuberculosis is considered rare and sporadic in sheep, but when occurring, it is often disseminated. Infection in sheep may present clinical signs (Van der Burgt *et al.* 2013) or may not (Marianelli *et al.* 2010). The low incidence of tuberculosis in sheep is argued to be a consequence of management and behavioural factors, which tend to reduce

their exposure to *M. bovis*. Sheep are extensively managed and grazed predominantly during daylight hours. In addition, they tend to flock together, both when resting and ruminating. As a result, close contact between sheep and infected cattle and wild animals is a rare occurrence (Marianelli *et al*. 2010).

1.1.6.5 Farmed or domesticated deer and camelids (camels, alpacas and llamas)

Farming red deer (*Cervus elaphus*) has been an emerging alternative livestock industry mainly in New Zealand, USA, China, Russia and Canada (De Lisle *et al.* 2001). Farmed red deer are in continuous contact with livestock and free ranging wildlife and therefore they are at an increased risk of getting and spreading infectious diseases such as BTB (Wadhwa *et al.* 2013). Camelids were not considered highly susceptible to tuberculosis (Fowler, 2010) and generally, the information on TB in camel is limited (Kasaye *et al*. 2013). Recently, there is a great concern about tuberculosis in New World camelids (NWC), particularly llamas and alpacas (Werney and Kinne, 2012). Tuberculosis was known to occur in dromedary camels (*Camelus dromedaries*) in Egypt in the early 1900s. Between 1910 and 1916, 2.9 % of camels slaughtered in Egypt were tuberculous and the bovine strain was implicated (Mason, 1917). In Ethiopia, 0.22 % of the camels slaughtered in a study to investigate the pathology of camel tuberculosis were found to be infected with *M. bovis* (Mamo *et al*. 2010). In another study, less than 1 % of dromedary camels' slaughtered at the abattoirs was infected with *M. bovis* (Kasaye *et al*, 2013). *M. bovis* infections in camels were also reported in Uganda (Oloya *et al.* 2007).

Tuberculosis is a well-recognised disease of South American camelids in the United Kingdom (UK) and the United States of America (USA) (Barlow *et al*. 1999), with an increasing number of farms being affected in recent years (Richey *et al*. 2011). In 2008, a first case of alpaca-to-alpaca transmission of *M. bovis* associated with movement of animals to a breeding herd was reported in Britain (Twomey *et al*. 2009). In 2009, a case of tuberculosis caused by *M. bovis* infection in the mammary gland of an alpaca in the UK was reported. In the majority of cases of TB in alpacas, the source of infection was linked to the spill-over of infection from other animals such as cattle and wildlife (Richey *et al*. 2011). *M. bovis* infections in alpaca herds were also documented in Ireland (Ryan *et al*. 2008). In Spain, García-Bocanegra and co-workers documented the description of *M. bovis* in alpacas in 2010 (García-Bocanegra *et al*. 2010). *M. bovis*

infections are endemic in cattle and wildlife in Spain (Lyashchenko *et al*. 2011). The disease in South American camelids has acquired importance since alpacas and llamas are being imported and kept in increasing numbers in many European countries (Barlow *et al*. 1999). Besides *M. bovis*, camelids are susceptible to other members of the MTBC like *M. tuberculosis* and *M. microti* (Oevermann *et al*. 2004) and *M. pinnipedii* (Moser *et al*. 2008).

1.1.6.6 Horses

Horses are considered naturally less susceptible to mycobacterial infections than other livestock, as such; infections like bovine tuberculosis are rarely diagnosed in horses (O'Reilly and Daborn, 1995). In the first part of the century, mycobacterial infections in horses were mainly caused by *M. bovis*. The situation has changed since the second part of the 20th century, and the most common cause of bacterial infections in horses are members of the *Mycobacterium avium* complex (MAC) (Pavlik *et al.* 2008). If BTB is diagnosed, the course of the disease appears to be chronic, with first symptoms being loss of body condition, despite normal appetite. Lesions are often found in the mesenteric lymph nodes. Liver and lung lesions are normally present (Cousins, 2001). Tuberculosis in horses was diagnosed in European countries like West Germany (Schutzler, 1954) and Czech Republic (Krejci, 1958). *M. bovis* was recently isolated from horses' faecal samples in Baghdad (Hamzah *et al.* 2013).

1.1.6.7 Dogs

It is generally believed that the susceptibility of dogs to *M. bovis* and *M. tuberculosis* is similar to that of humans (Feldman, 1934). The occurrence of BTB is usually related to exposure to *M. bovis* infected cattle (Snider, 1971). Spill-over of *M. tuberculosis* infection to dogs has been associated with close contact to human tuberculosis patient (Erwin *et al.* 2004). In naturally occurring cases, lesions are often found in the lungs, liver and kidneys. The absence of calcification has been considered characteristic of tuberculosis in dogs; however, exceptions do occur (Thoen *et al.* 2009). *M. bovis* infections in dogs were reported in New Zealand (NZ) (De Lisle, 1993; Gay *et al.* 2000). Parsons and co-workers reported a case of pulmonary caused by *M. tuberculosis* in a stray dog in South Africa (Parsons *et al.* 2008).

1.1.6.8 Cats

Cats appear to be very resistant to *M. tuberculosis* (Thoen, 1994), and among the domesticated animals, they are the most susceptible hosts of bovine tuberculosis (Underwood *et al.* 1999). Cats are more exposed to *M. bovis* than dogs, probably because a common pet owner's habit is to feed cats only with raw food such as milk, lung, liver etc. (Underwood *et al.* 1999; Colmegna *et al*. 2004). In NZ, by the year 1986, *M. bovis* was isolated from 73 domestic cats, and only two isolates of this bacterial pathogen was obtained from dogs (De Lisle, 1993). A further 19 *M. bovis* infected cats were identified between 1987 and 1992. *M. bovis* was isolated from carcases of 19 stray cats which died of unknown cases in Argentina from 1998 to 2006. These stray cats were fed daily with raw beef lung. In this study, researchers emphasized the importance of feeding cats with well-balanced food pellets or well cooked meat in order to reduce the risk of human infection (Zumárraga *et al*. 2009). In Great Britain, 15 % of the 339 cases of feline mycobacterial diseases studied was a result of *M. bovis* infection (Gunn-Moore *et al.* 2011). *M. bovis* infection was also reported in Turkey (Gökalp *et al.* 2011) in a two year old female household cat after 25 years of no reports of infections in cats. *M. bovis* infection of a cat was confirmed in Michigan, and the most likely source of tuberculosis was through the consumption of *M. bovis* infected wildlife carcasses or offal (Kaneene *et al.* 2002). In another study conducted to evaluate the infection in cats and dogs on farms with *M. bovis* infected cattle, Wilkins and co-workers could not find any evidence to indicate that pets residing on *M. bovis*-infected Michigan cattle farms pose a risk to human or *M. bovis*-free livestock, however, farm owners were given precautionary advice (Wilkins *et al.* 2008).

1.1.7 Transmission and routes of infection in domestic animals

Among domesticated animals, cattle, farmed buffalo and goats are considered reservoir hosts of *M. bovis* or *M. caprae*, whereas pigs, cats, dogs, horses and sheep are spill-over hosts (Biet *et al.* 2005; Rodrígues *et al.* 2011; Napp *et al*. 2013). In reservoir hosts, infection can persist through horizontal transfer in the absence of any other source of *M. bovis* and may as well be transmitted to other susceptible species. In contrast, spill-over hosts become infected with *M. bovis* but the infection only occurs sporadically or persists within these populations if a true maintenance host is present in the ecosysteM. However, if the source is removed, the prevalence for this disease is reduced and it can

only be maintained in the long term by re-infection from other sources (Haydon *et al.* 2002).

Routes of transmission of *M. bovis* are likely dependent upon the biology of the host animal as well as the environmental variables for areas occupied by the host (Walter *et al.* 2012). In cattle and other animal hosts, the route of transmission of *M. bovis* can be deduced from the pattern of lesions observed during *post mortem*. Animals with lesions restricted to the thoracic cavity are presumed to have been infected by inhalation of aerosols, while those with lesions in mesenteric lymph nodes are thought to have acquired the infection by ingestion (Pollock and Neill, 2002). Inhalation of *M. bovis* is considered the most probable route of infection due to majority of lesions found in the upper and lower respiratory tract and associated lymph nodes in field cases of cattle (Neill *et al.* 1994). The development of tuberculosis lesions which invade the airways was thought to be required to facilitate active excretion and aerosol spread of *M. bovis* in a study conducted in cattle from Northern Ireland (Menzies and Neill, 2000).

1.1.7.1 Transmission by inhalation

Respiratory excretion and inhalation of *M. bovis* is considered to be the main route through which cattle-to-cattle transmission occurs (Biet *et al.* 2005). Droplets of contaminated water, eructation while ruminating on infected pastures or inhalation of contaminated dust particles can also be an alternative way of facilitating aerogenous infection. Cattle could get infected in a contaminated environment this way (Phillips *et al.* 2003). The distribution of the lesions in a ewe described by Marianelli and co-workers suggested inhalation as the primary route of infection, probably due to contact with infected cattle sharing the same pastures or by trespassing on pastures where infected cattle grazed before it was brought to a new farm (Marianelli *et al.* 2010). Tuberculosis in cats can also be acquired through inhalation. The lungs and/or hilar lymph nodes are then affected (Greene and Gunn-Moore, 2006). In Egypt camels were often kept in close contact with cattle and the route of infection was usually the respiratory tract. The lungs and bronchial lymph nodes were always affected and lesions were restricted to these organs in 60 % of the cases (Mason, 1917).

1.1.7.2 Transmission by ingestion

Ingestion of *M. bovis* directly from infected animals or from contaminated pastures, water or fomites is considered secondary to respiratory spread, as deduced from minor presence of mesenteric lesions in cattle cases (Menzies and Neill, 2000). In general, mycobacterial infections in horses arise via ingestion, though primary respiratory infections do occur (Pavlik *et al.* 2008). Ingestion of contaminated materials including milk and offal from infected cattle or wildlife has been implicated as the most common source of infection in cats (Kaneene *et al.* 2002; Dean *et al.* 2006). Therefore, the primary site of infection is considered to be the alimentary tract (digestive route). Tuberculosis in cats is historically linked with tuberculosis in cattle and the pasteurization of milk resulted in a marked decline in the prevalence of disease seen in cats (Kaneene *et al.* 2002). Transmission of bovine tuberculosis in cats can be reduced by feeding them with cooked meat products (Zumárraga *et al.* 2009).

1.1.7.3 Transmission via direct contact

In most cases, *M. bovis* or *M. caprae* infection occurs as a direct result of contact with infected cattle. In many countries, caprine tuberculosis in both domestic and feral goats occurs as a result of contact with cattle and wildlife reservoirs infected with *M. bovis* (Morris *et al.* 1994). Infected goats with active pulmonary or udder lesions can spread infection within their cohort and other in contact susceptible species (O'Reilly and Daborn, 1995; Cousins, 2001).

1.1.7.4 Transmission via environmental contamination

Transmission via environmental contamination is considered a less effective means of disease transmission to cattle (Menzies and Neill, 2000), or probably negligible (Humblet *et al.* 2009) because the required infective dose is high, i.e. about 10^7 bacilli would be necessary for oral contamination (O'Reilly and Daborn, 1995). Faecal contamination of the environment due to dissemination via the intestines and mesenteric lymph nodes is also viewed as another possible route of transmission of *M. bovis* in goats (Daniel *et al.* 2009).

1.1.7.5 Congenital infection and vertical transmissions

Congenital infections and vertical transmissions to calves as well as genital transmission are uncommon in regions where intensive eradication programmes operate (Biet *et al.* 2005). However, congenital infections are likely to occur in developing countries with limited or no control measures implemented (Musoke *et al.* 2014). The oral route is the most important route of infection, most frequently caused by feeding milk, milk products or offal from infected cows.

1.1.7.6 Transmission through injection

Contact between open wounds (linked to an abscess, draining lymph node, etc.) of infected and an uninfected animal is able to transfer the disease. Transmission of *M. bovis* through injection (bites or open wound contamination) has also been reported (Dean *et al.* 2006); however, animals pose little likelihood of serving as a reservoir of diseases for other species (Thoen, 2009).

1.1.8 Bovine tuberculosis in wildlife species

1.1.8.1 Free-ranging wildlife

Cases of *M. bovis* have been reported in more than 40 free ranging wildlife species (Michel *et al.* 2010). The importance of BTB in wild animals has been acknowledged. Once infected, many wild animals have shown the potential to act as reservoirs of infection for both domestic cattle and other valuable wildlife species (De Lisle *et al.* 2002). Two categories of wildlife hosts have been distinguished, that is, the maintenance host and the spill-over host. The localization of lesions in infected animals may play a role in their characterization. Lesions that are mainly found in the thoracic cavity suggest an aerogenic infection, which is commonly observed in maintenance host like cattle (De Lisle *et al.* 2002). Digestive lesions suggest oral contamination by eating contaminated carcases, which is a characteristic of spill-over species, mostly the carnivores and omnivores.

Wildlife reservoirs are considered to constitute a major impediment to TB control or eradication programmes in some countries (Corner, 2006). These include the Eurasian badger (*Meles meles*) in the UK and Ireland (Delahay *et al.* 2002; Griffin *et al.* 2005).

The white-tailed deer (*Odocoileus virginianus*) is a reservoir of *M. bovis* in Michigan, USA (Schmitt *et al.* 1997) and served as a presumptive source of infection for cattle herds, and wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) in Spain (Vicente *et al.* 2007). The brush-tailed possums (*Trichosurus vulpecula)* are the primary wild maintenance host of bovine TB in NZ (Morris and Pfeiffer, 1995; Coleman and Caley, 2000; Barron *et al.* 2013) and are primarily responsible for the transmission of TB in livestock (Coleman and Caley, 2000).

In South Africa (SA), African buffaloes (*Syncerus caffer*) have been recognised for some time as important role players in the maintenance and transmission of a variety of economically important livestock disease like BTB at the wildlife and/or livestock interface (Michel *et al.* 2012). Bovine tuberculosis is endemic in African buffalo in the Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HiP). The infection had spilled-over to several other wildlife species including lion (*Panthera leo*), kudu (*Tragelaphus strepsiceros*), chacma baboon (*Papio ursinus*), leopard (*Panthera pardus*), cheetah (Acinonyx *jubatus*), hyena (*Crocuta crocuta*), honey badger (*Mellivora capensis*), spotted genet (*Genetta tigrina*), bushbuck (*Tragelaphus scriptus*) warthog (*Phacochoerus africanus*), bush pig (*Potamochoerus larvatus*) and impala (*Aepyceros melampus*) (Keet *et al.* 2000; Bengis *et al.* 2002; Michel *et al.* 2006; Michel *et al.* 2009). The transmission of *M. bovis* between buffalo herd members occurs most frequently by aerosol (Bengis *et al.* 1996). Predators and scavengers contract the disease mostly by ingestion of tuberculous tissues (Michel *et al.* 2006). In kudus, the first clinical sign of tuberculosis is the development of abscesses in the parotid lymph nodes. The abscesses increase with time and eventually rupture to intermittently discharge exudate. The *M. bovis* containing exudate discharges over long periods from the fistulous nodes below the ears and may contaminate the environment. This is suggested to be the mode of transmission between kudu as well as between kudu and cattle (Bengis *et al.* 2001). The Kafue lechwe (*Kobus leche*) antelope in Zambia has been described as a reservoir and source of BTB, and the high prevalence of the disease in the Kafue basin is reported to be a result of the contact that exist between cattle and wildlife species, particularly the lechwe antelopes (Munyeme *et al*. 2009). In Tanzania, scientists reported the occurrence of *M. bovis* in wildebeest (*Connochaetes taurinus*), topi (*Damaliscus lunatus*) and lesser kudu (*Tragelaphus imberbis*) sampled from protective areas in Northern Tanzania (Cleaveland *et al.* 2005).

The wood bison (*Bison bison*) and elk (*Cervus elaphus manitobensis*) are the primary reservoir hosts in and around Riding Mountain National Park in Canada (Lees *et al.* 2003). The maintenance of BTB in these wild ungulates is an important concern for the national park as well as the commercial livestock industry and people who may hunt animals from those infected populations (Nishi *et al.* 2006). Table 1 illustrates the different wildlife species from which *M. bovis* was isolated worldwide.

Table 1 Isolation of *M. bovis* in free-ranging wildlife species and their role as maintenance or spill-over hosts in the transmission of BTB (Adapted from Humblet *et al*. 2009)

Species	Country/Area	Epidemiological status	Reference
Cervids			
White-tailed deer (*Odocoileus virginianus*)	USA	Maintenance/ Spill-over	Bruning-Fann *et al.* 2001; Kaneene *et al*. 2002) Aranaz *et al.* 2004; Delahay *et al.* 2007,
Red deer (*Cervus elaphus*)	Spain, NZ, UK, Czech Republic, France	Maintenance/ Spill-over	Lugton *et al.* 1997, 1998; Zanella *et al.* 2008
Sika deer (*cervus nippon*)	UK	Maintenance/ Spill-over	Delahay *et al.* 2002
Fallow deer (*Dama dama*)	Spain, UK	Maintenance/ Spill-over	Aranaz *et al.* 2004; Delahay *et al.* 2002
Roe deer (*Capreolus capreolus*)	UK	Maintenance/ Spill-over	Delahay *et al.* 2007 Delahay *et al.* 2002; 2007
Elk (*Cervus Canadensis*)	Canada	Maintenance/ Spill-over	Lees *et al.* 2003
Muntjac (*Muntiacus reesi*)	UK	Spill-over	Delahay *et al.* 2007
Suids			
Wild boar (*Sus scrofa*)	Spain, Italy, Coatia, Slovakia, Hungary, France	Spill-over	Parra *et al.* 2006; Pavlik *et al.* 2002; Zanella *et al.* 2008
Feral pigs (*Sus domesticus*)	NZ	Spill-over	Corner, 2006
Warthog (*Phacochoerus aethiopicus*)	SA	Maintenance/ Spill-over	Michel *et al.* 2006; Woodford, 1982

Species	Country/Area	Epidemiological status	Reference
Carnivores			
Red fox (*Vulpes vulpes*)	USA, UK, Spain	Spill-over	Bruning-Fann *et al*. 2001; Delahay *et al.* 2002 and 2007; Martín-Atance *et al.* 2006
Feral ferret (*Mustela furo*)	NZ, UK	Spill-over	Coleman and Cooke, 2001; Delahay *et al.* 2002
Stoat (*Mustela ermine*)	NZ, UK	Spill-over	Coleman and Cooke, 2001; Delahay *et al.* 2007
Polecat (*Mustela potorius*)	UK	Spill-over	Delahay *et al.* 2007
Mink (*Mustela vison*)	UK	Spill-over	Delahay *et al.* 2001
Feral cat (*Felis catus*)	NZ, UK	Spill-over	Delahay *et al.* 2001; Ragg *et al.* 1995
Lion (*Panthera leo*)	Tanzania, SA	Spill-over	Coleman and Cooke, 2001; Michel *et al.* 2006
Cheetah (*Acinonyx jubatus*)	SA, Zambia	Spill-over	Keet *et al.* 2001; Zieger *et al.* 1998
Iberian lynx (*Lynx pardinus*)	Spain	Spill-over	Martin-Atance *et al.* 2006
Leopard (*Panthera pardus*)	Zambia	Spill-over	Zieger *et al.* 1998
Spotted hyena (*Crocuta crocuta*)	SA	Spill-over	Michel *et al.* 2006
Bobcat (*Lynx rufus*)	USA	Spill-over	Bruning-Fann *et al.* 2001
Coyotes (*Canis latrans*)	USA	Spill-over	Bruning-Fann *et al.* 2001
Black bear (*Ursus americanus*)	USA	Spill-over	Bruning-Fann *et al.* 2001
Racoon (*Procyon lotor*)	USA	Spill-over	Bruning-Fann *et al.* 2001
African wild dog (*Lycaon pictus*)	SA	Spill-over	Hlokwe, unpublished

Species	Country/Area	Epidemiological status	Reference
Wild ruminants			
African buffalo (*Syncerus caffer*)	SA, Uganda	Maintenance	Etter *et al.* 2006; Michel *et al*. 2006; Woodford, 1982
Asian water buffalo (*Bubalus arnee*)	Australia	Maintenance	Corner, 2006
Bison (*Bison bison*)	Canada, Poland	Maintenance	Nishi *et al.* 2006; Pavlik *et al.* 2002
Lesser Kudu (*Tragelaphus imberbis*)	Tanzania	Maintenance	Cleaveland *et al.* 2005
Greater kudu (*Tragelaphus strepsiceros*)	SA, Zambia	Maintenance	Keet *et al.* 2001; Zieger *et al.* 1998
Lechwe antelope (*kobus leche*)	SA, Tanzania	Maintenance	De Lisle *et al.* 2001; Zieger *et al.* 1998
Bushbuck (*Tragelaphus scriptus*)	Zambia	Maintenance	Zieger *et al.* 1998
Feral goat (*Capra hircus*)	NZ	Maintenance	Coleman and Cooke, 2001
Wildebeest (*Connochaetes taurinus*)	Tanzania	Spill-over	Cleaveland *et al.* 2005
Topi (*Damaliscus lunatus*)	Tanzania	Spill-over	Cleaveland *et al.* 2005
Small mammals			
Hedgehog (*Erinaceus europaeus*)	NZ	Spill-over	Coleman and Cooke, 2001
Mole (*Talpa auropaea*)	UK	Spill-over	Delahay *et al.* 2001 and 2002
Brown cat (*Rattus norvegicus*)	UK	Spill-over	Delahay *et al.* 2002
Common shrew (*Sorex araneus*)	UK	Spill-over	Delahay *et al.* 2007
Grey Squirrel (*Sciurus carolinensis*)	UK	Spill-over	Delahay *et al.* 2007
Bank vole (*Clethrionomys glareolus*)	UK	Spill-over	Mathews *et al.* 2006
Field vole (*Microtus agrestris*)	UK	Spill-over	Delahay *et al.* 2007
Yellow-necked mouse (*Apodemus flavicollis*)	UK	Spill-over	Delahay *et al.* 2007

Species	Country/Area	Epidemiological status	Reference
Others			
European badger (*Meles meles*)	UK, Ireland, Spain	Maintenance	Denny *et al.* 1999; Martín-Atance *et al.* 2006 and Griffin *et al.* 1993
Possum (*Trichosurus vulpecula*)	NZ	Maintenance	Jackson *et al.* 1995
Baboon (*Papio cynocephalus*)	Kenya	Spill-over	Tarara *et al.* 1985
Chacma baboon (*Papio ursinus*)	SA	Spill-over	Keet *et al.* 2000

There is conflicting evidence regarding the role of wild suids in the epidemiology of BTB (Di Marco *et al.* 2012). In Australia and NZ, the low prevalence of generalised lesions in feral pigs together with the management and genetic consideration has led to the conclusion that feral pigs are spill-over hosts rather than sources of BTB infection (Corner, 2006; Nugent *et al.* 2011a). Similarly, European wild boar (*Sus scrofa*) in northern Italy has been deemed unlikely to be a reservoir for BTB (Serraino *et al*. 1999). Researchers believed that wild boars were the spill-over hosts for *M. bovis* since lesions found were only limited to the lymph nodes of the head. However, wild boar was believed to act as a reservoir in Spain and Portugal (Aranaz *et al.* 2004). In this study, wild boars showed lesions in several body sites. The presence of tuberculous lesions in the lungs and associated lymph nodes usually reveal transmission by the aerogenous route, while lesions in the retropharyngeal and mandibular lymph nodes is associated with ingestion of contaminated offal or scavenging of carcasses. However, the behaviour of wild boars in Spain can be closely related to dual source of infection (Aranaz *et al.* 2004). In Sicily, researchers have suggested that the domestic black pig (*Sus scrofa*) might act as a bovine TB reservoir, however, additional studies are needed to establish the true epidemiological significance of the Sicilian black pig (Di Marco *et al.* 2012). Classification of wild animal species as maintenance host or spill-over host has been a controversial issue, based on the location of tuberculous lesions coupled with ecological factors such as population density, behavioural characteristics and interaction in the same environment (Aranaz *et al.* 2004). Based on aspects like these, it has been agreed that a wildlife species can be a spill-over host in the one ecosystem and a maintenance host in the other. Other wildlife species in Spain from which *M. bovis* infections were also detected include red deer (*Cervus elaphus*), fallow deer (*Dama dama*), Iberian lynx (*Lynx pardima*) and hare (*Lepus auropaeus*). Interspecies transmission between these wildlife species and cattle was confirmed (Aranaz *et al.* 2004).

Bovine tuberculosis is a multi-host infection with slow progression of disease in the individual infected. Effects of chronic infectious diseases such as bovine tuberculosis on wildlife buffalo population dynamics are often difficult to measure (Jolles, 2005). In addition, BTB causes some concerns with regard to the conservation of endangered species, for example, the Iberian lynx in the Donana National Park in Spain (Gortázar *et al.* 2008). Wildlife maintenance hosts allow the persistence of the infection in wildlife and livestock and enables the horizontal transmission of pathogen between species (De

Lisle *et al.* 2002), and limit the effectiveness of BTB eradication schemes in cattle which are largely based on testing and culling of infected animals (Aranaz *et al.* 2004). As a result, there is a need to better integrate conservation biology with livestock policy to develop management options (Nishi *et al.* 2002).

1.1.8.2 Captive wildlife species

Tuberculosis is one of the oldest and most frequent encountered problems in zoos in the USA and Europe. Until the 1960's, tuberculosis caused by *M. bovis* and *M. tuberculosis* had led to a loss of more than 40 % of some of the zoo collection (Ruch, 1959). *M. bovis* infections have been reported in many different captive species, including the threatened ones (De Lisle, 2001). The introduction of *M. bovis* into a zoo facility can lead to the spread of the infection to other species (Rocha *et al.* 2011). An outbreak of tuberculosis due to *M. bovis* infection was detected in the Dublin zoo, implicating a Mayotte lemur (*Lemur mayottensis mayottensis*), lion-tailed macaque (*Macacus silenus*), petas monkey (*Symphalangus syndactylus*), and a siamang (*Simphalangus syndactylus*). The infection source could not be linked to any zoo keeper. No strain typing was done however, the source of infection was suggested to be exogenous and the Siamang gibbon and a badger which were recently introduced were suspected to have been involved as carriers (Wilson *et al.* 1984). An outbreak of *M. bovis* in a conditioned colony of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques housed together in five different rooms in the USA (Garcia *et al.* 2004). *M. bovis* was isolated in captive waterbucks (*Kobus ellipsiprymnus*) located in the Sao Paulo Zoo. The necroscopy and laboratory examinations suggested that animals had developed lung disease due to inhaling aerosols containing *M. bovis* (Rocha *et al.* 2011).

In the National Zoological Garden of South Africa, Michel and co-workers recently reported a substantial increase in the number of tuberculosis cases caused by *M. tuberculosis* compared to a previous report in 2003. The number of species affected increased from eight to eleven (Michel *et al.* 2003; Michel *et al.* 2013). Authors suggested a countrywide trend of increasing spill-over of human tuberculosis to captive wild animals. In the same report, same strain of *M. tuberculosis* was repeatedly isolated in a Capuchin monkey colony on a private zoo, and it was suggested that the infection was either maintained within the Capuchin monkey colony or by a closely associated

person like the caretaker (Michel *et al.* 2013). Between 2001 and 2003 several species like giraffes, rhinoceros and buffaloes were found to be infected by different strains of *M. tuberculosis* in a Swedish zoo (Lewerin *et al*. 2005).

An unusual case of a *M. bovis* subsp. *caprae* infection causing pulmonary tuberculosis in a captive Siberian tiger (*Panthera tiger altaica*) from Spain was reported by Lantos and co-workers. In this report, the importance of early diagnosis of the disease in an endangered species was highlighted, which will assist in controlling the diseases and saving the animal species in particular (Lantos *et al.* 2003). *M. bovis* subsp. *caprae* was initially isolated in goats in that country (Aranaz *et al.* 1999). Disseminated tuberculosis due to *M. africanum* infection caused the death of two hyraxes (*Procavia capensis*) in the Zagreb zoo in Croatia. The source of infection could not be established. Due to long incubation period for tuberculosis and the fully developed inflammatory pattern observed, it was suspected that the hyraxes could have been infected before translocation from the United Arab Emirates (Gudan *et al.* 2008). Pinneped tuberculosis (causative agent is *M. pinnipedii*) has been reported in captive Australian sea lions (*Neophoca cinerea*) in Australia, captive New Zealand fur seals (*Arctocephalus forsteri*) in Australia and UK (Dunn *et al.* 2001; Cousins *et al.* 2003), captive South American sea lions (*Otaria flavescens*) in Uruguay and France (Dunn *et al.* 2001; Gomis *et al.* 2008; Moser *et al.* 2008). Recently, a multi-host species outbreak of *M. pinnipedii* infection involving Malayan tapirs (*Tapirus indicus*), Bactrian camel (*Camelus bactrianus bactrianus*) and Indian crested porcupine (*Hystrix cristata*) was reported (Jurczynski *et al.* 2011). *M. pinnipedii* can be transmitted from captive animals to human working in close contact with them, highlighting the zoonotic potential of this MTBC member (Dunn *et al.* 2001).

1.1.9 Bovine tuberculosis as a zoonosis and route of *M. bovis* transmission

The primary bacterium that causes tuberculosis in humans is *M. tuberculosis*; however, some other mycobacteria in the *M. tuberculosis* complex may also cause TB in humans and in animals. *M. bovis* has been shown to cause tuberculosis in both animals and humans, which makes this bacterium a potentially important zoonotic species (Biet *et al.* 2005). In general, there is an international agreement about underestimation of *M. bovis* as a cause of TB in humans (Milián-Suazo *et al.* 2010). Human tuberculosis caused by *M. bovis* has been reported in the UK (Smith *et al.* 2004; Mandal *et al.* 2011),

although the incidence remains low and the epidemiology is predominantly that of reactivated disease (Mandal *et al.* 2011). The study conducted in Croatia, the Czech Republic, Slovak Republic and Slovenia found that *M. bovis* infection rate in humans did not exceed 0.29 %. Bovine tuberculosis in cattle was eliminated from these countries during the second half of the 1960s and the incidence of outbreaks of the disease in cattle was very low, and thus cases of *M. bovis* infection in humans were unexpected (Pavlik *et al.* 2003). In South Siberia, a case of *Mycobacterium bovis* DNA in human remains from the iron age was first reported by Taylor and co-workers in 2007 (Taylor *et al.* 2007a). A retrospective analysis of TB cases surveillance data from San Diego in the United states from 1994 to 2005 indicated that *M. bovis* accounted for 45 % (62/138) of all culture positive TB cases in children less than 15 years of age, and 6 % (203/3, 153) of adult cases. This study highlighted that person with *M. bovis* were more than two times more likely to die during treatment than those with *M. tuberculosis* (Rodwell *et al.* 2008). Several other studies reported *M. bovis* infection in humans in the USA (Winters *et al.* 2005; Rodwell et al. 2010). Other countries in which *M. bovis* infection in humans were documented include Ireland (Ojo *et al.* 2008; Bilal 2010), France (Sunder *et al.* 2009; Aimé *et al.* 2012), Germany (Göbels *et al.* 2000), Mexico (Milián-Suazo *et al.* 2010), Taiwan (Jou *et al.* 2008) and Italy (Lari *et al.* 2010).

The extent of *M. bovis* involvement in the global TB burden in Africa is largely still unknown. This may be explained by the fact that pulmonary BTB is clinically, radiologically and pathologically indistinguishable from TB caused by *M. tuberculosis* (O'Reilly and Daborn, 1995). In addition, most laboratories in sub-Saharan Africa do not have the capabilities and resources to differentiate *M. bovis* from *M. tuberculosis* (Ayele *et al.* 2004). In Ethiopia, *M. bovis* was found to be a cause of tuberculous lymphadenitis in 17.1 % of the 35 human tuberculosis cases studied (Kidane *et al.* 2002). The incidence of zoonotic TB in Nigeria was reported by Cadmus and co-workers, who found 3 strains *of M. bovis* from a total of 60 strains cultured from human samples (Cadmus *et al.* 2006). Further reports from Nigeria indicated the presence of zoonotic TB in two human cases and the importance of conducting a nationwide survey of MTBC strain was highlighted (Jenkins *et al.* 2011). In Tanzania, 16 % of the 149 cases of human cervical adenitis were due to *M. bovis* infections. In a study conducted in Uganda, *M. bovis* was isolated from three of the 43 biopsies from human with cervical lymphadenitis in pastoral communities in the Karamoja region (Oloya *et al.* 2008). Another study conducted in Mbarara district in Uganda found the prevalence of less

than 4.2 % and confirmed the low level involvement of *M. bovis* in human TB (Byarugaba *et al.* 2009). In a very recent study, the median proportion of TB caused by *M. bovis* was 17 % in Ethiopia, 15.4 % in Nigeria and 26.1 % in Tanzania (Müller *et al.* 2013). This was in contrast with the median proportion of TB caused by *M. bovis* in another ten countries included in the same study, where less than 3.5 % median proportions were found. Overall, a median of 2.8 % of all tuberculosis cases in humans were caused by *M. bovis* in the 13 African countries studied. A median of 0.3 % was found for zoonotic tuberculosis in the Americas. Most of the countries in the Americas accounted for a negligible percentage of the tuberculosis cases; however, high proportions were reported for specific areas in Mexico and the United States (Müller *et al.* 2013). In some countries in Europe, zoonotic tuberculosis is caused by both *M. bovis* and *M. caprae* (Prodinger *et al.* 2002; Rodriguez *et al.* 2009). Müller and co-workers revealed a median proportion of 0.4 % of *M. bovis* or *M. caprae* infections among all confirmed tuberculosis cases reported. The median proportions of zoonotic tuberculosis were less than 0.1 % for both countries in the Eastern Mediterranean and Western Pacific. In general, data obtained from 61 countries suggested a low global zoonotic tuberculosis incidence (Müller *et al.* 2013). A study regarding the public health concerns of the transmission of bovine tuberculosis in the Kruger National Park, South Africa, did not find any evidence of *M. bovis* infection in 206 KNP employees assessed, not even those in the high risk group (Weyer *et al.* 1999).

The main route of transmission of *M. bovis* to humans is believed to be from cattle via consumption of unpasteurized milk and dairy products. As the lung is the main site of tuberculosis in cattle, another route of infection in humans, though less frequent, is the aerosol inhalation following close contact with infectious cattle on the farm or in abattoirs (Grange, 2001), or other animals including house hold pets (Shrikrishna *et al.* 2009). Another mode of *M. bovis* transmission is by contact with mucous membranes and broken skin (Bilal *et al.* 2010). Person-to-person transmission of *M. bovis*, although rare, has also been reported (Smith *et al.* 2004; Sunder *et al.* 2009). People who are at risk of *M. bovis* infection include animal workers, farmers, meat packers, veterinarians and zoo keepers. Immunosuppressed individuals are also at risk. This group includes people living with HIV/AIDS, and those receiving immunosuppressive medications (Ayele *et al.* 2004). Cases of *M. bovis* transmission from farm workers to cattle have been reported. The route of transmission of infection is by cough sprays (Bilal *et al.* 2010). An exceptional case of transmission from cattle to human and back to cattle was

previously described (Fritsche *et al.* 2004). Clinical presentation of TB due to *M. bovis* depends on the route of infection. Oral infection acquired by drinking unpasteurized milk from diseased cattle usually results in lymphadenitis of the cervical or mesenteric lymph nodes and other forms of non-pulmonary disease, whereas aerogenous infection from cattle or humans leads to pulmonary TB (Bilal *et al.* 2010).

In industrialized countries, animal TB control and eradication programmes together with milk pasteurization have drastically reduced the incidence of TB caused by *M. bovis* both in cattle and humans. However, in developing countries, animal TB is widely distributed, control measures are either applied sporadically or not at all and pasteurization is rarely practiced and limited information on the zoonotic impact is available (Cosivi *et al.* 1998). Tuberculosis in cattle remains an endemic infection in several countries despite several efforts to control the disease. It causes considerable loses of milk production on cattle dairy farms and poses health risks to the population that consumes products of animal origin (Amanfu, 2006). Despite the screening of cattle and pasteurization of milk, *M. bovis* has not been eradicated in a few European countries, including Ireland (Bilal *et al.* 2010). Although the proportion of *M. bovis* causing human tuberculosis is very low compared to *M. tuberculosis*, its potential impact on the population groups at the higher risk should not be underestimated (Michel *et al.* 2009).

1.1.10 Geographical distribution of bovine tuberculosis

The causative agent of BTB, *M. bovis*, is a highly adapted and “successful” pathogen and is distributed throughout the world. In many country countries, BTB remains a major problem and costly infectious disease of cattle and other domesticated, feral and wildlife populations (Skuce *et al*. 2012). The geographical distribution of BTB has changed drastically over the recent decades. Prior to the introduction of control measures and milk pasteurization in developed countries, tuberculosis was widely distributed throughout the world (FAO, 2012). *M. bovis* is suggested to have been distributed throughout the world through exportation of infected cattle mainly from the UK, the Netherlands and other countries to their former colonies (Renwick *et al.* 2007). It is also reported that cattle were exported from Europe into a majority of African countries during colonial times in a bid to improve dairy production in these countries (Müller *et al*. 2009). Furthermore, intensification of the dairy industry in combination

with cattle movements as well as cattle trades between neighboring countries and trading partners have led to the distribution of *M. bovis* strains (Cadmus *et al*. 2006; Müller *et al*. 2009). Further investigations on the relationships between European *M. bovis* strains and strains from other parts of the world are important to elucidate the global spread of *M. bovis* (Sahraousi *et al*. 2009). According to the World Animal Health Database (WAHID) of the OIE, 70 countries reported bovine tuberculosis in their cattle populations in 2010, and 49 countries in 2011 (OIE WAHID, 2010; 2011). Despite the disease being underreported in developing countries, there is adequate evidence that the prevalence is higher in the developed countries, and it is increasing in Africa, Asia and Latin America (Thoen *et al.* 2009).

1.1.11 Control of bovine tuberculosis

In cattle, existing strategies for long term bovine tuberculosis control or eradication campaigns are being reconsidered in many countries because of the development of new testing technologies, increased global trade, continued struggle with wildlife reservoirs of BTB, redistribution of international trading partner or agreements and emerging financial and animal welfare constraints on herd depopulation (Liebana *et al.* 2010). Efforts to control and eradicate BTB in wildlife species is hampered by a lack of validated *in vitro* and *in vivo* diagnostic tests of suitable sensitivity and specificity as well as procedures for handling and restraining animals under field conditions to conduct tuberculin skin test and/or to collect specimens for laboratory examination to confirm the diagnosis (Thoen and Ebel, 2006).

1.1.11.1 Control by whole herd test and slaughter programmes

Whole herd test and slaughter programmes have been used world-wide for the control of BTB. Monitoring of the cattle population for *M. bovis* infection depends on national programmes of herd tuberculin testing, supported by active abattoir surveillance (De la Rua-Domenech *et al*. 2006; Good *et al.* 2011), however, limited tuberculin sensitivity has contributed to under or over estimation of the impact of risk factors including cattle contact and movement (Skuce *et al.* 2012). Early diagnosis and intervention to interrupt transmission are the priority for control of BTB (Clegg *et al.* 2011). On cattle farms, the major source of *M. bovis* is infected cattle that either reside on the farm or are introduced to the herd from other facilities. Basic herd hygiene and biosecurity

practices have been found to reduce the risks of spread of *M. bovis* on cattle farms (Kaneene *et al.* 2002). Although very expensive, the application of test and slaughter policy has resulted in the reduction or elimination of BTB in domestic cattle in many developed countries (Ayele *et al.* 2004; Amanfu, 2006). However, there have been reports of sustained and unexplained increase in the incidence of BTB in the past 25 years in certain parts of the UK, although a test and slaughter strategy has been implemented (Carrique-Mas *et al*. 2008).

1.1.11.2 Control by abattoir surveillance methods

Monitoring of lesions consistent with tuberculosis by *post mortem* examination of cattle carcasses at abattoirs is a critical element of tuberculosis eradication. However, meat inspection procedures are estimated to detect only up to 50 % of cattle with tuberculosis lesions as compared to a detailed necropsy procedure that include slicing lymph nodes in a laboratory at 2 mm intervals and visual examination of the cut surfaces (Corner *et al*. 1990). The quality of meat inspection and hence the sensitivity of abattoir surveillance varies greatly between abattoirs. In a study conducted in Ethiopia, routine inspection missed about 90 % of the carcases with lesions suggestive of BTB as detected by the detailed inspection method (Bekele and Belay, 2011). Another study conducted in Ethiopia on five abattoirs indicated that routine abattoir inspection failed to detect approximately 70 % of carcasses with grossly visible lesions of tuberculosis. The findings indicated the magnitude of meat-borne zoonotic tuberculosis as an on-going risk to public health (Biffa *et al.* 2010). Effective monitoring requires the meat inspectors to show diligence and be well trained (Pocretcha *et al.* 1990).

1.1.11.3 Control by culling the wildlife reservoir

In NZ, lethal control (culling) of wildlife reservoirs to reduce TB incidences in cattle has been demonstrated. The intensive poisoning and culling programmes in combination with managed livestock disease control programs resulted in the national cattle herd TB reactor prevalence falling from 2.4 % in 1993 to 0.35 % by 2004 (Ryan *et al.* 2006). Significant declines in cattle reactor rates have been associated with widespread possum control, which suggests that in many areas the average density of possums has been reduced below the threshold for maintenance of BTB, however, there is still a need for concerted action to limit further spread of the disease (Caley *et al.* 1999). In

the UK and Ireland however, the impact of experimental badger culling on concurrent disease in domestic stock is more complex, with culling both increasing and decreasing cattle tuberculosis reactor rates depending on the circumstances (Donnelly *et al.* 2006; Cater *et al.* 2007; McDonald *et al.* 2008; Woodroffe *et al*. 2009). There are conflicting reports about the extent to which culling will reduce the spread of the disease. Animal welfare groups such as the Badger Trust and the Royal Society for the Prevention of cruelty against animals are opposed to badger culling as badgers are protected species in the UK and Ireland. The impact of badger culling is described as relatively small on the disease. However, other organizations including farmers organization and the Department for Environment, Food and Rural affairs (DEFRA) are in favour of a badger culling policy because of the mounting costs of the disease to farmers, and that other alternative for controlling the disease besides culling are not cost effective (Black, 2012).

1.1.11.4 Control by vaccination

Lethal control by poisoning of wildlife is controversial (Tompkins *et al.* 2009); as the wildlife in question may be valued for conservation or hunting purposes (Ryan *et al*. 2006; McDonald *et al.* 2008). This has driven a strong interest in non-lethal alternatives such as vaccination (Cross *et al.* 2007). Wildlife vaccination for TB control is being studied world-wide in several host reservoir models, including brush tail possum, ferret, and red deer in NZ (Griffin *et al.* 1999; Qureshi *et al.* 1999; Tompkins *et al*. 2009), white tailed deer in the USA (Nols *et al.* 2008), and African Buffalo in SA (De Klerk *et al.* 2010).

The only currently available vaccine against tuberculosis is the human vaccine bacillus Calmette-Guerin (BCG). BCG is a live vaccine derived from *M. bovis* via the loss RD1, the “region of deletion 1” that encodes the ESX-1 secretion system involved in virulence (Brosch *et al.* 2002). Vaccination against BTB is likely to become an important disease control strategy in developing countries which cannot afford the test and slaughter control programme or in countries where a wildlife reservoir of *Mycobacterium bovis* infection exists (Buddle *et al.* 2006). BCG has been used in cattle in a large number of experiments and trials with variable efficacies (Buddle *et al.* 2005). For example, field trials in Malawi (Berggren, 1981) and Madagascar (Waddington *et al*. 1972) indicated protective efficacies of 25 % and 45 % respectively. In Ethiopia, the overall protective

efficacy of BCG was between 56 % and 68 %, depending on parameters used (Ameni *et al.* 2010). Higher levels of protection (75 %) have been reported in the UK (Vordermeier *et al.* 2002). Sadagopal and co-workers have produced a modified BCG vaccine by removing BCG's antioxidants-producing capacity, which is known to suppress the immune response. Mice were vaccinated with a modified BCG and it was concluded that by targeting the antioxidants, it may be possible to restore BCG's ability to protect against pulmonary TB, however, clinical trials still needed to be conducted (Sadagopal *et al.* 2009). In addition, alternative approaches to BCG vaccination have been recently assessed for efficacy in humans and in cattle (Buddle *et al.* 2011; Tameris *et al.* 2013). The outcome of one of the studies in NZ indicated a significant protection of possum against tuberculosis and the vaccine efficacy was estimated to be 87-100 % in females and 82-99 % in males. Researchers suggested that wildlife vaccination, alongside other existing control methods, could be used to eradicate tuberculosis from domestic animals (Tompkins *et al.* 2009).

1.1.12 Molecular epidemiology of bovine tuberculosis

Molecular epidemiology is a field that has emerged largely from the integration of molecular biology, clinical/veterinary medicine, statistic and epidemiology. It focuses on the role of genetic and environmental risks at the molecular, cellular or biochemical level in disease aetiology and distribution among populations (Mathema *et al.* 2006). Molecular epidemiology of infectious diseases like BTB, attempts to use a multi-disciplinary approach to identify factors that determine disease causation, propagation/dissemination and distribution. This is achieved by associating epidemiologic characteristics with the biologic properties of clinical or veterinary isolates recovered from symptomatic individuals. Several molecular tools are available. It is however, critical to choose an appropriate method/s to address a particular question, e.g., transmission dynamics, outbreak, or phylogenetics. Generally, the key aspects in choosing an adequate molecular approach for studying TB epidemiology are the observed rate of polymorphism (stability of the marker) and the genetic diversity of strains in the population. That is, the rate of change of a marker must be adequate to distinguish epidemiologically unrelated strains and yet sufficiently "slow" enough to reliably link related cases. This factor, together with the general background information on TB prevalence should be taken into consideration when choosing molecular epidemiologic methods or in evaluating data (Mathema *et al.* 2006).

1.1.13 Methods for molecular epidemiological investigations

The development of molecular strain typing techniques for the differentiation of *M. bovis* isolates has greatly improved the ability to conduct epidemiological investigation of bovine tuberculosis in both domesticated and wildlife animals (Durr *et al.* 2000). Individual typing techniques commonly used for molecular characterizations of *M. bovis* strains are discussed below.

1.1.13.1 Restriction Fragment Length Polymorphism (RFLP)

To study the molecular epidemiology of bovine tuberculosis, genotyping (DNA fingerprinting) methods such as Restriction Enzyme Analysis (REA) and Restriction Fragment Length polymorphism (RFLP) using the internationally accepted standardized insertion sequence IS*6110*, and the polymorphic GC-rich sequence (PGRS) as probes, have provided useful epidemiological information regarding the differentiation and diversity of *M. bovis* isolates for studying the spatio-temporal distribution (Cousins *et al.* 1998; Michel *et al.* 2008). These techniques are however, laborious, time consuming, technically demanding and require large amounts of pure genomic DNA (Kremer *et al.* 2005). The biggest disadvantage of these methods is that results are generated as complex banding patterns. This presents difficulties for reproducible analysis of bands and exchange of data between laboratories (Van Embden *et al.* 1993). Reproducibility can however be overcome by the use of Gel Compare software (Streicher *et al.* 2007). Furthermore, *M. bovis* strains with less than two copies of IS*6110* could not be differentiated (Michel *et al.* 2008).

1.1.13.2 Spoligotyping

Spoligotyping (short form for spacer oligonucleotide typing) is a PCR based method exploiting polymorphism within the direct repeat region (DR) of the chromosome of *M. tuberculosis* complex bacteria (Kamerbeek *et al.* 1997). The technique is rapid, easy to perform and cost effective and results are easily interpreted using computer analysis. Another major advantage of spoligotyping is that *M. bovis* isolates worldwide can be compared in the international *M. bovis* database (http:www.mbovis.org). Some researchers have, however, found the method to give better epidemiological information when used in combination with other typing techniques, especially when typing *M. bovis* isolates with one IS*6110* copy (Lutze-Wallace *et al.* 2005; Allix *et al.* 2006; Müller *et al.*

2008; Romero *et al.* 2008). This is because spoligotyping relies on the amplification of DNA across a unique, highly polymorphic DR (direct repeat) locus available in the chromosomes of *M. tuberculosis* complex bacteria whereas other techniques detect changes within different regions of the chromosome (Cousins *et al.* 1998).

The classical spoligotyping procedure, also termed reverse line-blot hybridization, utilizes a nylon membrane carrying all 43 spacer-specific oligonucleotide probes (Kamerbeek *et al.* 1997). This membrane-based spoligotyping may be converted into DNA microarray format to qualify it for a high-throughput testing. The oligonucleotide sequences of the classical spoligotyping procedure may either be retained or adapted to the ArrayStrip platform by adding one to four 5'- or 3' located complementary nucleotides or by removing two nucleotides in order to adjust their thermodynamic parameters. Added advantages of the DNA microarray spoligotyping include a quick turn around time, ease of operation and use, automatic processing of measured data using online database instead of visually inspecting a chemiluminescent image, relatively low cost and the possibility of performing the test on cultured material as well as on the original tissue sample (Ruettger *et al.* 2012).

1.1.13.3 Variable number tandem repeat typing (VNTR)

More PCR-based genotyping methods have become available for rapid molecular epidemiology investigations. Initially, six variable number of tandem repeat (VNTR) loci/ markers described as exact tandem repeats (ETRs) A to F (ETR-A, -B, -C, -D, -E and -F) were reported to be more discriminatory than spoligotyping (Frothingham and Meeker-O'Connell, 1998). A novel class of genetic markers collectively known as MIRU-VNTR was later described. This includes the ETRs, mycobacterial interspersed repetitive units (MIRUs) (Supply *et al.* 2000) and VNTRs (LeFleche *et al.* 2002; Roring *et al.* 2000; Skuce *et al.* 2002). Several of these markers vary in copy number between the sequenced genomes for reference strains of MTBC bacteria and within test panels of clinical isolates of the MTBC bacteria including *M. bovis*. VNTR typing is highly discriminative for *M. tuberculosis* isolates and therefore has the potential to be the method of choice for typing *M. bovis* (Hilty *et al.* 2005). VNTR markers were described in several studies as highly discriminative for typing *M. bovis* isolates from cattle, especially those with low IS*6110* copy numbers (Hilty *et al.* 2005; Jeon *et al.* 2008; Martinez *et al.* 2008). VNTR typing allowed further differentiation of *M. bovis* strains

with the most frequent spoligotypes (Mignard *et al.* 2006; Müller *et al.* 2008; Sahraoui *et al.* 2009). It was also found to be more discriminatory than the gold standard IS*6110* RFLP typing in a study of *M. bovis* isolates found in Belgium with no known epidemiological links. It is faster than IS*6110* RFLP typing; *M. bovis* cell lysates can be utilized and has been adapted to high-throughput conditions (Supply *et al.* 2001; Allix *et al.* 2004). Unlike IS*6110*, VNTR typing has the advantage of typing identification being expressed by a digital numerical code and can be fully automated. In addition, the discriminatory power of tandem repeat typing can be increased by examining a larger number of loci/markers (Supply *et al.* 2001). Other advantages of genotyping using VNTR are that most markers are stable and the results can be compared between different laboratories. Several studies on the stability of VNTR markers have been conducted. Supply and co-workers tested the robustness and variability of 29 VNTR loci/markers on a standardized collection of 90 *M. tuberculosis* complex isolates from 38 different countries. Among the 29 markers, five were found to be unstable and thus subsequently excluded from the final collection. The remaining 24 loci were all amplified readily (Supply *et al.* 2006). In Italy, the stability of 24 VNTR markers was evaluated on two panels with a total of 91 isolates from different animals of the same herd and on isolates from epidemiologically linked herds. The most discriminatory and stable markers were identified and it was suggested that the markers can represent the first core set for *M. bovis* in different countries (Boniotti *et al.* 2009). Researchers in Ireland also evaluated the mycobacterial interspersed repetitive unit-VNTR markers. Their study revealed that a panel of only six loci was good enough to provide differentiation of *M. bovis isolates*, however, a combination of VNTR typing and spoligotyping offered more advantages over VNTR typing alone; a few VNTR profiles were common to more than one spoligotype (McLernon *et al.* 2010).

1.1.14 Whole genome sequencing

The field of microbial genomics provides exciting new opportunities in the control and prevention of a wide variety of veterinary diseases. In addition, genomics, and the functional analysis of genomic data are leading to novel approaches for vaccine discovery and improved methods for diagnosis and epidemiology (Gay *et al.* 2007). Genomics of several bacterial pathogens that influence veterinary medicine including *Mycobacterium* tuberculosis complex species have been sequenced (Cole *et al*. 1998; Garnier *et al.* 2003; Zhang *et.al.* 2013). DNA sequencing was first described by Sanger

and co workers in 1997, and has undergone a steady metamorphis from a cottage industry into a large-scale production enterprise that requires a specialized and devoted setup of bioinformatics and computer databases (Mardis, 2007). Since 2005, the so-called "next-generation" sequencing technologies have provided unprecedented opportunities for high thoroughput functional genomic research (Morozova and Marra, 2008).

1.1.14.1 Whole genome sequencing in the epidemiology of bovine tuberculosis

Genome sequencing is fundamentally changing the understanding with regard to ecology and epidemiology of important pathogenic microbes and provides new insights into the predictive biology and the discovery of effective counter measure for the disease control and eradication (Gay *et al*. 2007). Development and annotation of genome sequence establishes a framework through which targets for epidemiological analysis can be identified. The MTBC genome sequencing project at the Sanger Center and The Institute for Genomic Research have released valuable sequence data from genome sequences of *M. tuberculosis* H37Rv, *M. bovis* AF2122/97 and *M. tuberculosis* CDC1551 respectively, enabling the identification of various polymorphic loci-vavriable number of tandem repeats as described in section 1.1.13.3 (Cole *et al.* 1998; Garnier *et al*. 2003). The VNTR based tools are especially useful in the characterization of the MTBC (Le Fleche *et al*. 2002).

In the post genomic era, single nucleotide polymorphism (SNPs) have emerged as a robust tool for delineating phylogenetic relationships between closely related strains of pathogenic bacteria including the MTBC (Joshi *et al*. 2012). Thus, defining the extent of genetic diversity of the MTBC and providing insights into the evolution, pathogenicity and molecular epidemiology of tuberculosis globally (Joshi *et al*. 2012). It was shown recently that genomic variation is mainly driven by a high degree of diversity in the form of SNPs, with the majority of them being in the non-synonymous regions of the genome sequens with potential function (Hershberg *et al*. 2008; Comas *et al*, 2009). Besides being a rich primary source of genetic variation, SNPs are easy to assay and provide for large scale population genetic studies (Gutacker *et al*. 2002; 2006).

1.1.14.2 Bovine tuberculosis in South Africa

In South Africa, a case of BTB was first reported in cattle in 1880. It is assumed that infected cattle through European settlers (Hutcheon, 1880; Michel *et al.* 2008) introduced the disease. Many years later, cattle imported from countries such as Madagascar, Australia, and Argentina etc. (Cousins *et al.* 2004) could have introduced BTB. In 1929, Paine and Martinaglia reported the first case of bovine tuberculosis in wildlife species, i.e. common duiker and greater kudu on farmland in the Eastern Cape Province (Paine and Martinaglia, 1929). Bovine tuberculosis has a complicated epidemiology involving interactions between cattle, and between cattle and infected wildlife (Menzies and Neill, 2000; Johnston *et al.* 2005). In disease outbreaks, factors such as the movement of cattle, the spread of the disease from neighboring premises and the persistence of the infection on farms, together with the role of infected wildlife, most notably, the African buffalo (*Syncerus caffer*) in South Africa are of particular importance (Bengis, 2001; Pollock and Neill, 2002). The African buffalo serves as the main reservoir of the disease in the KNP and Hluhluwe-iMfolozi Park (HiP) and the initial diagnosis of the disease was made in 1990 (Bengis *et al.* 1996) and 1986 (Jolles, 2005) respectively. *M. bovis* infection was introduced into these two ecosystems by domestic cattle during the second half of the 20th century (De Vos *et al.* 2001), and the disease has major impacts on the ecological dynamics of the parks (Michel *et al.* 2006). Molecular typing of *M. bovis* isolates from KNP and a neighboring cattle herd located in the Southern border of the park suggested an epidemiological link (Michel *et al.* 2009).

Wildlife maintenance hosts of tuberculosis pose major difficulties to controlling infection in cattle they may interact with, and for other wild animals. In the KNP and HiP, *Mycobacterium bovis* has spilled-over to other wildlife animals, amongst others, lions, cheetah, chacma baboon, greater kudu, leopard, and warthog (De Vos *et al.* 2001; Jolles, 2005; Michel *et al.* 2006). Surveys in the KNP revealed that a majority of buffalo populations in the southern part are infected with *M. bovis* (Vos *et al.* 2001; Michel *et al.* 2009). Further surveys showed that the disease has reached the northern part of the park (De Garine-Wichatitsky *et al.* 2010). The establishment of the Greater Limpopo Transfrontier Park (GLTFP), which already links South Africa's KNP with the Limpopo National Park in Mozambique and a link is also envisaged with the Gonarezhou Park in Zimbabwe, might increase the risk of the disease spreading in the GLTFP and out of the park to neighboring livestock (Michel *et al.* 2006).

South Africa is one of only seven African countries that apply disease control measures and considers BTB as a notifiable and controlled disease (Cosivi *et al.* 1995). The test and slaughter scheme was implemented in 1969 and although it initially led to a drastic reduction of the infection in commercial cattle herds over the next 30 years, a steady increase in the prevalence has been noted during the past decade and the disease status in communal cattle herds is virtually unknown. Vaccination of cattle and wildlife alongside this control measure has a potential to make a significant contribution to the control of BTB where a lack of both veterinary field staff and compensation is a major constraint to eradication (Michel, personal communication). In South Africa vaccination has not been implemented but is still a subject of research (De Klerk *et al.* 2010)

Effective management and control of BTB in wildlife and ultimate eradication in livestock is very important for both wildlife conservation and socio-economic reasons, which requires the availability of tools to determine the manner of transmission within and between animal populations and the geographical spread of the disease. Epidemiological information is particularly important in the context of the establishment of the GLTFP where the potential spread of BTB from KNP into Limpopo National Park and Zimbabwe is of great concern.

1.2 Problem statement

In South Africa, *M. bovis* isolates from free-ranging wildlife from HiP including three smaller associated parks and the KNP and adjacent areas have previously been characterized using IS*6110*, PGRS and spoligotyping (Michel *et al.* 2009). IS*6110* and PGRS RFLP typing provided good discrimination; however, they are cumbersome, laborious and slow techniques. Spoligotyping is rapid but could not differentiate between KNP strain and its variants. Therefore, there is a need for a simpler technique that will incorporate the simplicity of PCR based methods with the discriminatory power of RFLP methods. In livestock, previous studies have indicated that *M. bovis* isolates comprising of only two copies of IS*6110* could not be resolved using this probe. In the same study, spoligotyping provided the lowest level of discrimination between isolates (Michel *et al.* 2008), hence better discrimination is needed.

1.3 Aims of the study

The current study aimed firstly on identifying an optimized set VNTR markers with a high discriminatory power to be able to distinguish between variants of the *Mycobacterium bovis strains* responsible for BTB epidemic in the KNP as well as between outbreak strains in other wildlife and domestic animal population in other regions of South Africa. Secondly, the resulting standardized, most discriminatory approach was used to study the spread of *M. bovis* in wildlife species in the GLTFP, HiP and other regions. The study also aimed at applying the same strategy to study the spread of BTB in domestic animals in SA. Furthermore, the study aimed at developing and evaluating novel VNTR markers from whole genome sequence data of local *M. bovis* strains with a view to improve VNTR typing tool.

1.4 Objectives

1. Isolate and identify *M. bovis* from wildlife and livestock diagnostic samples and to prepare template DNA.
2. Screen published VNTR markers on a selected panel of characterized, genetically diverse *M. bovis* isolates from SA and identify a set of VNTR markers with the highest discriminatory power, using data available from RFLP typing, spoligotyping and conventional epidemiological investigations as a frame of reference.
3. Determine the optimum discriminatory power of selected sets of VNTR markers for differentiation of *M. bovis* strains in South Africa.
4. Identify new and additional genetic markers using sequence data obtained from whole genome sequences of selected local strains, for optimized molecular epidemiological studies of BTB outbreaks in South African and the region.
5. Use the optimized genetic tools to genetically characterize and compare *M. bovis* isolates in wild and domestic animals in different infected ecosystems.

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Chapter 2
Molecular characterization of *Mycobacterium bovis* isolated from African buffaloes (*Syncerus caffer*) in Hluhluwe-iMfolozi Park in KwaZulu-Natal, South Africa

Hlokwe, T.M., Jenkins, A.O., Streicher, E.M., Venter, E.H., Cooper, D., Godfroid, J., Michel, A.L. 2011. Onderstepoort Journal of Veterinary Research. 78(1): 1-6

2.1 Abstract

Bovine tuberculosis (BTB), a chronic disease of mammals caused by *Mycobacterium bovis*, is a threat to South African wildlife. It has been reported that African buffaloes (*Syncerus caffer*) are reservoir hosts of BTB in South African wildlife populations. This study reports on the molecular identification and typing of 31 *M. bovis* isolates collected between 1993 and 2008 mainly from buffaloes including two lions and a bush pig in Hluhluwe-iMfolozi Park (HiP) in KwaZulu-Natal. To study the dynamics of bovine tuberculosis in the buffalo populations, a set of 28 *M. bovis* isolates from HiP and epidemiologically related parks were characterized using regions of difference RD4 and RD9 deletion analysis for species identification and spoligotyping, Variable Number of Tandem Repeats (VNTR), Polymorphic G-C rich Sequences (PGRS) and IS*6110* Restriction Fragment Length Polymorphism (RFLP) genotyping methods. The results indicated the circulation of at least three distinct *M. bovis* genotypes in HiP. The combination of VNTR typing (using a 16 loci panel) as well as IS*6110* RFLP revealed the presence of three additional genetic profiles in individual buffaloes, demonstrating that the highest level of discrimination was achieved by these typing methods. One of the spoligotypes obtained (SB0130) was dominant and represented 75 % of isolates (n = 24) from buffaloes and this pattern was also the dominant VNTR profile. A novel *M. bovis* spoligotype (SB1474), which is first reported in this study, was observed in 14.3 % (n = 4) of isolates from buffaloes and its origin is questioned. Based on their genetic relatedness the findings suggested independent introductions from at least three unrelated sources. These findings improved the previous knowledge on the diversity of circulating *M. bovis* strains in HiP.

2.2 Introduction

Bovine tuberculosis (BTB) is a chronic contagious disease caused by *Mycobacterium bovis,* a Gram-positive acid-fast bacterium. This pathogen is particularly noted for its diverse host tropism that includes humans, livestock and several wildlife species. The first report of BTB in cattle in South Africa (SA) was made by Hutcheon (1880), whilst BTB was first diagnosed in African buffaloes (*Syncerus caffer*) in Hluhluwe-iMfolozi Park (HiP) in 1986 (Jolles, 2004). Spill-over of BTB to other species has been documented (Michel *et al.* 2006; Michel *et al.* 2009). Hluhluwe-iMfolozi Park is situated in the KwaZulu-Natal Province of SA and its 100 000 ha area is almost entirely surrounded by communal farmland and livestock. It is the fourth largest game reserve in SA and has a buffalo population of approximately 3 500 heads. The molecular characterization of *M. bovis* is essential in understanding disease transmission between species and the spatial distribution of this infection in wildlife populations. In a previous study, molecular characterization of *M. bovis* isolated from wildlife in HiP was performed using a combination of three genetic tools; Restriction Fragment Length Polymorphism (RFLP), i.e. IS*6110* and Polymorphic G-C rich Sequences (PGRS) typing, and spoligotyping (Michel *et al.* 2009). In that study, *M. bovis* isolates, the majority of which was derived from buffaloes, had been collected in HiP and two epidemiologically related parks between 1993 and 2000 and presented the same IS*6110* RFLP banding pattern with only one exception (TB 328). This isolate had a shift in one of the bands (Michel *et al.* 2009). In addition, spoligotyping yielded only one type identical to SB0130 in the international *M. bovis* database (www.mbovis.org). This report also showed that epidemiologically unrelated strains are in circulation in HiP and in the Greater Kruger National Park complex (Michel *et al.* 2009).

Previous studies showed that variable number of tandem repeats (VNTR) typing is more discriminatory for *M. bovis* isolates than spoligotyping (Allix *et al.* 2006). In a study by Romero and co-workers, both spoligotyping and VNTR were found to display similar discriminatory capacity (Romero *et al.* 2008). Based on the above information, the aim of this study was to further investigate the circulating strains of *M. bovis* in HiP. Regions of difference (RD) deletion analysis was used for the identification of the isolates and spoligotyping, VNTR, PGRS and IS*6110* RFLP techniques were used for genotyping.

2.3 Materials and methods

2.3.1 Sample collection

Tissue samples, collected during BTB surveys between 1993 and 2008, from tuberculin skin test and gamma interferon test positive buffaloes were transferred to the Tuberculosis Laboratory at the Onderstepoort Veterinary Institute (OVI) for bacterial culture. A total of 28 buffaloes located in HiP (n = 20), Munyawana game reserve (n = 4), St Lucia Wetland game parks (n = 3) and a private game reserve (n = 1) were involved in the study. Buffaloes from Munyawana and St Lucia Wetland game parks (both in KwaZulu-Natal) originated from HiP, and were moved in 1997 and in 1997 to 2000, respectively. A buffalo from a private game reserve (situated in Mpumalanga Province) was born to a dam originating from HiP. Additional samples included in this study were collected during ad-hoc post mortem examinations from two lions (*Panthera leo*) from HiP and Munyawane game reserve, each, and a bush pig (*Potamochoerus larvatus*) from HiP.

2.3.2 Bacterial isolation and molecular identification

The tissue samples were processed at the Tuberculosis Laboratory of the Bacteriology Section, OVI, following standard procedures (Bengis *et al.* 1996; Alexander *et al.* 2002). The samples were inoculated onto Löwenstein-Jensen medium supplemented with pyruvate and incubated at 37 °C for up to ten weeks. Acid-fast bacterial isolates were subjected to Polymerase Chain Reaction (PCR) amplification using primers that target a sequence encoding the MPB70 antigen to identify *Mycobacterium tuberculosis* complex (MTBC) bacteria (Bengis *et al.* 1996; Alexander *et al.* 2002; Michel *et al.* 2009). *M. tuberculosis* complex bacteria were identified following the presence of a 372 bp PCR product. Deletion analysis was performed as described by Warren and co-workers (Warren *et al.* 2006) for species identification. The RD4 and RD9 genomic regions of difference were targeted using a multiplex PCR approach (Warren *et al.* 2006). The PCR products were separated on a 2-3 % agarose gel to allow for good definition of the bands. *M. bovis* isolates were confirmed by the presence of two specific bands of 268 bp and 108 bp for RD4 and RD9 respectively.

2.3.3 Genomic DNA extraction

A PUREGENE™ DNA extraction kit was used to extract genomic DNA from isolates following the manufacturer's instructions (Gentra Systems) with minor modifications. Overnight glycine-treated cells were heat killed at 94 °C for 10 min and allowed to cool down. Five hundred microliters of MSG extraction buffer (50 g/L Monosodium Glutamic Acid; 6.06 g/L Tris; 9.3 g/L EDTA) were added to the culture and cells were suspended by using an inoculating loop. Following centrifugation, 300 µl of lysis solution were added and 5 µl RNase A solution (4 mg/ml; Roche) were added to the sample and incubated at 37 °C for 30 min. A volume of 150 ul of protein precipitation solution was added and samples were centrifuged at maximum speed for 2 min, followed by precipitation of the aqueous phase with isopropanol (Merck). The resulting pellet was dissolved in 40 µl of TE buffer (10 mM Tris-HCl; 1 mM EDTA). The extracted DNA was stored at -20 °C until use.

2.3.4 Genotyping

Either pure genomic DNA (extracted using PUREGENE™ DNA extraction kit) or cell lysates (prepared by boiling the *M. bovis* cells for 25 min, and storage at -20 °C until use) were used for the PCR based typing methods i.e. spoligotyping and VNTR typing. For RFLP typing, pure genomic DNA was used.

2.3.4.1 Spoligotyping

Spoligotyping was done according to a standardized international method described by Kamerbeek *et al.* (1997) using a commercially available kit (Isogen Biosciences BV, Maarsen, The Netherlands). *M. tuberculosis* H37Rv, *M. bovis* BCG and sterile distilled water were used as controls. The spoligotype patterns were compared to those stored in the *M. bovis* spoligotype database (www.mbovis.org).

2.3.4.2 Variable number of tandem repeat (VNTR) typing

Cell lysate or genomic DNA was used for PCR amplification. Initially, VNTR analysis, also called MLVA (Multiple Locus Variable Analysis) was carried out using primers to amplify the ETR-A to ETR-F loci (Frothingham and Meeker-O'Connell, 1998). An additional VNTR on ten loci (Mtub 30, MIRU 10, Mtub 39, MIRU 16, Mtub 21, Mtub 12,

Mtub 2, MIRU 2, Mtub 29 and Qub-11a) was carried out as described by Le Fleche and co-workers (Le Fleche *et al.* 2002). PCR was carried out using an Eppendorf AG 22331 Hamburg thermo cycler (Merck). The PCR products were separated on a 2 % agarose gel at 85 V for three hours and visualized under UV light (Spectroline^{R} model TC-312A transilluminator 312 nm ultraviolet). A 100 bp ladder (Inqaba Biotechnology) was included as a marker. The band sizes were determined by using Quantity one 1-D Analysis software installed in the Gel Doc system (Bio-Rad), and were converted to copy numbers (ETRs: Frothingham and Meeker-O'Connell, 1998 and additional loci: Le Fleche *et al.* 2002) and saved in an excel format. The genotyping data obtained was exported from excel format into Bionumerics software (Applied Maths, Saint-Martens-Latum, Belgium). The categorical coefficient option was used for clustering analysis of the data to create a dendrogramme.

2.3.4.3 Polymorphic G-C rich Sequence (PGRS) and IS*6110* RFLP typing

Polymorphic G-C rich sequence and IS*6110* RFLP typing were performed as previously reported (Cousins *et al.* 1998; Michel *et al.* 2008). Analysis of the IS*6110* RFLP results was done using the GelCompar II system (Applied Maths, Sint-Martens-Latum, Belgium), using the similarity coefficient of Dice and the unweighted pair group method with arithmetic averages (UPGMA) for clustering maximum tolerance 1.2 %. PGRS RFLP patterns results were analyzed manually.

2.4 Results

2.4.1 *Mycobacterium bovis* isolation and molecular identification

Bacterial growth was observed on Löwenstein-Jensen culture slopes after 6-10 weeks of incubation. Ziehl-Neelsen staining of culture smears confirmed the presence of acid-fast bacilli. Isolates were classified as members of the *Mycobacterium tuberculosis* complex following detection of a 372 bp fragment by PCR reaction using primers targeting the sequence encoding the MPB70 antigen of *M. tuberculosis* complex. Deletion analysis using the multiplex PCR targeting the regions of difference RD4 and RD9 (Warren *et al.* 2006) further confirmed the isolates as *M. bovis* due to the amplification of DNA products with the expected band sizes of 268 bp and 108 bp, respectively (results not shown).

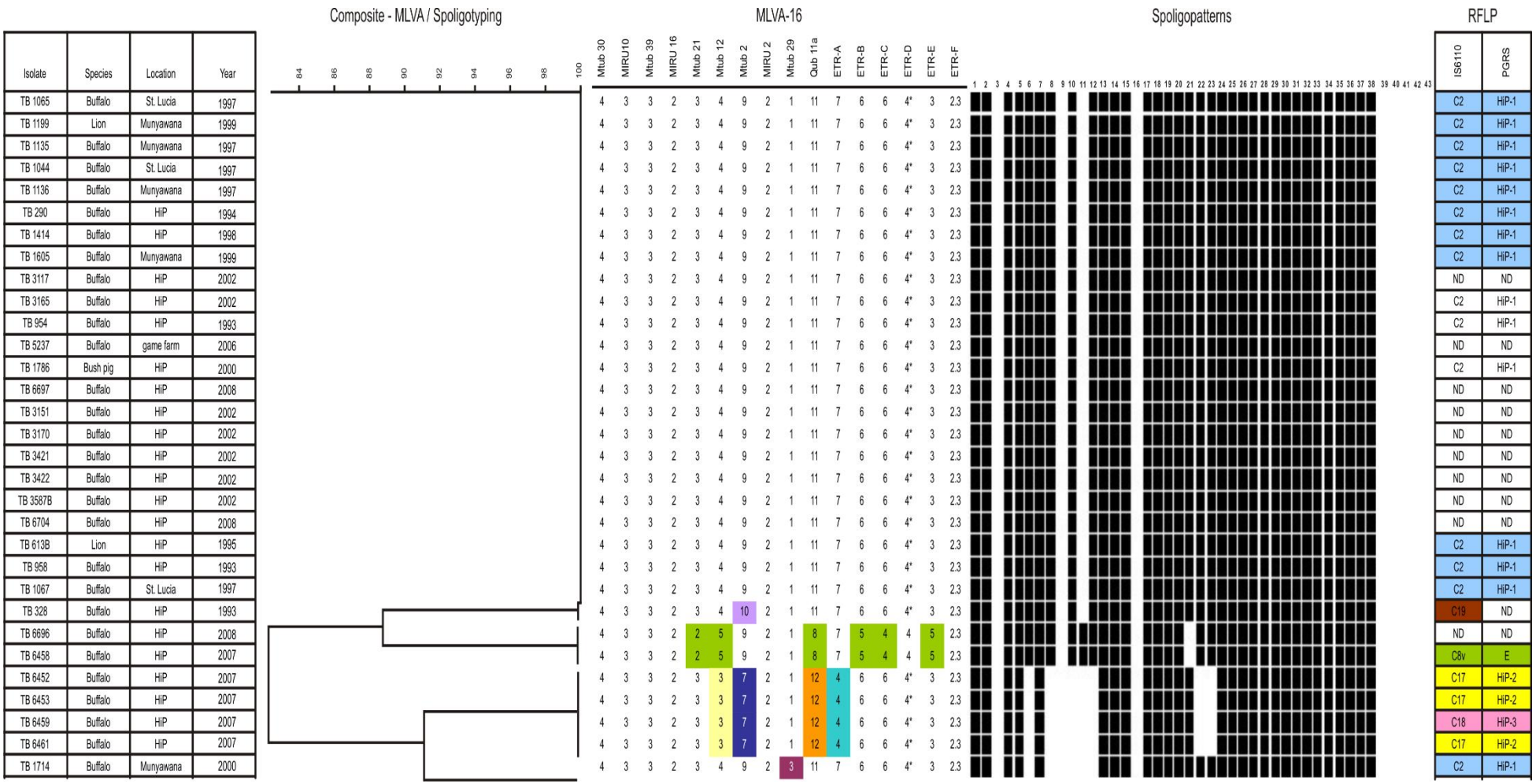
2.4.2 Spoligotyping

Spoligotyping was performed on all 31 *M. bovis* isolates from buffaloes (n = 28), lion (n = 2) and bush pig (n = 1). The results obtained (Figures 3 and 4) revealed four different spoligopatterns. The most prevalent spoligotype pattern was found in 24 of the 31 (77.4 %) *M. bovis* isolates, including 21 isolates from buffaloes, two isolates from lions and one bush pig. This spoligotype pattern was marked by the absence of spacers 3, 9, 11, 16 as well as spacers 39-43, which is consistent with SB0130 (www.mbovis.org). The strain type was identified in isolates collected in HiP throughout the period under examination (1993 to 2008) and was previously reported (Michel *et al.* 2009). A second spoligotype pattern was found in four buffaloes (TB 6452, TB 6453, TB 6459 and TB 6461) sampled in 2007. This *M. bovis* spoligopattern had not been described in the *M. bovis* database before and was allocated a new code SB1474. It was characterized by the lack of spacers 3, 6, 8, 9, 10, 11, 12, 16, 22, 23 as well as 39-43. A third spoligotype pattern was found in two *M. bovis* isolates from buffaloes sampled in 2007 and 2008, respectively (TB 6458 and TB 6696). The pattern, designated as SB0121, is delineated by the absence of spacers 3, 9, 16, 21 and lack of spacers 39-43. This pattern is similar to *M. bovis* BCG except that spacer 21 is present in *M. bovis* BCG. Another spoligotype pattern was isolated in 2 000 from a buffalo (TB 1714) that was translocated from HiP and was kept in a boma at Munyawane game reserve. This particular pattern was characterized by the absence of spacers 3, 6, 8, 9, 10, 11, 12-16 and by the lack of spacers 39-43 and is identical to spoligotype SB0140 in the international *M. bovis* database.

2.4.3 VNTR analysis

Results of tandem repeats analysis performed on all 31 isolates using a total of 16 loci are summarized in Figure 3. By comparing the copy numbers of the VNTR profile of each isolate, five profiles were observed. The most prevalent VNTR profile was found in 23 of the 31 *M. bovis* isolates from buffaloes, lions and a bush pig. This VNTR profile was essentially shared by TB 328 (second profile) and TB 1714 (third profile) with the exception of the copy numbers relating to locus Mtub 12 (in TB 328) and locus Mtub 29 (in TB 1714), which resulted in over 98 % homology. The remaining two VNTR profiles were found in four (TB 6452, TB 6453, TB 6459 and TB 6461) and two (TB 6458 and TB 6696) *M. bovis* isolates from buffaloes, respectively, collected during 2007 and 2008. The genetic diversity observed in this sample set resulted from the use of a

combination of the following loci: ETR-A, ETR-B, ETR-C, ETR-E, Mtub 21, Mtub 12, Mtub 2, Mtub 29 and Qub-11a.



Isolate	Species	Location	Year	Mtub 30	MIRU10	Mtub 39	MIRU 16	Mtub 21	Mtub 12	Mtub 2	MIRU 2	Mtub 29	Qub 11a	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	ETR-F	IS6110	PGRS
TB 1065	Buffalo	St. Lucia	1997	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1199	Lion	Munyawana	1999	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1135	Buffalo	Munyawana	1997	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1044	Buffalo	St. Lucia	1997	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1136	Buffalo	Munyawana	1997	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 290	Buffalo	HiP	1994	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1414	Buffalo	HiP	1998	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1605	Buffalo	Munyawana	1999	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 3117	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3165	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 954	Buffalo	HiP	1993	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 5237	Buffalo	game farm	2006	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 1786	Bush pig	HiP	2000	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 6697	Buffalo	HiP	2008	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3151	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3170	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3421	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3422	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 3587B	Buffalo	HiP	2002	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 6704	Buffalo	HiP	2008	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	ND	ND
TB 613B	Lion	HiP	1995	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 958	Buffalo	HiP	1993	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 1067	Buffalo	St. Lucia	1997	4	3	3	2	3	4	9	2	1	11	7	6	6	4*	3	2.3	C2	HiP-1
TB 328	Buffalo	HiP	1993	4	3	3	2	3	4	10	2	1	11	7	6	6	4*	3	2.3	C19	ND
TB 6696	Buffalo	HiP	2008	4	3	3	2	2	5	9	2	1	8	7	5	4	4	5	2.3	ND	ND
TB 6458	Buffalo	HiP	2007	4	3	3	2	2	5	9	2	1	8	7	5	4	4	5	2.3	C8v	E
TB 6452	Buffalo	HiP	2007	4	3	3	2	3	3	7	2	1	12	4	6	6	4*	3	2.3	C17	HiP-2
TB 6453	Buffalo	HiP	2007	4	3	3	2	3	3	7	2	1	12	4	6	6	4*	3	2.3	C17	HiP-2
TB 6459	Buffalo	HiP	2007	4	3	3	2	3	3	7	2	1	12	4	6	6	4*	3	2.3	C18	HiP-3
TB 6461	Buffalo	HiP	2007	4	3	3	2	3	3	7	2	1	12	4	6	6	4*	3	2.3	C17	HiP-2
TB 1714	Buffalo	Munyawana	2000	4	3	3	2	3	4	9	2	3	11	7	6	6	4*	3	2.3	C2	HiP-1

* indicates that there was a 24 bp deletion in one of the tandem repeats in VNTR-D locus

Figure 3 Results obtained from different typing methods applied i.e. spoligotyping VNTR, IS*6110* and PGRS typing of *M. bovis* isolates from HiP and epidemiologically related game reserves, including isolate identification, species involved, location and year of isolation.

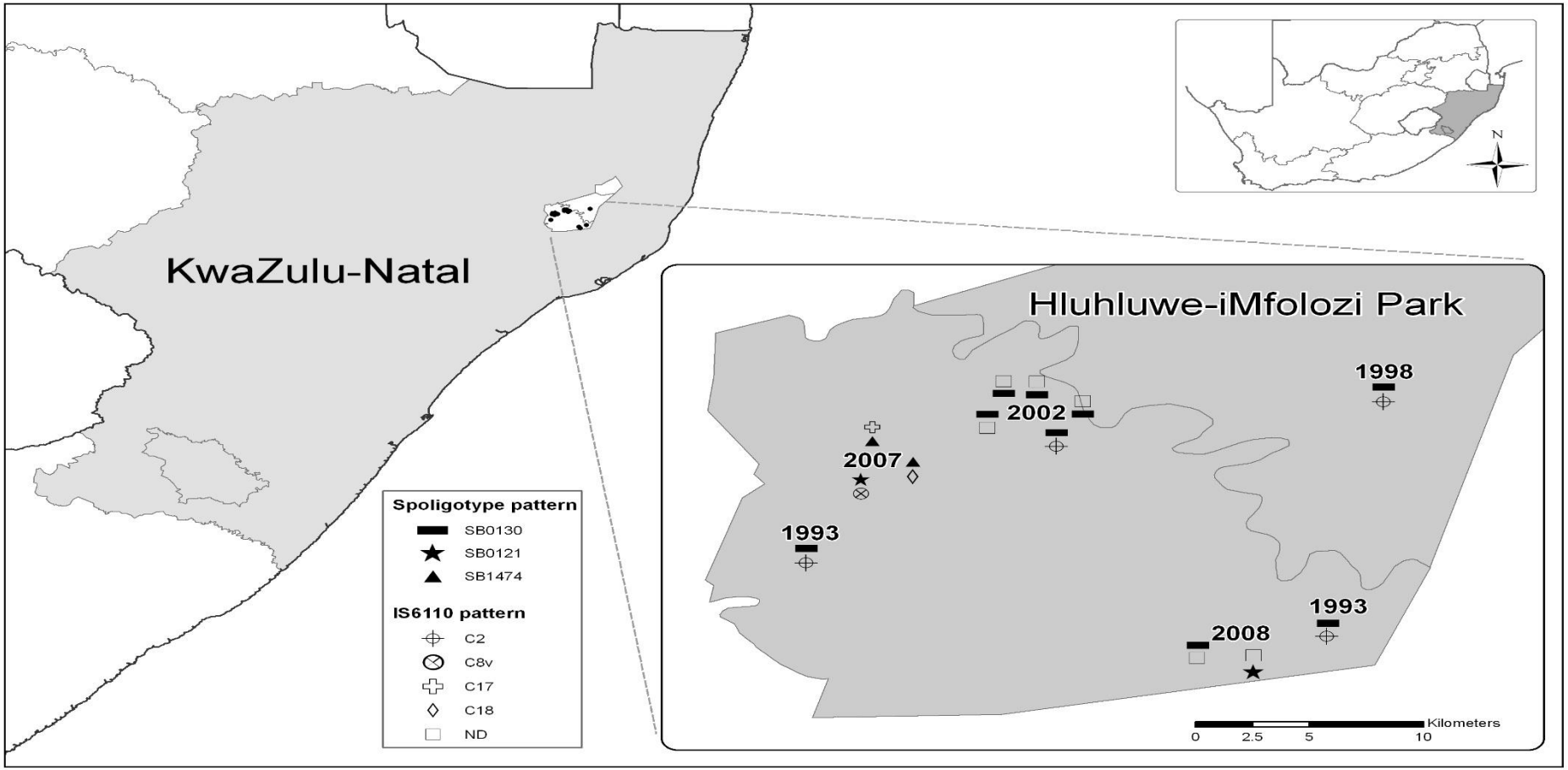


Figure 4 Geographical distribution of spoligotype patterns (SB0130, SB0121, and SB01474) and IS*6110* patterns (C2, C17, C18) of *M. bovis* isolates from HiP. Due to lack of information relating to coordinates, locations of some isolates are missing on the map. ND indicates that IS*6110* typing was not done.

2.4.4 Polymorphic G-C rich sequences (PGRS) RFLP typing

Polymorphic G-C rich sequences RFLP typing was performed on a total of five *M. bovis* isolates cultured from buffaloes sampled in 2007 (Figure 3: TB 6452, TB 6453, TB 6459, TB 6461 and TB 6458). The PGRS RFLP profiles of 16 isolates had been analyzed previously and were found to share a unique profile (Michel *et al.* 2009). In the present study, three PGRS DNA profiles were observed. One profile (HiP-2) was present in three of the five *M. bovis* isolates analyzed, while genetically different profiles (HiP-3 and E) were found in one of the isolates each (Figure 3).

2.4.5 IS*6110* RFLP typing

The same five *M. bovis* isolates (TB 6452, TB 6453, TB 6458, TB 6459, and TB 6461) as analyzed by PGRS RFLP typing were subjected to IS*6110* RFLP typing. Three IS*6110* patterns were identified, i.e. C17, C18, C8v. *M. bovis* isolates with identical PGRS RFLP patterns were at the same time found to have identical IS*6110* RFLP patterns (Figure 3).

2.5 Discussion

This study presents data obtained from *M. bovis* isolated from HiP and three epidemiologically related game reserves during the period between 1993 and 2008. PCR-based genotyping tools i.e. spoligotyping and VNTR typing were primarily applied to characterize 31 *M. bovis* isolates, with additional use of PGRS and IS*6110* RFLP typing on some of the samples for added discriminatory power.

The majority of *M. bovis* infections examined in this study were caused by a unique genotype represented in 24 out of 31 isolates (including isolates from two lions and a bush pig) which corresponded to a spoligopattern which was previously identified as SB0130 (Michel *et al.* 2009). These results were essentially in agreement with those produced by means of VNTR typing, with the exception that VNTR typing singled out one isolate (TB 328) on the basis of one difference in locus Mtub 2. This finding was supported by previous IS*6110* typing results (Michel *et al.* 2009). Based on the high homology between TB 328 and the dominant VNTR typing profile it is unlikely that

TB 328 was the result of a separate introduction of *M. bovis* into HiP but that it is linked with the dominant strain through an evolutionary process.

Spoligotyping and VNTR typing results were also found to be in full agreement regarding a unique genotype isolated in buffaloes sampled in 2007 (TB 6452, 6253, 6459, 6461). The spoligopattern was never described in HiP as well as in the international *M. bovis* database and was therefore designated a new code SB1474. However, when IS*6110* and PGRS RFLP typing data were taken into consideration for this group of isolates, both markers were able to discriminate between two profiles (e.g. C17 and C18). The genetic diversity of these strains collected in 2007 suggests that they did not evolve from the dominant HiP strain (SB0130) and therefore most likely represent an independent introduction of *M. bovis* into HiP.

During the period prior to 2 000 buffalo number TB 1714 was translocated from HiP and was kept in a boma at Munyawane game reserve where it was euthanized and sampled in 2000. Spoligotyping revealed a strain SB0140 which is related to the novel SB1474. The novel strain (SB1474) differs from SB0140 by deletion of spacers 22 and 23 in addition to the loss of spacers 3, 6, 8, 9, 10, 11, 12, 16 descriptive of the BOVIS2 family (Brudey *et al.* 2006) (Figure 3). The origin of this strain (SB0140) remains elusive despite identical PGRS and IS*6110* typing results with SB0130 (Figure 3).

Evidence of a further independent source of *M. bovis* to HiP was found in two buffaloes (TB 6458 and TB 6696) sampled in 2007 and 2008, which both carried the spoligotype SB0121. This particular spoligotype pattern was previously isolated in cattle in South Africa: in Mpumalanga and Limpopo provinces (Michel *et al.* 2008), as well as in buffaloes and other wildlife in the Kruger National Park (Michel *et al.* 2009). Additional IS*6110* and PGRS RFLP typing (only on TB 6458) confirmed that this genotype was a member of the C8 variant strain complex which is the cause of the BTB epidemic in the KNP (Michel *et al.* 2009). It is unclear whether the strain could have been introduced more recently from infected cattle or wildlife translocated from areas neighbouring the KNP to areas neighboring the HiP. Alternatively, the C8 outbreak strain in the KNP could have been more widely spread in the eastern parts of the country than previously known and undergone similar evolutionary processes elsewhere as described for KNP (Michel *et al.* 2009). It is also possible that, at one point in time, strains present in buffaloes sampled in 2007 (western part of HiP) and 2008 (eastern part of HiP) may not

have been present in buffaloes sampled before 2002 (eastern part of HiP). Indeed, earlier behavioral studies indicate that HiP buffaloes have relatively stable and small home ranges (compared to KNP) due to the abundant food and water availability in HiP, even in winter (Dora, 2004). It was also shown that there was very little if any mixing of breeding herds with adult bulls implicated as the main source of infection between herds.

The use of the above-mentioned methods proved to be useful in confirming the origins of *M. bovis* strains translocated to different locations, i.e. St Lucia Wetland game parks, in KwaZulu-Natal Province, and a private game reserve in Mpumalanga Province. In all cases, the *M. bovis* strain types were identical to the most prevalent type in HiP. The methods show that the prevalent strain has spilled-over to other wildlife species other than buffaloes in the cases of two lions and a bush pig as indicated by typing results of spoligotyping and VNTR in the present study, and by IS*6110* in the previous study by Michel and co-workers (Michel *et al.* 2009).

The identification of previously undetected *M. bovis* strains in the HiP is of great concern, since it indicates the possibility of multiple introductions in the past and a persistent risk for new *M. bovis* introductions into HiP, as well as a risk of spill-back to communal cattle farms which surround the HiP. This poses a huge challenge for controlling the disease in livestock, and has an impact on wildlife management and potentially on public health (Michel *et al.* 2002; Romero *et al.* 2008).

2.6 Conclusion

The molecular typing methods applied have been useful in studying the epidemiology of BTB in HiP and providing valuable information for future disease management strategies. This study further suggested that BTB detection surveys are needed around the HiP in communal cattle herds to identify and efficiently manage sources of *M. bovis* infection.

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Chapter 3
Evaluation of the discriminatory power of variable number of tandem repeat (VNTR) typing of *Mycobacterium bovis* isolates from southern Africa

Hlokwe, T.M., Van Helden, P., Michel, A. 2013. Transboundary and Emerging Diseases. 60(Suppl 1): 111-120

3.1 Abstract

The usefulness of variable number of tandem repeat (VNTR) typing based on limited numbers of loci has previously proven inferior compared to IS*6110*-RFLP typing when applied to the study of the molecular epidemiology of bovine tuberculosis in both livestock and wildlife in southern Africa. In this study, the discriminatory power of 29 published VNTR loci in the characterization of 131 *Mycobacterium bovis* strains isolated predominantly from wildlife and a smaller number from livestock in southern Africa was assessed. Allelic diversities calculated when loci were evaluated on a selected panel of 23 *M. bovis* isolates with identified varying degrees of genetic relatedness from different geographic origins as well as *M. bovis* BCG ranged from 0.00-0.63. Of the 29 loci tested, 13 were polymorphic (Qub-11a, Qub-11b, Qub-18, ETR-B and -C, Mtub 21, MIRU 16 and 26, ETR-E, Qub-26, MIRU 23, ETR-A, and Mtub 12). In addition, a comparative evaluation of the 13 loci on a panel of 65 isolates previously characterized by IS*6110* Restriction Fragment Length Polymorphism (RFLP) typing and further evaluation on 41 isolates with no typing history from Kruger National Park (KNP) highlighted that *M. bovis* from epidemiologically unrelated cases of bovine tuberculosis (BTB) in different geographic regions can be adequately distinguished. However, there is a need for improvement of the method to fully discriminate between the parental KNP strain and its clones to allow the detection of evolutionary events causing transmission between and within wildlife species.

Keywords: *Mycobacterium bovis*; VNTR typing; bovine tuberculosis; wildlife; Kruger National Park

3.2 Introduction

Bovine tuberculosis (BTB) was first diagnosed in the Kruger National Park (KNP) in African buffaloes (*Syncerus caffer*) in 1990. Over the past twenty years, the disease has spread throughout the park and there is evidence showing that it has spilled-over into at least 12 other small and large mammalian species (Michel and Bengis, 2012). The establishment of the Greater Limpopo Transfrontier Park (GLTFP), which already links South Africa's KNP with the Limpopo National Park in Mozambique and a future link is envisaged with the Gonarezhou National Park in Zimbabwe, might increase the risk of the disease spreading within the GLTFP and out of the park to livestock in neighbouring farming areas (Michel *et al*. 2006). In 2008, *M. bovis* was isolated from two African buffaloes in the Gonarezhou National Park, less than 45 km from the unfenced northern boundary of the KNP, raising questions regarding the origin of these cases (De Garine-Wichatitsky *et al.* 2010).

Bovine tuberculosis is a serious threat to livestock and wildlife species and is therefore controlled in many countries. A better understanding of transmission patterns and distribution of the disease will permit more precise targeting of control measures that will potentially benefit both wildlife and livestock (Aranaz *et al*. 2004; Michel *et al.* 2009). Molecular typing techniques are now useful tools that can be applied to *M. bovis* epidemiology in order to underpin control of BTB (Hilty *et al*. 2005; Allix *et al.* 2006; Boniotti *et al*. 2009; Hlokwe *et al.* 2011).

Restriction fragment length polymorphism (RFLP) typing techniques using IS*6110* and PGRS (Polymorphic G-C Rich Sequences) as probes as well as spoligotyping were previously applied to establish diversity of *M. bovis* strains within the KNP. The study revealed that the BTB epidemic was most likely caused by a single outbreak strain designated C8 (cattle strain). Due to a clonal expansion of the C8 strain in the wildlife population and accompanying changes in its genome, at least 21 C8 variant strains could be identified by IS*6110* RFLP typing. As expected, some variant strains of C8 were found to occur in spatial clusters and in wildlife species within these territories (Michel *et al.* 2009).

Although IS*6110* RFLP typing provided useful insight into the epidemiology of BTB in the KNP, this technique is time consuming, technically demanding and requires large amounts of pure genomic DNA and complex data analysis (Allix *et al.* 2006).

Spoligotyping on the other hand, though PCR based and therefore rapid, could not differentiate the parent C8 strain from its variant strains because of slow evolutionary rate of the direct repeat region (Smith *et al*. 2003). Spoligotyping is therefore considered unsuitable to study recent *M. bovis* transmission events within and between wildlife species in the KNP.

More PCR (Polymerase Chain Reaction (PCR) based genotyping methods have become available for rapid molecular epidemiology investigations. Initially, six variable number of tandem repeat (VNTR) loci/markers described as exact tandem repeats A to F (ETR-A, -B, -C, -D, -E, and -F) were reported to be more discriminatory than spoligotyping (Frothingham and Meeker-O'Connell,1998). A novel class of genetic markers collectively known as MIRU-VNTR was later described. This includes the ETRs, mycobacterial interspersed repetitive units (MIRUs) (Supply *et al.* 2000 and 2006) and VNTRs (Le Fleche *et al.* 2002; Roring *et al.* 2002; Skuce *et al.* 2002). Most of these differently named loci have received the reference names according to their position in the *M. tuberculosis* H37Rv genome (Supply *et al.* 2006). VNTR typing is highly discriminative for *M. tuberculosis* isolates and therefore has the potential to be a method of choice for typing *M. bovis* (Hilty *et al*. 2005; Allix *et al*. 2006).

In the current study, we have assessed VNTR typing using 29 loci for molecular epidemiological characterization of *M. bovis* isolates from southern Africa, with special emphasis on isolates from the KNP. The aim was to select a set of loci that provides sufficient discriminatory power to detect evolutionary events indicative of recent transmissions.

3.3 Materials and methods

3.3.1 Bacterial isolation and identification

Frozen *Mycobacterium bovis* isolates previously typed by RFLP and/or spoligotyping methods were used. These *M. bovis* isolates had been collected between 1993 and 2008 from different wildlife species and cattle from different regions in South Africa (SA) (Table 2). The isolates were sub-cultured on Löwenstein-Jensen medium supplemented with pyruvate and incubated at 37 °C until confluent growth was observed. Additional *M. bovis* isolates were isolated from tissue samples received in the Tuberculosis Laboratory of the Onderstepoort Veterinary Institute for routine

diagnostic purposes. These isolates were collected between the years 2004 and 2010 and no previous genetic analysis was done. Processing and isolation was performed according to standard procedures (Alexander *et al.* 2002). For identification, acid fast isolates were subjected to PCR amplification using primers that targets a sequence encoding the MPB70 antigen to identify *Mycobacterium tuberculosis* complex (MTBC) bacteria (Michel *et al.* 2009; Hlokwe *et al*. 2011). *M. bovis* was confirmed by deletion analysis PCR as described by Warren and co-workers (Warren *et al.* 2006). The PCR products were separated in 1.5-2 % agarose gels to allow a good separation of fragments. *M. bovis* isolates were confirmed by the presence of two specific bands of 268 bp and 108 bp for RD4 and RD9, respectively.

3.3.2 Bacterial DNA extraction

Depending on the amount of culture available, DNA from *M. bovis* isolates was extracted by either using a PUREGENE^{TM} DNA purification kit (Gentra Systems, Minneapolis, USA) or a crude preparation made by boiling *M. bovis* cells at 100 °C for 25 min and storage at -20 °C until use as previously reported (Hlokwe *et al*. 2011).

3.3.3 Variable number of tandem repeat (VNTR) typing of *M. bovis* isolates

The performance of available VNTR loci in the genetic characterisation of South African *M. bovis* isolates was evaluated in two stages. Initially, a total of 29 loci/markers were assessed for their ability to amplify the expected DNA product as reported by previous investigators, the repeatability of the amplification as well as for polymorphism within a selected panel of *M. bovis* strains with high genetic diversity. Markers which failed on any of these criteria were excluded from further evaluation. Markers considered suitable were then compared based on their allelic diversity and combined discriminatory power among wildlife isolates from the KNP. IS*6110* RFLP profiles were available for the majority of the isolates and have assisted in the overall evaluation of the VNTR typing method.

3.3.3.1 Stage 1

A set of 29 loci, including the Exact Tandem Repeats (ETR-A, -B, -C, -D, -E and -F), the Mycobacterial Interspersed Repetitive Units (MIRU 02, 10, 16, 23, 26, 27, 39, 40), the Queen's University of Belfast VNTRs (Qub-11a, 11b, 18, 26, 3232 and 3336) as well as Mtub 01, 02, 12, 21, 29, 30, 31, 38 and 39 were applied as described by other authors (Frothingham and Meeker-O'Connell, 1998; Supply *et al*. 2000; Skuce *et al*. 2002; Le Fleche *et al.* 2002; Roring *et al.* 2004; Supply *et al;* 2006). The PCR primer sequences are described in Table 3. The suitability of the loci was assessed on a genetically highly diverse panel (Panel 1) of 23 isolates from wildlife and livestock from southern Africa that were carefully selected based on profiling by RFLP typing, spoligotyping as well as traditional disease outbreak investigations (Hlokwe, T., unpublished results; Michel *et al.* 2008; Michel *et al*. 2009; Hlokwe *et al.* 2011). The panel comprised of epidemiologically related and unrelated strains from different geographical regions and host species (Table 2).

3.3.3.2 Stage 2

A set of 13 loci described in stage 1 (i.e. Qub-11a, 11b, 18, and 26; Mtub 12 and 21, MIRU 16, 23 and 26, and ETR-A, -B, -C and -E) were further subjected to a panel of 65 *M. bovis* isolates (Panel 2) from different wildlife hosts in the southern part of the KNP previously characterized by IS*6110* RFLP typing. The *M. bovis* isolates included the parent C8 type (cattle strain) and 12 of its unique variant strains (Michel *et al.* 2009).

The same 13 loci were used to analyse 43 *M. bovis* isolates (Panel 3) from different wildlife species (i.e. Forty one of these cases were from the northern part of the park and isolates were never characterized before, whereas two cases were from the Gonarezhou National Park in Zimbabwe (Table 2).

3.3.4 PCR amplification of VNTR loci

Primer sets as outlined previously (Frothingham and Meeker-O'Connell, 1998; Le Fleche *et al.* 2002) for the different loci were used for amplification. VNTR typing PCR was performed in a 20 µl reaction containing 2 µl of template DNA, 10 µl of the Qiagen master mix, 7 µl of DNA free water and 0.5 µl of each 20 pM primer. The cycling parameters were as follows: initial denaturation at 94 °C for 5 min, followed by

40 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, elongation at 72 °C for 1.5 min and a final elongation step at 72 °C for 10 min. PCR was done using an Eppendorf AG 22331 Hamburg thermo cycler (Merck Eppendorf, Hamburg, Germany).

3.3.5 Estimation of molecular size of the amplified DNA fragments and analysis of VNTR profiles

The PCR products were separated electrophoretically as outlined previously. The resulting fragment band sizes were estimated by comparison with the size marker (100 bp or 100 bp Plus DNA ladder, Fermentas Life Sciences, Vilnus, Lithuania) and/or by use of the Quantity One 1-D analysis software installed in the Gel doc system (Bi-Rad Laboratories, Milan, Italy) (Hlokwe *et al*. 2011). The sizes were converted to copy numbers (Le Fleche *et al*. 2002) and the resulting VNTR profiles were saved in a spread sheet and analysed using the Bionumerics software package (Applied Maths, St-Martin-Latem, Belgium).

3.3.6 Calculation of allelic diversity and construction of phylogenetic trees

Allelic diversity of each locus was calculated using the Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat (Hunter and Gaston, 1988). The genetic relationships of *M. bovis* isolates were deduced by construction of an UPGMA (Unweighted Pair Group Mean Average) tree as well as the maximum parsimony tree using the Bionumerics software package version 6.6 (Applied Maths, St-Martin-Latem, Belgium).

3.3.7 Spoligotyping

All isolates not spoligotyped before (n = 116) were subjected to spoligotyping for additional comparative evaluation of VNTR loci. Spoligotyping was done according to a standardized international method described by Kamerbeek and co-workers (Kamerbeek *et al.* 1997) using a commercially available kit (Ocimum Biosolutions, Indianapolis, United States of America). *M. tuberculosis* H37Rv, *M. bovis* BCG and sterile distilled water were used as controls. The spoligotype patterns were compared to those stored in the *M. bovis* spoligotype database (www.mbovis.org).

3.4 Results

3.4.1 *Mycobacterium bovis* isolates

In total, DNA from 131 *M. bovis* isolates was available for use in the study (Table 2). This panel included 77 IS*6110* RFLP typed isolates of wildlife species from the southern part of KNP, 11 isolates from wildlife species (n = 5) outside the KNP and cattle (n = 6) from different geographical regions of South Africa with varying degrees of genetic relatedness (as determined either by IS*6110* RFLP typing or a combination of IS*6110* RFLP typing, PGRS typing, spoligotyping or traditional outbreak investigation), as well as a panel of 41 isolates from different wildlife species from the northern part of KNP not characterized before, and two from Gonarezhou National Park which were only partially characterized using VNTR loci ETR-A to F (De Garine-Wichatitsky *et al.* 2010).

Table 2 Animal species and numbers of isolates from different regions analysed in this study

Animal species	Location							
	KNP	Gonarezhou	SNR	MP*	KZN	NW	HiP	Total
Cattle				4	1	1		6
Buffalo	51	2	1				4	58
Lion	42							42
Kudu	12							12
Baboon	6							6
Leopard	4							4
Cheetah	1							1
Warthog	1							1
Bushbuck	1							1
Total	**118**	**2**	**1**	**4**	**1**	**1**	**4**	**131**

KNP = Kruger National Park; HiP = Hluhluwe-iMfolozi Park; SNR = Spioenkop Nature reserve; MP* = Different regions in Mpumalanga Province; KZN = KwaZulu-Natal Province; NW = North West Province

3.4.2 Variable number of tandem repeat (VNTR) analysis of *M. bovis* isolates

Two-stage evaluation of VNTR loci:

3.4.2.1 Stage 1

Twenty nine loci (Table 3) were assessed individually and comparatively on a test panel of 23 genetically diverse *M. bovis* isolates (Panel 1: Figure 5). Of the 29 loci studied, 13 were found to be stable and polymorphic (Qub-11a, Qub-11b, Qub-18, ETR-B, ETR-C, Mtub 21, MIRU 26, MIRU 16, ETR-E, Qub-26, MIRU 23, ETR-A, Mtub 12) and 14 were monomorphic (Mtub 1, Mtub 2, Mtub 29, Mtub 30, Mtub 31, Mtub 38, Mtub 39, MIRU 2, MIRU 10, MIRU 27, MIRU 39, ETR-D, ETR-F, Qub-23). Two loci (Qub-3232 and Qub-3336) could not be studied, since we either could not amplify DNA or unreliable results with multiple DNA fragments were produced during repeat runs (Table 4). All monomorphic loci as well as Qub-3232 and Qub-3336 were regarded as unsuitable and therefore excluded from the evaluation.

The allelic diversity of individual loci was calculated and results are shown in Table 4. Ten loci (Qub-11a, Qub-11 b, Qub-18, ETR-B, ETR-C, Mtub 21, MIRU 16, MIRU 26, ETR-E and Qub-26) were regarded as highly discriminating, with allelic diversity ranging from 0.42-0.63 and three loci (MIRU 23, ETR-A and Mtub 12) were grouped as moderately discriminating (allelic diversity ranging from 0.16-0.33). Lastly, 14 loci (Mtub 1, Mtub 2, Mtub 29, Mtub 30, Mtub 31, Mtub 38, Mtub 39, MIRU 2, MIRU 10, MIRU 27, MIRU 39, ETR-D, ETR-F and Qub-23) were regarded as poorly discriminating, with allelic diversity of 0.0 per locus and results were discarded. Of the 13 polymorphic loci, only Qub-26 correctly identified isolates KNP 70 and KNP 171 as C8 variant strains (from a total of 9 variants strains), with two and three copies of tandem repeats, respectively, at this locus, while the other KNP isolates harboured four copies. Isolate TB 1464, which was previously classified as a C8 strain, was identified by ETR-C locus as a C8 variant strain (Figure 5). Other loci were useful in discriminating between isolates from different geographical regions, as well as identifying isolates with known epidemiological linkage. Isolates from distinct geographic localities were found to have unique VNTR profiles, whereas epidemiologically related isolates harboured identical VNTR profiles (Figures 5 and 6).

Table 3 Variable number of tandem repeat (VNTR) loci and PCR primer sequences used in this study

VNTR locus	VNTR alias	VNTR locus size (bp)	Forward primer	Reverse Primer
2163a	Qub-11a	69	CCCATCCCGCTTAGCACATTCGTA	TTCAGGGGGGATCCGGGA
2163b	Qub-11b	69	CGTAAGGGGGATGCGGGAAATAGG	CGAAGTGAATGGTGGCAT
1982	Qub-18	78	ATCGTCAGCTGCGGAATAGT	AATACCGGGGATATCGGTTC
2461	ETR-B	57	GCGAACACCAGGACAGCATCATG	GGCATGCCGGTGATCGAGTGG
0577	ETR-C	58	GACTTCAATGCGTTGTTGGA	GTCTTGACCTCCACGAGTGC
1955	Mtub 21	57	AGATCCCAGTTGTCGTCGTC	CAACATCGCCTGGTTCTGTA
2996	MIRU 26	51	CCCGCCTTCGAAACGTCGCT	TGGACATAGGCGACCAGGCGAATA
1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA	CCCGTCGTGCAGCCCTGGTAC
3192	ETR-C	53	ACTGATTGGCTTCATACGGCTTTA	GTGCCGACGTGGTCTTGAT
4052	Qub-26	111	AACGCTCAGCTGTCGGAT	GGCCAGGTCCTTCCCGAT
2531	MIRU 23	53	CAGCGAAACGAACTGTGCTATCAC	CGTGTCCGAGCAGAAAAGGGTAT
2165	ETR-C	75	ATTTCGATCGGGATGTTGAT	TCGGTCCCATCACCTTCTTA
1121	Mtub 12	15	CTCCCACACCCAGGACAC	CGGCCTACCCAACATTCC
0024	Mtub 1	18	GACAAACAGGAGGGCGTTG	TATTACGACGACCGCTATGC
0079	Mtub 2	9	CGTGCACAGTTGGGTGTTTA	TTCGTTCAGGAACTCCAAGG
2347	Mtub 12	57	AACCCATGTCAGCCAGGTTA	ATGATGGCACACCGAAGAAC
2401	Mtub 30	58	AGTCACCTTTCCTACCACTCGTAAC	ATTAGTAGGGCACTAGCACCTCAAG
2990	Mtub 31	55	GTGACGTTTACCGTGCTCTATTTC	GTCGTCGGACAGTTCTAGCTTT
3663	Mtub 38	63	GCCCAAAAAGCATGGGAACGTGCCCCT	GGTTGTCCCCGCAGTATCTC
3690	Mtub 39	58	AATCACGGTAACTTGGGTTGTTT	GATGCATGTTCGACCCGTAG
0154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT	TACTCGGACGCCGGCTCAAAAT
0959	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC	GCCACCTTGGTGATCAGCTACCT
3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA	GCGATGTGAGCGTGCCACTCAA
4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC	CGGAAACGTCTACGCCCCACACAT
0577	ETR-D	77	CAGGTCACAACGAGAGGAAGAGC	GCGGATCGGCCAGCGACTCCTC
1612	Qub-23	21	GCTGCACCGGTGCCCATC	CACCGGAGCCGGAACGGC
3239	ETR-F	79	CTCGGTGATGGTCCGGCCGGTCAC	GGAAGTGCTCGACAACGCCATGCC
3232	Qub-3232	56	CAGACCCGGCGTCATCAAC	CCAAGGGCGGCATTGTGTT
3336	Qub-3336	59	ATCCCCGCGGTACCCATC	GCCAGCGGTGTCGACTATCC

Table 4 Determination of heterogeneity at each of the loci among the 23 genetically diverse *M. bovis* from different geographic regions in southern Africa (Panel 1) and *M. bovis* BCG.

Locus	No. of isolates and Copy number													Allelic Diversity
	1	2	3	4	5	6	7	8	9	10	11	12	15	
Qub-11a								14		4	3	2	1	0.63
Qub-11b		14	5	5										0.60
Qub-18		6	4	14										0.60
ETR-B				14	10									0.50
ETR-C			14		10									0.50
Mtub 21		14	10											0.50
MIRU 26			1		16	7								0.49
MIRU 16		8	16											0.46
ETR-E			8	16										0.46
Qub-26		3	3	18										0.42
MIRU 23		5		19										0.33
ETR-A				1	1	20	2							0.30
Mtub 12			2	22										0.16
Mtub 1									24					0.00
Mtub 2							24							0.00
Mtub 29			24											0.00
Mtub 30				24										0.00
Mtub 31			24											0.00
Mtub 38			24											0.00
Mtub 39			24											0.00
MIRU 2		24												0.00
MIRU 10		24												0.00
MIRU 27			24											0.00
MIRU 39		24												0.00
ETR-D			24											0.00
Qub-23						24								0.00
ETR-F	24													0.00
Qub-3232	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Qub-3336	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = that no interpretable results were obtained

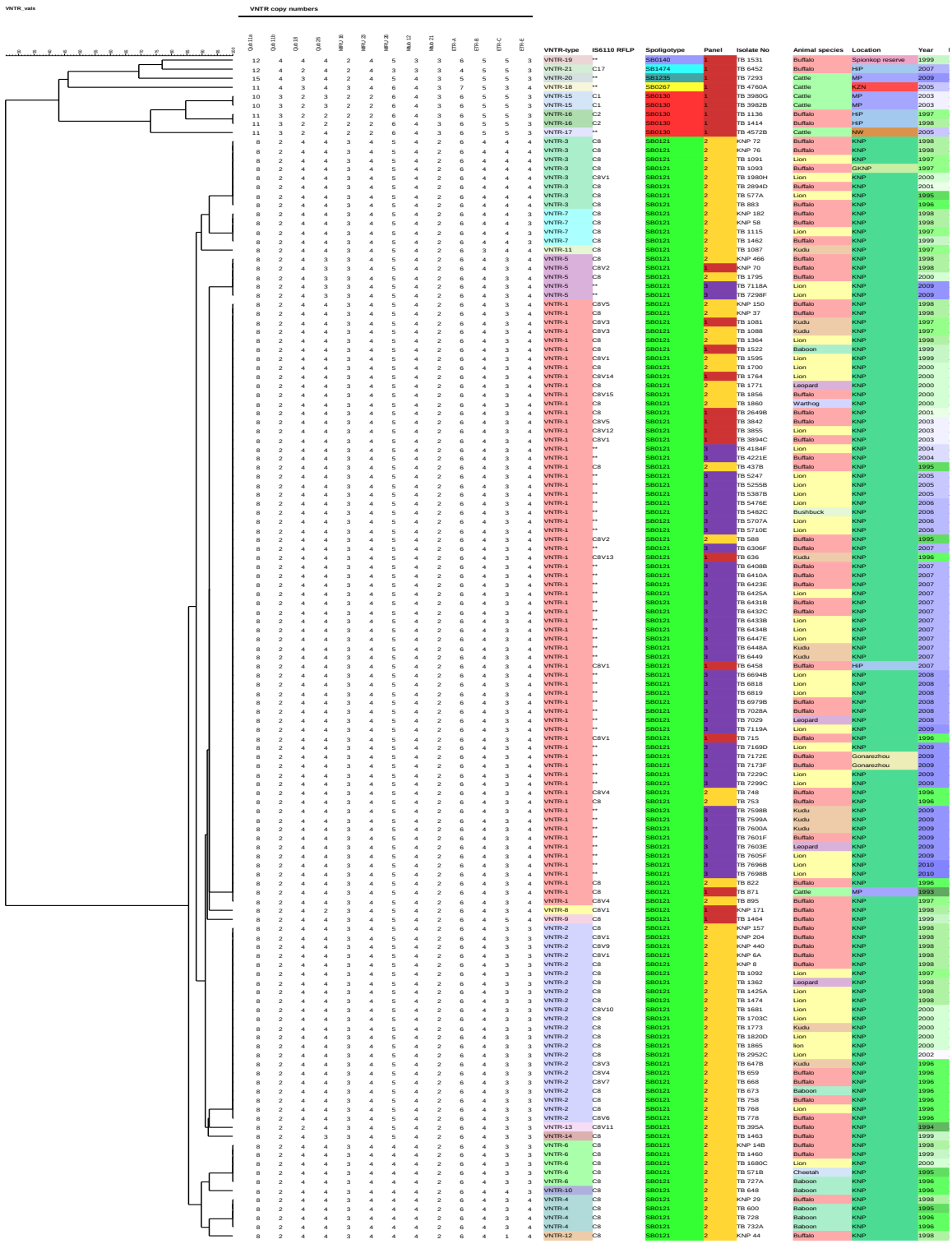

Figure 5 Dendrogramme and schematic representation of 131 *M. bovis* isolates typed with VNTR typing, IS*6110* RFLP typing and spoligotypes

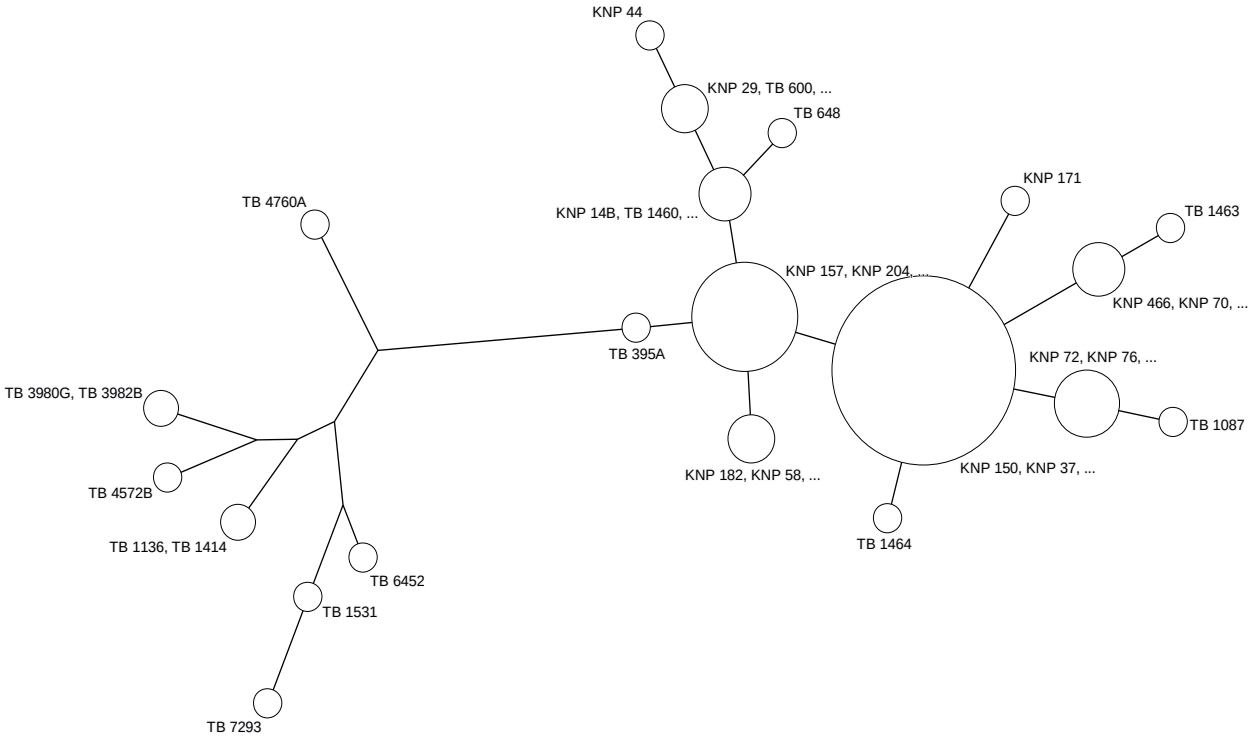

Figure 6 Maximum parsimony tree for the 131 isolates based on 13 polymorphic VNTR loci. The sizes of the circles differ according to the number of isolates represented.

3.4.2.2 Stage 2

The thirteen loci described above as stable and polymorphic were further evaluated on a panel of 65 IS*6110* RFLP typed isolates (Panel 2: Figure 5) from the southern part of the KNP, with the aim to compare the power of resolution of the combined loci to IS*6110* RFLP typing and spoligotyping. Among the 48 *M. bovis* isolates (73.8 %) previously classified as representatives of the C8 parent strain by IS*6110* RFLP typing (Michel *et al.* 2009), 11 VNTR profiles were identified (VNTR 1, 2, 3, 4, 5, 6, 7, 10, 11, 12 and 14). On the other hand, within the 17 *M. bovis* isolates (26.2 %) which were previously classified as representatives of the 12 unique C8 variant strains (C8V1-12) by IS*6110* RFLP typing (Figure 5), only four VNTR profiles (VNTR 1, 2, 3 and 13) were identified, three (VNTR 1, 2 and 3) of which matched profiles in the previous C8 group. In summary, 12 VNTR profiles were generated from this panel of isolates (Figure 5). Two most dominant profiles in this panel were found in 22 (33.8 % = VNTR 2) and 15 (23 % = VNTR 1) of the isolates respectively. A combination of only five of the 13 loci contributed to profile variation. ETR-E had the highest allelic diversity within this group

of isolates (h = 0.49), followed by ETR-C (h = 0.38), MIRU 26 (h = 0.33), Qub-26 (h = 0.09) and ETR-B (h = 0.03) (results not shown).

The 13 loci were further applied to 43 isolates (Panel 3: Figure 5) collected from different wildlife species from the northern part of KNP including two isolates from Gonarezhou National Park. Two VNTR profiles (VNTR 1 and 5) were detected in this group and both matched profiles detected within the C8 group of isolates. The most frequent profile (VNTR 1) was found in 41 (95.3 %) of the isolates. Qub-26 identified two isolates, i.e. TB 7118A and TB 7298F as C8 variants (Figure 5).

3.4.3 Spoligotyping

In this study, 116 *M. bovis* isolates were analysed by spoligotyping. All isolates, those analysed in this study including those previously characterized, from Greater Kruger National Park complex (GKNPC) and the Gonarezhou National Park yielded one spoligopattern, identified as SB0121, while *M. bovis* from buffalo and cattle in other locations revealed diverse spoligotypes (i.e. SB0130, SB0267, SB0140, SB1235 and SB1474).

3.5 Discussion

The importance of BTB in wildlife as a potential source of infection for domestic animals as well as a threat to valuable wildlife species is well documented (De Lisle *et al*. 2002; Aranaz *et al.* 2004; Michel *et al*. 2009; De Garine-Wichatitsky *et al.* 2010; Hlokwe *et al.* 2011). Knowledge of the epidemiology of bovine tuberculosis in free-ranging wildlife is an important factor in understanding the challenges associated with the management of the disease in this group of animals (De Lisle *et al*. 2002). The current study was aimed at evaluating the usefulness of the currently available loci used in VNTR typing for studying the epidemiology of bovine tuberculosis in South and Southern Africa. A correct analysis of the spatial and inter-species spread of bovine tuberculosis in the KNP is a particular challenge because of the clonal nature of the epidemic and typing tools have to be highly discriminatory to allow detection of recently mutated variants of the parent outbreak strain.

Several studies have indicated that increasing the number of VNTR loci increases the chances of detecting possible genetic variation and thereby improves the resolution of VNTR typing (Ojo *et al.* 2008; Millet *et al.* 2012). It is also cautioned that variability of the loci should be assessed as loci that proved to be discriminatory differ when tested in isolates from one country to another. Based on this information, a panel of 29 previously identified loci were evaluated individually and comparatively on a panel of 23 *M. bovis* isolates from South African wildlife species and cattle. The isolates were carefully selected and represented a broad spectrum of different levels of genetic relatedness. Of the 29 loci assessed, 13 loci were found to be polymorphic, with Qub-11a having the highest allelic diversity (0.63). Qub-11a was also found to be most discriminatory in studies conducted in Portugal (Duarte *et al*. 2010; Matos *et al*. 2010), Republic of Ireland (Mc Lernon *et al.* 2010), North America (Martinez *et al.* 2008), and also improved strain discrimination of *M. bovis* isolates from humans in Southwest Ireland (Ojo *et al.* 2008). In contrast, allelic diversity of this locus was found to be low in studies carried out in Spain (Romero *et al.* 2008). As in this study, Qub-11b, ETR-A, MIRU 26 and Qub-26 were also among the loci which provided good differentiation of strains in the Republic of Ireland (Mc Lernon *et al*. 2010) and Northern Ireland (Roring *et al*. 2004). ETR-B had the highest allelic diversity in *M. bovis* isolates from Chad (Hilty *et al*. 2005). ETR-D, MIRU 02, MIRU 10, MIRU 39 and ETR-F were amongst the 14 loci that were not informative in this study. Similar findings were obtained for *M. bovis* isolates from Chad. ETR-D, ETR-F, MIRU 39 and MIRU 40 were also not polymorphic for 41 North American *M. bovis* isolates (Martinez *et al*. 2008). DNA amplification of Qub-3232 and Qub-3336 (also named VNTR 3232 and VNTR 3336 in other studies) was not possible in this study. Either several non-specific PCR products were obtained or no product was generated in some of the isolates, making it difficult to interpret results. These findings are in consensus with findings by other researchers. In some of these studies, PCR amplifications had to be repeated in order to obtain interpretable amplification products (Martinez *et al.* 2008; Boniotti *et al.* 2009). In contrast to our findings, a study done on 68 *M. bovis* isolates from Belgium found Qub-3232 to be the most discriminative locus, with allelic diversity of 0.76. On the other hand, the same study found Qub-3336 to be difficult to amplify and was therefore not recommended for routine use, even though it was found to be the second most discriminative marker, with allelic diversity of 0.74. In Spain, Qub-3232 was also found to be the most discriminatory locus of *M. bovis* isolates from alpacas (Rodriguez-Campos *et al*. 2012).

In this study, a comparative evaluation of the 13 polymorphic loci identified on isolates from the south of the KNP which were previously characterized by IS*6110* typing had highlighted the discriminatory superiority of VNTR typing over IS*6110* typing within the isolates designated C8 type. The isolates were further resolved into 11 VNTR profiles. The ability to recognize recent evolutionary events is a powerful tool for analysing population structure and history (Smith *et al.* 2003). The resolution power of VNTR typing using the 13 loci was; however, lower for the C8 variant strains group, with the approach detecting only four of the 12 unique C8 variants identified by IS*6110* RFLP (Figure 5). A total of 12 VNTR profiles were generated on a panel of 65 isolates (panel 2), with only Qub-26, MIRU 26, ETR-B, ETR-C and ETR-E contributing to the variation seen amongst isolates. The fact that locus ETR-E demonstrated the highest allelic diversity (h = 49) within this panel of isolates highlighted its potential not only in discriminating genetically diverse isolates (panel 1) but also in discriminating *M. bovis* strains linked through clonal expansion. In contrast, Qub-11a was the most discriminatory locus within genetically diverse isolates, however, it was found to be monomorphic in strains linked through clonal expansion, and so was loci Qub-11b, Qub-18, ETR-A, MIRU 16 and 23, Mtub 12 and 21.

Previous typing reports of isolates from Greater Kruger National Park Complex (GKNPC) have indicated that there were more IS*6110* C8 variants in the central and northern regions than in the southern regions (Michel *et al.* 2009). This finding suggested that the IS*6110* C8 strain was transmitted progressively from the high prevalence southern region into previously uninfected buffalo herds further north which was accompanied by potentially cumulative mutational events. In the current study, analysis on a panel of 41 isolates from different host species from the northern part of the park collected over a period of six years, as well as two isolates from Gonarezhou National Park did not yield this expectation, as no new additional profiles were detected.

Overall, our results showed that isolates from the KNP and Gonarezhou National Park (120 isolates) can be classified into 14 VNTR profiles, with two most prevalent profiles detected in 54.2 % (VNTR-1) and 18.3 % (VNTR-2) respectively, of the total isolates analysed. These two strains seem to spread actively throughout the park infecting different wildlife species while individually undergoing some evolutionary changes as clearly illustrated by the maximum parsimony tree (Figure 6). All VNTR types in the KNP have one locus difference compared to their neighbour VNTR types and this entire

clonal complex corresponds to spoligotype SB0121. As reported previously, spoligotyping on its own lacked the ability to discriminate between strains within the GKNPC but successfully differentiated epidemiologically unrelated isolates from buffalo and cattle (Figure 5).

The value of the 13 VNTR loci was greatly highlighted when local isolates from different geographical areas within South Africa were characterized. All isolates with known genetic variations and those with identified epidemiological link were discriminated as such (Figures 5 and 6), making this typing approach an appropriate tool for molecular epidemiological studies of *M. bovis* isolates in SA.

In a recent study carried out on *M. bovis* isolates from Hluhluwe-iMfolozi Park, ETR-B, ETR-C and ETR-E were amongst the nine loci which contributed to the genetic diversity observed amongst the 31 isolates analysed, and there was no indication of the occurrence of mutational events (Hlokwe *et al.* 2011). These loci are amongst the loci described in this study as highly discriminative, highlighting their potential as tools to distinguish *M. bovis* isolates from SA.

With the limited sample size of isolates unrelated to the KNP epidemic included in this study, it was confirmed that a KNP strain and a non KNP strain can be clearly distinguished by application of the 13 loci (Figures 5 and 6). So far, our results confirm previous findings indicating that bovine tuberculosis in the KNP is a result of a single strain which seems to undergo evolutionary processes. In addition, this study further supports the emphasis on the importance of evaluating the different loci for each country or geographical setting. Although the composition of panels 1 and 2 was intentionally biased towards genetically diverse strains to allow best possible evaluation of the discriminatory power of VNTR loci, panel 3 was included for comparison of the marker performance in randomly selected, previously uncharacterised *M. bovis* from KNP.

3.6 Conclusion

The outcome of this assessment highlighted that molecular typing using 13 loci seems to be the best selection we can use far across regional South African *M. bovis* isolates. This combination of loci is good enough for application in epidemiological studies but not adequate for KNP, where active clonal expansion is observed. There is still room for further improvement of the VNTR typing method to be able to fully discriminate between the parent KNP strain and its variants to detect recent transmissions between and within wildlife species after evolutionary events. For this purpose, we recommend a search for new additional VNTR loci from whole genome sequence data of local *M. bovis* isolates.

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Chapter 3.1
Interlaboratory testing beyond diagnosis: Standardisation of a research tool in tuberculosis epidemiology

3.1.1 Abstract

Reliable and reproducible molecular methods which can be standardized between laboratories are required to monitor the spread of unique *M. bovis* strains beyond national borders for better understanding of bovine tuberculosis (BTB) transmission. This work presents the results of the interlaboratory testing performed using variable number of tandem repeat (VNTR) typing. To ensure quality control in research methodology, ten coded *M. bovis* isolates were analysed according to an internationally accepted VNTR typing protocol previously evaluated and standardised as part of a project aimed at setting up a network of laboratories involved in Mycobacterial diseases diagnosis in the European Union (VENoMYC). Six VNTR markers (VNTR 2165, VNTR 2461, VNTR 580, VNTR 3232, VNTR 2163a and VNTR 2163b) were utilized for their ability to optimally discriminate within this panel of isolates. VNTR typing was successful when using five of the six markers. The results serve as an example of how interlaboratory comparison can be integrated in and improve validity of epidemiological data analyses in a research laboratory.

Keywords: *Mycobacterium bovis;* Variable number of tandem repeat (VNTR) typing; genetic typing; interlaboratory comparison; laboratory quality assurance

3.1.2 Introduction

Validation of test results is an important control step in a microbiology laboratory quality assurance programme. In addition to the daily monitoring programmes, samples previously analysed by another laboratories are tested to evaluate the laboratory's ability to perform the analyses at an acceptable level of trueness and precision. This proficiency testing includes both intra-laboratory and interlaboratory sample examination. The interlaboratory tests are, for instance, used as part of the laboratory

accreditation process to estimate the comparability of results between laboratories (Garfield, 2000). The laboratory can use the outcome of an interlaboratory comparison to improve its performance in that specific method. Variable number of tandem repeat (VNTR) typing identifies polymorphism based on the copy number of VNTRs found at multiple loci along the bacterial chromosome (Allix *et al.* 2006; Hilty *et al.* 2005). VNTR typing is, however, unlike IS*6110* RFLP typing and spoligotyping, not yet widely standardised for *M. bovis* (Roring *et al.* 2002). It is, however, a challenge to compile standardised molecular fingerprinting patterns that originate from highly networked, multi-centric genotypic database for interlaboratory use and for further reference (Asgharzadeh and H.S Kafil, 2007). In an attempt to achieve this, the VENoMYC project was established in Europe, with the objective of setting up a network of veterinary laboratories for determining the efficiency of VNTR markers for typing of *M. bovis* strains from different geographical origins for epidemiological purposes (http://www.ucm.es/info/venomyc/workp.htm). Participation in interlaboratory testing will hence increase reliability of and confidence in the scientific findings and therefore ensures quality control of the entire process. This work presents the interlaboratory comparison results for ten *M. bovis* isolates typed blindly, based on the VNTR typing protocol of the VENoMYC VNTR Ring Trial of the EU-funded programme on the standardisation of *M. bovis* genotyping. The main purpose of the study was to confirm reproducibility of the standardised technique instituted by the VENoMYC project.

3.1.3 Materials and methods

3.1.3.1 Template DNA

Ten coded DNA samples were provided by the Molecular Microbiology and Genomic Analysis laboratory at Istituto Zooprofilattico Sperimentale del Piemonte in Turin, Italy, for genetic analysis. This laboratory participated in the VENoMYC VNTR proficiency testing scheme. The samples were used without any further treatment.

3.1.3.1.1 Primers

Six sets of primers (loci/markers) were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, Pretoria, South Africa). The loci names were VNTR 2165 (ETR-A), VNTR 2461 (ETR-B), VNTR 580 (ETR-D), VNTR 2163a (Qub-11a), VNTR 2163b (Qub-11b) and VNTR 3232 (Qub-3232).

3.1.3.1.2 PCR cycling parameters and analysis of results

VNTR typing PCR was performed in a 20 µl reaction containing 2 µl of DNA, 10 µl of the Qiagen master mix, 7 µl of DNA free water and 0.5 µl of each 20 pM primer. The cycling parameters were as follows: denaturation at 94 °C for 5 min, and 40 cycles of annealing at 62 °C for 1 min, elongation at 72 °C for 1.5 min (40 cycles) and a final elongation step at 72 °C for 10 min. PCR was carried out using an Eppendorf AG 22331 Hamburg thermo cycler (Merck). The PCR products were separated on a 2-3 % agarose gel stained with 20 µl ethidium bromide (10 µg/ml) and run at 80 V for three hours. A 100 bp ladder (Inqaba Biotechnical Industries) was included and used to estimate the sizes of the resulting PCR products. The band sizes were converted into number of tandem repeats at each locus based on the allele naming table provided (Le Fleche *et al.* 2002).

3.1.4 Results

DNA amplification was successful for five of the six loci used. The resulting PCR band sizes using five different loci were converted into tandem repeats numbers (Table 5) according to the allele calling table provided. VNTR 3232 (Qub-3232) could not give any convincing amplification under the PCR conditions outlined above. Multiple bands were observed on some isolates whereas no bands were observed on others. The results obtained in our laboratory were sent back to the Istituto Zooprofilattico Sperimentale del Piemonte. Results between both laboratories were 100 % in agreement for the calculated tandem repeats using 5 VNTR loci, i.e. VNTR 2165; VNTR 2461; VNTR 580; VNTR 2163a and VNTR 2163b for all ten *M. bovis* strains typed.

Table 5 VNTR typing results of the ten coded *M. bovis* isolates based on five VNTR loci

Isolate Number	Number of tandem repeats				
	VNTR 2165 (ETR-A)	VNTR 2461 (ETR-B)	VNTR 580 (ETR-D)	VNTR 2163a (Qub-11a)	VNTR 2163b (Qub-11b)
1	4	3	3	10	3
2	5	4	3	12	4
3	5	5	3	10	4
4	5	4	3	11	4
5	5	5	3	11	4
6	4	3	3	10	3
7	6	2	3	11	2
8	5	4	3	11	4
9	6	7	3	10	2
10	5	5	3	11	4

3.1.5 Discussion

Standardisation of molecular typing methods is undoubtedly essential in a bovine tuberculosis research field; however, typing data frequently suffer from a lack of interlaboratory comparability (Van Belkum, 2001). Standardisation of VNTR typing is important to allow valid comparison of typing information between countries all over the world and this will form a robust basis for determining genetic relationships of *M. bovis* strains causing bovine tuberculosis in different countries and allow tracing back of imported animals (Supply *et al.* 2006).

Ten coded *M. bovis* isolates were analysed using VNTR typing technique as part of interlaboratory testing with the aim of contributing to the validation and standardisation of VNTR typing, a research tool used for the epidemiological study of tuberculosis. DNA amplification was successful for five of the six loci tested (i.e. ETR-A, ETR-B, ETR-D, Qub-11a and Qub-11b) and the number of tandem repeat at each locus per an isolate was correctly calculated. The objective of proficiency/interlaboratory testing between research laboratories is to provide laboratories with information and support to enable them to demonstrate and improve the reliability of their test results. When specific information regarding the exact procedure followed by participants is noted, it is possible to draw conclusions regarding the optimum procedure to be followed (Boley,

1998). The use of this information by participants allows them to design either a customised testing strategy (consisting of markers highly discriminative for their epidemiological situation) or a primarily generic approach (a compromise between markers valid for all user environments). In all cases, it is important to ensure not only the intrinsic integrity of the panel of markers used for analysis, comparable to the requirements of a diagnostic assay, but also the integrity of the markers within a chosen epidemiological setting. The method validation process is therefore two-fold in this case and will result ultimately in an increase in the quality of the relevant research tool.

3.1.6 Conclusion

The outcomes of this interlaboratory test serve as an example of how interlaboratory comparison can be integrated in, as well as improve the validity of epidemiological data analyses in a research laboratory. The results have instilled greater confidence in the capability of laboratories to perform VNTR typing and interpret the results thereof. This will ensure that results or findings arising from the current research into bovine tuberculosis epidemiology using VNTR typing as a technique can be trusted by the international scientific research community and more importantly, that findings can be trusted by the decision and policy makers for BTB control and disease management for the country.

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Chapter 4
Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle ?

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4.1 Abstract

Tuberculosis caused by *Mycobacterium bovis* is recognized worldwide as a significant health risk in domestic cattle, farmed and wild animal species as well as in humans. We carried out spoligotyping and variable number of tandem repeat (VNTR) typing methods to characterize 490 *M. bovis* isolates from livestock (cattle, n = 230; pig n = 1) and wildlife species (n = 259) originating from different farms and regions in South Africa, with the aim to further establish the genetic diversity of the isolates, study the population structure of *M. bovis* and elucidate the extent of inter-species transmission of bovine tuberculosis (BTB). A total of ten spoligotype patterns were identified, two of which were novel (SB2199 and SB2200) and reported for the first time in the literature, while VNTR typing revealed a total of 97 VNTR profiles. Our results showed evidence of clonal expansion for some ancestral strains as well as co-infections with two or three *M. bovis* strains on some of the cattle and game farms, which suggested independent introductions of infected animals from epidemiologically unrelated sources. Five spoligotypes and nine VNTR profiles were shared between cattle and wildlife. Our findings showed that besides cattle, at least 16 different animal species in South Africa are infected with BTB, and highlight a strong evidence of intra and inter-species transmission of *M. bovis*. Infection of the blue wildebeest (*Connochaetes taurinus*) and a nyala (*Tragelaphus angasii*) with *M. bovis* is described for the first time in this report. This update in epidemiological information raises concerns that BTB has increased its spatial distribution in South Africa and is affecting an increasing number of wildlife species compared to ten years ago.

Keywords: Bovine tuberculosis; wildlife; spoligotyping; VNTR typing; South Africa

4.2 Introduction

Bovine tuberculosis (BTB) is still recognized worldwide as a significant animal health risk, primarily in domestic cattle and wildlife. The causative agent, *Mycobacterium bovis*, has a wide host range, which includes farmed and wild animals as well as humans (Neill *et al.* 2005). *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (MTBC), which includes mycobacterial species that cause tuberculosis in animals and humans (Brosch *et al.* 2002; Huard *et al.* 2006). In South Africa (SA), tuberculosis in cattle and free ranging wildlife species caused by *M. bovis* is well documented (Bengis *et al.* 2001; Michel *et al.* 2008 and 2009; Hlokwe *et al.* 2011). The prevalence of the disease in commercial cattle was reported to be less than 1 % in 1995, owing to the implementation of national BTB control and eradication scheme in 1969. The prevalence of the disease in communal cattle is currently unknown. Bovine tuberculosis in wildlife in the Kruger National Park (KNP) is endemic, with the highest disease prevalence in buffalo herds in the southern zone of the park. A single *M. bovis* strain was responsible for the epidemic and has subsequently spread progressively moving in a northern direction. It has infected at least 12 other wildlife species (Michel and Bengis, 2012) and has undergone evolutionary changes as described (Michel *et al.* 2009; Hlokwe *et al.* 2013). More recently, an epidemiological link between the KNP and the Gonarezhou National Park was confirmed, which has negative implications for the Greater Limpopo Transfrontier National Park (GLTFNP), De Garine-Wichatitsky *et al.* 2010; Hlokwe *et al.* 2013).

Bovine tuberculosis in the Hluhluwe-iMfolozi Park (HiP), which is geographically and epidemiologically distinct from KNP, is caused by at least three distinct *M. bovis* strains (Hlokwe *et al.* 2011). The prevalence of the disease in free ranging wildlife not associated with KNP or HiP, i.e. in private game reserves and game farms is currently unknown. Bovine tuberculosis in KNP and HiP was introduced by cattle from nearby communal farms and the persistence of the disease in these conservation areas as well as in communal farms pose a risk for ongoing transmission of the disease between wildlife and livestock. The situation may worsen if BTB prevalence in these ecosystems rises, since the disease in wildlife is generally not easy to control (Corner, 2006). Of further concern is that BTB poses a zoonotic risk, particularly in high HIV endemic communities surrounding the conservation areas (Thoen *et al.* 2006; Michel *et al.* 2010). An important factor for successful BTB control and eradication programs is contact

tracing and point source identification as unregulated and illegal movement of infected animals is considered the major constraint in such control strategies (Aranaz *et al.* 2004).

Molecular methods have become very tightly integrated with traditional epidemiological tracing of tuberculosis and provide a paradigm for such integration at both local and international levels (Achtman, 2001). In addition to traditional methods, typing methods such as IS*6110* restriction fragment length polymorphism (RFLP) and Polymorphic G-C Rich Sequences RFLP, spoligotyping and variable number of tandem repeat typing (VNTR) analyses are applied to characterize *M. bovis* isolates (Durr *et al*. 2000). Previous studies conducted in the KNP have shown that spoligotyping could not differentiate the parent C8 strain from its variants strains (Michel *et al.* 2009; Hlokwe *et al.* 2013) because of the slow evolutionary rate of the direct repeat region targeted. In addition, it generally has a lower discriminatory power for South African isolates as compared to other typing methods, i.e. IS*6110* typing, PGRS typing (Michel *et al.* 2008). Very recently, VNTR loci were assessed for their discriminatory power on isolates from South Africa. The results of this study led to a selection of a 13-locus VNTR panel for isolates from this region (Hlokwe *et al.* 2013).

The aim of the current study was to use spoligotyping and VNTR typing as described to characterize *M. bovis* isolated from livestock and wildlife species in South Africa to further establish their genetic diversity and assess the extent of intra- and inter-species transmission of BTB. The study also aimed to utilize the typing data to elucidate the population structure of *M. bovis* and generate a database to form the basis of back and forward tracing of sources of infection for improved surveillance and control of the disease in the country.

4.3 Materials and methods

4.3.1 Sample collection

The samples used in this study were received between 2002 and 2013 in the Tuberculosis Laboratory of the Onderstepoort Veterinary Institute for routine mycobacterial culture. Samples were collected from animals on livestock farms throughout SA and from different wildlife species from the KNP, HiP as well as private game ranches. They included tissue samples from lymph nodes, organs, and bronchial

fluids. The majority of the samples were collected from: (i) tuberculin skin test and gamma interferon test positive animals at slaughter, (ii) gamma interferon test was conducted together with skin test in buffaloes from game farms/reserves. In some cases, buffaloes testing positive in the gamma interferon assay but negative in the skin test were slaughtered and samples collected for culture, (iii) lesions detected in healthy cattle during routine slaughter from abattoir tuberculosis suspect animals, (iv) as part of passive BTB surveillance which was based on necropsy of all wild animals found dead in game parks/reserves and the collection of tissues showing pathological changes for specific testing. If tuberculous like lesions were found, the specimens were sent for tuberculosis culture. Bovine tuberculosis has been documented in all provinces of South Africa with a sporadic occurrence, irrespective of the size or density of the cattle population. Routine submissions formed part of the State Veterinary Service's strategy for confirming *M. bovis* infection in either skin test positive reactor cattle or slaughter cattle with suspect tuberculous lesions. Sample submission forms accompanied all samples with information relating to the animal, owner and precise location. In case where additional information was required, the responsible state veterinarian assisted with back tracing of animals and contacts. An additional two tissue samples from cattle originating from two different regions (i.e. Chimoio district in Manica Province and Guvuru district in Inhambane Province) in Mozambique were included for comparison purposes.

4.3.2 Bacterial isolation and *Mycobacterium bovis* identification

Briefly, tissue samples were processed and decontaminated using 4 % sodium hydroxide and 2 % hydrochloric acid methods. Bronchial fluids were decontaminated using 4 % sodium hydroxide. Samples were inoculated onto Löwensten-Jensen medium supplemented with pyruvate and incubated at 37 °C for up to ten weeks (Alexander *et al*. 2002). *M. tuberculosis* complex bacteria were identified by Polymerase Chain Reaction (PCR) using primers that target a sequence encoding the MPB 0 antigen as previously described (Alexander *et al.* 2002; Michel *et al*. 2009). Deletion analysis was performed using primers targeting the RD4 and RD9 regions of difference as previously described for *M. bovis* identification (Warren *et al.* 2006).

4.3.3 Template DNA preparation

4.3.3.1 Genomic DNA extraction from *M. bovis* cells

Genomic DNA from *M. bovis* isolates was extracted as previously described using a PUREGENE DNA extraction kit according to the manufacturer's instructions with minor modifications (Hlokwe *et al.* 2011).

4.3.3.2 *Mycobacterium bovis* lysate preparation

Mycobacterium bovis cells were suspended in 100 µl of sterile distilled water and heated to 100 °C for 25 min, cooled down briefly and stored at -20 °C until required (Hlokwe *et al.* 2013). Either pure genomic DNA or cell lysate prepared as described above was used for both the PCR-based methods, i.e. spoligotyping and VNTR typing.

4.3.4 Genotyping of *Mycobacterium bovis* isolates

4.3.4.1 Spoligotyping

Spoligotyping of all isolates was done according to a standardized international method (Kamerbeek *et al.* 1997), using a commercially available kit (Ocimum Biosolutions, Indianapolis, IN, USA). *M tuberculosis* H37Rv, *M. bovis* BCG and sterile distilled water served as the test controls. The resulting spoligopatterns were compared to those found in the *M. bovis* spoligotype database (www.mbovis.org).

4.3.4.2 Variable number of tandem repeat (VNTR) typing

PCR amplifications for VNTR typing were performed using a set of tandem repeat loci recently identified as stable and polymorphic for South African *M. bovis* isolates (i.e. ETR-A, -B, -C, and -E; Qub-11a, -11b, -18 and -26, MIRU 16, 23 and 26, as well as Mtub 12 and 21 (Hlokwe *et al.* 2013). The loci were amplified individually as previously described (Le Fleche *et al.* 2002) and the PCR primer sequences used are outlined in Table 6. The PCR products were separated on a 2 % agarose gels, run at 85 V for at least three hours and visualized under 312 nm UV light. A 100 bp or 100 bp ladder PLUS (Thermo Scientific, USA) were included to estimate the sizes of the products. In addition, the DNA fragment sizes were also determined by using Quantity One 1-D analysis software installed in the Gel Doc system (Bio-Rad laboratories, Hercules, CA).

These were converted into tandem repeat copy numbers according to Le Fleche and co-workers (Le Fleche *et al.* 2002) and the resulting VNTR profiles were saved in a spreadsheet.

Table 6 Variable number of tandem repeat (VNTR) loci and PCR primer sequences used for the typing of *M. bovis* isolates (Le Fleche *et al.* 2002)

VNTR locus	VNTR alias	VNTR locus size (bp)	Forward primer (5'-3')	Reverse Primer (5'-3')
2163a	Qub-11a	69	CCCATCCCGCTTAGCACATTCGTA	TTCAGGGGGGATCCGGGA
2163b	Qub-11b	69	CGTAAGGGGGATGCGGGAAATAGG	CGAAGTGAATGGTGGCAT
1982	Qub-18	78	ATCGTCAGCTGCGGAATAGT	AATACCGGGGATATCGGTTC
2461	ETR-B	57	GCGAACACCAGGACAGCATCATG	GGCATGCCGGTGATCGAGTGG
0577	ETR-C	58	GACTTCAATGCGTTGTTGGA	GTCTTGACCTCCACGAGTGC
1955	Mtub 21	57	AGATCCCAGTTGTCGTCGTC	CAACATCGCCTGGTTCTGTA
2996	MIRU 26	51	CCCGCCTTCGAAACGTCGCT	TGGACATAGGCGACCAGGCGAATA
1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA	CCCGTCGTGCAGCCCTGGTAC
3192	ETR-E	53	ACTGATTGGCTTCATACGGCTTTA	GTGCCGACGTGGTCTTGAT
4052	Qub-26	111	AACGCTCAGCTGTCGGAT	GGCCAGGTCCTTCCCGAT
2531	MIRU 23	53	CAGCGAAACGAACTGTGCTATCAC	CGTGTCCGAGCAGAAAAGGGTAT
2165	ETR-A	75	ATTTCGATCGGGATGTTGAT	TCGGTCCCATCACCTTCTTA
1121	Mtub 12	15	CTCCCACACCCAGGACAC	CGGCCTACCCAACATTCC
2347	Mtub 12	57	AACCCATGTCAGCCAGGTTA	ATGATGGCACACCGAAGAAC

4.3.5 Determination of the genetic relationships of the *Mycobacterium bovis* isolates

The genetic relationships of the isolates were deduced by the reconstruction of an unweighted pair group mean average (UPGMA) tree, as well as the minimum spinning tree (MST) using the Bionumerics software package version 7.1 (Applied Maths, Belgium).

4.3.6 Statistical analysis

Analysis and comparisons of counts for animal species, provinces, spoligopatterns, VNTR typing profiles and other statistical combinations were done using the Pivot tables (Microsoft Office Professional Plus 2010, Excel version 14.0.6129.5000-Pivot).

4.4 Results

4.4.1 Mycobacterial isolation and identification

A total of 492 isolates including 490 originating from 17 different animal species from South Africa (cattle, n = 232; pig, n = 1; wildlife from KNP, n = 151; wildlife from HiP, n = 28; wildlife from private game ranches, n = 80) and two isolates from cattle from Mozambique were recovered. All isolates were identified as MTBC organisms due to the amplification of a 372 bp PCR product from the MPB70 gene. *M. bovis* was confirmed in all cases by the presence of two specific DNA fragments of 268 bp and 108 bp for RD4 and RD9, respectively. The different animal species sampled as well as their locations are indicated in Table 7.

4.4.2 Spoligotyping

Where more than one animal with an isolate was found in one area, e.g. conservation area or farm, or there was known animal movement and contact between animals, we defined this as an “outbreak” in this report. Analysis was done for 490 *M. bovis* isolates from South Africa and yielded a total of ten different spoligopatterns (Figure 7). Two new patterns were designated the spoligopattern codes SB2199 and SB2200, respectively. Spoligopattern SB2199 had a close genetic relationship with SB1474 and was detected in cattle and a wildebeest. It was characterized by lack of spacers 3, 6, 8-12, 29-30 and 39-43. Spoligopattern SB2200 lacked spacers 1-3, 5, 9, 11, 16 and 39-43 (Figure 7). The geographic distribution of the different spoligotypes as determined in this study is shown in Figure 8. Of the 10 spoligopatterns, 5 were detected in both livestock and wildlife species (i.e. SB0120, SB0121, SB0130, SB0140 and SB2200), four were detected exclusively in livestock (i.e. SB0131, SB0134, SB0267 and SB02199) and a single spoligotype (SB1474) was detected in only 1 buffalo isolate. In total, 9 spoligopatterns were detected in 55 bovine tuberculosis outbreaks in cattle. Spoligopattern SB0130 was found to be geographically most widely distributed and was

detected in 34.3 % (168/490) of the total isolates. Spoligotyping results showed that the majority of the epidemiologically related outbreaks (90 %) were the result of infection with one *M. bovis* strain. Co-infection (infection with two or more genotypes on a farm or conservation area) was evident on five different cattle farms, (farm 1: SB0134 and SB0267; farm 2: SB0130 and SB0134; farm 3: SB0120 and SB0131; farm 4: SB0120 and SB0121; farm 5: SB0131 and SB0140) as well as in 2 private game reserves (reserve 1: SB0121 and SB2200; reserve 2: SB0120, SB0121 and SB0130) and in HiP (SB0130 and SB1474). The latter has been reported before (Hlokwe *et al.* 2011).

Table 7 Animal species, their origins and numbers of *M. bovis* isolated

Animal species	Location											
	EC	FS	GP	KZN	LP	MP		NC	NW	WC	MOZ	Grand Total
						KNP	Non-KNP					
Cattle	70	18		30	21		81	7	3	1	2	**232**
Buffalo				32	7	95	34		1			**170**
Baboon				1		3						**4**
Bushbuck							1					**1**
Cheetah					1							**1**
Eland		1										**1**
Hyena							4					**4**
Impala						1	5					**6**
Kudu						3	1					**4**
Leopard						1						**1**
Lion					1	47	8					**56**
Nyala					1							**1**
Porcine	1											**1**
Rhino					1							**1**
Warthog					1	1	5					**7**
Waterbuck							1					**1**
Wildebeest							1					**1**
Grant Total	**71**	**19**	**0**	**63**	**32**	**151**	**142**	**7**	**4**	**1**	**2**	**492**

EC = Eastern Cape Province; FS = Free State Province; GP = Gauteng Province; KZN = KwaZulu-Natal Province; LP = Limpopo Province; MP = Mpumalanga Province; NC = Northern Cape Province; NW = North West Province; WC = Western Cape Province; MOZ = Mozambique

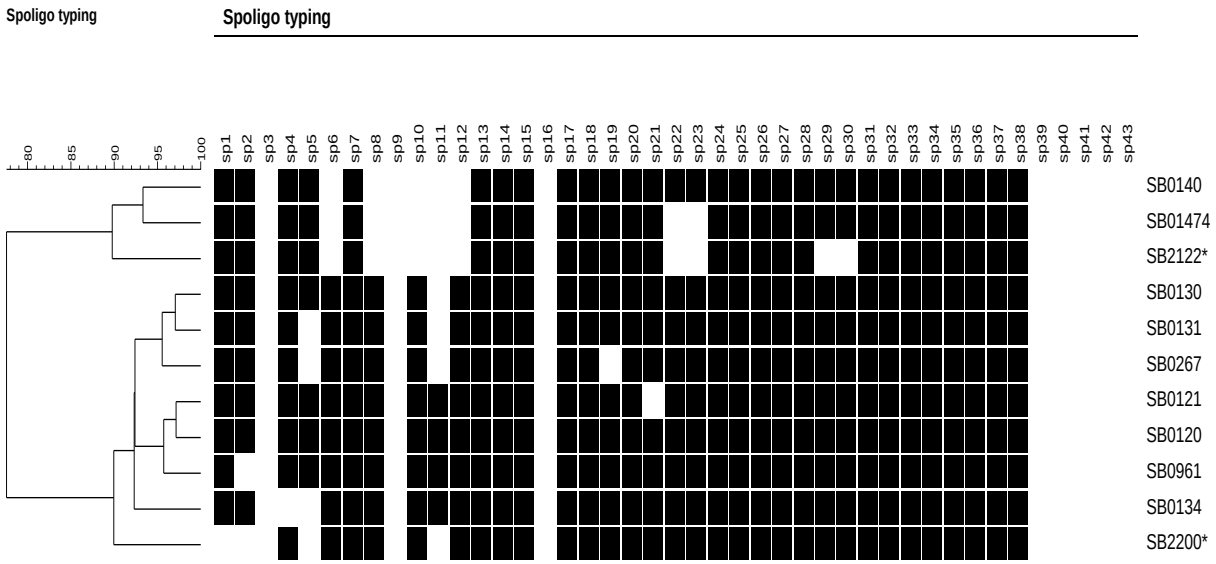

Figure 7 Dendrogramme and schematic representation showing relatedness of different spoligotype patterns found among the 490 livestock and wildlife *M. bovis* isolates from South Africa as well as two isolates from Mozambique analyzed. The newly identified spoligotypes are indicated with the asterisk. SB0961 was detected from cattle samples from Mozambique.

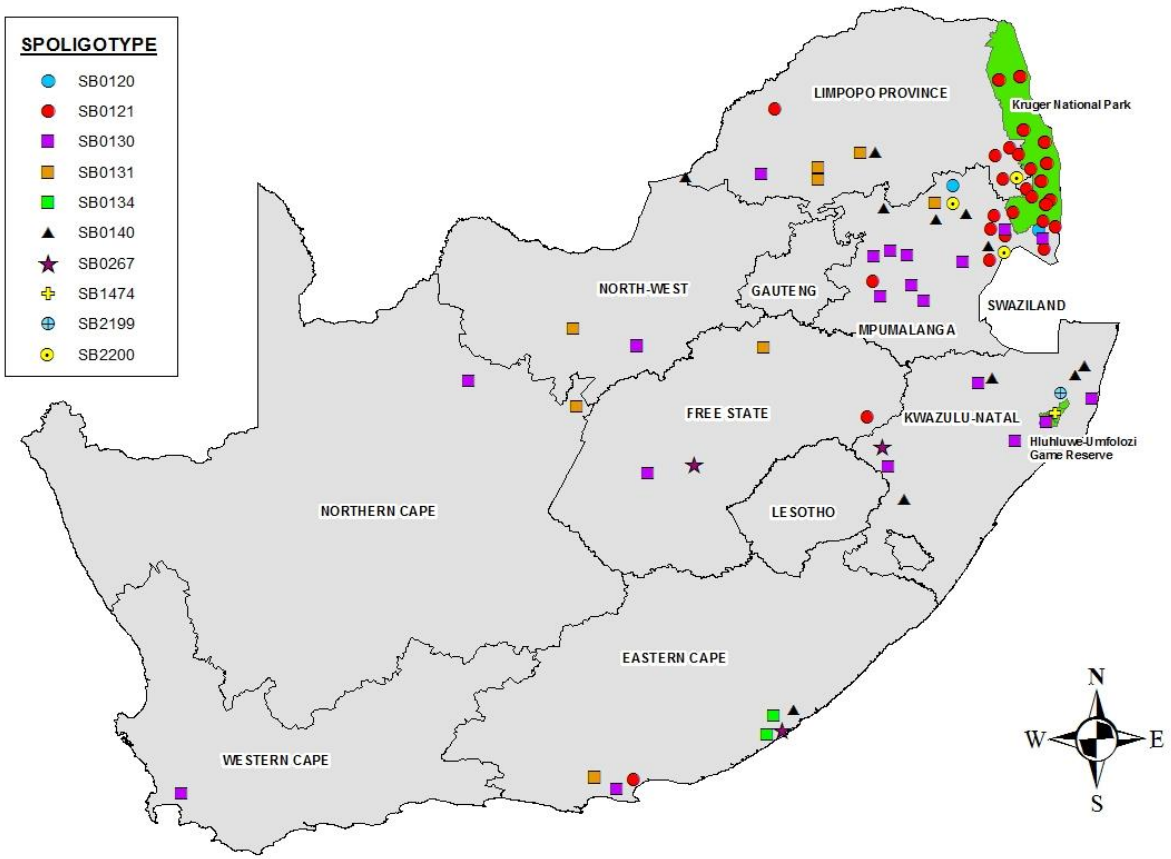

Figure 8 Geographical distribution of spoligotype patterns identified among the 490 *M. bovis* isolates from South Africa

4.4.3 Variable number of tandem repeat (VNTR) typing analysis

Analysis of typing results from 490 South African isolates was based on 13 VNTR loci and yielded a total of 97 VNTR profiles (Figures 9 and 10). In this study, we defined a cluster as a group of *M. bovis* genotypes which were ≥ 70 % similar according to the dendrogramme in Figure 10. The threshold used is unique to this study, and is based on empirical data derived from isolates from the Greater Kruger National Park Complex (GKNPC) where the epidemic can be traced back to one unique *M. bovis* strain. Of main interest was the KNP cluster consisting of approximately 40 % of the total profiles corresponding to SB0121 (Figures 9 and 10). Within this cluster, VNTR-1 was the KNP's dominant genotype. The genotypes of the KNP cluster were found in eight known infected wildlife species within the KNP as well as in private game reserves that form part of the Greater Kruger National Park complex (GKNPC). Overall, a total of nine VNTR profiles were shared among cattle and different wildlife species, whilst 46

and 42 VNTR types were found in cattle or wildlife species only, respectively. Some VNTR genotypes (e.g. genotypes corresponding to SB0130) were widely distributed.

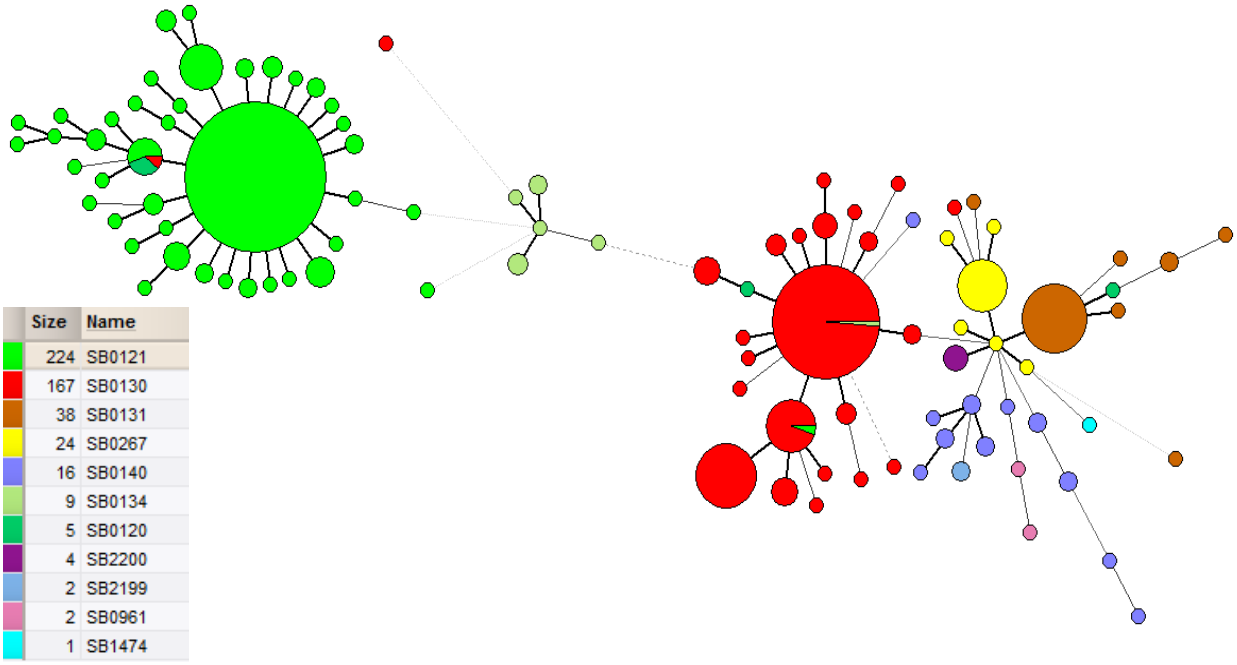

Figure 9 Minimum spanning tree (MST) for the phylogenetic relationship of the 492 *M. bovis* isolates analyzed from South Africa and Mozambique based on the VNTR profiles in relation to spoligotypes obtained

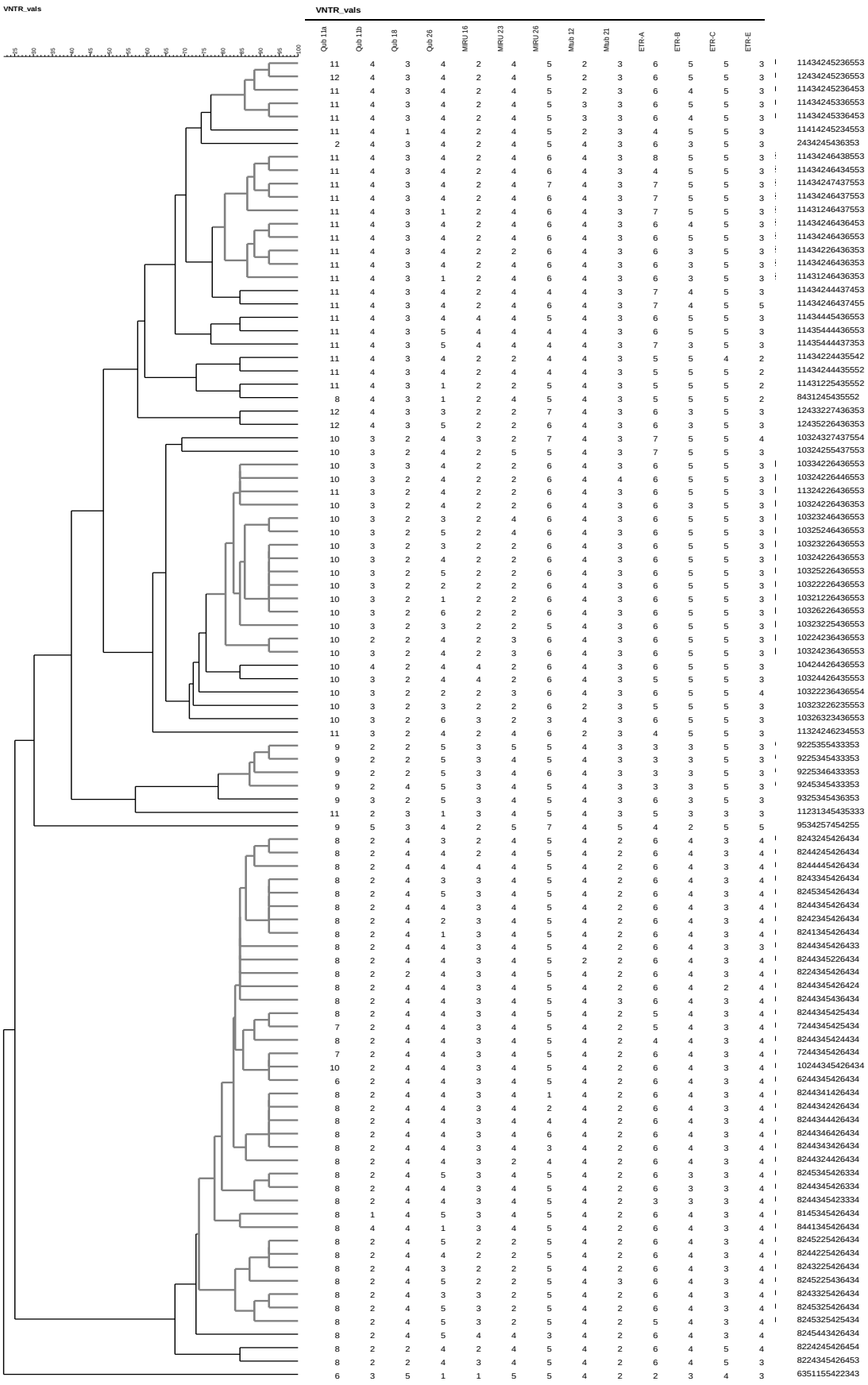

Figure 10 Dendrogramme and schematic representation demonstrating the genetic relatedness of the 97 VNTR profiles obtained from the *M. bovis* isolates

4.5 Discussion

Initial studies conducted in SA targeted specific ecosystems or species to determine the genetic diversity of *M. bovis* (Michel *et al.* 2008; 2009). The current study aimed to establish a database of *M. bovis* genotypes that allows the determination of genetic relationships between outbreaks not only among the 490 isolates characterized to date, but they can serve as reference for future isolates. The study aimed at further evaluation of the 13 VNTR loci panel across a variety of outbreaks in epidemiologically related and unrelated events. Information based on spoligopatterns is known to contain sufficient phylogenetic signals to construct recent events with some level of confidence (Smith *et al.* 2003). In addition, strains bearing the same spoligotype pattern are assumed to be a set of individuals derived relatively recently by clonal replication from a single ancestral cell. The VNTR typing tool applied here had the ability to recognize such clonal variants, allowing the genetic diversity of South African *M. bovis* strains to be studied at country level.

In this study, a total of ten spoligotype strains were detected, of which nine were detected in cattle isolates in 55 bovine tuberculosis outbreaks in different regions of the country. Most outbreaks (n = 25) occurred in Mpumalanga Province (Figure 8). The widespread distribution of some of the spoligotype strains (i.e. SB0130 and SB0131) could have started during the historical importation of cattle into SA from other countries in previous centuries. On the contrary, SB0120 and SB0134 appear to be geographically localized in Mpumalanga and Eastern Cape Provinces, respectively. It is of concern that outbreaks described in this study most likely resulted from the persistence of seven *M. bovis* strains (about 70 % of the *M. bovis* spoligotypes) previously detected in the country between 1993 to 2003 (Michel *et al.* 2008), suggesting that very few or none of the strains were effectively removed by control measures during these years. Movements of infected animals, possibly unknowingly, from one region to another seem to be the most likely route of transmission of the strains. Further noteworthy is the isolation of new strains, namely SB2199 and SB2200. SB2199 was detected in cattle originating from a research station in the Hluhluwe region, located approximately 80 km from HiP. SB2199 is genetically closely related to SB1474, which was detected for the first time in buffaloes from HiP. Both SB2199 and SB1474 seem to have evolved from SB0140 as their common ancestor (Figure 7). The research station exclusively farms and breeds Nguni cattle. Cattle from this research station were initially sourced from iShowe and Kokstad regions (both in KwaZulu-Natal

Province) in the 1980's. However, this particular animal from which SB2199 was detected, was born and bred in the station. In Mpumalanga Province, we detected spoligotype SB2200 from cattle on two epidemiologically unrelated farms, which may suggest that this novel strain could be more widespread than currently detected, as it was also detected in a blue wildebeest within the GKNPC. All infected cattle and the blue wildebeest also shared the same VNTR profiles. The persistence of the "old" strains and the detection of new emerging strains is an indication of active, ongoing BTB transmission, increasing the risk of spill-over to wildlife species in currently uninfected game farms and parks.

Mycobacterium bovis was also detected in tissues from a pig. This is only the second time in the past decade that *M. bovis* has been isolated from pigs in SA, reason being that pigs and cattle are rarely kept together commercially in SA. In contrast, *M. tuberculosis* is more frequently isolated from pigs, reflecting the high human tuberculosis incidence (Michel, unpublished data). On the other hand there is a complete lack of information regarding the occurrence of *M. bovis* in free-ranging pigs in communal farming areas as these animals are not marketed through abattoirs.

The majority of wildlife derived *M. bovis* isolates in this study were from the KNP (58 %), confirming the ongoing transmission of *M. bovis* between and within species in the park. The VNTR profiles detected, which all corresponded to spoligopattern SB0121, formed what we described as the KNP cluster. Genotype VNTR-1 dominated within this KNP cluster (Hlokwe *et al.* 2013). Spoligotype SB0121 clonal complex had originally descended from SB0120 through the loss of spacer 21. It is tempting to speculate that the success of SB0121 is at least partially caused by a higher transmissibility compared to its ancestor SB0120. This could, on the other hand, however, be severely flawed by the fact that SB0120 occurred only in farming areas where BTB control measures were applied. Hence there was a possibility of containment or even active elimination of SB0120 through test and slaughter, while SB0121 occurred within the GKNPC which is a free-ranging ecosystem with a high rate of intra-and interspecies contacts in the absence of any BTB control measures. We did not find any evidence suggesting an active spread of SB0121 clonal variants in other regions of the country except in private game reserves of the GKNPC. The possibility of direct spread of the disease from wildlife species in the GKNPC to cattle and other wildlife species in neighboring areas remains a risk to neighboring communities.

Our results showed that BTB had spread to previously uninfected (or unknown status) game farms and reserves in Mpumalanga, Limpopo, KwaZulu-Natal, Free State and North West Provinces. In addition, this study reports for the first time, *M. bovis* infection of a blue wildebeest (*Connochaetes taurinus*), and a nyala (*Tragelaphus angasii*) in SA. The wildebeest was on a private game reserve in the GKNPC and the former carried the novel *M. bovis* strain SB2200, indicating the introduction of a new *M. bovis* genotype in the GKNPC ecosystem, most probably effected by the repeated translocation of blue wildebeest into the reserve (Reininghaus, personal communication). Infection with *M. bovis* was first reported in two wildebeests in the Serengeti ecosystem in Tanzania (Cleaveland *et al.* 2005). Consistent with the previous report, the wildebeest in the current study did not present with any visible lesions during *post mortem* examination, but *M. bovis* was isolated from lung tissue. The animal was culled because it escaped from the reserve. In SA, movement control of any buffalo is instituted in order to prevent spread of BTB out of infected conservation areas (www.daff.gov.za/publications). Infected buffaloes escaping from the game parks/reserves due to compromised fences after floods etc. may also facilitate the spread of BTB in this country. Infected but undiagnosed wildlife species (as in the case of the wildebeest and other cases) can potentially play a role in the spread of the disease since their movement is not controlled. Over 50 % of the isolates in this study were from wildlife species, of which 65.6 % (170/259) were from buffaloes, emphasizing their role as carrier status and their role in the epidemiology of the disease in the country.

Genotyping methods applied in this study were useful in determining the possible sources of infection or true origins of buffaloes on a game farm located south of the KNP co-infected with three different strains of *M. bovis* (SB0120; SB0121 and SB0130). Our results confirmed that buffaloes had been initially sourced from HiP, based on the isolation of strains endemic in the latter conservation area. The resident SB0121 strain recovered from buffaloes and warthogs on the farm was likely to originate from the KNP. The source of infection could, however, not be established for one buffalo harboring SB0120 strain type and could either stem from a persisting infection in the immediate neighborhood or the strain could have been introduced during movement of game onto the farm.

We have analyzed two isolates from different regions in Mozambique, which borders SA in the east. Spoligopattern SB0961 was detected in both the cattle isolates and not recovered from any of the South African isolates. The absence of SB0961 in South Africa may indicate that the *M. bovis* populations in the two countries are largely unrelated based on historical livestock trading partners, a hypothesis which remains to be further investigated. The GLTFP links South Africa and Mozambique through the partial removal of park fences which has resulted in unrestricted movements of wildlife. In SA, only BTB test negative buffaloes can be moved between properties, while other wildlife species and cattle outside the foot and mouth disease control zone can be moved freely. Monitoring of BTB in wildlife and livestock populations including genetic characterization of *M. bovis* recovered from these populations in the transfrontier park is therefore of high importance for animal and human health at this wildlife/livestock/human interface.

4.6 Conclusion

We have analyzed a larger *M. bovis* sample size and it is suggested that both typing tools can be applied in future molecular epidemiological investigations of *M. bovis* infection in order to have a better knowledge of their genetic diversity and distribution in the country. Our findings showed that besides cattle, at least 16 different animal species in SA contracted the infection, and highlight a strong evidence of inter-species transmission of *M. bovis*. Although BTB is a controlled disease in SA in terms of animal health legislation, eradication of the disease in the near future is elusive. Despite the fact that national control of BTB is applied in commercial cattle, there is a high diversity of *M. bovis* persisting in the country, highlighting the importance and need for intensified diagnostic testing and consideration of alternative control measures such as vaccination. In the light of these most recent findings, screening of the BTB status of wildlife species before translocation is urgently recommended as prevention of the disease introduction remains the most effective control approach.

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Chapter 5
Wildlife sales: a new man-made wildlife/wildlife interface in South Africa

5.1 Abstract

In South Africa, African buffaloes *(Syncerus caffer)* serve as wildlife maintenance host for bovine tuberculosis (BTB) and play a role in the spread of the disease to other wildlife species and potentially back to cattle. In the present study, tuberculin skin and gamma interferon (IFN-γ) tests were conducted to diagnose BTB in African buffaloes in a private game reserve in the North West Province of South Africa (SA). *Mycobacterium bovis* was confirmed by culture and Polymerase Chain Reaction (PCR) tests in one of the 11 initial intradermal tuberculin test reactors and suspect reactor animals. Further disease investigations using intradermal tuberculin test, gamma interferon assay, culture and PCR tests confirmed *M. bovis* infection in two additional buffaloes. Molecular typing of the *M. bovis* isolates revealed a spoligotype and a variable number of tandem repeat (VNTR) genotype which were identical to a genotype isolated from wildlife in the KwaZulu-Natal Province of South Africa. This introduction of BTB into a previously uninfected buffalo population illustrates the emergence of a new man-made wildlife/wildlife interface in SA, which has been created by the large-scale translocation of plains game species throughout SA. The outcome of this work further served as an example of how BTB can remain unnoticed for many years in free-ranging buffaloes and emphasizes the need for development and validation of diagnostic tests for wildlife species, especially those in high demand for sale at game auctions.

Keywords: Bovine tuberculosis; African buffalo; wildlife; molecular typing; diagnostic tests

5.2 Introduction

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis* (*M. bovis*), was first reported in South African wildlife in kudus (*Tragelaphus strepsiros*) on commercial farms in the Eastern Cape Province of SA (Paine and Martinaglia, 1928). It was

speculated that kudus had contracted the disease from tuberculous cattle which were introduced into the area from the Western Cape Province (Thorburn and Thomas, 1940). Spill-over of BTB from buffaloes to other wildlife species in Hluhluwe-iMfolozi Park (HiP) and Kruger National Park (KNP) has been reported (Michel *et al.* 2009; Hlokwe *et al.* 2011). Bovine tuberculosis was also reported in private game reserves or parks in the Greater Kruger National Park (GKNPC) (Keet *et al.* 2001; Michel *et al.* 2009). The disease status in many other private game parks/reserves in the country is largely unknown; however, there is a growing number of newly infected game farms/reserves as well as introductions of new additional *M. bovis* strains (Hlokwe *et al.* 2014).

Movement of infected livestock and wildlife species is considered an important risk factor for facilitating the transmission of BTB (Skuce *et al.* 2012). In SA, movement control measures are only applied to African buffaloes. There are no restrictions for other wildlife animals and cattle outside the foot and mouth control zones. Wildlife is a major source of income, either directly for consumption or indirectly for tourism. Wildlife tourism is among the top revenue generating activities in many countries like SA, Tanzania and Kenya and generate an annual income of approximately half a billion United States (USA) dollars (Chardonnet *et al.* 2002). African buffaloes are amongst the “Big five” wildlife species and therefore of high economic value in the eco-tourism industry (Michel and Bengis, 2012). Unfortunately, their translocation and that of other wildlife species are associated with the spread of several high impact livestock diseases such as BTB (Woodform *et al.* 1993; De Vos *et al.* 2001).

This report describes the first diagnosis, isolation and genotyping of the *M. bovis* strain causing BTB in African buffaloes on a private game reserve situated in the North West Province of South Africa, next to the Botswana border. The main aim was to trace back the origin or source of the *M. bovis* infection using molecular typing techniques with the intent to better control the disease within the reserve and in neighbouring cattle farming communities.

5.3 Materials and methods

5.3.1 Case history

The private game reserve described in this report currently has approximately 1 000 African buffaloes roaming together with large numbers of other wildlife species, mostly antelopes. Most buffaloes came from the Addo Elephant National Park (Addo) in the Eastern Cape Province of SA, and they tested negative for BTB before introduction. Over the past years, the management of the reserve had introduced approximately 4 000 heads of game sourced from KwaZulu-Natal and other regions within SA. None of the animals were tested for BTB as the risk was considered low and for the lack of validated diagnostic tests. The reserve periodically held buffalo auction sales and no BTB positive reactors were detected during testing over the past years.

5.3.2 Single intradermal tuberculin testing

In June 2012, while preparing for an auction, 51 buffaloes were selected and put in a boma for routine testing for BTB, brucellosis, corridor and foot and mouth diseases. The initial BTB testing was a single intradermal tuberculin test using only bovine purified protein derivative (PPD) and two reactor animals were detected, based on an increase of 6 mm or more in skin fold thickness according to the guidelines of the World Organization for Animal Health (OIE, 2009).

5.3.3 Gamma Interferon testing

Fresh blood samples were collected from 11 of the animals for Gamma interferon testing a month after the initial intradermal tuberculin test. The samples were processed as previously described by Michel and co-workers by the Tuberculosis laboratory at ARC-Onderstepoort Veterinary Institute (Michel *et al.* 2011).

5.3.4 Comparative intradermal tuberculin testing

Three months following the single intradermal tuberculin test, a comparative intradermal tuberculin test using both bovine and avian PPDs was conducted. Five bovine reactor animals (two of which reacted positive during the initial testing) were detected based on the increase in skin thickness at the bovine site of injection which was more than 4 mm

greater than the reaction shown at the avian injection site. In addition, results for six animals were considered inconclusive based on the increase in skin thickness of 1 mm to 4 mm greater than the avian reaction (OIE, 2009).

5.3.5 Histopathology

The management agreed to slaughter one of the test positive buffaloes (an 18 months old bull; animal No. 713 in good body condition) which showed an enlargement of the prescapular lymph nodes. *Post mortem* examinations were conducted and several lymph nodes as well as a spleen and lung tissues were collected for histopathological examination and culture. Tissue samples for histopathology were fixed in 10 % buffered formalin and were processed for histopathological examinations using routine methods at the Department of Pathology, Faculty of Veterinary Science at the University of Pretoria.

5.3.6 *Mycobacterium* isolation and identification

Tissue samples from the tonsils, bronchial, mandibular, mesenteric, retropharyngeal, prescapular, prefemoral and inguinal lymph nodes as well as a spleen and a lung tissues were processed at the Tuberculosis laboratory of the ARC-Onderstepoort Veterinary Institute as previously described (Bengis *et al.* 1996). Ziehl Neelsen staining was used to confirm acid-fast bacterial isolates. *M. tuberculosis* complex bacteria were identified by polymerase chain reaction (PCR) using primers that target a sequence encoding the MPB70 antigen as previously described (Alexander *et al.* 2002; Michel *et al.* 2009). Deletion analysis was performed using primers targeting the RD4 and RD9 regions of difference as previously described for *M. bovis* identification (Warrren *et al.* 2006).

5.3.7 Follow up disease investigation

Approximately one year later (in June 2013), a follow up disease investigation was conducted based on gamma interferon testing. Of the 51 buffalo tested, two tested positive for *M. bovis*. The positive buffaloes (i.e. animal No. 4 and No. 32) together with three negative (i.e. animal No's. 33, No. 38 and No. 39) buffaloes were subsequently slaughtered for Mycobacterial culture and identification.

5.3.8 Molecular typing and construction of a dendrogramme and minimum spanning tree

Genotyping of *M. bovis* isolates from different tissue samples was carried out using spoligotyping (Kamerbreek *et al.* 1997) and variable number tandem repeat (VNTR) typing (Le Fleche *et al.* 2002; Hlokwe *et al.* 2013). The resulting VNTR data was compared to those already available in the database at the Tuberculosis laboratory at Onderstepoort Veterinary Institute. A dendrogramme and minimum spanning trees were constructed using the Bionumerics software version 7.1 (Applied Maths, Saint-Martens-Latum, Belgium) to determine the genetic relationships of the strains.

5.4 Results

5.4.1 Macroscopic and histopathological examination

Eighteen month old bull (No. 713): Typical tuberculous lesions were found in both tonsils of the 18-month bull. In addition, a few acid-fast bacilli were observed inside the multinucleated giant cells following Ziehl Neelsen (ZN) and IMP staining. The bull was diagnosed with an early and mild mycobacteriosis affecting both tonsils.

5.4.2 *Mycobacterium* isolation and identification

In total, tissue samples from six buffaloes (one buffalo from an initial and five from a follow up investigation) were cultured. *M. bovis* isolation was successful for three of the buffaloes, i.e. No. 713; No. 4 and No. 38 as confirmed by Ziehl Neelsen staining of the isolates followed by PCR tests. Buffalo No. 4 also tested positive on gamma interferon test. Buffalo No. 38 showed negative gamma interferon test results; however, was positive on culture. Animal No. 32, which reacted positive on gamma interferon testing, was negative on culture. No isolation was made on the other gamma interferon test negative buffaloes, i.e. animal No. 33 and No. 39 (see Table 8).

Table 8 Identification of the buffaloes from a private game reserve under study, results of different tests applied as well as different tissue samples from which *M. bovis* was isolated

Buffalo identification	Tuberculin skin test	Gamma interferon test	Culture test (sample type and identification)
No. 713 (18 months bull)	Positive	Positive	Positive (Tonsil = TB 8146)
No. 4	n/a	Positive	Positive (Prescapular LN = TB 8277C)
No. 38	n/a	Negative	Positive (Bronchial LN = TB 8278A); (Mediastinal LN = TB 8278B); (Right-Retropharyngeal LN = TB 8278C); (Left-Retropharyngeal LN = TB 8278D); (Mesenteric LN = TB 8278E); (Parotid LN = TB 8278F); (Lung = TB 8278G)
No. 32	n/a	Positive	Negative
No. 39	n/a	Negative	Negative
No. 33	n/a	Negative	Negative

n/a = not available; LN = lymph node

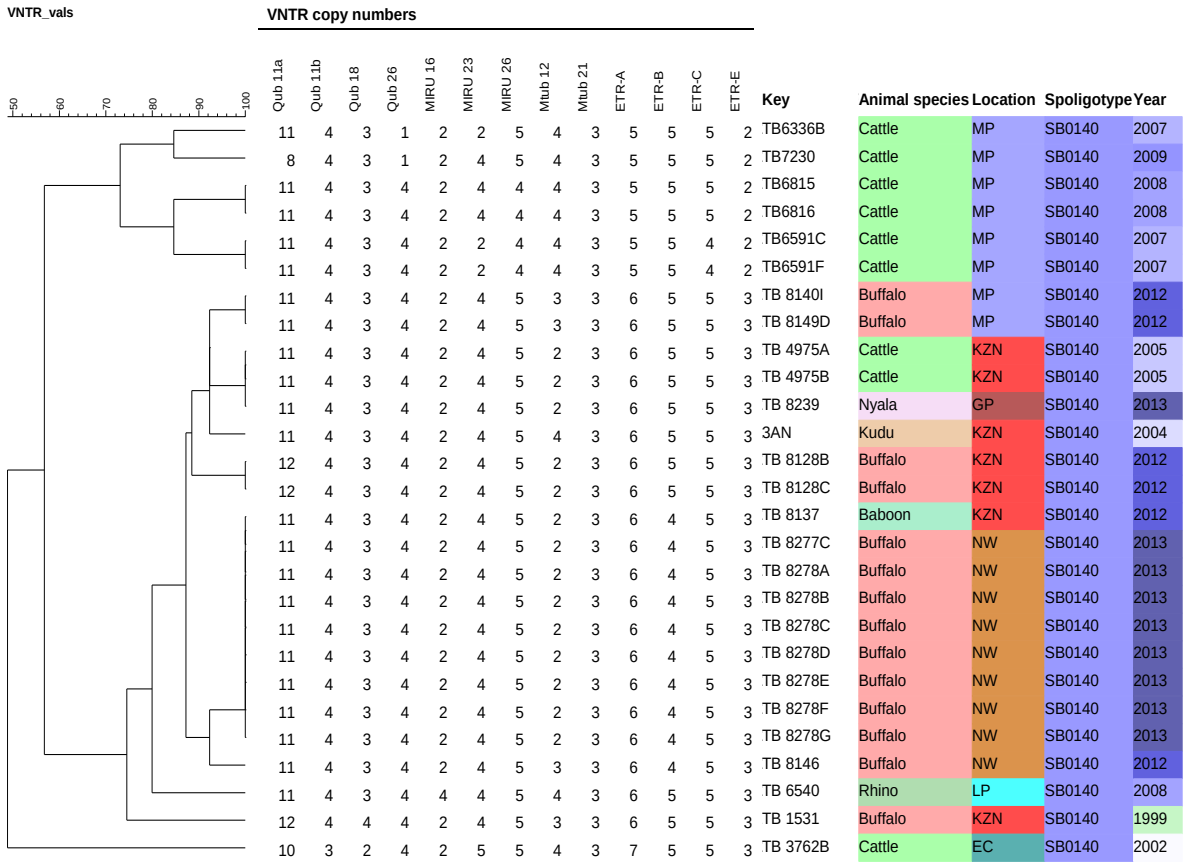
5.4.3 Genotyping

Spoligotyping of the nine isolates from different tissue samples from three buffaloes yielded a pattern corresponding to SB0140 in the international *M. bovis* database (www.mbovis.org). VNTR typing revealed two very closely related VNTR profiles (> 90 % similarity) differing only at one VNTR locus (Mtub 12) (Figure 11). One VNTR profile was detected in an isolate from an 18-year old buffalo bull, i.e. animal No. 713, while the other profile was detected in isolates from two other buffaloes (animals No. 4 and No. 38).

5.4.4 Comparison of genotypes recovered from new isolates with *M. bovis* database

In an attempt to establish the source or origin of infection, the genetic profiles of the *M. bovis* strain from a private game reserve were compared to those available in the recently established database at the Tuberculosis Laboratory of ARC-Onderstepoort Veterinary Institute. The database contains genetic profiles of approximately 650 *M. bovis* isolates from different regions and animal species in SA. No genetic

relationship could be established amongst genetic profiles of *M. bovis* strain isolated in North West Province, where the newly infected reserve is located. However, an exact match was obtained for the *M. bovis* isolated during follow-up disease investigation with a genetic profile from a baboon sampled in 2012 from Mkuze game reserve, situated in the KwaZulu-Natal (KZN) Province of SA. In addition, the isolates from the private game reserve under study had a close genetic relationship (≥ 85 % similarity) with strains isolated from the following animal species: (i) a buffalo (isolate No's. TB 8128B and TB 8128C) from a private game reserve in KwaZulu-Natal Province; (ii) a kudu (isolate No. 3AN) from the Spioenkop Nature reserve, also in the KwaZulu-Natal Province; (iii) a nyala (isolate No. TB 8239) recently translocated from unknown origin to a private game farm in Gauteng Province; (iv) bovine (isolate No's. TB 4975A and TB 4975B) from KZN; and lastly; (v) two buffaloes (isolate No's. TB 8140I and TB 8149D) from a private game reserve in Lydenburg (Mpumalanga Province). These buffaloes were initially sourced from Pongola Game Reserve in KwaZulu-Natal Province. The genetic profiles and evolutionary relationships of *M. bovis* strains from South Africa are illustrated in Figures 11 and 12 respectively.



VNTR copy numbers

Qub 11a	Qub 11b	Qub 18	Qub 26	MIRU 16	MIRU 23	MIRU 26	Mtub 12	Mtub 21	ETR-A	ETR-B	ETR-C	ETR-E	Key	Animal species	Location	Spoligotype	Year
11	4	3	1	2	2	5	4	3	5	5	5	2	TB6336B	Cattle	MP	SB0140	2007
8	4	3	1	2	4	5	4	3	5	5	5	2	TB7230	Cattle	MP	SB0140	2009
11	4	3	4	2	4	4	4	3	5	5	5	2	TB6815	Cattle	MP	SB0140	2008
11	4	3	4	2	4	4	4	3	5	5	5	2	TB6816	Cattle	MP	SB0140	2008
11	4	3	4	2	2	4	4	3	5	5	4	2	TB6591C	Cattle	MP	SB0140	2007
11	4	3	4	2	2	4	4	3	5	5	4	2	TB6591F	Cattle	MP	SB0140	2007
11	4	3	4	2	4	5	3	3	6	5	5	3	TB 8140I	Buffalo	MP	SB0140	2012
11	4	3	4	2	4	5	3	3	6	5	5	3	TB 8149D	Buffalo	MP	SB0140	2012
11	4	3	4	2	4	5	2	3	6	5	5	3	TB 4975A	Cattle	KZN	SB0140	2005
11	4	3	4	2	4	5	2	3	6	5	5	3	TB 4975B	Cattle	KZN	SB0140	2005
11	4	3	4	2	4	5	2	3	6	5	5	3	TB 8239	Nyala	GP	SB0140	2013
11	4	3	4	2	4	5	4	3	6	5	5	3	3AN	Kudu	KZN	SB0140	2004
12	4	3	4	2	4	5	2	3	6	5	5	3	TB 8128B	Buffalo	KZN	SB0140	2012
12	4	3	4	2	4	5	2	3	6	5	5	3	TB 8128C	Buffalo	KZN	SB0140	2012
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8137	Baboon	KZN	SB0140	2012
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8277C	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278A	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278B	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278C	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278D	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278E	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278F	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	2	3	6	4	5	3	TB 8278G	Buffalo	NW	SB0140	2013
11	4	3	4	2	4	5	3	3	6	4	5	3	TB 8146	Buffalo	NW	SB0140	2012
11	4	3	4	4	4	5	4	3	6	5	5	3	TB 6540	Rhino	LP	SB0140	2008
12	4	4	4	2	4	5	3	3	6	5	5	3	TB 1531	Buffalo	KZN	SB0140	1999
10	3	2	4	2	5	5	4	3	7	5	5	3	TB 3762B	Cattle	EC	SB0140	2002

Figure 11 A dendrogramme based on VNTR typing data illustrating the genetic relationships of *M. bovis* isolates corresponding to SB0140 from South Africa, including isolates from a private game reserve under study

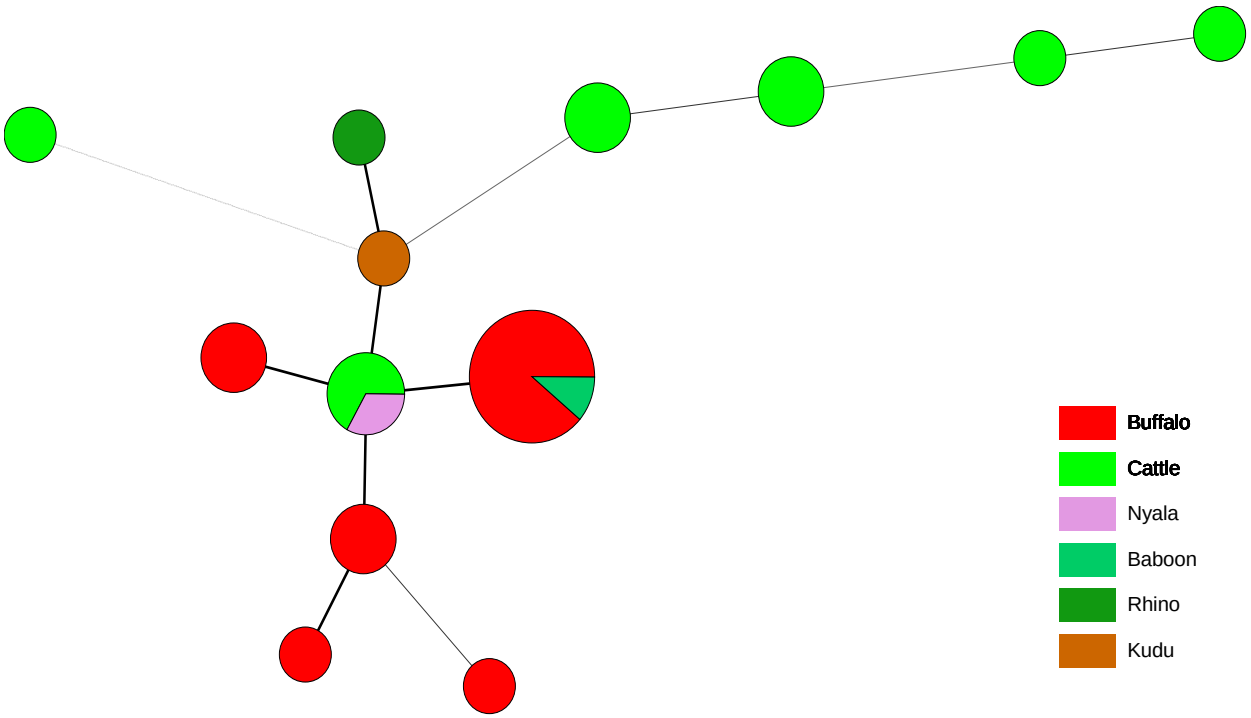


Figure 12 Minimum spanning tree analysis based on VNTR data illustrating the evolutionary relationships between *M. bovis* isolates corresponding to spoligopattern SB0140

5.5 Discussion

We have used molecular typing techniques to confirm and characterize *M. bovis* isolated from buffaloes in a newly infected private game reserve in the North West Province of SA. Buffaloes are known to be important wildlife reservoirs of the disease in Southern Africa; and play an important role in the transmission of the disease to other wildlife species with the risk of spread to livestock (De Vos *et al.* 2001; Michel *et al.* 2012). With this knowledge, it was of the utmost importance to identify the source of infection for the buffaloes in the game reserve under study.

Every time the management of the reserve held an auction, the animals were tested for BTB using a single intradermal tuberculin test, and no *M. bovis* reactors were detected over the past two decades. The exact time of introduction of this strain into the private game reserve remains unclear. The current diagnosis of BTB may suggest that control measures previously applied might have been inadequate for the insidious nature of the disease. The accuracy of the current *ad hoc* testing methods for the control of BTB in buffalo is questioned. For example, as shown in Table 8, animal No. 38 was the only

animal that appeared to have disseminated disease as detected by culture, however, no immune response to *M. bovis* infection was detected by gamma interferon test. Tests based on cell-mediated immune response such as gamma interferon assay can either be negative or inconclusive during the later stages of infection due to release of antibodies. These tests detect the presence of *M. bovis* organisms in the early stages of the disease. Additionally animal No. 32 reacted positive for *M. bovis* infection when using gamma interferon test, and was negative upon culture. Overall, the current findings emphasize that caution should be taken not to rely solely on *ad hoc* tests for the diagnosis of BTB in wild animals since the infection time is in most cases unknown.

The *M. bovis* strain detected could not be linked to any of the limited numbers of cattle strains isolated in the North West Province. The prevalence of BTB in the province is very low, with the latest reports indicating that out of more than a 1 000 heads of cattle tested; only one positive reactor was found. It is however, unknown whether the animal was slaughtered or if the diagnosis was substantiated any further (De Klerk-Lorist, personal communication). The report therefore ruled out the possibility that buffaloes on this particular game reserve could have contracted the disease from cattle in the neighbouring farms with reasonable certainty. In addition, the game reserve had a single electric fence bordering the communal grazing lands. The fence's main purpose is to prevent wild animals from escaping the reserve and therefore limits the risk of disease spill-over to neighbouring cattle farms. It was largely kept intact and buffalo breakouts were unlikely.

With further tracing, it was found that the VNTR genotype of the *M. bovis* strain was identical to the genotype of the strain isolated from a baboon which originated from Mkuze game reserve, located in the KwaZulu-Natal Province of SA. An epidemiological connection to this region may therefore be suggested, given that the management of the game reserve sourced large numbers of antelopes of different species from different regions in SA without prior BTB testing.

The possible transmission chain(s) and source of infection for the buffaloes in the private game reserve in North West Province are suggested as follows: (i) In 1992, tuberculosis was diagnosed in ten randomly culled kudus in the Addo Elephant National Park in the Eastern Cape Province (Weber and Van Hoven, 1992). According to the history provided for the private game reserve, majority of the buffaloes in this reserve

were sourced from Addo National Park (De Klerk-Lorist, personal communication). It is generally known, however, that a large proportion of "disease free" buffaloes in South Africa originate from Addo (Laubscher and Hoffmann, 2012) and as a result, only a single blood test is required from established disease-free herds before translocation. Bovine tuberculosis is difficult to detect and can often be present, undetected, in a herd, regardless of regular blood tests done (De Lisle *et al.* 2002). It is highly possible that not only kudus were infected with *M. bovis*, but buffaloes as well, of which some of them were translocated to the private game reserve in the North West Province where the *M. bovis* strain remained undetected for several years until recently. However, this is unlikely given that buffaloes were regularly tested; (ii) The disease was introduced into different game farms/reserves in KwaZulu-Natal Province from cattle, and later introduced into buffalo or other wildlife populations in the private game reserves in the North West Province through infected buffalo or other wildlife species translocation; (iii) The strain could be widely distributed amongst wildlife on different properties in SA. The private game reserve under study may have purchased the infected buffaloes or antelope species from any of the farms/reserves. However, the dendrogramme (Figure 11) showed that isolates with over 85 % similarity with the *M. bovis* strain in the private game reserve were from the KwaZulu-Natal, or were translocated from this province, suggesting KwaZulu-Natal Province as the source of the BTB outbreak strain in the private game reserve under study. According to the Minimum spanning tree (Figure 12), both the genotypes from the buffaloes in the game reserve under study, the baboon from Mkuze game reserve and a kudu from Spioenkop nature reserve are suggested to have evolved from the most recent common ancestor, which is a genotype from a bovine (TB 4975A and TB 4975B) and a nyala (TB 8239). These findings further support historical knowledge of the transmission of BTB between cattle and kudu.

The private game reserve described in this report is situated adjacent to the border of Botswana. It is surrounded by three cattle farms on the southern side of its border and communal villages on the other side. Detection and confirmation of *M. bovis* in this reserve calls for urgent intervention by Veterinary Service in the province to prevent the spread of the disease to other buffaloes as well as other wildlife species (over 60 large mammal species including the "Big five") within the reserve, as well as in livestock outside the reserve. The presence of BTB poses a risk on the survival of these species as well as the endangered ones. Another concern is the spread of the disease into neighbouring Botswana. The Botswana national cattle population is reported to be

officially free of BTB (Jori *et al.* 2013). The management of the reserve have, however, agreed to conduct passive surveillance on all wildlife species that die within the reserve and subject the carcases to *post-mortem* examinations (Madyibi, personal communication), an exercise which has some costly financial implications.

Knowledge that BTB infected buffaloes may have been sold to both national and international buyers during buffalo auctions further complicates the problem. It is possible that the regular sales held at the reserve might have facilitated the distribution of this *M. bovis* strain. Additionally, the detection of *M. bovis* has negative implications for the reserve's future income generation. African buffalo is most coveted trophy and essential asset on any game reserve and owners seek return on their investment through tourism revenue (Hunt, farmers weekly) and sales. The market prices for the BTB positive and negative buffaloes differ significantly, and as a result, the management needs to prove beyond doubt the negative status of the buffaloes to be sold. In 2010, one "disease free" buffalo cost over R 325 000 at the auctions (Cloete, 2011). In 2012, a "disease free" buffalo bull was sold for a price of R 26 000 000 (Laubscher and Hoffmann 2012). In 2013, a buffalo was sold for record price of R 40 000 000 (Michel, personal communication). The current developments signal the significance of *M. bovis* as a silent, growing risk factor in game translocation, especially in the absence of pre-movement testing.

5.6 Conclusion

This introduction of BTB into a previously uninfected buffalo population illustrates the emergence of a new man-made wildlife/wildlife interface in SA, which has been created by the large-scale translocation of plains game species throughout the country. There is a need for development and validation of diagnostic tests for wildlife species, especially those in high demand for sale at game auctions. The detection of *M. bovis* in the private game reserve calls for urgent disease surveillance in wildlife species within the park and in the surrounding communal cattle farms with a view to better control the disease.

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Chapter 6
CASE REPORT:
Isolation and molecular characterization of *Mycobacterium bovis* causing pulmonary tuberculosis in a horse from South Africa

6.1 Abstract

Tuberculosis caused by *Mycobacterium bovis* (*M. bovis*) is very uncommon in horses worldwide. In the current study, an eight year old male Thoroughbred in good body condition was admitted to the Equine Clinic at the Onderstepoort Veterinary Academic Hospital in 2005 due to bilateral epistaxis accompanied by coughing. Routine examinations were conducted to determine the cause of the condition. Endoscopic examination revealed the major source of the epistaxis as the trachea, whereas thoracic radiography indicated the presence of a primary pulmonary mass. *M. bovis* was isolated from a broncho-alveolar lavage sample collected. The pulmonary mass reduced in size three months later following an oral administration of enrofloxacin (7.5 mg/kg PO SID). Genetic fingerprinting by spoligotyping identified the *M. bovis* isolate as spoligotype SB0868 strain. This *M. bovis* strain type was never described previously in South Africa (SA), suggesting that the horse may have been imported into the country. This is the first case of *M. bovis* infection in a horse in SA which has been fully documented including clinical findings, isolation and genetic characterisation of the causative pathogen. This report indicates that horses may contract and harbour *M. bovis* despite their lower susceptibility compared to other domestic animals. It also suggests that the infection may be more easily contained and eliminated from the host.

6.2 Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) in a variety of mammalian hosts and a member of the *Mycobacterium tuberculosis* complex (MTBC). The worldwide status of this disease as a zoonosis is of great concern (Humblet *et al.* 2009). Horses are suggested to be more resistant to mycobacterial infections as

compared to other livestock animals (O'Reilly and Daborn, 1995). As result, tuberculosis incidence in horses is extremely low especially in countries implementing BTB control programs (Pavlik *et al.* 2004). Currently, the most common causes of mycobacterial infections in horses are members of the *Mycobacterium avium complex* (MAC) species (Pavlik *et al.* 2008). Amongst members of the MTBC, *M. bovis* has been more commonly isolated from horses than *M. tuberculosis (*Pavlik *et al.* 2004). Among the few cases documented in the literature, isolation of *M. bovis* was reported from a suspect lesion found on the left cheek of a nine year old horse from Nigeria (Garba *et al.* 2001). In another report, *M. bovis* was isolated form lymph nodes of a four year old horse in France living in close contact with infected cattle, highlighting that horses can be more easily infected when living under conditions of high *M. bovis* infection pressure (Keck *et al.* 2010). The most common site of infection due to *M. bovis* in horses is the gastro-intestinal tract, particularly caecum and colon, suggesting ingestion as a major route of transmission (Pavlik *et al.* 2004). *M. tuberculosis* infection was recently confirmed through Polymerase Chain Reaction (PCR) amplification of DNA extracts from formalin fixed paraffin embedded tissue samples of a 20 year old horse imported from Poland to Switzerland (Lyashchenko *et al.* 2012).

In this report, we describe for the first time, the isolation and molecular characterization of *M. bovis* in a horse in South Africa.

6.3 Materials and methods

6.3.1 Case history

6.3.1.1. Initial clinical examination

An eight year old male Thoroughbred was admitted at the Equine Clinic, at the Onderstepoort Veterinary Academic Hospital in Gauteng province on the 18th August 2005 with bilateral epistaxis. Mild bilateral epistaxis was first noted six days prior admission and had, however, increased in severity and frequency accompanied by coughing. Upon admission, the horse was in good body condition and appeared to be alert and bright. The patient weighed 563 kg, had good appetite and the body temperature was 37.9 °C. Respiratory rate was mildly elevated; however, there was no coughing upon rebreathing examination. Mucous membranes were pink with no evidence of petechiation. Diagnostic procedures (i.e. haematology and clinical

chemistry, endoscopy and thoracic radiography) were conducted to further determine the cause of the epistaxis.

6.3.2 Case progression

The horse was re-examined four days later with the intention of performing lung biopsies. Prior to performing lung biopsy, broncho alveolar lavage samples were collected form the site of haemorrhage for bacterial and fungal culture. Three Tru-cut® biopsies were collected from the left caudodorsal lung field under sedation at the site most affected by superficial pleuritis. The horse was re-examined three months later (14 November 2005).

6.3.3 Bacterial isolation and identification

A broncho-alveolar lavage sample collected prior to lung biopsy was subjected to mycobacterial culture according to the laboratory standard operating procedure. Briefly, the sample was decontaminated for 10 min using 4 % sodium hydroxide (NaOH), centrifuged at 3 500 rpm for 10 min and the pellet neutralised with sterile distilled water and centrifuged as before. The resulting pellet was inoculated onto Löwenstein-Jensen media slopes supplemented with glycerol and pyruvate, respectively. Mycobacterial identification of acid-fast colonies was done as previously described (Alexander *et al.* 2002).

6.3.4 Molecular characterization

Spoligotyping (spacer oligonucleotide typing) and variable number tandem repeat typing (VNTR) were used to characterize the isolate as previously described (Kamerbreek *et al.* 1997; Hlokwe *et al.* 2013). Spoligotyping detects variability in the direct repeat (DR) region in the DNA of *Mycobacterium tuberculosis* complex (MTCB) species including *M. bovis*. VNTR typing is based on variation in the number of tandem repeat sequences in the chromosomes of MTBC species.

6.4 Results

6.4.1 Haematology and clinical chemistry

Mild lymphopaenia and thrombocytopaenia were observed, but not of a scale sufficient to cause an increased bleeding tendency. Prothrombin and partial thromboplastin times were within reference range. Fibrinogen concentration was normal.

6.4.2 Endoscopy

Endoscopic examinations revealed petechiations in the medial compartment of the left guttural pouch; however, major source of the epistaxis was from the trachea. Using a 3 m Endoscope, fresh bleeding was noted from a focal section of the left sagittal dorsal lung lobe. A sample of fluid was collected from the site for cytology and bacterial and fungal cultures. The remainder of the respiratory tract was essentially normal.

6.4.3 Thoracic radiography

Lateral thoracic radiographs revealed a focal marked peribronchial infiltration in the caudo-dorsal lung field of approximately 12 x 15 cm in size with a dorsal gas cap; the fluid line was not completely straight, but more undulated indicative of a viscous content. In addition, there was marked peribronchial infiltration in the ventral lung field. Differential diagnosis included a primary pulmonary mass, fungal granuloma, bacterial abscess or foreign body abscess.

6.4.4 Histopathological examinations

Histopathological examinations of the collected lung samples showed signs of chronic fibrinous pleurisy of the superficial lung, but did not provide any further relevant diagnostic information.

6.4.5 Treatment and re-examination

The horse was discharged from the clinic on a course of oral enrofloxacin (7.5 mg/kg PO SID) and stable rest was recommended. Three months later, the horse was re-examined (14 November 2005) and was found to be in good body condition (563 kg) and the owner reported that there had been no epistaxis or coughing in the preceding

few weeks. Clinical examination was normal, and there was no coughing or respiratory abnormality on a rebreathing test. Clinical haematological and fibrinogen concentrations were within the reference range. Thoracic radiographs showed moderate reduction in size of the pulmonary mass (9 cm in diameter) of homogeneous soft tissue opacity with no gas cap evident. A mild to moderate focal brochio-interstinal lung pattern was still present caudodorsal to the cardiac silhouette, but was markedly improved compared to previous examination. In view of the reduction in size of the mass, neoplastic causes were considered unlikely, however, bacterial or fungal granuloma were still being the most likely differential diagnosis. In addition, the horse had bilateral bacterial conjunctivitis. A Schirmer test was performed on each side, with no presence of ulceration observed. The owner was requested to apply viscous tear gel to each eye once daily until completion of the course. A short course of Octin eye drops was prescribed as well for the treatment of infection. The horse was discharged with a further course of KI (10 g PO SID) to be administered orally for three months. Ridden walking exercises were recommended, however, was to be stopped if the horse coughs excessively. The horse was treated for a suspected bacterial granuloma in the left caudo-dorsal lung lobe. Treatment included enrofloxacin 7.5 mg/kg SID PO which was to continue for eight weeks; potassium iodide 10 g SID PO prescribed for six months. Complete rest of the horse was recommended, with restriction to free paddock exercise and no ridden work.

6.4.6 Mycobacterial isolation and identification

Mycobacterial growth was observed on Löwenstein-Jensen media slopes supplemented with pyruvate, which is known to promote growth of *M. bovis*, incubated at 37 °C. Microscopic examination of Ziehl Neelsen stained isolates revealed the presence of acid-fast organisms. The isolates were confirmed as members of the *Mycobacterium tuberculosis* complex (MTBC) due to the amplification of the 372 bp product and *M. bovis* was partially confirmed by amplification of the 206 bp product using PCR primers targeting RD9 region of difference.

6.4.7 Molecular characterization

Spoligotyping led to the confirmation of the isolates as *M. bovis*, due to lack of spacers 3, 9, 16, 39-43, characteristic of *M. bovis* strains. Additional spacers absent were 4, 5 and 13. This pattern was consistent with SB0868 according to the *M. bovis* database (www.mbovis.org). VNTR typing revealed a profile 6425346436553 based on a 13 loci VNTR panel (Qub-11a, Qub-11b, Qub-18 and Qub-26; MIRU 16, MIRU 23 and MIRU 26; Mtub 12 and Mtub 21; ETR-A, ETR-B, ETR-C and ETR-E).

6.5 Discussion

Tuberculosis is generally rare in horses even in countries where the disease is reported in other species (Garba *et al.* 2001). In this study, we report pulmonary tuberculosis due to *M. bovis* infection in an eight year old male Thoroughbred. Since the horse was not euthanized, the extent of spread of the infection to lymph nodes and other tissues is unknown, but it is unlikely that the organism caused disseminated lesions as the animals was in a very good body conditions, had good appetite and no longer coughing years following the initial diagnosis. Infection with *M. bovis* in horses appears to occur mostly by ingestion and the most common site of infection is the gastro intestinal tract (Pasvlik *et al.* 2004). This is unlikely in this case since the infection was associated with the trachea and the lungs, suggesting the respiratory route as the most probable mode of transmission. In consensus to our study, pulmonary infection in a four year old Camaguey stallion was also reported by Keck and co-workers. In this particular case, both ingestion and respiratory infections were suspected since the horse was in direct contact with infected cattle and shared potentially contaminated pasture (Keck *et al.* 2010).

The owner of the horse under study had bought the horse for the intended use of show jumping and showing one month before it presented bilateral epistaxis. Based on the time frames between the date of purchase and date of epistaxis presentation, it is highly unlikely that the horse had contracted the disease while in the possession of the new owner. Based on the owner's information, the horse originated from Cape Town in the Western Cape Province and was moved to Gauteng Province. No further details were available which could assist in tracing back the origin of the *M. bovis* strain type. Genotyping results of the *M. bovis* isolated however, revealed a spoligopattern (SB0860) and VNTR profile not previously identified in South African wildlife or

livestock, suggesting that the horse might have been imported into the country carrying a latent *M. bovis* infection. This suggestion is supported by the fact that both the Western Cape and Gauteng Provinces have the lowest incidences of bovine tuberculosis in the country. Spoligotype SB0860 had previously been isolated from mostly bovine samples in France during the period between 1979 and 2000 (Haddad *et al.* 2001).

M. bovis can be transmitted from horses to humans. In a previous report from Nigeria, a rare case of zoonotic transmission of *M. bovis* associated with the skin of a horse was described. This particular study also emphasized the importance of public awareness and control of the disease with special attention to those domesticated animals with close contact to humans such as horses (Garba *et al.* 2001). Horses, like cats, dogs and sheep, are considered incidental spill-over hosts and infection is not sustained within the population concerned (Cousins, 2001). *Mycobacterium bovis* was isolated from broncho alveolar lavage, indicating that the horse could have been shedding the organisms. Before diagnosis, the horse described in the current report had multiple contacts with other horses and humans raising the possibility of potential disease transmission. In 2012, then 15 year old, the horse was admitted again to the Equine Clinic at the Onderstepoort Veterinary Academic Hospital for colic surgery. Eight years following BTB diagnosis, the owner reported that the horse was in good condition, although no longer participating in show jumping. The horse seemed to have recovered from the clinical signs and possibly even the infection. Attempts are currently been made with the owner to subject the horse to further radiological, culture and DNA based tests, which are believed may give an insight into the nature of *M. bovis* infection in horses.

6.6 Conclusion

This report indicates that horses may contract and harbour *M. bovis* despite their lower susceptibility compared to other domestic animals. *M. bovis* was isolated from broncho alveolar lavage, indicating that the horse could have been shedding the organisms and thereby presenting the risk of transmission. Because of the zoonotic nature of the disease, it is of outmost importance to ensure that measures are taken to prevent transmission between the horse and persons with close contact.

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www.mbovis.org

Chapter 7
Identification and evaluation of tandem repeat sequences derived from whole genome sequences of *Mycobacterium bovis* isolates from South Africa

7.1 Abstract

Recent studies have indicated that a 13-locus variable number tandem repeat (VNTR) set was found suitable for molecular characterization of *Mycobacterium bovis* from South Africa. The same set could, however, not discriminate adequately between the parent strain and different variants isolates from wildlife species in the Kruger National Park (KNP) which evolved through clonal expansion. To improve the performance of this typing scheme, whole genome sequencing was carried out in the current study on three *Mycobacterium bovis* (*M. bovis*) isolates (i.e. TB 1474; TB 1771; KNP 440) originating from the KNP and one unrelated cattle isolate (i.e. TB 3912B) with the intent to identify new additional VNTR loci. Comparative genome analysis against the reference *M. bovis* AF2122/97 strain genome revealed significant differences based on the single nucleotide polymorphisms (SNPs). Seven tandem repeats of varying sizes (45 bp to 94 bp) were randomly identified from each genome sequence of isolates KNP 440 and TB 1474 using the Tandem Repeat Finder programme and exploited for polymorphism on selected panels of *M. bovis* isolates. Loci' individual allelic diversity ranged from 0.00-0.52 when assessed on a panel of genetically diverse *M. tuberculosis* complex strains including *M. bovis*. Five tandem repeats derived from genome sequence of isolate KNP 440 had higher allelic diversities and were subsequently further evaluated on three selected panels of *M. bovis* isolates. The outcome of this study highlighted the potential of two of the identified additional polymorphic loci in differentiating clonally linked isolates from the KNP. Further application of the polymorphic loci on a large collection of local *M. bovis* isolates and comparison of discriminatory powers with the currently utilized VNTR loci is recommended to confirm their usefulness in molecular epidemiological investigations.

7.2 Introduction

The identification of bacterial pathogens such as *M. bovis* at strain level is essential for epidemiological investigations and as a result efforts are undertaken to develop convenient, highly discriminatory, highly reproducible and high-throughput strain typing tools such as variable number tandem repeat (VNTR) typing that can be routinely applied in clinical laboratories (Le Fleche *et al*. 2002; Roring *et al.* 2004). In addition, investigation of the genetic diversity amongst bacterial populations is a promising approach to understand their evolutionary characteristics and pathogenicity (Fleischmann *et al.* 2002; Kato-Maeda *et al.* 2012). The whole genome sequences of *M. tuberculosis* strains H37Rv and CDC1551 (Cole *et al.* 1998; Fleischmann *et al.* 2002) as well as *M. bovis* AF2122/97 (Garnier *et al.* 2003) have been exploited to facilitate the identification of polymorphic tandem repeat DNA sequences used in VNTR typing (Roring *et al.* 2004). One of the main advantages of characterization using VNTR typing is that the technique can be further improved by exploiting additional tandem repeats present in the whole genome sequences of *M. tuberculosis* complex bacteria (Roring *et al.* 2002; Smittipat *et al.* 2005). However, it is important to use locally prevalent strains for local epidemiological applications (Roring *et al.* 2004; Bionotti *et al.* 2009). For example, in a study conducted on field *M. bovis* isolates from the Northern Ireland and Republic of Ireland, the discriminatory power of the existing VNTR typing was greatly improved by an application of a combination of novel VNTR loci. As a result, a highly discriminating and highly reproducible VNTR assay was developed (Roring *et al.* 2004).

In South Africa (SA), recent studies using VNTR typing for molecular characterization of local isolates indicated that the unique *M. bovis* strain linked to the epidemic in the KNP has been undergoing evolutionary changes resulting in a clonal expansion, hence the challenge to distinguish between the parent strain and its variants (Hlokwe *et al.* 2013). The goal of the current study was to identify variable number of tandem repeat loci from whole genome sequence data of local *M. bovis* strains. The polymorphism of the loci was assessed in an attempt to further improve the existing VNTR typing scheme for South African *M. bovis* in differentiating clonally linked isolates from the Greater Kruger National Park complex (GKNPC).

7.3 Materials and methods

7.3.1 Panel of *M. bovis* isolates for DNA extraction and sequencing

Mycobacterium bovis isolates including clonally linked isolates of wildlife species from the KNP as well as an unrelated cattle strain responsible for a bovine tuberculosis (BTB) outbreak involving epidemiologically linked farms were selected for full genome sequencing. All isolates had been previously genetically characterized using IS*6110* typing, variable number of tandem repeat (VNTR) typing as well as spoligotyping (Michel *et al.* 2008; Hlokwe *et al.* 2013). The isolates are listed in Table 9. DNA was extracted from these isolates according to a previously published protocol and stored at -20 °C until further use (Hlokwe *et al.* 2011).

7.3.2 Library construction and DNA sequencing

Libraries with insert sizes of approximately 250 bp were prepared using the Nextera DNA sample preparation kit and DNA sequencing was carried out in an Illumina genome analyser platform according to procedures adapted at the ARC-Onderstepoort Veterinary Institute, Biotechnology platform.

Table 9 *Mycobacterium bovis* strains selected for whole genome sequencing and comparative genome analysis

Isolate identification	Species	IS*6110* type	VNTR type	Spoligotype
TB 1474	Lion	C8[(a)]	VNTR-1[(b)]	SB0121[(b)]
TB 1771	Leopard	C8v[(a)]	VNTR-1[(b)]	SB0121[(b)]
KNP 440	Buffalo	C8v[(a)]	VNTR-2[(b)]	SB0121[(b)]
TB 3912B	Cattle	C1[(c)]	VNTR-15[(b)]	SB0130[(b)]

C1 = cattle strain; C8 = cattle strain (parent strain) which spilled over to KNP buffaloes from cattle; C8v = variant of the cattle strain
VNTR-1, 2 and 15 = published information defining unique profiles
[(c)]Michel *et al.* 2008; [(a)]Michel *et al*. 2009; [(b)]Hlokwe *et al.* 2013

7.3.3 Analysis of sequence data

A combination of software packages as well as in-house developed scripts was used to compile a customised pipeline for analysis of Mycobacterial genome sequences at Stellenbosch University. This pipeline was used to analyse the sequence data.

7.3.3.1 Quality control of the reads

Sequencing data for each isolate was subjected to quality control by trimming before being mapped to the reference strain. The quality of the sequence reads was inspected using the FastQC software programme (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Appropriate trimming of the 3' ends of the reads was carried out with a simple clipper function in the Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), while maintaining a good quality of the reads (phred score > 22) and reads length.

7.3.3.2 Genome assembly and annotation

Three independent mapping software packages utilizing different algorithms for were used for mapping the raw reads against *M. bovis* AF2122/97 reference strain downloaded from Sanger (ftp://ftp.sanger.ac.uk/pub/pathogens/mb/). The mapping software packages used included the BWA (that uses a Burrows-Wheeler transform algorithm), Novoalign (Needleman-Wunch algorithm) and Bfast - which employs a hash table-based algorithm.

7.3.3.3 Single nucleotide polymorphisms (SNPs) calling for comparative analysis

The three different mapping files obtained were analysed using two SNP callers, i.e. Samtools (http://samtools.sourceforge.net/) and GATK (http://www.broadinstitute.org/gatk/), which resulted in six variant files containing SNPs. SNPs that correspond in position and base change in all six files were found, and these high confidence SNPs were then used for further analysis. Regions with zero coverage indicating potential deletions were found on the alignment files.

7.3.3.4 Deletion determination

For each of the mapping files, genome wide depth of coverage was determined with a specialized function in the Bedtools software package (www://code.google.com/p/bedtools/). The depth coverage files were searched for regions with zero depth coverage (i.e. no reads mapped to these regions). To ensure regions of true deletions, every region was visually inspected. Large deletions present in at least two of the three mapping files were considered as high confidence deletions.

7.3.4 Construction of the phylogenetic tree

Using the high confidence SNPs found amongst the four *M. bovis* isolates, a neighbor joining phylogenetic tree was constructed using MEGA 5 software (http://www.megasoftware.net/mega). No out group isolate was included.

7.3.5 Identification of tandem repeats (VNTR loci)

Consensus DNA sequences in fasta format derived from the mapped reads of the genome sequences of *M. bovis* isolates KNP 440 and TB 1474 were subjected to a program called Tandem Repeat Finder version 4.07 (http:tandem.bu.edu/trf/output/59kXX6hmeIuCc.2.7.7.80.10.50.500.1.html) to locate tandem repeats in the DNA sequences (Benson, 1999). Primers flanking each side of the repeats were designed (Inqaba Biotechnologies, Pretoria, South Africa) to determine the number of repeats at each locus based on the sizes of the Polymerase Chain Reaction (PCR) products on agarose gels.

7.3.6 Typability and polymorphism evaluation of tandem repeats

Typability and discriminatory capabilities of individual loci was initially assessed across a panel of 16 genetically diverse *M. tuberculosis* complex species (panel 1, Table 11) including *M. bovis* (n = 12), *M. tuberculosis* (n = 3) and *M. orygis* (n = 1). Polymorphism testing was further conducted on a panel consisting of 12 *M. bovis* isolates collected from cattle on three epidemiologically linked different herds (panel 2, Table 12) (Michel *et al.* 2008; Hlokwe *et al.* 2014) as well as 13 *M. bovis* isolates collected from cattle on the same farm (panel 3, Table 13) (Hlokwe *et al*. 2014). In addition, loci were further subjected to a panel (panel 4) consisting of 21 isolates from the KNP linked through

clonal expansion. All isolates were previously characterized (Michel *et al.* 2009; Hlokwe *et al.* 2013; Hlokwe *et al.* 2014). VNTR polymorphism of each repeat locus was carried out by PCR amplification as previously described (Hlokwe *et al.* 2013) using primers outlined in Table 10.

7.3.7 Sequence verification

In order to verify repeat copy number variation, PCR products representing all the different alleles in the initial assessment for all polymorphic loci were sequenced using the forward primer (Inqaba Biotechnologies, Pretoria, South Africa). The resulting sequences were analysed by using Sequence scanner v1.0 (Applied Biosystems) to determine the PCR product sizes which were in turn used to predict repeat copy number for the other alleles.

7.3.8 Allelic diversity calculation

Allelic diversity of each locus was calculated using the Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat (Hunter and Gaston, 1988).

7.4 Results

7.4.1 Genome sequences of four selected *M. bovis* isolates

The genomes of *M. bovis* isolates TB 1474, TB 1771 and TB 3912B were of the same size (4 345 533 bp each), whereas *M. bovis* isolate KNP 440 was a single bp longer (4 345 534 bp). The genomes were all slightly greater than the genome sequence of the reference *M. bovis* AF 2122/97 strain (4 345 492 bp). Their GC content was 65 %.

7.4.2 Single nucleotide polymorphisms (SNPs) identification

Single nucleotide polymorhisms were identified on the four genomes based on the published and publicly available *M. bovis* AF2122/97 reference strain genome. For *M. bovis* isolate identified as KNP 440, a total of 527 high confidence SNPs were found. Among these SNPs were those found in the genic region (n = 461) and intergenic region (n = 66) distributed throughout the genome. Of the 461 genic SNPs, 175 were

synonymous changes and 286 were non synonymous changes. For isolate TB 1474, 333 SNPs were identified, 30 SNPs were intergenic, 121 were synonymous and 182 were non-synonymous. Isolate TB 3912 had 441 SNPs, of which 57 SNPs were intergenic, 149 were synonymous and 235 non-synonymous SNPs. The highest number of SNPs was identified in the genome sequence of isolate TB 1771 (i.e. 553 SNPs). Seventy of these SNPs were intergenic, 192 synonymous and 291 SNPs were nonsynomymous. Majority of the SNPs identified in all four *M. bovis* isolates were linked to intermediary metabolism and respiration (average approximately 20 %), cell wall and cell processes (average approx 18 %) as well as conserved hypothetic proteins (average approximately 19 %). Only a small percentage of SNPs was linked to virulence, detoxification and adaptation (average approximately 1.5 %). SNPs that were common in the genome of isolates derived from wildlife samples KNP 440, TB 1474 and TB 1771 and not in the genome from an unrelated cattle isolate (i.e. TB 3912) were identified. SNPs found in TB 3912 and not the other three strains were also identifed. Figures 13 and 14 illustrate total numbers of SNPs found in the synonymous, non-synonymous as well as intergenic regions for two of the four genomes, i.e KNP 440 and TB 3912B. Figure 15 illustrates a neighbor joining phylogenetic tree based on annotated high confidence SNPs.

7.4.3 Tandem repeat identification and evaluation

Only the whole genome sequences of isolate KNP 440 and TB 1474 were exploited for tandem repeats identification. A total of 979 tandem repeats of varying sizes ranging from 6 bp to 328 bp were identified on the entire genome sequence of *M. bovis* isolate KNP 440, and 971 repeats (sizes ranging between 9 bp to 327 bp) for *M. bovis* isolate TB 1474. The sizes and positions of the tandem repeat loci on the genome of KNP 440 and TB 1474 are indicated in Tables 12 and 13. The primers flanking regions of the loci were designed for only seven randomly selected tandem repeats from each genome and assessed for their potential value in a VNTR typing scheme for *Mycobacterium tuberculosis* complex including *M. bovis*. The tandem repeats selected varied in sizes between 45 bp and 94 bp. The list of primers designed and used for PCR amplification is outlined in Table 10. Within this panel of isolates, six loci, five of which were derived from the genome sequence of isolate KNP 440 (i.e. KNP 440 TR03, KNP 440 TR 07, KNP 440 TR 12, KNP 440 TR 15 and KNP 440 TR 16) and one loci from isolate TB 1474 (TB 1474 TR 03), were found to display size polymorphism (see Table 11 and

Figures 16 and 17 as examples). Both loci KNP 440 TR 03 and KNP 440 TR 07 had three allelic variants while the other three polymorphic loci had two allelic variants. Although amplification was possible for five loci (i.e. KNP 440 TR 14; TB 1474 TR 01, TB 1474 TR 02, TB 1474 04 and TB 1474 10), they could, however, not differentiate amongst the *M. tuberculosis* complex strains. Lastly, nonspecific product sizes and/or no amplification were observed when loci KNP 440 TR 13, TB 1474 TR 09 and TB 1474 TR 16 were amplified. Locus individual discriminatory index ranged from 0.00-0.52, with five loci derived from the genome of KNP 440 isolate having higher discriminatory indeces (see Tables 12 and 13). The study did not establish specific genes on which loci were housed.

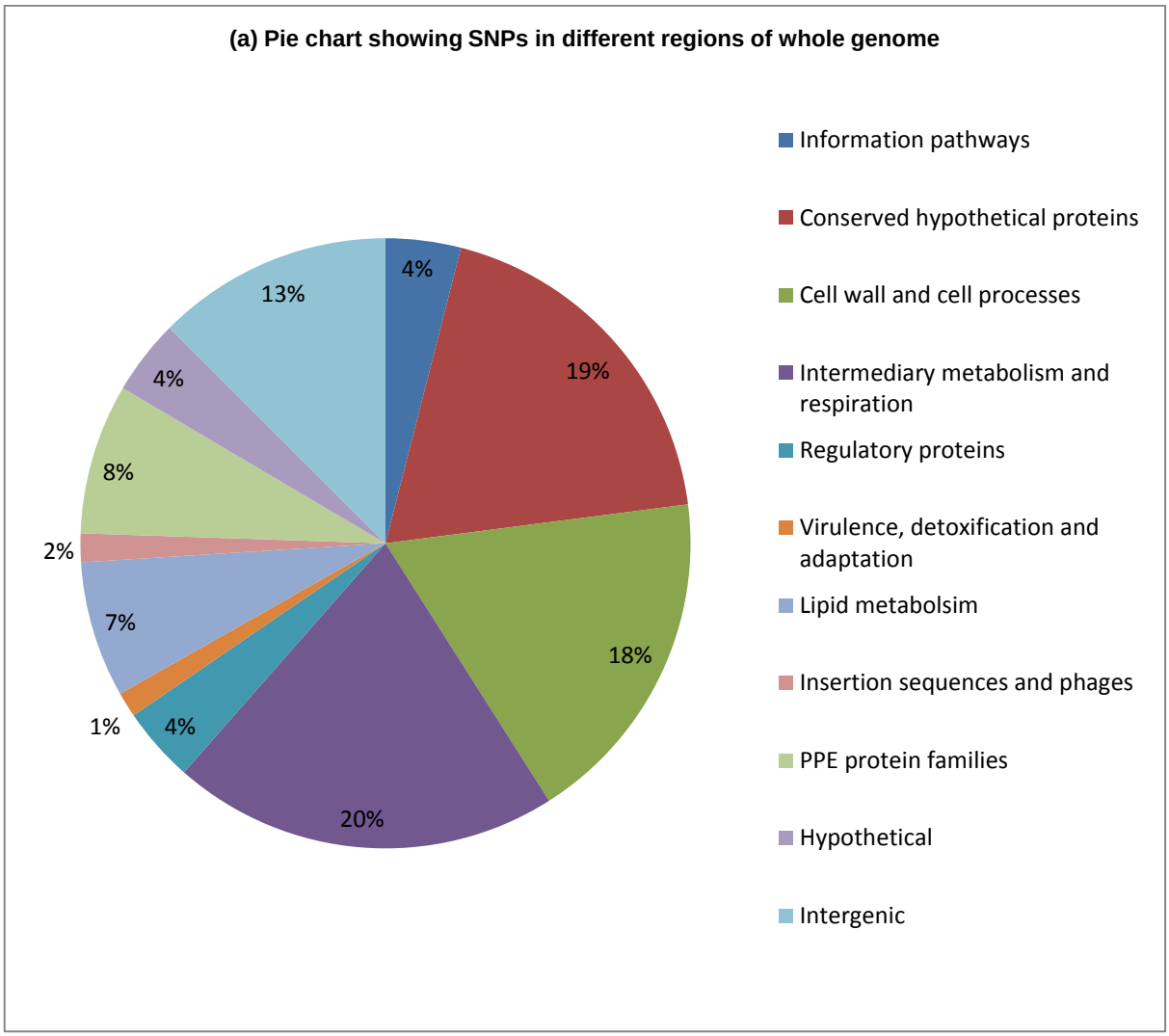

Figure 13 The total number of SNPs including intergenic, synonymous and non-synonymous SNPs detected in the whole genome sequence of *M. bovis* isolate KNP 440 collected from wildlife species

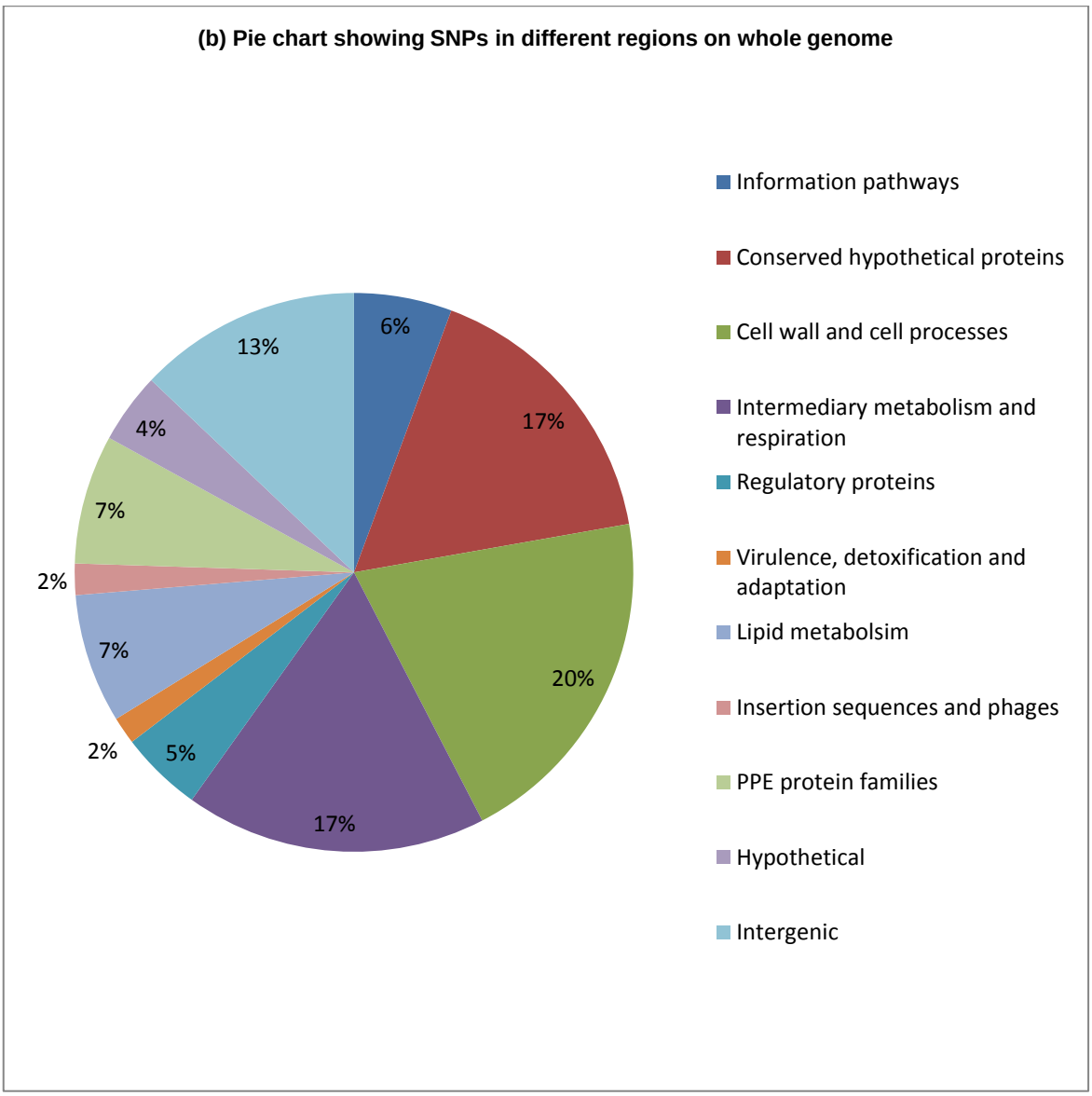

Figure 14 The total number of SNPs including intergenic, synonymous and non-synonymous SNPs detected in the whole genome sequence of *M. bovis* isolate TB 3912B collected from cattle

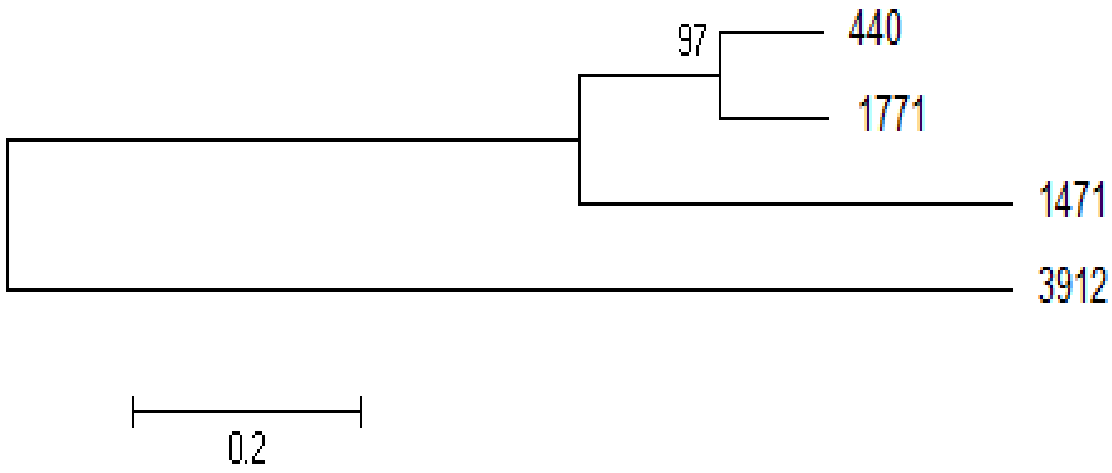

Figure 15 Neighbour joining phylogenetic tree based on the the high confidence SNPs found amongst the four *M. bovis* isolates (KNP 440; TB 1771; TB 1474; TB 3912 B) constructed using MEGA 5 software. The evolutionary history was inferred by using the maximum likelihood method based on the General Time Reversible model (Nei and Kumar, 2000).

Table 10 Outlines primer sequences used for PCR amplification of the tandem repeats derived from *M. bovis* isolates KNP 440 and TB 1474

Tandem repeat	Oligonucleotide primers	
	Forward Primer 5'-3'	**Reverse Primer 5'-3'**
KNP 440 TR03	TTA CAT ACC CCG CGA AGC C	ATT GCC CTG ACC TAC ACC
KNP 440 TR07	ACT GCC TCG AAC ACC AC	CAA ACA CCT CAA TCA CAC CC
KNP 440 TR12	TCG CTG TGG TCG CTT	TAG CGC GTC TGC CAT
KNP 440 TR15	GCC CGG ACA AGA AGA ACA	ATC AGC CAA CCG CCT
KNP 440 TR16	GGT TGG TCG TTG CTG TT	CTG TTG TGG TGT CGG TG
KNP 440 TR14	AAG GCG CGA TTC TGG	CGA CGG GAA GAA ACT GA
KNP 440 TR13	GTT TTC TGT TCG CCG ACT	TTG ATT TGC CCG GTT CCT
TB 1474 TR01	CCC TAT CTG CTG ACG CT	GCT CAA AAT CCC ATC CGA C
TB 1474 TR02	TTT GCT GCA GGG TGT G	AGT TGG CCA GGG CTT
TB 1474 TR03	TTG TTG CTG CCC GAG TT	GTT GGC AAA TCT GGG CG
TB 1474 TR04	CAG CGC GTA GGA GTA AC	AAC TTC GGC ATC GGC AA
TB 1474 TR09	TGC CGA TGC CGA TGT T	ATG TCT CGG GCT TGC T
TB 1474 TR10	TGC CGA TGC CGA TGT TT	ATG TCT CGG GCT TGC T
TB 1474 TR14	GTG ATG AAA GCC CCG GT	ATT TTG CTG GCG GCT G

Table 11 Evaluation of tandem repeat sequences derived from the whole genome sequences of *Mycobacterium bovis* isolates KNP 440 and TB 1474 on 16 *Mycobacterium tuberculosis* complex species (panel 1)

Isolate	*M. tb* species	Tandem repeat loci													
		KNP 440 TR03	KNP 440 TR07	KNP 440 TR12	KNP 440 TR13	KNP 440 TR14	KNP 440 TR15	KNP 440 TR16	TB 1474 TR01	TB 1474 TR02	TB 1474 TR03	TB 1474 TR04	TB 1474 TR09	TB 1474 TR10	TB 1474 TR14
TB 7655	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
B8	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 4760B	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 3980G	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 4572B	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
No.10	*M. bovis*	1.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
4841(Italy)	*M. tb*	2.2	6.2	1.9	n/a	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 7374	*M. tb*	2.2	5.2	1.9	-	2.2	2	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 3958A	*M. bovis*	1.2	5.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 7868A	*M. bovis*	1.2	6.2	3.9	n/a	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 8117	*M. bovis*	2.2	6.2	3.9	n/a	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 7942	*M. bovis*	2.2	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 6616B	*M. orygis*	3.2	4.2	3.9	n/a	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
TB 7876	*M. tuber-culosis*	2.2	6.2	3.9	n/a	2.2	2	5.1	2.4	2.4	4.3	2.2	-	3	-
***M. bovis* AF 2122/97**	*M. bovis*	1.1	6.2	3.9	-	2.2	3	5.1	2.4	2.4	2.3	2.2	-	3	-
KNP 440	*M. bovis*	2.2	6.2	3.9	-	2.2	3	3.1	2.4	2.4	2.3	2.2	-	3	-

- = no interpretable results; n/a = no amplification

Table 12 Variable number of tandem repeats loci derived from *Mycobacterium bovis* isolate KNP 440, repeat copy numbers and diversity index of the loci as evaluated across 16 *M. tuberculosis* complex isolates (Panel 1)

Locus name	Position on *M. bovis* KNP 440 genome	Locus size (bp)	No of alleles	Tandem repeat copy number in *M. bovis* KNP 440	Product size and (predicted copy numbers)	Diversity Index
KNP 440 TR03	1640994-1641112	53	3	2.2	300 bp (1.2); 353 bp (2.2); 406 bp (3.2); 459 bp (4.2)	0.52
KNP 440 TR07	1880747-1880985	57	3	4.2	286 bp (4.2); 353 bp (5.2); 410 bp (6.2); 467 bp (7.2)	0.51
KNP 440 TR12	2382213-2382439	58	2	3.9	334 bp (1.9); 392 bp (2.9); 450 bp (3.9); 508 bp (4.9)	0.33
KNP 440 TR15	2673597-2673786	94	2	2	320 bp (2); 415 bp (3); 509 bp (4); 603 bp (5)	0.23
KNP 440 TR16	2947064-2947232	55	2	3.1	320 bp (3.1); 375 bp (4.1); 430 bp (5.1); 585 bp (6.1)	0.24
KNP 440 TR14	2671959-2672082	57	1	2.2	300 bp (2.2); 357 bp (3.2); 414 bp (4.2); 471 bp (5.2)	0.00
KNP 440 TR13	2440351-2440649	57	n/a	5.2	n/a	n/a

n/a = not applicable as a result or no PCR amplification. The copy numbers for up to four alleles were predicted

Table 13 Variable number of tandem repeats loci derived from *M. bovis* isolate TB 1474, repeat copy numbers and diversity index of the loci as evaluated across 16 *M. tuberculosis* complex isolates (Panel 1)

Locus name	Position on *M. bovis* TB 1474	Locus size (bp)	No. of alleles	Tandem repeat copy number in *M. bovis* TB 1474	Product size and (predicted tandem repeat copy numbers)	Diversity Index
TB 1474 TR 01	154306-154430	53	1	2.4	410 bp (2.4); 463 bp (3.4); 516 bp (4.4)	0.00
TB 1474 TR 02	335982-336135	84	1	1.8	290 bp (1.8); 374 bp (2.8); 458 bp (3.8)	0.00
TB 1474 TR 03	367945-368081	60	2	2.3	210 bp (2.3); 330 bp (4.3); 390 bp (5.3)	0.125
TB 1474 TR 04	370371-370500	60	1	2.2	330 bp (2.2); 390 bp (3.2); 450 bp (4.2)	0.00
TB 1474 TR 09	426484-426653	90	n/a	1.9	n/a	n/a
TB 1474 TR 10	532526-532658	45	1	3	350 bp (3); 395 bp (4); 440 (5)	0.00
TB 1474 TR 14	581598-581755	77	n/a	2.1	n/a	n/a

n/a = not applicable and as a result no PCR amplification. Copy numbers of up to three alleles were predicted

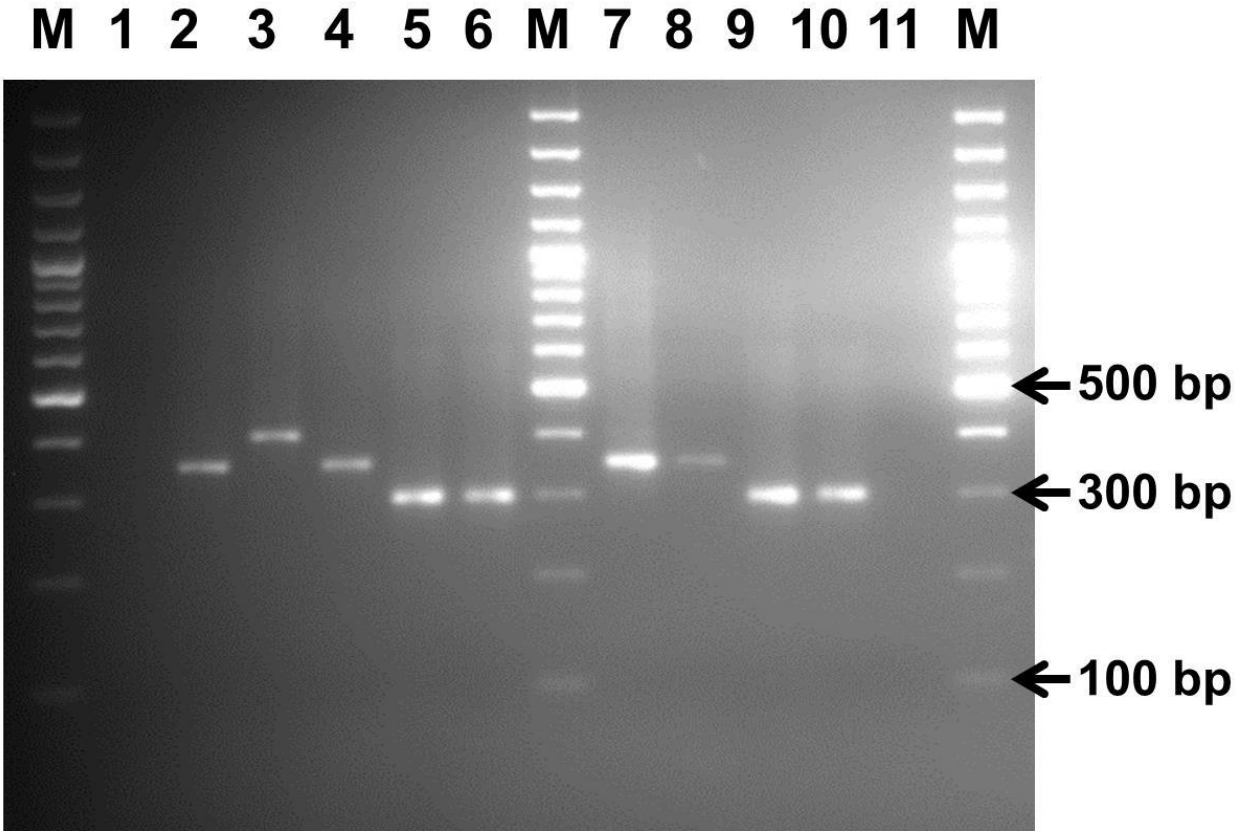

Figure 16 An electrophoresis gel picture showing three different alleles resulting from VNTR typing using KNP 440 TR03 locus. The gel lanes were loaded as follows: Lane M, 100 bp Plus DNA ladder; lanes 1-9: *M. tuberculosis* complex isolates (including 6 x *M. bovis* strains, 1 x *M. orygis* strain and 2 x *M. tuberculosis* strains, respectively); lanes 10 and 11: *M. tuberculosis* H37Rv (as positive control) and water (as a negative control), respectively.

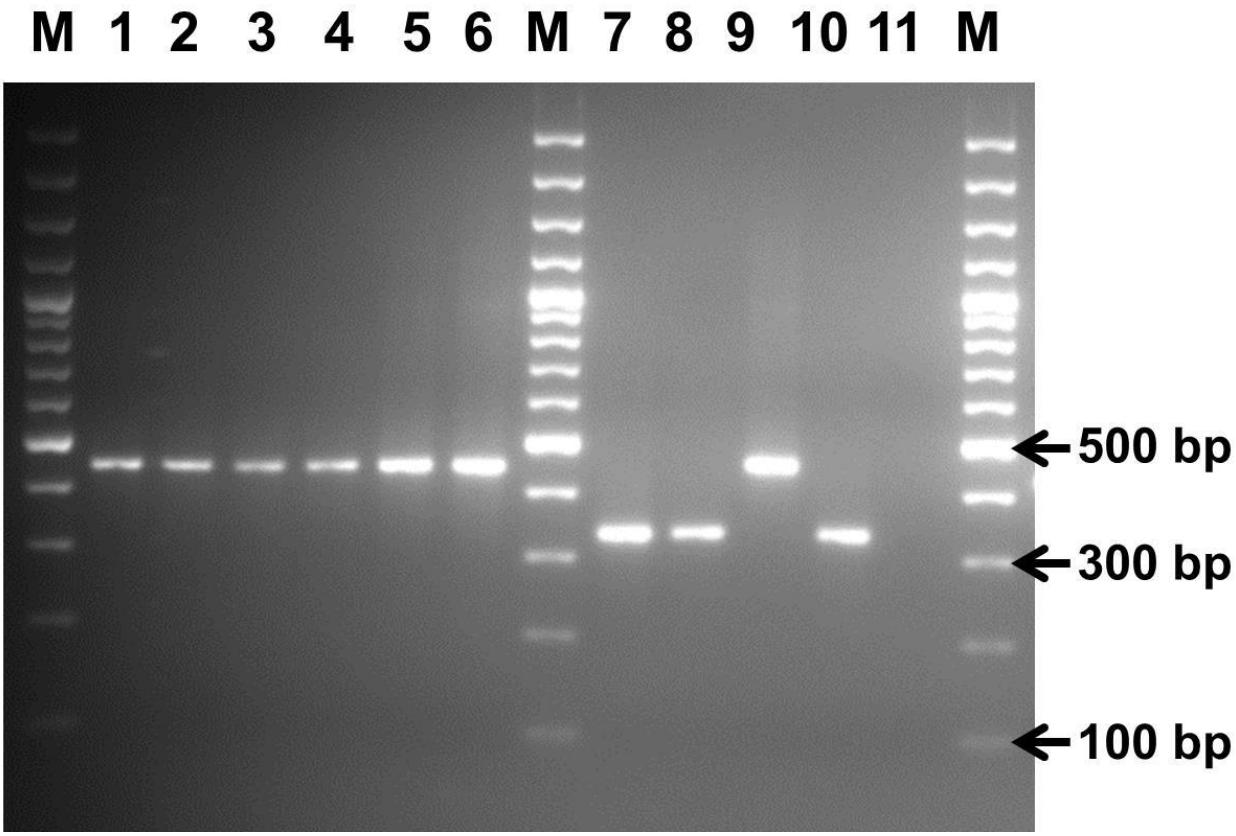

Figure 17 An example of an electrophoresis gel picture showing two different alleles resulting from VNTR typing using KNP 440 TR 12 locus. The gel lanes were loaded as follows: Lane M represents a 100 bp Plus DNA ladder, lane 1-9 is the *M. tuberculosis* complex isolates (including 6 x *M. bovis* strains, 1 x *M. orygis* strain and 2 x *M. tuberculosis* strains, respectively), lanes 10 and 11 is the *M. tuberculosis* H37Rv (as positive control) and water (as negative control) respectively.

7.4.4 Sequence verification of the PCR product size

The sizes of different alleles per locus as determined by sequencing of the PCR product and analysis using the Sequence scanner v1.0 was found to correspond satisfactorily to the sizes estimated by gel electrophoresis. The product sizes assisted in the determination of repeat copy number of each locus per isolate (see Tables 12 and 13).

7.4.5 Stability and further polymorphism evaluation

Based on their higher discriminatory index, five, tandem repeats derived from isolate KNP 440 genome (KNP 440 TR 03, KNP 440 TR 07, KNP 440 TR 12, KNP 440 TR 15 and KNP 440 TR 16) were further evaluated on additional three panels of isolates (i.e. panel 2, 3 and 4). Within isolates of panels two and three, none of the loci exhibited

allelic variation (Tables 14 and 15). However, three loci (i.e. KNP 440 TR 12, KNP 440 TR 15 and KNP 440 TR 16) were able to differentiate the *M. tuberculosis* H37Rv control from the *M. bovis* isolates. Locus KNP 440 TR 03 correctly identified isolate TB 7833D, TB 7834B and TB 7837D from the KNP as variant strains (Table 16); whereas KNP 440 TR 16 identified isolate KNP 440 as variant strains (results not shown).

Table 14 Stability and polymorphism evaluation of tandem repeats from the genome of *M. bovis* isolate KNP 440 across a panel of 12 isolates from epidemiologically linked cattle herds (Panel 2). *M. tuberculosis* H37Rv was included as a positive control.

Isolate No.	13-locus VNTR profile (Hlokwe *et al.* 2014)	VNTR locus and copy number				
		KNP 440 TR 03	KNP 440 TR 07	KNP 440 TR 12	KNP 440 TR 15	KNP 440 TR 16
TB 3932D	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3939G	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3940F	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3942B	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3958C	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3966D	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3968D	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3976E	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3979E	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3981C	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3982G	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
TB 3983A	10 3 2 3 2 2 6 4 3 6 5 5 3	1.2	6.2	3.9	3	5.1
***M.tb* H37Rv**	Not done	1.2	6.2	1.9	2	4.1

M. tb = M. tuberculosis

Table 15 Stability and polymorphism evaluation of tandem repeat sequences from the genome of *M. bovis* isolate KNP 440 across a panel of 13 isolates from the same cattle herd (Panel 3). *M. tuberculosis* H37Rv was included as a positive control.

Isolate No.	13-locus VNTR profile (Hlokwe *et al.* 2014)	VNTR locus and copy number				
		KNP 440 TR 03	KNP 440 TR 07	KNP 440 TR 12	KNP 440 TR 15	KNP 440 TR 16
TB 7905C	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
TB 7906B	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
TB 8135	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
TB 8136	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
TB 8042J	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 2	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 5	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 8	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 9	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 11	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 22	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 25	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
No. 10	11 4 3 4 2 4 6 4 3 6 3 5 3	1.2	6.2	3.9	3	5.1
***M. tb* H37Rv**	Not done	1.2	6.2	1.9	2	4.1

M. tb = M. tuberculosis

Table 16 Stability and polymorphism evaluation of five tandem repeat sequences from the genome of *M. bovis* isolate KNP 440 across a panel of 21 isolates epidemiologically linked through clonal expansion (Panel 4).

Isolate No.	13-locus VNTR Type (Hlokwe *et al*. 2013 and 2014)	VNTR locus and copy number				
		KNP 440 TR 03	KNP 440 TR 07	KNP 440 TR 12	KNP 440 TR 15	KNP 440 TR 16
TB 7831C	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
KNP 70	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7837B	8 2 4 3 3 4 5 4 2 6 4 3 4	1.2	6.2	3.9	3	3.1
TB 3852D	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 3887G	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 6892A	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7996A	8 2 4 3 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 3855D	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 8003G	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 1700	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7254D	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 3842	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7605B	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7655	8 2 4 4 3 4 5 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7833D	8 2 4 4 2 4 5 4 2 6 4 3 4	1.2	6.2	3.9	3	3.1
TB 7834B	8 2 4 4 2 4 5 4 2 6 4 3 4	1.2	6.2	3.9	3	3.1
TB 7170A	8 2 4 4 3 4 6 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 7830C	8 2 4 4 3 4 4 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
TB 3898E	8 2 4 4 3 4 3 4 2 6 4 3 4	2.2	6.2	3.9	3	3.1
KNP 6A	8 2 4 4 3 4 5 4 2 6 4 3 3	2.2	6.2	3.9	3	3.1
KNP 440	8 2 4 4 3 4 5 4 2 6 4 3 3	2.2	6.2	3.9	3	3.1

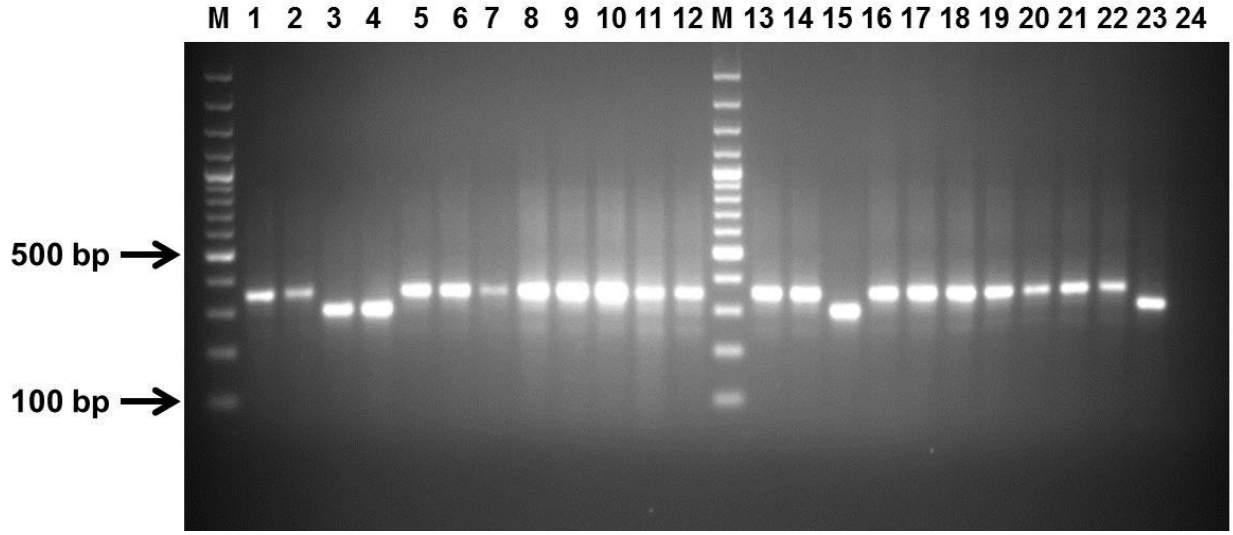

Figure 18 An electrophoresis gel picture showing two different alleles resulting from VNTR typing of 22 isolates from the Kruger National Park (KNP) using KNP 440 TR03 locus. Lane M represents a 100 bp Plus DNA ladder; lane 1-22 is the *M. bovis* isolates from KNP (one sample was run in duplicate); lane 23 and 24 is the *M. tuberculosis* H37Rv and water control respectively. Isolate TB 7833D and TB 7834B are in lane 3 and 4 respectively. Isolate TB 7837D is in lane 15.

7.5 Discussion

The availability of the genome sequences of *M. bovis* and *M. tuberculosis* strains have made it possible to address the genetic basis of key phenotypic traits of these pathogens as well as their evolution and pathogenesis (Pan *et al.* 2011; Kato-Maeda *et al.* 2012). Modern methods are usually genetic methods based on Polymerase Chain Reaction to identify variation directly at the DNA level (Le Fleche *et al.* 2002). In the current study, four *M. bovis* isolates from South African wildlife and cattle were subjected to whole genome sequencing, with the aim to identify additional new VNTR loci which can improve the currently applied VNTR typing regime in distinguishing among various clones of *M. bovis* strain circulating in the KNP. Comparative genomics analysis conducted for the four strains relative to *M. bovis* AF2122/97 revealed that the genomes derived from wildlife variant isolates of C8 strain, i.e. TB 1771 and KNP 440, had a 40 % and 37 % more SNPs, respectively, as compared to the parental C8 strain (i.e. TB 1474). These findings indicate that more mutations had occurred in these *M. bovis* strains, and provide alternative evidence to supports previous molecular typing results indicating that *M. bovis* strain causing BTB epidemic in the KNP is undergoing clonal expansion. In a study conducted in the United States of America, researchers had suggested that increased genomic deletions tend to reduce the pathogenicity of

M. tuberculosis species (Kato-Maeda *et al.* 2012). Increased mutations detected in isolate TB 1771 and KNP 440 as observed in the current study may also suggest that these strains are less virulent compared to their parental strain. In an earlier study conducted on the epidemiology of *M. bovis* infections in the KNP, it was found that VNTR-1 strain type, which is a C8 parental strain in the KNP, spreads more actively than its variant strains (Hlokwe *et al.* 2013). Previously, 73 % of the isolates characterized using IS*6110* typing shared this strain type, with the remaining 27 % represented the different variant strains of the parent strain (C8 strain) (Michel *et al.* 2009). Current findings suggest that not only is this strain more transmissible, but may also be more virulent than its variant strains. Future studies on these genomic variants including virulence assessments between the different strains will help to identify molecular factors that could provide a basis for the selection of vaccine candidate strains to be used in BTB prevention and control. Many successful vaccines target virulence factors, for example the toxins and capsular polysaccharides which are essential for the organism's potential to cause disease (Moxon and Rappuoli, 2002). It is noted with great interest that in all the genomes analysed, more SNPs were located in the non-synonymous regions. These regions are known to produce amino acid replacements, prompting the need for future investigations of the genes involved and their biological functions.

Based on their whole genome sequences, the polymorphisms of 14 tandem repeat sequences of a parental C8 strain from the KNP (TB 1474) and its variant strain (KNP 440) were investigated. The individual allelic diversity of the five loci derived from isolate KNP 440 genome on ten of the 16 *M. bovis* isolates (panel 1) was comparable to that of four of the 13 locus panel described in our previous study (Hlokwe *et al.* 2014), highlighting their usefulness in strain typing of local *M. bovis* isolates. Further evaluation on a larger panel is needed as the new loci may have potential to replace some of the currently used loci. No genetic variation was observed amongst isolates from two *M. bovis* panels consisting of epidemiologically linked cattle isolates, suggesting that loci are stable enough for use in contact tracing. Isolates of these two panels belonged to spoligotype patterns SB0131 and SB0130, respectively (Hlokwe *et al.* 2014). The five polymorphic loci identified in the current study failed to differentiate the two strains. Of great interest was the capability of KNP 440 TR03 and KNP 440 TR 16 loci to correctly identify some of the clonal variants in the KNP as characterised by a 13 locus panel used in a previously study. Same results were

obtained upon repeat typing of the isolates using locus KNP 440 TR03, highlighting the typing reliability of this particular marker. We had previously established that only five of the 13 loci (i.e. ETR-E, ETR-C, MIRU 26, QUB-26 and ETR-B) which were found to be polymorphic for South African *M. bovis* isolates contributed to genetic variation amongst isolates from the KNP (Hlokwe *et al.* 2013). Addition of tandem repeat locus KNP 440 TR03 to these five loci has the potential to increase the discriminatory power of the VNTR typing scheme for isolates from wildlife in this ecosystem. The current study did, however, not assess the *in vivo* stability of the loci. This study contributed local *M. bovis* genome information which will in future provide a greater opportunity for comprehensive sequence comparison able to assist in further deducing the phylogenetic and evolutionary relationships of *M. bovis* isolates in the country. In addition, the genomes will in future be screened for genes that encodes proteins with the attributes of good vaccine targets, for example, bacterial surface proteins (Masignani *et al.* 2002), which will ungergo further laboratory evaluation for immunogenicity.

Variable number tandem repeat loci had previously been found in both the intergenic and coding regions of the genomic DNA, and were found to function as molecular switches in microorganisms by regulating transcription and translation (Renders *et al.* 1999; Van Belkum, 1999). The current study did not investigate the position of the polymorphic loci based on the functional genes and proteins of which they encode, therefore further studies are needed in this regard. In addition, the similarity of these tandem repeats to previously reported *M. tuberculosis* H37Rv TR loci should be investigated to verify if they are not already included under alternative names in the 13 locus panel currently utilized. The SNPs identified in the four genomes also need to be verified through PCR amplification and DNA sequencing before they can be exploited in the future for genotyping analysis.

7.6 Conclusion

The outcome of this study provided alternative evidence to supports previous findings that *M. bovis* strain causing bovine tuberculosis epidemic in the KNP is undergoing clonal expansion. In addition, five of the loci investigated showed potential application in molecular typing of *M. tuberculosis* complex including *M. bovis*. The markers have proven to be sufficiently stable for characterization of epidemiologically linked *M. bovis* isolates from cattle and can therefore be applied in contact tracing investigations. Moreover, VNTR typing using two of the loci showed potential (discriminatory capability) for clonally linked isolates. Comparative evaluation of the current and previously selected set of loci on a larger collection of *M. bovis* isolates may allow further improvement of the VNTR typing method currently utilized in the KNP.

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Chapter 8
General discussion and conclusion

Bovine tuberculosis has been previously confirmed in livestock and wildlife species in South Africa; however, the population structure of *M. bovis* strains circulating in the region and spill-over dynamics was not adequately known. Progress towards eradication of the disease in many countries has been hampered by lack of epidemiological data. The current study was aimed at addressing an important knowledge gap in the field of bovine tuberculosis epidemiology in South Africa.

In this study, the utility of variable number of tandem repeat typing as a molecular epidemiological marker was evaluated against spoligotyping and restriction fragment length polymorphism typing to describe the molecular epidemiology of bovine tuberculosis in South Africa. The main aim was to identify a molecular typing technique as discriminatory as or superior to RFLP methods for *M. bovis* isolates in the country. A 13 VNTR locus panel was identified has been shown to be sufficiently discriminatory for application in epidemiological studies and the *M. bovis* population structure in South Africa was therefore deduced.

The study revealed that at least ten spoligotypes and 97 VNTR *M. bovis* genotypes are circulating in the country, of which five spoligotypes and 11 VNTR genotypes are shared between livestock and wildlife species, highlighting the on-going active transmission of the disease at the livestock/wildlife interface. Overall, the study showed that besides cattle, 17 species (16 wildlife and a pig) were affected, with infection in a wildebeest, nyala, giraffe first reported in this study. The findings highlighted a high *M. bovis* diversity and a strong evidence of intra and inter-species transmission of bovine tuberculosis in South Africa. The observed presence of BTB in a multi-host environment could impede on the success of the established national control and eradication programme, which on the other hand, seem not to be effective. It is also possible that the current testing protocol for control of BTB is suitable, however, not implemented accordingly, or not performed by well trained and competent personnel. The current findings indicated that 70 % of the *M. bovis* strain types identified over a decade ago is still circulating in the country. In addition, three novel *M. bovis* strains were also identified, the origin of which is questioned. This study recommends that

other control measures such as vaccination be considered in the future. Besides cattle, African buffaloes in South Africa are well known reservoirs of bovine tuberculosis. Although they are subjected to movement control measures between properties, movement of other wildlife species and cattle outside the foot and mouth control zones is not controlled. The outcome of the study showed the presence of co-infection with different *M. bovis* strain types in some of the livestock farms and game farms/reserves. In addition, the study showed that there is an emergence of bovine tuberculosis in previously uninfected (or unknown BTB status) game farms or reserves. Most of the game farms/reserves have a known history of sourcing wild animals of unknown BTB status from other reserves/game parks over a period of time. Some of the wildlife species such as greater kudus have long been suspected to be reservoirs of the disease. They are also popular at wildlife sales, and therefore pose a potential risk for transmission of the disease. Screening of the BTB status of wildlife species before translocation is urgently recommended as prevention of disease introduction remains the most effective control approach. This will however, be impeded by lack of validated diagnostic tests in different animal species. Therefore, there is an urgent need for the development and validation of species specific diagnostic tests.

In South Africa, there are instances where livestock and wildlife species often share habitats, especially in communal livestock communities bordering game farm/reserves. Buffaloes are notorious for escaping from the game farms/reserves through broken fences, while kudus and warthogs can easily jump fences. In this study, *M. bovis* was isolated from stray buffaloes as well as other wild animals reported to have escaped from game farms/reserves. These animal species pose a greater risk for BTB transmission to livestock and animals in other game farms/reserves. Regular maintenance of fences surrounding game parks/reserves plays a crucial role in reducing the escape of wildlife species.

The epidemiological information obtained in the current study is particularly important in the context of the established Greater Limpopo Transfrontier Park where the potential spread of the disease is of great concern. The VNTR typing regime has established that bovine tuberculosis due to the dominant Kruger National Park genotype, VNTR-1, has spread into the Gonarezhou National Park in Zimbabwe. Preliminary results based on very few isolates from Mozambique found that *M. bovis* population between South Africa and Mozambique may be unrelated, probably because of the different historical

livestock trading partners. However, characterization of a representative panel of *M. bovis* isolates from Mozambique will provide better insight into the hypothesis. Monitoring of BTB in wildlife and livestock populations including genetic characterization of *M. bovis* recovered from the three countries, i.e. South Africa, Zimbabwe and Mozambique, especially in the transfrontier park is therefore of high importance for animal and human health at this wildlife/livestock/human interface.

Additional new VNTR loci identified through whole genome sequence of local *M. bovis* isolated showed potential resolution power to differentiate among *M. bovis* isolates from the KNP which are epidemiologically linked through clonal expansion of the parental strain. This case of clonal expansion was further confirmed through whole genome comparison analysis of isolates from this ecosystem. Additional mutation observed in the genome sequence of variant strains analysed may suggest that these strains are less virulent and transmissible compared to their parental strain. Indeed, previous and current studies have demonstrated through IS*6110* RFLP and VNTR typing, respectively, that only the parental strain (C8 type, VNTR-1 type) seems to be more actively spreading than its variant strains. The current study contributed local *M. bovis* genome information which will in future provide a greater opportunity for comprehensive sequence comparison able to assist in further deducing the phylogenetic and evolutionary relationships of *M. bovis* isolates in the country. In addition, the genomes will in future be screened for genes that encode proteins with the attributes of good vaccine targets, for example, bacterial surface proteins, which will ungergo laboratory evaluation for immunogenicity. This information will be highly instrumental for the future development of vaccines.

The inclusion of interlaboratory comparison studies to ensure the quality of results of the VNTR typing scheme employed was also an important part of the study. The results obtained served as an example of how interlaboratory comparison can be integrated and improve validity of epidemiological data analyses in a research laboratory. The outcome has instilled greater confidence in the capability to perform VNTR typing and interpret results thereof. This will ensure that results or findings arising from the current research into bovine tuberculosis epidemiology using VNTR typing as a technique can be trusted by the international scientific research community and more importantly, that findings can be trusted by the decision and policy makers for bovine tuberculosis control and disease management for the country.

The study also reported for the first time in South Africa, a rare infection of a horse with an *M. bovis* strain never described in South Africa before, suggesting that the horse might have been imported into the country carrying a latent *M. bovis* infection. Isolation of the *M. bovis* from the broncho-alveolar lavage suggested that the horse was shedding the *M. bovis* organisms, and therefore presented a zoonotic risk. The study indicated that horses may contract and harbour *M. bovis* despite their lower susceptibility compared to other domestic animals and the zoonotic nature of the disease should be taken into consideration. The horse, however, now seemed to have recovered from the clinical signs.

With this study, a database with all typing profiles was established, as well as a culture bank for future reference and studies (for epidemiological purpose). Future studies will involve comparative evaluation of the newly identified VNTR loci and previously selected 13 panel set of loci on a larger collection of *M. bovis* isolates. It is anticipated that further improvement of the VNTR typing method currently utilized in the KNP may be obtained. The current study did not investigate the position of the polymorphic loci based on the functional genes and proteins of which they encode, therefore further studies are needed in this regard. This information will be highly instrumental in future for virulence assessments between different strains.

Appendix

Methodology

Tissue samples

Approximately 5 g of tissue samples were cut into small pieces and covered with 100 ml of sterile distilled water in a biohazard cabinet (Esco Class II BSC; Labotec, SA). The sample were homogenized at 17 500 rpm using the Ultra-Turrax® homogenizer (Separation Scientic, SA). 7 ml of the homogenates were poured into two separate 15 ml falcon tubes, and the remaining homogenates were poured into individual 50 ml centrifuge tubes and stored at -20 °C. The samples were decontaminated with 7 ml of 2 % HCL (final concentration of 1 %) and 7 ml of 4 % NaOH (final concentration of 2 %) respectively and left at room temperature for 10 min. This was followed by centrifugation (Heraeus Labofuge 400) at 3 500 rpm for 10 min. The supernatants were poured off and 7 ml of sterile distilled water was added. The samples were centriguged again for 10 min at 3 500 rpm. After centrifugation, most of the supernatants were poured off, keeping approximately 1 ml. The pellets were mixed well using a sterile inoculating loop. Two loops full of each of the pellets were spread evenly onto 2 Löwenstein Jensen (L-J) media slants supplemented with pyruvate (National Health Laboratory Service) and 1 L-J medium slant supplemented with glycerol (BD Dignostics), and incubated at 37 °C for upto ten weeks. The slants were monitored weekly for mycobacterial growth.

Fluids

The specimen were transferred into a 15 ml centrifuge tube and decontaminated by adding equal volumes of 4 % NaOH (final concentration of 2 %). The samples were left at room temperature for 10 min, then centrifuged at 3 500 rpm for 10 min. The supernatants were discarded and 7 ml of sterile distilled water was added to the pellets. The samples were centrifuged as above for 10 min. After centrifugation, most of the pellets were discarded, keeping approximately 1 ml. The pellets were mixed using a sterile inoculating loop. A loopfull of each sediment was inoculated onto 2 L-J slant supplemented with pyruvate and another loopfull was spread onto 1 L-J slant

supplemented with glycerol. The slants were incubated at 37 °C for upto ten weeks, and monitored weekly for mycobacterial growth.

Ziehl Neelsen (ZN) staining

Bacterial growth observed during monitoring was subjected to ZN staining to check for acid fast bacteria (AFB). Bacterial smears were prepared from colonies on a microscopic slide. The smears were heat-fixed by passing the slides through flame 3-4 times. The slides were place on a rack and covered with Carbolfuchsin, and then heat steamed for 5 min. The slides were rinsed thouroughly with tap water, and decolourized with acid alcohol for 2 min, then rinsed thoroughly with tap water. Malachite green was used for counterstaining for 1 min, the rinsed thoroughly with tap water. The slides were left to airdry at room temperature and were observed under the Microscope for AFB.

Genomic DNA extraction

A PUREGENE^{TM} DNA extraction kit was used to extract genomic DNA from isolates following the manufacturer's instructions (Gentra Systems) with minor modifications. Overnight glycine-treated cells were heat killed at 94 °C for 10 min and allowed to cool down. Five hundred microliters of MSG extraction buffer (50 g/L Monosodium Glutamic Acid; 6.06 g/L Tris; 9.3 g/L EDTA) were added to the culture and cells were suspended by using an inoculating loop. Following centrifugation, 300 µl of lysis solution were added and 5 µl RNase A solution (4 mg/ml; Roche) were added to the sample and incubated at 37 °C for 30 min. A volume of 150 ul of protein precipitation solution was added and samples were centrifuged at maximum speed for 2 min, followed by precipitation of the aqueous phase with isopropanol (Merck). The resulting pellet was dissolved in 40 µl of TE buffer (10 mM Tris-HCl; 1 mM EDTA). The extracted DNA was stored at -20 °C until use.

Preparation on cell lysates

Acid fast bacteria cell lysates were prepared by picking up individual colonies or several small colonies from the L-J media and emulsifying in 100 ul sterile distilled water. The suspension was boiled at 100 °C for 25 min, and allowed to cool down at room temperature, then transferred to 4 °C or -20 °C for long term storage.

Differentiation of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium* Other Than Tuberculosis (MOTTS)

For each AFB isolate, a master mix was prepared containing the following components: 25.5 µl Ultra pure water; 5 µl of 10 X reaction buffer (Separation Scientific), 3 µl of 25 mM $MgCL_2$ (Separation Scientific); 2.5 of 1 mM dNTP (Inqaba Biotechlonogies Industries); 2 µl of TB1A forward primer (5' GAACAATCCGGAGTTGACAA 3'); 2 µl of TB1B reverse primer (5' AGCACGCTGTCAATCATGTA 3') (Inqaba Biotechlonogies Industries) and 0.4 µl of SuperTherm Taq polymerase (Separation Scientific). The master mix was well-mixed while avoiding bubbles. 39 ul of was aliquoted into pre labelled microcentrifuge tubes. 10 µl of the template DNA (cell lysate) was dispensed into individual micro centrifuge tubes using filter tips. The cycling parameters were as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 seconds; annealing at 64 °C for 30 seconds, elongation at 72 °C for 2 min (40 cycles) and holding at 4 °C. PCR amplification was carried out using an Eppendorf AG 22331 Hamburg thermo cycler (Merck). The PCR products were separated on a 1.5 % agarose gel stained with 20 µl ethidium bromide (10 µg/ml) and run at 80 V for three hours. A 100 bp ladder (Inqaba Biotechnical Industries) was included. A product size of 372 bp was expected for MTBC species.

Differentiation of MTBC species

For each MTBC isolate, a PCR reactions contained 5 µl Q-buffer (Qiagen, Germany) 2.5 10 X reaction buffer (Separation Scientific); 2 µl 25 mM $MgCL_2$; 4 µl 10 mM dNTP 0.5 µl of each 50 pmol/µl primer (RD 4 and RD 9 forward, internal and reverse). 0.13 µl HotStarTaq DNA polymerase (Qiagen, Germany) and 2 µl DNA template. The mixture was made upto 25 µl with sterile distilled water. PCR amplification conditions were as follows: initial denaturation at 94 °C for 15 min, followed by 45 cycles at 94 °C for 1 min; annealing at 62 °C for 1 min; elongation at 72 °C for 1 min. After the last cycle, the samples were incubated at 72 °C for 10 min. PCR amplification was carried out using an Eppendorf AG 22331 Hamburg thermo cycler (Merck). The PCR products were separated on a 2-3 % agarose gel stained with 20 µl ethidium bromide (10 µg/ml) and run at 80 V for three hours. A 100 bp ladder (Inqaba Biotechnical Industries) was included. A product size of 372 bp was expected for MTBC species. Product sizes of 268 and 109 for RD 4 and RD 9 respectively were expected.

IS*6110* and PGRS RFLP typing

DNA digestion and gel electrophoresis

Approximately 1.5 µg of genomic DNA was digested with 1.5 units of *Pvu*II and *Alu*I for IS*6110* and PGRS RFLP typing respectively. The resulting fragments were separated by electrophoresis on a 0.8 % agarose gel at 8 V for approximately eight hours DIG-labelled molecular weight size marker (Roche) was also loaded.

Southern blotting

Transfer of DNA to membrane

For Southern blot transfer, the gel was submerged twice in Denaturation solution for 15 min, and then rinse with distilled water. The gel was submerged twice in Neutralization solution for 15 min at room temperature. The custom-made Perspex-platform was inserted into the buffer chamber and a glass plate was paced on top. Three filter paper wicks were inserted and 20 X SSC buffer was added to the brim of the chamber, ensuring that wicks are wet and all bubbles are removed. The gel was placed on top of the wicks, ensuring that the bottom topside and the wells are facing upwards, and a small piece from the top right cormer of the gel to indicate the top left hand first lane on the membrane. The Hybond N+ membrane (Amersham, SA) was placed on top of the gel and was wet with 20 X SSC. The edges of the gel were sealed with 4 X strips of parafilm. The filter paper pads were place on top of the gel, and the towelling blocks on top of the pads. A tray of approximately 1 kg was placed on top of the assembly and the transfer was allowed for overnight.

DNA fixation

The blotting apparatus was disassembled and the membrane was placed on 3 MM filter paper (Amersham) soaked in 2 X SSC and DNA was fixed to the blot by UV cross-linking (Spectroline^{R} model TC-312A transilluminator 312 nm ultraviolet) for 5 min on each side. The membrane was rinsed with distilled water afterwards and allowed to air dry. The gel was visualized under UV light to ensure that the entire DNA has been successfully transferred on to the membrane

Pre-hybridization

The blot was placed into the hybridization bag and sealed. 14 ml of DIG Easy Hyb (Roche diagnostics) was added to the bag, bubbles removed and the bag was sealed for to prevent leaks. The membrane was prehybridized for 30 min at 37 °C and 42 °C for IS*6110* and PGRS RFLP typing respectively, with gentle shaking.

Hybridization

14 ml of DIG-easy Hyb was heated to 37 °C and 42 °C for IS*6110* and PGRS RFLP typing respectively. 20 µl of the DIG labelled probe (IS*6110* or PGRS) was added to 50 µl of double distilled water in a microcentrifuge tube. The microcentifuge tube was placed in boiling water for 5 min to denature the probe, the chilled quickly on ice. The denatured probe was immediately added to the DIG Easy Hyb and mixed by inversion. The prehybridization solution was removed from the hybridization bag and replace with probe hybridization solution. The bag was sealed and checked for leaks and hybridized overnight at 37 °C or 42 °C for IS*6110* and PGRS RFLP typing respectively.

Post hybridization washes

The hybridization solution was poured off and the bag cut open. The membrane was removed and placed in a container with 200 ml of Wash buffer 1, then incubated at room temperature with mild shaking for 5 min. The step was repeated. The membrane was then washed twice with Wash buffer 2 at 65 °C for 15 min.

Detection

The membrane was transferred into a plastic container containing 200 ml Maleic acid buffer (Roche Diagnostics) and incubated for 2 min with shaking at room temperature. The Maleic acid buffer was poured off and 200 ml of 1 X Blocking solution (Roche Diagnostics) was added and incubated for 30 min with shaking at room temperature. With 5 min remaining, the diluted DIG alkaline phosphatase reagent (Roche Diagnostics) was prepared by spinning the antibody for 5 min at full speed, and added to 5 µl of the antibody conjugate to 50 ml Blocking solution, then mixed well and replaced the previous 1 X Blocking solution. This was incubated for 30 min with shaking at room temperature. The antibody solution was poured off and the membrane

was washed twice with DIG washing buffer (Roche Diagnostics) for 15 min. The DIG wash solution was poured off and 200 ml of Detection buffer (Roche Diagnostics) was added and incubated for 3 min at room temperature. The CSPD Working solution was prepared by adding 50 µl of CSPD 6 ml to 10 ml of DIG detection buffer and poured on the membrane in a new hybridization bag, with DNA side facing up. The hybridixation bag was sealed immediately and bubles removed. This was incubated for 5 min at room temperature. After incubation, excess liquid was squeezed out of the hybridization bag and the bag was sealed. The membrane was incubated for 10 min at 37 °C or 42 °C for IS*6110* or PGRS RFLP typing respectively. The hybridization bag was wiped out with 70 % ethanol to remove all electrostatic charges from the surface. The membrane was exposed to X-ray film for 15 min to one hour, and and film was developed.

Reagents and solutions

Acid alcohol

Thirty-two millilitres of concentrated HCL was mixed with 970 ml of ethyl alcohol (95 %). The solution was stored at room temperature

Carbolfuchsin stain

Basic fuchsin (0.3 g) was dissolved in 100 ml of ethyl alcohol. The stain was stored in a dark bottle at room temperature.

2 % HCL

HCL (133 ml of 32 % HCL) was mixed with 1 867 ml of sterile distilled water, mixed well and stored at room temperature.

4 % NaOH

Sodium Hydroxide (80 g) was dissolved in 2 000 ml sterile distlled water, mixed well and stored at room temperature.

0.2 M Filter Sterilized Glycine solution

MW Glycine (75.5) was mixed with 7.5 g of Glycine. Sterile distilled water (500 ml) was added. The solution was filter sterilized through 0.2 µm Cellulose filterand stored at 4 °C for approximately a year.

MSG Extraction buffer

The following were mixed together: 50 g Mono Sodium Glutamic acid (MSG); 6.06 g Tris and 9.3 g EDTA. 1 000 ml of sterile distilled water was added. The solution was filter sterilized through 0.2 µm Cellulose filter and stored at room temperature for approximately a year.

10% Sodium dodecylsulphate SDS (Merck)

Sodium dodecylphosphate (100 g) was dissolved in 1 000 ml of sterile distilled water, filter sterilized through 0.2 µm Cellulose filter and stored at room temperature for a month.

1M Tris-HCL

Tris (121 g) was dissolved in approximately 800 ml of sterile distilled water. pH was adjusted to 8.0 with HCL. The solution was autoclaved and stored at room temperature for approximately a year.

TE Buffer (1 X Solution)

Ten millimolar of 1 M stock was mixed with 1 mM EDTA, ensuring a pH of 8.0, autoclaved and stores for approximately a year.

50 X TAE Buffer

Tris (242 g) was dissolved in 500 ml of sterile distilled water, 100 ml of 0.5 M Na_2EDTA (pH 8.0) and 57.1 ml of glacial acetic acid (Merck chemicals) were added. The volume of the solution was adjusted to 1 000 µl with sterile distilled water, and stored at room temperature for approximately a year.

1 X TAE Buffer

50 X TAE Buffer (40 ml) was added to 2 000 ml sterile distilled water, mixed well and used immediately.

Denaturation solution

Twenty grams of NaOH was mixed with 87.66 g of NaCL and dissolved in 1 000 ml sterile distilled water, then stored at room temperature for approximately a year.

20 X SSC; pH 7.0

NaCL (350.6 g) was mixed with 176.4 g of Tri-Sodium Citrate (hydrous) and dissolved in 2 000 ml. The solution was stored at room temperature for approximately a year.

2 X SSC Solution

1:10 dilution of 20 X SSC was made by adding 100 ml of 20 X SSC to 1 000 ml sterile distilled water. The solution was stored at room temperature for approximately a year.

1.5 % agaose gel

Dissolve 6 g of agarose powder (Whitehead Scientific) in 1 X TAE Buffer to make upto 400 ml, then dissolved in a microwave for 6-10 min. The gel was allowed to cool down until it could be toached by hand. 20 ul of ethidium bromide (10 mg/ml) was added and mixed with a spatula. The gel was then poured into assembled gel tray apparatus and left at room temperature to solidify.

2 % or 3 % agarose gel

Dissolve 8 or 12 g of agarose powder (Whitehead Scientific) for 2 % and 3 % gel respectively in 1 X TAE Buffer to make upto 400 ml, then dissolved in a microwave for 10 min. The gel was allowed to cool down until it could be toached by hand. 20 ul of ethidium bromide (10 mg/ml) was added and mixed with a spatula. The gel was then poured into assembled gel tray apparatus and left at room temperature to solidify.