Characterization of tuberculous lesions in naturally infected African buffalo (*Syncerus caffer)*

by
Cláudio Laisse

Thesis presented in partial fulfilment of the requirements for the degree
Master of Science in Medical Sciences (Medical Biochemistry)
at the University of Stellenbosch

Supervisor: Prof Paul David van Helden
Co-supervisors: Prof Dolores Gavier-Widén
and Dr. Erik O. Ågren
Faculty of Health Sciences
Division of Molecular Biology and Human Genetics

December 2010

DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: November 2010

Cláudio João Mourão Laisse

ABSTRACT

Mycobacterium bovis has a wide host range and infects many wild and domestic animal species as well as humans. African buffalo (*Syncerus caffer)* is considered to be a wildlife reservoir of *M. bovis* in certain environments in South Africa, such as in the Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HiP).

A detailed pathological study was conducted on 19 African buffalos (*Syncerus caffer*) from a herd in the HiP in South Africa. The animals tested positive to the intradermal bovine tuberculin test and were euthanazed during a test-and-cull operation to decrease the prevalence of bovine tuberculosis (bTB) in the park. The superficial, head, thoraxic and abdominal lymph nodes and the lungs were examined grossly for presence of tuberculous lesions and were scored on a 1-5 scale for macroscopic changes. The gross lesions were examined histologically and scored I-IV according to a grading system used for bTB lesions in domestic cattle. Macroscopical lesions were limited to the retropharyngeal, bronchial, and mediastinal lymph nodes and the lungs. The most frequently affected lymph nodes were the bronchial (16/19) and mediastinal (11/19). All four grades of microscopic lesions were observed, although grade II lesions were the most frequent. Acid-fast bacilli were observed only rarely. Bovine tuberculosis was confirmed by PCR analyses.

All animals were in good body condition and most of the lesions were in an early stage of development, indicating an early stage of the disease. The absence of lesions in the mesenteric lymph nodes and the high frequency of lesions in respiratory tract associated lymph nodes suggest that the main route of *M. bovis* infection in African buffalo is inhalatory rather than alimentary. This study presents a systematic evaluation and semi-quantification of the severity and stages of development of tuberculous lesions in buffalo. The results may contribute to i) the understanding of the pathogenesis of the disease, ii) the evaluation of experimental models of *M. bovis* infection in *Syncerus caffer*, and iii) the interpretation of pathological data from vaccination trials.

OPSOMMING

Mycobacterium bovis het ‘n wye reeks van gashere en dit infekteer verskeie wilde en mak dierespesies, sowel as mense. Die buffel (*Syncerus caffer)* word beskou as die wild reservoir van *M. bovis* in sekere dele van Suid Afrika, soos in die Kruger Nasionale Park (KNP) en Hluhluwe-iMfolozi Park (HiP).

‘n Breedvoerige patologiese studie is uitgevoer op 19 buffels afkomstig vanaf ‘n trop in die HiP in Suid Afrika. Die diere het almal positief getoets vir die intradermale beestuberkulin toets en is uitgesit tydens ‘n toets-en-slag operasie met die doel om die voorkoms van beestuberkulose (bTB) in die park te bekamp. Die oppervlakkige, kop, toraks en abdominale limfknope en longe is oorsigtelik ondersoek vir die teenwoordigheid van tuberkulose letsels en was ‘n punt toegeken op ‘n skaal van 1-5 vir die teenwoordigheid van makroskopiese veranderinge. Die opsigtelike letsels is histologies ondersoek en ‘n I-IV punt toegeken volgens die gradering wat gebruik word vir bTB letsels in beeste. Makroskopiese letsels was beperk tot die retrofaringeale, brongiale, en mediastinale limfknope en in die longe. Die brongiale (16/19) en mediastinale (11/19) limfknope was meestal geaffekteerd. Al vier grade van mikroskopiese letsels is gevind, alhoewel graad II letsels die volopste was. Suur-vaste basille is slegs selde waargeneem. Beestuberkulose is bevestig deur PKR analises.

Al die diere was in ‘n goeie kondisie en meeste van die letsels was in ‘n vroeë stadium van ontwikkeling, wat aandui op ‘n vroeë fase van die siekte. Die afwesigheid van letsels in die mesenteriese limfknope en die hoë frekwensie van letsels in die lugweg geassosieerde limfkliere dui daarop dat the belangrikste roete van *M. bovis* infeksie in die buffel deur inaseming geskied eerder as deur opname in die spysverteringskanaal. Hierdie studie bied ‘n stelselmatige evaluering en semi-kwantifisering van die graad van erns en die stadia van ontwikkeling van tuberkulose letsels in buffels. Die resultate kan bydra tot i) die begrip van die patogenese van die siekte, ii) die evaluering van eksperimentele modelle van *M. bovis* infeksie in *Syncerus caffer*, en iii) die interpretasie van patologiese data van inentingsproewe.

ACKNOWLEDGEMENTS

This thesis results from work that was carried out at HiP, and in the Veterinary Faculty, Eduardo Mondlane University, Mozambique and Faculty of Health Science, Stellenbosch University, South Africa. Many people were involved, not only providing their scientific inputs but also their encouragement and assistance every time I asked. I wish to express my grateful acknowledgement to them.

My Supervisor, Prof. Paul van Helden and Co-supervisors, Prof Dolores Gavier-Widén and Erik Ågren for their invaluable guidance, advice and patience during the course of my master studies.

Special thanks to Dr Adelina Machado for transporting me for so many days to and from Hluhluwe IMfolozi Park and for her help with the sampling and also for her fundamental encouragement, advice and support during my studies.

Dr Dave Cooper, from Hluhluwe iMfolozi Park for providing the excellent facilities and appropriate environment for the sampling. My gratitude also goes to all the workers of the Hluhluwe iMfolozi Park slaughter house for their help during the slaughtering and sampling.

My colleague Doctor Custódio Bila for giving me the opportunity to be involved in this research project and also for his encouragement. Particular thanks to Dr Gaby Monteiro for her support and covering my absence at the University every time I had to travel. To Dr Roberto Insua for his encouragement and support at the beginning of this study.

Prof Rob Warren for assisting with the processing of paraffin-embedded material for the DNA extraction and the PCR and also for his assistance with the manuscript. I also extend my thanks to Ms Annemie Jordaan for her expert assistance on the DNA extraction.

Prof Fátima Gartner from the Pathology department of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal for providing me with the excellent facilities and appropriate environment for training in histochemistry techniques.

Prof Guillermo Ramis and Dr Juan J. Quereda at the Departamento de Producción Animal, Facultad de Veterinaria, Universidad de Murcia, Spain for their technical assistance on the method 2 used for DNA extraction and PCR.

To Mr. Gabriel Chaúque, Ms Joana Jamisse and Mr Leonardo Ngoca at the Pathology Section of the Veterinary Faculty, Eduardo Mondlane University for the processing, sectioning and staining of the histopathological sections.

Dr Borna Müller, for his comments on the manuscript and also for his friendship and support. My gratitude also goes to Ms Mandi Alblas, technician at the Division of Anatomy and Histology, Faculty of Health Science, Stellenbosch University for assisting me with the microscopic photographs.

My parents Joaquim Laisse and Teresa Sarmento for all the efforts, encouragement and support to ensure my academic and moral education. To them I dedicate this work. To my sisters Gercia and Sheila and brothers Edson and Gerdes to be friendly and supportive all the time. My cousins Albertina and Zefanias, for their affection and friendship. My uncle Ricardo Laisse for all his support.

My special gratitude goes to Delta Moisés for her encouragement, affection and friendship. Thank you very much.

I am also grateful for everyone not mentioned that direct or indirectly contributed for this study. Finally but specially I thank GOD for blessing and guiding me.

Funding

This study was funded by the Swedish International Development Cooperation Agency, Department for Research cooperation (Sida/SAREC)

This organization has been supporting research in Mozambique for many years. The overall objective of Sida's research support to Mozambique is to assist the country in its efforts to build sustainable conditions for research and research management and to support research of national relevance for the development of the country.

Sida/SAREC support to the University Eduardo Mondlane (UEM) began in 1978 and has been supporting the Veterinary Faculty since early 90's mainly through funding post-graduate and research projects, to build capacity in terms of research infrastructure and equipment.

Since 2006, Sida/SAREC has been funding the program "**Impact of Zoonotic Diseases in Animal Production and Public Health in Mozambique**" in which the present study is integrated.

LIST OF ABBREVIATIONS

^0C	degrees Celsius
µm	micrometer
µl	microliter
AIDS	Acquired immunodeficiency syndrome
AFB	Acid fast bacilli
B	bronchial
BCG	Bacille Calmette-Guérin
bp	base pair
bTB	Bovine tuberculosis
cm	centimeters
Cfu	Colony forming units
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene-diamine-tetra-acetic acid
EIA	Antibody Enzyme Immunoassay
EITB	Enzyme linked immunoelectrotransfer blot
ELISA	Enzyme-linked immunosorbent assay
F	female
FFPE	Formalin-fixed and paraffin-embedded
Km^2	Kilometer square
KZN	KwaZulu-Natal
ha	hectare
HE	Haematoxilin and Eosin
HiP	Hluhluwe iMfolozi Park
HIV	Human Immunodeficiency Virus
INFg	interferon gamma
KNP	Kruger National Park
L	lung
LN	lymph node
M	male

MAPIA	Multi-antigen printing immunoassay
min	minute
mm	millimeter
mg	milligram
$MgCl_2$	Magnesium dichloride
ML	mediastinal
MT	Masson's Trichrome
MTC	Mycobacterium tuberculosis complex
NTM	Non-tuberculous mycobacteria
NVL	Non visible lesions
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
PGRS	Poly G repeat sequence
pH	potential of hydrogen
PPD	Purified Protein Derivative
QENP	Queen Elizabeth National Park
RD	regions of difference
RFLP	Restriction fragment length polymorphism
rpm	rotations per minute
RT	room temperature
RT PCR	Real time polymerase chain reaction
sec	second
Sida/SAREC	Swedish International Development Cooperation Agency, Department for Research cooperation
TB	Tuberculosis
TE	Tris EDTA
TST	Tuberculin skin test
UEM	Universidade Eduardo Mondlane
WHO	World Heath Organization
VNTR	Variable number tandem repeat
ZN	Ziehl Neelsen

LIST OF FIGURES

CHAPTER 1

Figure 1.1. Map of Hluhluwe iMfolozi Park……. 3

CHAPTER 2

Figure 2.1. Dead animals before slaughtering ……...……... 26

Figure 2.2. Animals during the slaughtering process ……...…… 27

Figure 2.3. Inspection of the lung, liver and thoracic lymph nodes …….……..... 27

Figure 2.4. Inspection of the head lymph nodes ……...……... 28

CHAPTER 3

Figure 3.1. A bronchial lymph node with white caseogranulomatous lesions (Grade 2)…… 35

Figure 3.2. An enlarged retropharyngeal lymph node with white caseogranulomatous lesions (Grade 2)……...…….. 35

Figure 3.3. Bronchial lymph node with whitish granulomatous lesion replacing the lymphoid tissue (Grade 5)……..……. 36

Figure 3.4. Enlarged mediastinal lymph node with granulomatous white lesions replacing the lymphoid tissue. On cut surface the LN was gritty (Grade 5)……...…..…… 36

Figure 3.5. An enlarged mediastinal lymph node with a multinodular appearance (Grade 5)……..... 37

Figure 3.6. White multifocal to coalescing caseogranulomatous TB lesions in the lung (grade 3)... ……..... 37

Figure 3.7. Animal 79, mediastinal lymph node. Multiple stage I granulomas composed of epithelioid cells and multinucleated giant cells and lymphocytes. HE. 100X …….…… 41

Figure 3.8. Animal 79, mediastinal lymph node. Stage I granuloma, epithelioid cell and multinucleated giant cells surrounded by lymphocytes. HE. 200X……. 41

Figure 3.9. Animal 69, lung, Stage I granuloma: non-encapsulated lesion composed of epithelioid cells surrounded by lymphocytes. HE. 100X …… 42

Figure 3.10. Animal 77, mediastinal lymph node, Stage II granuloma: a central caseous necrosis, surrounded by epithelioid cells, giant cells and lymphocytes. HE. 200X……..... 42

Figure 3.11. Animal 69, lung, Stage II granuloma: central caseous necrosis, surrounded by epithelioid cells, multinucleated giant cells and lymphocytes. HE. 100X. ……..... 43

Figure 3.12. Animal 69, lung, Stage II granulomas. High magnification of the periphery of the granulomas. Necrotic area (bottom right) surrounded by epithelioid cells, multinucleated giant cells and lymphocytes. HE. 200X..... 43

Figure 3.13. Animal 57, bronchial lymph node, Stage III granuloma: central area of caseous necrosis and mineralization, surrounded by multinucleated giant cells and lymphocytes. HE. 200X…….. 44

Figure 3.14. Animal 57, bronchial lymph node**,** Stage IV granulomas: extensive confluent areas of necrosis with multiple foci of mineralization. HE. 100X. …….. 44

Figure 3.15. Periphery of stage IV granuloma. A necrotic area (left) surrounded by inflammatory cells and connective tissue with neovascularization. HE. 200X.…… 45

Figure 3.16. A single acid fast bacillus (white arrow) in a multinucleated giant cell. ZN. 1000X…….. 45

Figure 3.17. Agarose gel (2%) stained with ethidium bromide, showing RD polymerase chain reaction products……... 46

Figure 3.18. "Amplification plot" shows the amplification curve for the buffalo samples. The two lines at the left represent the positive control……. 47

Figure 3.19. "Melting curve" shows the melting temperature for the amplicons. The two lines at the right are for *Mycobacterium avium.* The curves *for Mycobacterium bovis* are in the group, together with the curves for the buffalo samples……... 48

LIST OF TABLES

CHAPTER 3

Table 3.1. Lymph node and lung samples collected from African buffalo with tuberculosis lesions…….…… 34

Table 3.2. African buffalo animal number, sex, age class, tissue samples taken and microscopic lesion scores and presence or absence of acid-fast bacilli in Ziehl-Neelsen stained sections……. 40

LIST OF ADDENDUM

TOPIC		PAGE
DECLARATION……		ii
ABSTRACT…….……....		iii
OPSOMMING……..……		iv
ACKNOWLEDGEMENTS ……		v
LIST OF ABREVIATIONS…….....		viii
LIST OF FIGURES……..……...		x
LIST OF TABLES……...		x
LIST OF ADDENDUM…….....		xiii
CHAPTER 1: INTRODUCTION……….....		1
1.1.	Background…….....	1
1.2.	Literature review…….....	6
1.2.1.	Causal agent and hosts…….....	6
1.2.2.	Disease manifestation……...	7
1.2.3.	Transmission of tuberculosis……	8
1.2.4.	Ante-mortem diagnosis of tuberculosis……...……	12
1.2.5.	Pathology of tuberculosis…….	15
1.2.5.1.	Necropsy findings…….	15
1.2.5.2.	Histopathology…….	17
1.2.6.	Bacteriology…….	19
1.2.7.	Molecular diagnosis…….	20
1.2.8.	Control of tuberculosis…….	21
CHAPTER 2: MATERIAL AND METHODS……..…..…….		25
2.1.	Animals……	25
2.2.	Post-mortem examination and sample collection…….	25
2.3.	Grading of macroscopic lesions……...	26
2.4.	Laboratory examinations……..	28

2.4.1. Histopathology……. 28
2.4.2. Bacteriology……. 29
2.4.3. Identification of *M. bovis* by polymerase chain reaction (PCR)…….. 30
2.4.3.1. Method 1……... 30
2.4.3.1.1. DNA Extraction procedure……... 30
2.4.3.1.2. PCR amplification…… 31
2.4.3.2. Method 2……... 31
2.4.3.2.1. DNA extraction……...…… 31
2.4.3.2.2. PCR amplification…… 32

CHAPTER 3: RESULTS……..…….... 33
3.1. Gross pathology…… 33
3.2. Laboratory examinations…….. 38
3.2.1. Histopathology……. 38
3.2.2. Bacteriology……. 46
3.2.3. PCR……..…… 46

CHAPTER 4: DISCUSSION……...…….. 49
4.1. Gross pathology……...…… 49
4.2. Histopathology……..…….. 53
4.3. PCR……..……... 55
4.4. Conclusion ……..…… 56
4.5. Limitations of the study……..……. 57

REFERENCES……. 58
APPENDIX 1…….…….. 71
APPENDIX 2……... 76
APPENDIX 3……... 78
APPENDIX 4……... 79
APPENDIX 5……... 80

CHAPTER 1: INTRODUCTION

1.1. Background

Tuberculosis has a wide host range and infects many wild and domestic animal species as well as humans (De Lisle et al., 2002). Bovine tuberculosis (bTB) is an important disease in many parts of the world because of its zoonotic potential and its economic and conservation impacts (Cheeseman et al., 1989; Cosivi et al., 1998; Caron et al., 2003).

The world Health Organization (WHO) estimates that human tuberculosis where the causative agent is *Mycobacterium tuberculosis* associated with HIV is one of the most important causes of death in the world (WHO, 2004). Often, this situation arises from poor control programs. Likewise, zoonotic tuberculosis (TB) caused by *Mycobacterium bovis* is present in animals in most developing countries where surveillance and control activities are often inadequate or unavailable (Cosivi et al., 1998). However, in industrialized countries, good animal TB control and eradication programs have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans (Cosivi et al., 1998).

Bovine tuberculosis is regarded as one of the most serious wildlife health issues currently confronting conservationists and veterinary regulatory officials in South Africa. In South Africa, the disease has been reported in wildlife species both in captivity (Michel et al., 2003) and in free-ranging animals (De Lisle et al., 2002). The Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HiP), respectively the largest and the third largest national park in South Africa are the most affected free ranging ecosystems (Michel et al., 2006).

The African buffalo (*Syncerus caffer)* is considered to be a wildlife reservoir of *M. bovis* in KNP and HiP (De Vos et al, 2001; Michel et al., 2006). In the KNP it was apparently first introduced from domestic cattle in the 1960's or 1980's (Bengis et al., 1996) and was first found in African buffalo in 1990. In 2001, an average bTB prevalence in buffalo herds of the southern region of the KNP was estimated to be 30%, while in the central and northern regions the prevalence

levels were 16% and 1,5% respectively (Rodwell et al., 2001a). This prevalence has probably increased since that time.

In Southern Africa, bTB in free ranging animals has also been reported in the Queen Elizabeth National Park (QENP)-Ruwenzori National park, Uganda (Guilbride et al., 1963; Kalema-Zikusoca et al., 2005), Tarangine National Park, Tanzania (Cleaveland et al., 2005) and Gonarezhou National Park, Zimbabwe (De Garine-Wichatitsky et al., 2010).

In QENP, *Mycobacterium bovis* in African buffalo was first diagnosed in the early 1960's and it was concluded that as in KNP and HiP, bTB in the buffalo originated after contact with infected cattle (Woodford, 1982). A most recent study curried out in QENP used the gamma interferon test as a diagnostic test and found that exposure to *M. bovis* was detected in 21.6% of the buffaloes (Kalema-Zikusoka et al., 2005). Serological assays detected antibodies to *M. bovis* in one of 17 (6%) of buffalo in Tarangine (Cleaveland et al., 2005).

The sampling for this thesis was done in HiP. This park consists of 960 km² (96,000 ha) of hilly topography in central Zululand, KwaZulu-Natal (KZN) province, on the east cost of South Africa and is known for its rich wildlife and conservation efforts. Due to conservation efforts, the park now has the largest population of white rhino (*Ceratotherum simum*) in the world. The park is surrounded by communal farm land. The area was originally a royal hunting ground for the Zulu kingdom, but was established as a park in 1895.

Hluhluwe is characterized by hilly topography, and this northern section of the park is noted for its wide variety of both bird and animal life. IMfolozi, the southern component of the park is generally hot in summer, and mild to cool in winter, although cold spells do occur. The park hosts a vast range of wild animals and is the only state-run park in KZN where all the Big Five Game occurs. The park was previously known as Hluhluwe-Umfolozi and can be found as such on many maps, such as the one shown below.

The park has a buffalo population of approximately 3000 animals (Michel et al., 2006). The relatively high susceptibility to *M. bovis* and the social herd structure and behavioral patterns

make African buffalo an ideal reservoir species that not only maintains the infection but harbours a high infection incidence (De Vos et al., 2001). In HiP, bovine TB was first definitely diagnosed in African buffalo in 1986 (Jolles, 2004), although it is generally thought, and historical records suggest, that it entered the park owing to contact with affected cattle in the 1950s and 1960s (Dr Dave Cooper, personal communication).

Figure 1.1. Map of Hluhluwe iMfolozi Park (http://en.wikipedia.org/wiki/Hluhluwe-Umfolozi_Game_Reserve)

Currently TB in HiP buffalo population occurs at a herd prevalence that can vary from 5% to 50% between herds (D. Cooper, unpublished data). Since 1999 annual test-and-cull operations have been carried out as part of a tuberculosis control program aimed at reducing the prevalence of bTB in buffalo within the park.

Infected buffalo can contaminate the environment (Rodwell et al., 2001a; De Vos et al., 2001, Michel, 2002) and are considered to be a source of infection to other wild animal species, including predators and scavengers, as well as livestock (Michel, 2002; Caron et al., 2003). In South Africa, bTB has been diagnosed in Greater Kudu (*Tragelaphus strepsiceros*), Lion (*Panthera leo*), Eland (*Taurotragus oryx*), Warthog (*Phacochoerus aethiopicus*), Bushpig (*Potamochoerus porcus*), Large spotted genet (*Genetta tigrina*), Leopard (*Panthera pardus*), Spotted hyaena (*Crocuta crotuta*), Cheetah (*Acinonyx jubatus*), Chacma baboon (*Papio ursinus*), Impala (*Aepyceros melamus*) Honey badger (*Mellivoca capensis*) (Michel et al., 2006) and Black rhinoceros (*Diceros bicornis minor)* (Espie et al., 2009).

Previous reports of bTB in African buffalo (Bengis et al., 1996; De Vos et al., 2001) describe lesions as being most often located in the lymph nodes of the head, in the bronchial and mediastinal lymph nodes, and in the tonsils and lungs. The affected lymph nodes are enlarged and show lesions of variable size, which may contain foci of caseous necrosis and mineralization. In the lungs, bTB-lesions can be presented as either disseminated lesions, diffuse pneumonia, or as individual granulomas. Generalized forms of bTB also occur and affect the pleura, peritoneum, intestinal tract, various other internal organs as well as visceral and peripheral lymph nodes (Keet et al., 1994; Bengis et al., 1996).

In South Africa, studies on the epidemiology, prevalence and controlling strategies of bTB in buffalo have been done (Keet et al., 1994; De Vos et al., 2001; Rodwell et al., 2001a; De Lisle et al., 2002). However as far as can be ascertained, no detailed macroscopic and microscopical studies have been conducted in African buffalo naturally infected by *M. bovis*. Detailed description of the pathology of bTB has been conducted in cattle (Wangoo et al., 2005), red and fellow deer (Martín-Hernado et al., 2010). Considering that African buffalo is a very important reservoir of bTB in free-ranging ecosystems in Africa, more studies are needed to better understand the pathology and pathogenesis of the disease in this species.

The aim of this study was to provide a systematic and detailed description of the macroscopical and histopathological lesions caused by natural *M. bovis* infection in African buffalo, and apply a scoring system to classify lesions according to their size, cellular composition and degree of

development. The resulting semi-quantitative data facilitates the comparison of severity of lesions between individual animals. The goals of this study were i) to contribute to the understanding of the pathogenesis of natural disease in African buffalo, ii) to provide a semi-quantitative evaluation of the severity of the lesions, which can be used as a comparative base for experimental infection and vaccine efficacy evaluation studies.

1.2. Literature review

1.2.1. Causal agent and hosts

Tuberculosis (TB) is a chronic infectious disease affecting humans and animals both in the wild and in captivity. TB in domestic and wild animals is usually caused by *Mycobacterium bovis. M. bovis* is a member of closely related group of mycobacteria referred to as Mycobacterium tuberculosis complex (MTC) which comprises *M. tuberculosis, M. africanum, M. bovis, M. microti, M. bovis bacilli Calmette-Guérine* (BCG), *M. canettii and M. caprae. M. tuberculosis* is the main causative agent of human tuberculosis, although it has been also isolated from domestic and wild animals living in prolonged close contact with humans (Michalak et al., 1998; Montali et al., 2001; Alexander et al., 2002; Ameni et al., 2010). Human beings can also be infected by *M. bovis* (Huchzermeyer et al., 1994; Thoen et al., 2006; de Kantor et al., 2008), which can progress to disease, although often as a non-pulmonary manifestation (Cosivi et al., 1998).

Tuberculosis has been reported in many domestic and wildlife species, including cattle (Corner, 1994; Asseged *et al*., 2004; Liebana et al., 2007), African buffalo (Keet et al., 1994; Keet et al., 1996; Bengis et al., 1996), kudu (*Tragelaphus strepsiceros*) (Keet et al., 2001), Eurasian badger (*Meles meles*) (Gavier-Widen et al., 2001), red deer (*Cervus elaphus*) (Griffen et al., 1994), brushtail possums (*Trichorusus vulpecula*) (Jackson et al., 1995a), lion (*Panthera leo*) (Keet at al., 1996; Keet et al., 2008), cheetah (*Acinonyx jubatus*), Chacma baboon (*Papio ursinus*) (Keet at al., 1996), Leopard (*Panthera pardus*) (De Vos et al., 2001) and black rhinoceros (*Diceros bicornis minor*) (Espie et al., 2009).

The African buffalo in South Africa (Bengis et al., 1996), brushtail possum (*Tricosurus vulpecula*) in New Zealand (O'Neil and Pharo, 1995), Eurasian badger (*Meles meles*) in Britain (Nolan and Wilesmith, 1994) and white-tailed deer (*Odocoileus virginianus*) in Michigan (O'Brien et al., 2006) have been shown to act as maintenance hosts. Through these maintenance hosts, the infection can persist within an affected population through horizontal transmission between individuals, even in the absence of spillover of the disease from other sources (De Lisle et al., 2002).

Many species are considered “spillover hosts” and they almost certainly contract TB by scavenging or predating on infected animals. Some examples include feral cats (*Felis catus*) (Ragg et al. 1995), ferrets (*Mustela furo*) (Caley and Hone, 2005) and European hedgehogs (*Erinaceus europaeus*) (Lugton et al., 1995), lions and cheetah (Keet et al., 1996).

1.2.2. Disease manifestation

During early stages of the disease, the majority of infected animals in a variety of species show no outward clinical signs. However, bTB is a chronic and progressive disease and visible signs are usually manifested several weeks, months or years after infection (De Lisle et al., 2002). In cattle, as in other species, the manifestation of this disease is determined by the route by which the animal is infected, together with the host immune response and the virulence of the organism (Neill et al., 1994).

Clinical signs may be exacerbated by environmental factors, such as lack of grazing during droughts (De Vos et al., 2001). Stress conditions, such as the post-calving interval, can also contribute to aggravate signs (Huchzermeyer et al., 1994). During advanced stages of the disease when the lesions are disseminated, the animals gradually become emaciated and anorexic, manifest fluctuating temperature, have a dull coat, and may become lethargic. Dyspnoea may be a consequence of extensive pulmonary lesions or of enlargement of bronchial lymph nodes causing obstruction of airways. In cattle, the esophageal pressure from enlarged mediastinal lymphnodes may lead to persistent ruminal bloat (Huchzermeyer et al., 1994).

Change of behavior may occur at advanced stages of tuberculosis in badgers (*Meles meles*), possums (*Trichosurus vulpecula*) and baboons (*Papio ursinus*). For example, baboons, which are normally social, became solitary. In brush-tailed opossums, bovine tuberculosis is usually a fulminating pulmonary disease that typically lasts for two to six months. In the final stage of the disease, animals become disoriented, cannot climb, and may be seen wandering about during daylight (OIE, 2009).

Rapid loss of body condition, gross enlargement of lymph nodes and sudden death has been observed in infected ferrets (Qureshi et al., 2000). Clinical signs of TB in suricate (*Suricata suricata*) infected by *M. tuberculosis* included emaciation, weakness and progressive cachexia, and dyspnoea with variable enlargement of the head, neck, and axillar lymph nodes. These animals in some cases can also present a pronounced lack of fear response to humans (Alexander et al., 2002).

Clinical signs in Greater kudu with bovine tuberculosis include bilateral abscessation of parotid lymph nodes, frequently accompanied by formation of draining fistules (Keet et al., 2001). Lameness in one hind leg was the only clinical sign observed in a cheetah infected by *M. bovis* (Hilsberg and van Hoven, 2000). Cheetah with tuberculosis can also present weight loss, dull coat, alopecia and poorly healing skin wounds (De Lisle et al., 2002).

In African buffalo, bTB manifests as a chronic and predominantly subclinical disease and clinical signs usually develop at a terminal stage of the disease (De Vos et al., 2001). The main clinical signs are weight loss (Bengis et al., 1996) hoarse or dry coughing, dyspnoea, dull coat, arched back, depression and lagging behind the herd (De Vos et al., 2001).

Chronic cough, body weight loss, abscessation of right inguinal lymph node, and discharging thick creamy pus has been seen in captive chimpanzees (*Pan troglodytes*) infected by *M. tuberculosis*. Another chimpanzee from which *M. tuberculosis* was cultured from a cerebellar abscess, developed signs of nervous disturbance, difficulty in walking, shaking and sporadic surges of apparent pain (Michel et al., 2003).

1.2.3. Transmission of tuberculosis

The introduction of infected animals into non-infected herds is the primary mode of transmission of bTB between herds. Possible routes of infection with *M. bovis* are respiratory, alimentary, congenital, cutaneous, venereal route and via the teat canal (Cousins et al., 2004).

Many studies suggest that the two main routes of infection for tuberculosis in animals and man are respiratory and alimentary. In cattle the best evidence for the transmission route of *M. bovis*

is the pattern of lesions observed on slaughtered animals. Most of the studies have found that the lesions are commonly located in the broncho-mediastinal and head lymph nodes as well as in the lung, suggesting that the route of infection is principally via the respiratory tract (Huchzermeyer et al., 1994; Philips et al., 2003).

The possibility of transmission of bTB within a cattle herd can be influenced by factors such as herd size, the number of infected animals, nature of diet, behavior, existing farm practices and the disease control measures taken (Jackson et al., 1995b; Cousin et al., 2004). It has been demonstrated that high density of cattle and high humidity provide an ideal environment for transmission of the organism (Philips et al., 2003).

In the KNP the movement of animals between herds was probably the primary mode of transmission of the disease between buffalo herds (De Vos et al., 2001). The lung associated TB lesions in African buffalo suggest that the transmission of *M. bovis* between herd members can occur via the aerosol route (Keet at al., 1994; Bengis et al., 1996). The gregarious nature of buffalo facilitates the spread of the disease within the herd. Physical contact by licking, grooming and suckling between animals over a long period of time is necessary for disease transmission to occur (De Klerk et al., 2008). An experimental study suggested that in a free-ranging ecosystem, contamination of surface water by infected buffalo is not likely to play a significant role in the spread of *M. bovis* infection, since diseased buffalo do not commonly shed the organism in high quantities in nasal and oral discharges (Michel et al., 2007).

In the KNP, the lions most probably contract TB by consuming infected buffalo carcasses (Keet et al., 1996). Lesions in lions are commonly found in the gastrointestinal tract which suggests that the main route of infection for this species is oral. Gastrointestinal lesions are also common in other predators and scavengers (Michel et al., 2006).

Specific behavior such as socializing or intra-species aggression between lions may facilitate and predispose to aerosol and percutaneous transmission (Michel et al., 2006). Lions can also become infected by inhalation of infective material while killing and feeding on the carcasses of infected buffaloes, since shedding of bacteria from open lesions is possible (Keet et al., 1996).

Dissemination of the infection within prides by droplet infection from tuberculous lions is also a possibility in free ranging animals (Keet et al., 1996).

The gross lesion distribution and the histological characteristics of *M. bovis* infection in meerkats (*Suricata suricata*) indicate that the infection in this species is acquired mainly via the respiratory and oral routes (Drewe et al., 2008). In European badger (*Meles meles*), the isolation of *M. bovis* from tracheal lavage indicated the importance of aeogenous excretion (Gavier-Widen et al., 2001).

In a cattle abattoir survey, in Tanzania (Cleaveland et al., 2007) it was reported that the majority of animals (791/1290 = 61,3%) slaughtered had lesions in the gastrointestinal tract. These authors suggested that the faecal contamination of the environment could be the main source of tuberculosis infection for cattle in that study area. Nevertheless, infection via the gastrointestinal route from licking of mucous discharge cannot be excluded. Because of the incidence of endometrial tuberculoses in cattle, the congenital route of transmission is of some importance in this species (Huchzermeyer et al., 1994).

Behavioral interactions including den sharing, sniffing of orifices and faeces, cannibalism and aggressive breeding behavior can play an important role in *M. bovis* transmission, for example, between red deer (Lugton et al., 1998) and ferrets (Qureshi et al., 2000). Furthermore, in red deer, significantly higher prevalence of bovine tuberculosis occurred in males probably as a result of aggression between male red deer which increased their contact and thus increased transmission potential (Lugton et al., 1998). In ferrets, lesions are most often seen in the mesenteric and retropharyngeal lymph nodes, suggesting that ingestion of infectious material is an important source of transmission (Lugton et al., 1997; Qureshi et al., 2000).

The observation of TB lesions in young possums was an evidence of probable pseudo vertical transmission via the respiratory route or ingestion of milk (Jackson et al. 1995b). In the same study, one tracheal washing, one urine and faecal sample from three terminally ill possums were culture-positive, suggesting that contact with these substances could also result in infection. Badgers are believed to transmit infection to livestock by contaminating feed and water via

excreta (Qureshi et al., 2000). In zoos, close contact in enclosures, drinking areas and close feeding, as well as close proximity and exposure to the public, favor the maintenance of tuberculosis (Michel et al., 2003).

Jackson et al. (1995b) demonstrated that possums with fistulae had numerous lesions per individual and numerous lung lobes affected by lesions, compared to possums without fistules. Infected exudate that can drain from infected lymph nodes can contaminate the environment or directly infect other animals. In this way ingestion or contamination of skin wounds may transmit *M. bovis* (Hilsberg and van Hiven, 2000)**.** A study on European badger concluded that bite wounds with exudates containing mycobacteria is a potential route of infection for other badgers (Gavier-Widen et al., 2001).

M. bovis infection in humans mostly occurs through the consumption of infected milk and the disease manifestation is reported to be most frequently characterized by localized extrapulmonary lesions (Huchzermeyer et al., 1994). This route of infection has become rare in countries where milk is regularly pasteurized and bovine tuberculosis in controlled in cattle. However, *M. bovis* still occurs in human and the occupational aerogenous exposure to TB cattle and their carcasses remain a source of infection (de Kantor et al., 2008; OIE, 2009). Slaughter house and dairy industry workers continue to be at risk, particularly in areas or countries with an extensive livestock industry, but in which the cattle population is infected (de Kantor et al., 2008). With regards to wildlife conditions the main risk for humans to be directly infected by *M. bovis* would be through close contact with infected animals in confined spaces such as crates and pens and by handling infected carcasses (De Vos et al., 2001).

Although much concern is expressed about the potential danger to humans via the wildlife/domestic stock interface in South Africa, there is little or no evidence to suggest that this is a real risk (De Vos et al., 2001). Compared to the burden of disease in humans from *M. tuberculosis* in high risk countries, the burden of disease from *M. bovis* is probably very low in most cases. In South Africa, for example, there are almost 500000 new cases of human tuberculosis per annum (Department of Health, RSA and WHO data). No known cases of *M. bovis* infection or disease have been confirmed for at least the last 10 years (personal

communication, Prof Paul van Helden, Dr Gerrit Coetzee, NHLS). However, if the diagnosis of tuberculosis in humans does not include mycobacterial culture to identify the type of mycobacteria involved (if *M. tuberculosis* or *M. bovis*), as is the case in the routine diagnosis in many countries, the frequency of bovine tuberculosis in humans may be underestimated (D. Gavier-Widén, National Veterinary Institute, Uppsala, Sweden, personal communication).

Tuberculosis due to *M. tuberculosis* infection has been sporadically reported in domestic and wild animals living in prolonged close contact with humans (Montali, 2001; Michel et al., 2003; Ameni et al., 2010). Humans suffering from active TB are the most likely source of *M. tuberculosis* with infection spread via sputum, and rarely urine and faeces (Thoen and Steele, 1995). Ameni et al. (2010) highlighted the possible risk of human-to-cattle transmission of *M. tuberculosis* though the practice of mouth-to-mouth feeding of tobacco juice. The presence of lesions in the retropharyngeal and mesenteric lymph nodes of such cattle at post mortem was the supportive evidence of the alimentary route of infection.

1.2.4. Ante-mortem diagnosis of tuberculosis

The presumptive diagnosis of tuberculosis may be obtained by the clinical examination of an animal suspected to suffer from TB. Information on the introduction of animals into the herd, the confirmation of positive cases in neighboring properties, abattoir reports on animals sent for slaughter and possible contact with other animals that may be suffering from tuberculosis is of importance (Cousins et al., 2004).

The clinical examination and history are applicable to domestic animals and not to free-ranging wild animals. It is difficult to make a clinical diagnosis of TB at the early stages, because the disease is asymptomatic, particularly if the animals are in good body condition and do not show outward clinical manifestations of the disease (De Lisle et al., 2002; Cousins et al., 2004). Clinical examination of an animal suspected to be suffering from bTB requires the palpation of all superficial lymph nodes, the udder in females, and percussion and auscultation of the pulmonary area (OIE, 2004). The clinical diagnosis in live animals must be confirmed by the application of the tuberculin skin test or by laboratory tests.

The tuberculin skin test (TST), using purified protein derivative (PPD) is the most frequently applied ante-mortem test for the diagnosis of tuberculosis (Keet et al., 2008; OIE, 2009). This test has been used successfully as the principal tool for identifying infected cattle herds in control programs (De Lisle et al., 1995; De Lisle, 2002; Woodroffe et al., 2005). The TST has also been used to detect TB in experimentally infected cattle (Palmer et al., 2002), and free-ranging animas, such as buffaloes (de Klerk et al., 2006) and lions (Keet et al., 2008).

The TST can be performed using bovine tuberculin alone or as a comparative test that compares immunological response to *M. bovis* antigens with response to antigens derived from environmental mycobacteria. Tuberculin is injected intradermally in the cervical region or caudal fold of the tail and a positive test is indicated by a local swelling, caused by a delayed hypersensitivity reaction. In the late stage of the disease, in animals with poor immune response and in those that have recently calved, false negative responses are sometimes observed (OIE, 2009). Cattle infected with the avian tubercle bacillus are sensitive to mammalian tuberculin, which may result in a false positive diagnosis of the intradermal test (Huchzermeyer et al., 1994).

When the test is used in other species, it may be necessary to change the test site, the dose of tuberculin and the test interpretation. When applied to free-ranging wildlife it has the disadvantage to have to re-examine the animal 72 h after the injection of tuberculin and animal holding facilities are therefore required (Jolles et al., 2005). There is the possibility of transmission to naive animals during this holding period, adding to the disadvantages of using TST in wildlife (De Lisle et al., 2002, D. Cooper, personal communication).

The lymphocyte proliferation assay is an *in-vitro* assay that compares the reactivity of peripheral blood lymphocytes to bovine tuberculin PPD and a PPD from *Mycobacterium avium*. The assay can be performed on whole blood or purified lymphocytes from peripheral blood samples (Griffen et al., 1994). The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation times and the use of radio-active nucleotides). However, the test may be useful in wildlife and zoo animals. A blood test comprising lymphocyte transformation assays and

ELISA has been reported to have a high sensitivity and specificity in diagnosis of *M. bovis* infection in deer (Griffen et al., 1994).

The interferon gamma (IFNg) assay is another blood-based laboratory test that can be used for the ante-mortem diagnosis of bTB. In this test, the release of lymphokine (IFNg) in a whole-blood culture system is measured. The assay is based on the release of IFNg from sensitized lymphocytes during a 16–24-hour incubation period after stimulation of target cells with specific antigen (PPD-tuberculin). The test makes use of the comparison of IFNg production following stimulation with avian and bovine PPD.

The quantitative detection of bovine gamma-interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine IFNg. The blood sample must be transported to the laboratory and the assay set up within 24–30 hours of collection. The test is considered to have a higher sensitivity than the skin test, but was shown to be less specific in a number of trials (OIE, 2004). This assay has been used in African buffalo and the necropsy and culture findings of all culled buffaloes showed excellent correlation with the results of the ante-mortem gamma-interferon test (Grobler et al., 2002). In this study the IFNg test showed 99.3% of specificity.

Enzyme-linked immunosorbent assay (ELISA) is a serological test that can be used to measure antibody titers to *M. bovis*. An advantage of the ELISA is its simplicity, but both specificity and sensitivity are limited in cattle, mostly due to the late and irregular development of the humoral immune response in cattle during the course of the disease. Infected animals with no visible lesions or only early tuberculous lesions may be missed when this diagnostic test is applied, resulting in false negative test results. ELISA may also be used in wildlife and zoo animals (OIE, 2004.

Other serological testes that can be used for TB diagnosis are the multi-antigen printing immunoassay (MAPIA) and Enzyme linked immunoelectrotransfer blot (EITB). The MAPIA is an antibody detection method that employs direct application of proteins sprayed onto nitrocellulose membranes in lines followed by classical detection of antibodies, typically using an enzyme conjugated anti-immunoglobulin and precipitating enzyme substrate. MAPIA permits

antibody detection of many unrelated antigens in a single assay (Lyashcenko et al., 2000). This test has been adapted for use in white-tailed deer, European badger, cattle, and asian and african elephants for the detection of TB-specific antibody (Lyashcenko et al., 2005).

1.2.5. Pathology of tuberculosis

1.2.5.1. Necropsy findings

Because of the limitation of the currently available ante-mortem diagnostic tests for wildlife, the post-mortem examination of free-ranging wildlife is the most commonly used method for surveying wildlife for tuberculosis and detailed information can be gathered from culling operations (De Lisle et al., 2002).

Bovine tuberculosis is characterized by the formation of granulomas (tubercles) that are usually yellowish and either caseous, caseo-calcarius or calcified. In some species such as deer, the lesions tend to resemble abscesses rather than typical tubercles (OIE, 2009).

In cases of general tuberculosis, there are miliary or large lesions in organs and lymph nodes throughout the body. Miliary lesions are small (up to 5 mm diameter) and translucent in the early stage but become caseous and calcified as they age. In advanced cases of tuberculosis, peripheral lymph nodes such as the submaxillary, prescapular, precrural and supramammary may be enlarged, and therefore easily palpable or visible (Huchzermeyer et al., 1994).

The distribution and severity of the lesions probably depend on the route of infection. The most common route to contract the mycobacterial infection are inhalation and granulomatous lesions are therefore found predominantly in the retropharyngeal, mediastinal and bronchial lymphnodes and the lungs (Huchzermeyer et al., 1994; De Lisle et al., 2002; Asseged et al., 2004). However in the past, abattoir inspection in cattle in Australia (Corner, 1994) and the USA (Whipple et al., 1996) recorded lesions more frequently in thoracic lymph nodes than the pulmonary parenchyma. In general, in large animals, tuberculous lung lesions often remain undetected at routine post-mortem examination due to the large volume of the lungs, whereas the lesions are

more easily found in the lymph nodes draining the lungs (D. Gavier-Widén, National Veterinary Institute, Uppsala, Sweden, personal communication). The predilection for tuberculous lesion development in caudal lung lobes has been reported in tuberculous cattle naturally infected (Liebana et al., 2007) and experimentally infected by aerosol exposure to *M. bovis* (Palmer et al., 2002), as well as in white-tailed deer inoculated intratonsilarly with 300 CFU of *M. bovis* (Palmer et al., 2002) and in black rhinoceros naturally infected (Espie, 2009).

However in a recent cattle abattoir survey, done in Tanzania, the gastrointestinal tract was the most affected by TB lesions (61.3%), whilst 35,3% were found in the thorax, 3.6% in the lymph nodes of the head and 5.1% in other sites, predominantly the pre-scapular lymph node (Cleaveland et al., 2007).

In more advanced cases the infection can be disseminated via several routes, including haematogenous, lymphatic, natural passages, body cavities and by direct extension (Neill et al., 1994; Lopez, 2001). Tuberculous lesions can be found in the liver, spleen, kidney, liver, joints, bone, mammary gland, testes and uterus (Huchzermeyer et al., 1994). Jackson et al. (1995a) considered that lesions in the liver, spleen and kidney indicate systemic haematogenous spread of infection due to the role of these organs in haemofiltration.

Early reports of bTB in African buffalo described lesions most often located in the lymph nodes of the head, in the bronchial and mediastinal lymph nodes, tonsils and lungs (Bengis et al., 1996; Grobler et al., 2002). The affected lymph nodes were enlarged and showed lesions of variable size, which may contain foci of caseous necrosis and mineralization. In the lungs, TB lesions were either disseminated or presented as individual granulomas. Generalized forms of bTB also occurred and affected the pleura, peritoneum, intestinal tract, various other internal organs and visceral and peripheral lymph nodes (Keet et al., 1994; Bengis et al., 1996). Lesions were found in the intestinal tract probably as a result of ingestion of coughed-up and swallowed exudates originating from open lesions in the lungs and tonsils (Keet et al., 1994). In the early stage of the disease, infected animals showed no macroscopical detectable lesions (De Vos et al., 2001).

In a detailed necropsy done on a black rhinoceros, firm discrete mottled tan to cream foci (40 mm diameter) TB lesions were seen in the left caudodorsal lung lobe. The thoracic and mesenteric lymph nodes were not affected (Espie et al., 2009). Fallow deer are significantly more likely to have thoracic lesions than red deer. In red deer the lesions were commonly observed in the retropharyngeal lymph nodes followed by the abdominal tissues, mainly the ileocaecal and mesenteric lymph nodes (Martín-Hernando et al., 2010).

Infection in the absence of gross visible lesions has also been described in many species. De Vos et al. (2001) studied TB in African buffalo in the KNP and found that 37% of infected animals which were culture positive did not show any macroscopical lesions. In another study done in KNP, the comparison of bacterial culture and pathology gave similar results (Rodwell et al. 2001a). In a study on cattle, 10.1% of the animals with no visible lesions (NVL) were shown to be infected using culture based diagnosis, and 6.7% were considered as tuberculous lesions by histopathology (Liebana et al. 2007). Moreover, in a study of natural infection in red deer (*Cervus elaphus*), 28% of the *M. bovis* positive cases on culture showed no detectable lesions (Lugton et al., 1998).

During inspection of carcasses at abattoirs, TB lesions may not easily be differentiated from those caused by other infectious agents such as staphylococci, fungi, Actinomyces or *Actinobacilus spp*, parasites, foreign bodies and abscesses. Confirmation of the diagnosis must be done by direct examination of smears made from the exudates after appropriate staining, and by histopathology and/or culture (Huchzermeyer et al., 1994).

1.2.5.2. Histopathology

The classic lesion in ruminants with tuberculosis is a typical granuloma containing a central area of necrotic tissue that in advanced cases are mineralized. The necrotic area is surrounded by epithelioid cells and Langhan's giant cells, and peripherally by lymphocytes, macrophages and varying degrees of fibrosis, which is common in the later stage of the disease (Wangoo et al., 2005).

Composition of the granulomatous lesions can vary between species. The presence of fibrosis surrounding the granuloma and the predominance of Langhan's multinucleated giant cells are common in cattle (Wangoo et al., 2005) and buffalo (Bengis et al., 1996). Studies in badgers (Gavier-Widen et al., 2001), ferrets (Lugton et al., 1997) and possums (Cooke et al., 1995) reported the lack of fibrosis surrounding the granuloma. In lions TB lesions typically consist of an amorphous, multifocal to coalescing, expansive non-encapsulated granulomatous inflammatory reaction, without necrosis, giant cells or calcification (Keet et al., 1996).

Keet *et al*. (1994) do not specify the type of giant cells observed in African buffalo TB lesions. On the other hand, Bengis et al. (1996) mention only Langhan's giant cells. The discrimination of the type and predominance of giant cells involved in bovine tuberculosis would be useful for comparative purposes among different wildlife species. For instance, giant cells of Langhan's type are not seen in badgers (Gallager et al., 1976) and giant cells are predominant in fallow and sika deer TB lesions compared to cattle, red and elk (Rhyan and Saari, 1995). Interestingly, the presence of multinucleated cells has not been described in wild felids (Keet et al., 1996).

In a black rhinoceros with TB, lesions consisted of a necrogranulomatous pneumonia with discrete to coalescing unencapsulated foci of central variably mineralized necrotic debris surrounded by loose aggregates of epithelioid macrophages, Langhans-type multinucleated giant cells, lymphocytes and plasma cells (Espie et al., 2009).

The difficulty to distinguish the lesions caused by *M. bovis* from those caused by other mycobacterial species is a limitation of histopathology (De Lisle et al., 2002). Acid fast bacilli may be identified in Ziehl-Neelsen stained sections prepared from tissues with suspected TB (Huchzermeyer et al., 1994). The number of the bacilli in sections can vary between species and individuals. Small numbers of acid fast colonies (range 3-18 colonies/animal sample) have been seen in buffalo (Bengis et al., 1996). Drewe et al. (2008) studying the pathology of *M. bovis* infection in wild meerkats (*Suricata suricatta*) detected a high number of acid fast bacilli within macrophages.

1.2.6. Bacteriology

Culture and identification of mycobacteria is the gold standard diagnostic method for the confirmation of tuberculosis (De Lisle, 2002). Several species of the non-tuberculous mycobacteria (NTM) do not usually cause disease in animals or humans. Under certain conditions, NTM's such as *M. kansassi, M avium-intracellulare, M. xenopi* and *M. fortuitum-chelonae* complex can cause disease (Ellis, 2004). In addition, pathogenic mycobacteria may cause disease in only a proportion of the infected hosts. A classic example being *M. tuberculosis* in humans, where in the absence of immunosupression or other high risk factors, only 11% of infected individuals will succumb to disease (Stoneburner et al., 1992). The immune response of the host is very important determining the outcome of the infection.

For primary bacteriological isolation, the sample is usually inoculated on to a set of solid egg-based media such as Lowenstein–Jensen, Coletsos base or Stonebrinks; these media should contain either pyruvate or glycerol or both. An agar-based medium such as Middlebrook 7H10 or 7H11 should also be used (OIE, 2004). Cultures are incubated for up to 8 weeks at 37°C with or without CO_2. When growth is visible, smears are prepared and stained by the Ziehl–Neelsen technique.

It is arguably important to distinguish *M. bovis* from the other members of the 'MTC', i.e. *M. tuberculosis*, *M. africanum* and *M. microti*. Characteristic growth patterns and colony morphology can provide a presumptive diagnosis of *M. bovis*, which can be confirmed by polymerase chain reaction (PCR) (Warren et al., 2006) and molecular typing techniques such as spoligotyping (OIE, 2004), for example.

As the bacteria may survive in heat-fixed smears or become aerosolized during specimen preparation, all the bacteriological procedures should be done in a biological safety cabinet (OIE, 2009). The total number of infectious organisms, state of preservation of tissues, destruction of viable organisms by overgrowth of other microorganisms and the use of a decontamination agent during tissue processing can lead to false negative results (Corner, 1994).

One of the disadvantages of this diagnostic method is the time taken to make a diagnosis which can be unacceptably long when disease management practices have to be adopted to control spread of the infection.

1.2.7. Molecular diagnosis

Over many years, diagnosis of TB using various techniques that depend on detection of macromolecules has been attempted. These include detection of antibodies or antigens, or detection of DNA. In the case of the latter, amplification by PCR is usually done (Warren et al., 2006).

One of the advantages of PCR over bacteriology is the reduction in time-to-diagnosis, which permits the rapid implementation of measures to prevent subsequent outbreaks. PCR also provides the possibility of detecting the presence of *M. bovis* in samples even if the organism is non-viable. The technique can provide rapid diagnosis of tuberculosis when it is applied to paraffin sections that have characteristic lesions and acid fast organisms (Miller et al., 1997; Cao et al., 2003; Coura et al., 2005). When examining formalin fixed tissues, care should be taken when interpreting negative PCR results on samples containing few acid-fast organisms (De Lisle et al., 2002) since there may be a low concentration of DNA in the samples and degradation of the DNA during formalin fixation, which will reduce the amplification efficiency (Vincek et al., 2003; Van Pelt-Verkuil et al., 2008).

If PCR is carefully designed to target species specific regions of the genome, it can also confirm the species of mycobacterium identified (Warren et al., 2006). After confirming the infection with laboratory diagnosis, the molecular characterization of *M. bovis* is essential for the study of spatial (De Lisle et al., 1995; Michel et al., 2006), temporal and inter-species transmission of *M. bovis* (Michel et al., 2006). From molecular typing of species together with traditional epidemiology traceback approaches, important insights can be gained regarding the sources of infection and the interpretation of practices or environments that may aid the spread and maintenance of tuberculosis (Hénault, 2006; Harris, 2006).

Genetic fingerprinting allows laboratories to distinguish between different strains of *M. bovis* and enables patterns of origin, transmission and spread of *M. bovis* to be described. The most widely used method is spoligotyping (from 'spacer oligotyping'), which allows the differentiation of strains within each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (Heifets and Jenkins, 1998).

Other new techniques are currently under development or used in order to differentiate more accurately the strains that have the same spoligotype. These include restriction fragment length polymorphism (RFLP) using IS*6110*, the direct repeat (DR) region and the poly G repeat sequence (PGRS) probe, RFLP using a combination of the DR and puce probes, and characterization of the VNTR profile (variable number tandem repeat) (Kamerbeek et al., 1997; Frothingham et al., 1998). The genome of *M. bovis* is currently being sequenced and this information should lead to improved methods of genetic fingerprinting.

1.2.8. Control of tuberculosis

Tuberculosis and Acquired Immunodeficiency Syndrome (AIDS) are two of the world's major pandemics in developing countries (WHO, 2004). Work on TB control in human beings has concentrated on massive case detection and treatment, although, Human immunodeficiency virus (HIV) infection has complicated the control of tuberculosis. Additional strategies need to be developed to control both this disease and HIV simultaneously. Such strategies would include active case-finding in situations where TB transmission is high, the provision of a package of care for HIV-related illness, and the application of highly active antiretroviral therapy (Harries et al., 2002).

Most industrialized countries implemented bTB control programs to eradicate the disease in cattle a long time ago. These control and eradication programs were based on testing and removal of infected animals under mandatory national bovine TB programs. In most of them where the disease in bovids was eradicated, human TB due to *M. bovis* has declined drastically (Huchzermeyer et al., 1994; Thoen et al., 2006).

Since *M. bovis* can be transmitted to humans from animals (LoBue, 2006) and because there are several potential routes of transmission, including gastrointestinal, airborne, and direct contact, education should be provided to persons at risk to prevent the disease in humans. The public health message should instruct such individuals to take appropriate precautions such as wearing gloves during handling of TB positive animal carcasses, cooking meat thoroughly and pasteurizing the milk (Cosivi et al., 1998; LoBue, 2006). With the practice of boiling milk, and the growth of milk pasteurization plants all over the world, the digestive route of infection to humans declined significantly (Thoen et al., 2006).

Tuberculosis due to *M. tuberculosis* has been reported in captive species (Michel et al., 2003) and in situation of increasing frequency of human tuberculosis and infections may therefore represent a serious threat to zoological collections. Urgent attention should be given to the management of such animal-human interactions to minimize risk of tuberculosis transmission (Michel et al., 2003).

In contrast, controlling tuberculosis in wildlife reservoirs has proven to be a major challenge. Strategies based on control of population densities as well as testing and culling of positive animals have shown variable results and are costly. Furthermore, culling of a threatened species can have conservation implications, and reduction in numbers of key species can have unknown but potentially serious ecological impact. One of the problems is the lack of a point-of-care test for affected species, which would be beneficial to the monitoring and control of tuberculosis in wildlife.

During recent years, vaccination of wildlife reservoirs has been considered a possible and acceptable control strategy (Buddle et al., 2000; Hope, 2008). Evaluating the efficacy of vaccines requires detailed knowledge of the natural disease in the species and the population that the vaccine is intended for. The Bacille Calmette-Guérin (BCG) vaccine has been used in many vaccination trials to try to control bTB in domestic and wild animals, however, the reported efficacy varies (Suazo et al., 2003). This vaccine was not effective in a study on captive African buffalo (De Klerk-Lorist, 2005). The BCG vaccine also failed to protect yearling African buffalo against intratonsilar challenge with *M. bovis* (De Klerk et al., 2008).

Cross and Getz (2006) used a model to assess the theoretical effectiveness of a vaccination program as a strategy to control bTB in buffalo and concluded that the programs would be more efficient if focused on young animals. To reduce the herd tuberculosis prevalence to less than 1%, it would be necessary to vaccinate more than 70% of the calves each year (Cross and Getz, 2006), assuming a vaccine efficacy of 70% (Buddle et al., 1995).

The high prevalence of bTB in some African buffalo herds, movement of animals between herds and spillover of bTB into other species makes it difficult to control the disease by vaccination alone (Cross and Getz, 2006). Testing and culling of the positive reactors is another management option to control the disease (Cross and Getz, 2006). This control strategy has been very successful for cattle but clearly has limitations for the control of bovine tuberculosis in free ranging wildlife (De Lisle at al., 2002). However, in buffalo for example, controlling TB by culling might be acceptable in high prevalence herds. In a low prevalence situation, a large sample size to detect the infection is needed. If many animals are culled, it can have adverse effects on the genetic diversity of the herd and can also have other ecological and ethical considerations, particularly if the culled animals show to be free of tuberculosis (Grobler et al., 2002).

The African buffalo is the maintenance host and the species most affected by bTB in South African conservation areas, although the disease has been confirmed in other species such as lion (*Panthera leo*), greater kudu (*Acinonyx jubatus*) and chacma baboon (*Papio ursinus*) which can act as "spillover hosts" (De Lisle et al., 2002). Since the disease is established in many species in the ecosystem of KZN and KNP, no matter how low the prevalence of the disease may be, eradication becomes virtually impossible, as maintenance hosts can perpetuate the infection (Keet et al., 1996).

The diagnosis of bTB in game species has severe implications on national and international trade in wildlife due to movement restriction and revenue losses for conservation parks (Michel et al., 2006). Clinical, serological and other investigative techniques should be used to ensure a minimum risk from the translocation operation to the existing or translocated animals at the

destination (Hilsberg and van Hoven, 2000). Based on the assumption that cattle acquire infection both from other badgers and from cattle, in Britain, control measures included restrictions on the movement of cattle from herds confirmed infected and testing of cattle on farms that either adjoin the farm or have recently received animals from restricted herds (Woodroffe et al., 2005).

In some Southern African countries and especially in South Africa's ecosystems, the African buffalo is considered to be the most important maintenance host of tuberculosis. Since the disease has spilled over into other wildlife, including several wildlife species, a clearer understanding of all aspects of the disease in the maintenance species could allow us to optimize control strategies and understand the value of diagnostic tools that may be used or that will come into use in the future.

This thesis aims to examine the pathology of bovine tuberculosis in African buffalo to gain insights into the pathogenesis of the infection, and routes of transmission and infectivity of infected animals.

CHAPTER 2. MATERIALS AND METHODS

2.1. Animals

The buffalo used in this study were acessed during test-and-cull operations, which were part of a tuberculosis control program aimed at reducing the prevalence of bTB in the species, in the Hluhluwe-iMfolozi Park (HiP) in Kwazulu/Natal, province of South Africa. The game reserve is the third largest in South Africa and it covers an area of 100 000 ha (Michel et al., 2006). This control program started in 1999 in HiP and involves annual buffalo capture for tuberculin skin testing and culling of bTB-positive buffalo (Jolles et al., 2005).

The buffalo in this study were selected from animals with a positive test result of the bovine component of the standard bovine comparative intradermal test using both avian and bovine Dutch tuberculin. All positive reactors in one test-and-cull operation (24 buffalo) were killed by a shot in the brain using a heavy caliber rifle .308 (7.62 mm) and transported to the abbatoir of the HiP. At slaughter inspection, 19 of 24 animals showed visible gross lesions suggestive of tuberculosis, and these buffalo were selected for further pathology studies.

2.2.Post-mortem examination and sample collection

The age category, sub-adult or adult, was determined based on tooth eruption patterns. The animal identification number, sex and age were registered. A detailed examination of the lungs and a selection of lymph nodes (LN) was done. The lungs were sliced at 2 cm intervals and each slice was inspected and palpated. The following LN were sliced thinly (approximately 2 mm thick slices), and each slice was visually inspected: head-associated LN (paired mandibular, parotid and medial retropharyngeal), thoracic LN (mediastinal and bronchial), abdominal LN (mesenteric, hepatic, omasal and abomasal) and paired peripheral superficial LN (superficial cervical, axillary and popliteal). Tissues with lesions suspecious of tuberculosis were split into two, and one half was sampled for histopathology and fixed in 10% neutral buffered formalin. The other half was used for culture and stored in the freezer at HiP until ready for transportation to the laboratory.

2.3. Grading of macroscopic lesions

Macroscopic lesions were considered as any focus or nodule, single, multifocal or confluent, yellow-white, circumscribed and solid, or necrotic, with or without apparent caseation and mineralization, that deviated from expected normal tissue. The LN were categorized in grades 1 to 5 according to the size and number of their gross lesions. Grade 1 was used for a single lesion, up to 1 mm in size; grade 2 for two to four lesions, sized 2 to 5 mm; grade 3 for five to eight lesions, up to 10 mm, or many small lesions affecting approximately 50% of the LN; grade 4 for confluent and extensive lesions in most slices but with some normal looking tissue left, and grade 5 for abundant lesions, with none or nearly no apparently healthy tissue left. The lungs and LN that did not show visible gross lesions were classified as grade 0 and were not sampled for further histopathology.

Figure 2.1. Dead animals before slaughtering

Figure 2.2. Animals during the slaughtering process

Figure 2.3. Inspection of the lung, liver and thoracic lymph nodes

Figure 2.4. Inspection of the head lymph nodes

2.4. Laboratory examinations

Histophatological analysis of field samples was done at the laboratory of Pathology, Veterinary Faculty, Eduardo Mondlane University (UEM), Mozambique and at the National Veterinary Institute (SVA), Uppsala, Sweden. The molecular techniques were performed at the MRC Centre for Molecular and Cellular Biology/ Division of Molecular Biology and Human genetics, Faculty of Health Science, Stellenbosch University, South Africa and at the Veterinary Faculty of Universidad de Murcia, in Spain.

2.4.1. Histopathology

The tissues were formalin-fixed for 30 days to render any mycobacteria non-infectious, and dehydrated, thereafter embedded in paraffin wax and sectioned at 4-5 µm. All sections were stained with hematoxylin and eosin (HE) and with Ziehl-Neelsen stain (ZN) to identify acid-fast

bacilli (AFB). The Masson's trichrome (MT) stain was applied to selected sections to visualize fibrous tissue.

The tuberculous granulomas in the LN and lungs were microscopically classified into four categories of development according to criteria previously described for cattle (Wangoo et al., 2005). The lesions were evaluated histologically for their size, type and extension of necrosis and mineralization, for abundance and distribution of inflammatory cell components, and for degree and architecture of fibrosis. Briefly, the granulomas were classified in four categories, namely: Stage I (initial or early lesions); Stage II (solid granulomas); Stage III (minimal necrosis); Stage IV (necrosis and mineralization).

The sections were examined using a Leica light microscope. Photographs we taken using a Zeiss Axioskop 2 microscope that is equipped with an Axiocam digital camera with a Axiovision 3,6 software.

2.4.2. Bacteriology

Samples from all the LN and lung sections that presented macroscopic lesions suggestive of TB were collected. Each sample was placed in a different receptacle for identification. The samples were stored in the freezer in HiP. On route from the park to the Veterinary Faculty, UEM, the samples were stored in a small freezer in a car. At the University, while waiting for delivery of the purchase and preparation of the reagents and medium for culture, the samples were stored in a -20^0C. Unfortunately, after the annual December vacation period, when the University closed, it was found that the freezer had failed and the samples had decomposed due to the extremely hot weather in Maputo. Because of this logistical difficulty, the samples could not be used for mycobacterial culture. To confirm the aetiological agent of the lesions, identification of *M. bovis* on paraffin sections was done by PCR.

2.4.3. Identification of *M. bovis* by polymerase chain reaction (PCR)

DNA was extracted from formalin-fixed and paraffin-embedded (FFPE) LN and lung tissue from 19 buffalo which tested positive by the bovine tuberculin test. All the animals presented with gross and microscopic lesions suggestive of TB. The tissue samples were fixed in 10% tamponated formalin for more than one month to inactivate the bacilli and reduce the probability of contamination during the histopathologic procedures.

Two different DNA extraction kits were used and for one of them the DNA amplification was done by a classic polymerase chain reaction (PCR) and for the other extraction method a Real Time (RT) PCR was performed.

2.4.3.1. Method 1

2.4.3.1.1. DNA Extraction procedure

Three to four slices of approximately 20 µm of formalin-fixed paraffin-embedded (FFPE) tissues were cut using a new scalpel blade for each sample and transferred to a 2 ml eppendorf tube. The samples were deparafinized with two xylene washes with shaking (1 ml, 30 minutes at 37 ^{0}C each step), two 100% ethanol washes (1 ml, 5 min at RT each step), and one 70% ethanol wash (1 ml, 5 min at RT). Each wash step was followed by centrifugation at full speed (3000 rpm) for 10 min and the wash solution was aspirated and discarded into organic waste.

Dewaxed tissue samples were rehydrated with 1 ml tris/EDTA (1 M, pH 7.5) for 5 minutes at room temperature. Thereafter the samples were pelleted by centrifugation and resuspended in 450 µl TE (1 M, pH 7.5) pH 7.5, 50 µl 10x proteinase K buffer and 150 µl proteinase K (10 mg/ml) and incubated at 45^{0}C overnight.

DNA was extracted using the Nuclisens extraction kit according to the manufacturer's instructions (bioMérieux bv, Bosein 15, 5281 RM Boxtel, The Netherlands).

2.4.3.1.2. PCR amplification

To identify the member of the mycobacterium complex present in the sample, extracted DNA was subjected to PCR amplification using four primer pairs, RD1, RD 2, RD 4 and RD 12 (Warren et al., 2006). Each PCR reaction contained 2 µl DNA template, 5 µl Q-solution, 2.5 µl 10 x buffer, 2 µl $10MgCl_2$, 4 µl 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl), 0.25 µl Taq DNA polymerase (Qiagen, Hilden, Germany) and was made up to 25 µl with H_2O. Amplification was initiated by incubation at 95^0C for 15 min. This was followed by 45 cycle of 94^0C for 1 min; 62^0C for 1 min and 72^0C for 1 min and a final cycle of 72^0C for 10 min.

To avoid cross contamination, all procedures were conducted in separate rooms and negative control (H_2O) were incorporated in each batch of amplifications. Positive controls included *M. bovis* and *M. tuberculosis*. PCR amplification products were analyzed by electrophoresis in a 2% agarose gel, and visualized by staining with ethidium bromide. Samples that produced a DNA fragment of the expected size compared to control DNA, were considered positive. Samples that had no fragments or fragments in any other position were considered negative.

2.4.3.2. Method 2

2.4.3.2.1. DNA extraction

DNA for PCR was extracted from three 20 µm sections cut from FFPE tissue wax blocks. Blades were changed between each sample to avoid contamination. The tissue sections were collected into a 1,5 ml steril DNAse-free microcentrifuge tube and pelleted by centrifugation at 4.000 rpm for 5 min. Next, 200 µl of Tween 20 (Sigma-Aldrich, St Louis, MO) diluted to 0.5% was added to each tube with a tissue sample, which were subjected to 3 consecutive cycles of heating for 10 min. The paraffin ring formed after centrifugation was removed, and 60 µl of the supernatant was taken and frozen at – 80^0C until PCR reaction was done. DNA extraction from the supernatant was carried out using a commercial kit (High pure PCR template preparation KIT, Roche Diagnostics GMBH, Mannheim, Germany) (Gómez-Laguna et al., 2010).

2.4.3.2.2. PCR amplification

RT-PCR was performed by using an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). The RT PCR mixtures for amplification of DNA consisted of 50 µl total volume containing: 25 µl 2X Power SYBR Green (Applied Biosystems, Warrington, UK), 5 µl template and 10 pmol of each oligonucleotide primer, myc1 and myc3 (Applied Biosystems, Warrington, UK) in a final 50 µl reaction volume. These primers can simultaneously amplify the *M. tuberculosis* complex (*M. bovis* and *M. tuberculosis*) and the *M. avium* complex (*M. avium* and *M. avium* subsp. *paratuberculosis*) producing different amplicon sizes of 178 bp and 257 bp, respectively (Coetsier et al., 2000).

All primers were manufactured by TIB Molbiol (Berlin Germany). Samples were heated at 95ºC for 10 min. Temperature cycling consisted of 45 cycles of 95ºC for 30 sec, 58ºC for 45 sec, and 72ºC for 30 sec, with an increase of 1 sec per cycle for the denaturation and extension steps. Dissociation curve analysis was performed to check the specificity of the amplified products. All reactions were run in duplicate. *M. tuberculosis* complex and *M. avium* complex PCR products were differentiated based on the melting temperatures as described elsewhere (Gómez-Laguna et al., 2010).

CHAPTER 3: RESULTS

3.1. Gross pathology

All animals appeared to be in good body condition, as assessed by external observation. Lesions consistent with tuberculosis were found in the lymph nodes (LN) of the head, thoracic cavity and lungs of 19 animals. Lesions suggestive of tuberculosis were not detected in other organs or LN. The severity scores of the lesions are shown in table 3.1.

The lesions in the LN varied from small white foci (1 mm in diameter) to multiple encapsulated granulomas of variably size with central caseation and mineralization, to diffuse, non-encapsulated lesions effacing and replacing normal lymphoid tissue, resulting in a spectrum of macroscopic grades 1-5 (Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6).

Most animals showed tuberculous lesions in the bronchial 84.2% (16/19) and mediastinal 57.9% (11/19) LN. Twenty one percent (4/19) of the animals presented TB lesions in the lung (Animals 58, 52, 97 and 69) and 21% (4/19) in the retropharyngeal LN (Table 3.1).

Forty seven percent (9/19) of the animals presented lesions in only one LN, 31.6% (6/19) in two LNs, 10.5% (2/19) in two LN as well as in the lungs and 10.5% (2/19) showed lesions in three different LN and in the lung. Lung lesions were most frequently located in the caudal lobes, particularly in the caudo-dorsal areas.

Most buffalo (73.7%) presented macroscopic tuberculous lesions of early stages (grade 1 and/or 2) while 26.3% (5/19) presented moderate to high severity gross lesions (grades 3, 4 and 5) and all of them were females. Grade 5 lesions were observed in only two (10.5%) animals: No. 58 (in the mediastinal LN) and animal No. 69 (in the bronchial, mediastinal LN and lung). Animal No. 69 presented the most severe macroscopic lesions, although only the bronchial LN presented lesions of all five grades (Table 3.1). Out of nineteen animals that presented lesions, 74% were females and the three animals that presented the most severe lesions (Animals 58, 69 and 97) were also females.

Table 3.1. Lymph node and lung samples collected from African buffalo with tuberculosis lesions.

Animal			Gross pathology †Tissue and lesion grade (1-5)			
*ID	**Sex	Age	**RP**	**B**	**ML**	**L**
49	F#	Adult	§0	3	3	0
52	M	Adult	1	1	2	2
53	F	Adult	0	1	0	0
57	F	Adult	0	2	0	0
58	F	Adult	0	3	5	3
61	F	Subadult	2	0	0	0
62	M	Adult	0	1	0	0
66	F	Subadult	0	2	2	0
69	F	Adult	0	5	5	5
77	F	Adult	0	2	2	0
79	M	Subadult	0	1	2	0
80	F#	Adult	0	0	2	0
84	F	Adult	0	0	2	0
85	F	Adult	0	2	2	0
89	F#	Adult	0	2	0	0
92	F	Adult	3	2	0	0
97	F	Subadult	3	4	4	3
98	M	Adult	0	2	0	0
Un	M	Subadult	0	2	0	0

Abbreviations:

* ID: Animal individual number. Un = Not numbered; ** Sex: M = male; F = female. F# = pregnant female; † Tissue: RP = medial retropharyngeal lymph node; B = bronchial lymph node; ML = mediastinal lymph node; L = lung. § 0 = without visible gross lesions (not sampled for histopathology)

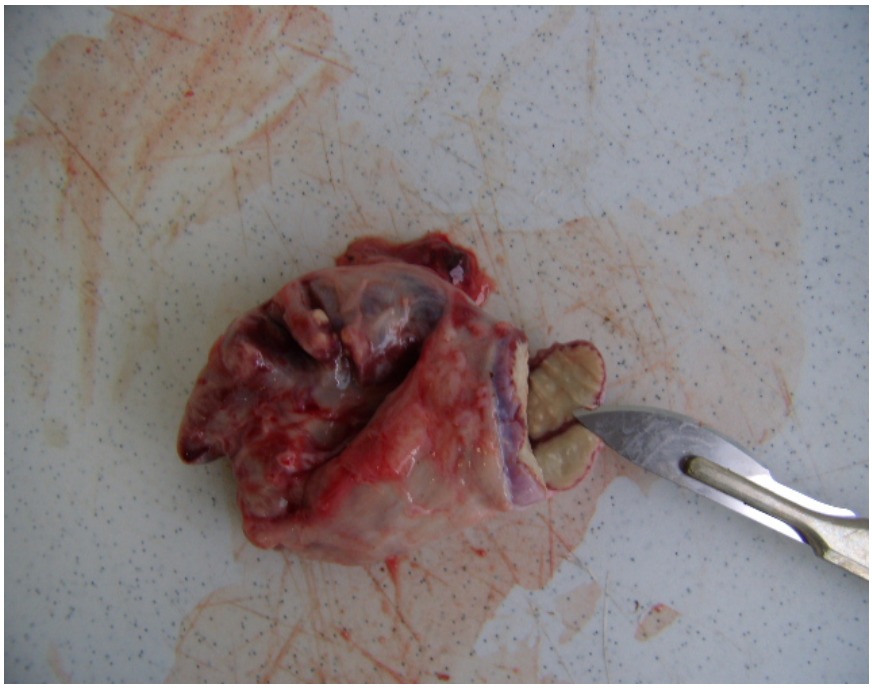


Figure 3.1. A bronchial lymph node with white caseogranulomatous lesions (Grade 2)

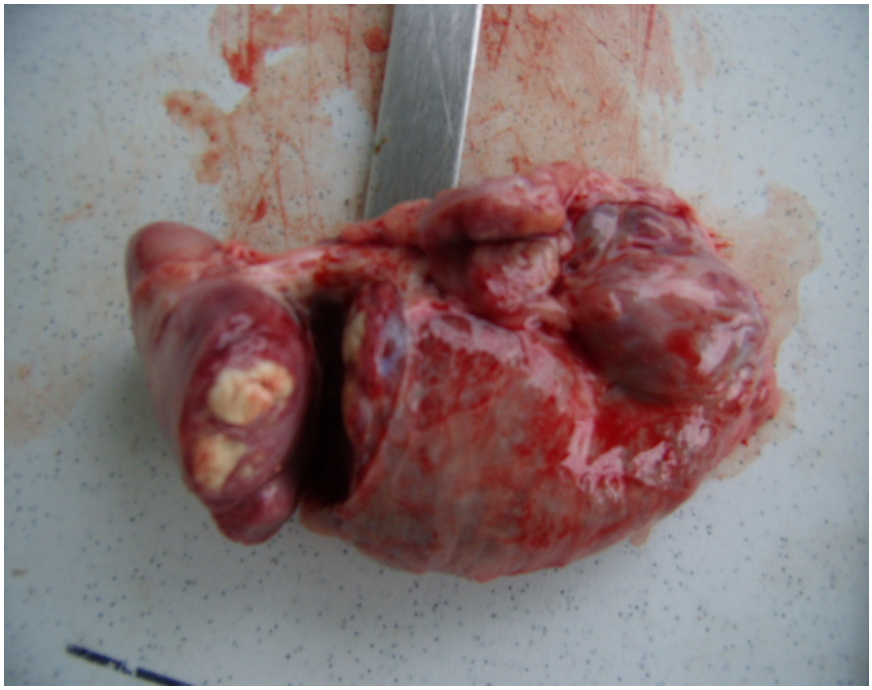


Figure 3.2. An enlarged retropharyngeal lymph node with white caseogranulomatous lesions (Grade 2).

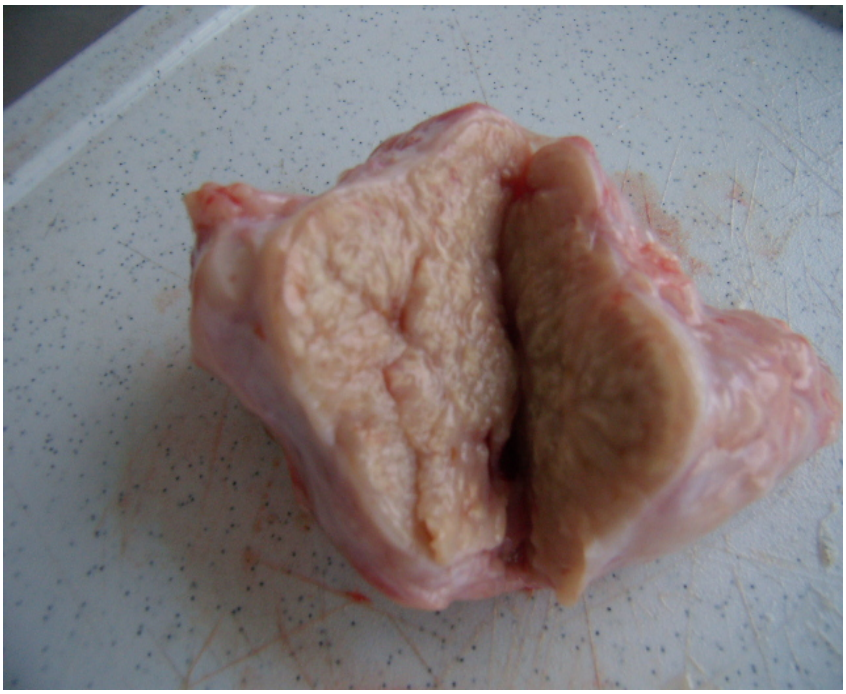


Figure 3.3. Bronchial lymph node with whitish granulomatous lesion replacing the lymphoid tissue (Grade 5).

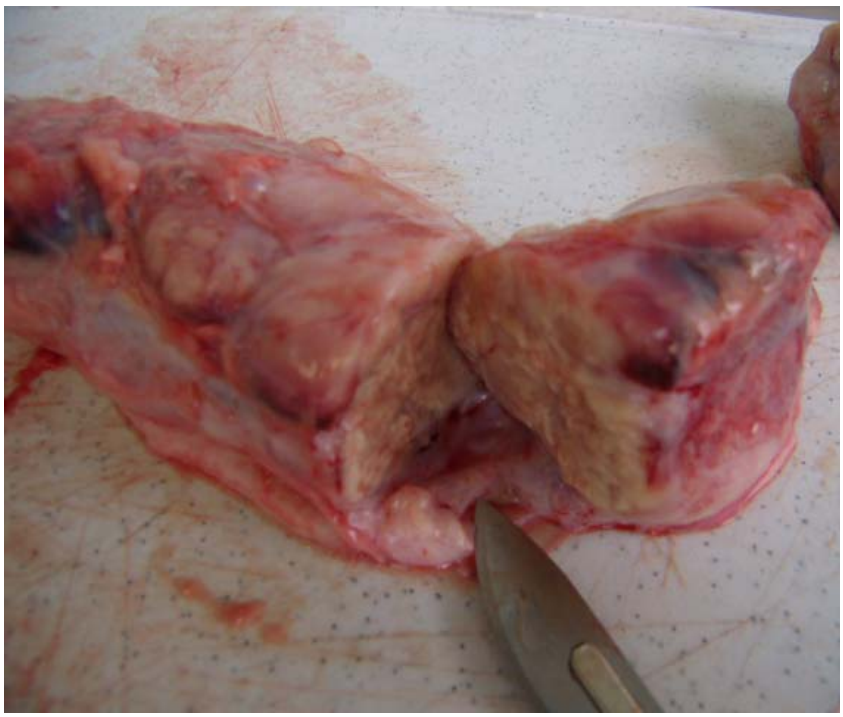


Figure 3.4. Enlarged mediastinal lymph node with granulomatous white lesions replacing the lymphoid tissue. On cut surface the LN was gritty (Grade 5).

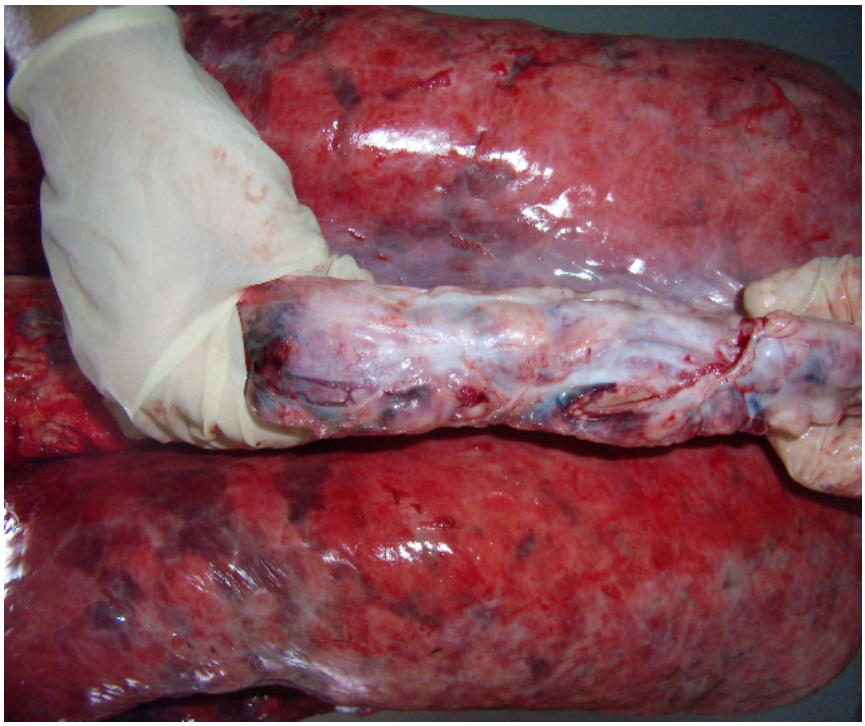


Figure 3.5. An enlarged mediastinal lymph node with a multinodular appearance (Grade 5).

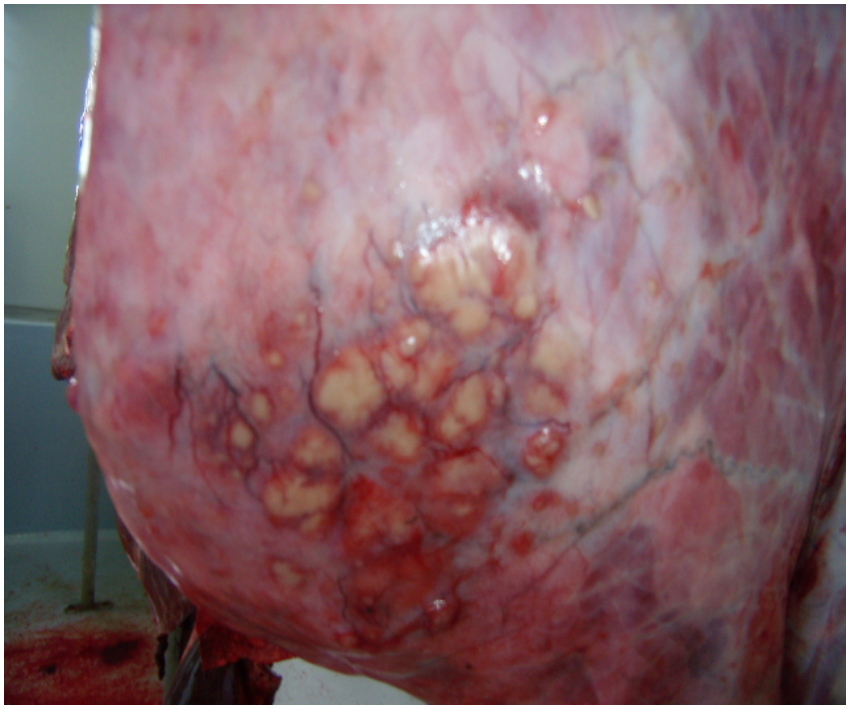


Figure 3.6. White multifocal to coalescing caseogranulomatous TB lesions in the lung (grade 3).

3.2. Laboratory examinations

3.2.1. Histopathology

Histopathologic examination showed a variation in size and composition of the granulomas, and all four different stages were found. Stage I (initial or early lesions) were characterized by irregular, unencapsulated clusters of predominantly epithelioid macrophages, lymphocytes and few multinucleated giant cells, without necrosis (Figures 3.7, 3.8 and 3.9); stage II (solid granulomas) were partly or completely thinly encapsulated granulomas formed by predominantly epithelioid macrophages, Langhan's giant cells and lymphocytes surrounding minimal areas of necrosis (Figures 3.10, 3.11 and 3.12); stage III (minimal necrosis) were partially or totally encapsulated granulomas with central necrosis, often mineralized, surrounded by epithelioid cells, Langhan's giant cells and lymphocytes at the periphery (Figure 3.13) and stage IV (necrosis and mineralization) were large, thickly encapsulated, multicentric granulomas with extensive necrosis and often multiple areas of mineralization surrounded by epithelioid cells, giant cells and lymphocytes (Figure 3.14) .

It was common to see extensive lesions with multifocal to coalescing stage I granulomas. Some granulomatous lung lesions involved the wall of the bronchioles (Animals no. 69 and 97), occasionally breaking through into the lumen. Other pulmonary changes observed were variably dispersed interstitial pneumonia, congestion, haemorrhage, proteinaceous-rich alveolar oedema, atelectasia and emphysema (appendix 3), and occasionally, metazoan parasites (appendix 3).

Most animals presented with stage II lesions. There were often several lesions in different stages of development within one LN. The most chronic or advanced stage (IV) was seen in five animals. One buffalo with severe macroscopic lesions presented microscopic granulomas of grade III. In LNs with macroscopic lesions of stages 4 and 5 it was common to find lesions of microscopic stage IV.

In Masson Trichome stained sections it was observed that the stage I granulomas were not surrounded by connective tissue. Some stage II granulomas demonstrated a delicate rim of fibroblasts and connective fibres. Commonly, the connective tissue surrounded the lesion of

stages III and IV granulomas partially or completely. The presence of marked neo-vascularization with small capillaries vessels (Figure 3.15) was a frequent finding in the connective tissue seen in the periphery of the stages III and IV granulomas.

With ZN staining, AFB were observed in tissues from seven animals (36.8%), most of which had macroscopic lesions of grades 3 to 4. The AFB were detected in mediastinal LN (4/7), lung (2/7), bronchial LN (1/7) and retropharyngeal LN (1/7) (Table 3.2). When present, acid-fast bacilli were found in low numbers only, mostly observed as a single organism in the cytoplasm of a multinucleated giant cell (Figure 3.16.).

Table 3.2. Microscopical lesions in African buffalo with tuberculosis

Animal			Histopathology				
			†Tissue and lesion stage (I-IV)				ZN
*ID	**Sex	Age	**RP**	**B**	**ML**	**L**	‡**AFB**
49	F#	Adult	-	II	I-II	-	+(ML)
52	M	Adult	II-III	I-II	I-II	I-II	Neg
53	F	Adult	-	II	-	-	Neg
57	F	Adult	-	I-IV	-	-	Neg
58	F	Adult	-	I-III	II-IV	I-III	+(ML,L)
61	F	Subadult	I	-	-	-	Neg
62	M	Adult	-	I	-	-	Neg
66	F	Subadult	-	I-II	II-III	-	+(ML)
69	F	Adult	-	I-III	I-III	I-IV	+ (L)
77	F	Adult	-	I	I-II	-	+(ML)
79	M	Subadult	-	I-II	I-II	-	Neg
80	F#	Adult	-	-	I	-	Neg
84	F	Adult	-	-	I-II	-	Neg
85	F	Adult	-	I-II	I	-	Neg
89	F#	Adult	-	I-II	-	-	Neg
92	F	Adult	III-IV	III	-	-	+(B)
97	F	Subadult	I-III	I-IV	I-IV	I-III	+(RP)
98	M	Adult	-	I-II	-	-	Neg
Un	M	Subadult	-	I-II	-	-	Neg

Abbreviations:

ID: animal individual number; Un=Not numbered; ** Sex: M = male; F = female. F# = pregnant female; † Tissue: RP = medial retropharyngeal lymph node; B = bronchial lymph node; ML = mediastinal lymph node; L = lung; ‡ AFB = acid-fast bacilli in Ziehl-Neelsen stained sections. + = positive, with tissue/s in brackets; Neg = negative; Hyphen (-) = tissue not examined

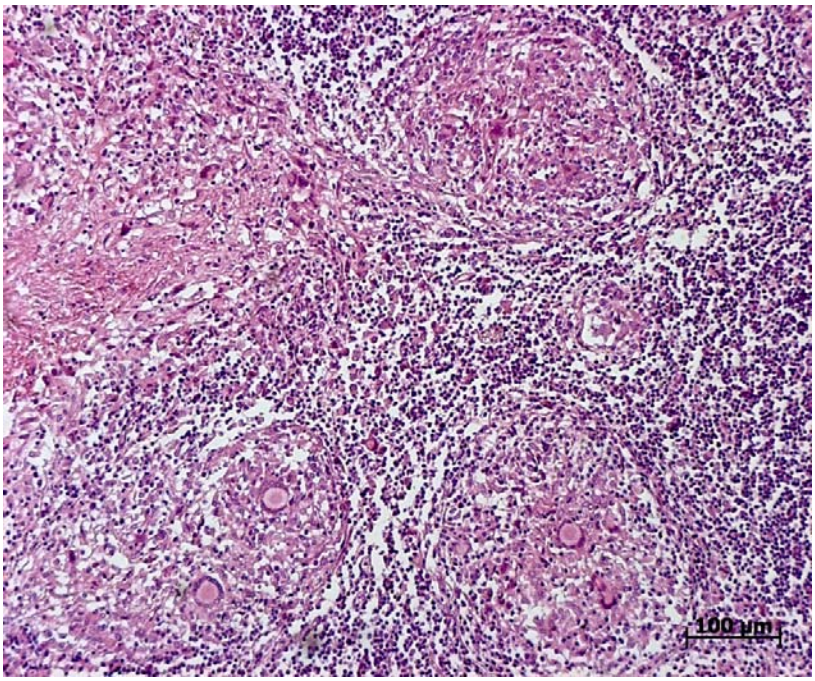


Figure 3.7. Animal 79, mediastinal lymph node. Multiple stage I granulomas composed of epithelioid cells and multinucleated giant cells and lymphocytes. HE. 100X.

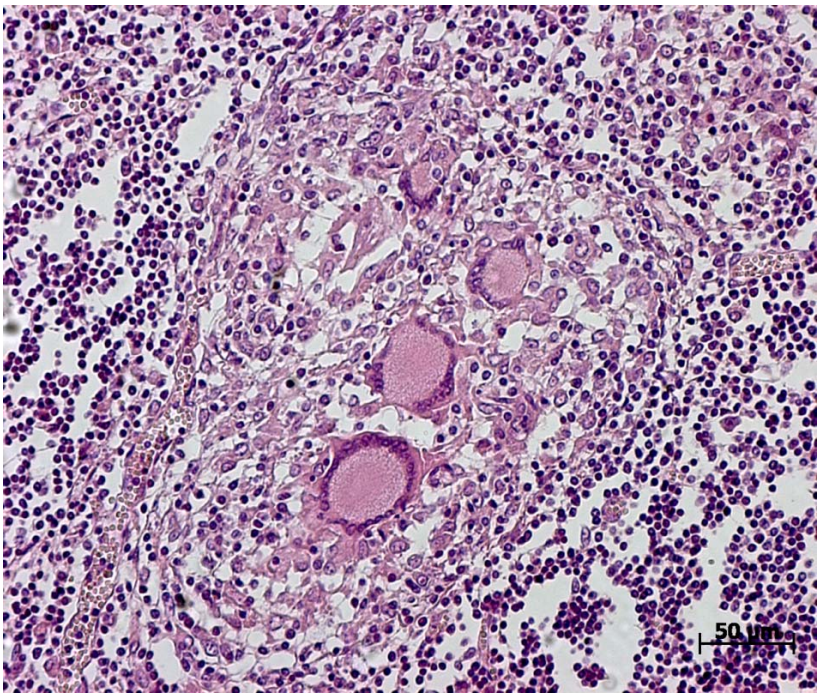


Figure 3.8. Animal 79, mediastinal lymph node. Stage I granuloma, epithelioid cell and multinucleated giant cells surrounded by lymphocytes. HE. 200X.

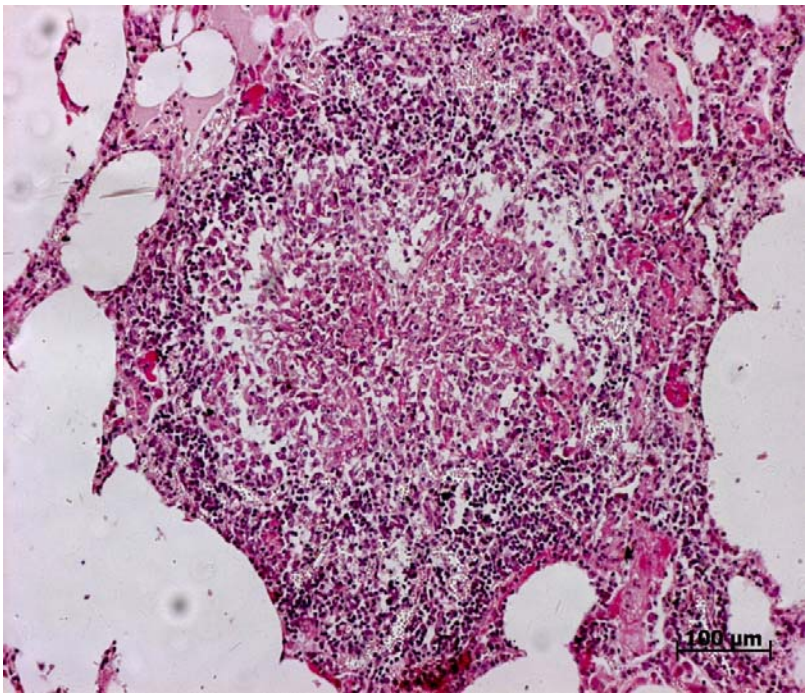


Figure 3.9. Animal 69, lung, Stage I granuloma: non-encapsulated lesion composed of epithelioid cells surrounded by lymphocytes. HE. 100X.

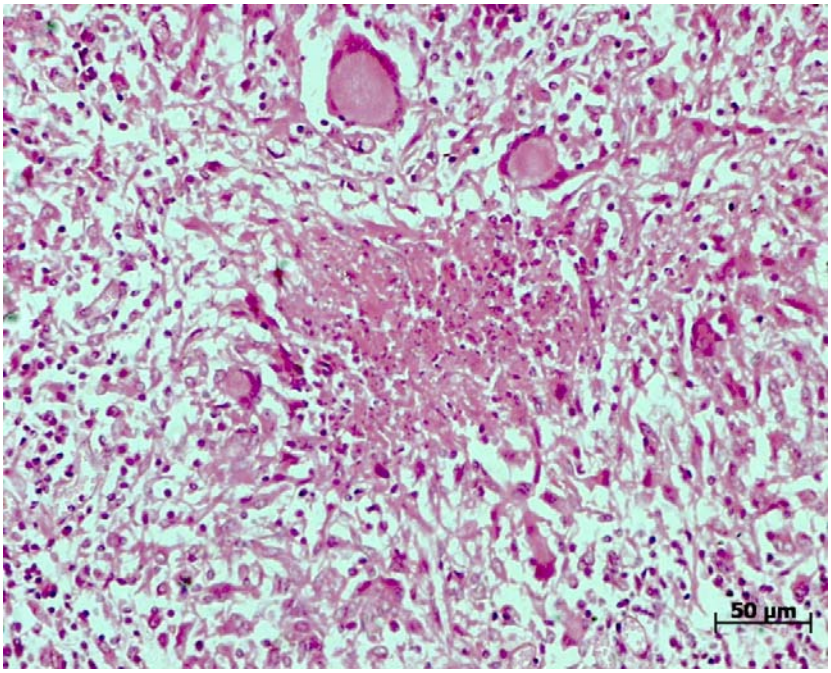


Figure 3.10. Animal 77, mediastinal lymph node, Stage II granuloma: a central caseous necrosis, surrounded by epithelioid cells, giant cells and lymphocytes. HE. 200X.

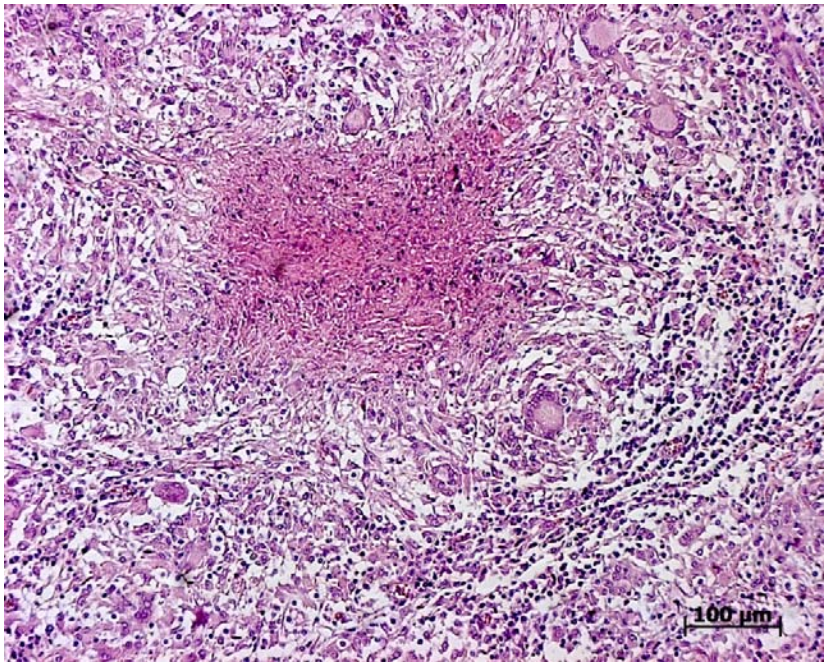


Figure 3.11. Animal 69, lung, Stage II granuloma: central caseous necrosis, surrounded by epithelioid cells, multinucleated giant cells and lymphocytes. HE. 100X.

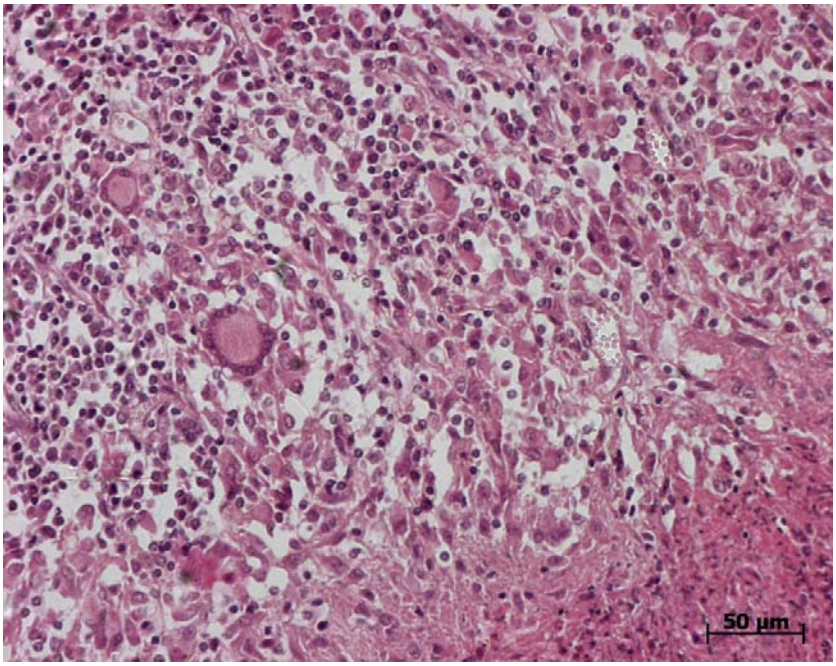


Figure 3.12. Animal 69, lung, Stage II granulomas. High magnification of the periphery of the granulomas. Necrotic area (bottom right) surrounded by epithelioid cells, multinucleated giant cells and lymphocytes. HE. 200X.

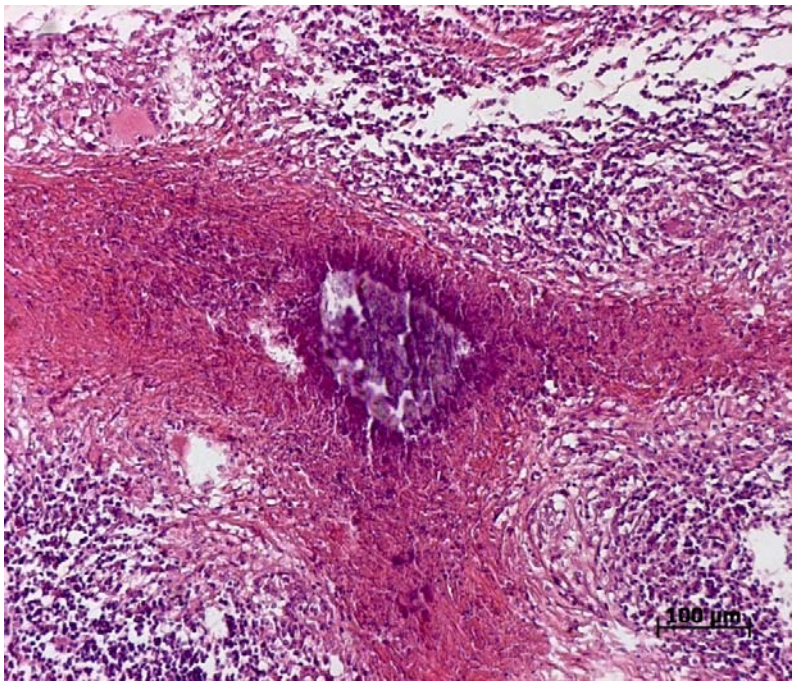


Figure 3.13. Animal 57, bronchial lymph node, Stage III granuloma: central area of caseous necrosis and mineralization, surrounded by multinucleated giant cells and lymphocytes. HE. 200X.

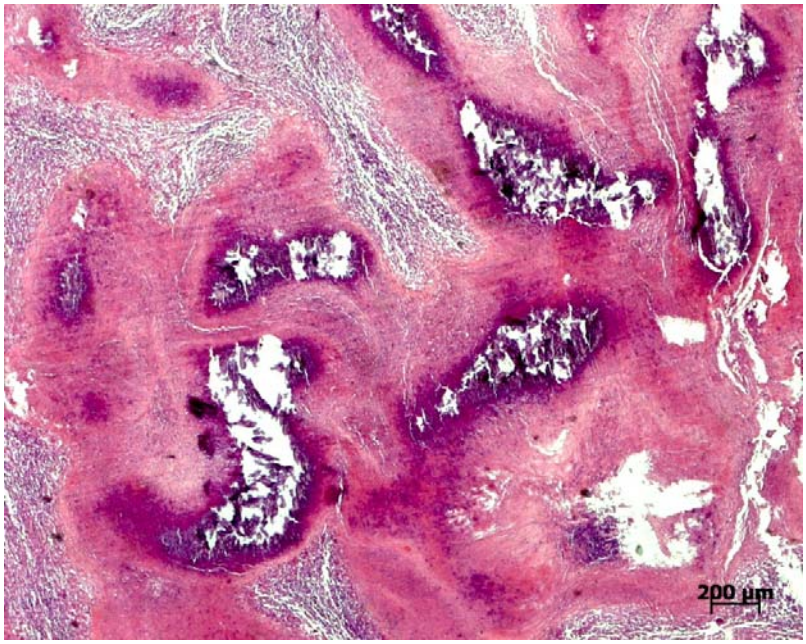


Figure 3.14. Animal 57, bronchial lymph node**,** Stage IV granulomas: extensive confluent areas of necrosis with multiple foci of mineralization. HE. 100X.

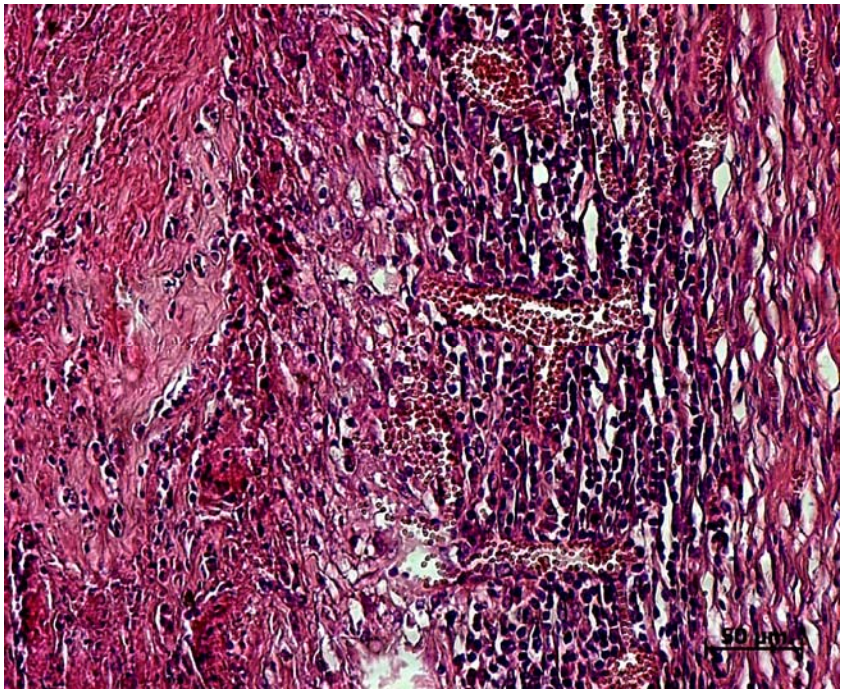


Figure 3.15. Periphery of stage IV granuloma. A necrotic area (left) surrounded by inflammatory cells and connective tissue with neovascularization. HE. 200X.

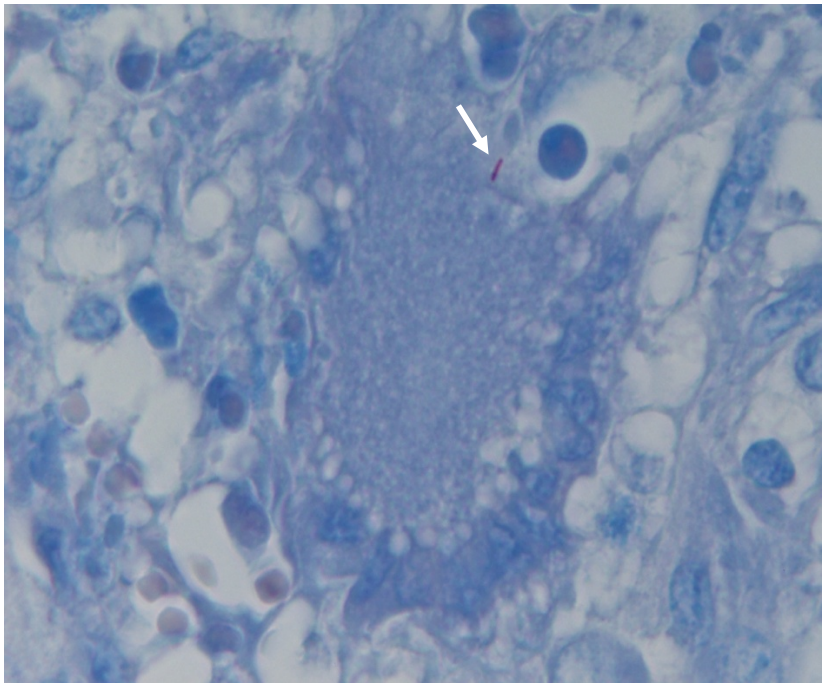


Figure 3.16. A single acid fast bacillus (white arrow) in a multinucleated giant cell. ZN. 1000X.

3.2.2. Bacteriology

Owing to power failure and freezer failure during storage of the samples, unfortunately fresh samples could not be used for mycobacterial culturing.

3.2.3. PCR

Method 1:

Of the 19 tissue specimens examined, only one specimen (A 77) demonstrated the presence of an amplification product (268 bp) suggestive of *M. bovis* infection (Figure 3.17).

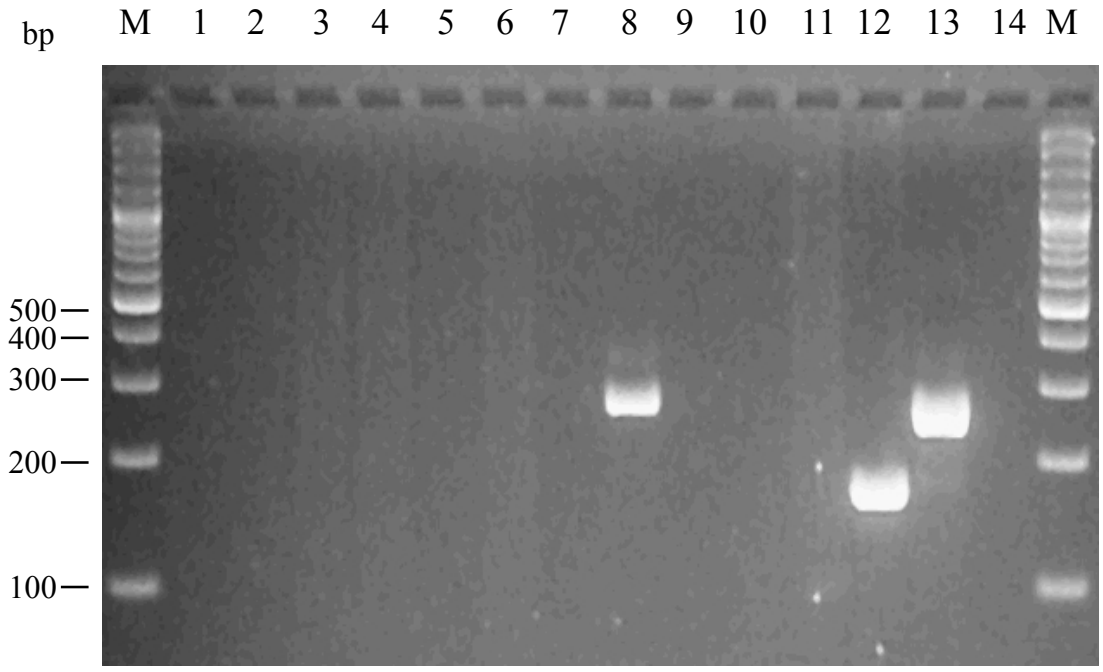


Figure 3.17. Agarose gel (2%) stained with ethidium bromide, showing RD polymerase chain reaction products. Lane M: Marker (100bp). Lane 1: 58 LM; Lane 2: 97 LM; Lane 3: 69 L; Lane 4: 79 LM; Lane 5: 97 LM; Lane 6: 62 LB; Lane 7: 52 LM; **Lane 8: 77 LB** (***M bovis* positive**); Lane 9: 61 L; Lane 10: 58 LRT; Lane 11: 52 LRT; Lane 12: Positive control *M tuberculosis*, Lane 13: Positive control *M bovis*, Lane 14: Negative control (H_2O). RD = regions of difference; bp = base pairs; LM = Mediastinal lymph node; LB = Bronchial Lymph node; L = lung; LRT = retropharyngeal lymph node.

Method 2:

Amplification of DNA by real-time PCR identified the genome of *M. bovis* in 8 of 19 African buffalos. Dissociation curve analysis showed that positive samples presented an amplicon melting temperature of 87.8 ± 0.3 º C. The technique was validated using positive controls of *M. bovis* culture (courtesy of Dr. Parra, Spain) and by means of sequencing (Figures 3.18 and 3.19).

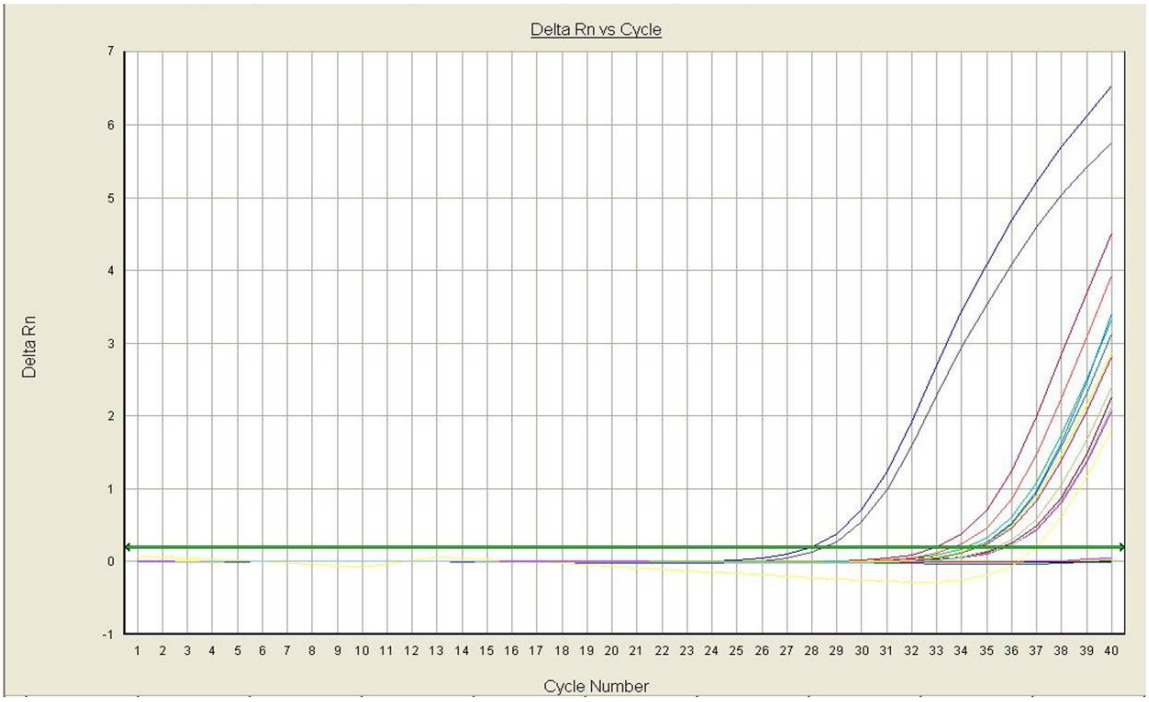


Figure 3.18. "Amplification plot" shows the amplification curve for the buffalo samples. The two lines at the left represent the positive control.

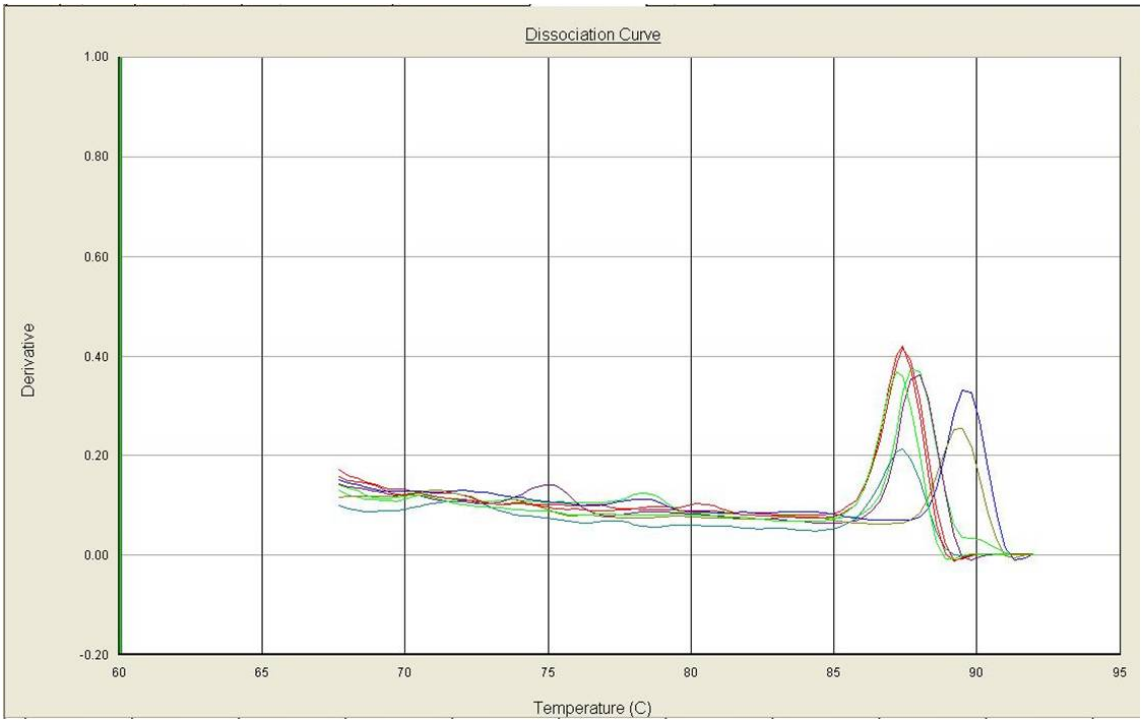


Figure 3.19. "Melting curve" shows the melting temperature for the amplicons. The two lines at the right are for *Mycobacterium avium.* The curves *for Mycobacterium bovis* are in the group, together with the curves for the buffalo samples.

CHAPTER 4: DISCUSSION

This study presents the gross and microscopical changes of bTB in the African buffalo, which is considered a wildlife reservoir of bTB in southern Africa. The macroscopical and histological appearance of the lesions in the buffalo closely resembled that of cattle. The microscopic classification described for domestic cattle (Wangoo et al., 2005), could be applied to the buffalo lesions with the limitation that immunohistochemistry and *in situ* hybridization for the identification of cell populations and cytokines were not done.

Due to logistical difficulties during the sample storage (including wide-scale power failure), fresh or frozen samples could not be used for mycobacterial culture. The diagnosis of bTB was based on the history of the herd, the positive tuberculin test and the characteristic appearance of the macroscopical and microscopical lesions, many with AFB. Bovine tuberculosis was confirmed by the PCR analyses in eight animals. Within the HiP, bTB test-and-culling program, *M. bovis* has regularly been isolated from culled buffalo (Jolles et al., 2005).

4.1. Gross pathology

Out of twenty four slaughtered animals that were positive for bTB skin test, 19 (79.2%) presented macroscopic tuberculous lesions. The animals were in good body condition and the lesions affected only a few LN, indicating an early or limited disease stage (Grobler et al., 2002). Bovine tuberculosis positive animals with poor body condition are usually found in herds with a high TB prevalence and mainly during the dry season (Caron et al., 2003). The time of sampling for this study was the rainy season in which there was an abundance of grazing in HiP, which could also have contributed to the good body conditions of the animals. Only three animals (no. 58, 97 and 69) presented more severe lesions in two or three LN, as well as in the lung. Furthermore, advanced cases were less likely to be found in the herd because it was subjected to a test-and-cull program. Therefore, many of the positive animals that could have reached advanced stages of the lesions were probably removed since the program in HiP began in 1999. Predation may also have eliminated animals with advance tuberculosis.

A study in HiP demonstrated that approximately 70% of bTB cases in buffalo were mild (Jolles et al., 2005), and it was considered unlikely that they had shown clinical signs. Bengis et al., 1996, de Klerk et al., 2006). This supports the opinion that bTB in buffalo is a disease of limited and slow development in the majority of the cases (Jolles et al., 2005). The test and culling TB control program in HiP has been successful in reducing the prevalence in some buffalo herds from previous 10-20% to below 10% and in high prevalence herds from approximately 55% in 2000/2001 to an estimated 20-30% (Michel et al., 2006, Jolles and Cooper, unpublished data).

In this study, five animals did not show any macroscopical visible lesions suggestive of bTB. Studies in cattle have shown that gross examination at slaughter fails to detect some of the *M. bovis* infected animals, mostly those with very small (approximately <1 mm in size) or non visible lesions (NVL) (Rohonczy et al., 1996; Asseged et al., 2004; Gavier-Widén et al., 2009). A study on cattle reported that 10.1% of the animals with NVL were culture positive, and 6.7% were considered as TB by histopathology (Liebana et al., 2007). De Vos and colleagues (2001) found that approximately 37 % of infected buffalo show no macroscopically detectable lesions. In the study reported here, such NVL cases may have occurred but this aspect was not investigated.

Forty seven percent (9/19) of the animals presented lesions in only one lymph node. This is in agreement with previous study in Ireland (Costello et al., 1997) and UK (Liebana et al., 2007) where gross lesions were confirmed to a single LN of cattle naturally infected by *M. bovis.* This is most likely due to the early stage of the disease, in which the lesions are still not disseminated to other lymph nodes and organs. Grade II lesions were the most commonly seen. Because of the small size of the grade I granulomas, it is possible that these could have been missed during the inspection.

The buffalo presented lesions most frequently in the LN associated with the respiratory tract (mediastinal and bronchial lymph nodes) and in the lungs, suggesting that the most likely route of infection was inhalation. These findings are consistent with previous descriptions in African buffalo (Keet et al., 1994; Bengis et al., 1996; De Vos et al., 2001; Lisle et al., 2002), water buffalo (Freitas et al., 2001) and are also similar to bTB in cattle (Corner, 1994; Whipple et al.,

1996; Palmer et al., 2002a; Asseged et al., 2004). In cattle it has been shown experimentally that infection via the respiratory route requires a lower dose than by the alimentary route (Neill et al., 2005).

Similarly to other studies in cattle (Palmer et al., 2002a; Liebana et al., 2007), buffalo (Keet at al., 1994; De Vos et al., 2001) and white-tailed deer (*Odocoileus virginatus*) (Palmer et al., 2002b), the tuberculous lesions in the lungs were most common in the caudal lobes. The reason for such a predilection in animals is unclear (Palmer et al., 2002b). The high occurrence of TB lesions in the upper lung lobes in humans is probably due to the increased oxygen tension and/or decreased lymphatic clearance in this area of the lung (McAdams et al., 1995). Likewise, similar factors may influence location of disease foci in animals.

Most of the buffalo (80%) did not show bTB lesions in the lungs, but had lesions in bronchial and mediastinal LN. This agrees with descriptions of early stages of bTB in cattle, and is most likely due to the difficulties of detecting very small lesions in the lungs at inspection, due to their large volume (Liebana et al., 2007). Small lesions in the lungs are more difficult to find than lesions of the same size in the lymph nodes (Cassidy, 2006). In this study, pulmonary lesions were detected in 21% of the buffalo and they were of the higher grades. The thoracic LN were involved in all the buffalo that presented TB lesions in the lungs.

An experimental study in which cattle were exposed to *M. bovis* by aerosol, revealed that 7 days post-infection the bacterial concentrations were high in lung-associated lymph nodes when compared with corresponding lung samples, suggesting that in the pre-adaptative immune stage, conditions in the lymph nodes were more favorable for *M. bovis* growth than conditions in the lung (Rodgers et al., 2007).

The absence of lesions in the gastrointestinal tract indicates that the oral route is not the main route of infection in buffalo (Keet et al., 1994). According to these authors, in advanced TB, lesions can be found in the intestinal tract probably as a result of ingestion of coughed-up and swallowed exudates originating from open lesions in the lung. In the study described here, this did not occur.

In an experimental intratonsilar model for bTB in African buffalo, the retropharyngeal LN was the most likely LN to develop tuberculous lesions (de Klerk et al., 2006). In this study, bTB lesions were present in retropharyngeal LN in 21% of the buffalo. Tuberculous lesions in buffalo are also common in tonsils (Keet et al., 1994; De Vos et al., 2001) but in this study the tonsils were not investigated.

As in other studies on African buffalo, most of the animals (73.7%) included in this study were adults (Rodwell et al., 2001b; Jolles et al., 2005). It has been reported that young buffalo have a lower risk of being positive for bTB than older buffalo, but that the risk for both age groups increases with increasing prevalence of the disease within the herd (Rodwell et al., 2001b).

In this study, the three animals that presented the most severe macroscopic and microscopic grade lesions were females (Animals 58, 69 and 97). A previous study in buffalo did not identify gender as a risk factor for developing bovine tuberculosis (De Vos et al., 2001). De Klerk et al. (2008) found that 9 out of 11 female buffalo challenged intratonsilarly with a field strain of *M. bovis* had tuberculous lesions, while only six out of 16 males showed lesions. These authors also found that females showed higher lesions scores than males. It is possible that continued social stress in the heifers due to competition with the older cows may play a significant role in the difference in lesions development between genders (De Klerk et al., 2008). A significantly high prevalence of bTB has been reported in male red deer, as a consequence of increased contact due to aggression (Lugton et al., 1998), illustrating the importance of the close contact between animals as a method of spread.

Twenty seven percent (3/11) of the adult females (A 49, 80 and 89) were pregnant. A study that evaluated the effect of bTB in free ranging African buffalo population in KNP, found that the proportion of pregnant and lactating buffalo cows infected with bTB was not significantly different from buffalo cows that were not infected, concluding that bovine bTB apparently do not affect fertility or lactation status during the 8 year study period (Rodwell et al., 2001b). Jolles et al. (2005) found that TB did not affect pregnancy rates in young adults females (6-8 years) but did affect pregnancy rates in subadults (< or = 5,5 years) and older adults (9-16 years).

4.2. Histopathology

All four developmental stages of the lesions were seen in the tissue examined. Some LN presented multiple stage I granulomas in the same section, which can represent the seeding of multiple bacilli in the same LN, resulting in the development of different foci. The stage II granulomas were the most frequent lesions in this study. Because of the small size of stage I granulomas, it is possible that these were present in organs or LN not sampled.

Animals with grades 3 to 5 lesions were the most likely to present up to four different microscopic grades in the same section. This result agrees with a previous report in naturally TB-infected cattle (Liebana et al., 2007) and it most likely represents repeated seeding of the LN by *M. bovis* with development of lesions at different times. Fenhalls et al. (2000) studying TB in humans, have shown that TB is a continual process of infection, granuloma formation, necrosis and reseeding and that each lesion represent an autonomous microenvironment.

Some stage III and IV granulomas were completely or partially surrounded by connective tissue. The degree of peripheral fibrosis in advanced tuberculous granulomas appears to be a feature related to the species of the host. Apart from buffalo, this lesion has also been reported in cattle (Rhyan and Saari, 1995; Wangoo et al., 2005). For instance, in badger (*Meles meles*) (Gavier-Widén et al., 2001) possums (*Trichosurus vulpecus*) (Cooke et al., 1995), and ferrets (*Mustela furo*) (Lugton et al., 1997) and cheetah (*Acinonyx jubatus*) (Keet et al., 1996) the tuberculosis lesions appear to have discrete fibrosis.

As reported in buffalos (Bengis et al., 1996, De Klerk at al., 2006) and cattle (Wangoo et al., 2005) the granulomas are composed mainly of lymphocytes and macrophages. Langhans multinucleated giant cells occurred in all four stages but were more frequent in grades III and IV. This finding is similar to the results reported in cattle (Wangoo et al., 2005). The latter cells have not been observed in species such as badgers (Gallager et al., 1976; Gavier Widen et al., 2001) or in wild felids (Keet et al., 1996). These differences of findings between species can be ascribed to the difference in the immune response against *M. bovis* infection between species.

Mineralization has been described in advanced TB lesions in African buffalo (Bengis et al., 1996; De Klerk et al., 2006) cattle (Rhyan and Saari, 1995; Cassidy et al., 1999), elk and red deer (Rhyan and Saari, 1995), sika deer (Rhyan and Saari, 1995) and white-tailed deer (*Odocoileus virginianus*) (Palmer et al., 2002b). As in cattle, the mineral deposits in African buffalo are commonly found in the center of the necrotic foci. These findings indicate that necrosis and calcification are common features of granulomas both in cattle (Liebama et al., 2007) and buffalo, even if the lesions in a given animal are limited to a small number of foci. Limited calcification of the necrotic debris was observed in cheetah and baboon and it was not seen in lion TB lesions (Keet et al., 1996).

Few AFB were observed in the ZN stained sections, indicating that the lesions were paucibacillary. The relatively few acid fast organisms observed in this study is similar to results reported in cattle (Liebana et al., 2007) and red and fallow deer (Martín-Hernando et al., 2010). Whether this is a reflection of a low bacterial load required to trigger lesion formation or a hostile environment for mycobacterial survival has not been elucidated (Liebana et al., 2007). Another reason is that the sensitivity of ZN staining for AFB is low and it is estimated that it needs to be at least 10^5 cfu/ml of tissue for the AFB to be detected microscopically (McCune and Tompsett, 1956). A study in lions concluded that none of the organ sections from culture-positive cases showed AFB in granulomas when stained with Ziehl Neelsen (Keet et al., 2008). Numerous AFB bacilli have been seen in sections of TB lesions from Springbok (*Antidorcas marsupialis*) (Gous, 2007). In this study, the AFB were usually located in the cytoplasm of giant cells, as previously reported in buffalo (de Klerk et al., 2006).

In this study early stage lesions seen in the lung were not encapsulated. The lack of encapsulation of lung TB lesions suggests that even very small lesions, are open and infectious. This possibly implies that buffalo can be infectious from a very early stage of infection (De Vos et al., 2001). In two animals (no. 69 and 97) with severe lung lesions, it was observed that the granulomas had eroded the wall of bronchioles, indicating that these animals could excrete *M. bovis* and be a source of infection for other animals (Mcllroy et al., 1986; Phillips et al., 2003). This finding suggests that individual animals may be responsible for most transmission (ie super-

shedders) and that if it as possible to identify these and remove them (as a priority), the epidemic could more easily be curbed.

This study also found the occasional occurrence of metazoan parasites in the lung of the buffaloes (See appendix 3). Parasites such as *Trichostrongilus* spp, *Trichurus* spp and *Fasciola* spp have been reported in buffalo in KNP (Caron et al., 2003). These authors found that herds with high bTB prevalence underwent the greatest increase in endoparasite load and the greatest decrease in body condition between early and late phases of the dry season.

4.3. PCR

PCR-amplification confirmed the presence of *M. bovis* in tissue specimen from eight animals. This low percentage of positivity could be due to the formalin fixation of the tissue samples (Vincek et al., 2003; Cao et al., 2003; Van Pelt-Verkuil et al., 2008). These samples were fixed in formalin for a period of 1 month to inactivate the bacilli and reduce the risk of contamination during the histopathologic procedures. This long fixation may have lead to DNA modification and degradation thereby preventing/inhibiting PCR amplification. It is well known that formalin fixation and embedding lead to DNA degradation which in turn may limit the number of intact TB DNA targets (fragments spanning the region complimentary to the PCR primers). This in turn will reduce the amplification efficiency. An alternative explanation could be that the xylene used in the extraction procedure could have inhibited the action of the proteinase K used for the sample digestion (Coura et al., 2005) thereby preventing the efficient release of DNA from the tissue sections.

The small number of AFB (paucibacillary infection) seen in the ZN staining could mean than the samples had an initial low concentration of DNA what could also explain the low sensitivity of the PCR (Gómez-Laguna et al., 2010). False negative PCR results may have occurred as a consequence of the small amount of tissue examined, compared to the amount generally used for bacteriology culture (Miller et al., 1997). Future genetic speciation of the MTC should be done on DNA extracted from frozen tissue specimens. To enhance the sensitivity of the method the amplification of smaller PCR products may be required.

The difference in the number of positive animals when comparing the two methods, method 1 (1/19 - 5.2 %) and method 2 (8/19 - 42%) could be due to the different efficiency of the paraffin wash method and the DNA extraction kits used in each method. The usage of xylene during the paraffin washing in method 1, could have influenced negatively the protease digestion of the samples. Another possible explanation is that the blocks used for the different methods may have had different bacterial (and therefore DNA) concentration. The other possibility is that in this case, the RT PCR presented more sensitivity than the convencional PCR.

4.4. Conclusion

This study was carried out in naturally infected animals. One experimental intratonsilar infection model for bovine tuberculosis in African buffalo has been done (de Klerk et al., 2006), although, more experimental studies in buffalo are needed to better understand the pathogenesis of bTB, specifically the time between the infection and the development of the different lesion stages. In this study, the granulomas were characterized based on their appearance in HE and MT stained sections. Immunohistochemistry and *in-situ* hybridization would have contributed to the identification of the populations of cells and cytokines involved in each stage of lesion development. Future studies will focuse on bovine lymphocyte and macrophage markers for immunohistochemistry studies on African Buffalo TB lesions, this will allow studying the cellular dynamic changes in TB lesions of different microscopic stages.

In conclusion, the gross and microscopic appearance of the lesions in the African buffalo closely resembles tuberculosis in cattle, showing that the grading system for microscopic lesions used for cattle is also valid for buffalo. This study outlines a systematic semi quantitative evaluation of stages of development of tuberculous lesions in buffalo. The results may contribute to i) the understanding of the pathogenesis of the disease, ii) the evaluation of experimental models of *M. bovis* infection in *Syncerus caffer*, and iii) the interpretation of pathological data from vaccination trials.

4.5. Limitations of the study

Due to logistical difficulties during the sample storage (including wide-scale power failure), fresh or frozen samples could not be used for mycobacterial culture. The confirmation of the causative agent was based on PCR analyses. Within the HiP bTB test-and-culling program, *M. bovis* has regularly been isolated from culled buffalo (Jolles et al., 2005). Other members of the MTC have not been identified as the causal agent of TB lesions HiP buffalo. To differentiate *M. bovis* from the other MTC bacteria using the PCR technique, the RD4 primer was used (Warren et al., 2006) and after that the identification of the MTC members was done by sequencing. If culture had been done, we predict that more *M. bovis* positive animals would have been identified, than the low positivity (8/19 = 42%) found with PCR (after DNA extraction from formalin fixed, paraffin embedded samples). It is known that formalin can degrade the DNA, reducing the sensitivity of PCR (Cao et al., 2005).

A further possible limitation of the study is that all the animals sampled, which were TST positive and presented gross lesions suggestive of TB, in general presented lesions reflecting early to middle stages of the disease. It would have been interesting to include animals with advanced or terminal stages of the lesions, to help to better understand the pathogenesis of the lesions of bTB in different stages of the disease. Such animals are seldom if ever seen in the wild, since they become targets for predation.

REFERENCES

Alexander KA, Pleydell E, Williams MC, Lane EP, Nyange JFC and Michel AL. 2002. *Mycobacterium tuberculosis*: An Emerging Disease of Free-ranging wildlife. Emerg Infect Disease. 8 (6): 1-7.

Ameni G, Vordermeier M, Firdessa R, Aseffa A, Hewinson G, Gordon SV and Berg S. 2010. *Mycobacterium tuberculosis* infection in grazing cattle in central Ethiopia. Vet J.doi:10.1016/j.tvjl.2010.005.

Asseged B, Woldesenbet Z, Yimer E and Lemma E. 2004. Evaluation of abattoir inspection for diagnosis of *Mycobacterium bovis* infection in cattle at Addis Ababa abattoir. Trop Anim Health Prod. 36: 537-546.

Bengis RG, Kriek NPJ, Keet DF, Raath JP, De Vos V and Huchzermeyer HFAK. 1996. An outbreak of bovine tuberculosis in a free-living African Buffalo (*Syncerus caffer*-Sparman) population in the Kruger National Park: a preliminary report. Onderstepoort J Vet Res. 63:15-18.

Buddle BM, Keen D, Thomson A, Jowett G, McCarthy AR, Heslop J, De Lisle GW, Stanford JL and Aldwell FE. 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae*. Res Vet Sci. 59: 10–16.

Buddle BM, Skinner MA and Chamber MA. 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. Vet Immunol Immunopathol. 74: 1-16.

Caley P and Hone J. 2005. Assessing the host disease status of wildlife and the implications for disease control: *Mycobacterium bovis* infection in feral ferrets. J Appl Ecol. 42: 708-19.

Cao W, Hashibe M, Rao JY, Morgenstern H, and Zhang ZF. 2003. Comparison of methods for DNA extraction from paraffin-embedded tissue and bucal cells. Cancer Detect Prev. 27: 397-404.

Caron A, Cross PC and du Toit JT. 2003. Ecological implications of bovine tuberculosis in African buffalo herds. Ecol Appl. 13 (5): 1338-1345.

Cassidy JP, Bryson DG and Neill, SD. 1999. Tonsillar lesions in cattle naturally infected with *Mycobacterium bovis*. Vet Record. 144(6): 139-142.

Cassidy JP. 2006. The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models. Vet Microbiol 112:151–161.

Cheeseman CL, Wilesmith JW and Stuart FA. 1989. Tuberculosis: the disease and its epidemiology in the badger: a review. Epidemiol Inf. 103:113-125.

Cleaveland S, Mlengeya T, Kazwala RR, Michel A, Kaare MT, Jones SL, Eblate E, Shirima GM and Paker C. 2005. Tuberculosis in Tanzanian Wildlife. J Wildlife Dis. 4(2): 446-453.

Cleaveland S, Shaw DJ, Mfinanga SG, Shirima G, Kazwala RR, Eblate E and Sharp M. 2007. *Mycobacterium bovis* in rural Tanzania: Risk factors for infection in human and cattle populations. Tuberculosis. 87: 30-43.

Cooke MM, Jackson R, Coleman JD and Alex MR. 1995. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): II. Pathology. N Z Vet J 43:128-132.

Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D, Robinson RA, Huchzermeyer HFAK, de Kantor, I and Meslin FX. 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg Infect disease. 4 (1): 59-70.

Corner LA. 1994. Post mortem diagnosis of *Mycobacterium bovis* infection in cattle. Vet Microbiol. 40: 53-63.

Costello E, Egan JW, Quigley FC, O'Reilly PF. 1997. Performance of the single intradermal comparative tuberculin test in identifying cattle with tuberculous lesions in Irish herds. Vet rec. 141: 222-224.

Coura R, Prolla JC, Meurer L and Ashton-Prolla P. 2005. An alternative protocol for DNA extraction from formalin fixed and paraffin embedded tissue. J Clin Pathol. 58: 894-895.

Cousins DV, Huchzermeyer HFKA, Griffin VFT, Bruckner GK, van Rensburg IBJ and Kriek NPJ. 2004. Tuberculosis in: Infectious disease of livestock. 2nd edition. Volume three. Edited by: J A W Coetzer & R C Tustin. Oxford University Press. pp 1973-2041.

Cross PC and Getz WM. 2006. Assessing vaccination as a control strategy in an ongoing epidemic: Bovine tuberculosis in African buffalo. Ecol model. 196:494-504.

De Kantor IN, Ambroggi M, Poggi S, Marcillo N, Telles MA, Ribeiro MO, Torres MCG, Polo CL, Ribón W, Garcia V, Kuffo D, Asencios L, Campos LMV, Rivas C and de Waard JH. 2008. Human *Mycobacterium bovis* infection in ten Latin American countries. Tuberculosis. 88: 358-365.

De Klerk-Lorist L. 2005. The evaluation of BCG vaccine against bovine tuberculosis in African buffaloes (*Syncerus caffer*). Master Thesis. University of Pretoria, Pretoria. pp 169.

De Klerk L-M, Michel AL, Grobler DG, Bengis RG, Bush M, Kriek NPJ, Hofmeyr MS, Griffin JFT and Mackintosh CG. 2006. An experimental intratonsilar infection model for bovine tuberculosis in African buffaloes, *Syncerus caffer*. Onderstepoort J vet Res 73:293-303.

De Klerk L-M, Michelo AL, Bengis RG, Krick NPJ and Godfroid J. 2008. BCG vaccination failed to protect yearling African buffalo (*Syncerus caffer*) against experimental challenge with *Mycobacterium bovis*. Vet Immunol Immunopath. Doi:10.1016/j.vetmm.2010.04.013.

De Lisle GW, Yates GE, Collins DM, Mackenzie R.W, Crews KB and Walker R. 1995. A study of bovine tuberculosis in domestic animals and wildlife in the Mackenzie Basin and surrounding areas DNA fingerprinting. N Z Vet J. 43: 226-271.

De Lisle GW, Bengis RR, Schmitt SM, and O'Brien DJ. 2002. Tuberculosis in free-ranging wildlife: detection diagnosis and management. Rev Sci Tech Off Int Epiz. 21 (2): 314-334.

De Garine-Wichatitsky M, Caron A, Gomo C, Foggin C, Dutlow K, Pfukenyi D, Lane E,Le Bel S,Hofmeyr M, Hlokwe T, and Michel A. 2010. Bovine Tuberculosis in Buffaloes, Southern Africa. Emerg Infect Dis. 16 (5).

De Vos V, Bengis RG, Kriek NPJ, Michel A, Keet DF, Raath JP and Huchzermeyer HFKA. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer)* in the Kruger National Park, South Africa. Onderstepoort J Vet Res. 68:119-130.

Drewe JA, Foote AK, Sutcliffe RL and Pearce GP. 2008. Pathology of Mycobacterium infection in wild Meerkats (*Suricata suricatta*). J. Comp Path. Doi:10.1016/j.jcpa.2008.09.04.

Ellis SM. 2004. The spectrum of tuberculosis and non-tuberculous mycobacterial infection. Eur Radiol. 14: 34–42.

Espie I W, Hlokwe TM, van Pittius NCG, Lane E, Tordiffe ASW, Michel AL, Múller A, Kotzer A and van Helden PD. 2009. Pulmonary infection due to *Mycobacterium bovis* in a Black Rhinoceros (*Diceros bicornis minor*) in South Africa. J Wildlife Dis. 45(4): 1187-1193.

Fenhalls G, Wong A, Bezuidenhout J, van Helden P, Bardin P and Lukey PT. 2000. *In situ* production of interferon-γ, interleukin-4, and tumor necrosis factor-α mRNA in human lung tuberculosis granulomas. Infect Immun. 68: 2827-2836.

Freitas, J de A, Guerra JL and Panetta JC. 2001. Características da tuberculose observeda em bufalos abatidos para consumo: aspectos patológicos e identificação de micobacterias [Characteristics of tuberculosis in slaughtered water buffaloes]. Braz Vet Res Anim Sci. 38 (4):170-176.

Frothingham R and Meeker-O'Connell WA. 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandom. Microbiology: 144: 1189–1196.

Gallagher J, Muirhead RH and Burn KJ. Tuberculosis in wild badgers (*meles meles*) in Gloucestershire: Pathology. Vet Rec. 98: 9-14.

Gavier-Widen D, Chamber MA, Palmer N, Newell DG and Hewinson RG. 2001. Pathology of natural *Mycobacterium bovis* infection in European badgerd (*Meles meles*) and its relationship with bacterial excretion. Vet Rec. 148: 299-304.

Gavier-Widén D, Cooke MM, Gallagher J, Chambers MA and Gortázar C. 2009. A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the "no visible lesions" presentation. N Z Vet J. 53 (3):122-131.

Gómez-Laguna J, Carrasco L, Ramis G, Quereda JJ, Gómez S and Pallarés FJ. 2010, Use of real-time and classic polymerase chain reaction assays for the diagnosis of porcine tuberculosis in formalin-fixed, paraffin-embedded tissues. J Vet Diag Invest. 22 (1): 123-127.

Gous TA. 2007. The pathology of tuberculosis caused by *Mycobacterium tuberculosis*, in a herd of semi free-ranging Springbok (*Antidorcas marsupialis*). Msc. Thesis. Pretoria University, Pretoria, South Africa.

Griffen JFT, Cross JP, Chinn DN, Rogers CR and Buchan GS. 1994. Diagnosis of tuberculosis due to *M. bovis* in New Zealand red deer (*Cervus elaphus*) using a composite blood test (BTB) and antibody (ELISA) assays. N Z Vet J. 42: 173–179.

Grobler DG, Michel AL, De Klerk LM and Bengis RG. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J Vet Res. 69(3): 221-7.

Guilbride PDL, Rollinson DHL, Mcanulty EG, Alley JG and Well EA. 1963. Tuberculosis in free living African (Cape) buffalo (*Syncerus caffer Sparrman*). J Comp Pathol Therap. 73:337-213.

Harries AD, Hargreaves NJ, Chimzizi R, Salaniponi FM. 2002. Highly active antiretroviral therapy and tuberculosis control in Africa: synergies and potential. Bull World Health Organ. 80(6): 469-70.

Harris NB. 2006. Molecular techniques: applications in epidemiologic studies. In: *Mycobacterium bovis* infection in animals and humans, second volume. Edited by: CQ Thoen, FH Steele and MF Gilsdorf. Blackwell Publishing. UK. pp 54-67.

Heifets LB and Jenkins PA. 1998. Speciation of *Mycobacterium* in clinical laboratories. *In:* Mycobacteria I. Basic Aspects, Gangadharam P.R. & Jenkins P.A., eds. Chapman and Hall, New York, USA, 308–350.

Hénault S, Karoui C and Boschiroli ML. 2006. A PCR-based method for tuberculosis detection in wildlife. Vannier P and Espeseth G (eds). New diagnosis technology: applications in animal health and biology controls. Dev Biol. Vol 126. pp 123-132.

Hilsberg S and van Hoves W. 2000. Tuberculosis in wild animals in Africa: a review with special reference to the Kruger National park. The infectious disease review. 1(4):248-252.

Hope JC and Villarreal-Ramos B. 2008, Bovine TB and the development of new vaccines. Comp Immun Microbiol Infect Dis. 31: 77-100.

Huchzermeyer HFKA, Brückner GK, van Heeerden A, Kleeberg HH, van Rensburg IBJ, Koen P and Loveday RK. 1994. Tuberculosis. In: JAW Coertzer, GR Thompson and RC Tustin, Editors; Infectious Diseases of liverstock. pp 1425-1445.

Jackson R, Cooke MM, Coleman JD and Morris R S. 1995a. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichorusus vulpecula*): I. An epidemiological analysis of lesions distribution. N Z Vet J. 43: 322-327.

Jackson R, Cooke MM, Coleman JD, Morris RS, de Lisle GW, Yaters GF. 1995b. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichorusus vulpecula*): III. Routes of infection and excretion. N Z Vet J. 43: 322-327.

Jolles AE. 2004. Disease ecology of bovine tuberculosis in African buffalo. PhD thesis. Princeton University.

Jolles AE, David C and Simon AL. 2005. Hidden effects of chronic tuberculosis in African buffalo. Ecology. 86:2358–2364.

Kalema-Zikusoko G, Bengis RG, Michel AL and Woodford MA. 2005. A preliminary investigation of tuberculosis and other diseases in African buffalo (*Syncerus cuffer*) in Queen Elizabeth National Park, Uganda. Onderstepoort J Vet Res. 72: 141-151.

Kamerbeek J., Schouls L., Kolk A., Van Agterveld M., Van Soolingen D., Kuijper S., Bunschoten A., Molhuizen H., Shaw R., Goyal M and Van Embden J. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 35: 907–914.

Keet DF, Kriek NPJ, Huchzermeyer H and Bengis RG. 1994. Advanced tuberculosis in an African buffalo (*Syncerus caffer* Sparrman). J S Afr Vet Assoc. 65:79-83.

Keet DF, Kriek NPJ, Penrith ML, Michel A and Huchzermeyer H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in Kruger National Park: spread of the disease to other species. Onderstepoort J Vet Res. 63:239-244.

Keet DF, Kriek NPJ, Bengis RG and Michel A. 2001. Tuberculosis in Kudus (*Tragelaphus strepsicerus*) in the Kruger National Park. Onderstepoort J Vet Res. 68: 225-230.

Keet DF, Michel AC, Bengis RG, Becker PS, van Dyk DS, van Vunren M, Rutten VPMG and Penzhorn BL. 2008. Intradermal tuberculin testing of wild African lion (*Panthera leo*) naturally exposed to infection with *Mycobacterium bovis*. Vet Microbiol. doi:10.a0a6/j.vetmic20/0.01.028.

LoBue P. 2006. Public heath significance of *M. bovis.* In: *Mycobacterium bovis* infection in animals and humans, second volume. Edited by: CQ Thoen, FH Steele and MF Gilsdorf. Blackwell Publishing. UK. pp 6-12.

Lopez A. 2001. Respiratory system, thoracic cavity and pleura. In: Thompson's Special Veterinary Pathology, 3rd edition. Edited by: M.D. Gavin, W.W. Carlton and J.F. Zachary. St. Louis, Mosby. Inc: 170-171.

Liebana E, Aranaz A, Mateos A, Vilafranca M, Gomez-Mampaso E, Tercero JC, Alemany J, Suarez G, Domingo M and Dominguez L. 1995. Simple and rapid detection of *M. Bovis* complex organisms in bovine tissue sampled by PCR. J Clin Mycrobiol. 3(1): 33-36.

Liebana E, Johson L, Gough J, Durr P, Jahans K, Clinfton-Hadley R, Spencer Y, Hewinson RG and Downs SH. 2007. Pathology of naturally occurring bovine tuberculosis in England and Wales. The Vet J. Doi:10.1016/j.tvjl.

Lugton IW, Johnstone AC and Morris RS. 1995. *Mycobacterium bovis* infection in New Zealand hedgehods (*Erinaceus europaenus*). N Z Vet J. 43: 342-5.

Lugton IW, Woberser G, Morris RS and Caley P. 1997. Epidemiology of *Mycobacterium bovis* infection in feral ferrets (*Mustela furo*) in New Zealand: I. Pathology and diagnosis. N Z Vet J. 45: 140-50.

Lugton IW, Wilson PR, Morris R S and Nugent G. 1998. Epidemiology and pathogenesis of *Mycobacterium bovis* infection of red deer (*Cervus elaphus*) in NewZealand. *N Z Vet J 46***,** 147-156.

Lyashchenko KP, Singh M, Colangeli R and Gennaro ML. 2000. A multiantigen print immunoassay for the development of serological diagnosis of infectious diseases. J Immunol Methods. 242:91-100.

Lyashcenko KP, Miller M and Waters WR. 2005. Application of mapia (multiple antigen print immunoassay) and rapid lateral flow technology for tuberculosis testing of elephants. In: Proceedings of American Association of Zoo Veterinarians. 2005 American Association of Zoo Veterinarians Annual Conference, October 15-18, 2005, Omaha, Nebraska.

MacCune Jr RM, Tompsett R. 1956. Fate of *Mycobacterium tuberculosis* in mouse tisssues as determined by the microbial enumeration technique. I. The persistence of drug-susceptible tubercle bacilli in the tissues sespite prolonged antimicrobial therapy. J Exp Med. 104: 737-762.

Martín-Hernando MP, Torres MJ, Aznar J, Negro JJ, Gandía A and Gostázar C. 2010. Distribution of lesions in Red and Fallow deer naturally infected with *Mycobacterium bovis*. J Comp Path. 142: 43-50.

McAdams HP, Erasmus J, Winter JA. 1995. Radiologic manifestations of pulmonary tuberculosis. Radiol Clin North Am J. 33(4): 655-678.

Mcllroy SG, Neill SD, McCracken RM. 1986. Pulmonary lesions and *Mycobacterium bovis* excretion from the respiratory tract of tuberculin reacting cattle. Vet Rec. 118:718-721.

Michalak K, Austin SK, Diesel S, Maichle Bancon J, Zimmerman P and Maslow JN. 1998. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Disease 4:283-287.

Michel AL. 2002. Implications of tuberculosis in African wildlife and liverstock. Ann N Y Acad Sci. 969:251-5.

Michel AL, Venter L, Espie IW and Coetzee ML. 2003. *Mycobacterium tuberculosis* infections in eight species at the National Zoological Gardens of South Africa, 1991-2001. J Zoo Wild Med. 34 (4): 364-370.

Michel AL, Bengis RG, Keet D F, Hofmeyer M, de Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte IJ, Buss P and Goldfroid J. 2006. Wildlife tuberculosis in South African conservation areas: Implications and challenges. Vet Microbiol. 112:91-100.

Michel AL, de Klerk L, van Pittius NCG, Warren RW and van Helden PD. 2007. Bovine tuberculosis in African buffaloes: Observation regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. BMC Vet Res. 3:23.

Michel AL, Coetzee ML, Keet DF, Maré L, Warren R, Cooper D, Bengis RG, Kremer K and van Helden P. 2009. Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South Africa game reserves. Vet Microbiol. 133: 335-343.

Miller J, Jenny A, Rhyan J, Saan D and Suarez D. 1997. Detection of *Mycobacterium bovis* in formalin-fixed embedded tissue of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. J Vet Diag Invest. 9: 244-249.

Montali RJ, Mikota SK and Cheng LI. *Mycobacterium tuberculosis* in zoo and wildlife species. 2001. Rev Sci Tech. 20:291-303.

Neill SD, Pollock JM, Bryson DB and Hunna J. 1994. Pathogenesis of *Mycobacterium bovis* infection in cattle. Vet Microbiol. 40: 41-52.

Nolan A and Wilesmith JW. 1994. Tuberculosis in badgers (*Meles meles*). Vet Microbiol. 40 (1-2). 179-191.

O'Brien DJ, Schmitt SM, Berry DE, Fitzgerald SD, Vanneste JR, Lyon , Magsig D, Fierke JS, Cooley TM, Zwick LS and Thomsen BV. Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan. J Wild Diseases. 40(1): 42-52.

OIE, 2004. Manual of diagnostic test and vaccines for territorial animals. 5th edition. Chapter 2.3.3.

OIE, 2009. Bovine tuberculosis. Center for food security and public health. pp 1-6.

O'Neil BD, Pharo HJ. 1995. The control of bovine tuberculosis in New Zealand. N Z Vet J. 43: 249-255.

Palmer MV, Waters WR and Whipple DL. 2002a. Aerosol delivery of virulent *Mycobacterium bovis* to cattle. Tuberculosis. 82(6): 275-282.

Palmer MV, Water WR and Whipple DL. 2002b. Lesion development in White-tailed Deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis.* Vet Pathol. 39: 334-340.

Phillips CJC, Foster CRW, Morris PA and Teverson R. 2003. The transmission of *Mycobacterium bovis* infection to cattle. Res Vet Sci. 74:1-15.

Qureshi T, Labes RE, Lambeth M, Montgomery H, Griffin JFT and Mackintosh CG. 2000. Transmission of *Mycobacterium bovis* from experimentally infected ferrets to non-infected ferrets (*Mustela furo*). N Z Vet J. 48: 99-104.

Ragg JR, Moller H and Waldrup KA. 1995. The prevalence of bovine tuberculosis (*Mycobacterium bovis*) infections in feral populations of cats (*Felis catus*), ferrets (*Mustela furo*) and stoats (*Mustela erminea*) in Otago and Southland, New Zealand. N. Z. Vet. J. 43: 33-37.

Rhyan JC and Saari DA. 1995. A comparative study of the histopathologic features of bovine tuberculosis in cattle, Fallow deer (*Dama dama*), Sika deer (*Cervus nippon*), and Red deer and Elk (*Cervus elaphus*). Vet Pathol. 32: 215-220.

Rodger JD, Connery NL, McNair J, Welsh MD, Skuce RA, Bryson DG, McMurray DN, Pollock JM. 2007. Experimental exposure of cattle to a precise aerosolized challenge of *Mycobacterium bovis*: A novel model to study bovine tuberculosis. Tuberculosis. 87: 405-414.

Rodwell TC, Kriek NP, Bengis RG, Whyte IJ, Viljoen PC, de Vos V and Boyce WM. 2001a. Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. J Wildl Dis. 37: 258-264.

Rodwell TC, Whyte IJ and Boyce WM. 2001b. Evaluation of population effects of bovine tuberculosis in free-ranging African buffalo (*Syncerus caffer*). J Mammal. 82: 231-238.

Rohonczy EB, Balachandran AV, Dukes TW, Payeur JB, Rhyan JC, Saari DA, Whiting TL, Wilson SA, Jarnagin JL. 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.

Steele JH. 1980. Human tuberculosis in animals. Vol2. CRC Press Inc. Boca Raton. Florida. USA.

Stoneburner R, Laroche E, Prevots R, Singh T, Blum S, Terry P, Reatrice S, Adler J. 1992. Survival in a cohort of human immunodeficiency virus-infected tuberculosis patients in New York City. Implications for the expansion of the AIDS case definition. Arch Intern Med. 152(10): 2033-2037.

Suazo FM, Escalera AMA and Torres RMG. 2003. A review of *M. bovis* BCG protection against TB in cattle and other animal species. Prev Vet Med. 58:1-Thoen CO and Steele JH. 1995. *Mycobacterium bovis* infection in animals and humans. Iowa State University Press. Iowa. USA.

Thoen C, LoBue P and de Kantor I. 2006. The importance of *Mycobacterium bovis* as a zoonosis. Vet Microbiol. 112: 339-345.

Van Pelt-Verkuil E, Van Belkum A and Hays JP. 2008, Ensuring PCR quality-laboratory organization, PCR optimization and controls. In: Principles and technical aspects of PCR amplification, ed. Van Pelt-Verkuil E, Van Belkum A, Hays JP, pp. 183–212. Springer Science, Dordrecht, The Netherlands.

Vincek V, Nassiri M, Nadji M and Morales AR. 2003, A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. Lab Invest. 83:1427–1435.

Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, Spencer Y, Hewinson G and Vorldermeier M. 2005. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, γδ (WC1+) T cells and CD 68^{+} cells. J Comp Path. 133: 223-234.

Warren RM, Gey van Pittius NC, Barnard M, Hessseling A, Engelke E, de Kock M, Gutierrez MC, Chege GK, Victor TC, Hoal EG and van Helden PD. 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of diference. Int J Tuberc Lung Dis. 10(7):818-822.

Whipple DL, Bolin CA, and Miller JM. 1996. Distribution of lesions in cattle infected with *Mycobacterium bovis*. J Vet Diag Invest. 8 (3): 351-354.

WHO (World Health Organization)**.** 2004**.** World Health Report 2004: Changing History. Geneva: WHO.

Woodroffe R, Donnelly CA, Johnston WT, Bourne FJ, Cheeseman CL, Clifton-Hadlen RS, Cox DR, Gettinby G, Hewinson RG, Le Fevre AM, Mcinerney JP and Morris WF. 2005. Spacial association of *Mycobacterium bovis* infection in cattle and badgers meles meles. J Appl Ecol. 42: 852-862.

APPENDIX 1

HEMATOXILIN- EOSIN STAINING

Solutions and reagents:

1. 1% Acid Alcohol Solution (for differentiation):
   Hydrochloric acid ---------- 1 ml
   70% ethanol ---------- 100 ml
   Mix well.

2. 0.2% Ammonia Water Solution (Bluing):
   Ammonium hydroxide (concentrated) -------- 2 ml
   Distilled water ---------- 1000 ml
   Mix well.

3. Lithium Carbonate Solution (Saturated):
   Lithium carbonate ---------- 1.54 g
   Distilled water ---------- 100 ml
   Mix well.

4. Eosin-Phloxine B Solution:

Eosin Stock Solution:
   Eosin Y ---------- 1 g
   Distilled water ---------- 100 ml
   Mix to dissolve.

Phloxine Stock Solution:
   Phloxine B ---------- 1 g
   Distilled water ---------- 100 ml
   Mix to dissolve.

Eosin-Phloxine B Working Solution:

Eosin stock solution ---------- 100 ml
Phloxine stock solution ---------- 10 ml
Ethanol (95%) ----------780 ml
Glacial acetic acid ---------- 4 ml
Mix well.

5. Hematoxylin Solution (Harris):

Potassium or ammonium (alum) ---------- 100 g
Distilled water ---------- 1000 ml

Heat to dissolve. Add 50 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute. Remove from heat and slowly add 2.5 g of mercuric oxide (red). Heat the solution until it becomes dark purple color. Cool the solution in a cold water bath and add 20 ml of glacial acetic acid (concentrated). Filter before use.

Staining procedure:

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Dehydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8 minutes.
6. Wash in running tap water for 5 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute.
10. Wash in running tap water for 5 minutes.
11. Rinse in 95% alcohol, 10 dips.

12. Counterstain in eosin-phloxine B solution (or eosin Y solution) for 30 seconds to 1 minute.
13. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
14. Clear in 2 changes of xylene, 5 minutes each.
15. Mount with xylene based mounting medium.

Results:

Nuclei ---------- blue
Cytoplasm ---------- pink to red

MASSON'S TRICHROME STAINING

Solutions and reagents:

1. Biebrich Scarlet 1% aq---------- 90ml
   Acid Fuchsin 1% aq---------- 10ml
   Glacial acetic acid---------- 1 ml

2. Phosphomolybdic-Phosphotungstic acid solution
   Phosphomolybdic acid---------- 5gm
   Phosphotungstic acid---------- 5gm
   Dist water ---------- 200ml

3. Aniline Blue solution
   Aniline Blue---------- 2.5 gm
   Glacial acetic acid---------- 2 ml
   Dist water---------- 100 ml

Staining procedure:

1. Dewax and hydrate.
2. Mordant in Bouin's solution for 1 hour at 60 ^{0}C, or overnight at room temperature if formalin fixed.
3. Cool and wash in running tap water until yellow colour disappears (This is very important).
4. Rinse in dist water
5. Stain nuclei in Weigert's A & B solution for 10 minutes. Wash in running water for 10 minutes. Rinse in dist water.
6. Stain in Biebrich Scarlet-Acid Fuchsin solution for 2 minutes.
7. Rinse in dist water.
8. Phosphomolybdic-Phosphotungstic acid solution for 10-15 minutes (use only once and discard the solution).
9. Stain in Aniline Blue solution for 5 minutes (for CNS 15-20 minutes).
10. Rinse in dist water.
11. 1% aq Acetic acid solution for 3-5 minutes.
12. Dehydrate in 96% and 100% alcohol, clear in xylol and mount.

Results

Nuclei---------- black
Cytoplasm, Keratin, muscle fibres and intercellular fibers---- red
Collagen---------- blue

ZIEHL-NEELSEN ACID FAST STAINING

Solutions and reagents:

1. Kinyoun's Carbol Fuchsin solution

   Basic Fuchsin---------- 4.0gm

   Phenol crystals, melted---------- 8.0ml

   Alcohol 96%---------- 300ml

   Distilled water---------- 100.0 ml

2. Loeffler's Alkaline Methylene Blue

   a) Methylene Blue---------- 3gm

      Alcohol 96%---------- 20 ml

   b) Potassium hydroxide 0.01% aq---------- 1000ml

      Dissolve separetly. Mix. Keep indefinitely
      Dilute 1-10 with dist water before use

Procedure

1. Dewax (only 2-3 minutes) and hydrate.
2. Stain in Kinyoun's for 1 hour at 60^0C.
3. Rinse in tap water.
4. Differentiate in 1% acid alcohol till light pink.
5. Rinse well in dist water.
6. Counterstain in diluted Methylene blue for 1 minute.
7. Rinse in dist water very quickly.
8. Dehydrate quickly in 96% alcohol and in 100% alcohol.
9. Clear in xylol and mount directly (do no leave in xylol).

Results

Acid fast bacilli---------- bright red

R.B.C.'s---------- yellowish orange

Background---------- pale blue

APPENDIX 2

PCR METHOD 1: NUCLISENS ISOLATION AND DNA EXTRATION

I. NUCLISENS ISOLATION

P3-Laboratory

Nuclisens lysis buffer contains the chaotropic agent guanidine thiocyanate that will lyse cells, denature many macromolecules such as proteins, and inactivate bacteria. Make sure no crystals are present in the lysis buffer tubes.

- If there is more than 500 µl of samples concentrate by centrifuging for 20 min at 12000g.
- Discard most of the supernatant-vortex
- Transfer specimen (50-500 µl) into lysis tube from the extraction kit.
- Incubate 10 min at room temperature
- Wipe outside of tubes

II. DNA EXTRACTION

1. Add 50 µl of vortex silica to samples.
2. Vortex – incubate room temperature 10 min
3. Centrifuge 2 min at 1500g
4. Remove supernatant by decantation
5. Add 400 µl Wash Buffer 1 to tube – pipet up and down – transfer to labeled 1,5 ml screw cap tube – place in Minimag.
6. Wash steps:

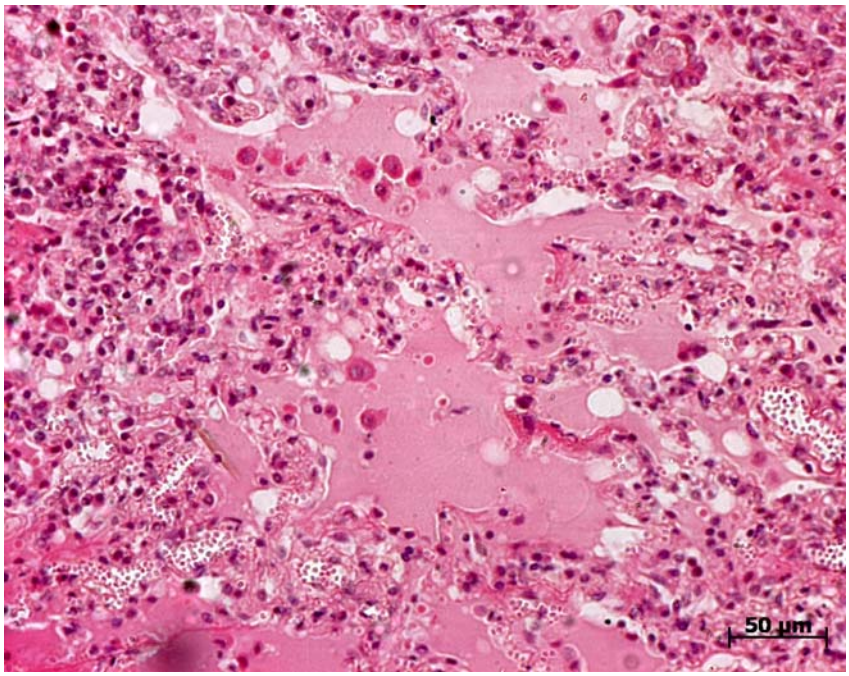
Wash buffer 1 – 400 µl	30 sec at STEP 1
Wash buffer 2 – 500 µl	30 sec at STEP 1
Wash buffer 2 – 500 µl	30 sec at STEP 1
Wash buffer 2 – 500 µl	15 sec at STEP 1

Make sure the last wash step is not more than 15 sec – longer washing can decrease the nucleic acid yield. Remove as much of SN as possible without disturbing silica pellet.

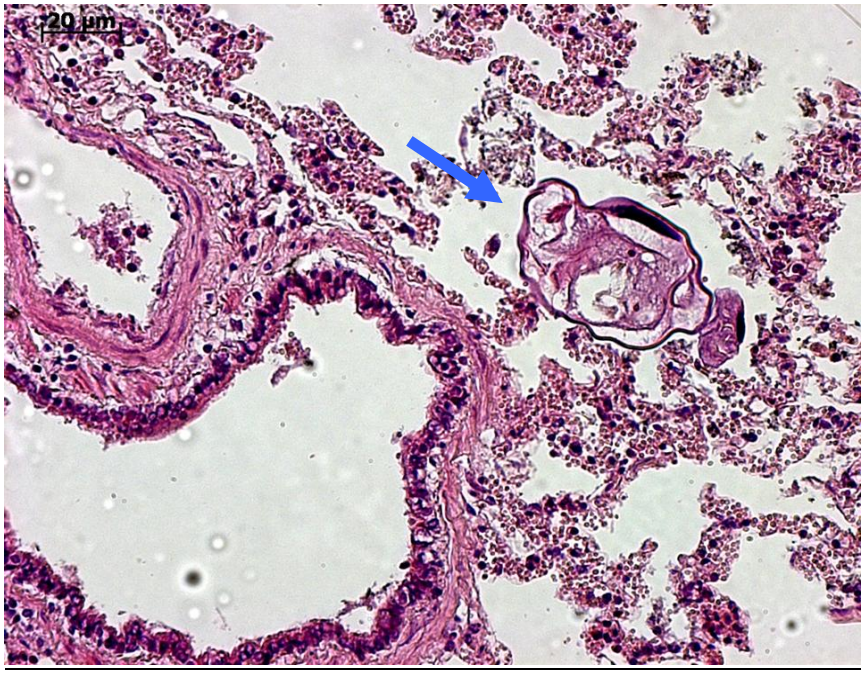
7. Add 50 µl elution buffer to tubes – collect silica at bottom of tube by tapping gently.
8. Place tubes in thermoshaker and incubate 5 min at 60^0C at 1400 rpm
9. Place tubes in magnetic rack and transfer extracted nucleic acid to clean tube – do not transfer any silica particles.

APPENDIX 3

OTHER FINDINGS OBSERVED IN THE LUNG



Lung. Protein rich alveolar edema. H&E. 200X



Lung. Metazoan parasite section in the lung (arrow). H&E. 200X

APPENDIX 4

RELEVANT COURSES AND TRAINING ATTENDED

September 2009 (2 weeks): Training in pathology diagnosis in zoo and domestic animals, at National zoological Gardens and Faculty of Veterinary Science, Pretoria University, Pretoria, South Africa.

July 2009 (2 weeks): Summer school in Veterinary Pathology organized by the European College of Veterinary Pathology and European Society of Veterinary Pathologists, Zaragoza University, Spain.

July 2008 (2 weeks): Summer school in Veterinary Pathology organized by the European College of Veterinary Pathology and European Society of Veterinary Pathologists, Zaragoza University, Spain.

Janeiro-February 2008 (7 weeks): Training in Veterinary Pathology at Abel Salazar Institute of Porto University, Portugal.

June 2007 (2 weeks): Training in histotechnology at Faculty of Veterinary Science, Pretoria University, South Africa.

February 2007 (2 weeks): Training in molecular diagnosis of tuberculosis, Faculty of Health Science, Stellenbosch University.

APPENDIX 5

OUTPUTS FROM THIS THESIS

A manuscript from this study has been submitted to the Journal for Veterinary Diagnosis and Investigation.

Manuscript title and co-authors:

Characterization of tuberculous lesions in naturally infected African buffalo (*Syncerus caffer)*

Cláudio J. M. Laisse[1,5], Dolores Gavier-Widén[2,3], Guillermo Ramis[4], Juan J. Quereda[4], Erik O. Ågren[2], Paul van Helden[5]

1. Division of Pathology, Veterinary Faculty, Eduardo Mondlane University, PO Box 257, Maputo Mozambique.
2. Department of Pathology and wildlife disease, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden.
3. Department of Biomedical Sciences and Veterinary Public Health; University of Agricultural Sciences (SLU), Uppsala, SE-751 89, Sweden.
4. Departamento de Producción Animal, Facultad de Veterinaria, Universidad de Murcia, 30100 Murcia, Spain.
5. DST/NRF Centre of Excellence for Biomedical TB Research/ US/ MRC Centre for Molecular and Cellular Biology/ Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg 7505, South Africa.

The work described was presented orally and published on the proceedings of the **13th Conference of the Association of Institutions for Tropical Veterinary Medicine (AITVM)**: Globalization of Tropical Animal Diseases and Public Health Concerns, which took place in Bangkok, Thailand, from 23 to 26 August 2010, pages 87-89 (Invitation letter and the first page of the publication next pages).

Association of Institutions for
Tropical Veterinary Medicine **(AITVM)**
Conference 2010

23rd - 26th August 2010. Bangkok, Thailand
Sofitel Centara Grand Bangkok Hotel

Organized by Chulalongkorn University, Kasetsart University,
Khon Kaen University and Chiang Mai University

26 June 2010

RE: Topic E : **Characterization of tuberculous lesions in naturally infected African buffalo (Syncerus caffer)** (A01C199)

To whom it may concern,

This letter is to confirm that the paper said above by Dr. Claudio Laisse, Veterinary Faculty, Eduardo Mondlane University, Maputo, Mozambique is accepted in 13th AITVM Conference: Globalization of Tropical Animal Diseases and Public health Concerns, held at Sofitel Centara Grand Hotel, Bangkok, Thailand during 23-26 August 2010.

Dr. Claudio Laisse has to present the said paper by himself in this conference. We, therefore, would much like to ask for your assistance to facilitate his participation in the conference that is aim to bring together leading researchers and practitioners to share and exchange ideas on the animal health and production in the tropics.

Your kind consideration and facilitation is much appreciated.

Yours sincerely,

Titinan Pocharasangkul, Conference Secretariat

For Dr. Annop Kunavongkrit, Chairperson of 13th AITVM Organizing Committee

Conference Secretariat:

Contact Person: Titinan Pojarasangkul
ABLE Conference & Travel Management
115 Payatai Road, Rajchataewi, Bangkok 10400
Telephone: 02-2519426, 081-4985413
Facsimile: 02-2512709
E-mail: ableconferences@gmail.com

13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Conference
23-26 August 2010 Bangkok,Thailand

Characterization of tuberculous lesions in naturally infected African buffalo (*Syncerus caffer)*

Cláudio Laisse[1,5*] Dolores Gavier-Widén[2,3] Guillermo Ramis[4] Juan Quereda[4] Erik Ågren[2] Paul van Helden[5]
[1]Division of Pathology, Veterinary Faculty, Eduardo Mondlane University, PO Box 257, Maputo Mozambique
[2]Department of Pathology and Wildlife Diseases, National Veterinary Institute, SE-751 89 Uppsala, Sweden
[3]Department of Biomedical Sciences and Veterinary Public Health; University of Agricultural Sciences, Uppsala, SE-751 89, Sweden
[4]Departamento de Producción Animal, Facultad de Veterinaria, Universidad de Murcia, 30100 Murcia, Spain
[5]Centre for Molecular and Cellular Biology/ Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg 7505, South Africa.
*Corresponding author
Keywords: Bovine tuberculosis, granuloma, *Mycobacterium bovis*, *Syncerus caffer*

Introduction

Mycobacterium bovis has a wide host range and infects many wild and domestic animal species as well as humans [1]. The African buffalo (*Syncerus caffer)* is considered to be a wildlife reservoir of *M. bovis* in certain environments of South Africa, such as in the Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HiP), which is the third largest national park in South Africa (2,3). Bovine tuberculosis (bTB) was first diagnosed in buffalo in the park in 1986 (4). Currently it occurs at a herd prevalence that can vary from 5% to 50% between herds (D. Cooper, *unpublished data*). Infected buffalo can contaminate the environment (1,5) and are considered to be a source of infection to other wild animal species, including predators and scavengers, as well as livestock (5). Previous reports of bTB in African buffalo (2,6) describe lesions as being most often located in the lymph nodes of the head, in the bronchial and mediastinal lymph nodes, and in the tonsils and lungs. Controlling tuberculosis in wildlife reservoirs has proven to be a major challenge. During recent years, elimination of wildlife reservoirs has been considered as a feasible and acceptable control strategy (7). The Bacille Calmette-Guérin (BCG) vaccine efficacy varied in many vaccination trials to control bTB in domestic and wild animals (8). Testing and culling of the positive reactors is another management option to control the disease (9). This study provides a systematic and detailed description of the macroscopic and histopathologic lesions caused by natural *M. bovis* infection in African buffalo, and applies a scoring system to classify lesions according to their size, cellular composition and degree of development. The purposes of this study were i) to contribute to the understanding of the pathogenesis of natural disease in African buffalo, ii) to provide a semi-quantitative evaluation of the severity of the lesions, which can be used as a comparative base for experimental infection and vaccine efficacy evaluation studies.

Materials and methods

Animals

The buffalo were obtained from test-and-cull operations part of a tuberculosis control program aimed at reducing the prevalence of bTB in the species, in HiP Province of Kwazulu/Natal, South Africa. This program started in 1999 and includes annual buffalo capture for tuberculin skin testing and culling of bTB-positive buffalo (10). The buffalo in this study were selected from animals with a positive test result of the bovine component of the standard bovine comparative intradermal test using both avian and bovine Dutch tuberculin. All positive reactors in one test-and-cull operation (24 buffalo) were sacrificed by a shot in the brain using a heavy caliber rifle (.308) and transported to the abbatoir of the HiP. At slaughter inspection, 19 of 24 animals showed visible gross lesions suggestive of tuberculosis, and these buffalo were selected for further pathology studies.

Post mortem examination and sample collection

A detailed examination of the lungs and a selection of lymph nodes (LN) was done. The lungs were sliced at 2 cm intervals and each slice was inspected and palpated. The following LN were sliced thinly (approximately 2 mm thick slices), and each slice was visually inspected: head-associated LN (paired mandibular, parotid and medial retropharyngeal), thoracic LN (mediastinal and bronchial), abdominal LN (mesenteric, hepatic, omasal and abomasal) and paired peripheral superficial LN (superficial cervical, axillary and popliteal). Tissues with lesions were sampled for histopathology and fixed in 10% neutral buffered formalin.

Grading of macroscopic lesions

Macroscopic tuberculous lesions were considered as any foci or nodules, single, multifocal or confluent, yellow-white, circumscribed and solid, or necrotic, with or without apparent caseation and mineralization. The LN were categorized in grades 1 to 5 according to the size and number of their gross lesions. Grade 1 was used for a single minimal lesion, up to 1 mm; grade 2 for two to four lesions of 2 to 5 mm; grade 3 for five to eight lesions, up to 10 mm, or many small lesions affecting approximately 50% of the LN; grade 4 for confluent and extensive lesions in most slices but with some normal looking tissue left, and grade 5 for abundant lesions, with none or nearly no apparently healthy tissue left. The lungs and LN that did not show visible lesions were classified as grade 0 and were not sampled for further histopathology.

Histopathology

The tissues were formalin-fixed for 30 days to render any mycobacteria non-infectious, dehydrated, embedded in