Molecular Characterization of Bovine Tuberculosis Strains in Swaziland

BY

MUZI MZWANDILE DUBE

Submitted in partial fulfilment of the requirements for the degree of Master of Science (Veterinary Tropical Diseases) in the Faculty of Veterinary Science, University of Pretoria

Acknowledgements

I wish to express my sincere appreciation and gratitude to the following people and institutions that made this work possible. First my wife, Bagezile Dube, Bongekile Ndwandwe (Central Veterinary Laboratory), Dr Patrick Dlamini (Epidemiology unit, Veterinary department, Swaziland), Blessing Marondera (Laboratory, Ministry of Health), Francis Makamba (University of Swaziland), Anna-Marie Bosman and MaMohale Chaisi (University of Pretoria).

List of Contents

List of Figures

List of Tables

List of Abbreviations

VNTR.....Variable Number Tandem Repeat

MIRU.....Mycobacterium Interspersed Repeat Unit

ETR .....Exact Tandem repeat

SD.....Swaziland

RSA.....Republic of South Africa

BTB.....Bovine Tuberculosis

MTBC.....*Mycobacterium tuberculosis* complex

ELISA.....Enzyme Linked Immunosorbent Assay

bp.....base pairs

EU.....European Union

TB.....Tuberculosis

PCR.....Polymerase chain reaction

Thesis Summary

Molecular Characterization of Bovine Tuberculosis Strains in Swaziland

By

MUZI MZWANDILE DUBE

Promoter: **Prof Anita L. Michel**

Co-promoter: **Dr Darshana Morar**

Degree: **MSc**

Department: **Tropical Veterinary Diseases**

The aim of the study was to gain knowledge on molecular techniques (spoligotyping and VNTR typing) in analysis of the *Mycobacterium tuberculosis* complex and characterize *M. bovis* isolates available in Swaziland.

Chapter 1

1.1 Introduction

Bovine tuberculosis (BTB) is a disease of cattle, characterised by progressive development of specific granulomatous lesions, or tubercles, in lung tissue, lymph nodes or other organs (Ayele *et al*. 2004). *Mycobacterium bovis* is the causative agent of BTB and belongs to the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related bacteria from the genus *Mycobacteria*, family *Mycobacteriaceae,* causing tuberculosis in various mammalian hosts (Sahraoui *et al.* 2009).

For practical purposes, *Mycobacteria* are differentiated into two groups known as the MTBC and the 'non-tuberculous' or 'atypical' *Mycobacteria*. MTBC comprises *M. tuberculosis, M. bovis, M. africanum, M .canettii, M. microti, M. pinnipedii, dassie bacillus, oryx bacillus and M. caprae.*

The most important *Mycobacterium* species in animals is *Mycobacterium bovis* and essentially infects cattle, sheep, goats in domesticated species and has been demonstrated in wildlife species, especially buffalo, but also other wildlife species including predators (lion, leopard, cheetah), herbivores (kudu) and omnivores (baboon, bush pig) (Michel *et al.* 2009, Vosloo *et al.* 2001). As a result of the wide range of hosts of *M. bovis,* this pathogen is a concern in areas where there is a wildlife (buffalo), cattle and human interface (Vosloo *et al.* 2001).

Mycobacterial infections of animals are of primary importance for both economic and public health reasons, as many have been recognized as causes of zoonoses (Durr *et al.* 2000). In developing countries, like in Africa, BTB is widely distributed, control measures are not applied or are applied sporadically and pasteurization is rarely practised (Cosivi *et al.* 1998). This is a concern as it poses a high risk of transmission to the human population, especially with the added burden of increased susceptibility as a result of the human immunodeficiency virus (HIV) in Africa.

Bovine TB is endemic in Swaziland (Cosivi *et al*. 1998) and control measures are hardly practised. Only in one abattoir in Swaziland is BTB specifically checked for at meat inspection. According to the 2008 annual report for the Ministry of Health and Social Welfare (MHSW), Swaziland National Tuberculosis Control Programme (SNTC), Swaziland is one of the countries with a high burden of human tuberculosis (TB). There has been a tenfold increase in cases from 1000 cases per year in 1987 to over 9,600 cases in 2007, with 79.6% of the TB cases also co-infected with HIV (MHSW, 2008). Bovine TB in Swaziland can only add to this problem since TB caused by *M. bovis* is clinically indistinguishable from TB caused by *M. tuberculosis* (Cosivi *et al.* 1998, Michel *et al*. 2010).

Since there is almost no control of BTB in Swaziland, there is a danger that the disease may be spreading in cattle herds and other animals. This is even more important in dairy herds where there is intensive rearing and thus easy spread. As a country it is important to control BTB, to protect the health of the human population and to ensure productivity of the cattle herds. This will ensure the country continues to export beef to the lucrative European market.

The objective of this research project is to gain knowledge of molecular techniques (Spoligotyping and Variable Number Tandem Repeat typing) in analysis of the *Mycobacterium tuberculosis* complex. This will be done by genotypically characterizing *M. bovis* isolates available in Swaziland using these techniques.

1.2 Literature

Swaziland is one of the countries where BTB is enzootic as reported by Cosivi *et al.* 1998. BTB is only checked at the export abattoir in Matsapa. About 60% of tissue samples submitted from the export abattoir to the Central Veterinary Laboratory (CVL) with suspect TB lesions, turn out positive on acid-fast staining. The only evidence of BTB in Swaziland is on slaughtered cattle and no further work is carried out on acid-fast positive tissues.

In countries where BTB is uncontrolled, like in Swaziland, most human cases can occur resulting from drinking or handling contaminated milk, inhaling aerosols from infected animals and eating undercooked meat (Cosivi *et al.* 1998). Zoonotic tuberculosis caused by *M. bovis* is present in animals in most developing countries where surveillance and control activities are often inadequate or unavailable (Cosivi *et al.* 1998, Milian-Suazo *et al.* 2008). Mycobacteriosis is a major opportunistic infection in HIV-infected persons, with the vast number of people carrying this dual infection living in developing countries, especially Sub-Saharan Africa (Cosivi *et al.* 1998). Dual HIV and *M. bovis* infection has been reported in industrialized countries. As such the HIV epidemic in developing countries, particularly those that have *M. bovis* infections present in animals, makes zoonotic TB a serious public health threat to persons at risk (Cosivi *et al.* 1998). Until recently, the majority opinion held that human-to-human transmission of *M. bovis* occurred infrequently (Grange *et al.* 1987). Several examples of this occurrence have now been documented generally in HIV positive patients, and typing has proved essential in providing evidence for transmission (Durr *et al.* 2000). *Mycobacterium bovis* has an intrinsic resistance to pyrazinamide and common resistance to isoniazid, two of the first line drugs recommended by the World Health Organization (WHO) for standard treatment of new TB cases in humans.

The primary mode of spread of BTB between herds is by introduction of infected animals into non-infected herds (Cousins 2001). The ability to detect and identify subtypes to distinct geographical regions is important in that it provides the advantage that if a particular type is isolated outside the area in which it predominates the investigation can focus upon the hypothesis of an introduced infection, and hence detection of the animal responsible for the introduction (Durr *et al.* 2000). Michel *et al.* (2009), using genotyping, suggested that kudus residing in Spioenkop Nature Reserve may have contracted *M. bovis* from cattle in adjacent farms and then transmitted the disease to a herd of newly introduced, BTB negative buffaloes. Furthermore this type of analysis has been used in the tracing of the origin of infected buffalo that have been moved to other parts of South Africa from their origin (Vosloo *et al.* 2001). The most important factors determining the occurrence and spread of tuberculosis within a herd of cattle are the number of infected individuals, the number of young stock exposed to the infected individuals, and the measures taken to prevent spread (Cousins 2001). Most cattle suffering from pulmonary BTB and tuberculous mastitis are infectious and milk, urine, vaginal secretions, semen or faeces in some infected individuals may also contain tubercle bacilli and act as means of disease transmission between individuals. However, *M. bovis* is primarily spread within cattle herds by inhalation of infectious aerosols, either from coughing or sneezing animals with open tuberculosis or from

infected dust particles (Cousins 2001). Since aerosol transmission predominates, infection is spread more rapidly in an intensive animal husbandry situation such as those in dairy establishments. Since aerosol transmission is effective over short distances (1 m to 2 m), cattle density is a significant factor in the rate of transmission (Cousins 2001). Traditional diagnosis of BTB has been done by the delayed type hypersensitivity skin tests. Other tests that are used less frequently include bacteriological culture, antibody based Enzyme Linked Immunosorbent Assay (ELISA), the bovine interferon-gamma assay, and molecular (nucleic acid-based) tests e.g. PCR. However, detailed epidemiological investigations of BTB have traditionally been hampered by the lack of easy-to-practice discriminating tests for typing *M. bovis* (Durr *et al.* 2000). Considerable advances have been made in developing techniques that discriminate between isolates of *Mycobacterium tuberculosis* complex, of which *M. bovis* is a member (Durr *et al.* 2000).

The use of these techniques that enable the analysis of the genome of the pathogen combined with statistical methods for deriving relationships between them, is being used increasingly to understand their epidemiology (Vosloo *et al.* 2001). Romero *et al*. (2008) used these tools to provide valuable insight into the importance of different hosts in the maintenance and spread of the infection. Comparative analysis of the 'genetic fingerprints' of BTB isolates from buffalo and cattle has aided in tracing the original source of infection of buffalo populations in the Kruger National Park in South Africa and Donana National Park in Spain (Romero *et el*. 2008, Michel *et al.* 2009). In Algeria, molecular typing (genotyping) has been used to link Algerian and European strains of *M. bovis* (Sahraoui *et al.* 2009). By far the most common use of *M. bovis* genotyping has been to find evidence for the role of wildlife reservoirs in outbreaks of BTB among cattle herds (Durr *et al.* 2000). In Michigan (USA) the re-emergence of BTB in cattle is thought to have been due to a reservoir in white-tailed deer, while in the UK and Ireland, maintenance of *M. bovis* infection has been attributed to the presence of infected badgers (Romero *et al.* 2008). Genotyping can also be used to trace outbreak strains in herds caused by cattle movements (Michel, personal communication).

According to Cosivi *et al.* (1998), molecular techniques have been used to study the epidemiology and role of *M. bovis* in human TB patients in several countries in Africa. In Egypt, the proportion of sputum-positive TB patients infected with *M. bovis* recorded in three observations were, 0.4%, 6.4%, and 5.4% and nine out of twenty randomly selected patients with TB peritonitis were infected with *M. bovis*. In Zambia, an association between BTB positive cattle and zoonotic TB was found (odds ratio = 7.6, p = 0.004). In Mexico, Uganda and Ethiopia epidemiological links between *M. bovis* infections affecting humans and cattle populations have been established through genotyping (Michel *et al.* 2009). In Uganda and Tanzania, *M. bovis* accounted for 18-30% of all *M. tuberculosis* complex strains isolated from humans in rural settings as compared to low prevalence rates of *M. bovis* found in urban populations (Michel *et al.* 2009).

Molecular techniques have been useful in preventing disputes between trade partner countries. Milian-Suazo *et al.* (2008) used genetic fingerprints to prove that the USA had its own source of *M. bovis* infection, dairy cattle and wildlife, other than through infected cattle bought from Mexico.

1.2.1. Underlying principles of genotyping

The basic assumption underlying the practical use of genotyping is the clonal model resulting from asexual reproduction. It is assumed that when an infectious bacterium is passed to a second host, the bacterium retains the same genetic makeup (Durr *et al.* 2000).

When two individuals have isolates with different genotypes, then infection has not spread from one host to the other, even if the hosts have had close contact. Similarly, two infection events would have occurred if two genotypes are found in the same host.

Although the assumption of clonality is central to the use of genotyping, this must be treated as a biological approximation. All bacteria undergo mutation of DNA sequences, and if these mutations do not affect the functioning of essential enzymes or structural proteins, the daughter generation should survive. If the typing technique used, recognizes these changes then a new clone is identified. However, if the technique does not recognize these changes, the daughter generation is classified as being the same as the clone. Optimally, a technique should balance in not being too excessive in sensitivity to genetic change and being insufficient in discrimination. This may be achieved by using more than one technique, provided that the techniques used targeted different areas of the genome.

1.2.2. The genome of *M. bovis*

The grouping of mycobacterial species pathogenic to mammals into the *M. tuberculosis* complex is due to the fact that there is high genomic similarity between these organisms (Rastogi *et al.* 2001). The genome of this complex in general is relatively difficult to elucidate due to difficulties related to extremely slow growth, unique cell wall composition, the need to protect personnel and paucity of cloning vehicles (Durr *et al.* 2000).

The genome of *M. bovis* consists of a double strand of DNA, consisting of approximately 4.4 million base-pairs (bp). This is comparatively large for an intracellular bacterial pathogen (Durr *et al.* 2000). The DNA of the genome has a high guanine and cytosine content of approximately 65% (Durr *et al.* 2000). A large portion of the genome is made up of mainly enzymes for the metabolism of lipids like mycolic acids, which are part of the distinctive hydrophobic, waxy cell wall of the mycobacteria. The genome is also rich in gene regulatory circuits. This is important for the organism so as to selectively activate genes, for survival for prolonged periods in the host macrophages and outside environment during transmission stages (Durr *et al.* 2000).

The *M. tuberculosis* complex in general lacks inter-strain genetic diversity, reason of which may mean recent evolution as a pathogen. Alternatively, it may be due to lack of genetic exchange mechanism between pathogenic mycobacteria as neither conjugation nor transformation is known to occur. Much of the genomic polymorphism that has been detected is associated with mobile genetic elements, particularly insertion sequences (Durr *et al.* 2000). These are small segments of DNA that insert themselves at multiple sites within the genome. This may provide genetic variability by deactivating genes (Durr *et al.* 2000). Recent *M. tuberculosis* genome sequencing revealed that these are mainly clustered in intergenic and non-coding regions, in which case functional gene activation would be unlikely.

1.2.3. Genotyping techniques

The basic understanding of the *M. tuberculosis* complex genome has led to development of several typing techniques. These use specific genetic elements, upon which the techniques are classified. Broadly, the techniques can be classified into those that use the whole genome for typing and those that use only part of the genome.

1.2.3.1. Whole genomic techniques

The whole genome techniques have an advantage that all potential genetic information is used. The only drawback is that these techniques are technically demanding and thus are less popular (Durr *et al.* 2000).

i) Restriction endonuclease analysis (REA).

This technique was first developed specifically for intraspecific typing of *M. bovis* (Collins & de Lisle 1985). After cell extraction genomic DNA is digested with 3 restriction endonuclease enzymes, i.e *BstEII, PvuII* and *BclI*. These cleave DNA strands at highly specific nucleotide sequences. The resulting fragments of DNA are separated by agarose gel electrophoresis. Strains are characterized based on the patterns of segments in the gel. To visualize the patterns, a stain is used e.g. ethidium bromide. This is then recorded photographically after transillumination with ultraviolet light. The added disadvantage to this method is that fragment patterns are difficult to interpret, and that there is no method for numerically cataloging types thus limiting comparison between laboratories (Collins *et al*. 1993).

ii) Pulsed field gel electrophoresis (PFGE)

This technique produces few fragments compared to REA. All steps are similar to REA, except for the use of restriction endonuclease enzymes, to get few large DNA fragments. These fragments are not separated by conventional electrophoresis but are subjected to a constantly changing (pulsed) electrical field (Durrr *et al.* 2000). This technique has been rarely used for typing bacteria of the MTBC, due to its difficulty specifically regarding this pathogen.

1.2.3.2. Partial genomic techniques not based on the polymerase chain reaction

These techniques mainly use polymorphism within specific regions of the *M. bovis* genome, the earliest being restriction (RFLP) fragment length polymorphism analysis. DNA is digested by using a restriction enzyme, either (*PvuII* or *AluI*). The fragments are then separated by agarose gel electrophoresis. The DNA patterns are determined by using the Southern blot technique by using a nitro-cellulose or nylon filter upon which labeled/marked probes are added. The probes are complementary to a fraction of the DNA isolate, thus not all the fragments are visualized. The essential step is to select a probe that reveals DNA differences between isolates. Probes based upon repetitive DNA elements are preferred to ensure binding. The repetitive elements that have been used for RFLP include insertion

sequences, polymorphic G-C rich repeat sequences (PGRS) and the direct repeats (DR) sequence.

i) RFLP using IS*6110*

This is the most important RFLP probe used in the analysis of the *M. tuberculosis* complex. It is a 1361 bp DNA fragment. It was also known as IS*986* or IS*987* (Poulet & Cole 1995). Up to 20 copies of IS*6110* are present in the genome of *M. tuberculosis*, but *M. bovis* generally has fewer copies of IS*6110*, majority having just a single copy especially isolates from cattle, and are located in the DR region (Collins *et al.* 1993). The current recommendation is that if strains contain more than three copies of the sequence, then IS*6110*- RFLP should be considered the method of choice.

ii) RFLP using IS*1081*

In studies where IS*1081*-RFLP was applied on *M. bovis* isolates, limited polymorphism was detected, thus strain differentiation was not possible (Collins *et al.* 1993). However, IS*1081*-RFLP can distinguish between *M. bovis* BCG from other strains of *M. bovis* (Van Soolingen *et al.* 1992). This technique, however, is no longer used as PCR based tools can distinguish much easier between *M. bovis* and BCG.

iii) RFLP using GC-rich repeat sequences

Within the genome, short, repeated sequences with a GC composition in excess of 80% are present (Ross *et al.* 1992). These polymorphic GC rich sequences (GRS) occur in multiple clusters throughout the genome. The probe currently in use has a set of oligonucleotide primers comprising several PGRS sequences (Cousins *et al.* 1998). PGRS – RFLP for *M. bovis* resulted in good strain differentiation, when DNA was digested using *AluI* (Cousins *et al.* 1993). The strain differentiation was even higher than in spoligotyping, IS*6110*-RFLP or DR-RFLP for large sample of isolates (Cousins *et al.* 1998). PGRS –RFLP has been recommended as the method of choice when maximum strain differentiation is required and multiple copies of IS*6110* are absent (Cousins *et al.* 1998). This technique is extremely complex and can only be analyzed manually due to the large number of bands.

iv) DR-RFLP

The DR cluster is unique to isolates of MTBC. The cluster consists of multiple 36bp 'DR' sequences interspersed with non-repetitive spacer sequences ('spacers'), from 35bp to 41bp in length. Strains vary in number of DRs and presence or absence of particular spacers (variable in length and sequence) (Durr *et al.* 2000).

Discrimination is similar to spoligotyping, consistent with the fact that both techniques target the same chromosomal locus (Cousins *et al.* 1998).

1.2.3.3. Partial genomic techniques based on the polymerase chain reaction

The techniques were developed out of the need for rapid automated techniques that will enable a high throughput of samples (Durr *et al.* 2000). These techniques depend on

the polymerase chain reaction (PCR), which is an essential method for amplification of sequences of DNA (Durr *et al.* 2000).

i) Random amplified polymorphic deoxyribonucleic acid analysis (RAPD)

The technique uses short oligonucleotide primers that target sequences randomly within a genome. This is also referred to as 'arbitrary primer PCR' or 'DNA amplification finger-printing (Durr *et al.* 2000). Fragments are not all amplified with equal efficiency thereby raising concern about reproducibility and validity of methodology. The technique has been rarely used to type *M. bovis*, with poor differentiation between isolates.

ii) Ampliprinting using IS*6110*

The major polymorphic tandem repeat (MPTR) sequence was first described in 1992 (Hermans *et al.* 1992). It is structurally similar to the DR cluster, consisting 10bp DRs separated by 5bp DNA spacer. It is estimated there are eight copies of MPTR per genome. This sequence is not in *M. tuberculosis* complex only, but occurs in *M. kansasii* and *M. gordonae*. For typing of the *M. tuberculosis* complex MPTR has not been used alone, but with IS*6110* in a unique typing technique named 'ampliprinting (Durr *et al.* 2000). The basis for this technique is the variable distance between IS*6110* elements and copies of MPTR sequences, which are amplified by PCR (Durr *et al.* 2000). The technique is more sensitive in isolates with multiple copies of IS*6110*.

iii) Spoligotyping

The technique is based on detection of DNA polymorphism within the DR locus, which is a relatively large tandem repeat (Le Fleche *et al*. 2002), and is the first PCR technique to be widely used for genotyping (Durr *et al.* 2000). The DR locus belongs to a class of genomic structures called clustered repetitive interspersed palindromic repeats (CRISPR), which have been shown to play a role in bacterial defence against viruses (Reyes & Tanaka 2010). One half of the repeat is conserved, whereas the other half, called spacer element, is highly diverged (Le Fleche *et al*. 2002). The DR marker is more stable than the gold standard IS*6110* marker *(*Haddad *et al*. 2004). The microevolution of this region is unidirectional i.e. oriented towards deletions accumulation, without acquisition of new spacers or recovery of previously lost spacers (Haddad *et al*. 2004). The polymorphism between two isolates is in the fact that one or more spacers can be absent/present in the DR region of one isolate and not of the other (Haddad *et al*. 2004). Spoligotyping takes advantage of these internal variations to distinguish the different alleles at this locus, which has been reported for in the *M. tuberculosis* complex. The amplified products are hybridized to a set of immobilized oligonucleotides, each corresponding to one of 43 potential unique spacer DNA sequences within the DR of *M. tuberculosis* complex. For each subspecies some spacers are constantly absent, allowing differential identification at the level of subspecies (Haddad *et al*. 2004). In the case of *M. bovis,* spacers 3, 9, 16, 39-43 are always lacking (Haddad *et al*. 2004). The potential polymorphism for *M. bovis* therefore is of 2^{35} different spoligotypes (Haddad *et al*. 2004). This technique is also known as 'reverse' line blot hybridization. Detection of hybridized DNA then follows, using chemiluminescent system. The output is the result of hybridization of the forty-three sequences as 'positive or negative' according to presence or absence of each of the spacers. A word processor is then used to compare the forty-

three results. The technique does enable the grouping of affected mammalian host species. Spoligotyping is recommended for rapid screening of isolates followed by a more discriminatory technique. Since this technique does not need large amounts of high quality DNA, it can be used directly on pathological samples, thus allowing for simultaneous diagnosis and typing of the bacterium (Durr *et al.* 2000, Haddad *et al*. 2004). Another advantage for this technique is the use of two primers, *a* and *b*, specific to the two extremities of the DR sequences (Haddad *et al*. 2004).

iv) Variable Number of Tandem Repeat Typing

Genetic loci containing variable numbers of tandem repeats (VNTR loci), also called minisatellites (Le Fleche *et al*. 2002), form the bases for human gene mapping, forensic analysis and paternity testing (Durr *et al.* 2000). In mycobacteria, VNTR of different classes of interspersed genetic elements called mycobacterial interspersed repetitive units (MIRUs) is genotyped (Supply *et al.* 2006). DNA with variable numbers of tandem repeats is amplified by PCR and size of products determined by gel electrophoresis, capillary or non-denaturing, high-performance liquid chromatography. Most repeat units are more than 50 bps long and allele sizes rarely exceed 1000 bps, thus precision which can be obtained by ordinary agarose gel electrophoresis is sufficient to estimate the number of units in an allele (Le Fleche *et al.* 2002). The resolution of VNTR typing is cumulative i.e. the inclusion of more markers in the typing assay can, when necessary, increase the identification resolution (Le Fleche *et al*. 2002).

Traditionally, VNTR typing based on 12-loci has been used for *M. tuberculosis* isolates in combination with spoligotyping, though a proportion of unrelated isolates remained clustered (Supply *et al*. 2006). For improved resolution, Supply *et al*. (2006) described 24-loci including the 12, with a subset of 15 loci with the highest evolutionary rates. They proposed the 15-loci system as the standard for routine epidemiological discrimination of *M. tuberculosis* complex and the 24-locus system as a high-resolution tool for phylogenetic studies. Not all the 24 or 15-loci are always necessary to define all the isolates in any given situation, as 9 loci from the 15-loci set can be sufficient (Supply *et al.* 2006). Generally, loci with highest evolutionary rates within the set of 15 can be applied in the first place depending on the lineages prevalent in the population of interest. Variation is driven mainly by DNA replication slippage, causing changes in the numbers of repeats, a process that can be modeled using the stepwise mutation model (Reyes & Tanaka. 2010). There may also be micro-deletions or insertions within some repeats (Le Fleche *et al*. 2002).

Since tandem repeats can be unstable, for particular strains under consideration in a geographical location, the markers must be evaluated using representative collections of strains before they can be used (Le Fleche *et al.* 2002). VNTR typing by PCR has several advantages (Frothingham *et al.* 1998). It is a rapid and reproducible method. It can be performed on mycobacteria killed by heat or alcohol, reducing biohazards also applicable to crude DNA extracts from early cultures (Supply *et al*. 2006). Results are intrinsically digital, simplifying the comparison of large numbers of strains.

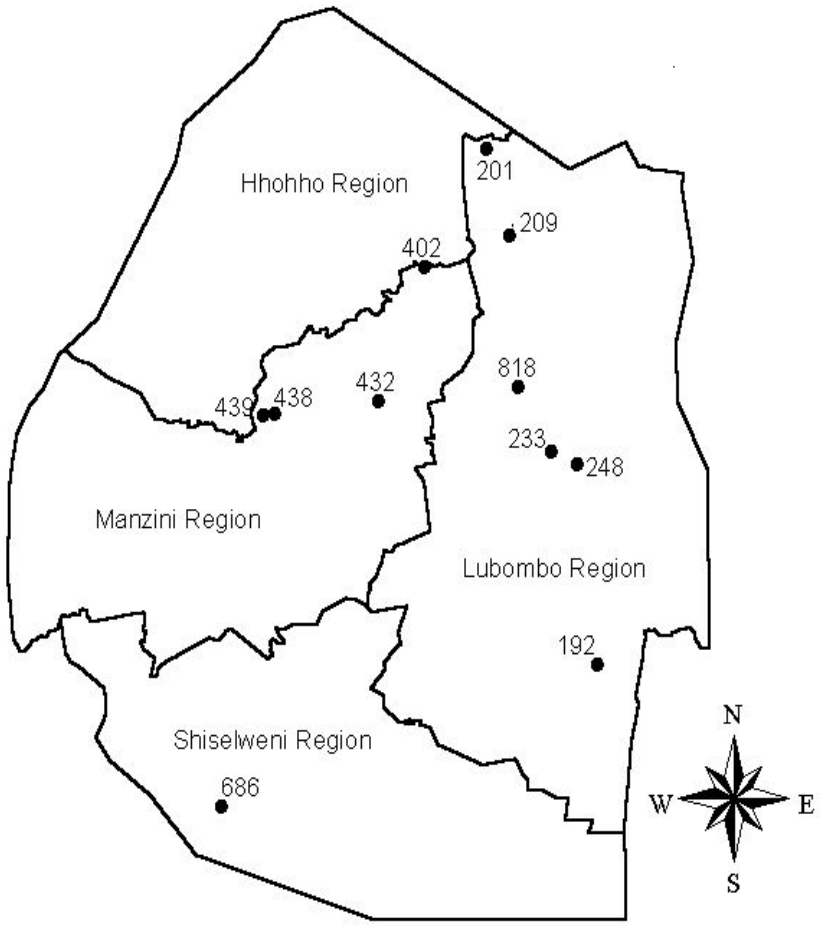
Chapter 2

2.1 Materials and methods

2.1.1 Sample collection

Tissue samples were collected at the Swaziland Meat Industries (EU-export) abattoir in Swaziland. This is the only abattoir in the country that screens for bovine TB and has the personnel capacity to do so. The sampling was opportunistic as it targeted the carcasses with specific lesions. A total of 39 tissue samples were used for this project owing to the frequency of lesions in the carcasses. These were mainly lymph nodes which showed characteristic BTB lesions upon meat inspection. The samples were collected from November 2008 to July 2010, mainly from mixed breed cattle slaughtered for beef. These animals came from different parts of the country i.e. Manzini region, Shiselweni region and Lubombo region as illustrated in figure 1 below.

Figure 1. Map of Swaziland showing dip tanks of origin of animals sampled



Numbers on the map represent different dip tanks.

Dip tank 749 from Shiselweni was represented by dip tank 686 on the map as a nearest dip tank

2.1.2 Tissue preparation, culture, identification of isolates and DNA extraction

The samples were first tested for presence of acid-fast bacilli using the Ziehl-Neelsen staining technique at Central Veterinary Laboratory in Manzini, Swaziland.

Acid-fast samples were then cultured at the Referral Hospital (human) Laboratory, Tuberculosis division. Specimens were first homogenized manually using a mortar. The samples were then decontaminated and digested by adding equal volume to the sample (5 ml) of a mixture of sodium hydroxide and trisodium citrate. This mixture was prepared by mixing equal quantities of 4% NaOH and 2.9% sodium citrate, in a volume that was needed for that day. 5 ml of this mixture was added to each sample. To achieve a final concentration of 0.5%, 0.5 g NALC powder was then added per 100 ml of the mixture. Phosphate buffer, pH6.8, was added up to a total volume of 50 ml. After centrifugation, 2 ml of phosphate buffer was added to the sediments. A total of 500 µL of the resultant solution was cultured in Modified Middlebrook 7H9 broth. Added into this was 800 µL of a mixture of growth supplements (bovine albumin,dextrose, catalase, oleic acid and polyoxyethyline state) and antimicrobial agents (polymixin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin). This was incubated in a MGIT 960 incubator until growth was observed or for a maximum of 42 days. Growth was automatically indicated by the machine on fluorescence in the tube. Presence of *Mycobacterium spp* was checked by *M. tuberculosis* complex gene probe. The protocol used was that provided with the commercial kit for the AccuPROBE Mycobacterium Tuberculosis Complex Probe kit, 102896 Revision L, by GEN-PROBE®.

DNA extraction from positive samples was done using a commercial kit, Extract-N-Amp™ Tissue PCR Kit, from SIGMA. The protocol used was provided by the manufacturer with the kit.

2.1.3 Spoligotyping

A commercial spoligotyping kit by ISOGEN Biosolutions was used, which included a protocol by the manufacturer. The PCR reactions were performed as follows: initial denaturing at 96ºC for 3 min, 30 cycles of 96ºC for 1 min, 55ºC for 1 min, and 72ºC for 30 sec, followed by 72ºC for 5 min. The PCR product was then hybridized to a set of 43 immobilized oligonucleotides. The patterns were identified photographically and analysed in comparison with data from other publications, especially that by Michel *et al*. (2008).

2.1.4 Variable Number Tandem Repeat (VNTR) typing

VNTR typing was performed according to the method of Frothingham *et al*. (1998). A set of 9 primers as published by Frothingham *et al.* (1998) and Supply *et al*. (2006) were used (Table 1a). The primer pairs used were ETR loci A, B, C and E, MIRU loci 16, 23 and 26, M. tub 21 and Qub 11b. Each PCR reaction consisted of 12.5 µl Fermentas mastermix, 0.5 µl of each of the primers for each locus and 2 µl of DNA sample in a final volume of 25 µl. Following a denaturing step at 94ºC for 5 min, each sample was subjected to 40 cycles of 94ºC for 5 min, 62ºC for 1 min, 72º C for 90 sec. This was followed by a final extension annealing at 72ºC for 10 min. A known positive isolate (7472B) from South Africa was used as a positive control. PCR products were separated using agarose gel electrophoresis. Copy numbers for each locus in an isolate were interpreted based on the product length using tables published by Le Fleche *et al*. (2002) as shown in table 1b.

Table 1a. Primers and their sequences

Primer	Sequence
ETR A	AAATCGGTCCCATCACCTTCTTAT (fwd) CGAAGCCTGGGGTGCCCGCGATTT (rev)
ETR B	ATGGCCACCCGATACCGCTTCAGT (fwd) CGACGGGCCATCTTGGATCAGCTAC (rev)
ETR C	CGAGAGTGGCAGTGGCGGTTATCT (fwd) AATGACTTGAACGCGCAAATTGTGA (rev)
ETR E	ACTGATTGGCTTCATACGGCTTTA (fwd) GTGCCGACGTGGTCTTGAT (rev)
MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA (fwd) CCCGTCGTGCAGCCCTGGTAC (rev)
MIRU 23	CTGTCGATGGCCGCAACAAAACG (fwd) AGCTCAACGGGTTCGCCCTTTTGTC (rev)
MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC (fwd) CATAGGCGACCAGGCGAATAG (rev)
Qub 11b	CGTAAGGGGGATGCGGGAAATAGG (fwd) CGAAGTGAATGGTGGCAT (rev)
M tub 21	AGATCCCAGTTGTCGTCGTC (fwd) CAACATCGCCTGGTTCTGTA (rev)

Table 1b. Product length and locus copy number

Locus	Product Length (copy number)										
ETR A	247 (1)	322 (2)	397 (3)	472 (4)	547 (5)	622 (6)	697 (7)	772 (8)	847 (9)	922 (10)	
ETR B	178 (1)	235 (2)	292 (3)	349 (4)	406 (5)	463 (6)	634 (9)				
ETR C	230 (2)	288 (3)	346 (4)	404 (5)	462 (6)	520 (7)	684 (10)				
ETR E	545 (1)	598 (2)	651 (3)	704 (4)	757 (5)	810 (6)	863 (7)				
MIRU 16	618 (1)	671 (2)	724 (3)	777 (4)	829 (5)	882 (6)	1147 (11)				
MIRU 23	607 (1)	661 (2)	714 (3)	767 (4)	820 (5)	873 (6)	926 (7)	979 (8)			
MIRU 26	511 (1)	562 (2)	613 (3)	664 (4)	715 (5)	766 (6)	817 (7)	868 (8)	919 (9)		
Mtub 21	149 (1)	206 (2)	263 (3)	320 (4)	377 (5)	434 (6)	491 (7)	548 (8)	605 (9)	662 (10)	719 (11)
Qub 11 b	136 (1)	274 (3)	343 (4)	412 (5)	826 (11)						

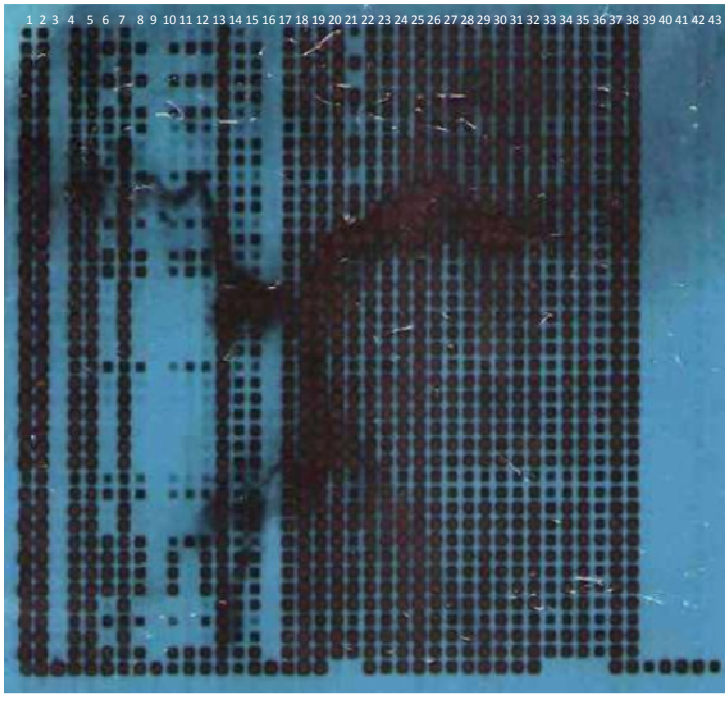
Chapter 3

3.1. Results

3.1.1. Spoligotyping

The spoligotype patterns of all 39 *M. bovis* DNA isolates were found to be representative of *M. bovis* strains as they had the characteristic deletions in loci 3,9,16, 39-43 (Haddad *et al*. 2004) as shown in Figure 2. As shown in Table 2 (page 21), 12 different spoligotypes were identified from the 39 *M. bovis* isolates analysed in this study. The most frequently observed spoligotype, SMD 1, which accounted for 23 (59%) of the 39 samples, lacked spacers 6, 8, 10, 11 and 12 in addition to the typical *M. bovis* deletion pattern. All the other types were less frequently observed with the next highest, SMD 3, accounting for only 3 (8%) of the 39 *M. bovis* DNA isolates done. Sample 13 and 30 had a pattern similar to the *M. bovis* BCG P3 control supplied with test kit.

Figure 2. Spoligotype patterns detected among 39 *M. bovis* DNA isolates from cattle in Swaziland



Columns represent the 43 spoligo loci and rows represent isolates. Bottom 1st and 2nd rows represent the positive control isolates from the kit used (*M. tuberculosis* strain H37Rv and BCG P3 respectively).

Table 2. Spoligotypes detected among Swazi *M. bovis* isolates

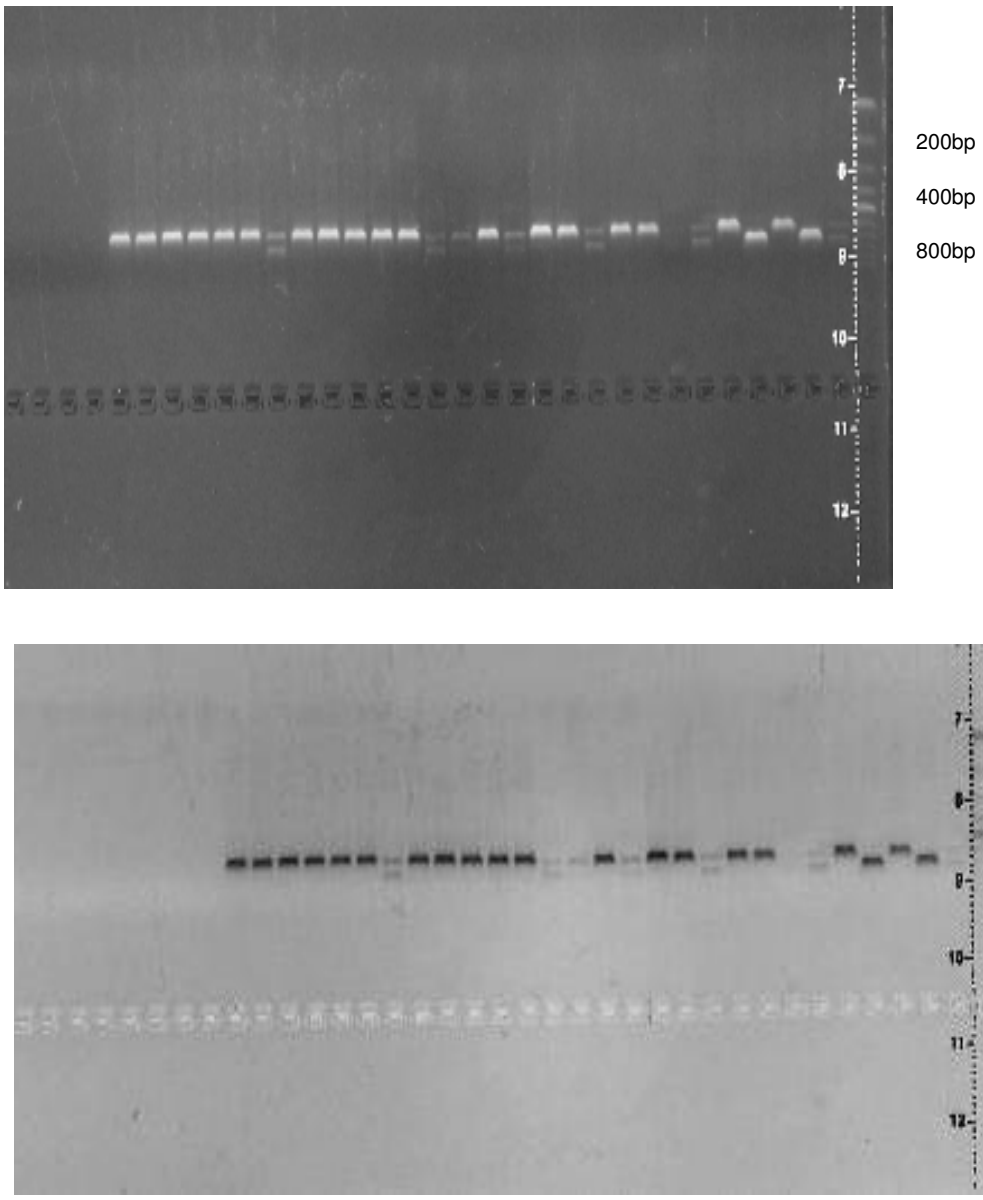
Spoligo type	SB CODE*	Deleted loci	%	Region/-Frequency		
				Manzini	Shiselweni	Lubombo
SMD 1	SB 0140	6,8,10,11, 12	56	16	3	3
SMD 2	SB 1184	6,11	5	0	0	2
SMD 3	SB 0121	21	8	1	0	2
SMD 4	SB 0265	6,21	5	1	0	1
SMD 5	SB 1269	10,14,15,21	3	0	0	1
SMD 6	SB 0332	14,15,21	3	1	0	0
SMD 7	SB 0290	6,8,10,11, 12,14,15	3	1	0	0
SMD 8	SB 1324	6	3	1	0	0
SMD 9	BCG	3,9,16	5	0	0	2
SMD 10	SB 0127	14,15	3	1	0	0
SMD 11	SB 0130	11	3	0	0	1
SMD 12	SB 0678	11,15	3	1	0	0

Spoligotypes of 39 *M. bovis* isolates and their geographical distribution are listed. All the samples have the typical *M. bovis* deleted loci at 3,9,16, 39-43 (Haddad *et al*. 2004). *http://www.mbovis.org. SMD – Swaziland Mycobacterium Division. Isolate 26 was SMD 1 but had no information on region of origin.

3.1.2. VNTR typing

After amplification and electrophoresis, bands for the different VNTR loci were photographed as in Figure 3a, ETR E loci. For all other loci the results are in the annex (Figures 4 – 11). According to the VNTR patterns of the 9 chosen loci, 19 VNTR types were identified for 38 isolates as shown in table 5. Isolate number 7 did not produce any amplification product for all the primer sets and thus was not included. The most dominant type, VNTR type 1, accounted for 11 (29%) of the 38 isolates analysed, while the next highest, VNTR type 4, was represented in 4 (11%) isolates. When only 4 loci were used (ETR A, B, C, E), only 11 VNTR patterns emerged, with the dominant type accounting for 23 (63%) of 38 isolates analysed. When only the 3 MIRU loci (MIRU 16, 23, 26) were used, 6 VNTR patterns emerged, with the dominant pattern accounting for 15 (39%) isolates.

Figure 3a. PCR amplification of the ETR E marker from 28 bovine *M. bovis* isolates



The ladder (L) indicates that the DNA bands are 600bp and 700bp long, indicating that the isolates either had 2 copies or 4 copies of tandem repeat sequences in the allele at the ETR E locus, as indicated in Table 1b.

Table 3. VNTR allelic profiles detected among Swazi *M. bovis* isolates

Isolate	ETR Locus				MIRU Locus			M. tub	Qub
	A	B	C	E	16	23	26	21	11b
1	6	0	0	0	0	0	0	3	4
2	7	0	3	4	3	1	2	2	3
3	6	0	6	2	3	1	2	3	4
4	7	0	3	4	3	1	2	2	3
5	6	0	6	2	0	1	2	3	4
6	7	0	3	0	0	1	2	2	0
8	6	0	6	2	3	1	2	3	4
9	6	0	6	2	3	1	2	3	4
10	7	0	0	0	0	0	0	0	3
11	6	0	6	2	3	1	0	3	4
12	6	0	0	2	3	1	0	3	4
13	6	0	0	0	0	1	0	3	4
14	6	0	6	2	3	1	0	3	4
15	0	0	3	0	0	1	0	2	0
16	7	0	3	0	0	0	0	3	4
17	6	0	6	2	3	1	0	3	4
18	6	0	6	2	3	1	2	3	4
19	6	0	6	2	3	1	2	3	4
20	6	0	6	2	3	1	2	3	4
21	6	0	6	2	3	1	2	3	4
22	6	0	6	0	0	0	2	0	0
23	6	0	6	2	0	1	2	3	4
24	6	0	6	2	3	1	2	3	4
25	6	0	6	2	0	1	2	3	4
26	6	0	6	2	3	1	2	3	4
27	6	0	6	2	3	1	2	3	4
28	6	0	6	2	3	1	2	3	4
29	6	0	6	2	0	0	0	3	0
30	6	0	6	2	0	0	0	3	0
31	6	0	6	2	0	1	0	3	3
32	6	0	6	2	0	1	2	3	4
33	0	0	3	2	0	0	0	0	0
34	7	0	6	4	0	1	0	3	2
35	7	0	6	4	3	1	2	3	2
36	7	0	6	4	3	1	2	3	2
37	6	0	6	2	0	1	0	3	3
38	0	0	6	4	0	0	0	3	0
39	6	0	6	2	0	1	0	0	3

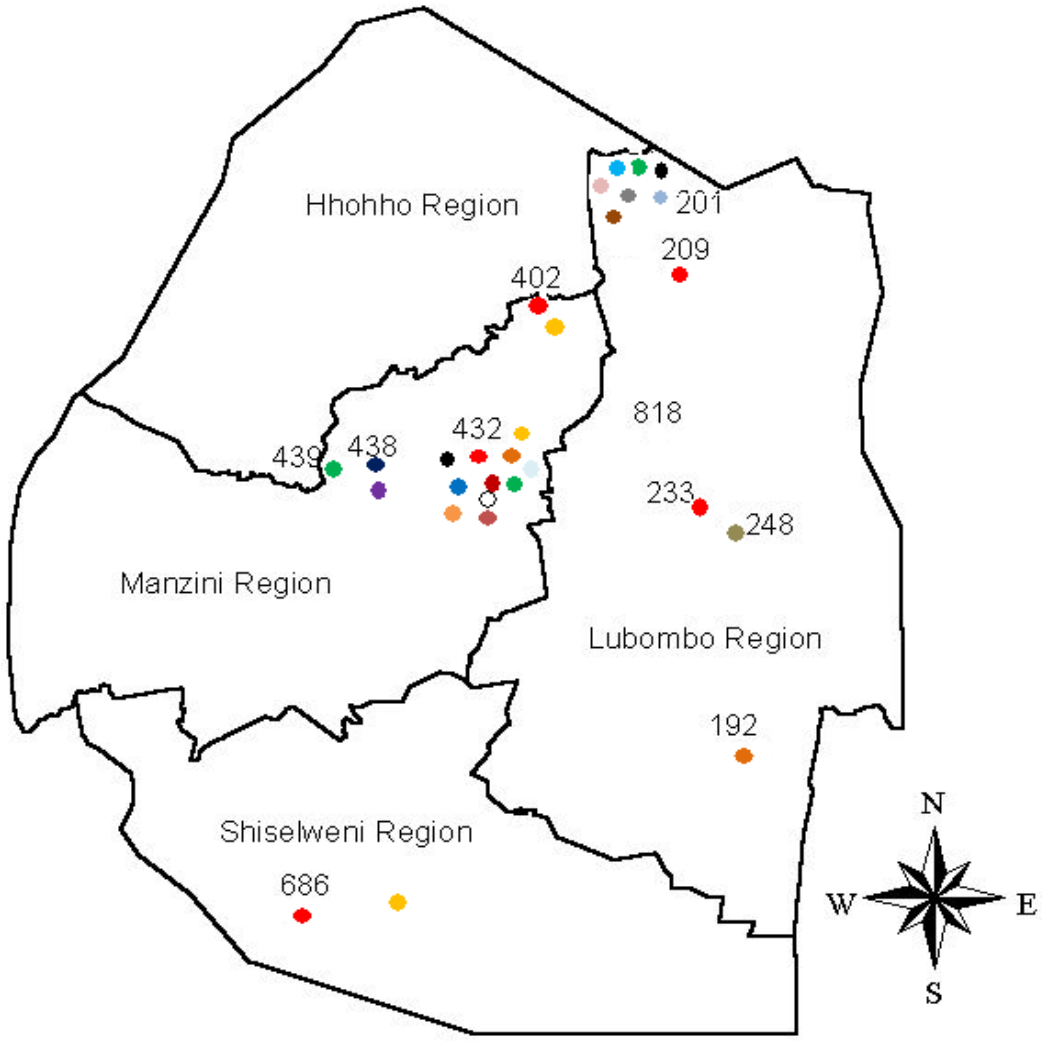
Allelic Profiles of 38 *M. bovis* DNA isolates depicted by their copy numbers of VNTR loci.

Table 4. VNTR types detected among Swazi *M. bovis* isolates

VNTR type	ETR A	ETR B	ETR C	ETR E	MIRU 16	MIRU 23	MIRU 26	M.tub 21	Qub 11b	Freq	%	Colour
1	6	0	6	2	3	1	2	3	4	**11**	29	
2	7	0	3	4	3	1	2	2	3	**2**	5	
3	6	0	0	0	0	0	0	3	4	**1**	3	
4	6	0	6	2	0	1	2	3	4	**4**	11	
5	6	0	6	2	3	1	0	3	4	**3**	8	
6	6	0	6	0	0	0	2	0	0	**1**	3	
7	6	0	0	2	3	1	0	3	4	**1**	3	
8	7	0	3	0	0	1	2	2	0	**1**	3	
9	7	0	0	0	0	0	0	0	3	**1**	3	
10	7	0	3	0	0	0	0	3	4	**1**	3	
11	0	0	3	0	0	1	0	2	0	**1**	3	
12	6	0	0	0	0	1	0	3	4	**1**	3	
13	6	0	6	2	0	1	0	0	3	**1**	3	
14	6	0	6	2	0	0	0	3	0	**2**	5	
15	7	0	6	4	3	1	2	3	2	**2**	5	
16	7	0	6	4	0	1	0	3	2	**1**	3	
17	6	0	6	2	0	1	0	3	3	**2**	5	
18	0	0	3	2	0	0	0	0	0	**1**	3	
19	0	0	6	4	0	0	0	3	0	**1**	3	

Summary of the different VNTR types for the 38 *M. bovis* DNA isolates depicted by colour and their copy numbers at the different VNTR loci analysed**.**

Figure 3b. Geographical distribution of VNTR types



VNTR type	1	2		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

The different colours on the map represent the different VNTR types as shown above

3.1.3 VNTR/Spoligotying

When the outcomes of both typing methods results, VNTR typing and spoligotyping (Table 5), were analysed in parallel, 21 V-S types emerged (Table 6) for the 38 isolates tested with both techniques. The most dominant type was V-S type 3, with 7 (18%) of the samples. This was followed by type 4 with 4 (10.5%) of isolates. V-S type 6 was from isolate 7 and as already mentioned it does not have a defined VNTR genotype but it is spoligotype 5. The discriminatory power of combining the techniques was better than spoligotyping alone, but was comparable with VNTR typing.

Table 5. Combined analysis of VNTR and Spoligotyping results by region and dip tank

M- Manzini H- Hhohho S- Shiselweni L- Lubombo U- unknown

Isolate	VNTR type	Spoligo (SMD)	Region	Dip tank
1	3	7	MZ	432
2	2	3	MZ	432
3	1	1	MZ	432
4	2	3	L	192
5	4	1	MZ	432
6	8	3	L	201
7	U	5	L	818
8	1	1	MZ	432
9	1	1	MZ	402
10	9	6	MZ	438
11	5	1	MZ	439
12	7	1	MZ	438
13	12	9	L	248
14	5	1	L	201
15	11	4	L	201
16	10	4	MZ	432
17	5	1	MZ	432
18	1	1	MZ	402
19	1	1	SH	749
20	1	1	SH	749
21	1	1	MZ	209
22	6	8	MZ	432
23	4	1	MZ	402
24	1	1	MZ	402
25	4	1	MZ	402
26	1	1	U	U
27	1	1	L	233
28	1	1	MZ	402
29	14	1	MZ	432
30	14	9	L	201
31	17	1	MZ	432
32	4	1	SH	706
33	18	10	MZ	U
34	16	11	L	201
35	15	2	L	201
36	15	2	L	201
37	17	1	MZ	U
38	19	12	MZ	U
39	13	1	L	201

Table 6. Different V-S genotypes detected among Swazi M. bovis isolates

V-S genotype	Pattern		Freq	Sample I.D
	VNTR	Spoligo		
1	3	7	1	1
2	2	3	2	2,4
3	1	1	11	3,8,9,18,19,20,21,24,26,27,28
4	4	1	4	5,23,25,32
5	8	3	1	6
6	U	5	1	7
7	9	6	1	10
8	5	1	3	11,14,15
9	7	1	1	12
10	12	9	1	13
11	11	4	1	15
12	10	4	1	16
13	6	8	1	22
14	14	1	1	29
15	14	9	1	30
16	17	1	2	31,37
17	18	10	1	33
18	16	11	1	34
19	15	2	2	35,36
20	19	12	1	38
21	13	1	1	39

V-S genotype – genotype identified by parallel analysis of VNTR types and Spoligotypes

Chapter 4

4.1 Discussion

The present study is the first to investigate DNA polymorphism among *M. bovis* isolates from cattle in Swaziland. Two commonly used molecular techniques, spoligotyping and VNTR typing, were used for this purpose. The techniques used differed in terms of their discriminatory level and reproducibility. With the exception of isolate 29 and 30, VNTR typing was equal to or more discriminatory than spoligotyping, as observed by Michel *et. al* 2008. In the present study, combining the two methods did not provide a significant improvement from the discrimination provided by VNTR typing alone.

Though spoligotyping was less discriminatory than VNTR typing, it was useful to classify all the isolates as *M. bovis* DNA, which VNTR typing could not do for isolate 7. This again is testimony that spoligotyping is simple, robust and does not need a high quantity or quality of DNA (Haddad *et al*. 2004). The most dominant spoligotype, SMD1, which accounted for 23 isolates, was further discriminated into 6 different genotypes by VNTR typing i.e. VNTR type 1, 4, 5, 13 and 17.

As seen in Table 5, VNTR typing also clustered isolates from epidemiologically unrelated outbreaks. Since movement of cattle between dip tanks is regulated and thus minimal, a disease outbreak in one dip tank is unlikely to be related to another outbreak in a geographically different dip tank. This is seen with isolates 29 and 30 which were clustered into VNTR type 14, but were classified as spoligotypes 1 and 9 respectively and subsequently into 2 different V-S types (Table 6). These isolates came from dip tank 432 and 201 respectively, which are in 2 different regions (Figure 3b).

Spoligotyping was able to discriminate isolates with more than one deletion, forming patterns which are not found in the South African isolates. This is especially true for type SMD 8 and 10. SMD 10 had a deletion at locus14, and SMD 8 had a deletion at locus 6 only, which none of the South African isolates had. These strains most probably either originated from within Swaziland and have evolved within the country over time, or could, alternatively, have been eradicated or remained undetected to date in South Africa. However SMD 1, 2, 7, 11 and 12 were related to isolates from the Kruger area in South Africa because of loss of spacer 11. The phylogenetically oldest isolate here, which has the least deletions with only one deletion at spacer 11, originated from Lubombo, dip tank 201 (Figure 1). SMD 3, 4, 5 and 6 were related to the Hluhluwe area in South Africa because of loss of spacer 21. The phylogenetically oldest isolate here, with only one deletion at spacer 21, came from Manzini (dip tank 432) and Lubombo (dip tank 201 and 192) (Figure 1).

The isolates analysed in this study were not related to the African 1 (deletion in spacer 30) or African 2 (deletion in spacer 3 – 7) clonal complexes dominant in East Africa and West Africa, as shown by previous studies (Berg *et al.* 2011; Muller *et al.* 2009). However, some of the isolates were related to the clonal complex Europe 1 (deletion in spacer 11). This is in line with findings from Smith *et al*. (in press) who classified an isolate from Swaziland into this genotype, and also found the genotype in former British colony areas. Swaziland, as a former British colony, may thus have received this genotype through cattle imports from Britain.

The pattern of deletion also classified the isolates according to age with the oldest having the least deletions e.g. SMD 11(isolate 34 with one deletion at spacer 11) was older than SMD 1, 2, 7 and 12. SMD 3 (isolate 2, 4, 6 with one deletion at spacer 21) was older than SMD 1, 4, 6 and 5. (Haddad *et al*. 2004).

The pattern of spoligotype distribution in the regions, as depicted in Table 6, suggests that only the dominant spoligotype (SMD1/SB0140) is widely distributed in the country. According to this study this genotype was found mainly in Manzini, but was also found in Shiselweni and Lubombo. SMD8/BCG genotype was only found in Lubombo region for the isolates done in this study. The BCG genotype was found only in Lubombo region, but in different dip tanks i.e. 201 and 248 (Figure 1).

All the chosen loci for VNTR typing were discriminatory for the set of isolates, except for the ETR B locus, where amplification of the product was not obtained for any of the isolates (Table 3) . The problem was unlikely to be due to reagent or technical failure since the positive control, which was a South African isolate, did amplify. This may be due to a complete deletion of the locus in the local strains or that the marker sequence is short at this locus, as a result of micro-deletion within the repeat (Le Fleche *et al*. 2002), especially at the point where it complements the primer, thus no annealing and no amplification was possible. The VNTR pattern of distribution follows that of spoligotyping in that the most dominant pattern, VNTR type 1 was found in all the regions (Figure 3b, Table 5). VNTR 5 was found in dip tanks both in Lubombo and Manzini. VNTR type 8, 11 and 12 were found only in Lubombo and VNTR 3, 4, 6, 7 and 9 were only found in Manzini in this study.

The study revealed a high genetic diversity among the 38 *M. bovis* isolates, since according to Michel *et al*. (2008), strains with different VNTR patterns most likely represent genetically distinct strains. This may not be expected in a country as small as Swaziland and especially using such small number of isolates. Michel *et al*. (2008), using isolates from South Africa, found 12 spoligotypes from 50 isolates and 13 VNTR types from 43 isolates from six provinces. This study revealed there were 12 spoligotypes and 19 VNTR types in Swaziland. An important aspect in understanding the reason for this high diversity is the immigration rate (introduction of infected animals from different sources) and population structure and type (Reyes and Tanaka 2010). As such, the diversity may be due to purchase of infected animals from different areas, or, alternatively persistence and evolution of “old” *M. bovis* strains within the cattle population in the country (Michel *et al*. 2008). Historically , especially in the last 60 years, cattle import records showed that Swaziland has almost exclusively received import cattle, both beef and dairy, from South Africa (Eastern Cape, Gauteng, KwaZulu Natal and Mpumalanga), with no evidence of export in the opposite direction. In this study, four spoligotypes, which includes the dominant and second dominant types, as shown in the Table 7 below, are the same as those classified by Michel *et al.* (2008) from different areas and outbreaks in South Africa. Michel *et al*. (2008) classified a 1996 isolate from Swaziland into the SP 8 group from South Africa. Although the four shared spoligotypes are not unique to South Africa but occur in many countries worldwide, these findings would suggest that these isolates may have come with infected cattle imports from South Africa or via South Africa, when this country imported large numbers of cattle from Australia, Europe and Madagascar long before the TB control scheme was implemented in 1969 (Michel *et al*. 2008).

When analysing the VNTR genotypes and spoligo genotypes in parallel, the 21 “V-S” types that emerged were similar to the VNTR type patterns, except for the two isolates which were clustered by VNTR typing and the one isolate which did not amplify for any of the chosen loci as already mentioned (Table 5). This suggests that VNTR typing was sufficient in classifying the isolates and the V-S typing may not be necessary given the extra effort needed to do it.

Table 7. Similar spoligotypes (Swaziland and South Africa)

Spoligotype				
Swaziland	**South Africa**	**SB CODE**	**Area in S.A**	**Deleted loci (3, 9, 16, 39 – 43)**
SMD 1	SP 8	SB 0140	EC, WC	6, 8, 10, 11, 12
SMD 3	SP 1	SB 0121	LP, GP, MP	21
SMD 4	SP 9	SB 0265	MP, WC	21,6
SMD 11	SP 4	SB 0130	KZN, MP, GP	11

EC- Eastern Cape WC- Western Cape LP- Limpopo Province GP- Gauteng Province MP- Mpumalanga Province KZN- KwaZulu Natal

4.2 Conclusion

Practical knowledge and application of the two molecular techniques, spoligotyping and variable number tandem repeat typing, used in this study was adequately gained. All the 39 isolates used in this study were successfully characterised using these techniques.

Though spoligotyping was less discriminatory, it can be used as a diagnostic and screening tool for *M. bovis* in Swaziland. For more detailed epidemiological studies like molecular characterisation, e.g. intra strain differentiation, the VNTR typing technique could be used. The VNTR typing technique was very discriminatory for the chosen isolates, though the ETR B locus needs to be investigated further for suitability in isolates from Swaziland.

The pattern of strain distribution in the regions was expected because the largest ranch (dip tank 432) which has a feedlot from which the surveyed abattoir collects cattle from, is located in Manzini (Figure 3b). Therefore 73% (16/22) of the dominant spoligotype and 73% (8/11) of the dominant VNTR type originate from Manzini. Lubombo also has another large ranch (dip tank 201) with a high diversity of strains (Figure 3b). Both these ranches keep large numbers of cattle from all over the country and from South Africa, for purposes of breeding and feedlotting. This is believed to account for the high strain diversity in these two regions.

It can also be concluded that the polymorphism pattern indicates that Swaziland and South Africa do share at least four spoligotypes suggesting either a common source of infection or movement of infected cattle between the two countries. The data is also suggestive that Swaziland received infected cattle from Europe at some point in the past, a point which is supported by her colonial history. The high genetic diversity, in such a small country, points to inadequate control measures to an extent that the disease in Swaziland is allowed to evolve into new strains or there is continued importation of BTB from outside the country.

Currently legislation on BTB control is inadequate and there are no active programmes on the ground to control the transmission of this disease.

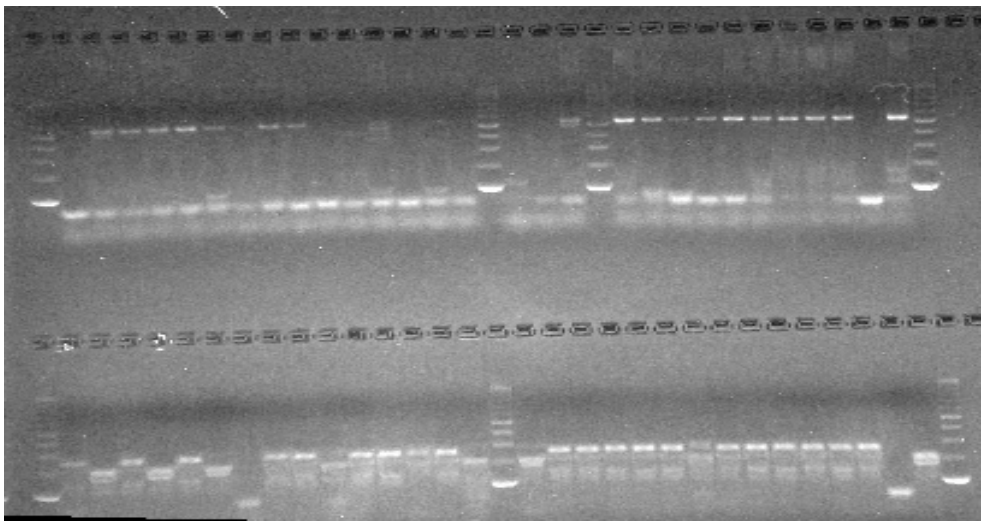
Only 39 samples were used in this study and further work should continue so that more strains can be isolated and characterised to obtain a better understanding of the epidemiology of the disease in Swaziland. Spoligotyping on human TB cases can now be proposed to determine the presence and a potential link of BTB with zoonotic TB in Swaziland.

Chapter 5

Annex

Figure 4. MIRU 26 and M. tub 21 primer products (isolates 1 -28)

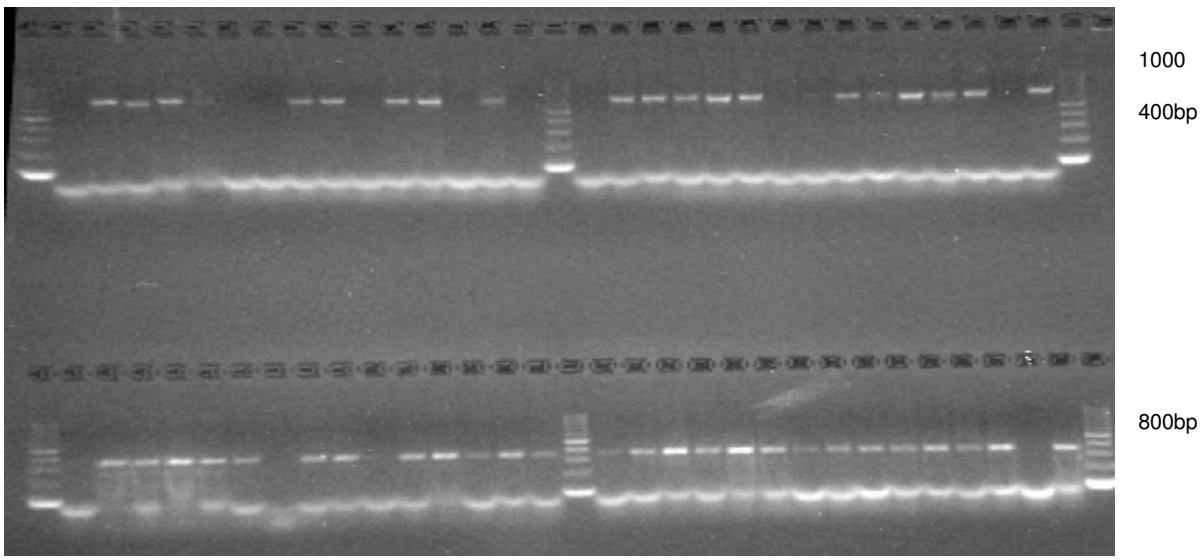
L 1 2 3 4 5 6 7 8 9 101112131415 L161718 L20212223 2425262728 - + L



MIRU 26 (top row), M. tuberculosis 21 (bottom row)MIRU 26 - 2 copies (550bp) *M. tuberculosis* 21 – 2 and 3 copies (200bp and 250bp).

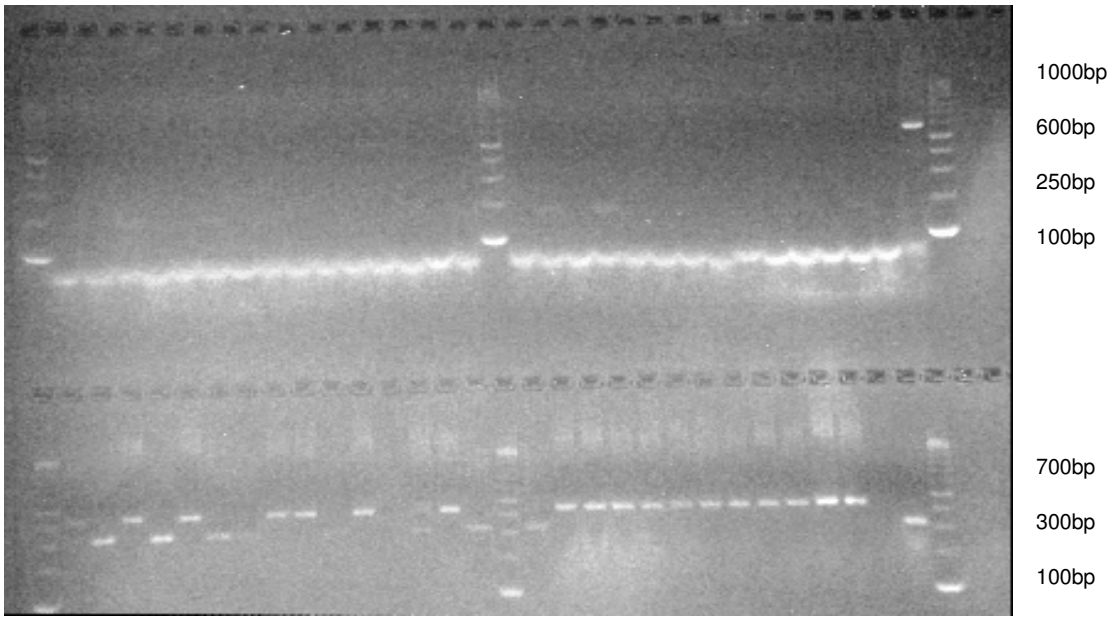
Figure 5. MIRU 16 and MIRU 23 primer PCR products for isolates 1 – 28

L 1 2 3 4 5 6 7 8 9 10 11 1213 14 15 L 16 17 18 19 20 21 22 23 24 25 26 27 28 - + L



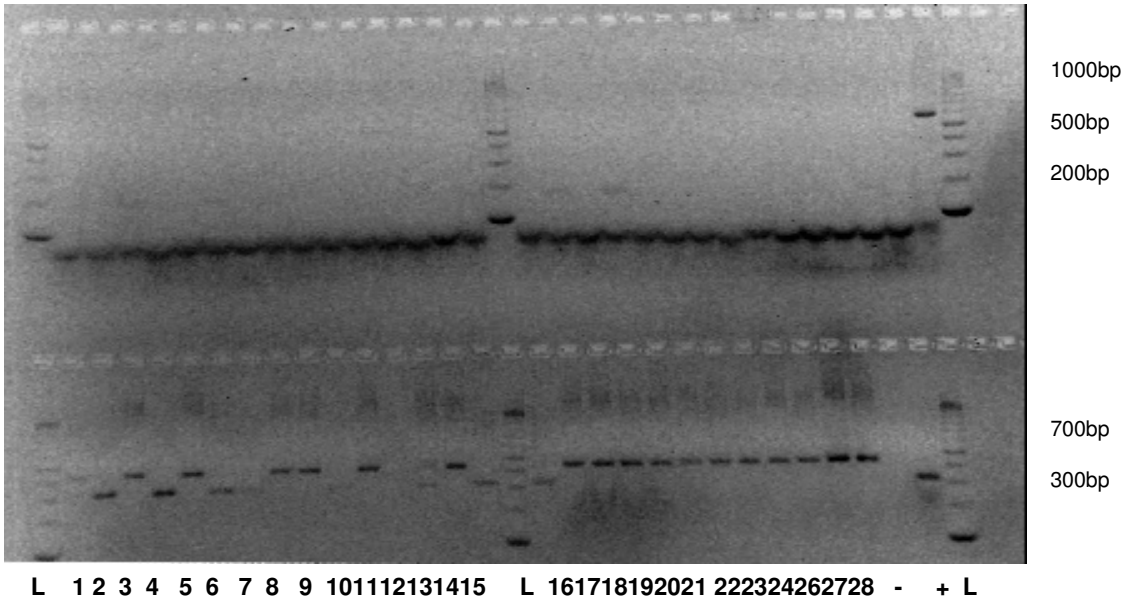
MIRU 16 – 3 copies (750bp) MIRU 23 – 1 copy (350bp). MIRU 16 (top row), MIRU 23 (bottom row)

Figure 6a. ETR B (top row) primer products (isolates 1-28)



ETR B – 0 and 9 copies (0 and 600bp)

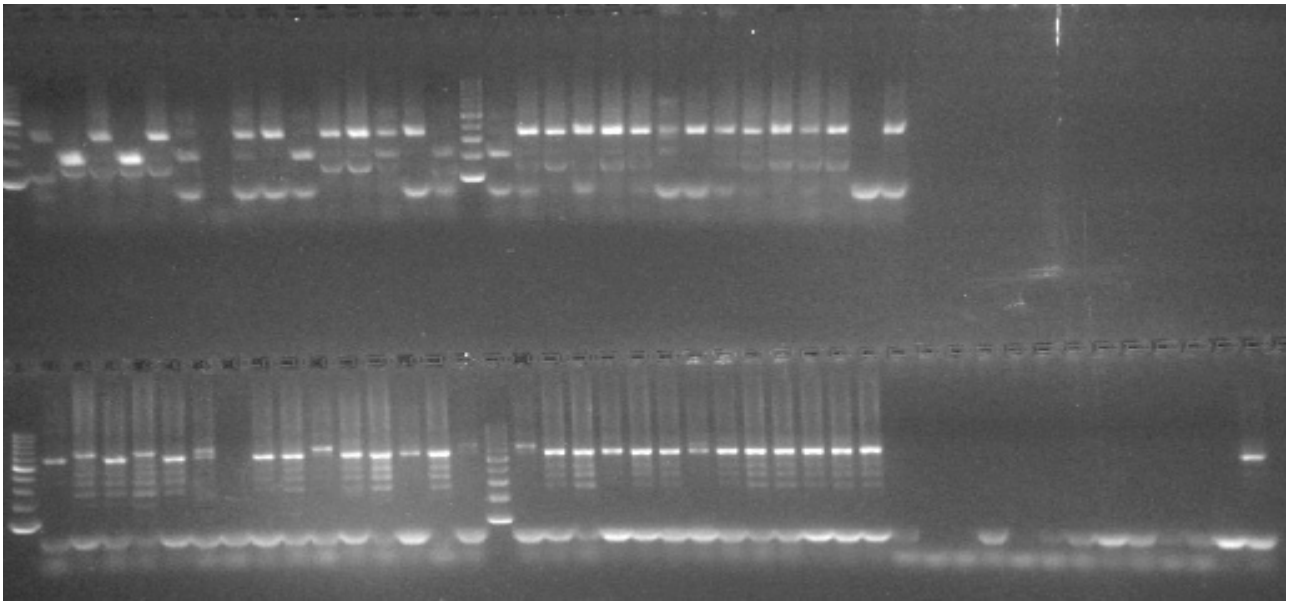
Figure 6b. ETR C (bottom row) primer products (isolates 1 – 28)



ETR C – 3 and 6 copies (300bp and 450bp)

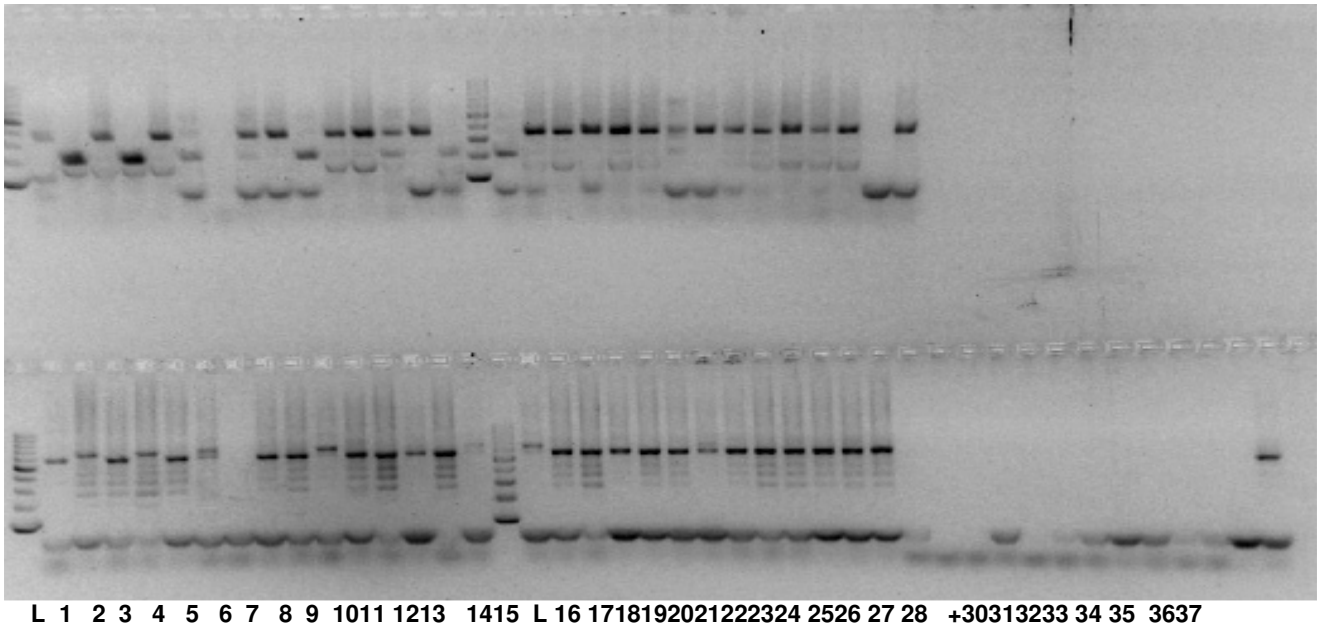
Figure 7a. Qub 11b primer products (isolates 1 – 28)

L 1 2 3 4 5 6 7 8 9 101112131415 L161718 19 2021222324 252627 28 - +



Qub 11b – 3 and 4 copies (200bp and 400bp). Qub 11b (top row, samples 1 – 28).

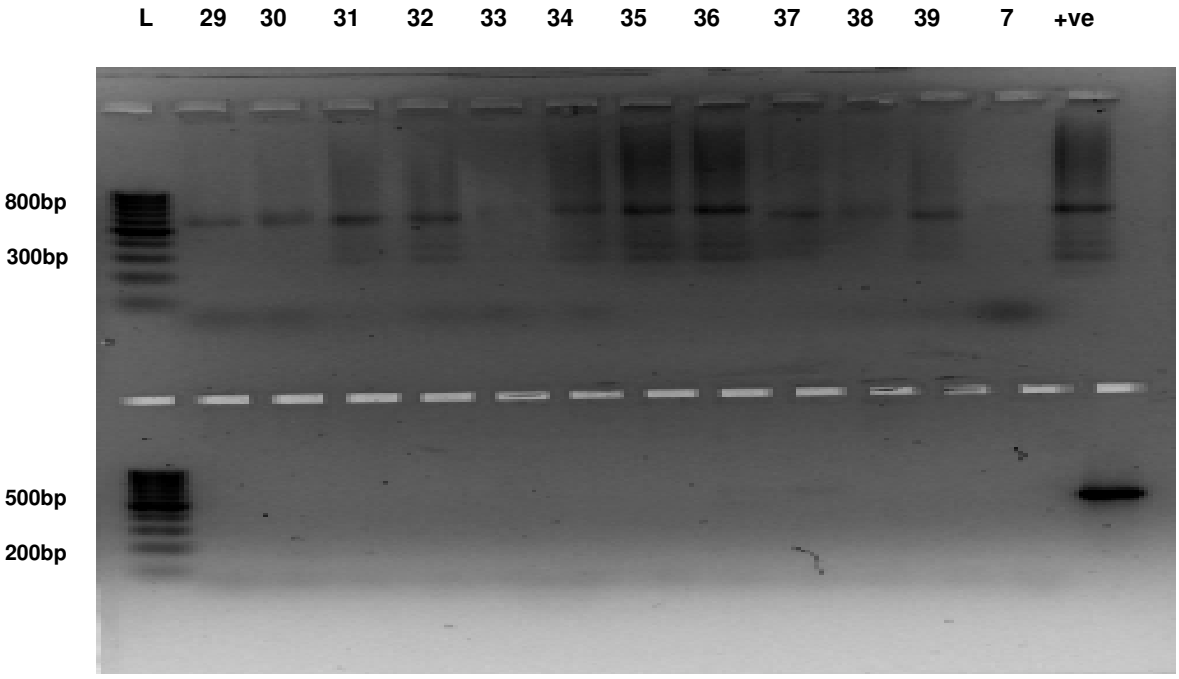
Figure 7b. ETR A primer PCR products (isolates 1-39)



L 1 2 3 4 5 6 7 8 9 1011 1213 1415 L 16 17181920212223 24 2526 27 28 +30313233 34 35 3637

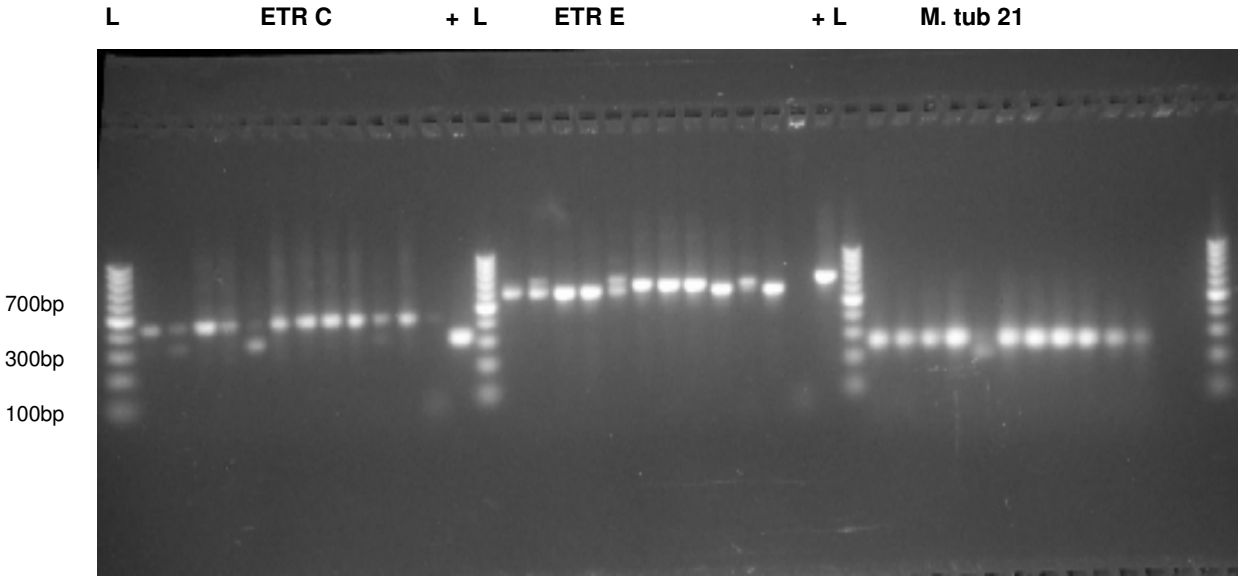
ETR A primer PCR products (bottom row). ETR A – 6 and 7 copies (600bp and 700bp).

Figure 8 ETR A and ETR B primer PCR products (isolates 29 – 39, 7)



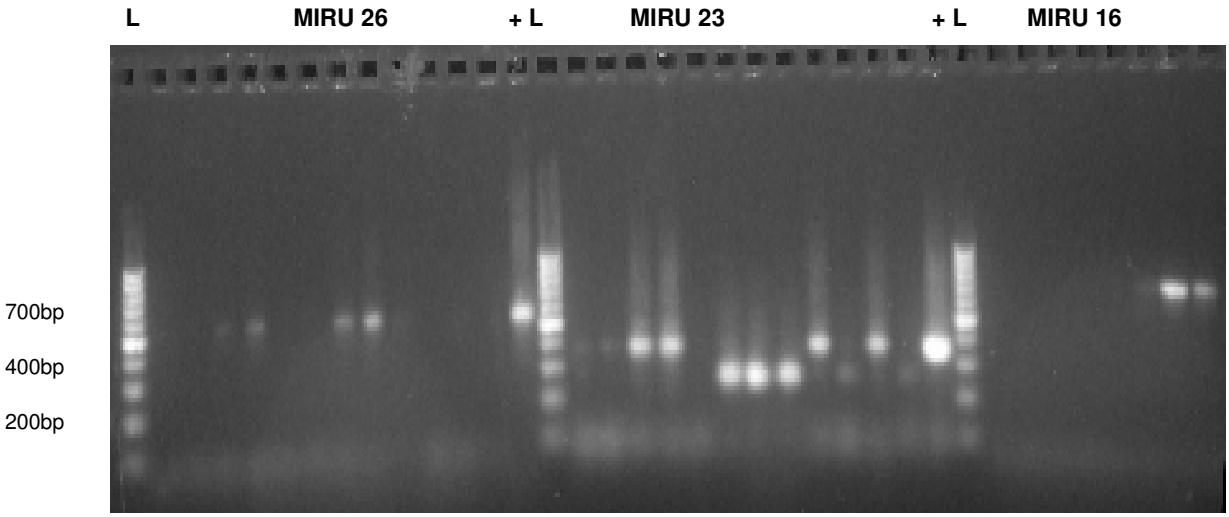
ETR A (top), ETR B (bottom) primer PCR products.

Figure 9. ETR C, ETR E and M. tub 21 primer PCR products (isolates 29 – 39, 7)



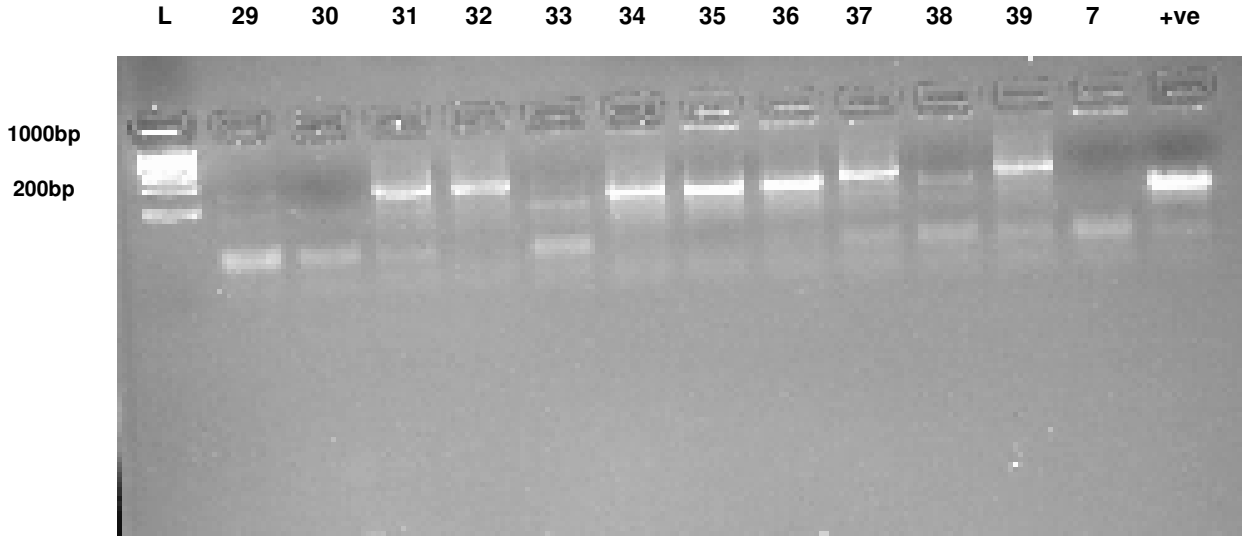
L – ladder + positive control isolate

Figure 10. MIRU 26, MIRU 23, MIRU 16 primer PCR products (isolates 29 -39, 7)



L – ladder + - Positive control isolate

Figure 11. Qub 11 b primer PCR products (isolates 29 – 39, 7)



Chapter 6

References

1. Ayele, W.Y., Neill, S.D., Zinsstag, J., Weiss, M.G. & Pavlik, I. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. *International Journal of Tuberculosis and Lung Disease*, 8: 924-937.

2. Berg, S., Garcia-Pelayo, M.C., Muller, B., Hailu, E., Asiimwe, B., Kremer, K., Dale, J., Boniotti, M.B., Rodriguez, S., Hilty, M., Rigouts, L., Firdessa, R., Machado, A., Mucavele, C., Ngandolo, B.N., Bruchfeld, J., Boschiroli, L., Muller, A., Sahraoui, N., Pacciarini, M., Cadmus, S., Joloba, M., van Soolingen, D., Michel, A.L., Djonne, B., Aranaz, A., Zinsstag, J., van Helden, P., Portaels, F., Kazwala, R., Kallenius, G., Hewinson, R.G., Aseffa, A., Gordon, S.V., Smith, N.H. 2011. African 2, a Clonal Complex of *Mycobacterium bovis* Epidemiologically Important in East Africa. *Journal of Bacteriology*, 193: 670-678.

3. Collins, D.M., Erasmuson, S.K., Stephens D.M., Yates G.F. & de Lisle, G.W.1993. DNA fingerprinting of *Mycobacterium bovis* strains by restriction fragment analysis and hybridization with insertion elements IS1081 and IS6110. *Journal of Clinical Microbiology,* 31: 1143-1147.

4. Collins, D.M. & de Lisle, G.W. 1985. DNA restriction endonuclease analysis of *Mycobacterium bovis* and other members of the *M. tuberculosis* complex. *Journal of Clinical Microbiology,* 21: 562-564.

5. Cosivi, O., Grange, J.M., Daborn, C.J., Ravinglion, M.C., Injikura, T., Cousins, D., Robinson, R.A., Huchzermeyer, A.K., de Kantor, I. & Meslin, F.X. 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerging Infectious Diseases*, 4,1: 59-70.

6. Cousins, D.V. 2001. Mycobacterium bovis infection and control in domestic livestock. *Revue Scientific et Technique, 20,* 1: 71-85.

7. Cousins, D.V., Williams, S., Liebana, E., Aranaz, A., Bunschoten, A., van Embden, J. & Ellis, T. 1998. Evaluation of four DNA typing techniques in epidemiological investigations of bovine tuberculosis. *Journal of Clinical Microbiology*, 36: 168-178.

8. Cousins, D.V., Williams, S.N., Ross, B.C.& Ellis, T.M. 1993. Use of a repetitive element isolated from *Mycobacterium tuberculosis* in hybridization studies with *Mycobacterium bovis* – a new tool for epidemiological studies of bovine tuberculosis. *Veterinary Microbiology*, 37: 1-17.

9. Durr, P.A., Hewinson, R.G. & Clifton-Hadley, R.S. 2000. Molecular epidemiology of bovine tuberculosis I. Mycobacterium bovis genotyping. *Revue Scientifique et Technique*, 19, 3: 675-688.

10. Durr, P.A., Hewinson, R.G. & Clifton-Hadley, R.S. 2000. Molecular epidemiology of bovine tuberculosis II. Application of genotyping. *Revue Scientifique et Technique*, 19, 3: 689-701.

11. Frothingham, R. & Meeker-O'Connell, W.A. 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology*, 144: 1189-1196.

12. Grange, J.M. & Collins, C.H. 1987. Bovine tubercle bacilli and disease in animals and man. *Epidemiology and Infection*, 92: 221- 234.

13. Haddad, N., Masselot, M. & Durand, B. 2004. Molecular differentiation of *Mycobacterium bovis* isolates. *Review of main techniques and applications. Research in Veterinary Science,* 76: 1-18.

14. Hermans, P.W.M., van Soolingen, D. & van Embden, J.D.A. 1992. Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae. Journal of Bacteriology*, 174: 4157-4165.

15. Le Fleche, P., Fabre, M., Denoeud, F., Koeck, J.L. & Vergnaud, G. 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BioMed Central Microbiology,* 2,37: //www.biomedcentral.com/147-2180/2/37.

16. Michel, A.L., Muller, B. & Helden, P.D. 2010. *Mycobacterium bovis* at the animal-human interface: a problem, or not?. *Veterinary Microbiology,* 140: 371-381.

17. Michel, A.L., Coetzee, M.L., Keet, D.F., Mare, L., Warren, R., Cooper, D., Bengis, R.G., Kremer, K. & van Heden, P. 2009. Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South Africa game reserves. *Veterinary Microbiology*, 133: 335-343.

18. Michel, A.L., Hlokwe, T.M., Coetzee, M.L., Mare, L., Connoway, L., Rutten, V.P.M.G. & Kremer, K. 2008. High *Mycobacterium bovis* genetic diversity in low prevalence setting. *Veterinary Microbiology*, 126: 151-159.

19. Milian-Suazo, F., Harris, B., Diaz, C.A., Torres, C.R., Tuber, T., Ojeda, G.A., Loredo, A.M., Soria, M.P. & Payeur, J.B. 2008. Molecular epidemiology of *Mycobacterium bovis*: Usefulness in international trade. *Preventive Veterinary Medicine*, 87: 261-271.

20. Ministry of Health and Social Welfare. 2008. Swaziland National tuberculosis control programme, 2006 treatment outcomes and 2007 TB Morbidity. *Annual report*.

21. Muller, B., Hilty, M., Berg, S., Garcia-Pelayo, M.C., Dale, J., Boschiroli, M.L., Cadmus, S., Ngandolo, B.N., Godreuil, S., Diguimbaye-Djaibe, C., Kazwala, R., Bonfoh, B., Njanpop-Lafourcade, B.M., Sahraoui, N., Guetarni, D., Aseffa, A., Mekonnen, M.H., Razanamparany, V.R., Ramarokoto, H., Djonne, B., Oloya, J., Machado, A., Mucavele, C., Skjerve, E., Portaels, F., Rigouts, L., Michel, A., Muller, A., Kallenius, G., van Helden, P.D., Hewinson, R.G., Zinsstag, J., Gordon, S.V., Smith, N.H. 2009. African 1, an epidemiologically important clonal complex of *Mycobacterium bovis* dominant in Mali, Nigeria, Cameroon, and Chad. *Journal of Bacteriology*, 191: 1951-1960.

22. Poulet, S. & Cole, S.T. 1995. Repeated DNA sequences in mycobacteria. *Archives of Microbiology*, 163: 79-86.

23. Rastogi, N., Legrand, E. & Sola, C. 2001. The Mycobacteria an introduction to Nomenclature and Pathogenesis. *Revue Scientifique et Technique*, 20, 1: 21-54.

24. Reyes, J.F. & Tanaka, M.M. 2010. Mutation rates of spoligotypes and variable numbers of tandem repeat loci in *Mycobacterium tuberculosis. Infection, Genetics and Evolution,* 10: 1046-1051.

25. Romero, B., Aranaz, A., Sandoval, A., Alvarez, J., de Juan, L., Bezos, J., Sanchez, C., Galka, M., Fernandez, P., Mateos, A. & Dominguez, L. 2008. Persistance and molecular evolution of *Mycobacterium bovis* population from cattle and wildlife in Donana National Park revealed by genotype variation. *Veterinary Microbiology*, 132: 87-95.

26. Ross, B.C., Raios, K., Jackson, K. & Dwyer, B. 1992. Molecular cloning of high repeated DNA element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. *Journal of Clinical Microbiology*, 30: 942-946.

27. Sahraoui, N., Muller, B., Guetarni, D., Boulahbal, F., Yala, D., Ouzrout, R., Berg, S., Smith, N.H. & Zinsstag, J. 2009. Molecular characterization of Mycobacterium bovis strains isolated from cattle slaughtered at two abattoirs in Algeria. *BMC Veterinary Research,* 5, number 4.

28. Smith, H.N., Berg, S., Dale, J., Allen, A., Rodriguez, S., Romero, B., Matos, F., Ghebremichael, S., Karoui, C., Donati, C., Machado, A., Mucavele, C., Kazwala, R.R., Hilty, M., Cadmus, M.,Ngandolo, B.N.R., Habtamu, M., Oloya, J., Muller, A., Milian-Suazo, F., Andrievskaia, O., Projahn, M., Barandiaran, S., Macias, A., Muller, B., Zanini, M.S., Ikuta, Y.C., Rodriguez, C.A.R., Pinheiro, R.S., Figueroa, A., Cho, S., Mosavari, N., Chuang, P., Jou, R., Zinsstag, J., va Soolingen, D., Costello, E., Aseffa, A., Proano-Perez, F., Portaels, F., Rigouts, L., Cataldi, A.A., Collins, D.M., Boschiroli, M.L., Hewinson, R.G., Neto, J.S.F., Surujballi, O., Tadyon, K., Botelho, A., Zarraga, M.A., Buller, N., Skuce, R., Michel, A., Aranaz, A., Gordon, S.V., Jeon, B., Kallenius, G., Niemann, S., Boniotti, B.M., van Helden, P.D., Harris, B., Zumarraga, M.J. & Kremer, K. 2011. Europe 1: a globally important clonal complex of *Mycobacterium bovis*. *Infection, Genetics and Evolution*, 11, 6: 1340-1351.

29. Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepin, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C. & van Soolingen, D. 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit- variable number tandem repeat typing of *Mycobacterium tuberculosis. Journal of Clinical Microbiology, 44, 12*: 4498-4510.

30. Van Soolingen, D., Hermans, P.W.M., de Haas, P.E.W. & van Embden, J.D.A. 1992. Insertion element IS*1081*-associated restriction fragment length polymorphism in *Mycobacterium tuberculosis* complex species: a reliable tool for recognizing *Mycobacterium bovis* BCG. *Journal Clinical Microbiology*,30: 1772-1777.

31. Vosloo,W., Bastos, A.D.S., Michel, A. & Thomson, G.R. 2001. Tracing Movement of African Buffalo in Southern Africa. *Revue Scientifique et Technique, 20,* 2: 630-639.