Optimisation of the lion *(Panthera leo)* specific interferon gamma assay for detection of tuberculosis in lions in South Africa

NOZIPHO LINDIWE KHUMALO

Submitted in accordance with the requirements for the degree of

MASTER OF SCIENCE

IN AGRICULTURE

at the

UNIVERSITY OF SOUTH AFRICA

Supervisor : Mr J Oosthuizen

Co- Supervisor : Dr J Crafford

February 2017

Abstract

Optimisation of the lion *(Panthera leo)* specific interferon gamma assay for detection of tuberculosis in lions in South Africa

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) which has a diverse host range. The maintenance host of BTB in South Africa is the African buffalo (*Syncerus caffer*). It is believed that lions get infected by feeding on infected buffalo or through wounds. The spread of the disease amongst lions has raised concern regarding the future of the animals and the impact on tourism in the country. Diagnoses of tuberculosis in free ranging wildlife is often dependent on post-mortem samples due to logistical challenges, the use of the lion specific interferon gamma release assay as an antemortem test offers a simpler methodology to testing live animals. The aim was to optimise an already developed assay by Maas *et al.,*2012 and to harmonise it with the Rhinoceros specific interferon gamma assay developed by Morar-Leather *et al* 2007. Optimisation of the interferon gamma specific ELISA included: determination of optimal concentrations for the capture and detection monoclonal antibodies; optimal concentrations for the conjugate and evaluation of alternative blocking agents. Different mitogens and incubation times were evaluated for the stimulation of whole blood as positive control in the assay. The optimum concentration for coating the plates with the capture monoclonal antibody was 2 μg/ml. An optimum dilution of 1:5000 was selected for both the biotinylated detection monoclonal antibody and the streptavidin horseradish peroxidase conjugate. The assay was optimised using recombinant lion interferon gamma and the lower detection limit was calculated to be 109 pg/ml. Phosphate buffered saline with 1% bovine serum albumin was found to be

a suitable blocking agent. Native interferon gamma was detected in whole blood samples from 5 lions and a 24 hour incubation time with PMA and ionomycin was selected as the optimal mitogen positive control. This assay system demonstrated good potential as an ante mortem test for the diagnosis of tuberculosis in lions.

In conclusion, the assay can detect IFN-γ from supernatants harvested from whole blood cultures stimulated with specific antigens and mitogens

Key words: *Mycobacterium bovis,* Interferon gamma; Bovine tuberculosis; ELISA; Lion; recombinant lion; Optimisation

Table of Contents

List of Tables

List of Figures

List of abbreviations

Ab	Antibody
ANOVA	Analysis of variance
av PPD	Avian Tuberculin purified protein derivative
bov PPD	Bovine Tuberculin purified protein derivative
BTB	Bovine tuberculosis
Cal	Calcium ionophore
CD^4	Cluster of differentiation
C-ELISA	Capture enzyme-linked immunosorbent assay
CITT	Comparative cervical intradermal tuberculin test
CMI	Cell-mediated immunity
CO_2	Carbon dioxide
CPC	Cetylpyridinium chloride
Cv	Coefficient variation
DAFF	Department of Agriculture Forestry and fisheries
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay

FIV	Feline immune deficiency virus
H	Hour
HIP	Hluhluwe ImFolozi Park
HRPO	Horseradish peroxidase
H_2So_4	Sulphuric acid
IELISA	Indirect enzyme-linked immunosorbent assay
IFN-γ	Interferon - gamma
IGRA	Interferon gamma release assay
IL-12	Interleukin-12
IUCN	International Union for Conservation of Nature
KNP	Kruger National Park
M. bovis	*Mycobacterium bovis*
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
ML	Millilitre
MTB	Mycobacterium Tuberculosis
NaOH	Sodium hydroxide
NZG	National Zoological Gardens

OD	Optical density
OIE	Office International des Epizooties (The World Organization for Animal Health)
PMA	Phorbol 12- Myristate 13- Acetate
PWM	Pokeweed Mitogen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pg	Picogram
PPD	Purified protein derivative
rLIFN-γ	Recombinant lion interferon gamma
Rpm	Rotations per minute
SD	Standard deviation
SICCT	Single intradermal comparative cervical tuberculin test
SICT	Single intradermal cervical test
SIT	Single intradermal test
SITT	Single intradermal cervical tuberculin test
TB	Tuberculosis
Th1	T-helper 1

Th2	T-helper 2
TNF-ά	Tumour necrosis factor-ά
µl	Microliter
µg	Microgram
WHO	World Health Organisation

Acknowledgments

I would like to acknowledge and appreciate the following people:

Firstly I would like to thank Professor Anita Michel who financially supported the study.

My Supervisor, Mr Johan Oosthuizen and co-supervisor Dr Jannie, Crafford for their continued supervision and encouragement that helped me to complete this work. I would also like to thank them for their patience and time and most of all their dedication in the study.

The University of South Africa (UNISA) Department of Agriculture (Animal Health) and the University of Pretoria, department of Tropical disease Ondersterpoort, for providing a platform to conduct the study.

Peter Van Kooten thank you very much for your time and patience most of all your dedication in the lab, you taught me a lot.

Thank you to the National Zoological Gardens for allowing me to advance my career while working for them, I really appreciate it a lot. This opportunity has taught me a lot. I

met lovely people in the journey, made friends and learned to smile at all times regardless of the situation.

My deepest gratitude goes to Dr Darshana Morar Leather and her Husband who have also taught me a lot and were supportive through the study, not also forgetting Dr Emily Mitchel who has been exceptionally supportive through the years of my study I would also like to thank the division of state veterinary services of Skukuza from the Kruger National Park who helped with all relevant information on request, thank you for your time and efforts. I would also like to thank Dr Michelle Miller for added information when I needed it.

Declaration

I Nozipho Lindelwa Khumalo, hereby declare that all information written in the document is my original work except the stimulation of whole blood and mitogen stimulation which was done by Dr D Morar and Dr M Maas University of Pretoria, Ondersterpoort. Result analysis was supported by Dr J Crafford. Neither the full dissertation nor any part of it has been submitted for another degree at this or any other University.

This dissertation is submitted in fulfilment of the requirements for the degree of Master of Science in Agriculture in the Department of Agriculture and Animal Health, University of South Africa.

1 Chapter 1

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB). The maintenance host of BTB in South Africa is the African buffalo (*Syncerus caffer*). During August 1990, BTB was reported in a significant proportion of the African buffalo population in the Kruger National Park (Bengis, *et al.,* 1996). Cases of BTB in lions have been described in captive (Eulenberger *et al.,* 1992; Morris *et al.,* 1996) and free-ranging lion populations (Keet *et al.,* 1996; Cleveland *et al.,* 2005; Trinkel *et al.,* 2011) and the prevalence of the disease could be as high as 79% (Maas *et al.,* 2012). BTB is a chronic disease (de Lisle *et al.,* 2002; Michel *et al.,* 2006), and affects mostly the lungs and other organs (Keet *et al.,* 1996, 2000; Kirkberger *et al.,* 2006) and can eventually lead to fatalities (Keet *et al.,* 2010). Transmission of *M. bovis* in lions has not been proven. However, it has been proposed that prolonged eating of meat from *M. bovis* infected cattle and African buffaloes may be the mode of transmission in lions (Eulenberger, *et al.,* 1992; Jones, 1953). Currently, the only validated test for the diagnosis of BTB in live animals is the Single intradermal comparative cervical Tuberculin test for cattle and it has been applied in lions (Keet *et al.,* 2010). The disadvantage of this test is that it requires recapture of animals after 72hrs which is labour intensive, stressful for the animal and impractical to use in free-ranging populations.

To know the BTB status of lions is important for disease control purposes, especially in case of translocations between zoo's or wildlife parks and for international trade.

Other tests like the interferon-gamma (IFN-γ) release assay (IGRA) are currently being investigated to replace the skin test. This assay is based on the detection of IFN-γ in whole-blood culture which is released in response to stimulation with BTB specific antigens (Rothel *et al.*, 1992; Wood *et al.,* 1990a). Advantages of this assay include a single capture of the animals, an increased sensitivity, early detection of infection, a

more standardised procedure and more objective interpretation (Aagaard *et al.,* 2006; de la Rua-Domenech *et al.,* 2006; Schiller *et al.,* 2010).

In this study optimisation of the lion specific IGRA was the main objective, adapting the protocol by Maas *et al.,* (2012) using opportunistic available lion samples in South Africa.

1.1 Problem statement

Tuberculosis (TB) is an infectious lethal disease causing a high number of deaths in humans and animals. Lions are cooperative group hunters compared to other cats. They prey on African buffalo and their method of hunting ultimately requires the suffocation of the prey animal through the occlusion of the respiratory tract. If an advanced case of an *M. bovis* infected animal falls prey, it can be assumed that the lion doing the suffocating may inhale large numbers of infectious bacilli. After the prey has died, lions may also ingest numbers of infectious bacilli while eating tuberculous lesions.

When feeding on carcasses, lions have their heads in close proximity to one another and heavy breathing and growling is a constant feature of these sessions. It is also during these sessions that lions may infect other lions in the pride. From infected individuals transmission can occur through horizontal transmission, indicating the lion as a possible maintenance host (Eulenberger, *et al.,* 1992). According to the IUCN list of threatened species, the lion population trend is decreasing and it was listed as vulnerable during 1996, 2002, 2004, and 2008. On news for National Geographic, September 30, 2005, interview done by Nicholas Bakalar with Dewald Keet, chief veterinarian at Kruger National Park, said that bovine tuberculosis is an ever-increasing threat to Kruger lions but because TB is a chronic disease, people may have the mistaken impression that it has stabilized. Regardless of the efforts done to control BTB, the disease persists, with serious implications for human health and the economy in the context of global trade. The IGRA is currently the best method to be used to detect infected animals in the early stages of the disease.

1.2 Study aim

The aim of this study was to optimize the lion specific interferon-gamma release assay for lions in South Africa.

1.3 Study objectives

- Optimisation of the IFN-γ ELISA
    - Optimization of the IFN-γ monoclonal capture antibody
    - Optimization of the IFN-γ monoclonal detection antibody and conjugate
    - Optimization of the blocking buffer
- Evaluation of different mitogens and incubation times

1.4 Limitations of the study

The most challenging aspects of the current study were the difficulties in obtaining fresh samples from captive lions, semi-captive and free-ranging lions, therefore, the sample size used in this study was insufficient. Ideally information for a capture had to be conveyed a week before in order to prepare for the trip, unfortunately, information would only be received late and thus missing an opportunity of obtaining fresh samples for the study. This also played a huge role in budgeting for the captures planned.

1.5 Conclusion

Major improvements in diagnostics, prevention and control of BTB in wildlife have been made due to the increased awareness of the disease and its potential environmental and socio-economic implications. Though BTB eradication in wildlife is very unlikely to happen sooner and resulting in the threat of re-infection of domestic livestock from wildlife as a source will remain. A number of options are available to contain, control and eradicate BTB, but many are considered unacceptable on economic, moral and environmental grounds.

The optimized protocol has provided results that show the assays efficiency. Findings provide data that one can obtain results within a shorter period (24 hours) of detecting interferon gamma in whole blood, but one could still go up to 48 hours testing for detection of interferon gamma The level of detection was found at 109 pg/ml.

With the findings of the current study, the assay can be used as a potential diagnostic tool by parks and zoos for testing during veterinary check-ups, translocations and for research purposes.

2 Chapter 2

Literature Review

2.1 General Overview of Mycobacteria

2.1.1 Introduction

This chapter gives a general overview of tuberculosis in lions and the different *ante mortem* diagnostic tests that are currently available.

Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis* (*M.bovis*) which is a chronic infection in cattle, and still remains an economic problem and has a zoonotic risk for a number of countries. Because of the economic impact that the disease has on international trade and risks to human health, initiations have led to global programs to try and eradicate the disease (Cousins DV., 2001) being implemented all over the world. The presence of maintenance hosts in wildlife presents a transmission risk at the wildlife-livestock-human interface and may threaten the conservation of susceptible wildlife species like lions.

Primarily known as a disease of livestock, bovine tuberculosis has also been reported in African buffalo, lions, cheetahs, chacma baboons, greater kudu, leopard, hyena, large-spotted genet, warthog, bush pig, and eland (Huchzermeyer *et al*., 1994; Thoen, 1994; de Lisle *et al.,* 2001; Michel, 2002; Cleveland *et al*., 2005). Bovine tuberculosis is thought to have been imported into South Africa through cattle of the European settlers towards the end of the eighteenth century (Henning, 1956) and has since spread to the buffalo population in the Kruger National Park (KNP) (Bengis *et al*, 1996). The importance of TB in wildlife has only been recognized recently (de Lisle *et al*., 2002). Once animals have been infected they tend to act as reservoirs of infection. Some of the wild animals that can pose as maintenance host of the disease include the African buffalo (*Syncerus caffer*), Kafue lechwe (*Kobus leche*), and White-taled deer (*Odocoileus virginianus*) (de Vos *et al*., 2001).

2.1.2 Scientific classification of Mycobacteria

Mycobacterium is a genus of Actinobacteria, belonging to the *Mycobacteriaceae family*. The genus includes pathogens that can cause serious diseases in mammals; these include tuberculosis caused by *Mycobacterium tuberculosis* and leprosy caused by *Mycobacterium leprae* (Ryan KJ, Ray CG., 2004).

2.1.3 Microbiological characteristics

Mycobacteria are aerobic, mesophilic, non-motile, non-spore forming and host-associated bacteria, that are characteristically acid fast (Ryan KJ, Ray CG., 2004). They can grow up to 1.0 to 10µm long and 0.2 to 0.6µm wide. *M. bovis* is similar in structure and metabolism to *M. tuberculosis*. It is a rod-shaped bacterium that stains red with acid-fast stains like Ziehl-Nielsen.

Mycobacteria have an outer membrane, (Niederweis M, *et al.,* 2010) and do not have capsules, and most of them form endospores. *Mycobacterium marinum* and feasibly *M.bovis* have shown to form spores (Ghosh JL, *et al.,* 2009). The distinctive characteristic of Mycobacterium species is that the cell wall is thicker than in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acids.

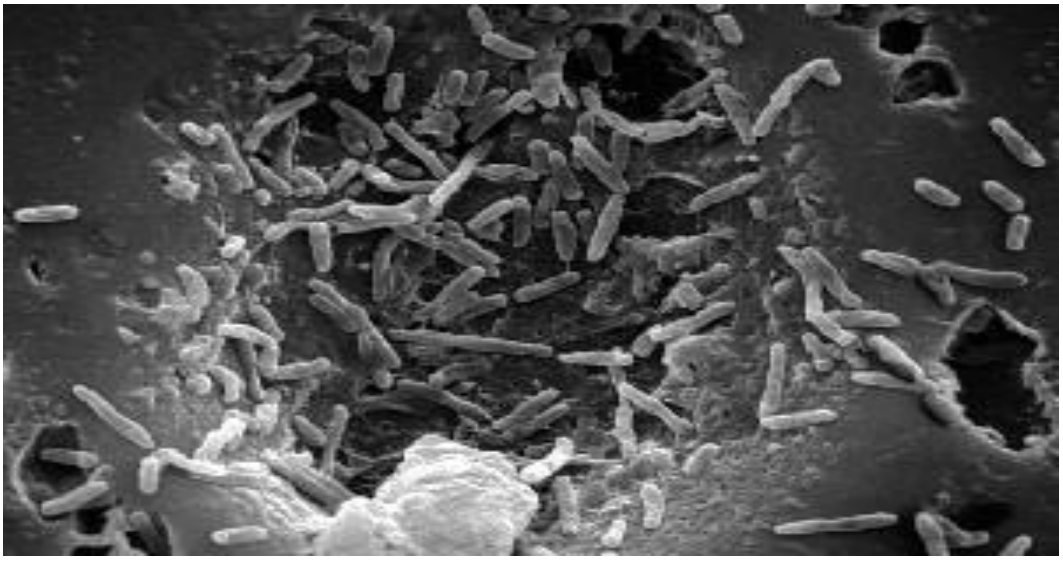

Figure 2.1 *Mycobacterium bovis* (Photo Courtesy of CDC 3 June 2007; microbewiki.kenyon.edu; downloaded on the internet)

The maintenance host of BTB is South Africa is the African buffalo (*Syncerus caffer*). During August 1990, BTB was reported in a significant proportion of the African buffalo population in the KNP (Bengis *et al.,* 2006). *M. bovis* infection has been reported in a wide range of animals that include elephants, non-human primates, rhinoceros, tapir, marine animals, non-domestic ungulates and carnivores, and domestic animals (Thoen *et al.,* 1977; Forshaw and Phelps, 1991; Montali *et al.,* 2001; Ghodbane *et al.,* 2013).

The first case of tuberculosis (MTB) in South African captive animals was recorded at the National Zoological Gardens of South Africa by Robinson in 1953. Though pure cultures of *M. tuberculosis* were obtained in two cases of springbok at that time, *M bovis* was found in a guinea pig that was inoculated with affected lung tissue and therefore the identity of the causative pathogen is uncertain. Another case reported in South Africa of MTB infection in a wild animal was of a chacma baboon from a free-ranging troop in a rural region of Limpopo Province (Kovalev, 1980). Another case reported in Chachma baboons was in 1998 in a single troop of free-ranging in the KNP that fed on infected carcasses scavenged in the wild or from a post-mortem facility. Necropsy findings presented severe miliary lesions of the lungs and spleen, indicating that the disease enters the blood stream and then disseminates to distal sites (Keet *et al*., 2000). The isolations of *Mycobacterium tuberculosis* found were similar to that found in African buffalo in the KNP.

Another *M. tuberculosis* case reported at the IThemba laboratories was from a semi-free-ranging springbok living on the grounds of IThemba Laboratory for Accelerator-Based Science in Cape Town (Gous and Williams, 2009). A study done in Kenya, on a rehabilitated elephant that was released back to the wild showed histological and gross pathology of MTB infection after its fifth year in the wild (Obanda *et al.,* 2013).

Michel and co-workers (2006) also reported an *M. bovis* strain in kudu that was genetically similar to that found in buffalo. Other cases with genetically unrelated *M. bovis* strains were also detected, suggestive of different sources of infection. Keet *et al.,*

2001) also suggested another possible mode of transmission of *M. bovis* amongst kudu, and between cattle and kudu being the secretion of infectious pus from draining fistulae of parotid lymph nodes.

The majority of tuberculosis in wildlife is caused by *M. bovis* infection, reflecting its broad host range. It has been reported in a wide range of animals that include *inter alia*: African buffalo (*Syncerus caffer)*, wildebeest *(Connochaetes*), lions (*Panthera leo*), cheetahs (Ac*inonyx jubatus*), leopard (*Panthera pardus*), chacma baboons (*Papio ursinus*), greater kudu (*Tragelaphus strepsiceros*), hyena (*Hyaenidae*), impala (*Aepyceros melampus*), large-spotted genet (*Genetta tigrina*), warthog (*Phacochoerus africanus*), bush pig (*Potamochoerus larvatus*), eland (*Taurotragus oryx*), spotted hyena (*Crocuta crocuta*), lechwe (*Kobus leche*), common duiker (*Sylvicapra gremial*), honey badger (*Mellivora capensis*) (Keet *et al.,* 1996; de Vos *et al.,* 2001; Cleveland *et al.,* 2005; Michel *et al.,* 2006; Trinkel *et al.,* 2011; OIE, 2012)

A control programme for managing BTB was initiated at the Hluhluwe- imfolozi Park in 1999 because of the increasing BTB prevalence, which aimed at reducing the number of buffalo herds infected with *M. bovis* to below 10% as well as the spill-over into key species and domestic livestock in the surrounding areas (Michel *et al.,* 2006).

2.1.4 An Outline of tuberculosis in lions

The first BTB cases in lions were first reported in 1995; then it was reported in cheetahs. Leopards were later reported in 1998, since then 50 more cases of lion infections have been reported.

Tuberculosis has been reported in many felid species in captivity and in the wild, including leopard (*Panthera pardus*) (Eulenberger *et al.,*1992), cheetah (*Acinonyx jubatus*) (Keet *et al.,* 1996), ocelot (*Leopardus pardalis)* (Eulenberger *et al.,* 1992), common genet (*Genetta genetta*) (de Lisle *et al.,* 2002), snow leopard (*Uncia uncia*) (Eulenberger *et al.,* 1992, Thorel 1994) and Amur leopard (*Pathera pardus orientalis*) (Thorel.,1994).

It is well established that lions are susceptible to *M. bovis* infection. Tuberculosis is a chronic disease and can affect multiple organs of the body specifically the lungs (de Lisle et al., 2002; Keet et al., 2000), and can lead to fatalities (Keet et al., 2010). The prevalence of BTB in lions requires disease surveillance in events of translocations between zoo's or wildlife parks or for research purposes. Lions have been categorized as spill-over hosts (Keet DF., 2000) in their populations as this depends on a persistent alternate source of infection (de Lisle *et al*., 2002). The risk imposed by BTB infections in lions may be increased by simultaneous infections with Feline immune deficiency virus (FIV), potentially increasing their susceptibility to BTB (Maas *et al*, 2012).

According to the IUCN Red List of Threatened Species, lions are listed as vulnerable and have experienced a 43% population decline over the past 21 years. The KNP populations and the Serengeti are two of the few remaining strongholds of lions that are considered to be genetically viable (Bauer *et al*., 2008). The decline is primarily due to the killing of lions to protect human life and livestock as well as reduction in wild pray availability and habitat loss, in addition to this, tuberculosis is also considered a threat to lion populations (Nowell *et al.,* 2012)

The first reported cases of lions contracting tuberculosis were from animals in captivity (Eulenberger *et al.,* 1992; Morris *et al.,* 1996). Cases of tuberculosis in primates and felid species and the management thereof in the Leipzig Zoological Gardens in Germany were reported between 1951 and 1990 by Eulenberger *et al.,* 1992. The report included all felid species housed at the zoo including leopard (*Panthera pardus*), tiger (*Panthera tigris*), puma (*Puma Concolor*), lynx (*Lynx lynx*) but lions were the most often diagnosed with tuberculosis (Eulenberger *et al.,* 1992). In 1996 the second report from another zoo was published and reported on tuberculosis due to *M. bovis* in lions. In Knoxville Zoo, the USA, an eight- year- old male lion was euthanized in 1985 due to continuous deteriorating health (Morris *et al.,* 1996).

In free-ranging African lions the first report of *M. bovis* was in 1996 in two lionesses in the KNP, South Africa (Keet *et al.,* 1996). The approximate age of these two lionesses was about 10 years with one emaciated to a point that it couldn't even stand. Both of the

females had similar lung lesions. Since the publishing of this report, many more lions with tuberculosis have been identified in the KNP (Keet *et al.,* 2000, 2010). More cases of lions with tuberculosis were reported in KwaZulu Natal in the Munyawana Game Reserve (Michel *et al.,* 2006, 2009; Trinkel *et al.,* 2011). In Hluhluwe ImFolozi Park (HIP), post-mortem inspection and culture samples from lions that had died (most likely from tuberculosis) or that were euthanized due to advanced emaciation were used as confirmation of infection of *M.bovis* (Trinkel *et al.,* 2011).

Tuberculosis might not just be a problem in Kruger National Park lions but may also have an impact in other parks in Africa. *M.bovis* infection was reported in free ranging lions in the Serengeti National park in Tanzania (Cleveland *et al.,* 2005). Serum samples collected over the period 1984-2000 were subjected to *M. bovis* antibody ELISA. The results indicated that mycobacterial infection was present in the Serengeti lions from as early as 1984. Though the results could not classify the species of the Mycobacterium Tuberculosis Complex involved, isolation of *M. bovis* from lion prey species suggested it is a likely candidate (Cleveland *et al.,* 2005).

Currently, the only validated test for use as a diagnostic tool in lions is the tuberculin skin test (Keet *et al*., 2010). Although the test has been adapted for use in lions it is very impractical in wild populations and expensive as it requires two visits to the farm. Other diagnostic methods have been investigated to replace the skin test which includes the interferon gamma release assay (IGRA) which compliments the tuberculin tests in both humans and cattle (Shiller *et al*., 2010; Gormeley *et al*., 2006). The IGRA is based on tuberculin specific stimulation (bov PPD and av PPD) of cell mediated-immunity (CMI) and measures production of IFN-γ. Advantages of IGRAs are its increased sensitivity, detection of infection at an earlier stage of infection, the opportunity repeat testing, requires only one capture of the animal and more objective test procedures and interpretation [Chambers M. A., 2009; Rhodes *et al*., 2008; Rhodes *et al*., 2011)

2.1.5 Routes of infection

Inhalation is considered to be the primary route of infection in cattle and in many other species, with primary lesions occurring in the lungs and associated lymph nodes and/or in lymphoid tissues of the head (Eulenberger *et al*., 1992). This appears to be the main route of infection for big cats in Kruger National Park, presumably associated with suffocating their prey (Keet *et al*., 2002). Ingestion is the important route for some species, particularly carnivores, and the transcutaneous transmission has occurred occasionally in humans with cuts/ abrasions handling infected carcasses, and biting is known as the route of infection, for example meles-Eurasian badger.

2.1.6 The spread of bovine tuberculosis to lions

The spread of infection in lions and other wildlife species became evident in 1995 (Keet *et al.,* 1996). This poses a direct health consequence for the lions but also causes a threat to the biggest tourist attraction of the park (de Lisle *et al.,* 2002, Kirkberger, Keet & Wagner 2006). In the KNP buffaloes are one of four preferred prey species of lions and get preyed on extensively more in dry seasons (Keet, Michel & Meltzer 2000). Funston 1998 stated that weak animals are an easy target for lions because they are easy to kill. There seems to be a connection between increasing prevalence of BTB in buffalo herds and a decrease in overall body condition score (Caron, *et al.,* 2003). This mechanism of spread has led to increased exposure of lions to *M. bovis.* In areas where the occurrence of BTB in buffaloes is high the infection is also more prevalent in lions. Therefore, prides in the south of the park are more affected than prides in the north. Keet *et al.,* 2000 stated that adult lions are more likely to be infected than sub-adults and cubs. Since the prevalence of BTB amongst buffalo is still increasing, so is the threat of BTB to lions. It is likely that the majority of the KNP lions may become infected in the future (Keet *et al.,* 2000).

2.1.7 Mycobacterium bovis transmission between lions

Although lions have the potential to be spill-over hosts (de Lisle *et al.,* 2002), additional evidence has shown that lions are likely to be maintenance hosts, managing to maintain the infection within their prides in different ways. Factors that can play a role in the propagation of infection amongst lions include social behaviour like sociality, intraspecies aggression as well as a preference for hunting buffaloes *(Keet et al., 2001).* Aerosol droplets have been suggested to also cause horizontal transmission for *M. bovis* bacteria (Eulenberger *et al.,* 1992). Fine aerosol suspensions of low viscosity carrying the organism are very effective for transmission. Food and water can also be contaminated with the organism. Another form of spread is simultaneous or sequential feeding after contamination of the carcasses/food sources with infected oral secretions.

Inhalation is considered to be the primary route of infection in cattle and in many other species, with primary lesions occurring in the lungs and associated lymph nodes and/or in lymphoid tissues of the head. This appears also to be the main route of infection for big cats presumably associated with suffocating their prey (Keet *et al*., 2002).

Aerosol transmission is not the only route of transmission, infection of young animals suggests that transmission by milk is also a likely route, young animals have shown signs of severe infection and *M. bovis* has been isolated from the mammary lymph nodes of three lionesses (Keet, Michel and Meltzer 2000). This transmission hasn't been proven yet, though studies with badgers have shown that in that species, the most important route of transmission is via aerosol, and this may already begin when cubs are very young, getting the bacteria from their mother (Clifton-Hadley, *et al.,*1993). Fighting amongst male badgers seems to be a notable route of transmission and this can also apply for male lions (Cheeseman, *et al.,* 1989).

2.2 Pathogenesis

Tuberculosis is known primarily to be an infection of the respiratory tract, where bacteria uses macrophages as primary host cells for duplication of cells (Pollock, *et al.,* 2006). The general accepted mode of transmission of *M. bovis* to cattle is by inhalation of

tubercle bacilli, in an aerosol droplet (Neill *et al*.,1994) that lodges within the respiratory tract, most probably the alveolar surface of the lung (Pritchard, 1988). Aerosol transmission depends on the size and consistency of the aerosolized droplets. Pollock and Neill (2002) state that fine aerosol suspensions of small viscosity appear to be most efficient for delivering mycobacterial content, however only a very small portion of those droplets contain viable bacilli up to one hour after release. After deposition of bacilli on the respiratory surface, phagocytosis ensures interaction with innate immune response and CMI take place, B-lymphocytes get activated and antibody titres amplify in later stages of the infection (Pollock *et al*., 2006). Infection with mycobacteria such as *M. bovis* results in the development of granulomas, comprised of a core of infected and killed macrophages surrounded and infiltrated by T-lymphocytes within lungs and lymph nodes. The role of the granuloma is to control and restrict the spread of infection, but results in major tissue damage (Widdison *et al.,* 2006). The size of the challenge dose is positively correlated with severity of the disease (Pollock and Neill 2002).

2.3 Clinical signs

Tuberculosis is a chronic infection; initially clinical signs are not evident which makes diagnosis difficult (de Lisle *et al.,* 2002). Lions that test positive for the skin test can be found in good condition. Eulenberger *et al.,* 1992, described BTB infection in zoo lions as a progression where clinical signs could develop very suddenly after latency. Signs seen were increased emaciation and a lack of enthusiasm to move, after short exercises animals showed severe dyspnoea (Eulenberger *et al.,* 1992). Keet *et al.,* 2000 also mentioned other clinical signs like emaciation, depression, alopecia, and unilateral ocular lesions. The skin also shows a dull, rough appearance and dermatitis where lions were bitten. In progressed stages swollen joints and elbow hygromas are often seen in younger animals (Keet, Michel & Meltzer 2000). Macroscopic and microscopic lesions are seen throughout the lymphatic system especially the mesenteric, peripheral and head lymph nodes. Lesions found are usually well advanced, but do not show signs of caseation or calcification and their appearance are unlike those of traditional TB lesions of ruminants and primates (Bengis RG and Keet DF., 2000).

2.4 Ante mortem test for testing BTB specifically to lions

M. bovis incidences in free-ranging lions have urged the need for new diagnostic methods for the lion species. The use of single blood-based tests are tempting for use in wildlife testing, since it only requires a once of handling of animals, which assist in decreasing stress for the animal and reduces chances of injuries during captures (Waters *et al*., 2005) The chronic nature of the disease makes diagnosis of *M.bovis* in lions complicated as initially clinical signs may not be seen and infected lions may be found to be in good condition (de Lisle *et al*., 2002).

Clinical signs can be observed to tell if an animal might be infected but apart from that ante mortem tests for the detection of BTB in lions are available and they include the skin test (SICT: Single Intradermal Cervical Test) (Keet *et al*., 2010) and serology (ELISA). The SICT currently used for diagnosis of BTB has a sensitivity of 86.5% and a specificity of 81% (Keet *et al*., 2010). It is capable of detecting infection in earlier stages, but its disadvantage is that animals need to be recaptured after three days (72h), which makes it expensive to use as it is time and money consuming and it also causes stress for the animals and difficult to implement as often lions cannot be recaptured. The IGRA also based on CMI seem to provide good results in cattle and buffaloes, but its disadvantage is that blood needs to be processed within a stipulated period after collection and that might not be practicable to do in the field. Although the uses of serological assays has the advantages of logistics, lower costs, and are easily applicable, Serology also has its disadvantages like the lack of sensitivity in early stages of infection.

Due to the chronic nature of the disease, it can persist subclinical for extended periods (de la Rua-Domenech *et al*., 2006). Because CMI usually develops mostly within weeks it can then be measured using the FN-γ assay and the tuberculin skin test. But when CMI deteriorates, the FN-γ response will be reduced, this is called the anergic stage, which can be induced by stress (Pollock and Neill 2002). Slowly humoral response develops meaning that the animal starts producing antibodies, which can be measured using serological tests. Recently new diagnostic assays have been developed for a

number of wildlife species that seem to have satisfactory sensitivity and specificity best for routine testing (Lyashchenko *et al.*, 2008). Preliminary studies done with new serologic assays have the potential to be used as diagnostic tests (Miller *et a*l., 2012).

2.5 Immune responsiveness after *M. bovis* infection

In tuberculosis, the immune response aims to contain an intra-cellular bacterium that does not make toxins (de Lisle *et al.,* 2002). According to Pollock *et al.,* 2001 there is a general agreement that CMI plays a major role in controlling the infection. There are quite a few ways in which the infection may develop when an animal is exposed to *M. bovis*. The diverse immune responses can lead to killing the bacterium, a dormant state of the bacterium and to development of active tuberculosis *(*Welsh, *et al.,* 2005). External factors such as poor nutritional status, pregnancy or stress can lessen resistance to bovine tuberculosis, but there is little definite information on the impact of these factors on the progression of the disease (Pollock and Neill 2002).

Infection progression in the host is depended on the balance between different anti-mycobacterial immune responses (Welsh *et al.,* 2005). Referring to unpublished data it shows that the initial challenge dose of *M. bovis* also influences the result (Pollock and Neill): higher challenge doses are linked with cell-mediated immune response that develops within weeks with rapid generation of circulating antibodies. Lower challenge doses produce a steadier development of the CMI and little or no antibody response (Pollock and Neill 2002).

The most important macrophage that hosts the bacteria is the effector cell for control of mycobacterial infections; however, the T-lymphocyte is the main inducer of the protective acquired immune response (Buddle *et al.,* 2002). T-cells can be divided into T-helper cells expressing the $CD4^+$ marker and cytotoxic T-cells expressing the $CD8^+$ marker. The $CD4^+$ T-helper cells recognise antigen presented on class II major histocompatibility complex (MHC) molecules, while $CD8^+$ cytotoxic T-cells only recognise antigen presented on class I MHC molecules (Gajewski, *et al.,* 1989, Howard, Zwilling 1999). T-helper cell can be differentiated into two types, T-helper 1 (Th1) or T-

helper 2 (Th2) cells, depending on the type of cytokines and signals from the antigen presenting cells that activate them. Th1 cells secrete INF-γ and another type 1 cytokine such tumour necrosis factor-α (TNF- α) (Finkelman *et al.,* 1990, Hernandez-Pando *et al.,* 1996).

Cell-mediated immunity develops after an animal is infected. $CD4^+$ Th1 cells are first mediated and then become activated under the influence of interleukin-12 (IL-12). The Th1 cells are considered essential to activate macrophages and are able to contain the mycobacterial bacilli (Hernandez-Pando *et al.,* 1996, Buddle *et al.,* 2002). Th2 cells secrete IL-4, a type 2 cytokine that assists with B-cell activation and antibody production and is associated with the chronic and progressive phase of the infection (Finkelman *et al.,* 1990, Hernandez-Pando *et al.,* 1996).

2.6 Pathology

Lions tend to show completely different macroscopic lesions from the ones described in ungulates and non-human primates and are difficult to identify, except for the pulmonary lesions. No abscesses or calcification are present (de Lisle *et al.,* 2002). In more advanced cases pulmonary lesions are usually seen and only macroscopic lesions can be pathognomic (Keet, Michel & Meltzer 2000). In cases where the lung changes are most noticeable, an aerosol infection is most likely to have been the transmission route (Eulenberger *et al.,* 1992). Keet described the tuberculosis lesions in the lungs which showed signs of bronchiectasis and numerous fibrous but fairly thin-walled cavities in the lungs with small quantities of opaque, greyish-white mucoid exudates. These do not essentially need to contain many acid-fast bacilli (Keet *et al.,* 1997). Cases where histopathology was performed, showed granulomatous pneumonia, but no necrosis occurred in the inflammatory reaction (Keet, Michel & Meltzer 2000). The multinucleated massive cells that are important in the granulomatous reactions of buffaloes, kudus and baboons, were lacking from the lesions in lions (Keet *et al.,* 1997).

2.7 Current diagnostic methods used for the detection of bovine tuberculosis in wildlife according to the OIE

The diagnosis of tuberculosis in wildlife often relies on post-mortem because of the logistical challenges and lack of field-friendly techniques for live animal testing. The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the Office International des Epizooties (OIE) lists several diagnostic tests that can be used to detect *M. bovis* infection in cattle. Increasing perception has raised awareness that no single method is adequate for detecting BTB infected animals (Salfinger and Pfyffer 1994). As a result, a multidisciplinary approach is essential, based on the currently available methods. Below, are some of a various antemortem tests employed as diagnostic methods currently used for diagnosing BTB.

Identification of the agent:

- Microscopic examination of smears stained with Ziehl Neelsen stain method provides presumptive evidence
- Isolation of mycobacteria on selective culture media
- Nucleic acid recognition methods by identification and biochemical tests or Gen-Probe TB complex DNA probe or polymerase chain reaction (PCR)

- Delayed Hypersensitivity test

    - Single Intradermal test (SIT)
    - Single intradermal comparative cervical tuberculin test (SICCT)

- Blood-based Laboratory tests

    - Lymphocyte proliferation assay

        - This test is done in-vitro and is based on the comparison of the reactivity of blood lymphocytes to avian and bovine purified protein derivative, however, the test has scientific value, but it is time-

consuming, and laboratory procedures are complicated and expensive so it is not used for routine purposes.

- Gamma-interferon release assay
- Enzyme-linked immunosorbent assay (ELISA)

2.7.1 The best Test to use

When the aim is to eradicate infection, tests based on cellular immunity are the most important to use, as the infection can be detected in the early stages. If controlling infection is the aim, it is important to identify shedders of the bacteria earlier, e.g. the use of serology is important. The major disadvantage of using the ELISA test for detection of antibody to *M. bovis* infection in cattle is that there is usually the late and irregular development of humoral immune response in cattle during the course of the disease. In wildlife absence of routine testing and test and slaughter policy, infected animals have the chance to reach the Th2 stage different to cattle that is removed before the stage (Pollock *et al.,* 2001).

Single blood–based tests (IGRA and ELISA) are tempting to use for wildlife, since they are cheap in terms of logistics, due to the stability of antibodies during transport, storage and handling, which reduces costs and increases the ease of application. This is less stressful for the animals and reduces chances of injuries (Waters *et al.,* 2005). Assays that are done *in vitro*, cell-based assays like the IGRA seem to provide good results for cattle and buffalo, their disadvantage is that blood needs to be processed within a certain period of time in conditions that might not be possible in the field. Ab detecting doesn't have this disadvantage, but it has advantages in terms of logistics, cost, and they are easy to apply. Up to this day, no *M. bovis* specific antibody detecting assay has shown sufficient sensitivity or specificity suitable for routine diagnostic use and more information is needed about antigens recognized by antibodies produced during infection (Cousins D.V and Florisson 2005, Waters *et al.,* 2005). According to Pollock *et al.,* 2001, regular testing is not possible in wildlife, most animals are most likely to reach

the advance state with the humoral immune response, and serology might be practical in wildlife, where the test would not be valuable in regularly tested animals.

2.7.2 Culture (Golden standard)

Culture is regarded as a golden standard for diagnosing BTB, but the process is long, and obtaining samples is difficult on living animals. Veterinary and medical laboratories use different culture systems to isolate *mycobacteria*. The reason for this is that strains of *M. bovis* grow poorly or they may even not grow at all on the glycerol-based media traditionally used to culture *M. tuberculosis*. Due to the lack of poor growth, sodium pyruvate media is used instead of glycerol for isolation of *M. bovis* (WHO, 1996). In addition, it has been acknowledged that mycobacteria grow faster in liquid medium (Salfinger and Pfyffer 1994). When comparing the genus Mycobacterium to other microorganisms, it has very high nutritional needs compared to other microorganisms. To detect the mycobacteria from contaminated samples like sputum, milk or tissue lesions, decontamination methods should be used to eliminate competitive microorganisms. Decontamination of samples involves the addition of 1 to 5% sodium hydroxide (NaOH), which is followed by additional treatment with oxalic acid (C2H2O4), or quaternary ammonium compounds (Young J.S *et al., 2005*). Another method of decontamination is by using 0.75% cetylpyridinium chloride (Corner L.A., 1994; OIE, 2008). Although decontamination is important, the toxic effects caused by it may affect mycobacterial viability, therefore interfering with culturing the organism (Holanda, *et al.,* 2002). The growth rate of *M. bovis* may take up to 6-8 weeks (Wards, *et al.,* 1995). If *M. bovis* is grown on a suitable pyruvate-based solid medium, the colonies come out smooth and off-white. Although culture is regarded as a golden standard for confirming BTB it lacks specificity and sensitivity (Coetzer, Tustin 2004).

2.8 Assays based on cellular immunity

2.8.1 Intradermal tuberculin test

The intradermal tuberculin test has been used for more than 100 years, and it is still the most used test to diagnose BTB in cattle (Monaghan *et al.,* 1994) it is also called the

single intradermal cervical tuberculin test (SITT). The test is done in vivo and it is based on an intra-dermal injection of a purified protein derivative (PPD) of *M. bovis* origin (bov PPD). When done in conjunction with the injection of PPD of *M. avium* origin (av PPD), the test is identified as the comparative cervical intradermal tuberculin test (CITT). The test requires a second visit seventy-two hours from the day of injection (3 days), the skin thickness is measured and the skin swelling is used as a measure of hypersensitivity to the antigens used (Brasil, 2006). Cattle infected with *M. avium, M. tuberculosis, M. avium paratuberculosis, Nocardia farcinica,* or other mycobacteria could be reactive to bov PPD, leading to false-positive results. Regardless of the broad usage of this test, its sensitivity ranges from 68 to 95%. Specificity for the CITT ranges from 96 to 99%. The major disadvantage of this test is that it requires two visits to see the same animals within 3 days (Monaghan *et al.*, 1994), making it expensive and time-consuming (Lilenbaum *et al.,* 2001). Using the skin test as an exclusive diagnostic tool does not detect all infected animals the bacteria can live in anergic animals that serve as reservoirs, which could compromise the removal of the source of infection (Liebana *et al.,* 2008).

2.8.2 Interferon-gamma release assays

IFN-γ is a cytokine produced by lymphocytes, and in small amounts produced by natural killer cells, in response to intracellular antigen or mitogen stimulation (Farrar, Schreiber 1993). Pollock *et al.,* 2001 states that it stimulates the macrophages by up-regulation of the MHC-expression, thereby augmenting antigen presentation. It is known to have a major role in anti-mycobacterial immunity, and is produced by both $CD4^+$ and $CD8^+$ T-cells

Immune response of mycobacterial infections is mainly cellular. Wood and Rothel 1994 recommended that primary diagnostic techniques should be based preferably on the measurement of T lymphocyte responses. Due to the limitations of the intradermal tuberculin tests a rapid (24 hour) in-vitro cellular assay based on the detection of IFN-γ which is released in response to specific antigens in whole-blood culture for the detection of bovine tuberculosis was developed by (Wood *et al.,* 1990a).

The production of monoclonal antibodies for specific bovine IFN-γ facilitated the development of sandwich enzyme immunoassays (ELISA) for bovine IFN-γ (Rothel *et al.,* 1992, Wood *et al.,* 1990a). Since the assay is able to detect cases in very early stages of infection, it helps in identifying some tuberculosis skin test-negative cattle (Gormley *et al.,* 2006; Neill *et al.,* 1994). Cagiola *et al.,* 2004 did a study in which they reported a higher specificity with ranges from 97.3% to 98.6% than the skin test, depending on the tuberculin used. Another study in Brazil where the comparison of the IGRA and the tuberculin test was done under field conditions, for the diagnosis of bovine tuberculosis, reported a higher sensitivity of the IGRA than the skin test, sensitivities ranged from 100 and 88.3%, respectively (Lilenbaum *et al.,* 1999b). The IGRAs primary advantage is that animals are handled once. In addition to that, the test can be repeated as often as wanted as the status of the animals remains unchanged since stimulation of lymphocytes is carried out *ex vivo* and the animal is not injected with PPD (Whipple *et al.,* 1995). The interpretation of results of the assay makes it ideal to use since results are based on numerical values, instead of hypersensitivity reactions measured in the skin. The use of the IGRA has a disadvantage in terms of processing samples. It requires processing samples in a well-equipped laboratory, making the cost of conducting tests higher than the skin test (Lilenbaum *et al.,* 1999).

Although the ELISA shows low sensitivity, it has the potential to be used as a diagnostic tool for BTB diagnosis by complementing CMI and determining anergic animals. This is ideal to use in cattle as they are tested regularly, whereas in wildlife where testing is not done as frequently more animals reach the Th2 stage of infection. Serology then plays a role of testing shedders. Animals that have a defined infection status should be tested more and serology results should be correlated to CMI results.

2.8.2.1 The principle of the IGRA

Antibodies specific to IFN-γ are used to coat plates. Supernatants of lymphocyte cultures stimulated with PPD's or other relevant antigens are added. IFN-γ in the supernatants will bind to the monoclonal antibodies (capture antibodies), the samples are discarded, monoclonal or polyclonal antibodies specific for IFN-γ are added

(detecting antibodies). Lastly, secondary antibodies with specificity for the detection antibodies are added; the substrate is added and a colour change is measured spectrophotometrically. Colour development represents the presence of IFN-γ or an indication that the animals tested have been sensitised to the relevant antigen.

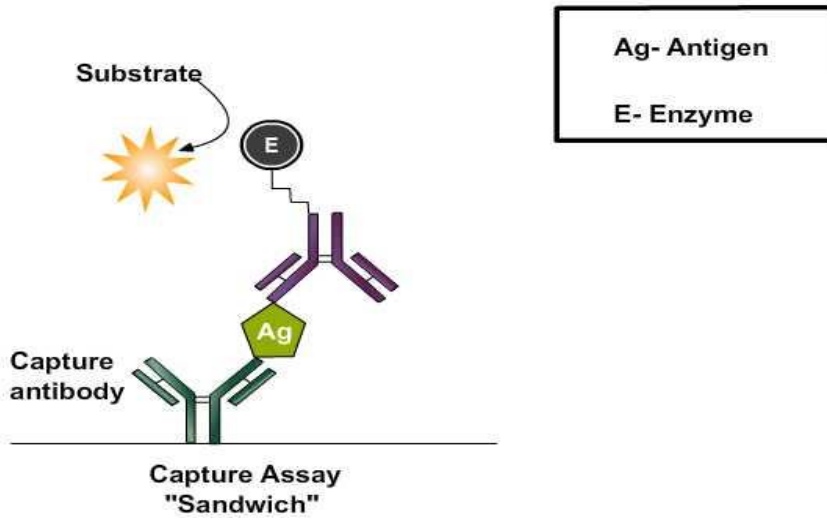

Figure 2.2 Principle of the capture ELISA; vlab.amrita.edu, (2011). Retrieved 1 July 2016, from vlab.amrita.edu

2.9 Assays based on antibody detection

Serological assays are based on antibody detection. Although they cannot be considered as first choice diagnostic methods for diagnoses of tuberculosis, a number of researchers have described strategic approaches for their use (de la Rua Domenech *et al.,* 2006; Lilenbaum, Fonseca 2006, and Silvia 2001).

A report stated that researchers recommended the use of serological assays, were mostly based on the presence of anergic animals and in advance stages of the disease increased antibody titres are observed (Pollock & Neill 2002; Welsh *et al.,* 2005).Eighteen herds of cattle involved in a tuberculosis control programme were tested by Lilenbaum & Fonseca 2006 using the ELISA antibody detection assay in order to

identify infected cows, which they later confirmed infection of *M. bovis* from lung lesions. In order to improve the control of tuberculosis by identifying anergic cows, antibody detection was used as a complementary diagnostic test.

The disadvantage of serology is that it is less effective in identifying infected cattle in the early stages of infection as antibody levels are low, (Wood *et al., 1994*). There are several technical advantages for using antibody detection methods e.g. ELISA, which assists in the diagnosis of BTB: animals are handled once and only one visit to the farm is required, blood sampling can be repeated as often as required without altering the immune status of the animal, results interpretation is based on numerical values and it makes it more ideal than the measurement of swelling of the skin (Lilenbaum *et al.,* 2001).

The ELISA measures the binding of specific antibodies to an antigen (Lilenbaum *et al.,* 1999). There are specific antigens used to diagnose cattle infected by *M. bovis*, and they are called purified antigens from *M. bovis* (Fifis *et al.,* 1994, Lilenbaum, Fonseca, 2006; Lilenbaum *et al.,* 1999; Silvia, 2001; Waters *et al.,* 2006). Wiker and Harboe, 1992 reported that antibodies directed against these antigens may also be present in non-infected individuals leading to false positives.

Lilenbaum *et al.,* 2001 conducted a study in Brazil and used an ELISA with Ag85 as antigen and obtained a sensitivity of 91.3% and specificity of 94.8%. Although in other studies, reports showed high specificity of antibody detection. The sensitivity of ELISA employing this antigen was lower (18%) (Fifis *et al.,* 1994; Harboe *et al., 1998;* Juarez *et al*., 2001*;* Pollock *et al.,* 1994)

The MPB70 protein represents approximately 10% of the PPD, and has been identified as a B-cell target in cattle with tuberculosis, Wiker and Harboe., 1992; Lightbody *et al.,* 2000) (Billman-Jacobe *et al.,* 1990; Harboe *et al.,* 1998; Radford *et al.,* 1990). MPB70 and MPB83 are secreted mycobacterial proteins which are regarded as highly homologous with limited species distribution (Chambers *et al.,* 2004). In *M.bovis* cases the two proteins are expressed highly, but less in *M. tuberculosis,* both *in vitro* and *in*

vivo (Wiker and Harboe., 1992). Diagnostic potential of these proteins was established by Wiker *et al.,* 1998, they reported that several monoclonal antibodies identify shared epitopes on these molecules and that the linear epitopes of MPB70 were the major antibody targets, both upon immunization with protein preparations and during infection with *M. bovis*. When comparing MPB83 with MPB70, there were slight differences. Current studies demonstrated the effectiveness of the MPB83 to diagnose tuberculosis earlier than MPB70 in experimentally infected animals. MPB83-ELISA detected specific antibodies four weeks after being infected, whereas MPB70-ELISAs detect specific antibodies 18-22 months after experimental infection (Wiker 2009).

The use of MPB70 as an antigen on antibody ELISAs, yielded results with good specificity ranging from 88 to 96%, whereas the sensitivity was quite inconsistent, ranging from 18 to 73% (Wiker., 2009). The drive to test specific epitopes of the protein MPB70 to increase the test specificity was due to cross-reactivity with Nocardia (Lightbody *et al.,* 2000; Radford *et al.,* 1990). In addition, rM70-83-E6, a combination of MPB70, MPB83 and ESAT-6 antigens, specifically reacts with bovine tuberculosis-positive sera, also improving ELISA sensitivity and specificity (Liu *et al.,* 2007).

Other studies done on antibody detecting ELISA‘s used bov PPD as an antigen, and produced good sensitivity (90%) and specificity of (89.9%) (Ritacco *et al*., 1987) In addition, in Mexico a study done which was using the same test, reported on sensitivity of 76.5% (Casillas *et al.,* 1995), while Lilenbaum *et al.,* 1999a reported 86.7% sensitivity and 90.6% specificity in Rio de Janeiro, Brazil. The sensitivity of antibody ELISAs depends on the duration and severity of herd infection. (Silvia, 2001).

2.9.1 The principle of the indirect ELISA (iELISA):

The indirect enzyme-linked immunosorbent assay (iELISA) is a semi-quantitative serological test that measures antibodies in serum samples. Plates are first coated with the specific antigen. Serum samples are added, if the serum has antibodies, it will bind to the antigen in the wells. An anti-species conjugate is added and attaches to bound

antibodies. It causes the substrate to change colour. The colour development is proportional to the concentration of antibodies for the tested antigen.

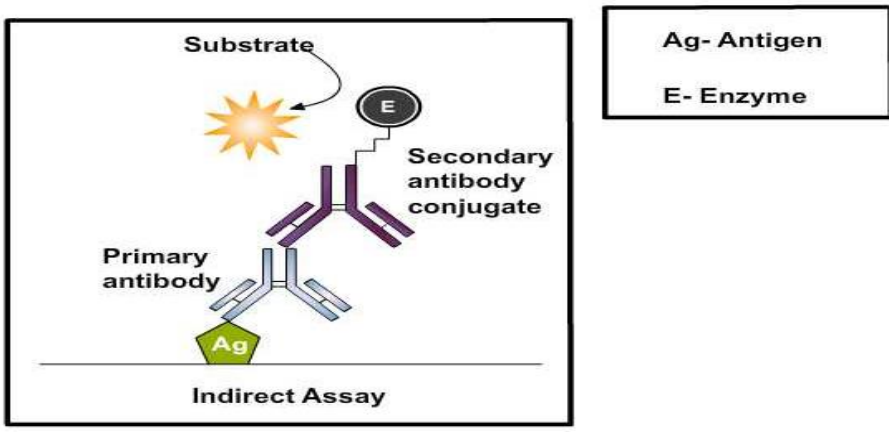

Figure 2.3 Representation of an indirect ELISA; vlab.amrita.edu, (2011). Retrieved 1 July 2016, from vlab.amrita.edu

3 Chapter 3

Materials and Methods

3.1 Sampling and handling of samples

Blood was collected in heparinized tubes from free-ranging and captive lions. From each lion, 10 ml of blood was collected and stimulated with mycobacterial antigens and mitogens.

The majority of samples were collected from the KNP in Mpumalanga (n=17); all samples were opportunistically collected during routine immobilisation of lions. Animal ethics clearance was obtained from UNISA (2014/CAES/187) and the University of Pretoria (Section 20 approval was obtained from the Department of Agriculture (DAFF) (12/11/1/1/18)

Samples were kept at ambient temperature, and packaged using the 3 layer packaging system; samples were collected in Vacutainer tubes, placed in plastic bag and put in a Styrofoam box with a tightly secured lid and transported to the laboratory where they were processed within 8 hours from collection.

3.2 Evaluation of mitogens as positive cell control.

Preparation of the mitogens

Pokeweed (PWM): 5% in NaCl solution was made up and 5 ml of 0.9% NaCl to PWM 10mg to make up a final concentration of 2 mg/ml (stock solution and store at -20 ºC.

Phorbol 12- Myristate 13- Acetate (PMA): An aqueous solution of PMA (1 mg) powder was made by using a concentrated solution of 100 % DMSO (1 ml) and then diluted in a small aliquot with water to a final concentration of 1mg/ml.

Calcium Ionophore (CAI): 1ml of DMSO (100%, Sigma) was added to 1mg of Ionomycin (lyophilized powder) to a final concentration of 1mg/ml.

3.2.1 Whole blood processing

1.5 ml heparinised blood (mixed thoroughly by inverting four times) was dispensed into 2 ml tubes (Eppendorf) and stimulated using one of the following conditions: PMA/CaI at different concentrations (100 ng/ml/2 µg/ml & 50 ng/ml/1 µg/ml) (Sigma, South Africa), (PWM 10 µg/ml, Sigma, South Africa) , av PPD (20 µg/ml) 1 and bov PPD (20 µg/ml) (Ondersterpoort Biological products) and incubated at 37 °C in 5% CO_2 for 24 and 48 hours respectively. The negative control consisted of blood stimulated with RPMI medium and the positive control consisted of blood stimulated with a mitogen. Plasma was harvested after incubation, and stored at -80 ºC until further use in the lion-specific IFN-γ capture ELISA.

3.3 Optimisation of the IFN-γ ELISA

The lion-specific IFN-γ ELISA developed my Maas *et al.* (2012) was re-evaluated and further optimized to harmonize the procedure with a similar rhino-specific interferon-γ ELISA developed by Morar *et al.* (2007). Harmonization was done in order to ensure that the assay test results of the lion specific IFN-γ ELISA are equivalent to what has previously described on the rhino-specific interferon-γ ELISA as it has already been optimized.

3.3.1 Optimization of the IFN-γ monoclonal capture antibody

The first step was to evaluate 2 different concentrations for coating of the IFN-γ capture monoclonal antibody (mAb) (LI2B7.2G7) (Maas *et al*, 2012) using (Greiner Bio One Microlon , extra high binding, 655061, E120700P) plates . Wells were coated with 50 µl volumes using either 2 µg/ml or 4 µg/ml mAb diluted in phosphate buffered saline (PBS; ph 7.2). Plates were incubated for one hour on an orbital shaker (250 rpm) at 37 °C. Coating buffer was discarded and 200 µl of PBS with 1% bovine serum albumin (BSA; Sigma, South Africa) (blocking buffer) was added. Plates were incubated as above and the blocking buffer was discarded, washing was optional after blocking. Recombinant lion IFN-γ (rLIFN-γ) produced by U-Protein Express BV, Utrecht The

Netherlands (Maas *et al*., 2010) was titrated in duplicate over 7 wells in a 2-fold dilution series (3 500- 54 pg/ml), 50 μl/well in blocking buffer, from row A to row G, and row H received only diluent as negative control. Plates were incubated as above and washed three times with wash buffer (PBS with 0.1% Tween-20 (Sigma). Biotinylated detecting antibody mAb (Li7A9B4) (Maas *et al*, 2012) (50 µl/well) was added at 1:5 000 dilution in blocking buffer. Plates were incubated for 30 minutes at 37 °C on a shaker. After incubation plates were washed as described above and 50 μl/well of 1:5 000 streptavidin conjugated to horseradish peroxidase (strep-avidin-HRP 80; Stereospecific Detection Technologies, Germany) in blocking buffer was added. Plates were incubated for 15 minutes on a shaker at 37 °C and then washed six times before 50 µl/well ready-made TMB substrate (Sigma, T4444)) was added, colour reaction was stopped after 20 minutes by adding 50 µl/well 2 M H_2So_4. Plates were read at a wave length of 450 nm with a reference read at 650 nm.

3.3.2 Optimization of the IFN-γ monoclonal detection antibody and conjugate

The second step was to evaluate different concentrations of biotinylated IFN-γ detection mAb (Li7A9B4) (Maas *et al*, 2012) and strep-avidin-HRP 80. Plates were coated with 2 μg/ml of IFN-γ capture mAb and rLIFN-γ was titrated as described previously in 3.1.1.The detection antibody and streptavidin-HRP 80 was diluted in the blocking buffer and added in 50 μl volumes to respective wells as indicated in Table 3.1

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Dilution for IFN-γ detection mAb (Li7A9B4)	1:5 000	1:10 000	1:5 000	1:10 000	1:5 000	1:10 000

Dilution for strep-avidin HRPO-conjugate	1:2 500	1:2 500	1:5 000	1:5 000	1:10 000	1:10 000

Table 3.1 Different concentrations of biotinylated detecting mAb and strep-avidin- horse radish peroxidase conjugate were tested in the following combinations.

The assay was then performed as previously described in

3.3.3 Optimization of the blocking buffer

The initial assay was developed using 1.3% Universal Casein Diluent (Stereospecific Detection Technologies, Germany) in PBS as a diluent and blocking agent. The third step was to evaluate two different blocking agents: casein and BSA to harmonize with the rhino IGRA protocol (Morar *et al*., 2007).

ELISA plates were coated with 2μg/ml of IFN-γ capture mAb and rLIFN-γ was titrated as described previously in 3.1.1. RLIFN-γ was titrated in a two-fold dilution series in all columns (1 to 12) of the plate starting at a concentration of 3500 pg/ml in row A and ending at a concentration of 54 pg/ml in row G. Row H received only diluent as negative. The assay was performed as previously described in 3.3.1 but PBS with 1% BSA was used for blocking and as diluent on the first half of the plate (columns 1 to 6) and PBS with 1.3% casein (universal casein diluent SDT, Germany) was used for blocking and diluent on the other half of the plate (columns 7 to 12).

3.3.4 Lion IFN-γ capture ELISA optimized protocol

A final protocol was optimized. Extra high binding ELISA plates (655061, E120700P Greiner Bio-One Microlon) were coated with 50 µl/well of capture mAa (LI2B7.2G7) diluted in PBS to a concentration of 2µg/ml. The plate was incubated for an hour at 250

rpm. After incubation, coating buffer was discarded, and 200 µl of the diluent (PBS 1% BSA) was added to each well, the plate was incubated for another hour at 37 °C. After blocking, the buffer was discarded, and the plate was washed three times using wash buffer (PBS 0.1% Tween-20). Fifty µl/well of supernatant/plasma was added in duplicate to appropriate wells according to plate layout, leaving two columns empty for the standard curve. For the standard curve, rIFN-γ was used at a starting concentration of 3 500 pg/ml followed by a two-fold dilution series to 54 pg/ml. The last well served as the blank containing only diluent. The plate was incubated for an hour at ambient temperature. Washing was done as described above. Biotinylated detecting mAb (Li7A9B4) was added to the diluent at a concentration of 1:5 000 (50 µl/ well) and incubated for 30 minutes on a shaker at ambient temperature. After the wash step, streptavidin-HRPO80 (Sigma) was added (1:5 000 dilution) and a 15 minutes incubation at 37 °C followed. Substrate TMB (Sigma) reagent (50 µl/well) was added, the colour reaction was stopped after 10 to 20 minutes depending on how fast the reaction was by adding 50 µl per well of a 2 molar H_2So_4 solution and plates were read at an optical density at 450 nm with a reference read at 650 nm.

3.4 Data Analysis

Samples were analysed in duplicate and results were expressed as the averaged OD_{450}.The statistical comparison used to compare different incubation time and mitogen was performed using the ANOVA and Student t-test in Microsoft excel.

3.4.1 Precision (reproducibility)

Precision is defined as the quality of being thorough or accurate and reproducibility is the measurement of an experiment if whether it can be reproduced as a whole. In this regard, the assay was tested to present its reproducibility and its accuracy.

Four ELISA plates tested on different days were used to determine the precision of the assay. This was done by calculating the mean, standard deviation (SD) and coefficient of variation (CV).

3.4.2 Results calculation

The lower limit of detection of the assay was calculated based on the OD_{450} reading higher than the average OD_{450} of the negative control plus three times the standard deviation. Stimulated whole blood supernatants were assayed in duplicate and 4-parameter logistic curve fitting using rLIFN-γ was used to calculate concentration levels (pg/ml) of FN-γ detected.

Whole blood cultures from animals originating from BTB free areas were tested for measuring FN-γ production. The FN-γ response of each individual to the two different stimulation antigens is listed under the results Table 4.2.

4 Chapter 4

Results

4.1 Optimization of the IFN-γ monoclonal capture antibody

The titration curves for rLIFN-γ using two different coating concentrations for the capture mAb are presented in fig 4.1. The limit of detection was calculated as 3 times the optical density (OD) of the negative. The detection limit for coating the plates with 4 and 2 μg/ml of mAb was 219 and 109 pg/ml respectively. Using the mAb at 2 μg/ml resulted in more colour development and it was best to use as it saves reagents.

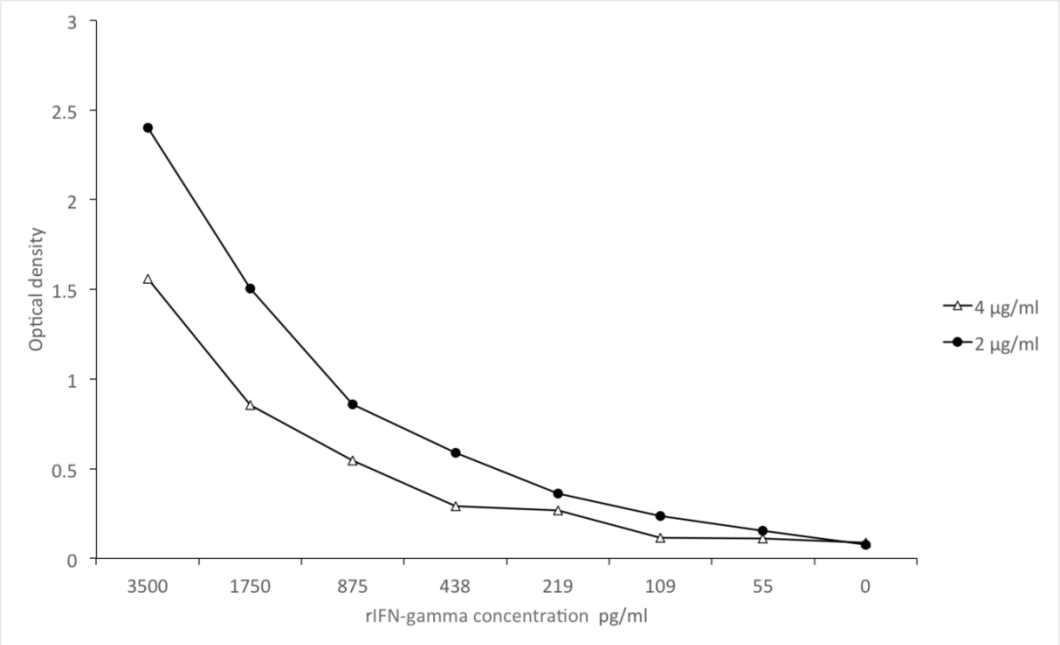

Figure 4.1 The titration curves of two different concentrations of IFN-γ capture mAb are shown. Plates were coated with 4 μg/ml or 2 μg/ml. Each of the lines represent a serial 2-fold titration of rLIFN-γ ranging from 3 500 pg/ml to 55 pg/ml. The lower detecting limit of the assay was determined by calculating the negative value three times the average OD value and it was found at 109 pg/ml rLIFN-γ.

4.2 Optimization of the IFN-γ monoclonal detection antibody and conjugate

The analytical sensitivity of different concentrations of biotinylated detecting mAb and streptavidin conjugated to horse radish peroxidase were tested. The results are shown in the table below; the table also represents detection limits obtained from comparing the two blocking buffers Figure 4. shows two titration curves for rLIFN-γ using different blocking agents. When PBS with 1% BSA as blocking agent was used, the assay developed higher overall OD values when compared to PBS with 1.3% casein as blocking agent. The assay with the BSA as blocking agent was more sensitive detecting up to109 pg/ml while the assay with casein as blocking agent detected 219 pg/ml of the rLIFN-γ.

Table 4.1 Detection limits for rIFN-γ at different concentrations of biotinylated detecting mAb and strep-avidin-horse radish peroxidase conjugate are shown below. Two different blocking agents were also tested. These constituted 1% BSA in PBS and 1.3% Casein in PBS. Columns showing the other results not shown on below graph fig 4.2.

	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6
Dilution for IFN-γ detection mAb (Li7A9B4)	1:5 000	1:10 000	1:5 000	1:10 000	1:5 000	1:10 000
Dilution for strep-avidin HRPO-80	1:2 500	1:2 500	1:5 000	1:5 000	1:10000	1:10 000
rLIFN-γ detection with 1% BSA blocking	219 pg	219 pg	109 pg	219 pg	109 pg	438 pg
rIFN-γ detection with 1.3% casein blocking	440 pg	220 pg	220 pg	220 pg	440 pg	220 pg

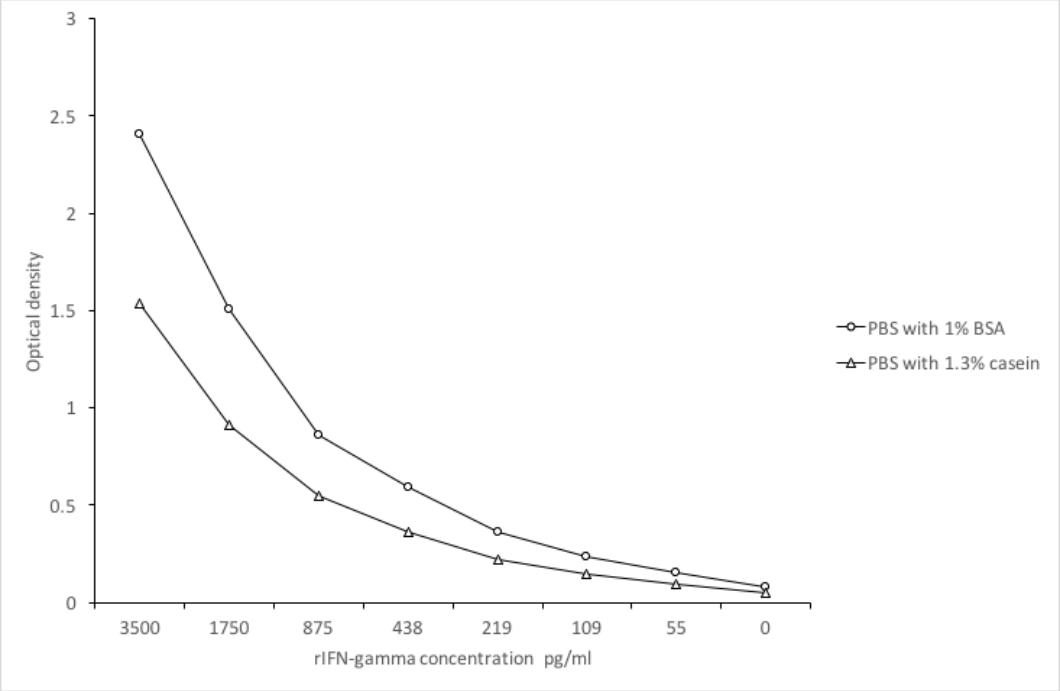

Figure 4.2 Titration curves of 2 different blocking buffers are shown. Plates were blocked with 1% BSA or 1.3% casein. Each of the lines represent a serial 2-fold titration of rLIFN-γ ranging from 3 500 pg/ml to 55 pg/ml

4.3 Whole blood stimulation with mitogen for 24 h and 48 h

Two incubation times were evaluated on five lion blood samples; blood was incubated for 24h and 48h respectively before harvesting supernatants. All five of the lion samples stimulated with PMA/CaI, produced strong IFN-γ signals following the 24h and 48h incubation. Table 4.1 presents the comparison between PMA and PWM. PMA gives a significantly stronger reaction ($p < 0.05$). No significant difference of FN-γ (ng/ml) production was found after antigen stimulation with bovine and avian PPD.

Table 4.1 Concentration values obtained from OD_{450} readings. Whole blood stimulation was 24h and 48h assayed in duplicates respectively. Samples were stimulated with PMA/CaI with concentrations of 100 ng/ml and 2µg/ml and PWM. IFN-γ responses to PWM and PMA had a significant difference with a

($p<0.0002$) at 24 and at 48h ($p< 0.0198$) ANOVA. The mean OD_{450} value of the samples stimulated with PMA/Ionomycin differed significantly.

		24 hrs			**48 hrs**	
Lion	**Neg**	**PMA**	**PWM**	**Neg**	**PMA**	**PWM**
N3	22.72	3675	1100.597	102.03	2790.87	633.25
N5	0.00	3675	831.82	0.00	2790.88	633.25
Maisha	32.53	3675	132.6245	50.343	3675	149.02
Lion 4	104.79	3675	971.37	140.77	3675	732.59
Lion 5	8.836	3675	165.4185	2.93	3675	137.52
Mean	33.78	3675.00	640.37	59.80	3321.35	457.13
Standard deviation	41.62	0.00	458.64	61.72	484.26	289.39
p (24h vs 48h)	0.102	0.2411	0.4867	0.102	0.2411	0 0.4867
p (PMA vs PWM)		0.0002	0.0002		0.0198	0.0198

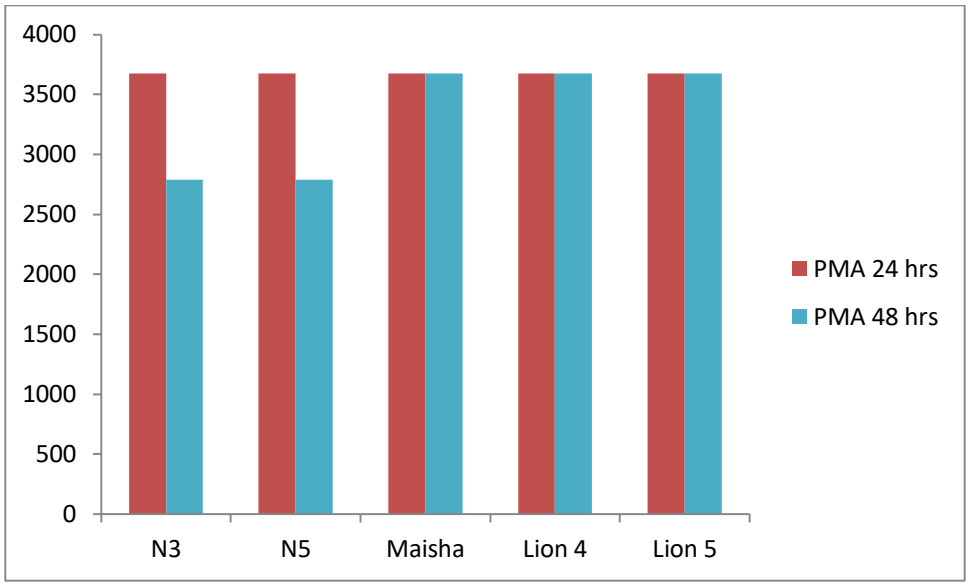

Figure 4.1 IFN-γ response in whole blood obtained from 5 lions in response with PMA 24 and 48h respectively. The concentration value of the samples stimulated with PMA/ionomycin were highly produced in the incubation interval 24 h. P=0.0002

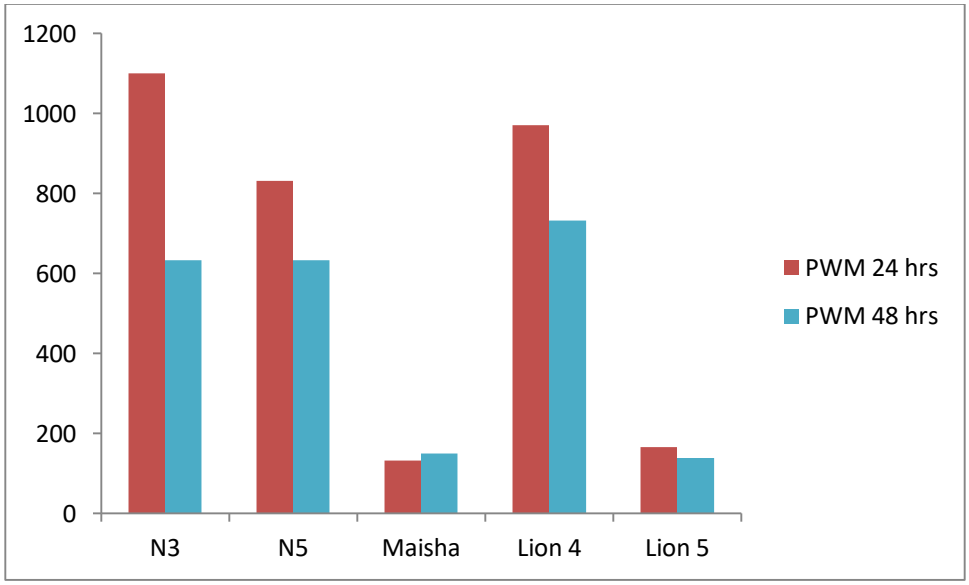

Figure 4.2 IFN-γ response in whole blood obtained from 5 lions in response with PWM 24h and 48h respectively. Both graphs show IFN-γ responses to PWM had a significant difference ($p<0.0002$) after 24h to 48h compared to the levels observed with PMA/Cal after 24h to 48h respectively. P=0.0198

4.4 Precision

The range and detection limit of the lion IFN-γ ELISA was determined by testing replicates of rILFN-γ concentrations (0- 3 500 pg/ml). A precision profile was constructed by plotting the %CV against the analyte concentration. The working range of the assay can be defined as the range where imprecision is below a preset level such as 15% as shown on the graph below.

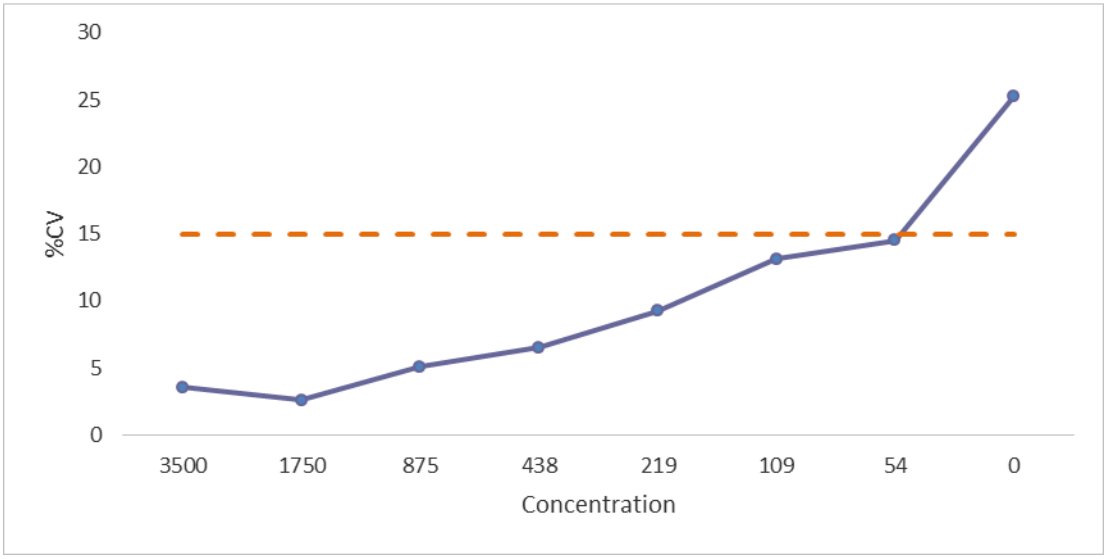

Figure 4.6 The precision profile of the rLIFN-γ an assay showing nonuniform error. The %CV is plotted against the analyte concentration. The working range of the assay can be defined as the range where imprecision is below a preset level such as 15%, as shown by the red line.

4.5 Detection of IFN-y responses

To determine the effectiveness of whole blood culture for detection of IFN-γ, blood samples from 5 lions were tested. The values from the negative control (medium only) were subtracted from the antigen stimulated values to obtain a corrected value. IFN-γ response of each individual are listed in Table 4.2. The mean concentrations of IFN-γ of blood from individual lions stimulated with bv PPD and av PPD for 24h and 48h were 18.75; 17.69 and 13.39 and 13.03 respectively.

Table 4.2 Corrected concentration values of whole blood samples of 5 lions from BTB free areas that were stimulated with bovine PPD avian PPD and PMA/CaI.

	24 hrs			48 hrs		
Lion	**PMA/CaI**	**Bv ppd**	**Av ppd**	**PMA/CaI**	**Bv ppd**	**Av ppd**
N3	3652.28	62.45	64.91	2768.15	4.79	36.81
N5	3675	0	0	2790.88	19.04	22.41
Maisha	3642.47	11.89	3.89	3642.47	13.02	6.53
Lion 4	3570.21	28.29	28.53	3570.21	27.87	-4.62
Lion 5	3666.164	-8.836	-8.836	3666.164	2.26	4.05
Mean	3641.22	18.75	17.69	3287.5748	13.39	13.03
Standard deviation	41.62	28.10	29.80	465.20	10.48	16.49

5 Chapter 5

Discussion, conclusion and recommendation

5.1 Discussion

The complex nature of diagnosis of tuberculosis has resulted in limited diagnostic tests available for wildlife. Studies previously done in lions have used intradermal tuberculin test or antibody detection (Keet D. F *et al* 2010). The intradermal tuberculin tests have proved to be impractical for use in lions because it is time consuming based on two sedations within a three-day interval (72h), they cause stress for the animal, and recapturing after 3 days is not guaranteed.

The aim of this study was to optimise an IGRA for detection of tuberculosis in free ranging and captive lions.

For optimisation, specific conditions linked to the performance of the assay were determined and evaluated. The conditions included the optimal concentrations of capture and detecting antibody, optimal concentrations of the conjugate and optimal concentrations of the blocking buffer. The detection limit of rLIFN-γ was determined and it was used as a standard curve on each plate to normalise the data. Selection of suitable mitogens for use as an indicator of cell viability and determination of optimum incubation times for cell stimulation was performed.

The optimal concentration of the capture antibody was 2 µg/ml. When 4 µg/ml was used it resulted in overall lower OD values. The use of 2 µg/ml will result in a significant saving of reagent.

The detecting antibody and conjugate could be used at 1:5000 or 1:10000 dilutions and could detect rLFN-γ at109 pg/ml. The detection limit obtained from the current study is in agreement with the 160 pg/ml that was previously described by Maas *et al*.

(2012).Though there was no significant difference when using 1:5000 and 1:10000 combinations of detecting and conjugate, it was decided to use the 1:5000 combination of both detecting and conjugate in further ELISAs. The use of 1% BSA in PBS yielded results with less background and provided an increased signal of the lower detection limit of the assay.

PMA/CaI and PWM were used as cell stimulators for the effectiveness of IFN-γ production in whole blood cultures. The best performing mitogen was based on the strongest OD response and the most consistent when repeating testing using the same sample, PMA/CaI was recognized as the best performing mitogen for use as a positive control in stimulation of whole blood cultures. Whole blood samples stimulated with PMA/CaI and PWM from five lions were tested and IFN-γ production was found to be produced in similar quantities for both 24h and 48h when stimulated with PMA/CaI as shown in Figure 4.1. The results also show that responses to PMA/CaI were greater than those of PWM in all 5 lions, this supports the use of PMA/CaI as a viability control as what was used by Rhodes *et al*., 2008 in his feline assay and Maas *et al*., 2012 in the development of this assay.

The assay test performance was assessed by analysing the repeatability of the assay. This was done by determining the precision profile which was used to determine the valid performance range of the assay. This was done in accordance to the guidelines described by (Crowther, J. R. 2001). The working range of the assay can be defined as the range where imprecision is below a preset level such as 15%, as shown in Figure 4.5. When the cut-off for the standard curve is determined as 2 standard deviations above the mean of the negative control, the assay has a limit of detection of 55 pg/ml, however there is very little variation between OD values towards the end of the titration curve (as the line flattens out) and therefore little significant difference between consecutive concentration values. Therefore, we opted to select the cut-off as 3 times the mean of the negative control which gives a cut-off of 109pg/ml. This decision is supported when evaluating the precision profile in figure 4.5 when the %CV of each dilution of the positive control obtained over several runs and on different days, is plotted against the concentration range; a profile of the precision of the assay is

obtained. According to this profile the assay performs well in the range of 3500 to 109 pg/ml with %CV below 15%.

The results obtained in this study demonstrate the ability of the assay to detect IFN-γ responses when stimulated with mitogen. Whole blood culture samples that were stimulated with PMA/CaI produced higher responses of IFN-γ. Samples stimulated with both bovine ppd and avian ppd at 48h produced as much IFN-γ responses as compared to responses observed at 24h as shown in Table 4.2. Nonetheless, none of the animals reacted well to the mycobacterial antigens as compared to the reactions produced by the mitogen. More samples of infected and un-exposed lions are needed to determine cut-off values for the assay.

The ELISA described by Maas was adapted and re-evaluated, in this study. The original ELISA was developed in line with the Rhino protocol (D. Morar., 2007) and used 1.3% universal casein diluent at 75 µl/well as a blocking buffer, which means only a quarter of the well was blocked. In this revised protocol PBS 1% BSA was used as a blocking buffer at 200 ul/well, this means that the whole well was blocked and less non-specific binding occurred.

In the original protocol supernatants of stimulated blood were diluted 1:1 in 1.3% casein buffer with bovine serum, with bovine serum added to a final concentration of 10%. In the revised protocol supernatants were only diluted in 1% BSA in PBS and no bovine serum was added.

The incubation stage of the revised protocol is half of what was initially described (1h), the same incubation time after addition of the supernatants was used in the rhino protocol. Combination of detecting antibody and streptavidin- HRP80 dilutions were evaluated done by comparing 1:10000 diluted in 0.43% casein at a concentration of 10% and 1:20000 (initial protocol) and at 1:5000 and 1:10000 for the current study.

Results show that the 1:5000 and the 1:10000 combinations were similar, though there was no significant difference between the two. We chose to use the 1:5000 dilutions for both detecting antibody and conjugate.

Washing was done six times in the reviewed protocol as opposed to eight times in the initial protocol. Colour reactions were only read after 20 minutes incubation. The final optimal conditions are thoroughly described in chapter 3 (3.1.1).

On the initial protocol, the coating step was followed by the washing step, which we opted to skip when optimizing the assay; this was decided based on ways to shorten the assay as the step did not make a difference if it was done after the coating step or the blocking step.

5.2 Conclusion and recommendation

In conclusion, the aim of the study was to optimise the lion specific IGRA specific to lions and the work done on the current study contributes to the optimisation of the IGRA for detecting tuberculosis in lions. The assay can detect IFN-γ from supernatants harvested from whole blood cultures stimulated with specific antigens and mitogens. Though, the main challenge is obtaining the fresh samples from lions with known tuberculosis status. Recommendation for future work is further validation of the assay for diagnostic testing in wildlife is still required.

References

1. Aagaard, C., Govaerts, M., Meikle, V., Vallecillo, A.J., Gutierrez-Pabello, J.A., Suarez-Güemes, F., McNair, J., Cataldi, A., Espitia, C., Andersen, P. and Pollock, J.M., 2006. Optimizing antigen cocktails for detection of Mycobacterium bovis in herds with different prevalences of bovine tuberculosis: ESAT6-CFP10 mixture shows optimal sensitivity and specificity. Journal of clinical microbiology, 44(12), pp.4326-4335.
2. Alito, A., McNair, J., Girvin, R.M., Zumarraga, M., Bigi, F., Pollock, J.M. and Cataldi, A., 2003. Identification of Mycobacterium bovis antigens by analysis of bovine T-cell responses after infection with a virulent strain. Brazilian journal of medical and biological research, 36(11), pp.1523-1531.
3. Bauer, A., Hogue, I., Marino, S., & Kirschner, D., 2008. The Effects of HIV-1 Infection on Latent Tuberculosis. Mathematical Modelling of Natural Phenomena, 3(7), 229-266. doi:10.1051/mmnp:2008051
4. Bengis RG, Kock RA, Fischer J. Infectious animal diseases: the wildlife/livestock interface. Rev Sci Tech. 2002 Apr; 21(1) 53-65. PMID: 11974630.
5. Bengis, R.G. and Keet, D.F., 2000, June. Bovine tuberculosis in the Kruger National Park. In Proceedings of the North American Veterinary Conference, Jan15th–19th (pp. 1034-1036).
6. Bengis, R.G., 2002. Infectious diseases of wildlife: detection, diagnosis and management-Conclusion.
7. Billman-Jacobe, H., Radford, A.J., Rothel, J.S. and Wood, P.R., 1990. Mapping of the T and B cell epitopes of the Mycobacterium bovis protein, MPB70. Immunology & Cell Biology, 68(6).
8. Buddle, B.M., Skinner, M.A., Wedlock, D.N., Collins, D.M. and De Lisle, G.W., 2002. New generation vaccines and delivery systems for control of bovine tuberculosis in cattle and wildlife. Veterinary immunology and immunopathology, 87(3), pp.177-185.

9. Buddle, B.M., Wedlock, D.N., Denis, M. and Skinner, M.A., 2005. Identification of immune response correlates for protection against bovine tuberculosis. Veterinary immunology and immunopathology, 108(1), pp.45-51.
10. BRASIL. (2006). Ministério da Agricultura Pecuária e Abastecimento do Brasil. Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal (PNCEBT)/ National Program for the Control and Eradication of Bovine Brucellosis and Tuberculosis – MAPA/DAS/DAS, 188p.
11. Cagiola, M., Feliziani, F., Severi, G., Pasquali, P. and Rutili, D., 2004. Analysis of possible factors affecting the specificity of the gamma interferon test in tuberculosis-free cattle herds. Clinical and diagnostic laboratory immunology, 11(5), pp.952-956.
12. Caron, A., Cross, P.C. and Du Toit, J.T., 2003. Ecological implications of bovine tuberculosis in African buffalo herds. Ecological Applications, 13(5), pp.1338-1345.
13. Casillas, C.R., Elizondo, G.V. and Díaz, C.A., 2012. Comparacion del elisa con la tuberculinizacion en el diagnostico de la tuberculosis bovina. Revista Mexicana de Ciencias Pecuarias, 33(3).
14. Chambers, M.A., 2009. Review of the Diagnosis and Study of Tuberculosis in Non- Bovine Wildlife Species Using Immunological Methods. Transboundary and Emerging Diseases, 56(6- 7), pp.215-227.
15. Cheeseman, C.L., Wilesmith, J.W. and Stuart, F.A., 1989. Tuberculosis: the disease and its epidemiology in the badger, a review. Epidemiology & Infection, 103(1), pp.113-125.
16. Cleaveland, S., Mlengeya, T., Kazwala, R.R., Michel, A., Kaare, M.T., Jones, S.L., Eblate, E., Shirima, G.M. and Packer, C., 2005. Tuberculosis in Tanzanian wildlife. Journal of Wildlife Diseases, 41(2), pp.446-453.
17. Clifton-Hadley, R.S., Wilesmith, J.W. and Stuart, F.A., 1993. Mycobacterium bovis in the European badger (Meles meles): epidemiological findings in tuberculous badgers from a naturally infected population. Epidemiology & Infection, 111(1), pp.9-19.

18. Coetzer, J.A.W. and Tustin, R.C., 2004. Infectious diseases of livestock. Volume Three (No. Ed. 2). Oxford University Press.
19. Corner, L.A., 1994. Post mortem diagnosis of Mycobacterium bovis infection in cattle. Veterinary microbiology, 40(1-2), pp.53-63.
20. Cousins, D.V. and Florisson, N., 2005. A review of tests available for use in the diagnosis of tuberculosis in non-bovine species. Revue scientifique et technique-Office International des Epizooties, 24(3), p.1039.
21. Crowther, J.R., 2001. Titration of Reagents, The ELISA Guidebook, 149.
22. De la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H. and Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Research in veterinary science, 81(2), pp.190-210.
23. De la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H. and Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Research in veterinary science, 81(2), pp.190-210.
24. de Lisle, G.W., Mackintosh, C.G. and Bengis, R.G., 2001. Mycobacterium bovis in free-living and captive wildlife, including farmed deer. Revue Scientifique et Technique-Office International des Epizooties, 20(1), pp.86-111.
25. De Vos, V; Bengis, R G; Kriek, N P J; Michel, A; et al. The Onderstepoort Journal of Veterinary Research; Onderstepoort 68.2 (Jun 2001): 119-30.
26. Eulenberger, K., Elze, K., Schuppel, K.F., Seifert, S., Ippen, R. and Schroder, H.D., 1992. Tuberkulose und ihre Bekampfung bei Primaten und Feliden im Leipziger Zoologischen Garten von 1951-1990. In Erkrankungen der Zootiere. Verhandlungsbericht des 34. Internationalen Symposiums uber die Erkrankungen der Zoo-und Wildtiere, Santander, Spain 1992 (pp. 7-15).
27. Farrar, M.A. and Schreiber, R.D., 1993. The molecular cell biology of interferon-gamma and its receptor. Annual review of immunology, 11(1), pp.571-611.

28. Fifis, T., Corner, L.A., Rothel, J.S. and Wood, P.R., 1994. Cellular and humoral immune responses of cattle to purified Mycobacterium bovis antigens. Scandinavian journal of immunology, 39(3), pp.267-274.
29. Fifis, T., Corner, L.A., Rothel, J.S. and Wood, P.R., 1994. Cellular and humoral immune responses of cattle to purified Mycobacterium bovis antigens. Scandinavian journal of immunology, 39(3), pp.267-274.
30. Fifis, T., Rothel, J.S. and Wood, P.R., 1994. Soluble Mycobacterium bovis protein antigens: studies on their purification and immunological evaluation. Veterinary microbiology, 40(1), pp.65-81.
31. Finkelman, F.D., Holmes, J., Katona, I.M., Urban Jr, J.F., Beckmann, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R. and Paul, W.E., 1990. Lymphokine control of in vivo immunoglobulin isotype selection. Annual review of immunology, 8(1), pp.303-333.
32. Forshaw, D. and Phelps, G.R., 1991. Tuberculosis in a captive colony of pinnipeds. Journal of Wildlife Diseases, 27(2), pp.288-295.
33. Gajewski, T.F., Joyce, J. and Fitch, F.W., 1989. Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. The Journal of Immunology, 143(1), pp.15-22.
34. Ghodbane, R. and Drancourt, M., 2013. Non-human sources of Mycobacterium tuberculosis. Tuberculosis, 93(6), pp.589-595.
35. Ghosh, J., Larsson, P., Singh, B., Pettersson, B.F., Islam, N.M., Sarkar, S.N., Dasgupta, S. and Kirsebom, L.A., 2009. Sporulation in mycobacteria. Proceedings of the National Academy of Sciences, 106(26), pp.10781-10786.
36. Good, M., Clegg, T.A., Murphy, F. and More, S.J., 2011. The comparative performance of the single intradermal comparative tuberculin test in Irish cattle, using tuberculin PPD combinations from different manufacturers. Veterinary microbiology, 151(1), pp.77-84.

37. Gormley, E., Doyle, M.B., Fitzsimons, T., McGill, K. and Collins, J.D., 2006. Diagnosis of Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam®) assay. Veterinary microbiology, 112(2), pp.171-179.
38. Gous, T.A. and Williams, M.C., 2009. The pathology of tuberculosis caused by Mycobacterium tuberculosis in a herd of semi-free-ranging springbok (Antidorcas marsupialis). Onderstepoort Journal of Veterinary Research, 76(4), pp.419-441.
39. Grange, J.M., Yates, M.D., Kantor, I.N.D. and World Health Organization, 1996. Guidelines for speciation within the Mycobacterium tuberculosis complex.
40. Harboe M., Malin A. S., Dockrell H. S., Wiker H. G., Ulvund G., Holm A., Jørgensen M. C.,Andersen P, 1998 B-cell epitopes and quantification of the ESAT-6 protein ofMycobacterium tuberculosis. Infect. Immun. 66, 717–723.
41. Henschel, P., Hunter, L., Breitenmoser, U., Purchase, N., Packer, C., Khorozyan, I., Bauer, H., Marker, L., Sogbohossou, E. and Breitenmoser-Wursten, C., 2013. Panthera pardus. The IUCN Red List of Threatened Species. Version 2012.2.
42. Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J.M. and Madrid, M.V., 1996. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology, 89(1), p.26.
43. Holanda, E.D., Lobato, F.C.F., Mota, P.M.P.C. and Abreu, V.L.V., 2002. Avaliação de métodos de descontaminção para isolamento de Mycobacterium bovis. Rev. Bras. Med. Vet, 24(2), pp.54-57.
44. Holanda, E.D., Lobato, F.C.F., Mota, P.M.P.C. and Abreu, V.L.V., 2002. Avaliação de métodos de descontaminção para isolamento de Mycobacterium bovis. Rev. Bras. Med. Vet, 24(2), pp.54-57.
45. Howard, A.D. and Zwilling, B.S., 1999. Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. Clinical and experimental immunology, 115(3), p.428
46. International Office of Epizootics. Biological Standards Commission and International Office of Epizootics. International Committee, 2008. Manual of

diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees (Vol. 2). Office international des épizooties.

47. Jones, O.G., 1953. Tuberculosis in a cheetah: The use of antibiotics and modern chemotherapeutic agents. The Veterinary Record, 65, pp.453-454.
48. Juárez, M.D., Torres, A. and Espitia, C., 2001. Characterization of the Mycobacterium tuberculosis region containing the mpt83 and mpt70 genes. FEMS microbiology letters, 203(1), pp.95-102.
49. Junker, K., Lane, E.P., McRee, A.E., Foggin, C., van Dyk, D.S. and Mutafchiev, Y., 2013. Two new species of Cylicospirura Vevers, 1922 (Nematoda: Spirocercidae) from carnivores in southern Africa, with validation of the related genera Gastronodus Singh, 1934 and Skrjabinocercina Matschulsky, 1952. Folia parasitologica, 60(4), p.339.
50. Keet, D.F., Kriek, N.P.J. and Huchzermeyer, H.F., 1996. Tuberculosis in buffaloes (Syncerus caffer) in the Kruger National Park: spread of the disease to other species.
51. Keet, D.F., Kriek, N.P.J., Bengis, R.G. and Michel, A.L., 2001. Tuberculosis in kudus (Tragelaphus strepsiceros) in the Kruger National Park. The Onderstepoort journal of veterinary research, 68(3), p.225.
52. Keet, D.F., Kriek, N.P.J., Bengis, R.G., Grobler, D.G. and Michel, A., 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. The Onderstepoort journal of veterinary research, 67(2), p.115.
53. Keet, D.F., Michel, A. and Meltzer, D.G.A., 2000, September. Tuberculosis in free-ranging lions (Panthera leo) in the Kruger National Park. In Proceedings of the South African Veterinary Association Biennial Congress, Durban, Kwazulu-Natal (Vol. 20, No. 0, p. 0).
54. Keet, D.F., Michel, A.L., Bengis, R.G., Becker, P., Van Dyk, D.S., Van Vuuren, M., Rutten, V.P.M.G. and Penzhorn, B.L., 2010. Intradermal tuberculin testing of wild African lions (Panthera leo) naturally exposed to infection with Mycobacterium bovis. Veterinary microbiology, 144(3), pp.384-391.
55. Kirberger, R.M., Keet, D.F. and Wagner, W.M., 2006. Radiologic abnormalities of the appendicular skeleton of the lion (panthera leo): incidental findings and

mycobacterium bovis- induced changes. Veterinary Radiology & Ultrasound, 47(2), pp.145-152.

56. Kohn, B., 1994. Zoo animal welfare. Rev. Sci. Tech. 3, 233–245.
57. Kriek, N.P.J., Keet, D.F., Raath, J.P., De Vos, V., Huchzermeyer, H.F. and Bengis, R.G., 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (Syncerus caffer-Sparrman) population in the Kruger National Park: a preliminary report.
58. Kriek, N.P.J., Keet, D.F., Raath, J.P., De Vos, V., Huchzermeyer, H.F. and Bengis, R.G., 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (Syncerus caffer-Sparrman) population in the Kruger National Park: a preliminary report.
59. Liebana, E., Johnson, L., Gough, J., Durr, P., Jahans, K., Clifton-Hadley, R., Spencer, Y., Hewinson, R.G. and Downs, S.H., 2008. Pathology of naturally occurring bovine tuberculosis in England and Wales. The Veterinary Journal, 176(3), pp.354-360.
60. Lightbody, K.A, McNair, J, Neill, S.D, Pollock, J,M. IgG isotype antibody responses to epitopes of the Mycobacterium bovis protein MPB70 in immunised and in tuberculin skin test reactor cattle. Vet Microbiol 2000; 75: 177–88.
61. Lilenbaum, W.; Schettinni, J.; Souza, G.N.; Ribeiro, E.R.; Moreira, E.C. 1999. Comparison between a gama- IFN assay kit and intradermal tuberculin test for the diagnosis of bovine tuberculosis on field trials in Brazil. J. Vet. Med. 46 (5), 353-358.
62. Lilenbaum, W., Ribeiro, E.R., Souza, G.N., Moreira, E.C., Fonseca, L.S., Ferreira, M.A.S. and Schettini, J., 1999. Evaluation of an ELISA-PPD for the diagnosis of bovine tuberculosis in field trials in Brazil. Research in veterinary science, 66(3), pp.191-195.
63. Lilenbaum, W., Pessolani, M.C.V. and Fonseca, L.S., 2001. The use of Ag85 complex as antigen in ELISA for the diagnosis of bovine tuberculosis in dairy cows in Brazil. Zoonoses and Public Health, 48(3), pp.161-166.

64. Lilenbaum, W. and de Souza Fonseca, L., 2006. The use of ELISA as a complementary tool for bovine tuberculosis control in Brazil. Brazilian Journal of Veterinary Research and Animal Science.
65. Liu, S., Guo, S., Wang, C., Shao, M., Zhang, X., Guo, Y. and Gong, Q., 2007. A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis. Tuberculosis, 87(3), pp.212-217.
66. Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Chambers, M.A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B.M., Jackson, R. and O'brien, D.J., 2008. Animal-side serologic assay for rapid detection of Mycobacterium bovis infection in multiple species of free-ranging wildlife. Veterinary microbiology, 132(3), pp.283-292.
67. Maas, M., Keet, D.F., Rutten, V.P., Heesterbeek, J.A.P. and Nielen, M., 2012. Assessing the impact of feline immunodeficiency virus and bovine tuberculosis co-infection in African lions. Proceedings of the Royal Society of London B: Biological Sciences, 279(1745), pp.4206-4214.
68. Maas, M., van Kooten, P.J.S., Schreuder, J., Morar, D., Tijhaar, E., Michel, A.L. and Rutten, V.P.M.G., 2012. Development of a lion-specific interferon-gamma assay. Veterinary immunology and immunopathology, 149(3), pp.292-297.
69. McNair, J., Corbett, D.M., Girvin, R.M., Mackie, D.P. and Pollock, J.M., 2001. Characterization of the early antibody response in bovine tuberculosis: MPB83 is an early target with diagnostic potential. Scandinavian journal of immunology, 53(4), pp.365-371.
70. Michel, A. L. 2002. Implications of Tuberculosis in African Wildlife and Livestock. Annals of the New York Academy of Sciences, 969: 251–255. doi:10.1111/j.1749-6632.2002.tb04387.
71. Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., De Klerk, L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P. and Godfroid, J., 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. Veterinary microbiology, 112(2), pp.91-100.
72. Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., De Klerk, L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P. and Godfroid, J., 2006. Wildlife

tuberculosis in South African conservation areas: implications and challenges. Veterinary microbiology, 112(2), pp.91-100.

73. Michel,A.L., Coetzee, M.L., Keet, D.F., Maré, L., Warren, R., Cooper, D., Bengis, R.G., Kremer, K., van Helden, P., 2009. Molecular epidemiology of Mycobacterium bovis isolates from free-ranging wildlife in South African game reserves, In Veterinary Microbiology, Volume 133, Issue 4, Pages 335-343.
74. Michel, A.L., Hlokwe, T.M., Espie, I.W., Zijll Langhout, M., Koeppel, K. and Lane, E., 2013. Mycobacterium tuberculosis at the human/wildlife interface in a high TB burden country. Transboundary and emerging diseases, 60(s1), pp.46-52.
75. Miller, M., Joubert, J., Mathebula, N., De Klerk-Lorist, L.M., Lyashchenko, K.P., Bengis, R., van Helden, P., Hofmeyr, M., Olea-Popelka, F., Greenwald, R. and Esfandiari, J., 2012. Detection of antibodies to tuberculosis antigens in free-ranging lions (Panthera leo) infected with Mycobacterium bovis in Kruger National Park, South Africa. Journal of Zoo and Wildlife Medicine, 43(2), pp.317-323.
76. Miriam Maas, Ildiko Van Rhijn, Maria T.E.P. Allsopp, Victor P.M.G. Rutten, Lion (Panthera leo) and cheetah (Acinonyx jubatus) IFN-γ sequences, In Veterinary Immunology and Immunopathology, Volume 134, Issues 3–4, 2010, Pages 296-298.
77. Montali, R.J., Mikota, S.K. and Cheng, L.I., 2001. Mycobacterium tuberculosis in zoo and wildlife species. Revue scientifique et technique (International Office of Epizootics), 20(1), pp.291-303.
78. Morar, D., Tijhaar, E., Negrea, A., Hendriks, J., Van Haarlem, D., Godfroid, J., Michel, A.L. and Rutten, V.P.M.G., 2007. Cloning, sequencing and expression of white rhinoceros (Ceratotherium simum) interferon-gamma (IFN-γ) and the production of rhinoceros IFN-γ specific antibodies. Veterinary immunology and immunopathology, 115(1), pp.146-154.
79. Morris, P.J., Thoen, C.O. and Legendre, A.M., 1996. Pulmonary tuberculosis in an African lion (Panthera leo). Journal of Zoo and Wildlife Medicine, pp.392-396.

80. Muller, B. and Van Helden, P.D., 2010. Mycobacterium bovis at the animal–human interface: A problem, or not?
81. Neill, S.D., Bryson, D.G. and Pollock, J.M., 2001. Pathogenesis of tuberculosis in cattle. Tuberculosis, 81(1-2), pp.79-86.
82. Neill, S.D., Cassidy, J., Hanna, J., Mackie, D.P., Pollock, J.M., Clements, A., Walton, E. and Bryson, D.G., 1994. Detection of Mycobacterium bovis infection in skin test-negative cattle with an assay for bovine interferon-gamma. The Veterinary Record, 135(6), pp.134-135.
83. Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C. and Engelhardt, H., 2010. Mycobacterial outer membranes: in search of proteins. Trends in microbiology, 18(3), pp.109-116.
84. OIE, World Organization for Animal Health. Terrestrial Manual, (2008). http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.07_BOVINE_TB.pdf. Accessed 11 May 2009
85. OIE Terrestrial Manual. 2012: Chapter 1.1.5: Principles and methods of validation of diagnostic assays for infectious diseases. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.05_VALIDATION.pdf (accessed February 01, 2013).
86. Palmer, M.V., Waters, W.R., Thacker, T.C., Greenwald, R., Esfandiari, J. and Lyashchenko, K.P., 2006. Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with Mycobacterium bovis. Clinical and Vaccine Immunology, 13(3), pp.387-394.
87. Poester, F.P., Figueiredo, V.C.F.D., Lôbo, J.R., Gonçalves, V.S.P., Lage, A.P., Roxo, E., Mota, P.M.P.C., Müller, E.E. and Ferreira Neto, J.S., 2009. Estudos de prevalência da brucelose bovina no âmbito do Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose: Introdução.
88. Pollock, J.M. and Neill, S.D., 2002. Mycobacterium bovis infection and tuberculosis in cattle. The Veterinary Journal, 163(2), pp.115-127.
89. Pollock, J.M., McNair, J., Welsh, M.D., Girvin, R.M., Kennedy, H.E., Mackie, D.P., Neill, S.D., 2001. Immune responses in bovine tuberculosis, In Tuberculosis, Volume 81, Issues 1–2, 2001, Pages 103-107, ISSN 1472-9792.

90. Pollock, J.M., Welsh, M.D. and McNair, J., 2005. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Veterinary immunology and immunopathology, 108(1), pp.37-43.
91. Pollock, J.M., Rodgers, J.D., Welsh, M.D. and McNair, J., 2006. Pathogenesis of bovine tuberculosis: the role of experimental models of infection. Veterinary microbiology, 112(2), pp.141-150.
92. Pritchard DG (1988). A century of bovine tuberculosis 1888-1988, conquest and controversy. Journal of Comparative Pathology, 99: 357-399.
93. Radford, A.J., Wood, P.R., Jacobe, H.B., Geysen, H.M., Mason, T.J. and Tribbick, G., 1990. Epitope mapping of the Mycobacterium bovis secretory protein MPB70 using overlapping peptide analysis. Microbiology, 136(2), pp.265-272.
94. Rhodes, S.G., Gruffydd-Jones, T., Gunn-Moore, D. and Jahans, K., 2008. Adaptation of IFN-gamma ELISA and ELISPOT tests for feline tuberculosis. Veterinary immunology and immunopathology, 124(3), pp.379-384.
95. Rhodes, S.G., Gunn-Mooore, D., Boschiroli, M.L., Schiller, I., Esfandiari, J., Greenwald, R. and Lyashchenko, K.P., 2011. Comparative study of IFNγ and antibody tests for feline tuberculosis. Veterinary immunology and immunopathology, 144(1), pp.129-134.
96. Ritacco, V., Barrera, L., Nader, A., Bernardelli, A., Torrea, G., Errico, F. and Fliess, E., 1987. Assessment of the sensitivity and specificity of Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of mycobacterial antibodies in bovine tuberculosis. Zoonoses and Public Health, 34(1- 10), pp.119-125.
97. Robinson, E.M., 1953. Journal Of The South African Veterinary Medical Association Vol-XXIV.
98. Rothel, J.S., Jones, S.L., Corner, L.A., Cox, J.C. and Wood, P.R., 1992. The gamma- interferon assay for diagnosis of bovine tuberculosis in cattle:

conditions affecting the production of gamma- interferon in whole blood culture. Australian Veterinary Journal, 69(1), pp.1-4.

99. Ryan, K.J. and Ray, C.G., 2004. Medical microbiology. McGraw Hill, 4, p.370.
100. Salfinger, M. and Pfyffer, G.E., 1994. The new diagnostic mycobacteriology laboratory. European Journal of Clinical Microbiology and Infectious Diseases, 13(11), pp.961-979.
101. Schiller, I., Oesch, B., Vordermeier, H.M., Palmer, M.V., Harris, B.N., Orloski, K.A., Buddle, B.M., Thacker, T.C., Lyashchenko, K.P. and Waters, W.R., 2010. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transboundary and emerging diseases, 57(4), pp.205-220.
102. Silbereisen, A., 2015. Monoclonal antibodies as tools in antigen detection assay and vaccine development: design of a sensitive detection test for Brucella bacteria and profiling of the malaria vaccine candidate antigen reticulocyte-binding homolog 2 (PfRH2) (Doctoral dissertation, University_of_Basel).
103. Stärk, K.D., 2013. First reported case of fatal tuberculosis in a wild African elephant with past human–wildlife contact. Epidemiology & Infection, 141(7), pp.1476-1480.
104. Thoen C. 1994. Tuberculosis in Wild and Domestic Mammals, p 157-162. In Bloom B (ed), Tuberculosis. ASM Press, Washington, DC. doi: 10.1128/9781555818357.ch11.
105. Thoen, C.O., Richards, W.D. and Jarnagin, J.L., 1977. Mycobacteria isolated from exotic animals. Journal of the American Veterinary Medical Association, 170(9), pp.987-990.
106. Thorel, M.F., 1994. Le role du laboratoire dans le contrôle de la tuberculose chez les animaux. Le Point Vet, 26(159), pp.35-40.
107. Trinkel, M., Cooper, D., Packer, C. and Slotow, R., 2011. Inbreeding depression increases susceptibility to bovine tuberculosis in lions: an experimental test using an inbred–outbred contrast through translocation. Journal of Wildlife Diseases, 47(3), pp.494-500.
108. Anonymous 1997

109. Wards, B.J., Collins, D.M. and De Lisle, G.W., 1995. Detection of Mycobacterium bovis in tissues by polymerase chain reaction. Veterinary microbiology, 43(2-3), pp.227-240.

110. Wards, B.J., Collins, D.M. and De Lisle, G.W., 1995. Detection of Mycobacterium bovis in tissues by polymerase chain reaction. Veterinary microbiology, 43(2-3), pp.227-240.

111. Waters, W.R., Palmer, M.V., Bannantine, J.P., Greenwald, R., Esfandiari, J., Andersen, P., McNair, J., Pollock, J.M. and Lyashchenko, K.P., 2005. Antibody responses in reindeer (Rangifer tarandus) infected with Mycobacterium bovis. Clinical and diagnostic laboratory immunology, 12(6), pp.727-735.

112. Waters, W.R., Palmer, M.V., Thacker, T.C., Bannantine, J.P., Vordermeier, H.M., Hewinson, R.G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J.M. and Andersen, P., 2006. Early antibody responses to experimental Mycobacterium bovis infection of cattle. Clinical and Vaccine Immunology, 13(6), pp.648-654.

113. Whipple DL, Bolin CA, Davis AJ, Jarnagin JL, Johnson DC, Nabors RS, Payeur JB, Saari DA, Wilson AJ, Wolf MM. Comparison of the sensitivity of the caudal fold skin test and a commercial gamma-interferon assay for diagnosis of bovine tuberculosis. Am J Vet Res. 1995 Apr; 56(4) 415-419. PMID: 7785813.

114. Widdison, S., Schreuder, L.J., Villarreal- Ramos, B., Howard, C.J., Watson, M.T.J.C. and Coffey, T.J., 2006. Cytokine expression profiles of bovine lymph nodes: effects of Mycobacterium bovis infection and bacille Calmette–Guérin vaccination. Clinical & Experimental Immunology, 144(2), pp.281-289.

115. Wiker, H.G. and Harboe, M., 1992. The antigen 85 complex: a major secretion product of Mycobacterium tuberculosis. Microbiological reviews, 56(4), pp.648-661.

116. Wiker, H. G., K. P. Lyashchenko, A. M. Aksoy, K. A. Lightbody, J. M. Pollock, S. V. Komissarenko, S. O. Bobrovnik, I. N. Kolesnikova, L. O. Mykhalsky, M. L. Gennaro, and M. Harboe. 1998. Immunochemical characterization of the MPB70/80 and MPB83 proteins of Mycobacterium bovis. Infect. Immun. 66:1445-1452.

117. Wiker, H.G., 2009. MPB70 and MPB83–major antigens of Mycobacterium bovis. Scandinavian journal of immunology, 69(6), pp.492-499.

118. Wood, P.R. and Rothel, J.S., 1994. In vitro immunodiagnostic assays for bovine tuberculosis. Veterinary microbiology, 40(1-2), pp.125-135.

119. Wood, P.R., Corner, L.A. and Plackett, P., 1990. Development of a simple, rapid in vitro cellular assay for bovine tuberculosis based on the production of gamma interferon. Research in veterinary science, 49(1), pp.46-49.

120. Young, J.S., Gormley, E. and Wellington, E.M., 2005. Molecular detection of Mycobacterium bovis and Mycobacterium bovis BCG (Pasteur) in soil. Applied and environmental microbiology, 71(4), pp.1946-1952.