# Typing of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues from selected wildlife species in the Kruger National Park, South Africa

**By**

**Kutlwano A. Hutamo**

Submitted in partial fulfillment of the requirements for the degree of
Magister Scientiae (Veterinary Tropical Diseases) in the
Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science,
University of Pretoria.


| | |
|---|---|
| **Supervisor:** | **Prof. J. Godfroid** |
| | Department of Veterinary Tropical Diseases |
| | University of Pretoria |
| **Co-supervisors:** | **Prof. Estelle H. Venter** |
| | Department of Veterinary Tropical Diseases |
| | University of Pretoria |


April 2012

© University of Pretoria

## DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of MSc (Veterinary Tropical Diseases) has not been previously submitted by me or anyone for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly cited.

Signature

..........................................................................................

*(Kutlwano Aggrineth Hutamo)*

Pretoria, ___/____/2012

***This dissertation forms part of the requirements for a web-based MSc degree research project in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. These projects carry a weight of approximately 100 credits, and are therefore smaller than projects required for a research-based MSc degree with a weight of 240 credits. It would be appreciated if reviewers could evaluate the dissertation in that context.***

## ACKNOWLEDGEMENTS

Thank God and my ancestors for all the spiritual fulfilment and guidance in my life.

Many thanks to the National Research Foundation and the Department of Veterinary Tropical Diseases, University of Pretoria for providing the financial support necessary for me to complete this degree. Additionally, I would like to thank the Departmental Head and Staff of the Department of Veterinary Tropical Diseases and the Department of Zoology and Entomology for providing support and an environment conducive to the completion of my research.

I would like to thank my supervisors, Professor Jacques Godfroid and Estelle Venter very much. Your invaluable input and support is much appreciated. Thanks also to Professor Armanda Bastos for allowing me to use the facilities of the Molecular Epidemiology and Zoology research programme as well as the vital advice you gave me.

A special thank you to Professor NJ Kriek of the Wildlife Unit, Department of Production Animal Clinical Studies, Faculty of Veterinary Science, for providing me with most of the samples used in my research.

I would like to thank Glen Malherbe, Akin Jenkins, Ayesha Hassim, Anne-Marie Bosman, and Lisma Streicher for all the help and support during my research.

Lastly, I would like to thank my family for their support and understanding throughout my studies, especially my mom, Constance Hutamo who helped me to believe…not forgetting Makale Hutamo, thank you for all the coffee. Tumi I haven't forgotten you either, thanks for putting up with the "slimes".

## TABLE OF CONTENTS PAGE


ACKNOWLEDGEMENTS iii

LIST OF ABBREVIATIONS vi

LIST OF FIGURES vii

LIST OF TABLES x

SUMMARY xii

## CHAPTER 1

**1.1 INTRODUCTION** 1

**1.2 LITERATURE REVIEW** 3

**1.2.1 CLASSIFICATION OF *MYCOBACTERIUM BOVIS*** 3

**1.2.2 TRANSMISSION** 5

**1.2.3 DIAGNOSIS** 6

1.2.3.1 INDIRECT TESTS USED FOR DIAGNOSIS 6

1.2.3.1.1 INTRADERMAL TUBERCULIN (SKIN) TEST 6

1.2.3.1.2 THE GAMMA INTERFERON ASSAY 7

1.2.3.2 DIRECT TESTS USED FOR DIAGNOSIS 7

1.2.3.2.1 NECROPSY 7

1.2.3.2.2 HISTOPATHOLOGY 7

1.2.3.2.3 CULTURE AND PHENOTYPIC IDENTIFICATION 8

1.2.3.2 MOLECULAR (DIRECT) TECHNIQUES USED FOR DIAGNOSIS AND TYPING 9

1.2.3.2.1 IS*6110* TYPING 9

1.2.3.2.2 GENOMIC REGIONS OF DIFFERENCE 10

1.2.3.2.3 SPOLIGOTYPING 11

1.2.3.2.4 VARIABLE NUMBER TANDEM REPEAT TYPING 11

1.2.3.3 DIAGNOSIS OF *MYCOBATERIUM BOVIS* IN FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE 13

**1.3 AIMS OF THE STUDY** 15

## CHAPTER 2

## MATERIALS AND METHODS 16

**2.1 SAMPLES COLLECTED** 16

**2.1.1 SAMPLES FROM BELGIUM** 16

**2.1.2 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES FROM THE KNP** 16

**2.1.3 FRESH TISSUE SAMPLES** 16

**2.2 POLYMERASE CHAIN REACTION** 18

**2.2.1 EXTRACTION OF DNA** 18

2.2.1.1 SAMPLES FROM BELGIUM 18

2.2.1.2 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE 18

2.2.1.3 FRESH TISSUE SAMPLES 19

**2.2.2 MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS** 19

2.2.2.1 OPTIMIZATION OF THE POLYMERASE CHAIN REACTION USING THE SAMPLES FROM BELGIUM 19

2.2.2.2 ANALYSIS OF FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE 19

**2.2.3 GEL ELECTROPHORESIS ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCTS** 20

**2.2.4 SPOLIGOTYPING** 20

2.2.4.1 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE 20

2.2.4.2 FRESH TISSUE SAMPLES 21

**2.2.5 IDENTIFICATION OF *M. BOVIS* ISOLATES BY DELETION ANALYSIS OF THE REGIONS OF DIFFERENCE** 21

# CHAPTER 3

## RESULTS 23

**3.1 POLYMERASE CHAIN REACTION** 23

**3.1.1 EXTRACTION** 24

**3.1.2 MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS AMPLIFICATION** 24

**3.1.3 SPOLIGOTYPING ANALYSIS** 28

**3.1.4 DELETION ANALYSIS OF THE REGIONS OF DIFFERENCE** 30

# CHAPTER 4

## DISCUSSION 33

## REFERENCES 37

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BCG | Bacille Calmette Guérin |
| BTB | Bovine tuberculoses |
| CERVA-CODA | Centre d'Etude et de Recherches Vétérinaires et Agrochimiques |
| DNA | Deoxyribonucleic acid |
| DR | Direct repeat |
| ELISA | Enzyme linked immunosorbent assay |
| ETRs | Exact tandem repeats |
| FFPE | Formalin-fixed paraffin-embedded |
| H & E | Haematoxylin and eosin |
| HiP | Hluhluwe iMfolozi Park |
| HIV | Human Immunodeficiency virus |
| LJ | Lowenstein-Jensen |
| KNP | Kruger National Park |
| $MgCl_2$ | Magnesium chloride |
| MIRUs | Mycobacterial Interspersed Repetitive Units |
| MLVA | Multiple locus variable number tandem repeat analysis |
| MTBC | *Mycobacterium tuberculosis* Complex |
| MTUB | *Mycobacterium tuberculosis* |
| PPD | Purified protein derivative |
| RD | Region of difference |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| SpolDB4 | International Spoligopattern database |
| SICTT | Single Intradermal Comparative Tuberculin Test |
| SIT | Single intradermal test |
| TB | Tuberculosis |
| UV | Ultra violet |
| VNTR | Variable number of tandem repeats |
| WGA | Whole genome amplification |
| ZN | Ziehl Neelsen |

## LIST OF FIGURES


| FIGURE | | PAGE |
|---|---|---|
| **Figure 1**: | The proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes (the distances between certain branches may not correspond to actual phylogenetic differences calculated by other methods). Source: (Brosch *et al.*, 2002) | 4 |
| **Figure 2**: | DNA extraction from FFPE tissue using the QIAamp® DNA mini kit. In lanes 9, 18, 27 and 37 is the Fermentas 100 bp ladder. Lanes 3, 5, 6, 8, 10, 12, 14, 16, 19, 21, 22, 24, 26, 29 and 31 show extracted samples extracted with xylene and Lanes 2, 4, 7, 11, 13, 15, 17, 20, 23, 25, 28, 30 and 32 showed the same samples without the use of xylene. Lanes 1, 33 and 35 were not used. | 23 |
| **Figure 3**: | DNA extraction from fresh tissue obtained from three adult female buffalo. Lanes 1 to 6 show samples 1 to 6 where RNAse was not used. Lanes 7 to 12 show samples 7 to 12 where RNAse was used during extraction. Samples 1, 5, 7 and 11 are from animal B1; samples 2, 3, 8 and 9 are from animal B2; and samples 4, 6, 10 and 12 are from animal B3. | 24 |
| **Figure 4A**: | The ETRC locus was used to differentiate strains. Lane 6 is the 100 bp ladder. | 25 |
| **Figure 4B**: | The Mtub21 locus was used to differentiate strains. Lane 1 is the 100 bp ladder and in Lane 2 is the negative control. | 25 |
| **Figure 5** | MLVA typing of strains obtained from Belgium using the MIRU40 locus to differentiate strains. Lane 1 is the Fermentas 100 bp ladder and in lane 2 is the negative control. | 25 |
| **Figure 6**: | MLVA typing of strains obtained from Belgium (Allix *et al.* 2006) using the ETR-A locus to differentiate strains. In Lane 1 is the 100 bp ladder (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd., South Africa). | 26 |

**Figure 7A**: Lane 1 is the Fermentas 100 bp ladder, Lanes 2 to 7, 9 to 14 are samples 1500-96, 2936-96, 3086-95 (Table 1) (DNA extracted with the QIAamp® DNA mini kit) and samples 268-98, 269-98, 2871-96 (Table 1) (DNA extracted using the boiling method). Each sample was amplified twice, first with extra $MgCl_2$ and then without any extra $MgCl_2$. The samples in Lanes 2 to 7 contain an extra 0.5 µl of $MgCl_2$ (in addition to the $MgCl_2$ in the mastermix). Lanes 8 and 15 contain the positive control *M. bovis* AN5. 26

**Figure 7B**: Lane 1 is the Fermentas 100 bp ladder, lanes 2 to 5 are 3086-95, 268-98, 269-98 and 2871-96 (Table 1) (DNA extracted using the boiling method). These samples contain extra $MgCl_2$. In lane 6 is the positive control. 26

**Figure 8A**: MLVA typing of DNA from FFPE tissue using the Mtub38 locus to differentiate strains. In Lane 1 is the Fermentas 100 bp ladder. In Lane 2 is the negative control and Lane 3 is the positive control for the Mtub38 locus. In Lanes 4 to 16 are samples 3086-95, 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub38 locus). The positive control was loaded at regular intervals after every six samples. 27

**Figure 8B**: MLVA typing of DNA from FFPE tissue using the MIRU40 locus to differentiate strains. In lane 1 is the negative control and Lane 2 is the positive control for the MIRU40 locus. In Lanes 3 to 14 are samples 3086-95, 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (MIRU40). In Lanes 15 and 16 are the negative and positive controls of the Mtub21 locus. In Lane 17 is sample 3086-95 (Mtub21 locus). In Lane 18 is the Fermentas 100 bp ladder. As with amplification of the Belgium samples, amplification of FFPE tissue using the primer based on MIRU40 yielded multiple bands. 27

**Figure 8C**: MLVA continued. In Lane 1 is the Fermentas 100 bp ladder. In Lanes 2 to 13 are samples 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub21 locus). The positive control was loaded at regular intervals after every six samples. 28

**Figure 8D**: MLVA typing of DNA from FFPE tissue using the Mtub02 locus to differentiate strains. In Lanes 1 to 12 are samples 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub02 locus). 28

**Figure 9**: Spoligotyping results of FFPE tissue extracted using the boiling method and the QIAamp® DNA mini kit. In Lanes 1 to 17 is samples 1500-96, 400-98, 981-96, 268-98, 269-98, 1504-96, 269-98, 3086-95, 1006-06, 1009-06, 981-06, 269-98, 1022-06, 3086-96, 2871-96, 268-98 and 977-06. 28

**Figure 10**: Spoligotyping of DNA isolated from fresh tissues. Samples 1, 5, 7 and 11 are from animal B1; samples 2, 3, 8 and 9 are from animal B2; and samples 4, 6, 10 and 12 are from animal B3. The DNA samples from the lymph node tissue of animal B2 yielded unreadable results. 29

**Figure 11**: Deletion analysis of FFPE tissue amplifying RD9. In Lanes 1, 12, 24 and 36 is the Fermentas 100 bp ladder, in Lanes 2 and 3 is the positive control and negative control, in Lanes 4 to 11 is samples 2871-96 (xylene), 2871, 400-98 (xylene), 400-98, 1006-06 (xylene), 1504-96 (xylene), 1504-96, 1009-06 (xylene); Lane 13 is empty; in Lanes 14 to 23 is samples 1022-06 (xylene), 1022-06, 2936-96 (xylene), 2936, 1500-96 (xylene), 1500-96, 2934-96 (xylene), 2934-96, 3086-95 (xylene), 3086-95; and in Lanes 25 to 35 is samples 3086-95 (xylene); 268-98 (xylene), 268-98, 972-06 (xylene), 972-06, 977-06 (xylene), 977-06, 981-06 (xylene), 981-06, 269-98 (xylene) and 269-98. 31

**Figure 12**: Deletion analysis of fresh tissue samples amplifying RD4 and RD9 regions. In Lane 1 is the Bioline Hyperladder II 100 bp marker (Bioline, Celtic Molecular Diagnostics (Pty) Ltd., South Africa), Lane 2 is the negative control, Lane 3 is the positive control for deletion analysis of RD4, Lane 4 is sample 1 of animal B1, Lane 5 is sample 2 of animal B2, Lane 6 is the marker, Lane 7 is sample 1 of animal B1 (no RNAse), Lane 8 and 11 is sample 4 of animal B3, and in Lanes 13 to 17 are the results for the deletion analysis amplifying RD9. The samples are 1 (B1), 2 (B2), 3 (B2), 4 (B3), and 6 (B3). 31

# LIST OF TABLES


| TABLE | | PAGE |
|---|---|---|
| **Table 1:** | FFPE in full samples manifesting typical BTB infection when using histology | 17 |
| **Table 2:** | List of primers used for *M. bovis* strain typing | 22 |
| **Table 3:** | Spoligopatterns of strains obtained from fresh tissue of three adult buffalos | 30 |
| **Table 4:** | Summary of the results obtained for typing of *M. bovis* strains using different PCR-based techniques | 32 |

# SUMMARY

Typing of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues from selected wildlife species in the Kruger National Park, South Africa

**By**

**Kutlwano A. Hutamo**

**Supervisor:** Prof. J. Godfroid

**Co-supervisors:** Prof. Estelle H. Venter

**Department:** Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

**Degree:** MSc (Veterinary Tropical Diseases)

## SUMMARY

*Mycobaterium bovis* is the causative agent of bovine tuberculosis (BTB) and it is a member of the *Mycobacterium* tuberculosis complex (MTBC). This bacterium has a wide host range of which, cattle is considered as the maintenance host. Humans, goats, wildlife, cats, dogs and lions are also susceptible to the bacterium and are considered putative spillover hosts as infection is not confined in these hosts. *Mycobacterium bovis* is prevalent in developing countries especially in farmed animals. This presents a problem since BTB is a zoonosis. People living in close contact with infected cattle or those who drink unpasteurized milk are at risk of infection. About 10% of cases of human tuberculosis are thought to be caused by *M. bovis*. In some instances, wildlife provides a reservoir for the pathogen and transmits it to cattle in farms and poses further risk to humans at the wildlife/livestock/human interface. Certain countries like the United Kingdom where BTB was previously eradicated are experiencing substantial increase in BTB infection. This is thought to be a result of wildlife reservoirs that infect farmed animals, especially cattle. Such reservoirs make eradication of the disease extremely difficult and require programmes to be put in place to control spread of the disease. This makes *M. bovis* a pathogen of economic importance since the programmes may be costly. In addition, wildlife that is infected cannot be exported and this further affects the economy negatively.

In order to control the spread of the pathogen, it is essential to determine the source of infection. However, it is difficult to determine the source or to track the spread of BTB especially in wildlife where animals have unrestricted movement. The inability to conduct epidemiological studies of BTB may be a result of the lack of molecular typing methods that allow bacteria to be identified to strain level rapidly and fairly simpler than culture, thus providing much needed information about the pathogen. In recent years, typing of *M. bovis* isolates to strain level has been made possible by the development of PCR-based technologies such as *IS6110* typing and spoligotyping. These technologies were however, found to be unsuitable for differentiating certain species in the MTBC. Newer technologies based on the variable number of tandem repeats (VNTRs) in organisms have been developed and allow for the differentiation of members in the MTBC, which have a high level of genome homology. These technologies include multiple-locus variable number tandem repeat analysis (MLVA) and mycobacterial interspersed repetitive unit (MIRU)-VNTR analysis. It was also discovered that mycobacteria have genomic regions of difference (RD) that could be used to identify the different species of bacteria in the MTBC.

Retrospective studies may play a key role in tracing the source of diseases and following the pattern of transmission. However, in most instances, no fresh samples are available for such studies. For this reason, formalin-fixed paraffin-embedded (FFPE) tissue from wildlife in the Kruger National Park (KNP) was used for conducting a retrospective study aimed at determining the epidemiology of *M. bovis* in the KNP. However, amplification of DNA derived from FFPE tissue for PCR based techniques has been found to be a difficult exercise and not many standard protocols have been developed and validated for the use of such DNA. In this study, different methods of extraction were used to obtain DNA from FFPE tissue since it is difficult to obtain high quality DNA from such tissue, which is degraded. Formaldehyde, the main component of formalin which is used to fix tissue samples, causes degradation and cross-linking of DNA. In addition, previous studies are inconsistent with regards to the best method to use when extracting DNA from FFPE tissue.

Three PCR-based techniques were used to type or identify the isolates in order to standardize a protocol for use in typing isolates from FFPE tissue. These techniques included analysis of the RDs, VNTR based methods i.e. MLVA and MIRU-VNTR and spoligotyping. Since there are many factors that influence the quality of FFPE tissue, samples confirmed BTB positive by VNTR analysis, spoligotyping and *IS6110* analysis were used in order to optimize a PCR for FFPE tissue. Furthermore, in order to serve as control samples for spoligotyping and analysis of the RDs, DNA obtained from fresh tissue was also used in the study.

Despite the various methods used to extract and to type DNA, the DNA from FFPE tissue provided unspecific results that did not allow for an informative retrospective study of *M. bovis*. This may be due to the fact that the DNA used had a high degree of degradation from prolonged fixation in formalin. Although *M. bovis* could not be typed in FFPE tissues, it could be identified by analysis of the regions of difference, more specifically the RD9 region. Amplification of RD9 is thus recommended for use in retrospective studies for diagnostic purposes, especially in cases where highly degraded DNA is used. This region (RD9) should however, only be used as a presumptive diagnosis since RD9 also identifies *M. africanum, M. microti, M. pinnipedii, M. caprrae* and *M. bovis BCG*. However, RD9 specifically excludes *M. tuberculosis*. In the SA context, particularly in the KNP, this allows for some sound inferences since the animals are likely to be infected with *M. bovis* as opposed to *M. tuberculosis*.

This study highlighted statements in previous studies where it was stated that fixation of tissue in formalin should be done in such a way to reduce degradation of DNA in FFPE

tissue in order to allow for its use in retrospective molecular studies which may be very insightful in determining the epidemiology of diseases that are difficult to track and/or control.

# CHAPTER 1

## 1.1 INTRODUCTION

Tuberculosis is a clinical or pathological diagnosis that refers to the clinical signs caused by infection with bacteria of the *Mycobacterium tuberculosis* complex (MTBC) (de la Rua-Domenech *et al.*, 2006).This complex comprises of numerous species, some of which are economically important pathogens such as *M. bovis*, *M. bovis* BCG, *M. tuberculosis*, *M. africanum*, *M. microti*, *and M. bovis* subspecies *caprae* (Haddad *et al.*, 2004). These strains are mostly host-adapted to animals and were found to form a nested lineage characterized by the absence of a specific chromosomal region known as RD9 (Brosch *et al.*, 2002, Smith *et al.*, 2006). An antelope clade associated with known oryx strains was further identified (Smith *et al.*, 2006).

Although *M. bovis* is predominantly host-adapted to animals, it has a very broad host range, which includes humans. The bacterium can sustain itself by transmission between the same species (de Lisle *et al.*, 2002). Species in which the bacterium sustains itself are called maintenance hosts, an example of which is the African buffalo (*Syncerus caffer)(*de Lisle *et al.,* 2002*)*. In some instances the bacterium infects other species that do not sustain the infection within their population. Such species are referred to as spill-over hosts and include kudu (*Tragelaphus strepsiceros*), eland (*Taurotragus oryx*), impala (*Aepyceros melampus*), lion (*Panthera leo*) and Chacma baboon (*Papio ursinus*) amongst others (de Lisle *et al.*, 2002, Amanfu, 2006). The fact that *M. bovis* has such a wide host range contributes greatly to its survival and spread (Allix *et al.*, 2006).

*Mycobacterium bovis* causes bovine tuberculosis (BTB) in cattle and it is a serious zoonosis in developing countries (de la Rua-Domenech *et al.*, 2006). People living in close proximity to game parks affected by BTB are prone to infection via inhalation and drinking unpasteurised milk. This is due to the fact that cattle grazing in close proximity to infected buffalo may be infected via aerosols and the infected cattle may then transmit the disease to farmers (Michel *et al.*, 2006). Humans are considered as putative spillover hosts since infection is not confined in these hosts (Allix *et al.*, 2006). Infection rates in people could be exacerbated by the high number of HIV infected individuals in developing countries (Amanfu, 2006), who are immunocompromised and are more susceptible to infection than others (Raviglione *et al.*, 1995). A study conducted in South Africa has shown that some *M. tuberculosis* infected patients may have multiple infections as different strains were isolated from the sputum of a single patient (Warren *et al.*, 2004). This may also be true for buffalo in the Kruger National Park (KNP) as no control programme exists in the KNP and infected buffalo are not treated. It is thus possible that a single buffalo may be infected by different strains of *M. bovis* (Michel *et al.*, 2006).

Infection of game by *M. bovis* results in major economic losses in the KNP and Hluhluwe iMfolozi Park (HiP) due to movement restrictions (Michel *et al.*, 2006). This in turn influences the country's economy adversely due to decreases in livestock production and trade restrictions (Hilty *et al.*, 2005).

Apart from having an adverse effect on the country's economy, BTB is also a threat to the country's biodiversity. The KNP is home to a large number of wildlife species including 147 mammal species and as a result it is a very important biodiversity resource (Michel *et al.*, 2006). However, over the years, the incidence of *M. bovis* has increased significantly in the KNP and the organism has been isolated in more than ten species in the KNP alone (Michel *et al.*, 2006). In 1992 the prevalence of *M. bovis* in buffalo was 27.1% in the southern part of the KNP, 4.4% in the central part, and 0% in the northern part (Michel *et al.*, 2006). The prevalence has been increasing substantially over the years. In 1998 the prevalence of BTB was estimated at approximately 1.5%, 16%, and 38.2% in the north, central, and south zones, respectively (Rodwell *et al.*, 2001). Bovine tuberculosis is spreading northward from its initial introduction in the southern border of the KNP and buffalo maintain BTB at a high prevalence (from about 60% to 92% in some herds) (Cross and Getz, 2006, Montali, 2007). The spread was estimated at 6 km per year in 2001 and it was proposed then that if the spread is maintained at this rate, the entire KNP may be affected in less than 30 years (Montali, 2007, De Vos *et al.*, 2001).

The Hluhluwe iMfolozi Park (HiP) is the third largest game reserve in the country and covers a substantial area of KwaZulu-Natal and comprises a buffalo population of about 3000. This game reserve is completely surrounded by communal farm land (Michel *et al.*, 2006). Species that are often implicated in disease outbreaks at the wildlife/livestock interface are those from the ungulate group, more specifically from the family *Bovidae.* These species include buffalo, cattle and bovine antelope (Kock, 2004). Buffalo have been sighted grazing in close proximity to cattle from the communal farmlands surrounding HiP (Michel *et al.*, 2006).

In 1999, a programme aimed at reducing the prevalence of BTB to below 10% in the HiP was launched. This programme includes the mass capture of buffalo which are then tested for the presence of *M. bovis* using the skin test (Michel *et al.*, 2006). This has reduced BTB prevalence in individual herds significantly (Michel *et al.*, 2006). It is essential to confirm infection in animals via laboratory diagnosis, which, when used in combination with molecular identification provides a robust tool for tracking the transmission of *M. bovis* within species and between different species (Michel, 2002). A study of interspecies transmission is especially critical in cases where endemically infected wildlife is suspected of providing a reservoir for domestic animals (Allix *et al.*, 2006).

Bovine tuberculosis is a chronic disease and can remain asymptomatic for long periods and infected cattle can transmit the disease long before manifestation of symptoms (Paylor, 2007). The lack of appropriate tests that can detect BTB in its early stages (especially in asymptomatic carriers) can result in infection of a large number of wildlife in herds. It is essential to trace the origin of infection in order to track the spread of disease and to inhibit transmission of the disease in animals. In most cases, fresh or frozen tissue is not available for conducting epidemiological studies and other sources of DNA must be used. Formalin-fixed paraffin-embedded (FFPE) tissue can be a powerful tool for the retrospective study of diseases because paraffin blocks have been collected for over a century and as a result provide a history of numerous diseases (Greer *et al.*, 1994, Michalik, 2008).

Therefore the aim of this study was to optimize a PCR-based technique which would facilitate a retrospective study aimed at detecting and typing strains of *M. bovis* from FFPE tissue originating from wildlife.

## 1.2 LITERATURE REVIEW

### 1.2.1 CLASSIFICATION OF *MYCOBACTERIUM BOVIS*

The causative agent of BTB, *M. bovis*, is a Gram-positive irregular non-spore forming obligate parasite which has been demonstrated to have the ability to survive for significant periods in the environment under suitable conditions (Morris *et al.*, 1994, van Soolingen, 2001). *Mycobacterium bovis* is considered an acid-fast bacillus because, like other mycobacterial species, it is impermeable by certain dyes and stains. *Mycobacterium bovis* is acid-fast stained using the Ziehl-Neelsen (ZN) stain (Todar, 2008). When subjected to acid-fast staining, mycobacteria, if present in the sample, take up the stain and appear as red or pink rods when viewed under the microscope (Todar, 2008).

Mycobacteria are divided into two groups, MTBC and non-MTBC (atypical) (van Soolingen, 2001). The species belonging to the non-tuberculous mycobacteria group are more genetically heterogenous than the MTBC mycobacteria (van Soolingen, 2001).

Bacteria in the MTBC have a very high conservation of housekeeping genes and have 99.9% homology at the nucleotide level as well as identical 16S RNA sequences (Boddinghaus *et al.*, 1990, Sreevatsan *et al.*, 1997, Le Fleche *et al.*, 2001, Le Fleche *et al.*, 2002, Pourcel *et al.*, 2004). Due to the high level of homology in the species of the MTBC, it was suggested that polymorphism in the variable number tandem repeats (VNTR's) could be the main source of phenotypic variation in members of the MTBC. Tandem repeats are powerful genetic markers as some have multiple alleles at a single locus (Lindstedt, 2005). Size differences between species and strains are also easily resolved through the use of electrophoresis (Le

Fleche *et al.*, 2006). Tandem repeats have been found to be highly efficient for classifying pathogenic bacteria with very high sequence homology (Le Fleche *et al.*, 2001, Le Fleche *et al.*, 2002, Pourcel *et al.*, 2004).

The high level of homology might be a result of an evolutionary bottleneck that mycobacteria in the MTBC underwent about 15 000 to 20 000 years ago (Sreevatsan *et al.*, 1997). It was originally proposed that *M. tuberculosis* evolved from *M. bovis*. However, the use of comparative genomics techniques such as subtractive hybridization, bacterial artificial chromosome arrays or DNA microarrays using the DNA sequence of *M. tuberculosis* H37Rv has allowed researchers to identify sixteen regions of difference (RD 1 to RD 16), deleted in some members of the MTBC except *M. tuberculosis* (Parsons *et al.*, 2002). These RDs, ranging in size from 2 to 12.7 kb, represent the loss of genetic material in some members of MTBC compared to *M. tuberculosis* (Huard *et al.*, 2003).

The loss of genetic material in *M. bovis* appears to suggest that it is the last member of the *M. africanum* lineage to have branched from progenitor *M. tuberculosis* isolates as illustrated in Figure 1 (Brosch *et al.*, 2002).

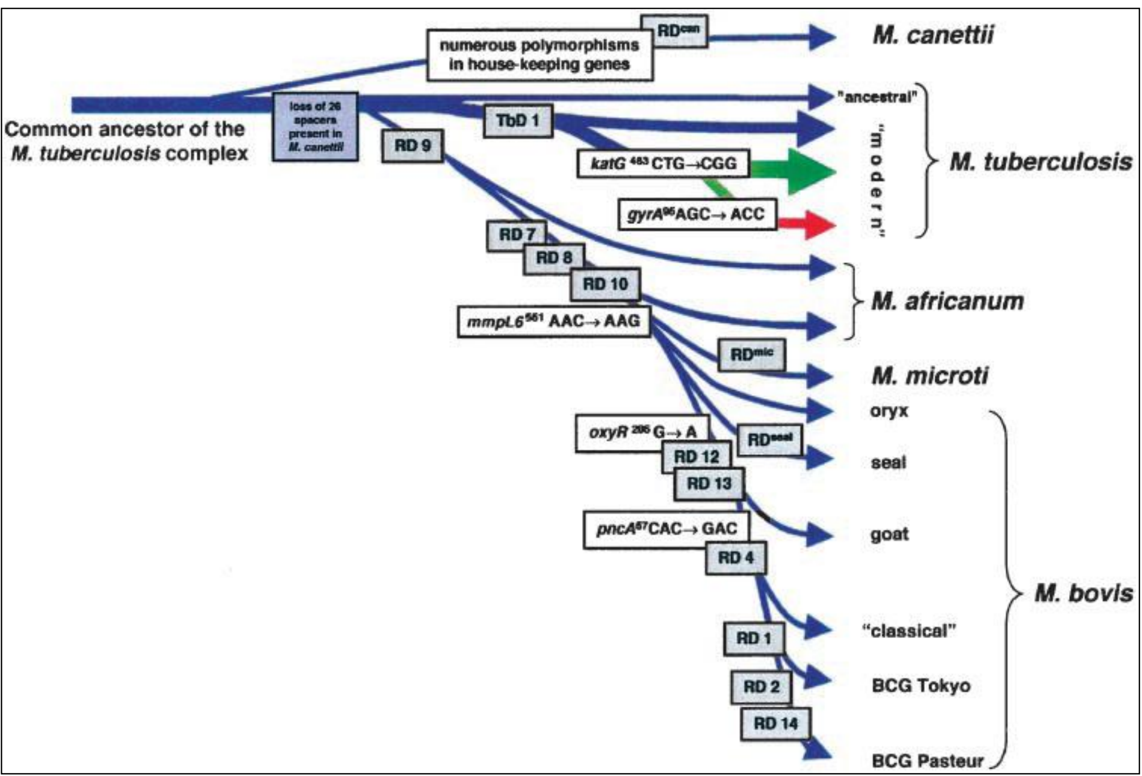



**Figure 1**: The proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes (the distances between certain branches may not correspond to actual phylogenetic differences calculated by other methods). Source: (Brosch *et al.*, 2002)

### 1.2.2 TRANSMISSION

Under optimal conditions, the different animal species in KNP and other game parks live in spatially discrete small family groups or in larger herds. Animals in these herds maintain contact but they may occasionally separate (Prins, 1987). A herd may come into close contact with other herds depending on social and environmental factors such as rain, drought, breeding and migration, thus promoting the spread of the disease within and between herds of the same species (Kock, 2004).

Although rare, herds of different species may also come into close contact with each other, especially during adverse conditions such as drought (Kock, 2004). In these adverse conditions, herds of different species come into close contact and although they may be a few feet apart, aerosol borne infections such as BTB can be transmitted at such distances via grazing, water and bodily fluids (Kock, 2004). A retrospective study suggested that cattle, at barriers between communal farm land and the south east corner of the KNP, may have transmitted *M. bovis* to African buffalo (*Synceruscaffer*) between 1950 and 1960 (Kloeck, 1998). The Crocodile River forms a natural barrier between the KNP and a farmland area adjacent to the southern part of the KNP. However, the presence of cattle grazing in close proximity to buffalo in the southern part of the KNP is not uncommon (Michel *et al.*, 2006). These cattle are suspected to have transmitted *M. bovis* to the buffalo and infection has most probably occurred via aerosols (Michel *et al.*, 2006).

Transmission of *M. bovis* via the aerosol route occurs most frequently between herd members, and spillover to other species occurs via different means of transmission (Michel *et al.*, 2007). Lions may be infected by feeding on infected buffalo carcasses (Michel *et al.*, 2006). Pus secreted from draining fistulae of parotid lymph glands was suggested to be the mode of transmission in the greater kudu (Michel *et al.*, 2007). Once BTB is established in a herd, it can be spread via aerosols, contact between animals and sharing of feed.

*Mycobacterium bovis* can be isolated for about six weeks from carcasses and four weeks from buffalo faecal matter, depending on weather conditions (Tanner and Michel, 1999). Heavy rains can also disperse *M. bovis* from game parks to grazing sites for domestic cattle, thus making the cattle more susceptible to infection from wildlife in the neighbouring game parks (Tanner and Michel, 1999).

Michel *et al.* (2007) established that shedding of *M. bovis* in nasal and oral discharges of African buffalo is not common (perhaps more particularly in animals with clinical signs or animals with low bacterial loads). This finding suggests that under free-ranging conditions, buffalo pose a very low risk of transmitting *M. bovis* via water.

### 1.2.3 DIAGNOSIS

Identification of *M. bovis* is based mostly on physiochemical and biological characteristics (Amanfu, 2006). The bacterium is difficult to isolate from humans, resulting in poor diagnosis in most instances and an underestimation of disease incidence (Amanfu, 2006). A few tests have been developed for the detection of mycobacteria in cattle, some of which have been adapted for use in wildlife. Some of these tests can be used prior to clinical manifestation (Adams, 2001) and include: The enzyme linked immunosorbent assay (ELISA), *in vivo* intradermal tuberculin (skin) tests, the gamma interferon assay, necropsy, histopathology and mycobacterial culture (de la Rua-Domenech *et al.*, 2006).

#### <u>1.2.3.1 INDIRECT TESTS USED FOR DIAGNOSIS</u>

##### 1.2.3.1.1 INTRADERMAL TUBERCULIN (SKIN) TEST

The skin tests is the gold standard for the ante mortem diagnosis of BTB in cattle and it is preferred to other available tests due to the ease with which it is executed as well as its affordability (de la Rua-Domenech *et al.*, 2006). The simplest of the skin tests is the single intradermal test (SIT), however, the single intradermal comparative tuberculin test (SICTT), which although time consuming, is more discriminatory than the SIT. The SICTT is able to differentiate *M. bovis* from other mycobacteria (de la Rua-Domenech *et al.*, 2006).This test involves shaving two sites on opposite sides of the animal's neck followed by measuring of the thickness of the neck on both sites. A certain volume of bovine purified protein derivative (PPD) is then injected intradermally in the centre of the left shaved site and a certain volume of avian PPD is injected on the right side of the neck using a McClintock syringe (de la Rua-Domenech *et al.*, 2006). If an animal is not sensitised to tuberculin antigens, then no significant local inflammatory response will be observed when PPD is injected into the animal.

However, should an animal be sensitised to tuberculin by *M. bovis* infection, an inflammatory response is triggered and swelling at the injection site is observed (de la Rua-Domenech *et al.*, 2006). The skin at each site is then examined and measured after 48 to 72 hours which is the period within which the inflammatory response reaches its greatest intensity. The advantages of this test are that it is an old, well-established diagnostic method, that it can be used in several species, that it can be used in live animals, and that it has good sensitivity and specificity (de la Rua-Domenech *et al.*, 2006). The drawbacks are that there is a long interval between test and result, that it is expensive (more so in wildlife, as these animals need to be immobilized twice), and that this test is unlikely to detect super shedders with poor cellular immunity (anergic animals) (de la Rua-Domenech *et al.*, 2006).

#### 1.2.3.1.2 THE GAMMA INTERFERON ASSAY

With the gamma interferon (IFNγ) assay cell-mediated immunity in cattle is measured. The principle is that animals infected with *M. bovis* have lymphocytes in the blood that detect mycobacterial antigens specific to bovine PPD (de la Rua-Domenech *et al.*, 2006). Compared to the skin test, the period between test and result is shorter, being about 36 hours. This assay can be used in live animals and it has good sensitivity and specificity.

Sensitisation of animals by environmental mycobacteria was found to cause false positive results in free-ranging buffalo when using the standard IFNγ protocol (Michel, 2008). In order to circumvent this, the IFNγ assay was modified to include IFNγ produced in response to sensitin from *M. fortuitum* (fortuitum PPD) (Michel *et al.*, 2011). In addition to the use of bovine and avian tuberculin PPD, fortuitum PPD is used in this modified protocol. The modified IFNγ assay may help in the detection of non-specific sensitisation of free-ranging buffalo and improve test specificity in BTB negative herds (Michel *et al.*, 2011). Improved test specificity may reduce the unnecessary culling of false positive animals which is disadvantageous financially and ethically objectionable (Michel *et al.*, 2011). The disadvantages of this assay are that it is expensive and it is also unlikely to detect anergic animals (de la Rua-Domenech *et al.*, 2006).

### 1.2.3.2 DIRECT TESTS USED FOR DIAGNOSIS

#### 1.2.3.2.1 NECROPSY

This test is performed on animals that have been euthanized, killed at an abattoir or died as a result of a disease (i.e. the carcass is submitted for necroscopy) (Corner, 1994). Different parts of the animal are then examined for common signs of BTB infection like lesions and granulomas (Corner, 1994). *Mycobacterium bovis* forms characteristic lesions manifested in different forms in different animals. However, there are some bacteria that may sometimes form lesions that are very similar to those formed by *M. bovis*. These bacteria may then be wrongly diagnosed as *M. bovis* (de Lisle *et al.*, 2002). An example is the discovery of *M. bovis*-like lesions in buffalo which were later found to be caused by *M. fortuitum* (de Lisle *et al.*, 2002). Following the detection of macro lesions, histopathology is used to establish a diagnosis (de Lisle *et al.*, 2002).

#### 1.2.3.2.2 HISTOPATHOLOGY

Tissue samples are collected and stored in formalin (even when there are no gross lesions) and embedded in paraffin wax (Liebana *et al.*, 2008). Thin sections are then cut from the tissue embedded in formalin and stained using haematoxylin and eosin (H & E) and ZN methods (Rohonczy *et al.*, 1996). After processing of the samples, a diagnosis is made based on the histological changes of cells caused by infection of *M. bovis*. Some of these characteristics in cattle include central necrosis with mineralization surrounded by a granulomatous inflammatory response of macrophages and an outer walling off with fibrous

connective tissue (de Lisle *et al.*, 2002). The histological appearances may differ in different species and different mycobacteria may be histologically indistinguishable (de Lisle *et al.*, 2002). Animals at the early stages of infection may not have any visible lesions, thus resulting in false negatives since they are mostly classified as negative (Rohonczy *et al.*, 1996). Live animals are not used - the animal is either dead or must be killed in order to perform this test.

Histopathology can be used to make a presumptive diagnosis of *M. bovis* infection. This is advantageous in that it may be used to circumvent the long delay in making a diagnosis based on bacterial culture (Corner, 1994). Generally, the outcomes that can occur when conducting a histopathology diagnosis are as follows:

(a) Tissues may not have any lesions or acid-fast organisms associated with *M. bovis;*
(b) Tissues may have *M. bovis* associated lesions, but acid-fast organisms are absent; and
(c) Tissues may have *M. bovis* associated lesions and acid-fast organisms are present (Durmaz *et al.*, 1997, Miller *et al.*, 2002).

Outcome (b) may be due to the fact that other bacteria can cause lesions similar to those associated with *M. bovis* in ruminants (Miller *et al.*, 2002). Another cause of outcome (b) may be attributed to the fact that acid-fast bacilli occur in low numbers in tissues and may not be detected (Durmaz *et al.*, 1997).

Histopathology has a low sensitivity and provides no information on the mycobaterial strains causing TB. It is therefore necessary to conduct further tests that provide a definitive diagnosis (Miller *et al.*, 2002, Warren *et al.*, 2006). A definitive diagnosis requires the isolation of *M. bovis*. Usually this is achieved by culture, which is a lengthy process and may be disadvantageous for disease control. Consequently, more rapid and sensitive molecular based tests have been developed to identify *M. bovis* and distinguish it from other species in the MTBC.

#### 1.2.3.2.3 CULTURE AND PHENOTYPIC IDENTIFICATION

Culturing of mycobacteria is still routinely practiced in diagnostic laboratories (Amanfu, 2006). It involves the culturing of processed lesions in Lowenstein-Jensen (LJ) egg-based medium with glycerine, LJ medium without glycerine and LJ medium with 0.5% pyruvate (Corner, 1994). If any colonies are observed, they are sub-cultured onto the same medium that supported the initial growth (Corner, 1994). The isolates are then identified by growth characteristics such as preference for LJ medium with pyruvate (Corner, 1994). The key difference between *M. bovis* and *M. tuberculosis* is that *M. bovis* requires addition of pyruvate to the growth media, reflecting a defective glycerol metabolism (Hewinson *et al.*, 2006).

Due to the fact that identification of MTBC bacteria based on their growth characteristics on egg-based media is subjective and often unreliable, biochemical tests have been used as well in the speciation of MTBC bacteria (Grange *et al.*, 1996). The biochemical tests comprise nitratase activity, oxygen preference (aerobic or micro-aerophilic), susceptibility to thiophen-2-caboxylic acid hydrazide (the isoniazid analogue) and susceptibility to pyrazinamide (PZA) or the pyrazinamidase test (Grange *et al.*, 1996). Culture and phenotypic identification can detect *M. bovis* in an animal at the early stages of infection even though the animal tested negative in all other tests. However, mycobacterial culture takes up to three months before final results are available since the organism may take about one to two months to grow (Kamerbeek *et al.*, 1997).

Mycobacteria have a very high level of homology, thus making identification at the strain level very difficult. Many methods have been developed to ameliorate this difficulty. Typing of mycobacterial strains using most of these methods relies on isolation of *M. bovis* DNA from culture. With the advent of molecular techniques, isolation of DNA from culture is no longer necessary for strain typing. Typing has become possible directly from tissue, body fluids and blood, thus reducing the time within which mycobacteria can be identified or typed (Boddinghaus *et al.*, 1990, Sreevatsan *et al.*, 1997, Le Fleche *et al.*, 2001, Le Fleche *et al.*, 2002, Pourcel *et al.*, 2004).

Typing forms of bacteria can either be pathotyping or epityping (van Belkum, 2008). Pathotyping is largely concerned with the pathogenic potential of microorganisms and relies on the differences in genomic content of the organisms to differentially analyze each organism's pathogenic potential (van Belkum, 2008). Epityping, also known as strain fingerprinting, is used for tracing the spread of bacterial lineages (van Belkum, 2008). The availability of whole genome sequences of most microorganisms has led to the development of a plurality of new epityping techniques. Some of the frequently used techniques which include insertion element IS*6110* typing, VNTR typing and spoligotyping, are discussed below.

### 1.2.3.2 MOLECULAR (DIRECT) TECHNIQUES USED FOR DIAGNOSIS AND TYPING

#### 1.2.3.2.1 IS*6110* TYPING

The mobile insertion element IS*6110* is, when used as a hybridization probe, able to differentiate between different restriction fragment length polymorphism (RFLP) patterns of the various mycobacteria in the MTBC (Razanamparany *et al.*, 2006). Since the IS*6110* insertion element is present in variable copy numbers in MTBC bacteria, it is used to identify species within the complex (Razanamparany *et al.*, 2006). This highly discriminatory method yields stable and reproducible results and has been used successfully to type strains of *M. bovis* and *M. tuberculosis* in samples with high copy numbers and can be used to trace outbreaks (Frothingham and Meeker-O'Connell, 1998). This technique was the golden

standard for the molecular typing of MTBC species. It is, however, not an ideal method for typing strains of *M. bovis* and *M. tuberculosis* in samples with no or low copy numbers of the IS*6110* insertion element (van Embden *et al.*, 1993, Frothingham and Meeker-O'Connell, 1998). Consequently, the use of other typing techniques which can differentiate such isolates is necessary.

In addition to having very low discriminatory power in isolates having low copy numbers of IS*6110*, this method also presents a few other difficulties. Firstly, prior cultivation of isolates is necessary before IS*6110* typing can be carried out and this causes a delay in results since the growth of *M. bovis* may take from one to two months (Kamerbeek *et al.*, 1997). The handling of live organisms increases the risk of infection of laboratory workers. Secondly, IS*6110* typing is relatively expensive (Hilty *et al.*, 2005) and may thus be inappropriate for use in developing countries with financial constraints. Lastly, IS*6110* typing is labor intensive and requires skilled laboratory personnel for culturing and analysis of results (Hilty *et al.*, 2005). This may pose a problem in developing countries, where in most cases, skilled workers are a scarcity.

#### 1.2.3.2.2 GENOMIC REGIONS OF DIFFERENCE

Chromosomal evolution of species in the MTBC have been largely driven by deletions in their genomes (Rao *et al.*, 2005). It has been suggested that the causes for such deletions include recombination between adjacent insertion sequence (IS) elements and strand slippage errors of DNA polymerase amongst others (Rao *et al.*, 2005). *Mycobacterium tuberculosis* has about 40 ISs and mobile genetic elements that could facilitate such deletions (Cole *et al.*, 1998).

The region of difference (RD) patterns of bacteria in the MTBC have been exploited as an identification tool for differentiating the species of the complex (Huard *et al.*, 2003). A PCR based identification technique was developed using six RD’s. These regions include RD 1, which is absent in *M. bovis* BCG; RD 5, which is absent in *M. bovis* and *M. bovis* BCG; RD 3 and RD 11, which allow differentiation of *M. bovis* and *M. microti*; and RD 9 and RD 10, which are absent in *M. africanum* ( Huard *et al.*, 2003).

More recently, Warren *et al.* (2006) developed a two-step multiplex technique based on RD 1, RD $1^{mic}$, RD $2^{seal}$, RD 4, RD 9 and RD 12. The first primer set (primer set 1) included RD 1, RD 4, RD 9 and RD 12; the second primer set (primer set 2) included RD $1^{mic}$ and RD $2^{seal}$ primers. Primer set 1 differentiates between *M. canettii*, *M. tuberculosis*, *M. caprae*, *M. bovis* and *M. bovis* BCG. Primer set 2 differentiates between *M. africanum*, *M. microti*, and *M. pinnipedii*. Due to the fact that amplification of non-MTBC DNA does not generate the desired bands, this technique is unique to species of the MTBC complex only (Warren *et al.*, 2006).

#### 1.2.3.2.3 SPOLIGOTYPING

Spacer oligonucleotide typing (spoligotyping) is a PCR-based method used to type bacteria in the MTBC (Kamerbeek *et al.*, 1997). Spoligotyping constitutes a reverse hybridization technique which is based on polymorphism at a single locus known as the direct repeat (DR) locus (Kamerbeek *et al.*, 1997). The DR locus, which is present in MTBC bacterial species, consists of a series of well conserved DR sequences, which are 36-bp long (Song *et al.*, 2007, Hilty *et al.*, 2005). The DR sequences are separated by unique, non-repetitive spacer sequences which are between 35 to 41 bp in size (Kamerbeek *et al.*, 1997, Mathuria *et al.*, 2008). One or more spacer sequences may be present in one isolate, but absent in another, this variation forms polymorphism in the DR locus (Haddad *et al.*, 2004). Previous studies have indicated that spoligotypes of *M. bovis* lack spacers 3, 9, 16 and 39 to 43, the absence of spacers 39 to 43 makes it possible to distinguish *M. bovis* strains from *M. tuberculosis* (Milian-Suazo *et al.*, 2008, Razanamparany *et al.*, 2006, Romero *et al.*, 2008).

Spoligotyping, unlike IS*6110* typing, is easy to perform and does not require prior cultivation of isolates, thus reducing the risk of infection to laboratory workers (Hilty *et al.*, 2005). Minimal amounts of DNA are required for spoligotyping, thus isolates with no or low copy numbers of IS*6110* may be differentiated using this method (Song *et al.*, 2007). An international spoligotype database (http://www.mbovid.org) has been established and it allows rapid comparison of spoligotyping results between laboratories (Duarte *et al.*, 2008).

The disadvantage of spoligotyping is that it shows variation at a single locus, which reduces its discriminatory power (Kamerbeek *et al.*, 1997). As a result, a number of studies have proposed that spoligotyping be associated with other PCR-based techniques, for example MLVA targeting, VNTR's at multiple loci such as exact tandem repeat (ETR) loci and the mycobacterial interspersed repetitive units (MIRU) loci for clinical practice applications or epidemiological studies (David *et al.*, 2007, Gori *et al.*, 2005). In the United States of America, MIRU-VNTR associated with spoligotyping has been implemented as a first-line approach for typing strains of *M. tuberculosis* (Maes *et al.*, 2008).

#### 1.2.3.2.4 VARIABLE NUMBER TANDEM REPEAT TYPING

Minisatellites (repeats range from 10-100 bp) and microsatellites (repeats range from 1-13 bp) are simple sequence repeats (SSR) and are also known as VNTRs due to the hypervariability in the repeat numbers in organisms (Tautz and Renz, 1984). These tandem repeat motifs occur in coding and noncoding regions in both eukaryotes and prokaryotes (Zane *et al.*, 2002). Due to their high degree of length polymorphism, VNTRs have been used as powerful genetic markers, permitting fingerprinting and paternity testing in humans for decades (Frothingham and Meeker-O'Connell, 1998). Variable number tandem repeat loci were discovered in bacteria by Andersen *et al.* (1996) and were used to type several bacterial pathogenic species with very high homology, such species include *Bacillus anthracis*,

*Yersinia pestis*, *Brucella* and species within the MTBC (Andersen *et al.*, 1996, Le Fleche *et al.*, 2001, Le Fleche *et al.*, 2002, Le Fleche *et al.*, 2006, Pourcel *et al.*, 2004).

Regions containing VNTR's are firstly amplified using different primer sets corresponding to a specific locus or several loci (Haddad *et al.*, 2004). The differences in amplicon length are then determined, such differences can be translated into differences in the number of repeat units present at each locus (van Belkum, 2008). The VNTRs present may be characterized by using agarose gel electrophoresis (AE). Alternately, multiplex PCRs can also be visualized simultaneously using an automated technique that makes use of a fluorescent-based DNA analyzer coupled to a computerized automation of each genotype (Haddad *et al.*, 2004). This technique is known as capillary electrophoresis (CE). The number of repeats present in each locus is determined by the size of the band visualized (Haddad *et al.*, 2004).

Although previous studies suggested that AE was less reliable and less sensitive than CE when typing isolates from the MTBC, Jenkins *et al.*, (2010) conducted a study which showed that there were no differences in copy numbers observed when using the AE VNTR analysis technique and the CE VNTR analysis technique. Furthermore, sensitivity of VNTR typing was not compromised by use of AE analysis (Jenkins *et al.,* 2010). The use of the AE analysis technique is ideal for laboratories with limited finances since the CE analysis technique is more costly.

A technology, known as MLVA, allowing the observation of VNTR's at multiple repeat loci has been developed (van Belkum, 2008). Some loci containing VNTR's include the ETR loci, MIRU loci and the Queen's University Belfast (QUB) novel loci amongst others.

Frothingham and Meeker O'Conell (1998) identified five ETR loci (ETR-A, -B, -C, -D, -E and -F) by searching *M. tuberculosis* cosmid sequence data whilst one ETR locus was identified in a previous study. Exact tandem repeats contain large tandem repeats which are unique to each locus and repeat units vary in size from about 53 to 79 bp (Frothingham and Meeker-O'Connell, 1998). Of all six ETR loci, ETR-A and ETR-B were found to be the most discriminatory loci (Roring *et al.*, 2004).

Mycobacterial species in the MTBC have MIRUs which are distributed throughout the genome either as single copies or in multiple tandem repeats (Neonakis *et al.*, 2008, Bull *et al.*, 2003b). Mycobacterial interspersed repetitive units are known as minisatellite sequences since they are about 40 to 101 bp in size (Bull *et al.*, 2003, Supply *et al.*, 2000). A significant difference between MIRUs and other VNTR's is that MIRUs contain regulatory elements such as start and stop codons and consensus sequences (Romano *et al.*, 2005). In most instances, MIRU's overlap the start and stop codons of the genes flanking them (Romano *et al.*, 2005).

Forty one MIRU loci have been identified, however, only 12 of these loci have been shown to display polymorphism in the number of tandem repeats (Supply *et al.*, 2000). Mycobacterial interspersed repetitive units-VNTR involves the use of these 12 polymorphic loci for genotyping MTBC species (Neonakis *et al.*, 2008). The size of the amplicons is used to calculate the number of repeats (Neonakis *et al.*, 2008). Although MIRU-VNTR has been found to have significant discriminative power, it has been suggested that the addition of more polymorphic loci to the existing panel of 12 loci would make it more discriminatory (Cowan *et al.*, 2002, Sola *et al.*, 2003).

Variable number tandem repeat typing is more rapid and reproducible. Dead organisms can be used for this technique, thus reducing the risk of laboratory infections as laboratory workers do not have to manipulate live, infectious agents (Frothingham and Meeker-O'Connell, 1998). Another advantage of using VNTR's is that they are stable, thus allowing traceability of strains that may be responsible for TB outbreaks on farms and game parks (Hilty *et al.*, 2005).

However, despite the great potential that molecular diagnosis of *M. bovis* has, there are still drawbacks regarding the sensitivity and reliability of molecular techniques. As a result older methods such as mycobacterial culture, which is still the gold standard for BTB diagnosis, are preferred (de la Rua-Domenech *et al.*, 2006).

#### 1.2.3.3 DIAGNOSIS OF *MYCOBATERIUM BOVIS* IN FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE

Molecular studies of archival specimens have increased largely because of developments in PCR technology. The long-term preservation of archival specimens includes formalin fixation and paraffin embedding (Lin *et al.*, 2009). Although these samples serve as an important resource for the study of diseases in cases where DNA from fresh or frozen tissue is unavailable, extraction of DNA from such samples is difficult (Lin *et al.*, 2009). Other factors that affect the quality of DNA isolated from FFPE tissue are summarized as follows:

1) the chemical composition of the fixative;
2) the duration of fixation;
3) the duration of tissue hypoxia, which is proportional to DNA degradation;
4) the size of the specimen and hence its permeability to the fixative; and
5) the length of the storage time (Crisan and Mattson, 1993, Schander and Halanych, 2003).

The chemical composition of the fixative plays a key role especially in cases where formalin is used. This is because the main component of formalin, formaldehyde, denatures proteins in a

primary reaction where functional groups of some amino acids are combined, the oxygen atoms form hydrogen bonds with amines and cross-link the proteins (Schander and Halanych, 2003). Formaldehyde also causes fragmentation of DNA. Cross-linking impedes PCR amplification of DNA, however, DNA from FFPE tissue is still used in a wide variety of PCR applications. DNA from FFPE tissue is especially suitable for PCR amplification that requires short fragments (Lin *et al.*, 2009).

In a study conducted by Zsikla *et al.* (2004), the use of buffered formalin was shown to be significantly better for DNA preservation in embedded tissue than the use of non-buffered formalin. This is because non-buffered formalin oxidizes to formic acid, creating an acidic environment (pH 4) which results in DNA degradation due to the hydrolysis of the β glycosidic bonds in the purine bases. Buffered formalin, on the other hand, stabilizes the solution for a longer period than non-buffered formalin (Ferrer *et al.*, 2007). Renewal of the buffered formalin at regular intervals also decreases DNA degradation (Ferrer *et al.*, 2007).

Several independent studies confirmed that in addition to the type of fixative used and the period of fixation, the quality of DNA used for analysis is also greatly influenced by the type of extraction method used (Legrand *et al.*, 2002, Ferrer *et al.*, 2007). The cell wall of mycobacteria, which includes lipids and fatty acids, allows these pathogens to resist most environmental pressures (Loeschke *et al.*, 2005). This impermeable cell wall is also responsible for the difficulty encountered during DNA extraction procedures from mycobacteria (Boddinghaus *et al.*, 1990, Loeschke *et al.*, 2005). In order to obtain mycobacterial DNA, the cell wall must be opened and the methods used have to be fairly abrasive. As a result, DNA might be fragmented further and PCR sensitivity might be reduced (Loeschke *et al.*, 2005). The use of a silicagel product such as that produced by Qiagen was shown to yield good quality DNA from FFPE tissue. The type of tissue from which DNA was extracted and the thickness thereof also influence DNA quality – a large section of tissue has a greater likelihood of containing PCR inhibitors than a smaller section of tissue (Legrand *et al.*, 2002).

Storage time of the tissues was found to affect DNA integrity and PCR amplification. DNA from tissues that was stored for significantly long periods were found unsuitable for PCR amplification of long target sequences, only short fragments of less than 300 bp could be amplified (Hosek *et al.*, 2006, Lin *et al.*, 2009). Fragments longer than 500 bp are rarely amplifiable after storage time of 1 or 2 years (Hosek *et al.*, 2006).

## 1.3 AIMS OF THE STUDY

The aims of this study include:

a. To compare four different methods of DNA extraction in order to determine an optimal extraction procedure for DNA from FFPE tissue.

b. To conduct a retrospective study aimed at typing strains of *M. bovis* from FFPE tissue originating from buffalo, kudu, and lion, using MLVA and spoligotyping methods. Gross and histopathological evidence showed evidence of *M. bovis* infection in these tissues.

c. To determine the efficacy of deletion analysis of the regions of difference when used to identify *M. bovis* in DNA extracted from FFPE tissue.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 SAMPLES COLLECTED

Three sets of samples were used in this study. The first set was obtained from a previous study conducted by Allix *et al.* (2006). These samples were used to optimize a PCR for the second set of samples which comprised of FFPE tissue from wild animals from the KNP. The third set of samples were fresh tissue samples also obtained from wild animals in the KNP.

### 2.1.1 SAMPLES FROM BELGIUM

The DNA samples obtained from Belgium comprised of 127 *M. bovis* isolates from 77 BTB positive herds collected during 1995 to 2003. These *M. bovis* isolates were selected from a total of 451 samples from 204 herds characterized by IS*6110* RFLP and spoligotyping methods. Some samples were typed using the MIRU-VNTR method (Allix *et al.,* 2006). The samples were not subjected to formalin fixation prior to extraction.

### 2.1.2 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES FROM THE KNP

The FFPE tissue samples were provided by Professor NJ Kriek of the Wildlife Unit, Department of Production Animal Clinical Studies, Faculty of Veterinary Science, who extensively has studied BTB in wild animals. The samples (Table 1) were isolated from a number of wild animals from a couple of game parks, but mainly from the KNP. All the animals, with the exception of the black rhino (sample ID 2871-96), were confirmed BTB positive by culture and histopathology.

Several samples from different tissues with visual lesions were excised from each animal. A set of 15 samples, each with a subset of about 3, 5 or 6 cuttings (a total of 103 samples) were used. Non-buffered formalin was used to fix the tissues before they were embedded in paraffin. The time of fixation of the tissues could not be determined.

### 2.1.3 FRESH TISSUE SAMPLES

To draw a comparison between the results obtained from FFPE tissue, fresh tissue samples were used. The fresh tissue samples were also provided by Professor NJ Kriek. The samples were obtained (in early August 2009) from three adult female buffalo from game parks close to the KNP. The tissue samples were kept at -20°C before DNA was extracted in early December 2009.

Two samples were obtained from each buffalo i.e. a total of six samples were obtained. The first buffalo (B1) was a ten year old female and lymph node tissue was excised from the

carcass. Hard, white lesions could be seen on one of the samples (labelled sample number 1), whilst the second sample had white puss-like lesions (labelled sample number 5).

The second buffalo (B2) was an adult female (the exact age is unknown) from which lymph node tissue was excised. The first sample (labelled sample number 2) had no visible lesions and the second sample (labelled sample number 3) had small hard lesions.

The third buffalo (B3) was a ten year old female and it was confirmed positive for BTB. The first sample (labelled sample number 4) from this animal was excised from lung tissue and it had soft medium sized white lesions. The second sample (labelled sample number 6) was excised from the retropharyngeal lymph node and had a thick cotex.

**Table 1: Formalin-fixed paraffin-embedded tissue samples manifesting typical BTB infection when using histology**

| SAMPLE ID | YEAR | OBSERVED CHANGES | ISOLATED FROM |
|---|---|---|---|
| 3086-95 | 1995 | Lung: florid granulomatous inflammatory reaction and focal necrosis; extensive neutrophil infiltration | Baboon (KNP) |
| 1500-96 | 1996 | Lung: Severe interstitial and alveolar granulomatous inflammatory reaction in association with marked bronchitis/bronchiolitis | Lion (OVI)** |
| 1504-96 | 1996 | Lymph node: Extensive multi-focal caseo-necrotic granulomatous | Baboon (Skukuza) |
| 2871-96 | 1996 | Lymph node: Multifocal granulomatous lymphadenitis with scant necrosis and fibrosis | Black Rhino (NPB)* |
| 2934-96 | 1996 | Lung and lymph node: Extensive caseo-necrotic granulomatous lymphadenitis and pneumonia; multifocal with mild calcification | Kudu (KNP) |
| 2936-96 | 1996 | Lymph node: Extensive caseo-necrotic granulomatous lymphadenitis; multifocal with mild calcification | Kudu (KNP) |
| 268-98 | 1998 | Lymph node: Granulomatous lymphadenitis with no necrosis (no acid-fast organisms can be observed in lymph nodes of BTB lions) | Lion (NPB)* |
| 269-98 | 1998 | Lymph node: Granulomatous lymphadenitis with no necrosis | Lion (NPB)* |
| 400-98 | 1998 | Lymph node: Granulomatous lymphadenitis with no necrosis | Lion (NPB)* |
| 972-06 | 2006 | Lymph node: Florid granulomatous lymphadenitis and accompanying necrosis involving about 50% of the diseases tissue | Buffalo (KNP) |
| 977-06 | 2006 | Granulomatous reaction with extensive pyogranulomatous inflammation, necrosis and calcification | Buffalo (KNP) |
| 981-06 | 2006 | Granulomatous reaction with extensive necrosis | Buffalo (KNP) |
| 1006-06 | 2006 | Lymphnode: Granulomatous reaction with extensive necrosis | Buffalo (KNP) |
| 1009-06 | 2006 | Florid inflammatory reaction with extensive necrosis and calcification; liquefaction of necrotic tissue due to infiltration of large numbers of neutrophils | Buffalo (KNP) |
| 1022-06 | 2006 | Extensive caseous necrosis in association with peripheral granulomatous inflammation; extensive calcification | Buffalo (KNP) |

*Natal Parks Board
**Onderstepoort Veterinary Institute

## 2.2 POLYMERASE CHAIN REACTION

### 2.2.1 EXTRACTION OF DNA

#### 2.2.1.1 SAMPLES FROM BELGIUM

The DNA samples from Belgium were obtained from samples collected in a previous study by Allix *et al.,* (2006) and provided in pellet form. The DNA was isolated from mycobacterial strains grown in Middlebrook 7H9 liquid medium supplemented with oleic acid-albumin-dextrose-catalase (Becton Dickinson,Belgium), Tween 80, penicillin, and Fungizone for 3 weeks (Allix *et al.*, 2006) as follows: 1.5 ml of the concentrated culture was heated at 80°C for 20 min to kill the cells and then centrifuged. After centrifugation, the cells were resuspended in 500 µl of 1 x TE buffer (0.01 M Tris-HCl, 0.001 M EDTA at pH8.0). Lysozyme was added to a final concentration of 1 mg/ml, and the tube was incubated for 1 hour at 37°C. Seventy microliters of 10% sodium dodecyl sulfate (SDS) and 6 µl of proteinase K (at a 10 mg/ml concentration) were added, and the mixture was incubated for 10 min at 65°C. An 80 µl volume of *N*-acetyl-*N,N,N*-trimethyl ammonium bromide was added. The cups were vortexed briefly and incubated for 10 min at 65°C. An equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was added, and the mixture was vortexed for 10 s. After centrifugation for 5 min, 0.6 volume of isopropanol was added to the supernatant to precipitate the DNA. After 30 min at -20°C and centrifugation for 15 min, the pellet was washed once with 70% ethanol and the air-dried pellet was redissolved in 20 µl of 0.1 x TE buffer (0.001 M Tris-HCl, 0.0001 M EDTA pH 8.0) (van Soolingen *et al.*, 1991). A 100 µl of TE buffer was added to the pellet to prepare the DNA for PCR amplification. The DNA was maintained at -20°C in Qiagen® TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA buffer) until used.

#### 2.2.1.2 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE

Since previous studies have indicated that the type of method used to extract DNA plays a major role in the quality of DNA obtained from FFPE tissue (Legrand *et al.*, 2002, Ferrer *et al* 2007), three methods were used to determine which method yielded the best results.

The boiling method as described by Miller *et al.* (1997) was the first extraction method used. The FFPE tissue was cut in thin sections and placed in 1.5 ml tubes, 200 µl 0.5% Tween 20 was then added to the samples. The tubes were placed on a heating block at 100 °C for 10 minutes. This boiling step was followed by snap freezing the samples in liquid nitrogen. The heating and snap freezing steps were repeated followed by an additional heating step for 10 minutes. The samples were centrifuged and the supernatant containing the DNA was transferred to a new tube. Since there are a lot of PCR inhibitors in FFPE samples, further DNA purification is generally recommended, this also allows the breaking of the cross-link matrix and liberates more DNA (Greer *et al.*, 1994). After extraction with the boiling method, the pellet was therefore subjected to further extraction and purification using the QIAamp® DNA mini kit. This was the second extraction method used.

In a third method, the FFPE tissue was cut in thin sections, which were then placed in 1.5 ml tubes. DNA was extracted from the FFPE tissue using the QIAamp® DNA mini kit and following the protocol for DNA extraction from fixed tissues. With this method, there is an optional step of adding xylene to the tissue samples. In cases where there was more than one tissue sample from one animal, two samples from each animal were used and xylene was used in one of the sample, and not in the other.

2.2.1.3 FRESH TISSUE SAMPLES

DNA extractions were carried out at the Onderstepoort Veterinary Institute (OVI) using the QIAamp® DNA mini kit and following the user manual. Two sets of samples were extracted from each buffalo. RNAse was used in one set of the samples from each buffalo and not in the second set.

**2.2.2 MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS**

2.2.2.1 OPTIMIZATION OF THE POLYMERASE CHAIN REACTION USING THE SAMPLES FROM BELGIUM

The 1 X Fermentas PCR Mastermix (Inqaba Biotechnical Industries (Pty) Ltd., South Africa) was used for the PCR assays instead of the Qiagen Hotstart *Taq* DNA polymerase kit as used by Allix *et al.*, (2006). All PCRs were performed in 25 µl containing 2.5 µl of the DNA, 12.5 µl of 1 x Fermentas PCR Mastermix, 9.0 µl of RNA free water and 0.5 µl of each flanking primer at a concentration of 20 pM. The primers used were in accordance with the proposed set of 27 loci for the typing of MTBC species and as recommended by Le Fleche *et al.* (2002). Twenty one of the 27 loci were used to type *M. bovis* strains (Table 2).

An Applied Biosystems 2720 thermal cycler was used. An initial denaturation at 94°C for 5 minutes was followed by 40 cycles of denaturing at 94°C for 1 minute, annealing at 62°C for 1 minute (except for H37Rv_0079 and H37Rv_2387: annealing temperature 55°C), elongation at 72°C for 90 seconds, followed by a final extension step of 10 minutes at 72°C.

2.2.2.2 ANALYSIS OF FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE

All PCRs were performed in 25 µl containing 2.5 µl of the DNA, 12.5 µl of 1 x Fermentas PCR Mastermix, 9.0 µl of RNA-free water and 0.5 µl of each flanking primer at a concentration of 20 pM (Table 2). In some reactions, the amount of each primer used varied between 0.5 and 0.6 µl depending on the sample used. Some samples could only be amplified when 0.6 µl of each primer was added along with 0.5 µl of $MgCl_2$ at a concentration of 25 mM.

The primers used correspond to the proposed set of 21 loci recommended by Le Fleche *et al.* (2002). However, since FFPE tissue was used and DNA in such tissue is degraded, resulting

in only short sequences being amplified (Bonin *et al.*, 2003), a total of 13 loci were selected for optimization (Table 2). These loci were selected on the basis of the amplicon size. The loci with tandem repeat motifs of 500 bp and less were selected. The PCR cycles used were the same as the cycles as discussed in 2.2.2.1.

Further optimization reactions were conducted using reagents similar to those used by Allix *et al.*, 2006. Polymerase chain reactions were performed in 25 µl containing 2.5 µl of the DNA, 5 µl of Q buffer, 2.5 µl of 10 x buffer, 2 µl of 25 mM $MgCl^2$, 0.125 µl of Taq polymerase (Qiagen HotStarTaq DNA Polymerase®), 0.2 mM of each dNTP, RNA-free water and 0.5 µl of each flanking primer at a concentration of 20 pM. An initial denaturation at 95°C for 15 minutes was followed by 40 cycles of denaturing at 94°C for 1 minute, annealing at 62°C for 1 minute, elongation at 72°C for 1 minute, followed by a final extension step of 10 minutes at 72°C

**2.2.3 GEL ELECTROPHORESIS ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCTS**

A volume of 5 µl of the PCR products were run on 3% agarose gels in 1 x TAE buffer at a voltage of 120 for 30 minutes for analysis of the samples from Belgium. The gel length used was 6 cm. The amplicons from FFPE DNA were run on 3% agarose gels in 1 x TAE buffer at a voltage of 80 for 3 hours. Five microlitres of ethidium bromide at a concentration of 10 mg/ml per 100 ml of gel was used for staining. The stained gels were visualized under UV-light and then photographed. The allele size was estimated using a Fermentas 100 bp ladder and the Bioline Hyperladder II as markers of size.

**2.2.4 SPOLIGOTYPING**

2.2.4.1 FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE

Some of the DNA samples extracted from FFPE tissue were spoligotyped in accordance with a method standardized by Kamerbeek *et al.,* (1997).

In short, the method includes the use of 50 µl reaction mixture for the PCR containing T*th* buffer (5 mM Tris-HCl, 5 mM KCl, 0.7 mM $MgCl_2$, pH 9.0), each deoxynucleoside triphosphate at 200 mM, 20 pmol each of DRa and DRb primers, 10 ng of DNA, and 0.5 U of T*th* polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The mixture was heated at 96°C for 3 minutes followed by 20 cycles of 1 minute at 96°C, 1 minute at 55°C, and 30 s at 72°C. The amplicons were hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. The oligonucleotides (sequence included in Table 1 of Kamerbeek *et al.*, 1997) were covalently bound to a membrane. The membrane was activated by using 16% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The oligonucleotides were then applied to the membrane

in parallel by using a mini blotter system. Following a brief incubation period, the membrane was inactivated by using 100 mM NaOH and washed in 2X SSPE (1 x SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7]) supplemented with 0.1% sodium dodecyl sulfate (SDS). For hybridization, 20 µl of the amplified PCR product was diluted in 150 µl of 2 x SSPE–0.1% SDS and heat denatured. A 130 µl volume of the diluted samples was pipetted into the parallel channels in such a way that the channels of the mini blotter apparatus were perpendicular to the rows of oligonucleotides deposited previously. Hybridization was done for 60 minutes at 60°C. After hybridization, the membrane was washed twice in 250 ml of 2 x SSPE–0.5% SDS for 10 minutes each time at 60°C and then incubated in 1:4,000-diluted streptavidin-peroxidase conjugate for 45 to 60 minutes at 42°C. The membrane was washed twice, for 10 min each time, in 250 ml of 2 x SSPE–0.5% SDS at 42°C and rinsed with 250 ml of 2 x SSPE for 5 minutes at room temperature. Detection of hybridizing DNA was done by using chemiluminescent ECL detection liquid, followed by exposure to X-ray film (Kamerbeek *et al.*, 1997).

#### 2.2.4.2 FRESH TISSUE SAMPLES

The fresh tissue samples were also spoligotyped using the method of Kamerbeek *et al.,* (1997) as described in 2.2.4.1 above.

### 2.2.5 IDENTIFICATION OF *M. BOVIS* ISOLATES BY DELETION ANALYSIS OF THE REGIONS OF DIFFERENCE

The primers used for the deletion PCR were as previously described by Warren *et al* (2006). However, only primers RD 4 and RD 9 (from primer set 1) were used, the primer set consists of two flanking primers and an internal primer.

The PCR mix contained 12.5 µl of 1 X Fermentas PCR Mastermix (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 5 µl of the Q-solution (Qiagen), 3.5 µl of the RNAse free water and 0.5 µl of each 50 pM primer. The reaction was run at: denaturation temperature of 94°C for 5 minutes, and 40 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute, a final elongation step at 72°C for 10 minutes and a holding step at 4°C until used. The PCR products were then separated by electrophoresis using a 3% agarose gel at 10V/cm for 2 hours.

## Table 2: List of primers used for *M. bovis* strain typing

| Locus name and size and location of tandem repeat motif | Forward primer | Reverse primer |
|---|---|---|
| ***H37Rv_0024_18 bp*** | GAGAAACAGGAGGGCGTTG | TATTACGACGACCGCTATGC |
| ***H37Rv_0079_9 bp*** | CGTGCACAGTTGGGTGTTTA | TTCGTTCAGGAACTCCAAGG |
| ***H37Rv_0154_53 bp*** | TGGACTTGCAGCAATGGACCAACT | TACTCGGACGCCGGCTCAAAAT |
| ***H37Rv_0577_58 bp*** | GACTTCAATGCGTTGTTGGA | GTCTTGACCTCCACGAGTGC |
| ***H37Rv_0580_77 bp*** | CAGGTCACAACGAGAGGAAGAGC | GCGGATCGGCCAGCGACTCCTC |
| ***H37Rv_0802_54 bp*** | AAGCGCAAGAGCACCAAG | GTGGGCTTGTACTTGCGAAT |
| ***H37Rv_0959_53 bp*** | GTTCTTGACCAACTGCAGTCGTCC | GTGGGCTTGTACTTGCGAAT |
| ***H37Rv_1121_15 bp*** | GTTCTTGACCAACTGCAGTCGTCC | CGGCCTACCCAACATTCC |
| ***H37Rv_1644_53 bp*** | TCGGTGATCGGGTCCAGTCCAAGTA | CCCGTCGTGCAGCCCTGGTAC |
| ***H37Rv_1955_57 bp*** | AGATCCCAGTTGTCGTCGTC | CAACATCGCCTGGTTCTGTA |
| ***H37Rv_2163_a_69 bp*** | CCCATCCCGCTTAGCACATTCGTA | TTCAGGGGGGATCCGGGA |
| ***H37Rv_2165_75pb*** | ATTTCGATCGGGATGTTGAT | TCGGTCCCATCACCTTCTTA |
| ***H37Rv_2347_57 bp*** | AACCCATGTCAGCCAGGTTA | ATGATGGCACACCGAAGAAC |
| ***H37Rv_2401_58 bp*** | AGTCACCTTTCCTACCACTCGTAAC | ATTAGTAGGGCACTAGCACCTCAAG |
| ***H37Rv_2461_57 bp*** | GCGAACACCAGGACAGCATCATG | GGCATGCCGGTGATCGAGTGG |
| ***H37Rv_2531_53 bp*** | CAGCGAAACGAACTGTGCTATCAC | CGTGTCCGAGCAGAAAAGGGTAT |
| ***H37Rv_3006_53 bp*** | TCGAAAGCCTCTGCGTGCCAGTAA | GCGATGTGAGCGTGCCACTCAA |
| ***H37Rv_3192_53 bp*** | ACTGATTGGCTTCATACGGCTTTA | GTGCCGACGTGGTCTTGAT |
| ***H37Rv_3663_63 bp*** | GCCCAAAAAGCATGGGAACGTGCCCCT | GGTTGTCCCCGCAGTATCTC |
| ***H37Rv_3690_58 bp*** | AATCACGGTAACTTGGGTTGTTT | GATGCATGTTCGACCCGTAG |
| ***H37Rv_4348_53 bp*** | CGCATCGACAAACTGGAGCCAAAC | CGGAAACGTCTACGCCCCACACAT |

Table obtained from (Le Fleche *et al.*, 2002). A total of 13 loci (highlighted in blue) were selected for amplification of DNA from FFPE tissue. These loci were selected on the basis of the amplicon size. The loci with tandem repeat motifs of 500 bp and less were selected.

# CHAPTER 3

# RESULTS

## 3.1 POLYMERASE CHAIN REACTION

### 3.1.1 EXTRACTION

DNA was extracted from FFPE tissue using three different methods:

DNA extracted from FFPE tissue using the boiling method provided samples that had DNA concentrations of more than 100 ng, however, no bands or smears were visualised when gel electrophoresis was carried out (results not shown).

DNA extraction using the QIAamp® DNA mini kit (following the protocol for DNA extraction from fixed tissues) yielded smears with no definitive bands when analysed by gel electrophoresis (Figure 2). The use of xylene in DNA extraction does not appear to have improved the quality of DNA obtained from the FFPE tissue since both samples (with xylene and without) appear to be of the same quality. However, in Figure 2, two samples displayed a marked difference between the xylene and non-xylene extracted DNA. For tissue sample 972-06 (Lane 28, blue arrow), DNA obtained without using xylene in the extraction method appeared to be of better quality than DNA obtained using xylene. On the other hand, the inverse was true for tissue sample 269-98 (Lane 34 black arrow), DNA obtained using xylene during extraction appeared to be of a better quality than DNA obtained without using xylene during extraction. Since these results are contradictory, it appears that the difference in DNA quality may rather be as a result of the difference in the amount of mycobacterial DNA present in each tissue section.

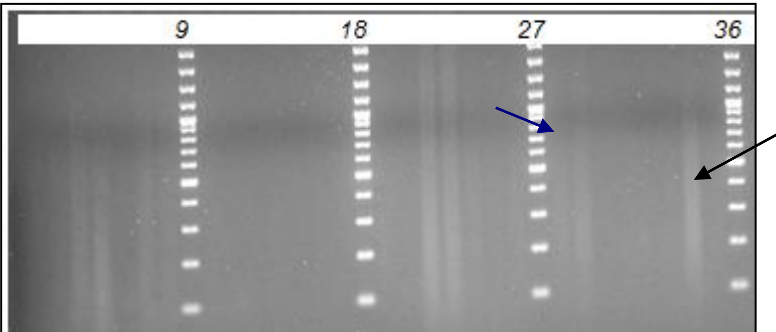



**Figure 2**: DNA extraction from FFPE tissue using the QIAamp® DNA mini kit. In Lanes 9, 18, 27 and 37 is the Fermentas 100 bp ladder. Lanes 3, 5, 6, 8, 10, 12, 14, 16, 19, 21, 22, 24, 26, 29 and 31 show extracted samples extracted with xylene and Lanes 2, 4, 7, 11, 13, 15, 17, 20, 23, 25, 28, 30 and 32 show the same samples without the use of xylene. Lanes 1, 33 and 35 were not used.

Results of DNA extraction from fresh samples are indicated in Figure 3. Samples 1 to 6 comprise of the first set of samples where RNAse was not used and samples 7 to 12 comprise the second set of samples where RNAse was used. RNAse was used to avoid copurification of RNA. This is however not necessary since RNA does not inhibit PCR. Samples 1, 5, 7 and 11 are from animal B1. Samples 2, 3, 8 and 9 are from animal B2. Samples 4, 6, 10 and 12 are from animal B3. DNA extraction from the fresh tissue was largely successful (Figure 3). However, samples 1 (animal B1) and 2 (animal B2) (no RNAse used) did not yield any visible bands when DNA extracted from the tissue samples was analysed by gel electrophoresis. Sample 1 (animal B1) may have had a low DNA concentration that could not be visualised by gel electrophoresis. Sample 2 (animal B2) on the other hand had no visible lesions and might not have had any mycobacterial DNA.

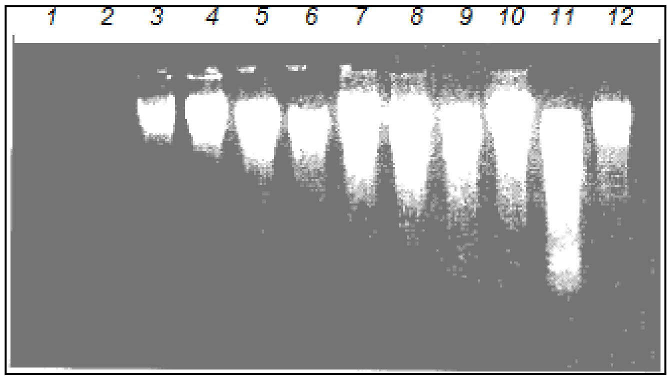



**Figure 3**: DNA extraction from fresh tissue obtained from three adult female buffalo. Lanes 1 to 6 show samples 1 to 6 where RNAse was not used. Lanes 7 to 12 show samples 7 to 12 where RNAse was used during extraction. Samples 1, 5, 7 and 11 are from animal B1; samples 2, 3, 8 and 9 are from animal B2; and samples 4, 6, 10 and 12 are from animal B3.

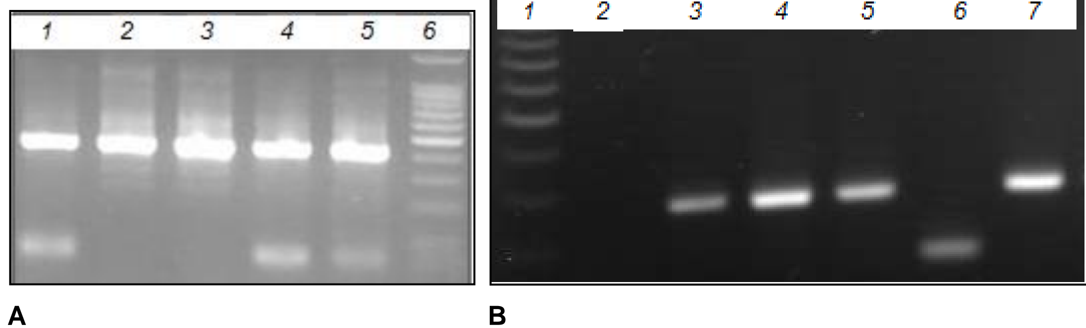
Host DNA co-purifies with mycobacterial DNA, thus gel electrophoresis analyses as seen in Figures 2 and 3 are not clear indications of how much bacterial DNA was extracted. Measurements of DNA concentration using a spectrophotometer also provide total DNA concentration and not just bacterial DNA. As a result, the success or failure of *M. bovis* DNA extraction is better seen when conducting a PCR assay.

### 3.1.2 MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS AMPLIFICATION

During MLVA typing of the samples from Belgium, most of the samples yielded the expected results (as previously obtained by Allix *et al.,* 2006) (Figures 4 A and B and 6). However, the primer based on the MIRU40 locus yielded unspecific bands (Figure 5). After multiple attempts at optimizing the PCR for this primer, it still yielded multiple bands. Use of the Qiagen Hoststart *Taq* DNA polymerase kit (as used by Allix *et al.*, 2006) produced the same results as the Fermentas master mix during amplification of DNA samples from Belgium and

DNA from FFPE tissue. As a result, the Fermentas master mix was used for the majority of the reactions to reduce costs.

Amplification of the samples from Belgium yielded results consistent with *M. bovis* infection when band sizes were converted to repeat number using conversion table which facilitates the conversion of band size to VNTR number (Le Fleche *et al.,* 2002). The samples used were found to be BTB positive, as established previously by Allix *et al.* (2006).




**A** **B**

**Figure 4:** MLVA typing of strains obtained from Belgium and used in a previous study by Allix *et al.* (2006). **Figure 4A**, the ETRC locus was used to differentiate strains. Lane 6 is the 100 bp ladder. **Figure 4B**, locus Mtub21 was used to differentiate strains.

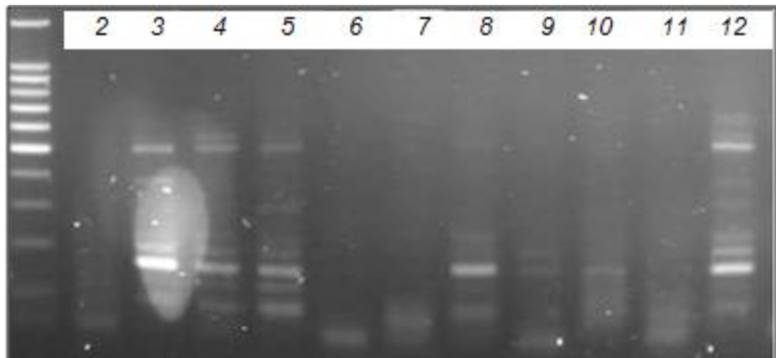



**Figure 5:** MLVA typing of strains obtained from Belgium using the MIRU40 locus to differentiate strains. Lane 1 is the Fermentas 100 bp ladder and in lane 2 is the negative control.

When MLVA was carried out using DNA obtained from the boiling method and the QIAamp® DNA mini kit, there was no marked difference in the results obtained (Figure 7 A and B). Polymerase chain reaction amplification of DNA from these two methods yielded unspecific products of less than 100 bp. DNA extraction using the QIAamp® DNA mini kit and following the protocol for DNA extraction from fixed tissues (including the optional step of adding xylene to the tissue samples) also yielded unspecific results.

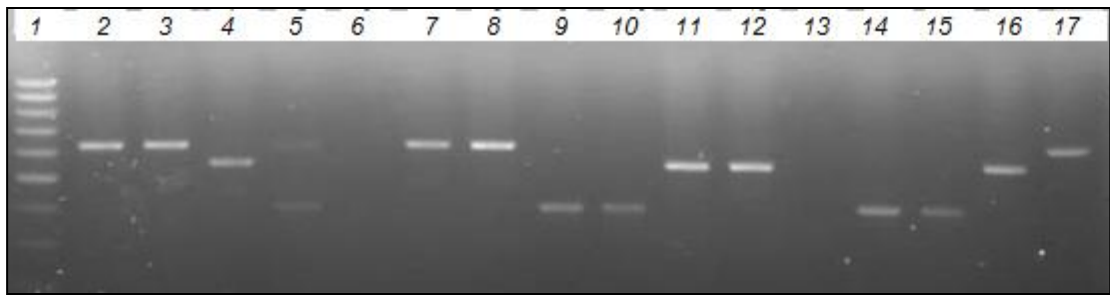
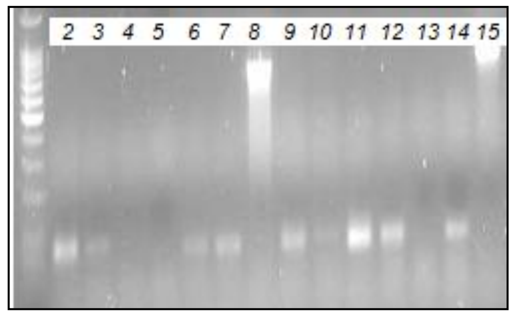
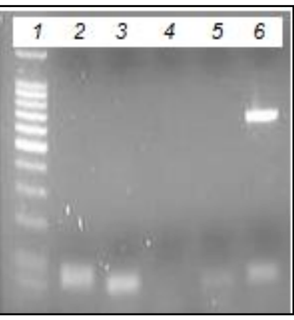



**Figure 6:** MLVA typing of strains obtained from Belgium (Allix *et al.* 2006) using the ETR-A locus to differentiate strains. In Lane 1 is the 100 bp ladder (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd., South Africa)**.**

There are many variables that can influence the outcome of amplification using DNA from FFPE tissue. This necessitates the optimization of techniques that are suitable for DNA extracted from FFPE tissue. In this study, varying concentrations of DNA were used to determine the optimal DNA concentration at which PCR should be conducted. The results of the optimization reactions are shown in Figures 7A, 7B, 8A to 8D.




**A**




**B**

**Figure 7**: MLVA analysis of DNA extracted from FFPE tissue using either the boiling method or the QIAamp® DNA mini kit. This was an optimization reaction wherein three PCR reactions were run per sample under different conditions. **Figure 7A**, Lane 1 is the Fermentas 100 bp ladder, Lanes 2 to 7, 9 to 14 are samples 1500-96, 2936-96, 3086-95 (Table 1) (DNA extracted with the QIAamp® DNA mini kit) and samples 268-98, 269-98, 2871-96 (Table 1) (DNA extracted using the boiling method). Each sample was amplified twice, first with extra $MgCl_2$ and then without any extra $MgCl_2$. The samples in Lanes 2 to 7 contain an extra 0.5 µl of $MgCl_2$ (in addition to the $MgCl_2$ in the mastermix). Lanes 8 and 15 contain the positive control *M. bovis* AN5. **Figure 7B**, Lane 1 is the Fermentas 100 bp ladder, lanes 2 to 5 are 3086-95, 268-98, 269-98 and 2871-96 (Table 1) (DNA extracted using the boiling method). These samples contain extra $MgCl_2$. In lane 6 is the positive control.

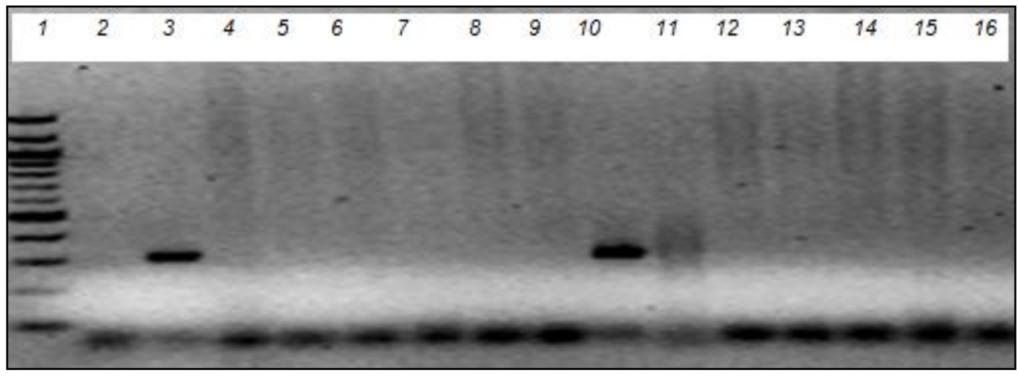



**Figure 8A:** MLVA typing of DNA from FFPE tissue using the Mtub38 locus to differentiate strains. In Lane 1 is the Fermentas 100 bp ladder. In Lane 2 is the negative control and Lane 3 is the positive control for the Mtub38 locus. In Lanes 4 to 16 are samples 3086-95, 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub38 locus). The positive control was loaded at regular intervals after every six samples.

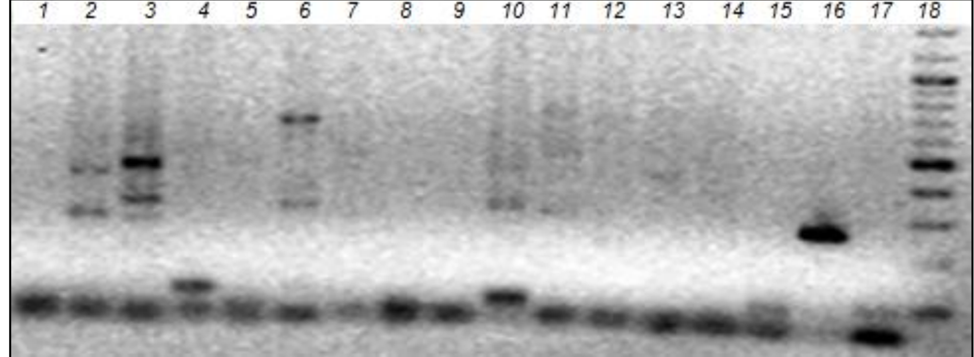
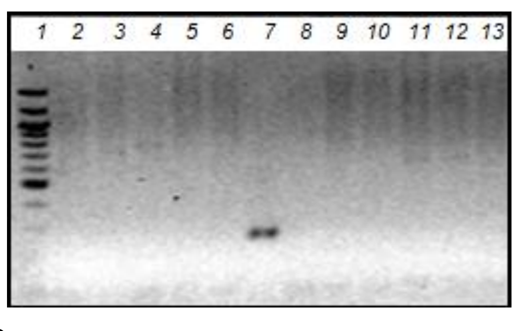
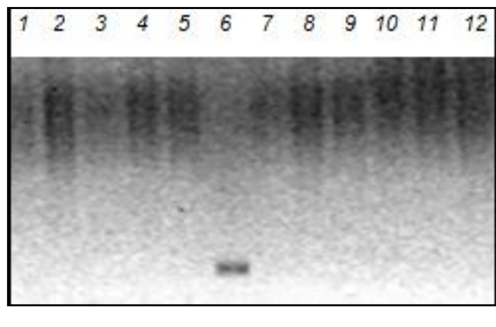



**Figure 8B:** MLVA typing of DNA from FFPE tissue using the MIRU40 locus to differentiate strains. In lane 1 is the negative control and Lane 2 is the positive control for the MIRU40 locus. In Lanes 3 to 14 are samples 3086-95, 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (MIRU40). In Lanes 15 and 16 are the negative and positive controls of the Mtub21 locus. In Lane 17 is sample 3086-95 (Mtub21 locus). In Lane 18 is the Fermentas 100 bp ladder. As with amplification of the samples from Belgium, amplification of FFPE tissue using the primer based on MIRU40 yielded multiple bands.




**C** **D**

**Figure 8C:** Lane 1 is the Fermentas 100 bp ladder. In Lanes 2 to 13 are samples 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub21 locus). The positive control was loaded at regular intervals after every six samples. **Figure 8D:** Lanes 1 to 12 are samples 1009-06, 1500-96, 1006-06, 268-98, 2871-96, 1504-96, 1022-06, 977-06, 2936-96, 977-06B and 3086-95 (Mtub02 locus).

### 3.1.3 SPOLIGOTYPING ANALYSIS

DNA from FFPE tissue was also spoligotyped using the method standardized by Kamerbeek *et al.* (1997). The spoligotype patterns obtained were unspecific, much like the results obtained from using the MLVA method. DNA extracted using the boiling method, the QIAamp® DNA mini kit and the Qiagen REPLI-g FFPE kit all yielded unspecific results (Figure 9).

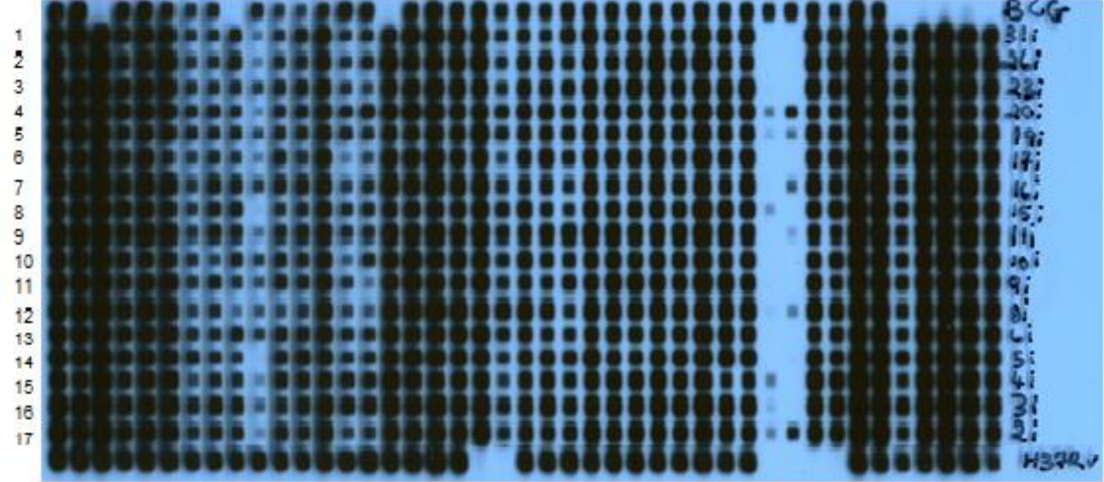



**Figure 9**: Spoligotyping results of FFPE tissue extracted using the boiling method and the QIAamp® DNA mini kit. In Lanes 1 to 17 are samples 1500-96, 400-98, 981-96, 268-98, 269-98, 1504-96, 269-98, 3086-95, 1006-06, 1009-06, 981-06, 269-98, 1022-06, 3086-96, 2871-96, 268-98 and 977-06.

The spoligotyping results of DNA extracted from fresh tissue are shown in Figure 10. The DNA sample from the lymph node tissue of animal B1 (labelled sample number 1) yielded readable results, whilst the DNA from the second sample of animal B1 (labelled sample number 5) did not yield any readable results.

Both DNA samples from the lymph node tissue of animal B2 did not yield any readable results (Figure 10). The first tissue sample of animal B2 (labelled sample number 2) did not have any visible lesions, as a result, there could have been no *M. bovis* organisms in the tissue or there were too few to be detected by PCR. The second tissue sample of animal B2 (labelled sample number 3) had small hard lesions, however, the sample did not prove BTB positive. This could have been due to the fact that the second buffalo was not BTB positive and the TB-like lesions were caused by an organism other than *M. bovis*. This would also explain the lack of results from the first tissue sample.

The first and the second DNA samples (labelled samples number 4 and 6, respectively) from animal B3 yielded readable results.

As seen in Table 3, the DNA extracted from the tissue samples of animals B1 and B3 were identified as BOVIS1 according to the spolD4 database.

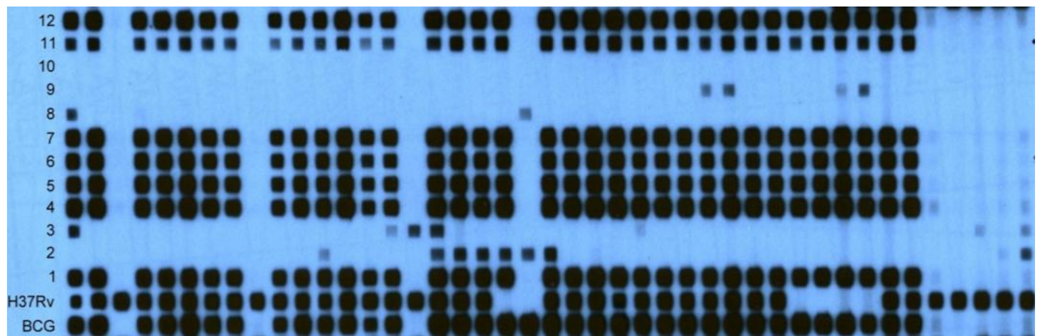



**Figure 10**: Spoligotyping of DNA isolated from fresh tissues. Samples 1, 5, 7 and 11 are from animal B1; samples 2, 3, 8 and 9 are from animal B2; and samples 4, 6, 10 and 12 are from animal B3. The DNA samples from the lymph node tissue of animal B2 yielded unreadable results.

## Table 3: Spoligopatterns of strains obtained from fresh tissue of three adult buffalos

| Isolate | SpolDB4 type | SpolDB4 classification | Spoligotype pattern |
|---|---|---|---|
| 6 | 481 | BOVIS1 | |
| 5 | 481 | BOVIS1 | |
| 4 | 481 | BOVIS1 | |
| 3 | | | no result |
| 2 | | | no result |
| 1 | 481 | BOVIS1 | |
| Control: H37Rv | 451 | H37Rv | |
| Control: BCG | 482 | BOVIS1_BCG | |

### 3.1.4 DELETION ANALYSIS OF THE REGIONS OF DIFFERENCE

*Mycobacterium bovis* isolates are expected to produce a band size of 268 bp when amplifying the RD4 region and 108 bp when amplifying the RD9 region. Deletion analysis amplifying the RD4 region using DNA extracted from FFPE tissue did not produce the expected results. Amplification of the RD9 region produced the expected band size of 108 bp (Figure 11). The samples used were those extracted with the use of xylene and those extracted without the use of xylene.

Deletion analysis of the DNA obtained from FFPE tissue of the black rhino (sample ID 2871-96) yielded a 108 bp band when amplifying the RD9 region. Although the sample was declared BTB positive by histology, it was not confirmed BTB positive by culture and thus it is not clear which mycobacteria infected it since other MTBC bacteria can cause *M. bovis*-like lesions. However, from the results obtained during RD9 analysis, it appears that the rhino was negative for *M. tuberculosis*. This is because RD9 identifies *M. bovis*, *M. africanum, M. microti, M. pinnipedii, M. caprrae* and *M. bovis* BCG and specifically excludes *M. tuberculosis*. The rhino was more than likely infected by *M. bovis* than another MTBC bacterium since *M. bovis* is prevalent in the KNP and HiP (where the rhino was kept).

Deletion analysis amplifying the RD4 region using DNA extracted from fresh tissue produced results similar to the spoligotyping results (Figure 12). This is because sample 1 of animal B1 and sample 4 of animal B3, which displayed spoligopatterns typical of *M. bovis*, produced results typical of *M. bovis* during deletion analysis whilst none of the samples of animal B2 produced any results.

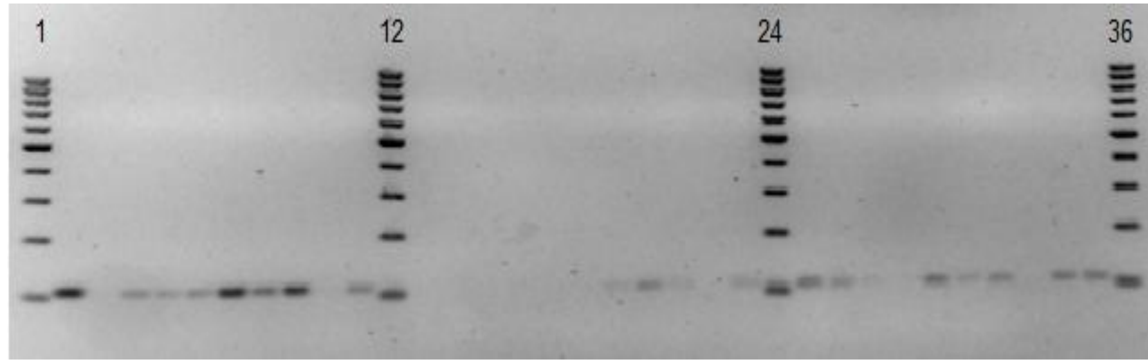



**Figure 11**: Deletion analysis of FFPE tissue amplifying RD9. In Lanes 1, 12, 24 and 36 is the Fermentas 100 bp ladder, in Lanes 2 and 3 is the positive and negative controls, in Lanes 4 to 11 is samples 2871-96 (xylene), 2871, 400-98 (xylene), 400-98, 1006-06 (xylene), 1504-96 (xylene), 1504-96, 1009-06 (xylene); Lane 13 is empty; in Lanes 14 to 23 is samples 1022-06 (xylene), 1022-06, 2936-96 (xylene), 2936, 1500-96 (xylene), 1500-96, 2934-96 (xylene), 2934-96, 3086-95 (xylene), 3086-95; and in Lanes 25 to 35 is samples 3086-95 (xylene); 268-98 (xylene), 268-98, 972-06 (xylene), 972-06, 977-06 (xylene), 977-06, 981-06 (xylene), 981-06, 269-98 (xylene) and 269-98.

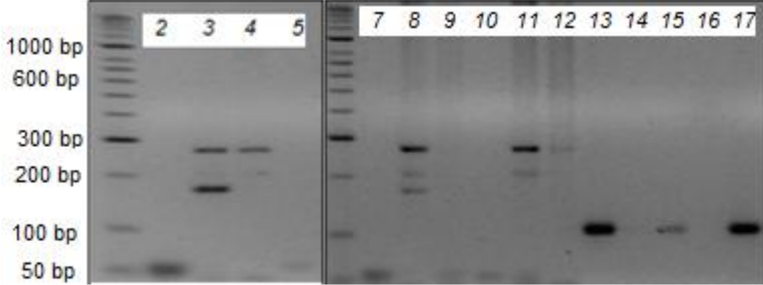



**Figure 12**: Deletion analysis of fresh tissue samples amplifying RD4 and RD9 regions. In Lane 1 is the Bioline Hyperladder II 100 bp marker (Bioline, Celtic Molecular Diagnostics (Pty) Ltd., South Africa), Lane 2 is the negative control, Lane 3 is the positive control for deletion analysis of RD4, Lane 4 is sample 1 of animal B1, Lane 5 is sample 2 of animal B2, Lane 6 is the marker, Lane 7 is sample 1 of animal B1 (no RNAse), Lane 8 and 11 is sample 4 of animal B3, and in Lanes 13 to 17 are the results for the deletion analysis amplifying RD9. The samples are 1 (B1), 2 (B2), 3 (B2), 4 (B3), and 6 (B3).

Table 4 summarizes the results obtained in the current study when the different PCR-based techniques were used for typing *M. bovis* strains.

**Table 4: Summary of the results obtained for typing of *M. bovis* strains using different PCR-based techniques**

| | MLVA | Spoligotyping | Deletion PCR |
|---|---|---|---|
| DNA from FFPE tissue | Typing unsuccessful | Typing unsuccessful | RD4 amplification unsuccessful<br><br>RD9 amplification successful (108 bp band observed) |
| DNA from fresh tissue animal B1* | | Successfully identified as BOVIS1 | RD4 and RD9 amplification successful (268 bp and 108 bp bands observed, typical of BOVIS1) |
| DNA from fresh tissue animal B2* | | Typing unsuccessful | RD4 and RD9 amplification unsuccessful |
| DNA from fresh tissue animal B3* | | Successfully identified as BOVIS1 | RD4 and RD9 amplification successful (268 bp and 108 bp bands observed, typical of BOVIS1) |

*The DNA from fresh tissue samples was not typed using MLVA since PCR assays of the samples from Belgium established that samples that are not fixed and paraffin embedded can be typed successfully using MLVA.

# CHAPTER 4

## DISCUSSION

Formalin-fixed tissue is an invaluable source of DNA especially in cases where no gross lesions are visible and fresh tissue is thus not saved for bacterial culture (Miller *et al.*, 1997). The use of FFPE tissue also circumvents the need for laboratory workers to handle infectious material (Suresh *et al.*, 2007). In some cases, FFPE tissue is the only source of DNA available (Santos *et al.*, 2009). Formalin-fixation of DNA however, cause the fragmentation of DNA and allows PCR amplification of only relatively short target sequences (Gillio-Tos *et al.*, 2007). Due to the presence of numerous variables involved in tissue fixation and processing as well as variable primer efficiency, it is recommended that pilot studies be conducted to assess DNA quality (Greer *et al.*, 1994). For this reason optimization reactions were carried out in the current study.

It is not clear for how long samples used in this study were kept in formalin before processing. However, from the poor quality of DNA that was obtained (even from samples stored for two years) it may be that samples were fixed for long periods in formalin. Legrand *et al.* (2002) found that high DNA yields were obtained from tissues that were fixed for seven days whereas DNA yields from tissues that were fixed for 16 days decreased by more than 50%. Only small amounts of DNA could be obtained from tissues after 32 days of formalin fixation (Legrand *et al.*, 2002).

The samples used for this study were obtained mainly from animals in the KNP and a few were obtained from HiP. The samples may have thus required prolonged fixation and storage before they could be analyzed in a laboratory since tissue sampling happened in a relatively remote area (Greer *et al.*, 1994). The Pathology laboratory at the Faculty of Veterinary Science, University of Pretoria, Onderstepoort, makes use of non-buffered formalin as a matter of routine. The samples may have been exposed to formalin for significantly long periods, thus resulting in a high level of DNA degradation, especially since non-buffered formalin was used. In that instance, the DNA quality would be poor and only PCR amplification of fragments less than 200 bp would be achieved (Greer *et al.*, 1994)

It is not clear how long the specimens were fixed in formalin before they were embedded in paraffin. However, the storage time after embedding appeared not to have affected DNA integrity or PCR amplification in this study due to the fact that tissues that were stored for 13 years and those that were stored for two years yielded the same results i.e. unspecific bands. These results are consistent with previous studies by Legrand *et al.* (2002) who found that research using DNA from FFPE tissue stored for up to 15 years was not affected by storage time. Rather, it was suggested that the degradation of DNA was related to the formalin

fixation time (Legrand *et al.*, 2002). The fixation time is inversely proportional to the size of alleles that can be amplified because it was established that the longer the fixation time, the shorter the alleles that can be amplified and vice versa (Legrand *et al.*, 2002). A study by Rumph and Williams (1986) found that less formaldehyde could be eluted from tissue that was stored for about 100 days than tissues stored from 50 to 75 days, suggesting that prolonged exposure of tissues to formaldehyde results in further secondary reactions taking place (Rumph and Williams, 1986, Schander and Halanych, 2003).

The method of DNA extraction used is as equally important as the fixative and period of fixation when it comes to the quality of DNA obtained from FFPE tissue. In the majority of cases, one has insufficient details regarding the procedure used on archival specimens (Gillio-Tos *et al.*, 2007). As a result, it is desirable to use an optimized extraction protocol that will yield good quality DNA (Legrand *et al.*, 2002, Gillio-Tos *et al.*, 2007). It was reported that the Chelex® 100 extraction method performed best on tissues that were fixed for 3 and 7 days i.e. less degraded DNA and that the QIAamp (Qiagen SA) procedure performed best on tissues that were fixed in formalin for longer periods (Legrand *et al.*, 2002). Furthermore, Sethusa (2006) found that using the boiling method as established by Miller *et al.* (1997), one could obtain amplifiable DNA fragments from tissues up to 22 days post fixation. The expected amplicon sizes ranged from 123 bp to 229 bp (Sethusa, 2006).

The different extraction methods used in this study did not yield DNA greater than 200 bp that could be used for MLVA or deletion analysis of sequences. The boiling method and further extraction and purification using the QIAamp® DNA mini kit yielded amplicons of 100 bp during MLVA PCR amplification. This was smaller than the expected fragment sizes of about 215 bp to 508 bp. Qiagen have manufactured a REPLI-g FFPE kit specifically for extracting DNA from FFPE tissue. The kit was used in the study. However, the results were negligible and thus not shown. Although DNA extracted using this kit yielded products greater than 100 bp, the products were unspecific. This is due to the fact that REPLI-g FFPE kit is based on WGA. Although the technique has a high genomic coverage and high product yield, it is less specific in the analysis of short tandem repeat loci as found by Sun *et al.*, 2005. additionally, the accuracy and yield of the WGA method would decrease with increased DNA fragmentation (Wang *et al.*, 2007). When DNA was extracted using the QIAamp® DNA mini kit (following the protocol for DNA extraction from fixed tissues) products of 100 bp were amplified, (data not shown). The results obtained from using the various methods of extraction suggest that the DNA obtained from FFPE tissue was very fragmented and as a result, could not yield target sequences of more than 200 bp.

Furthermore, Miller *et al.*, (1997) amplified a 123 bp fragment of IS*1160* obtained from FFPE tissue. It was also found that PCR amplification targeting a β-globin DNA fragment of 152 bp was 69% successful, whereas PCR amplification targeting 268 bp and 676 bp DNA fragments

was 17% and 5%, respectively successful (Gillio-Tos *et al.* 2007). The primers used in the current study were based on loci having short target sequences ranging from 108 bp to 508 bp. If the level of degradation was high due to prolonged exposure to formalin, no amplification would have been possible for MLVA or deletion analysis for bands of more than 200 bp. The amplification of the 108 bp fragment indicating absence of the RD9, is consistent with the previous findings where band sizes of less than 200 bp were successfully amplified using DNA from FFPE tissue exposed to formalin for long periods (Gillio-Tos *et al.,* 2007, Lin *et al.*, 2009).

Multiple-locus variable number tandem repeat analysis of DNA from FFPE tissue yielded unspecific bands that could not identify the different strains of *M. bovis*. The expected band sizes for the thirteen loci used to type the *M. bovis* isolates ranged from 215 bp to 508 bp. Deletion analysis of FFPE tissue amplifying the RD4 region yielded unspecific bands. The absence of the RD4 region would result in a band size of 268 bp in *M. bovis*. This was the expected band size since all the FFPE samples (except sample 2871-96) were confirmed BTB positive by culture and histology. However, the isolates yielded unspecific bands or no bands in some cases. Deletion analysis of FFPE tissue amplifying RD9 is expected to yield a band size of 108 bp which is indicative of the absence of the region. In this study, amplification of the RD9 region using DNA from FFPE tissue yielded the expected band of 108 bp typical of *M. bovis*. Spoligotyping of the samples yielded unspecific results and no spoligopatterns could be determined using the DNA from FFPE tissue (Table 4). These results are consistent with the suggestion that the DNA used in this study was very fragmented. The fragmentation of DNA was exacerbated by the use of non-buffered formalin amongst other things. Non-buffered formalin creates an acidic environment which hydrolyses β glycosidic bonds in the purine bases and heavily fragments DNA (Ferrer *et al.,* 2007).

The alleles used in molecular fingerprinting of organisms are largely stable, thus allowing one to epidemiologically trace the origin of infections as well as to determine whether or not interspecies transmission occurs (Perumaalla *et al.*, 1999, Milian-Suazo *et al.*, 2008). Since the whole genome sequence of *M. bovis* is available, it would be advisable to design primers with target sequences of less than 200 bp for DNA from FFPE tissue for use in MLVA.

As previously recommended, improvement in the fixation procedure of tissues may greatly assist in the extraction of quality DNA from FFPE tissue (Lin *et al.*, 2009). This may be done by using buffered formalin as opposed to non-buffered formalin, shorter fixation periods or use of alternative fixatives that are better suited to preservation of DNA (Srinivasan *et al.*, 2002).

Despite the difficulties encountered when using FFPE tissue in PCR applications, many studies have been conducted using these tissue samples. This is because FFPE tissue

makes it possible to conduct retrospective studies which may provide an affordable and rapid way to obtain epidemiological information on infectious diseases (Greer *et al*., 1994). For this reason, it is worthwhile to continue developing optimal methods of extracting and amplifying DNA from FFPE tissue.

# REFERENCES

ADAMS, L. G. (2001) *In vivo* and *in vitro* diagnosis of *Mycobacterium bovis* infection *Revue Scientifique et Technique Office International des Epizootis,* 20, 304-324.

ALLIX, C., WALRAVENS, K., SAEGERMAN, C., GODFROID, J., SUPPLY, P. & FAUVILLE-DUFAUX, M. (2006) Evaluation of the epidemiological relevance of variable-number tandem-repeat genotyping of *Mycobacterium bovis* and comparison of the method with IS*6110* restriction fragment length polymorphism analysis and spoligotyping. *Journal of Clinical Microbiology,* 44, 1951-1962.

AMANFU, W. (2006) The situation of tuberculosis and tuberculosis control in animals of economic interest. *Tuberculosis,* 86, 330-335.

ANDERSEN, G. L., SIMCHOCK, J. M. & WILSON, K. H. (1996) Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. *Journal of Bacteriology,* 178, 377-384.

BODDINGHAUS, B., ROGALL, T., FLOHR, T., BLOCKER, H. & BOTTGERÎ, E. C. (1990) Detection and identification of mycobacteria by amplification of rRNA. *Journal of Clinical Microbiology,* 28, 1751-1759.

BONIN, S., PETRERA, F., NICCOLINI, B. & STANTA, G. (2003) PCR analysis in archival postmortem tissues. *Journal of Clinical Pathology,* 56, 184-186.

BROSCH, R., GORDON, S. V., MARMIESSE, M., BRODIN, P., BUCHRIESER, C., EIGLMEIER, K., GARNIER, T., GUTIERREZ, C., HEWINSON, G., KREMER, K., PARSONS, L. M., PYM, A. S., SAMPER, S., VAN SOOLINGEN, D. & COLE, S. T. (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Sciences of the United Staes of America,* 99, 3684-3689.

BULL, T. J., SIDI-BOUMEDINE, K., MCMINN, E. J., STEVENSON, K., PICKUP, R. & HERMON-TAYLOR, J. (2003) Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. *Molecular and Cellular Probes,* 17, 157-164.

COLE, S. T., BROSCH, R., PARKHILL, J., GARNIER, T., CHURCHER, C., HARRIS, D., GORDON, S. V., EIGLMEIER, K., GAS, S., BARRY III, C. E., TEKAIA, F., BADCOCK, K., BASHAM, D., BROWN, D., CHILLINGWORTH, T., CONNOR, R., DAVIES, R., DEVLIN, K., FELTWELL, T., GENTLES, S., HAMLIN, N., HOLROYD, S., HORNSBY, T., JAGELS, K., KROGH, A., MCLEAN, J., MOULE, S., MURPHY, L., OLIVER, K., OSBORNE, J., QUAIL, M. A., RAJANDREAM, M.-A., ROGERS, J., RUTTER, S., SEEGER, K., SKELTON, J., SQUARES, R., SQUARES, S., SULSTON, J. E., TAYLOR, K., WHITEHEAD, S. & BARRELL, B. G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from complete genome sequence. *Nature,* 393, 537-544.

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

COWAN, L. S., MOSHER, L., DIEM, L., MASSEY, J. P. & CRAWFORD, J. T. (2002) Variable-Number Tandem Repeat Typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS6110 by using Mycobacterial Interspersed Repetitive Units. *Journal of Clinical Microbiology,* 40, 1592-1602.

CRISAN, D. & MATTSON, J. C. (1993) Retrospective DNA analysis using fixed tissue specimens. *DNA Cell Biology,* 12, 455-464.

CROSS, P. C. & GETZ, W. M. (2006) Assessing vaccination as a control strategy in an ongoing epidemic: Bovine tuberculosis in African buffalo. *Ecological Modelling,* 196, 494-504.

DAVID, S., RIBEIRO, D. R., ANTUNES, A., PORTUGAL, C., SANCHO, L. & DE SOUSA, J. G. (2007) Contribution of spoligotyping to the characterization of the population structure of *Mycobacterium tuberculosis* isolates in Portugal. *Infection, Genetics and Evolution,* 7, 609-617.

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

DE LISLE, G. W., BENGIS, R. G., SCHMITT, S. M. & O'BRIEN, D. J. (2002) Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue Scientifique et Technique-Office Internatiobal des Epizooties,* 21, 317-334.

DE VOS, V., BENGIS, R. G., KRIEK, N. P., MICHEL, A., KEET, D. F., RAATH, J. P. & HUCHZERMEYER, H. F. (2001) The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research,* 68, 119-130.

DUARTE, E. L., DOMINGOS, M., AMADO, A. & BOTELHO, A. (2008) Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates. *Veterinary Microbiology,* 130, 415-421.

DURMAZ, R., AYDIN, A., DURMAZ, B., AYDIN, N. E., AKBASAK, B. S. & GUNAL, S. (1997) Sensitivity of two-stage PCR amplication for detection of *Mycobacterium tuberculosis* in paraffin-embedded tissues. *Journal of Microbiological Methods,* 29, 69-75.

FERRER, I., ARMSTRONG, J., CAPELLARI, S., PARCHI, P., ARZBERGER, T., BELL, J., BUDKA, H., STROBEL, T., GIACCONE, G., ROSSI, G., BOGDANOVIC, N., FAKAI, P., SCHMITT, A., PETER, R., AL-SARRAJ, S., RAVID, R. & KRETZSCHMAR, H. (2007) Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: A BrainNet Europe Study. *Journal Compilation (c) 2007 International Society of Neuropathology.Brain Pathology*, 297-303.

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.

GILLIO-TOS, A., DE MARCO, L., FIANO, V., GARCIA-BRAGADO, F., DIKSHIT, R., BOFFETTA, P. & MERLETTI, F. (2007) Efficient DNA extraction from 25-year-old paraffin-embedded tissues: study of 365 samples. *Pathology,* 39, 345-348.

GORI, A., ESPOSTI, A. D., BANDERA, A., MEZZETTI, M., SOLA, C., MARCHETTI, G., FERRARIO, G., SALERNO, F., GOYAL, M., DIAZ, R., GAZZOLA, L., CODECASA, L., PENATI, V., RASTOGI, N., MORONI, M. & FRANZETTI, F. (2005) Comparison between spoligotyping and IS6110 restriction fragment length polymorphisms in molecular genotyping analysis of *Mycobacterium tuberculosis* strains. *Molecular and Cellular Probes,* 19, 236-244.

GRANGE, J. M., YATES, M. D., DE KANTOR, I. N. (1996) Guidelines for speciation within the Mycobacterium tuberculosis complex. Second edition. *World Health Organization: Emerging and other communicable diseases, surveillance and control. http://whqlibdoc.who.int/hq/1996/who_emc_zoo_96.4.pdf*

GREER, C. E., WHEELER, C. M. & MANOS, M. M. (1994) Sample preparation and PCR amplification from paraffin-embedded tissues. Cold Spring Harbor Laboratory Press.

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.

HEWINSON, R. G., VORDERMEIER, H. M., SMITH, N. H. & GORDON, S. V. (2006) Recent advances in our knowledge of *Mycobacterium bovis*: A feeling for the organism. *Veterinary Microbiology,* 112, 127-139.

HILTY, M., DIGUIMBAYE, C., SCHELLING, E., BAGGI, F., TANNER, M. & ZINSSTAG, J. (2005) Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of *Mycobacterium bovis* strains. *Veterinary Microbiology,* 109, 217-222.

HOSEK, J., SVASTOVA, P., MORAVKOVA, M., PAVLIK, I. & BARTOS, M. (2006) Methods of mycobacterial DNA isolation from different biological material: a review. *Veterinarni Medicina,* 51, 180-192.

HUARD, R. C., DE OLIVEIRA LAZZARINI, L. C., BUTLER, R. W., VAN SOOLINGEN, D. & HO, J. L. (2003) PCR-based method to differentiate the subspecies of *Mycobacterium tuberculosis* complex on the basis of genomic deletions. *Journal of Clinical Microbiology,* 41, 1637-1650.

JENKINS, A. O., VENTER, E. H., HUTAMO, K., GODFROID, J. (2010) Comparison of the capillary and agarose electrophoresis based multiple locus VNTR (variable number of tandem repeats) analysis (MLVA) on *Mycobaterium bovis* isolates. *Veterinary Microbiology*, 145, 172-176.

KAMERBEEK, J., SCHOULS, L., KOLK, A., VAN AGTERVELD, A., VAN SOOLINGEN, D., KUIJPER, S., BUNSCHOTEN, A., MIOLHUIZEN, H., SHAW, R., GOYAL, M. & VAN EMDEN, J. (1997) Simultaneous detection and strain differentiation of *Mycobacterium*

*tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology,* 35, 907-914.

KLOECK, P. E. (1998) Tuberculosis of Domestic animals in areas surrounding the Kruger National Park. *Proceedings of the challenges of managing tuberculosis in wildlife in Southern Africa.* Nelspruit, South Africa.

KOCK, R. A. (2004) What is this Infamous "Wildlife/Livestock Disease Interface?" A review of Current Knowledge for the African Continent. *In: IUCN Species Survival Commission report on Conservation and Development Interventions at the Wildlife/Livestock Interface Implications for Wildlife, Livestock and Human Health.* S Osofsky (Ed.) pp. 1-14).

LE FLECHE, P., HAUCK, Y., ONTENIENTE, L., PRIEUR, A., DENOEUD, F., RAMISSE, V., SYLVESTRE, P., RAMISSE, F. & VERGNAUD, G. (2001) A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BMC Microbiology,* 1.

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. *BMC Microbiology,* 2.

LE FLECHE, P., JACQUES, I., GRAYON, M., AL DAHOUK, S., BOUCHON, P., DENOEUD, F., NOCKLER, K., NEUBAUER, H., GUILLOTEAU, L. A. & VERGNAUD, G. (2006) Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiology,* 6.

LEGRAND, B., DE MAZANCOURT, P., DURIGON, M., KHALIFAT, V. & CRAINIC, K. (2002) DNA genotyping of unbuffered formalin fixed paraffin embedded tissues. *Forensic Science International,* 125, 205-211.

LIEBANA, E., JOHNSON, L., GOUGH, J., DURR, P., JAHANS, K., CLIFTON-HADLEY, R., SPENCER, Y., HEWINSON, R. G. & DOWNS, S. H. (2008) Pathology of naturally occurring bovine tuberculosis in England and Wales. *The Veterinary Journal,* 176, 354-360.

LINDSTEDT, B. A. (2005) Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis,* 26, 2567-2582.

LOESCHKE, S., GOLDMANN, T. & VOLLMER, E. (2005) Improved detection of mycobacterial DNA by PCR in formalin-fixed, paraffin-embedded tissues using thin sections. *Pathology Research and Practice,* 201, 37-40.

MAES, M., KREMER, K., VAN SOOLINGEN, D., TAKIFF, H. & DE WAARD, J. H. (2008) 24-locus MIRU-VNTR genotyping is a useful tool to study the molecular epidemiology of tuberculosis among Warao Amerindians in Venezuela. *Tuberculosis,* 88, 490-494.

MATHURIA, J. P., SHARMA, P., PRAKASH, P., SAMARIA, J. K., KATOCH, V. M. & ANUPURBA, S. (2008) Role of spoligotyping and IS6110-RFLP in assessing genetic diversity of *Mycobacterium tuberculosis* in India. *Infection, Genetics and Evolution,* 8, 346-351.

MICHALIK, S. S. (2008) Overcoming poor quality DNA. *Drug Discovery*, 24.

MICHEL, A. L. (2002) The epidemiology of *M. bovis* infection in South African wildlife. *Abstracts of the Veterinary European Network on Mycobacterium (VENOM) Symposium: DNA Fingerprinting of Bovine TB strains.* Belfast, Northern Ireland.

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

MICHEL, A. L., DE KLERK, L. -M., VAN PITTIUS, N. C. G., WARREN, R. M.,VAN HELDEN, P. D. (2007) Bovine tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. *BMC Veterinary Research,* 3.

MICHEL, A.L. (2008) Mycobacterium fortuitum infection interference with mycobacterium bovis diagnostics: natural infection cases and a pilot experimental infection. *Journal of Veterinary Diagnostic Investigation*, 20, 501–503.

MICHEL, A.L., COOPER, D., JOOSTED, J., DE KLERKE, L.-M., JOLLES, A. (2011) Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine 98 (2011) 142-151*.

MILIAN-SUAZO, F., HARRIS, B., DIAZ, C. A., TORRES, C. R., STUBER, T., OJEDA, G. A., LOREDO, A. M., PEREZ SORIA, M. & PAYEUR, J. B. (2008) Molecular epidemiology of *Mycobacterium bovis*: Usefulness in international trade. *Preventive Veterinary Medicine,* 87, 261-271.

MILLER, J., JENNY, A., RHYAN, J., SAARI, D. & SUAREZ, D. (1997) Detection of *Mycobacterium bovis* in formalin-fixed, paraffin embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* organisms. *Journal of Veterinary Diagn Invest,* 9, 244-249.

MILLER, J. M., JENNY, A. L. & PAYEUR, J. B. (2002) Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and Mycobacterium avium organisms in formalin-fixed tissues from culture-negative ruminants. *Veterinary Microbiology,* 87, 15-23.

MONTALI, R. J. (2007) Tracking Emerging Wildlife Diseases on Three Continents: Implications for Conserved Species and Zoonotic Potentials. *24th annual pathology meeting of the west coast subdivision of the CL Davis DVM Foundation.* Monterey, California.

MORRIS, R. S., PFEIFFER, D. U. & JACKSON, R. (1994) The epidemiology of *Mycobacterium bovis* infections. *Veterinary Microbiology,* 40, 153-177.

NEONAKIS, I. K., GITTI, Z., KRAMBOVITIS, E. & SPANDIDOS, D. A. (2008) Molecular diagnostic tools in mycobacteriology. *Journal of Microbiological Methods,* 75, 1-11.

PARSONS, L. M., BROSCH, R., COLE, S. T., SOMOSKOVI, A., LODER, A., BRETZEL, G., SOOLINGEN, D. V., HALE, Y. M. & SALFINGER, M. (2002) Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. *Journal of Clinical Microbiology,* 40, 2339-2345.

PAYLOR, R. (2007) Animal disease diagnostic laboratory. IN MOUSER, P. (Ed.) *Bovine Tuberculosis.*

PERUMAALLA, V. S., ADAMS, L. G., PAYEUR, J., BACA, D. & FICHT, T. A. (1999) Molecular fingerprinting confirms extensive cow-to-cow intra-herd transmission of a single *Mycobacterium bovis* strain. *Veterinary Microbiology,* 70, 269-276.

POURCEL, C., ANDRE-MAZEAUD, F., NEUBAUER, H., RAMISSE, F. & VERGNAUD, G. (2004) Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BMC Microbiology,* 4.

PRINS, H. H. T. (1987) Nature Conservation as an integral part of optimal land use in East Africa: The case of the Masai Ecosystem of Northern Tanzania. *Biological Conservation,* 40, 141-161.

RAO, K. R., KAUSER, F., SRINIVAS, S., ZANETTI, S., SECHI, L. A., AHMED, N. & HASNAIN, S. E. (2005) Analysis of genomic downsizing on the basis of region-of-difference polymorphism profiling of *Mycobacterium tuberculosis* patient isolates reveals geographic partitioning. *Journal of Clinical Microbiology,* 43, 5978-5982.

RAVIGLIONE, M. C., SNIDER JR, D. E. & KOCHI, A. (1995) Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *Journal of the American Medical Association,* 273.

RAZANAMPARANY, V. R., QUIRIN, R., RAPAOLIARIJAONA, A., RAKOTOARITAHINA, H., VOLOLONIRINA, E. J., RASOLONAVALONA, T., FERDINAND, S., SOLA, C., RASTOGI, N., RAMAROKOTO, H. & CHANTEAU, S. (2006) Usefulness of restriction fragment length polymorphism and spoligotyping for epidemiological studies of *Mycobacterium bovis* in Madagascar: Description of new genotypes. *Veterinary Microbiology,* 114, 115-122.

RODWELL, T. C., KRIEK, N. P., BENGIS, R. G., WHYTE, I. J., VILJOEN, P. C., DE VOS, V. & BOYCE, W. M. (2001) Prevalence of bovine tuberculosis in african buffalo at kruger national park. *Journal of Wildlife Diseases,* 37, 258-264.

ROHONCZY, E. B., BALACHANDRAN, A. V., DUKES, T. W., PAYEUR, J. B., RHYAN, J. C., SAARI, D. A., WHITING, T. L., WILSON, S. H. & JARNAGIN, J. L. (1996) A comparison of gross pathology, Histopathology, and mycobacterial culture for the diagnosis of tuberculosis in elk (*Cervus elaphus*). *Can J Vet Res,* 60, 108-114.

ROMANO, M. I., AMADIO, A., BIGI, F., KLEPP, L., ETCHECHOURY, I., NOTO LLANA, M., MORSELLA, C., PAOLICCHI, F., PAVLIK, I., BARTOS, M., LEAO, S. C. & CATALDI, A. (2005) Further analysis of VNTR and MIRU in the genome of *Mycobacterium avium* complex, and application to molecular epidemiology of isolates from South America. *Veterinary Microbiology,* 110, 221-237.

ROMERO, B., ARANAZ, A., SANDOVAL, A., ALVAREZ, J., DE JUAN, L., BEZOS, J., SANCHEZ, C., GALKA, M., FERNANDEZ, P., MATEOS, A. & DOMINGUEZ, L. (2008) Persistence and molecular evolution of *Mycobacterium bovis* population from cattle and wildlife in Donana National Park revealed by genotype variation. *Veterinary Microbiology,* 132, 87-95.

RORING, S., SCOTT, A. N., GLYN HEWINSON, R., NEILL, S. D. & SKUCE, R. A. (2004) Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland. *Veterinary Microbiology,* 101, 65-73.

RUMPH, P. F. & WILLIAMS, J. C. (1986) A comparison of the efficiency of water and ethanol at removing formaldehyde from immersion fixed muscle tissues. *Anatomia Histologia Embryologia,* 15, 269-276.

SANTOS, S., SA, D., BASTOS, E., GUEDES-PINTO, H., GUT, I., GARTNER, F. & CHAVES, R. (2009) An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues. *Research in Veterinary Science,* 86, 421-426.

SCHANDER, C. & HALANYCH, K. M. (2003) DNA, PCR and formalinized animal tissue - a short review and protocols. *Organisms Diversity and Evolution,* 3, 195-205.

SETHUSA, T. M. (2006) Evaluation of a method used to detect *Mycobaterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* in formalin fixed paraffin embedded tissues of domestic and wild animals. Msc. dissertation. Department of *Veterinary Tropical Disease.* University of Pretoria.

SMITH, N. H., KREMER, K., INWALD, J., DALE, J., DRISCOLL, J. R., GORDON, S. V., VAN SOOLINGEN, D., GLYN HEWINSON, R., SMITH, J. M. (2006) Ecotypes of the *Mycobacterium tuberculosis* complex. *Journal of Theoretical Biology,* 239, 220-225.

SOLA, C., FILLIOL, I., LEGRAND, E., LESJEAN, S., LOCHT, C., SUPPLY, P. & RASTOGI, N. (2003) Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infection, Genetics and Evolution,* 3, 125-133.

SONG, E. J., JEONG, H. J., LEE, S. M., KIM, C. M., SONG, E. S., PARK, Y. K., BAI, G.-H., LEE, E. Y. & CHANG, C. L. (2007) A DNA chip-based spoligotyping method for the strain identification of *Mycobacterium tuberculosis* isolates. *Journal of Microbiological Methods,* 68, 430-433.

SREEVATSAN, S., PAN, X., STOCKBAUER, K. E., CONNELL, N. D., KREISWIRTH, B. N., WHITTAM, T. S. & MUSSER, J. M. (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proceedings of the National Academy of Sciences of the United Staes of America,* 94, 9869-9874.

SRINIVASAN, M., SEDMAK, D. & JEWELL, S. (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *American Journal of Pathology,* 161, 1961-1971.

SUN, G., KAUSHAL, R., PAL, P., WOLUJEWICZ, M., SMELSER, D., CHENG, H., LU, M., CHAKRABORTY, R., JIN, L., DEKA, R. (2005) Whole-genome amplification: relative efficiencies of the current methods. *Legal Medicine,* 7, 279-286.

SUPPLY, P., MAZARS, E., LESJEAN, S., VINCENT, V., GICQUEL, B. & LOCHT, C. (2000) Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Molecular Microbiology,* 36, 762-771.

SURESH, N., ARORA, J., PANT, H., RANA, T. & SINGH, U. B. (2007) Spoligotyping of *Mycobacterium tuberculosis* DNA from Archival Ziehl-Neelsen-stained sputum smears. *Journal of Microbiological Methods,* 68, 291-295.

TANNER, M. & MICHEL, A. L. (1999) Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park *Onderstepoort Journal of Veterinary Research,* 66, 185-190.

TAUTZ, D. & RENZ, M. (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research,* 12, 4127-4138.

TODAR, K. (2008) Todar's online textbook of bacteriology. *http://www.textbookofbacteriology.net/.*

VAN BELKUM, A. (2008) Newer methods for bacterial strain typing. *Clinical Microbiology Newsletter,* 30, 63-69.

VAN EMBDEN, J. D. A., DONALD CAVE, M., CRAWFORD, J. T., DALE, J. W., EISENACH, K. D., GICQUEL, B., HERMANS, P., MARTIN, C., MCADAM, R., SHINNICK, T. M., SMALL, P. M. (1993) Strain identification of mycobacterium tuberculosis by DNA fingerprinting: Recommendations for a standardized methodology. *Journal of Clinical Microbiology,* 31, 406-409.

VAN SOOLINGEN, D., HERMANS, P. W. M., DE HAAS, P. E. W., SOLL, D. R., VAN EMBDEN J. D. A. (1991) Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: Evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *Journal of Clinical Microbiology,* 29, 2578-2586.

VAN SOOLINGEN, D. (2001) Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. *Journal of Internal Medicine,* 249, 1-26.

WANG, F., WANG, L., BRIGGS, C., SICINSKA, E., GASTON, S. M., MAMON, H., KULKE, M. H., ZAMPONI, R., LODA, M., MAHER, E., OGINO, S., FUCHS, C. S., HADER, C. & MAKRIGIORGOS, M. G. (2007) DNA degradation test predicts success in whole-genome amplification from diverse clinical samples. *Journal of Molecular Diagnostics,* 9, 441-451.

WARREN, R. M., VAN PITTIUS, N. C. G., BARNARD, M., HESSELING, A., ENGELKE, E., DE KOCK, M., GUTIERREZ, M. C., CHEGE, G. K., VICTOR, T. C., HOAL, E. G. & VAN HELDEN, P. D. (2006) Differentiation of *Mycobacterium tuberculosis* complex by

PCR amplification of genomic regions of difference. *International Journal of Tuberculosis and Lung Disease,* 10, 818-822.

WARREN, R. M., VICTOR, T. C., STREICHER, E. M., RICHARDSON, M., BEYERS, N., GEY VAN PITTIUS, N. C. & VAN HELDEN, P. D. (2004) Patients with Active Tuberculosis often Have Different Strains in the Same Sputum Specimen. *American Journal of Respiratory and Critical Care Medicine,* 169, 610-614.

ZANE, L., BARGELLONI, L. & PATARNELLO, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology,* 11, 1-16.

ZSIKLA, V., BAUMANN, M. & CATHOMAS, G. (2004) Effect of buffered formalin on amplification of DNA from paraffin wax embedded small biopsies using real-time PCR. *Journal of Clinical Pathology,* 57, 654-656.