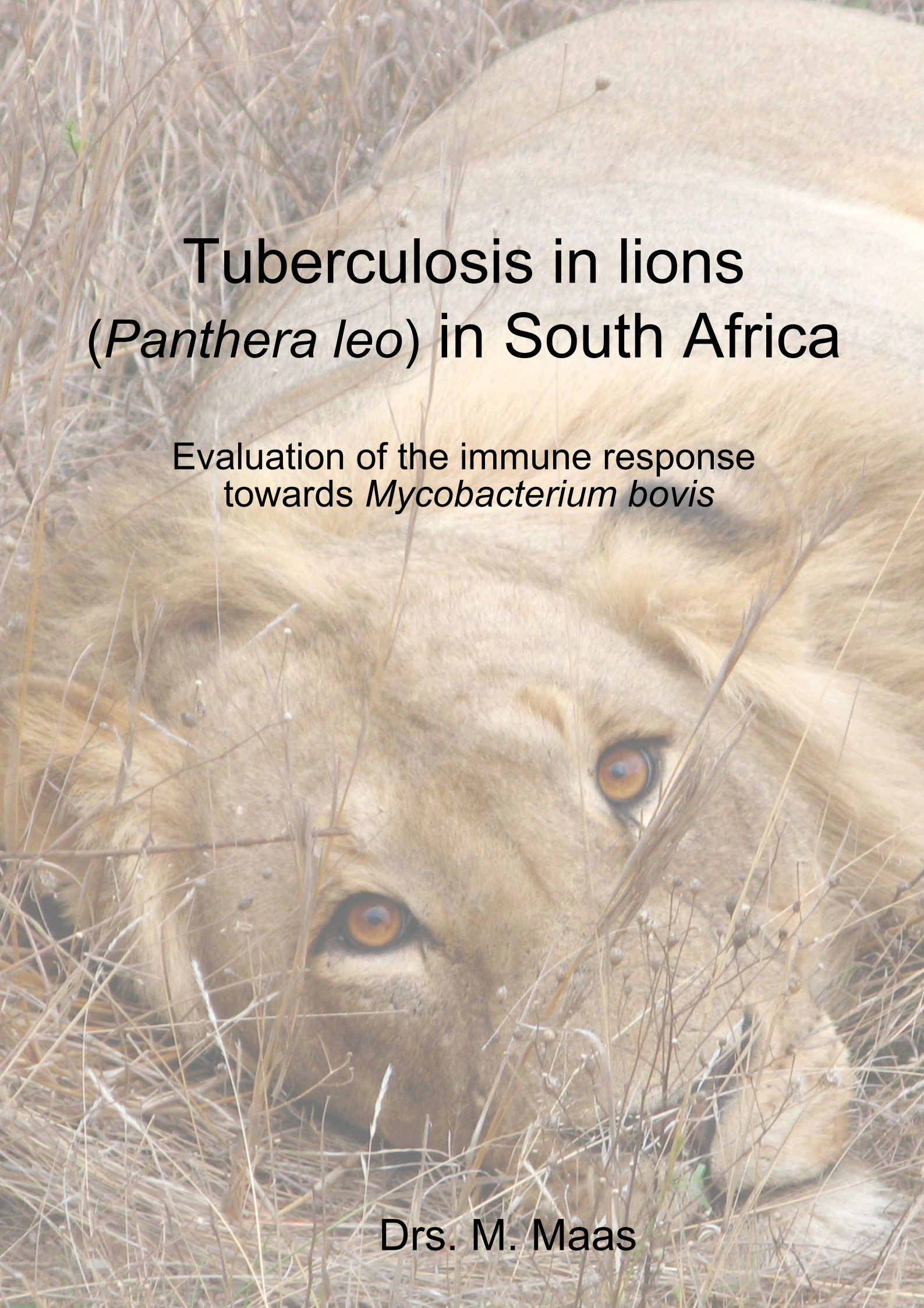

Tuberculosis in lions (*Panthera leo*) in South Africa

Evaluation of the immune response towards *Mycobacterium bovis*

Drs. M. Maas

Universiteit Utrecht
Faculty of Veterinary Medicine

University of Pretoria

An Excellent Track Research on

Tuberculosis in lions (*Panthera leo*) in South Africa

Evaluation of the immune response towards *Mycobacterium bovis*

by

Drs. Miriam Maas

Division of Immunology, Department of Infectious Diseases and Immunology -
Faculty of Veterinary Medicine, University of Utrecht, the Netherlands

Department of Veterinary Tropical Diseases -
Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

Research title: Tuberculosis in lions (*Panthera leo*) in South Africa – Evaluation of the immune response towards *Mycobacterium bovis*

Research conducted as an Excellent Track Research

Drs. Miriam Maas (M.Maas3@students.uu.nl)
Kwartelstraat 12bis, 3514 EV Utrecht, The Netherlands
tel. nr.: ++31 6 48 3138 71

Under the joint supervision of
Prof. Dr. V.P.M.G. Rutten (v.rutten@uu.nl)
Department of Infectious Diseases and Immunology - Division of Immunology,
Faculty of Veterinary Medicine, University of Utrecht, Utrecht
The Netherlands
And
Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science, University of Pretoria, Onderstepoort
South Africa

Prof. Dr. J. Godfroid (jacques.godfroid@veths.no)
Department of Food Safety and Infection Biology,
Norwegian School of Veterinary Science, Tromsø
Norway
And
Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science, University of Pretoria, Onderstepoort
South Africa

Prof. Dr. J.A.P. Heesterbeek (J.A.P.Heesterbeek@uu.nl)
Department of Farm Animal Health - Theoretical Epidemiology, Faculty of Veterinary Medicine Utrecht
Faculty of Veterinary Medicine, University of Utrecht, Utrecht
The Netherlands

Dr. D.F. Keet (dewaldkeet@vodamail.co.za)
Chief State Veterinarian, Kruger National Park
National Department of Agriculture, Phalaborwa, South Africa

July 2007- September 2008

Table of contents v

List of abbreviations

Amplification rate (AR)
Bacillus Calmette-Guérin (BCG)
Body condition score (BCS)
Bovine tuberculosis (BTB)
Cell-mediated immunity (CMI)
Concanavalin A (Con-A)
Culture Filtrate Protein 10 (CFP10)
Early Secretory Antigenic Target 6 (ESAT-6)
Enzyme-linked Immunosorbent Assay (ELISA)
False Negative (FN)
False Positive (FP)
Feline immunodeficiency virus (FIV)
Human immunodeficiency virus (HIV)
Interferon-γ IFN-γ
Kruger National Park (KNP)
Lion lentivirus (LLV)
Major Histocompatibility Complex (MHC)
Mycobacterium bovis (*M. bovis*)
Mycobacterium Tuberculosis Complex (MTC)
Optical density (OD)
Peripheral blood mononuclear cells (PBMC)
Polymerase Chain Reaction (PCR)
Purified protein derivatives (PPD)
Avian Purified Protein Derivative (PPD A)
Bovine Purified Protein Derivatives (PPD B)
Single Intradermal Test (SIT)
Single Intradermal Comparative Cervical Tuberculin Test (SICCT)
T-helper 1 (Th1)
True Negative (TN)
True Positives (TP)
Tuberculosis (TB)

Chapter 1

Introduction to *Mycobacterium bovis*

1.1 Introduction to *Mycobacterium bovis*

Mycobacteria are aerobic, non-motile, non-spore forming, straight or slightly curved rods that are 1.5 to 4.0 um long and 0.3 to 0.5 um wide (Coetzer, Tustin 2004). *Mycobacterium bovis* is a member of the Mycobacterium Tuberculosis Complex (MTC), which contains a variety of closely related mycobacteria, including *M. bovis*, *M. tuberculosis*, *M. africanum* and *M. canetti* (Mishra et al. 2005). The genome sequence of *M. bovis* is >99.95 % identical to that of *Mycobacterium tuberculosis*, but deletions have caused a reduced genome size. The *M. bovis* genome AF2122/97 is 4.345.492 base pairs in length, arranged in a single circular chromosome. It has an average G + C content of 65.63% and contains 3.952 genes encoding for proteins (Garnier et al. 2003).

Most mycobacteria are non-harmful bacteria, but several, for example *M. tuberculosis* and *M. bovis*, can cause serious health problems and even death, by resulting in the disease tuberculosis. In 2006, human tuberculosis caused 1.7 million deaths and 9.2 million cases (World Health Organization 2008). *Mycobacterium bovis* is the cause for a worldwide annual loss to agriculture of 3 billion dollar (Garnier et al. 2003).

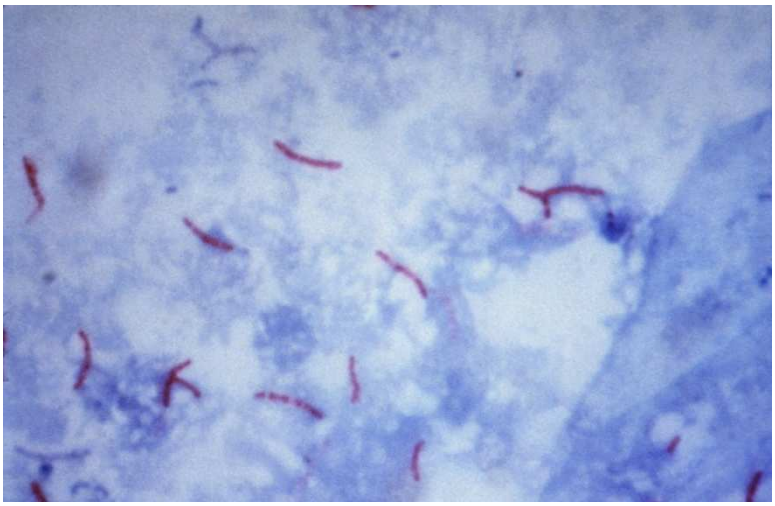

Figure 1.1. Mycobacterium tuberculosis bacteria, acid-fast Ziehl-Neelsen stain; Magnified 1000 X. (Dr. George P. Kubica)

Pathogenesis

Tuberculosis is primarily an infection of the respiratory tract, where the agent uses the macrophage as primary host cell for intracellular replication (Pollock et al. 2006). Aerosol transmission is the most important way mycobacteria are spread for human and cattle. The efficiency of aerosol transmission depends on the size and

consistency of the aerosolized droplets. Fine aerosol suspensions of low viscosity appear to be most effective for delivering mycobacterial content, though only a very small fraction of those droplets contain viable bacilli up to one hour after release (Pollock, Neill 2002). After deposition of bacilli on the respiratory surface, phagocytosis ensures interaction with the innate and acquired immune system. In the first phase of the immune response an innate immune response and a cell-mediated immune response (CMI) take place, in later stages of the infection B-cells get activated and antibody titers increase (Pollock et al. 2006). Infection with mycobacteria such as *M. bovis* results in the formation of granulomas, comprised of a core of infected and killed macrophages surrounded and infiltrated by T-lymphocytes within the lungs and lymph nodes. The granuloma acts to control and restrict the spread of infection, but also results in significant tissue damage (Widdison et al. 2006). The size of the challenge dose not only influences the proportion of the infected animals, but is also positively correlated with the severity of the disease (Pollock, Neill 2002).

Survival in the environment

Several studies have been conducted on the survival of mycobacteria in the environment. Morris et al. (1994) report about various studies that show that sunlight has an indirect effect on survival of *M. bovis* by causing desiccation. Organisms on naturally contaminated material tend to die faster than similar organisms on artificially contaminated material that is otherwise equivalent (Morris, Pfeiffer & Jackson 1994). Coetzer and Tustin (2004) found *M. bovis* can live up to 49 days on grass under moderate conditions (Coetzer, Tustin 2004). Tanner and Michel (1999) performed experiments on the survival of *M. bovis* in different environmental conditions in the Kruger National Park (KNP) and found that *M. bovis* in tissue specimens can survive for a maximum of six weeks in winter under moist and shady conditions. In spiked faeces –experimentally infected faeces– the maximal survival was four weeks, which –as mentioned above– is likely to be shorter in naturally infected faeces. The survival was mainly determined by moisture and temperature. In their study, sunlight was not found to have a limiting effect, if the surrounding environment was moist (Tanner, Michel 1999).

1.2 Tuberculosis in South Africa and the Kruger National Park

Bovine tuberculosis (BTB), caused by *M. bovis*, was most likely introduced to South Africa by the first imported European cattle breeds during the 18th and 19th century. The earliest reports of bovine tuberculosis in wildlife hosts date from 1929 and from that time on it was occasionally diagnosed in wildlife. It was officially first discovered in African buffaloes (*Syncerus caffer*) in the Kruger National Park in 1990. It is suggested that the infection has spread from livestock to the buffaloes in the park, starting in a single entrance point in the south of the park, and spreading progressively northwards, a process continuing today. The regional prevalence of the infection in buffalo in 1998 was 38.2% in the Southern district and 1.5% in the northern district (Keet, Michel & Meltzer 2000). The most recent known (August 2007) percentages of infection are 30-40% infected buffalo in the south of the park, 15-24% in the central area, and 10-16% in the northern area (de Klerk, personal communication). It is likely that these percentages will rise in the near future (Rodwell et al. 2001), probably to an equilibrium. It is unlikely that the infection can be eradicated, because it has become established now in the African buffalo population, a native, free-ranging maintenance host (Michel et al. 2006).

Consequences of bovine tuberculosis in South Africa

The spread of BTB in the KNP has severe (agro-)economical and environmental implications, besides direct health consequences (de Lisle et al. 2002, Kirkberger, Keet & Wagner 2006). In 1969 a national control programme for bovine tuberculosis was developed in South Africa. The presence of bovine tuberculosis in wildlife makes it more difficult to control the disease (Michel et al. 2006). The disease counts for national and international trade restrictions in South Africa, and it is thus of great economical importance. Spill-over to the cattle living in the surrounding area of the park are being minimised by putting big fences around the park, but occasionally these are destroyed by natural disasters and elephants, and greater kudu are able to jump over the fences (Michel 2002). Thus the potential risk of transmission of *M. bovis* to cattle in surrounding farm communities cannot be prevented.

The wildlife-livestock-human interface also causes other concerns, regarding public health. The number of people with human immunodeficiency virus (HIV) in South Africa is increasing considerably (Dorrington et al. 2001), and it has been shown that the risk for humans to get tuberculosis increases greatly in HIV infected people

(Raviglione, Snider & Kochi 1995). Humans are as susceptible to *M. bovis* infection as to *M. tuberculosis* infection, and the course of the infection will be the same once the lesions develop in an organ (Coetzer, Tustin 2004). The risk of contracting zoonotic tuberculosis caused by *M. bovis* is especially present for the people living in the rural communities and who often have close contact with their animals, drinking unpasteurised milk (Michel 2002).

1.3 Development of a wildlife vaccine

Treatment possibilities such as antibody immuno-therapy are mentioned in the literature (Glatman-Freedman, Casadevall 1998), but especially because of the difficulties of delivering these therapies in wildlife, the development of a wildlife vaccine is an important research goal. A lot of research has been done and is still continuing on vaccines, of which an overview is presented here.

BCG vaccination in human and cattle: Protection by vaccination is likely to be achieved by activating macrophages through stimulation of a Th1 type immune response (Buddle et al. 2002). The bacillus Calmette-Guérin (BCG) vaccination has since the 1920s been the only commercially available vaccine against *M. tuberculosis* and *M. bovis* infection. In humans, the efficacy of the vaccine is very variable, protection ranging from 0% to 80%. Trials in cattle showed some reduction in the severity of the disease, but it appeared to be ineffective in field trials as a vaccine against natural infection. It also causes a positive result with the tuberculin skin test, especially when a single cervical or caudal fold test is performed (Buddle, Wedlock & Denis 2006). These characteristics make the BCG vaccination less suitable for use in human and cattle, and BCG vaccination for cattle now occurs only when adjuvantia are added.

DNA vaccines: Recent trials have been performed with DNA vaccine, but this generally produced disappointing results when used on its own in cattle (Buddle, Wedlock & Denis 2006). Maue et al. (2004) improved results by adding co-stimulatory molecules. Protein vaccines, when combined with BCG, can result in strong antigen-specific antibody responses, but so far no sub-unit vaccine has induced strong antigen specific responses and protection against tuberculosis (Maue et al. 2004). The most promising results have been seen when DNA or protein vaccines are combined with BCG vaccination (Buddle, Wedlock & Denis 2006). New

human vaccines are being developed, but to date the BCG vaccination is the only available vaccination (Nol et al. 2008).

BCG vaccination for wildlife: For wildlife, the aim of vaccination would ideally be to eliminate tuberculosis, but a more feasible goal is control of the infection, e.g. slowing down or halting spread of the infection by reducing severity of the disease and bacterial excretion. Cross et al. (2007) wrote an extensive overview of the viability of the use of wildlife vaccines for various major wildlife diseases, including BTB. They conclude that oral vaccination is the most widely used and most feasible approach for wildlife. The diversity of wildlife maintenance hosts for *M. bovis* emphasizes the need for a pleiotropic vaccine. The BCG vaccine has shown to confer a degree of protection against BTB in most studies, and has been shown to be effective as a human oral vaccine as well, which supports its use as a wildlife oral vaccine (Cross, Buddle & Aldwell 2007).

Live vaccination of replicating immunogen would be necessary to generate effective protection. This is essential because of the delivery via the mucosal route (oral) and because of the difficulty of administrating more than a single dose (Buddle, Wedlock & Denis 2006, Cross, Buddle & Aldwell 2007). Live vaccination poses a potential problem, because the immunizing bacilli have to remain viable during oral delivery to the point of immune induction in order to induce a Th1-type immunity (Cross, Buddle & Aldwell 2007). A lipid-based formulation that can serve as a vehicle has been developed that allows passage through the stomach and proximal small intestine, to reach the sites of immune induction in the small intestine, and was tested in brushtail possums (Aldwell et al. 2003). In New Zealand oral vaccinations have been trialed with possums and protection against virulent *M. bovis* challenge was demonstrated in field and captive animals (Corner et al. 2002, Aldwell et al. 2003, Cross, Buddle & Aldwell 2007). In several countries oral-delivered BCG vaccination is considered for a diversity of wildlife species (Cross, Buddle & Aldwell 2007). Studies of vaccination of buffaloes in South Africa have up till now given no significant positive results (Buddle, Wedlock & Denis 2006, De Klerk et al. 2008).

Once a suitable wildlife vaccination has been developed, characteristics of the vaccine, the success of the administration and the infection rate (R_0) will determine what fraction of the population needs to be vaccinated. The smaller the protection of the vaccine for spread of the infection is, the larger the fraction of animals should be

that is vaccinated, to cross the population threshold for spread of the infection (Lloyd-Smith et al. 2005).

Chapter 2

Immune responsiveness in the course of time after *M. bovis* infection: cattle as an example

2.1 Immune responsiveness of cattle in the course of time after *M. bovis* infection

The immune responses in tuberculosis are those aiming to contain an intra-cellular bacterium that does not produce toxins (de Lisle et al. 2002). In the literature there is a general agreement that the cell-mediated immunity (CMI) plays the major role in controlling the infection (Pollock et al. 2001). When an animal is exposed to *M. bovis*, there are several ways in which the infection may develop. The different immune responses can lead to killing of the bacterium, a dormant state of the bacterium and to development of active tuberculosis (Welsh et al. 2005). This is illustrated in figure 1. External factors as poor nutritional status, pregnancy or stress can reduce resistance to bovine tuberculosis, but there is little conclusive information on the impact of these factors on the progression of tuberculosis (Pollock, Neill 2002).

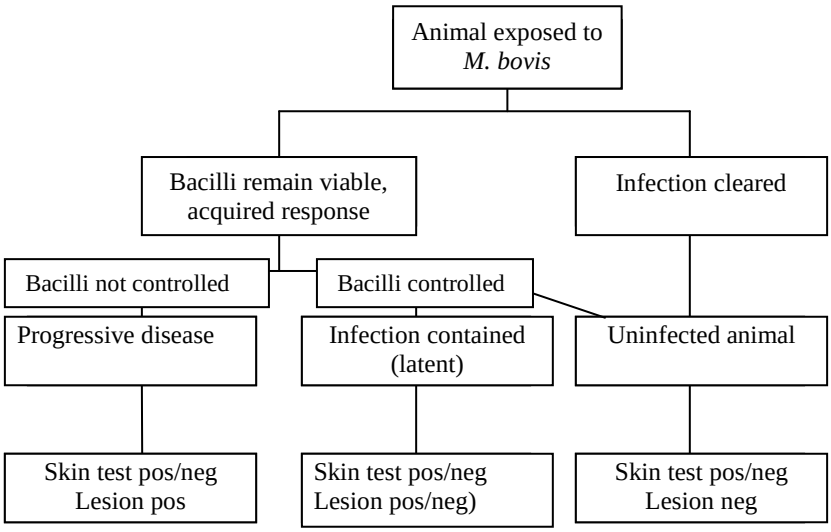

Figure 2.1 Progression of BTB, adapted from Pollock and Neill (Pollock, Neill 2002)

The progression of the infection in the animals depends on the balance between evolving anti-mycobacterial immune responses (Welsh et al. 2005). Pollock and Neill refer to unpublished data that show that the initial challenge dose of *M. bovis* also influences the outcome: higher challenge doses are associated with the cell-mediated immune response that develops within weeks and with rapid generation of circulating antibodies. Lower challenge doses produce a more gradual development of the CMI and little, or no, antibody response (Pollock, Neill 2002).

The macrophage, that “hosts” the bacteria, is the main effector cell for control of mycobacterial infections, but the T-lymphocyte is the major inducer of the protective acquired immune response (Buddle et al. 2002). T-cells can be divided phenotypically into cells expressing the $CD4^+$ marker and cells expressing the $CD8^+$ marker. The first subset is considered to be cytokine-producing helper T-cells and exhibits an antigen response restricted to class II MHC (major histocompatibility complex) molecules, whereas the second subset are cytotoxic cells, antigen-bearing, usually only class I MHC and also able to produce cytokines (Gajewski et al. 1989, Howard, Zwilling 1999).

After infection, first the cell-mediated immunity develops. It is mediated by T-lymphocytes that respond to peptide antigens that are presented by the MHC II present on all cells in the body. The cellular immune response is characterized by $CD4^+$ T-cells, which become activated under the influence of IL-12. $CD4^+$ lymphocytes are divided into two classes according to the type of the cytokines produced: T- helper 1 (Th1) and Th2 lymphocytes (Finkelman et al. 1990, Hernandez-Pando et al. 1996). Th1 cells are Class II MHC restricted and secrete interferon- γ (IFN-γ) and other type 1 cytokines. They are thought to make the major contribution in acquired immunity to tuberculosis, activating macrophages, and are essential for containing the tubercle bacilli (Hernandez-Pando et al. 1996, Buddle et al. 2002). Th2 cells induce B-cell differentiation and antagonize the Th1 response and are associated with the chronic and progressive phase of the infection (Hernandez-Pando et al. 1996). A type 2 cytokine is IL-4 (Finkelman et al. 1990).
In the more advanced, non-infectious, chronic stage of the infection, a subset of Th0 cells arises, that are characterized by the production of both type 1 and type 2 cytokines (Finkelman et al. 1990, Hernandez-Pando et al. 1996, Howard, Zwilling 1998).

Scanga et al. (2000) found an important role for $CD4^+$ T-cells in maintaining a latent infection, though the mechanism was unknown. Depletion of $CD4^+$ T-cells in mice resulted in marked reactivation of *M. tuberculosis*, but the generally believed production of IFN-γ was not the mechanism for this, since IFN-γ was the same level as in control mice (Scanga et al. 2000). Van Pinxteren et al. (2000) on the other side showed results suggesting a predominant role for the IFN-γ-producing $CD4^+$ T-cells

during the acute stage of the infection, and a predominant role for the IFN-γ-producing $CD8^+$ T-cells for prevention of reactivation during the latent phase of the infection (van Pinxteren et al. 2000). Others have described this as well, finding a predominance of MHC I-restricted $CD8^+$ T-cells during the chronic phase and depletion of $CD8^+$ T cells facilitating infection (Hernandez-Pando et al. 1996, Vordermeier et al. 2002). These $CD8^+$ T cells also produce cytokines in a Th1 and Th2-like pattern (Hernandez-Pando et al. 1996), produce IFN-γ during the latent phase (van Pinxteren et al. 2000) and have cytotoxic activities too, though IFN-γ is produced predominantly by the Th1 $CD4^+$cells.

The main stimulators of the humoral immune response that follows the cell-mediated immune response are the Th2-cells. The humoral response is formed by the combined actions of B-lymphocytes and T-lymphocytes. B-lymphocytes present pieces of the antigen with the MHC II molecule, stimulating T-cells to secrete cytokines that stimulate the B-lymphocytes to divide, differentiate and start secreting antibodies.

Howard and Zwilling (1998) performed experimental infections of mice with *M. tuberculosis* and found that T-cells in the lung produced a predominantly type 1 cytokine response, whose appearance coincided with the establishment of latency.
During the period of active growth of the bacillus, not a substantial number of Th0 cells in either the $CD4^+$ or $CD8^+$ population was found. However, by week 12 after infection, at a time when the period of latency begins, significant numbers of Th0 naïve $CD4^+$ and $CD8^+$ cells appeared in the lungs, as did a large number of Th0 $CD4^+$ memory cells (Howard, Zwilling 1998).

2.2 Tuberculosis and cytokine expression

Immunity to mycobacteria is dependent mainly on a cell-mediated immune response involving macrophages, dendritic cells and an adaptive T-cell response. The functions of these cells are modulated by cytokines (Widdison et al. 2006). Cytokines are regulatory proteins, secreted by many different cell types, of which the most prominent are the leukocytes, and play a central role in the immune system by modulating immune responses (Giulietti et al. 2001). The roles played by cytokines vary widely; some are pro-inflammatory, activating cells of the immune system to kill

mycobacteria and inducing a type I immune response, while others, such as IL-4 and IL-10, are anti-inflammatory, down-regulating the pro-inflammatory immune response to control tissue damage. Of the pro-inflammatory cytokines, IFN-γ is considered to be critical for the control of mycobacterial infection, although others such as IL-12, IL-6 and tumour necrosis factor (TNF) also play a significant role.

The helper ($CD4^+$) T-cells are divided into different subpopulations based on differential cytokine profiles. The early phase of the infection is dominated by the Th1 cells, which are characterized by the production of interleukin (IL) 2, IL-12, TNF-α, TNF-β and IFN-γ (Howard, O'Garra 1992). The Th2 cells produce cytokines like IL-4, IL-5, IL-10, transforming growth factor-beta (TGF-β) and other cytokines that promote humoral immune responses (Howard, Zwilling 1999). During the development of the infection, the Th0 subpopulation rises and produces a combination of both Th1 and Th2 cytokines and cytokine secretion is balanced (Hernandez-Pando et al. 1996, Howard, Zwilling 1999). In a successive study from Hernandez-pando, mycobacterial growth was reactivated, resulting in a shift from a type 1 to a type 2 cytokine pattern in both $CD4^+$ and $CD8^+$ T-cells. Control of mycobacterial growth resulted in a return to the type 1 cytokine pattern found during latent infection (Howard, Zwilling 1999).

Elevated levels of mRNA for Th1 cytokines as IFN-γ and the antagonistic splice variant of IL-4δ2 can be found in latently infected healthy humans, who are defined by Demissie et al. (2006) as individuals who respond to *M. tuberculosis* virulence factor ESAT-6, without showing clinical signs of illness (Demissie et al. 2006).
Seah *et al*. (2000) show the principle that in chronic, advanced cases, high amounts of IL-4 mRNA were produced for human TB cases, and finds a larger rise of IL-4 in TB patients than in tuberculin-positive controls (Seah, Scott & Rook 2000).
From human cytokine response studies it can be concluded that in humans infected with *Mycobacterium* the immune response is predominantly of the pro-inflammatory Th1 type, promoting killing of infected cells and intracellular bacteria. A reduction in levels of IL-4 and IL-10 has been demonstrated in patients with TB in comparison to the control group. Although many of the principles established for *M. tuberculosis* will hold for *M. bovis*, there must be differences that affect each species' ability to infect their hosts.

Rhodes *et al*. (2000) found that in cattle experimentally infected with *M. bovis* an early increase of IFN-γ could be measured, that would stay high throughout the study period of 20 weeks. There was a delayed response of IL-4. Both could be used to differentiate infected cattle from uninfected cattle (Rhodes et al. 2000).

Results indicate that the infected lymph nodes of animals challenged with *M. bovis* show suppression of IL-4, IL-6, IL-10 and TNF expression when compared to healthy animals, but no change was seen in the expression levels of IL-12 or IFN-γ (Widdison et al. 2006).

Thacker et al. (2007) performed an experiment on cytokine gene expression in response to *M. bovis* in cattle to identify correlates of immunity. He found that expression of IFN-γ, TNF-α and IL-4 increased in response to infection, whereas IL-10 expression decreased in infected animals compared to the controls. This difference was greatest at 30 days post-infection, and animals that showed many pathological lesions had a more pronounced difference in cytokine expression than animals with less severe pathology. Both Th1 and Th2 type cytokines were expressed at early time points. More robust immunological responses –a high IFN-γ and IL-4 gene expression- in the beginning of the infection were associated with increased pathology. As the infection progressed however, the differences in gene expression between the high and low pathology group were indistinguishable. It suggested that *M. bovis* induces a Th1 response early after infection, with a correlation between Th1 cytokine gene expression and high pathology, though the gene expression waned as infection progressed. IFN-γ and TNF-α expression were positively correlated (Thacker, Palmer & Waters 2007).

In a murine mouse model by Hernandez-Pando, high amounts of IL-4 were produced in chronic, advanced cases. The highest mRNA levels of both IFN-γ and IL-4 were measured at the end of the study at four months of infection, when gross pathology was most severe. Therefore, chronicity and severity are not necessarily associated with a quantitative decline of Th1 cytokines, but could be due to high levels of Th2 cytokines that reduce the functional efficiency of Th1 cytokines (Hernandez-Pando et al. 1996).

Thacker et al. (2007) doubts the often suggested Th1 to Th2 shift as for example described by Hernandez-Pando (1996), that should correlate with increased pathology as infection progresses. He shows that peak expression of IL-4 occurred

30 days after infection and then declined, similar to the Th1 cytokines. The ratio of IFN-γ to IL-4 at the mRNA level did not change as infection progressed during the three months of the study. However, the study period of Thacker was only three months, so the Th1 to Th2 shift could occur after these three months (Thacker, Palmer & Waters 2007).

Cytokines as regulatory proteins

IL-10 is produced by the Th2 $CD4^+$ T-cell subset, as well as by B-cells and macrophages. It suppresses both cytokine production and antigen-specific proliferation of culture clones of Th1 cells by an indirect action on the subpopulation of macrophages, instead of a direct action on the Th1 cells. IL-10 also augments proliferation and differentiation into antibody-secreting cells of activated B-cells (Howard, O'Garra 1992).

IFN-γ has direct effects on B-cells, inhibiting class II expression on B cells (Mond et al. 1986) and it regulates isotype switching on B-cells (Snapper, Paul 1987). IL-10 and IFN-γ are thought to have opposing effects on the development of the immune response: IL-10 inhibits the IFN-γ production by T-cells, changing the response to a humoral response (see figure 2.2.). IFN-γ inhibits the expansion of Th2-like T-cells, and by doing so, eliminates an important source of IL-10 (Farrar, Schreiber 1993).

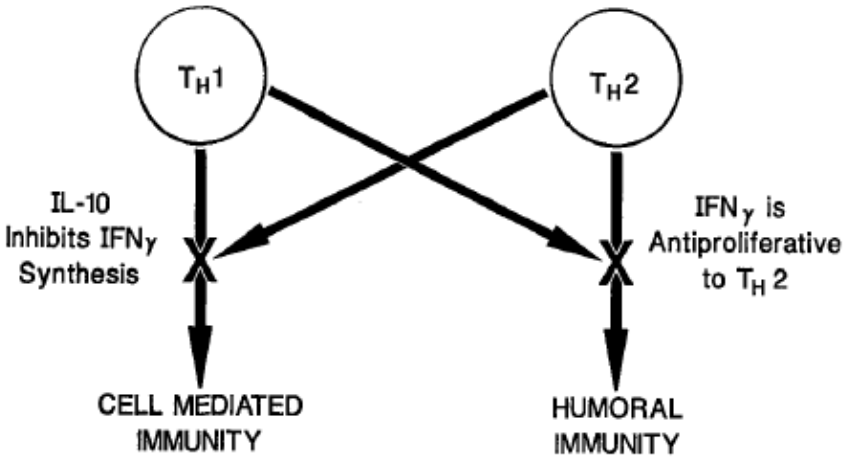

Figure 2.2. The influence of IFN-γ and IL-10 on the development of the immune response (Farrar, Schreiber 1993).

A same opposing relationship exists for IFN-γ and IL-4, where IL-4 induces B-cells to secrete IgG1 and IgE, where IFN-γ inhibits this activity, and the relative amount of IL-4 and IFN-γ thus decide what isotypes of antibody are produced (Finkelman et al. 1990).

2.3 BTB infection kinetics in cattle

Infection of cattle with *M. bovis* is often chronic and can remain subclinical for long periods (de la Rua-Domenech et al. 2006). When cattle is infected, both tests based on the CMI and serology/humoral response (for example Enzyme-linked Immunosorbent Assay (ELISA)) will be negative in the beginning. The CMI that develops most of the times within weeks, can be measured with the IFN-γ assay and the comparative tuberculin skin test. When CMI declines, the responses of the tests will diminish as well, and below the cut-off level, the tests will be negative. This state of the animal is called ‘anergic’ and can also be temporarily induced by stress, for example in cows that have just calved (Pollock, Neill 2002). Gradually the animal develops a humoral response, which means it starts producing antibodies. These can be measured by serologic tests. This is also the period that the animal may start shedding bacteria and that it enters the clinical stage.
This means that in the state of shedding, the ELISA will have a positive outcome, and the CMI is negative. This can also be seen in Figure 2.3. There is an intermediate period that both tests are positive.

Recently, similar infection kinetics have been detected for humans infected with *M. tuberculosis* (see figure 2.4.) (Andersen et al. 2007). In that model, the IFN-γ response is maintained throughout the course of the infection, though it can wane in individuals who develop very severe TB.

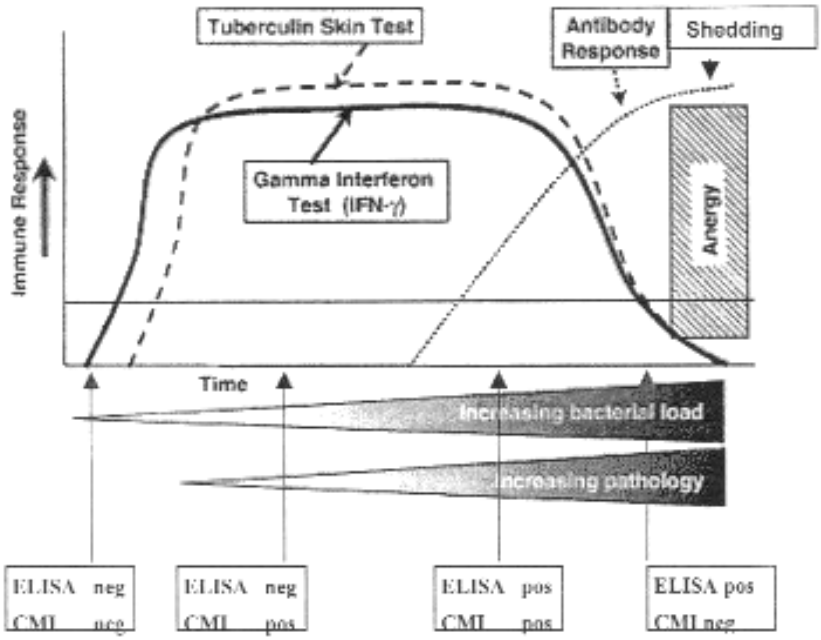

Figure 2.3. Immune response in cattle (de la Rua-Domenech et al. 2006).

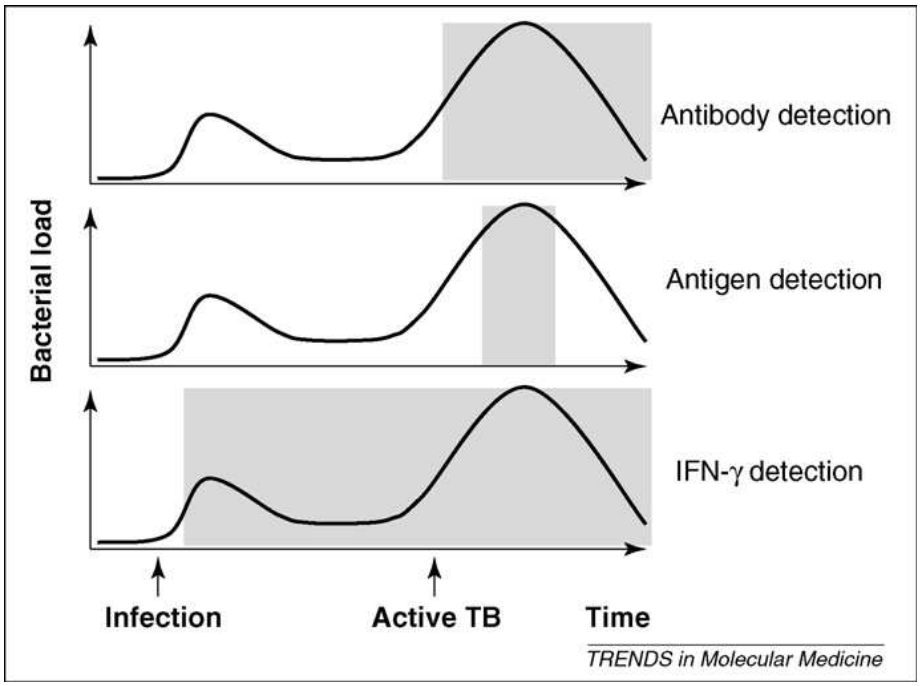

Figure 2.4. Schematic representation of the immune response during the course of human TB infection. The shade areas illustrate when it is possible to detect a response using the given test (Andersen et al. 2007).

Chapter 3

TB diagnostics

3.1 Test characteristics

Diagnostic tests are characterized by their sensitivity and specificity. Test sensitivity is defined as the proportion of infected animals (true positives TP) detected as positive in the diagnostic assay (de la Rua-Domenech et al. 2006) (see table 3.1.). It can be calculated as TP/ (TP+FN) x 100%.

Specificity is defined as the proportion of non-infected animals (true negatives TN) detected as negative in the diagnostic assay. Specificity can be calculated by TN/ (TN+FP) x 100%.

Tabel 3.1. Basic principles of sensitivity and specificity

	Infected	**Non-infected**
Test result positive	True Positive (TP)	False Positive (FP)
Test result negative	False Negative (FN)	True Negative (TN)

Sensitivity and specificity of a test are defined by cut-off levels, which are arbitrary. The values of the sensitivity and specificity have a reverse relationship, so increasing the sensitivity would mean decreasing the specificity and vice versa. Moreover, the values of sensitivity and specificity are not static. Most of the times, the ranges are defined by the normal range containing 95% of the results.

The positive predictive value of the test can be calculated by TP/ (TP+FP) x 100% and defines the proportion of the positive test results that are correct. If cut-off levels are set higher, fewer animals will be false positive, and the positive predictive value will increase. The same can be said the other way around for the negative predictive value. The predictive values are not only heavily influenced by sensitivity and specificity, but are also a function of the prevalence of *M. bovis* in the study population (de la Rua-Domenech et al. 2006).

Ideally a test would have 100% sensitivity and 100% specificity in all stages of an infection/disease, but no such tests exist. For tuberculosis control in cattle the aim is to eliminate all the animals that are carriers of *M. bovis*: i.e. a high sensitivity is needed in order to eliminate all animals. This means, as few as possible false negatives are wanted, and false positives are accepted. For tuberculosis in wildlife the aim is control, since large culling operations are unwanted. For this purpose a high specificity is wanted, to know that animals that are tested positive have a high chance of being really infected, and no false positive animals are culled. The

disadvantage of a high specificity is that more infected animals will be missed, as the cut-off level is raised, i.e. the level of false negatives rises.

3.2 Diagnostic tests according to the OIE

In the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the Office International des Epizooties (OIE) several diagnostic tests are listed that can be used to detect *M. bovis* infection. These are divided in several categories: identification of the agent (for example microscopic examination of the agent of smears, or polymerase chain reaction (PCR) techniques), delayed hypersensitivity tests (skin test) and blood-based laboratory test (IFN-γ test and ELISA for example). For a short version of the list, see table 3.2.

Table 3.2. Summary of official diagnostic methods for *M. bovis*

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition

Diagnostic tests to diagnose *M. bovis* infection:

- Identification of the agent:
  - Microscopic examination of smears stained with Ziehl-Neelsen method, provides presumptive confirmation
  - Isolation of mycobacteria on selective culture media
  - Subsequent identification by cultural and biochemical tests or DNA probe and PCR techniques
  - *Animal inoculation, preferable not done, only when histopathology lesions are compatible with infection, and culture is negative*
- Delayed hypersensitivity test
  - Single intradermal test, SIT
  - Single intradermal comparative cervical tuberculin test, SICCT
- Blood-based laboratory tests
  - Lymphocyte proliferation assay
    - This in-vitro assay is based on the comparison of the reactivity of blood lymphocytes to avian and bovine PPD. Though the test has scientific value, it is time-consuming, logistics and laboratory execution are complicated and expensive and so not used for routine diagnostics.
  - Gamma-interferon assay
  - Enzyme linked immunosorbent assay (ELISA)

As a follow up of gross pathology and histopathology, the gold standard for tuberculosis diagnosis is bacterial culture. A PCR can be used to confirm presence of bacterial DNA. Culture however is a very time consuming method, generally only

used at necropsy, so other options for tuberculosis diagnostics have been and are being investigated.

3.3 Deciding what test to use

In case eradication of the infection is the aim, tests based on the cellular immune response are the most important, since animals can be detected in an early stage. When control is the aim, it is important to identify shedders as early as possible, e.g. the serology is important, because this seems to be related to lesions and shedding. One disadvantage of the ELISA test when using it for detection of *M. bovis* infection in cattle is that many animals don't get to the stage where they show humoral response, because they are detected earlier with tests based on CMI. In wildlife surveillance is less frequently, and infected animals have the chance to reach the Th2 stage – in contrary to cattle that is removed before that stage. Therefore, the chances will be bigger to find animals that are in the humoral response stage, i.e. have a fading CMI response. Since the Kruger National Park has a role in conservation of animals, eradication of the infection will likely be impossible and the main goal thus is to control the infection. Until a proper tuberculosis vaccine for wildlife has been developed, control and surveillance of the infection will be the main infection management tools.

Single blood-based tests are appealing to use for wildlife, since only one handling is necessary, which reduces stress for the animal and reduces chances for injuries (Waters et al. 2005). In vitro cell-based assays like the IFN-γ test seem to provide good results for cattle and buffaloes, but they have the disadvantage that blood needs to be processed within a certain time in circumstances that might not be achievable in the field. This is a disadvantage serology doesn't have, but though serological assays have advantages in terms of logistics, lower cost, and ease of application, to date no assay detecting circulating antibody to *M. bovis* has shown adequate sensitivity or specificity suitable for routine diagnostic use and more information is needed about the antigens recognized by antibodies produced during infection (Cousins, Florisson 2005, Waters et al. 2005). Since with wildlife regular testing is not possible and more animals will be likely to have reached an advanced state with humoral immune response, serology might be useful in wildlife, where the same test would not be useful in regularly tested animals (Pollock et al. 2001).

Identification of *M. bovis*

Microbiology: Ziehl-Neelsen staining

Cell walls of mycobacteria differ from other cells because they contain “waxes”: complex glycolipids and peptidoglycolipids which are characterized by mycolic acids. Using normal dyes, the cell walls would be difficult to stain, but with the Ziehl-Neelsen method staining is possible. The method used is described in the fifth chapter. Failure to see mycobacteria does not rule out infection with mycobacteria. Finding acid-fast bacteria however can give a presumption of infection.

Culture

Bacterial culture is the gold standard for diagnosis of *M. bovis* infection, but has the disadvantage that it takes sometimes up to ten weeks before a positive result shows and has a low sensitivity. All the members of the *M. tuberculosis* complex grow slowly and are non-pigmented. *M. bovis* colonies have a white, flat, smooth and moist appearance and are easily emulsified (Coetzer, Tustin 2004). Culture should be done at 37℃, on a specific enriched medium, such as Löwenstein-Jensen medium.

Polymerase chain reaction

Polymerase chain reaction (PCR) was developed in 1983 and is a technique based on DNA amplification by DNA polymerase. PCR can be performed on a range of samples such as nasal mucus, milk and blood, and on environmental samples. It can be used for a range of biomolecular applications, of which detection of infectious agents and quantifying cytokine gene expression are two. However, of though PCR offers flexibility and speed, PCR is not yet superior to culture in terms of sensitivity, specificity and reliability –there is especially a high rate of false positives because of contaminating DNA. In a study where several laborotories tested the same samples, rates of false-positive PCR results ranged from 3 to 20%, with one extreme value of 77%. The levels of sensitivity also ranged widely: a positive PCR result was reported for 2 to 90% of the samples with 10^3 mycobacteria (Noordhoek et al. 1994). Other problems with the PCR test are low numbers in clinical samples, intermittent shedding, inefficient DNA extraction of mycobacteria or the presence of PCR inhibitors in the samples (de la Rua-Domenech et al. 2006).

PCR is used to amplify specific regions of a DNA strand, which can be a gene, part of a gene or a non-coding sequence. Its main component is the DNA polymerase, which has to be heat stable. One of the often used DNA polymerases is Taq polymerase. This DNA polymerase uses nucleotides to assemble a new DNA strand and also possesses 5'→3' exonuclease activity, which is of importance for using it in the real-time PCR (Holland et al. 1991).
As the reaction continues and more thermal cycles are performed, more template DNA will be available, thus the reaction will amplify exponentially, generating up to millions of copies of the DNA piece.
Besides DNA polymerase, also DNA oligonucleotides, more often called DNA primers, are necessary for the PCR; they have to be specific for a region.

The PCR technique may be used for many biomolecular applications, for example sequencing. It may also be used for cytokine gene expression, starting with mRNA that is reverse transcribed in cDNA, instead of DNA as described above. This may be done with the Quantitative Real-Time PCR (RT qPCR). This can be performed in a so-called two-tube system (with separate tubes for the cDNA synthesis and Real-Time PCR), or in a one-tube system (a single reaction). It is based on fluorescence resonance energy transfer (FRET), where a quencher dye silences a reporter dye (Cardullo et al. 1988, Giulietti et al. 2001), which is illustrated in figure 3.1.

Delayed hypersensitivity

Skin test

From the various tests available for the diagnosis of BTB, the most widely known is the tuberculin skin test. The test makes it possible to diagnose infected animals before they show signs and has been used for many years now.

Tuberculin was developed in 1890 by Koch, called Koch´s old tuberculin, and was a concentrated sterile culture filtrate of tubercle bacilli grown on synthetic media. The tuberculins that are currently used for the skin test are called purified protein derivates (PPD) and are a result of perfection of the old method of tuberculin production. They are prepared from heat-inactivated culture filtrates of mycobacteria but are still poorly defined, complex mixtures of proteins, including a great variety of

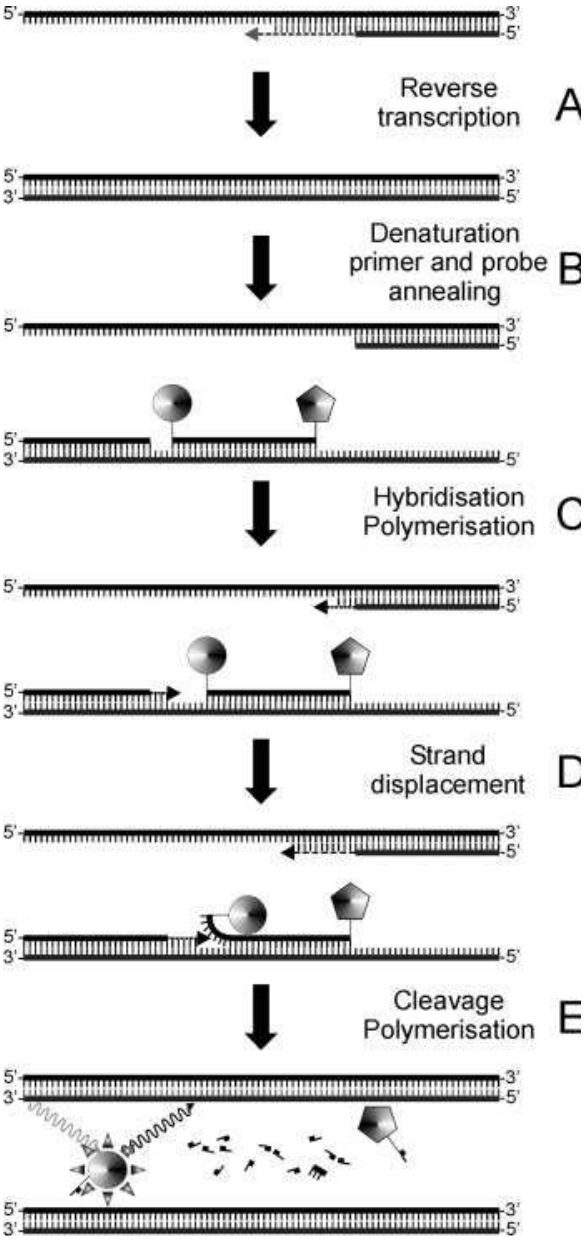

Figure 3.1. The Taqman assay (Bustin 2000).

(A) The RT step synthesises a cDNA copy of the mRNA.

(B) After denaturation, primers and probe anneal. The proximity of the two dyes quenches the signal from the fluorescent dye at the 5' end of the probe.

(C) Polymerisation proceeds at the same temperature as the annealing step.

(D) The polymerase displaces and hydrolyses the labelled probe.

(E) The fluorescent dye is released from its proximity to the quencher, and fluorescence is detected. This signal is directly proportional to the number of molecules present at the end of the previous or beginning of the current cycle.

antigens, many of which are common to several mycobacterial species (Monaghan et al. 1994).
To improve specificity of the PPDs, specific mycobacterial proteins from *M. bovis* and *M. avium* are used in tests to distinguish between infection by *M. bovis* or environmental infections, respectively called PPD B and PPD A (Buddle et al. 2001).

The skin test is based on a delayed-type hypersensitivity response, i.e. CMI (Welsh et al. 2005). After injection of the PPD, a positive reaction will cause an inflammation, and a swelling can be measured 72 hours later (see figure 3.2.). The skin test has two variants: the single intradermal test (SIT), where a small volume of bovine PPD is injected into the skin –most often the mid-cervical or the caudal region (respectively the cervical SIT or the caudal fold SIT, the first one being more sensitive, but less specific than the second), and the single intradermal comparative cervical tuberculin test, (SICCT) where both bovine and avian PPD are injected side-by-side into the skin of the neck (Monaghan et al. 1994, de la Rua-Domenech et al. 2006). Several reviews on sensitivity and specificity of the skin test with cattle have been published and estimates of the sensitivity range from 63.2% to 100% for the cervical and caudal fold SITs with a median value of 83.9%. For the SICCT the sensitivity lies between 52.0% and 100%, with median values of 80.0% for standard interpretation. Sometimes a more severe interpretation is used by lowering the cut-off value, which increases the median value to 93.5%. The specificity of the SIT lies between 75.5 and 99.0% (median of 96.8%) and studies conducted in TB-free cattle populations show that specificity lies between 78.8% and 100% for SICCT (de la Rua-Domenech et al. 2006).

Testing anergic animals lowers the sensitivity, since these are false-negative (Monaghan et al. 1994). Specificity can be reduced by non-specific reactions to non-pathogenic environmental mycobacterial species, even though the specificity improved by using PPD with defined mycobacterial proteins. To reduce this problem, the SICCT can be used, injecting also PPD A, to be able to distinguish between responses to non-harmful environmental mycobacteria and *M. bovis* (Gormley et al. 2006). This is more time-consuming, but a comparison can be made between the two reactions. *M. bovis* infected animals tend to show a greater response to bovine tuberculine than to avian, whereas with infections with other mycobacteria a greater

swelling of the avian injection side is registered (Pollock et al. 2003). Therefore, the SICCT test allows a better discrimination than the SIT between animals that are infected by *M. bovis* or other mycobacteria (Monaghan et al. 1994).

The decision what skin test to use, depends generally on the prevalence of tuberculosis and on the prevalence of exposure to other sensitizing, environmental mycobacteria (Monaghan et al. 1994). In field situations, *M. avium* might be common and reactions to avian PPD might mask the reactions to bovine PPD in the SICCT. This can be solved by ignoring the avian result, or using the SIT, but this will result in a lower specificity (de la Rua-Domenech et al. 2006).

The skin test however is not practical to use for wildlife, because two sedations within a three-day interval are required. Not only is this time-consuming, it causes stress for the animal twice, and it is also not guaranteed that the animals will be re-captured after three days.

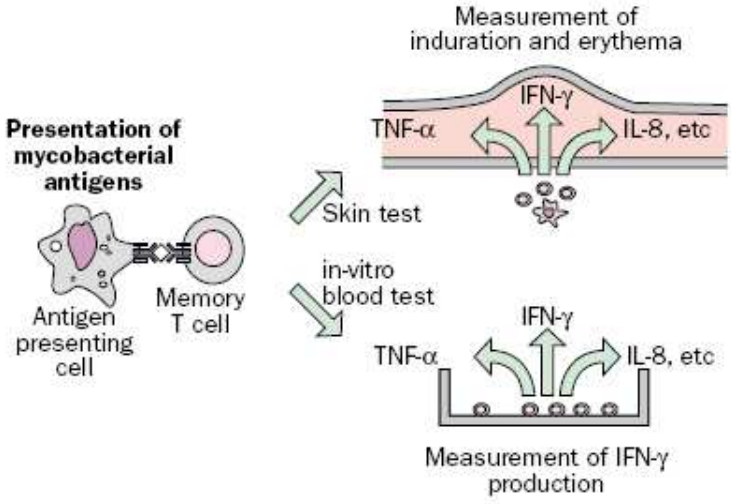

Figure 3.2. Representation of in-vivo and in-vitro diagnostic tests from Andersen, 2000. In the skin test, represented by the upper illustration, antigen-specific lymphocytes infiltrate, producing inflammatory cytokines and causing the typical swelling. In the in-vitro test, shown in the lower illustration, blood or peripheral blood mononuclear cells (PBMC) are stimulated and start producing IFN-γ, which can be measured by ELISA (Andersen et al. 2000).

Blood-based laboratory tests

IFN-γ test

Another test based on the CMI is the IFN-γ test, see figure 3.2.

IFN-γ is a cytokine produced by lymphocytes, and in smaller amounts by natural killer cells, in response to antigenic or mitogenic stimulation, for example in case of infection with intracellular pathogens (Farrar, Schreiber 1993). It stimulates the

macrophages by up-regulation of the MHC-expression, thereby augmenting antigen presentation. It is recognized to have a key role in anti-mycobacterial immunity, and is produced significantly by both $CD4^+$ and $CD8^+$ T-cells (Pollock et al. 2001).

In the IFN-γ test, blood is stimulated and in reaction to avian and bovine PPD or other more specific antigens of *Mycobacterium* spp, sensitized T-cells start producing IFN-γ. The following quantitative measurement of the cytokine is carried out in a sandwich ELISA.

The IFN-γ test is considered at least as sensitive as the skin test and has the advantage that it can detect animals in an earlier stage of the infection than the skin test, where positive results are only seen after one to nine weeks after infection (de la Rua-Domenech et al. 2006). Since the IFN-γ test is based on the CMI response, it has the same time limits as the tuberculin skin test, because the CMI response fades away. In a study performed by Harington et al. (2007) the CMI response decreased at four to six months and IFN-γ assays did not detect BTB infected deer anymore (Harrington et al. 2007).

The most important advantage of the IFN-γ test compared to the skin test for wildlife is that only one capture is needed to know the infection status of an animal, whereas for the skin test two captures within 72 hours are necessary, without guarantee that the same animal can be recaptured. An additional advantage of the IFN-γ test is that it is more likely to be independent of BCG vaccination (Dinnes et al. 2007).

*The principle of the IFN-γ test (capture ELISA):*The plate is coated with capture antibodies specific for IFN-γ. Samples are added from supernatants of lymphocyte cultures stimulated with PPD's or other relevant antigens. IFN-γ in the supernatant will bind to the monoclonal antibodies (capture antibodies), the samples are discarded, after which monoclonal or polyclonal antibodies specific for IFN-γ are added (detecting antibodies). Finally secondary antibodies with specificity for the detection antibodies are added, that will cause the substrate that is added next, to change colour. Colour development indicates presence of IFN-γ or an indication that the animals tested is infected with *M. bovis*. The principle is illustrated in figure 3.3.

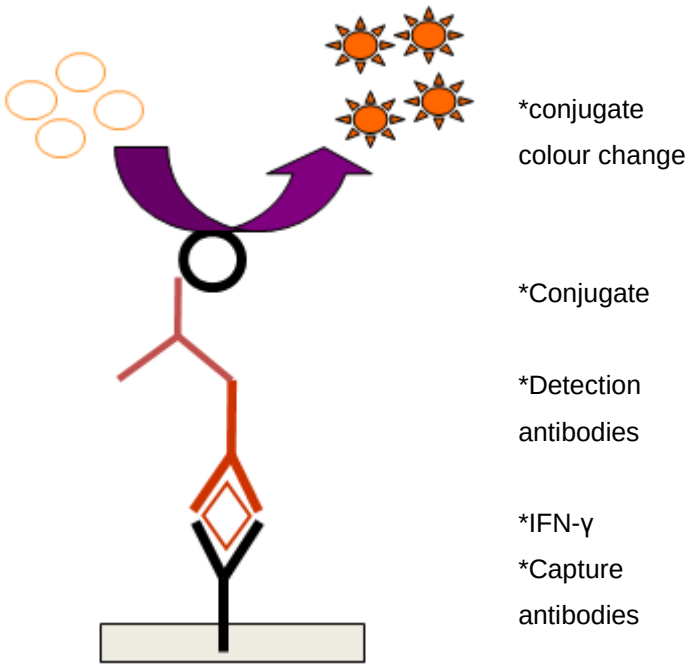

Figure 3.3. Principle of the capture ELISA

ELISA

The most frequently used diagnostic test based on the humoral response is the Enzyme-linked Immunosorbent Assay (ELISA). It can complement the tests based on CMI, and can be helpful in anergic animals, since chronically infected animals gradually loose the capacity to mount CMI.

Tests to detect the infection in an early stage are now based on CMI, because of the poor sensitivity for the early detection of *M. bovis* of antibody based tests. However, Waters *et al*., (2006) found that if sensitive immunoassays are applied in cattle, responses to MPB83 were detectable already after 7 weeks (60% of the experimentally infected cattle) and after 18 weeks 96%. MPB83-specific IgM was detected prior to MPB83-specific IgG detection; however, these early IgM responses rapidly waned, suggesting a benefit of tests that detect both IgM- and IgG-specific antibodies (Waters et al. 2006). Further research has to be done to find out if this will be the same under natural infection circumstances and if results can be extrapolated to lions.

In the UK, extensive research has been done to develop a serology-based diagnostic test, which resulted in the Brock test (a blocking ELISA). This test had a specificity of

98%, but only 37% sensitivity, which was too low to be useful for a control programme based on a test and cull strategy (de Lisle et al. 2002).

The principle of the indirect ELISA (iELISA): The indirect Enzyme Linked Immunosorbent Assay (iELISA) is a serological test that measures quantitatively antibodies in serum samples. The plate is first coated with the desired antigen. Serum of the animals is added; if this serum carries antibodies, these will bind to the antigen in the wells. Conjugate is added and attaches to bound antibodies. It causes the substrate to change colour. The colour development indicates if an animals had antibodies for the tested antigen and can be an indication that the animal is infected with *M. bovis*. Figure 3.4. shows the principle of an iELISA

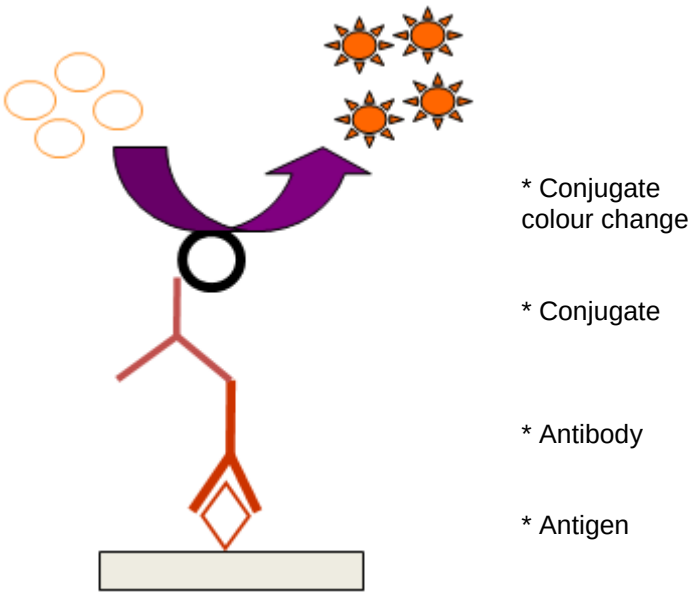

Figure 3.4. Principle of an iELISA

An indirect ELISA has a positive correlation between the amount of antibody and intensity of the colour (Wright et al. 1993).

Use of different antigens in the ELISA

CFP10 and ESAT-6: The antigens CFP10 (culture filtrate protein 10) and ESAT-6 (early secretory antigenic target 6) and the hybrid molecule ESAT-6-CFP10 have given promising results as diagnostic antigens as well (Vordermeier et al. 2001). Mahairas et al. (1996) defined regions of differences (RD) of the *M. tuberculosis* genome that are missing from the BCG vaccine and most environmental

mycobacteria and named them RD-1, RD-2 and RD-3 (Mahairas et al. 1996). The genes for both ESAT-6 and CFP10 can be found in these deleted regions (Andersen et al. 2000). ESAT-6 and CFP10 can be found in *M. tuberculosis, M. africanum* and *M. bovis.* Both are also found in environmental mycobacteria *M. kansasii, M. marinum* and *M. szulgai*, the first two not giving tuberculosis signs and the last one only causing clinical signs similar to tuberculosis in 0.5% of the environmental infections in a study in Denmark (Andersen et al. 2000). Thus no interference with vaccinations is to be expected (Harboe et al. 1996) nor with environmental bacteria usually involved in tuberculosis infection, since these do not contain these antigens.

ESAT-6 is an antigen that can be found in short-term culture filtrates of pathogenic mycobacteria of the *M. tuberculosis* complex (Vordermeier et al. 2001) and is essential for the bacteria to survive and spread in vivo (Demissie et al. 2006). It is highly expressed in early stages of the tuberculosis infection and most human *M. tuberculosis* patients respond to it (Andersen et al. 2000, Demissie et al. 2006). The immune response to the ESAT-6 is known to correlate with the progression and severity of the disease (Demissie et al. 2006).

In humans, a recent comparative study has indicated that –in comparison with the tuberculin skin test– assays based on RD1-specific antigens ESAT-6 or CFP10, correlate better with intensity of exposure than the skin test or PPD-based assays and are therefore more likely to detect latent tuberculosis infection (Andersen et al. 2007, Dinnes et al. 2007).

ESAT-6 shows a great potential for the diagnosis of *M. bovis* infection in cattle as well, especially when combined with other *M. bovis*-specific antigens, for example CFP10 (Andersen et al. 2007). Andersen (2000) refers to unpublished data where sensitivity of 76.3% and specificity of 99.2% are achieved with the ESAT-6 antigen, and in combination with the CFP10 antigen, a sensitivity of 84% is achieved (Andersen et al. 2000).

MPB70 and MPB83: Two of the early antigens with which improvements have been made in the recent past are the recombinant early antigens MPB70 and MPB83. These are both highly expressed in *M. bovis* and minimally expressed in *M.*

tuberculosis in vitro and probably in vivo (Wiker et al. 1998). They are also minimally expressed in BCG strains that have lost the RD2 region, but are major constituents of PPD B. MPB70 is a secreted protein and MPB83 is a cell wall-associated glycolipoprotein (Lyashchenko et al. 2004). Antibody activity against MPB70 is seen in a later stage in assays than antibody activity against ESAT-6-p (Koo et al. 2005).

Purified Protein Derivates (PPDs): PPD's have also been used in ELISAs as the antigen. They do, however, contain components that show much antigenic cross-reactivity with many mycobacterial species. Therefore ELISAs with PPD's have a lower specificity than modern ELISAs with highly specific *M. bovis* antigens like MPB70 and MPB83 (Lightbody et al. 1998) and have not been included in this study.

Chapter 4

Bovine tuberculosis in lions

4.1 Tuberculosis in Felids

Tuberculosis has been found in many Felid species in zoos and in the wild, including lions (*Panthera leo*) (Eulenberger et al. 1992, Keet et al. 1997), Bengal tigers (*P. tigris tigris*) (Eulenberger et al. 1992), 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).

4.2 Spread of BTB to the lions in the Kruger National Park

In 1995, spread of infection to lions (*Panthera leo*) and other wildlife species became evident (Keet et al. 1996). This has direct health consequences for the lions, but also causes a threat to the main tourist attraction of the park (de Lisle et al. 2002, Kirkberger, Keet & Wagner 2006). A correlation between increasing prevalence of BTB in buffalo herds and a decrease in overall body score was found by Caron et al. (2003)(Caron, Cross & Toit 2003). Buffaloes are considered to be one of the four preferential prey species of lions in the KNP and get preyed on significantly more in dry seasons (Keet, Michel & Meltzer 2000). Weak prey animals have been proven to be a likely target for lion predation, because they are easy to kill (Funston 1998). Hence the buffaloes worst affected by the disease are the most likely targets for lion predation (Caron, Cross & Toit 2003). This mechanism has led to a frequent exposure of lions to *M. bovis*. In areas where the prevalence of BTB in buffaloes is high, the infection is also more prevalent in lions. This means prides in the south of the park are more affected than prides in the north of the park. Adult lions are more likely to be infected than sub adults and cubs (Keet, Michel & Meltzer 2000). No precise data is available on the life expectancy once lions are infected, but infected lions tend to die at a younger age than uninfected animals (Keet, personal communication). Since the prevalence of BTB amongst buffalo is still growing, and so is the prevalence for BTB amongst lions, it is very likely that the majority of the Kruger Park lions will be infected in the future (Keet, Michel & Meltzer 2000).

In humans it has been shown that the risk of getting tuberculosis greatly increases in human immunodeficiency virus (HIV) infected people (Raviglione, Snider & Kochi 1995). However, no such correlation has been proven yet for lions with the lion

lentivirus (LLV) infection, a species specific lentivirus very similar to feline immunodeficiency virus (FIV) of the domestic cat (Van Vuuren et al. 2003).

Transmission of M. bovis amongst lions

Though in the past years lions were often labelled as spillover hosts (de Lisle et al. 2002), more evidence suggests that lions are likely to be maintenance hosts, maintaining the infection in their prides in several ways. The social behaviour of lions –sociality, intraspecific aggression and a predilection for hunting buffaloes– promote infection of lions (Keet, Michel & Meltzer 2000). Keet et al. (1997) states that infected animals can infect other members of the group horizontally (Keet et al. 1997). Transmission mutually by lions is most likely predominantly via the aerosol route, but also occurs percutaneously (biting) and the oral transmission takes place when feeding on infected buffaloes (Keet, Michel & Meltzer 2000). During the suffocating when killing buffalo, a lion is prone to inhale a large number of mycobacteria. When feeding later on the carcass the lions are in close proximity and can spread the bacteria by aerosols by heavy breathing and growling (Keet et al. 1997). Eulenberger *et al.* (1992) found that mycobacterium infection of zoo felids that was obtained by eating infected carcasses could cause infection of other animals and cubs through pulmonary lesions and subsequent aerosol transmission. Most common pathology were lung lesions, what points to aerosol transmission as the main route of infection (Eulenberger et al. 1992).

Besides aerosol transmission, infection of very young animals suggest the transmission by milk is also a likely route; young animals have been already found to show severe signs of infection and *M. bovis* has been isolated from the mammary lymph nodes of three lionesses (Keet, Michel & Meltzer 2000). Keet et al. (2000) found the youngest cub responding to be seven months old, and a ten months old cub in poor condition showed signs of advanced tuberculosis (Keet, Michel & Meltzer 2000). This transmission route hasn't been proven yet, however.

Studies with badgers have shown that in that species the most important route of transmission is also via aerosol transmission, and this may already start when cubs are really young, getting the bacteria from their mother (Clifton-Hadley, Wilesmith & Stuart 1993). Fighting seems a notable transmission route as well in male badgers (Cheeseman, Wilesmith & Stuart 1989), and this could also be true for male lions.

Clinical signs and pathology

Descriptions of clinical signs of BTB in lions are not abundant in the literature. Tuberculosis is a chronic infection and in the beginning clinical signs are non-existent, which makes the diagnosis difficult (de Lisle et al. 2002). Lions that are positive for the TB skin test, can often be found to be in good condition.

Eulenberger et al. (1992) describe tuberculosis infection with zoo lions as a process where clinical signs could develop very suddenly after latency. There was an increasing emaciation and lack of willingness to move. After short exercise, the animals showed severe dyspnoea (Eulenberger et al. 1992). Keet et al (2000) mention emaciation, depression, alopecia and unilateral ocular lesions. Also the skin has a dull, rough appearance and dermatitis was seen where lions were bitten. Swollen joints and elbow hygromas are often seen, the latter especially in younger animals (Keet, Michel & Meltzer 2000).

Macroscopic lesions in lions seem to be completely different from the ones described in ungulates and non-human primates and are very difficult to identify, except for the pulmonary lesions. Contrary to the situation in omnivores and herbivores, no abscessation, caseation or calcification is present (de Lisle et al. 2002). Pulmonary lesions were usually seen in more advanced cases and are the only macroscopic lesions that can be called pathognomonic. (Keet, Michel & Meltzer 2000). In cases where the lung changes are most obvious, an aerosol infection is most likely to have been the transmission route (Eulenberger et al. 1992). In tuberculosis cases described by Keet, the lungs showed signs of bronchieactasis and numerous fibrous but fairly thin-walled cavities in the lungs with small quantities of an opaque, greyish-white mucoid exsudate. These do not necessarily need to contain many acid-fast bacilli (Keet et al. 1997). Histopathology showed granulomatous pneumonia, but no necrosis occurred in the inflammatory reaction (Keet, Michel & Meltzer 2000). The multinucleated giant cells that are prominent in the granulomatous reactions of buffaloes, kudus and baboons, were absent from the lesions in lions (Keet et al. 1997).

In a radiologic study performed by Kirkberger (2006), almost half of the tuberculosis positive lions had visible lesions, that were suspected to be caused by *M. bovis*. The osseus lesions were mainly proliferative and were more likely to involve the joints, in particular the tarsal joint (Kirkberger, Keet & Wagner 2006).

Besides the lungs, a variety of organs gets infected, including deep and superficial lymph nodes, mammary lymph nodes, eyes, bones and kidneys, where an amyloidosis was found (Keet et al. 1997, Keet, Michel & Meltzer 2000). Eulenberger et al. (1992) mentions liver and spleen lesions, pleuritis, and intestinal changes – especially in the intestinal lymph nodes were caseation could be noticed. These signs could be signals for infection by eating (Eulenberger et al. 1992).
Advanced lion tuberculosis cases in the Kruger National Park showed anaemia, hypoalbuminaemia and hyperglobulinaemia. The subclinically infected animals had significantly lower albumin and cholesterol, but higher gamma globulin levels, when compared to non-infected animals (Keet, Michel & Meltzer 2000).

The tuberculin skin test in lions

In cats, the tuberculin skin test is considered unreliable (Willemse, Beijer 1979, Tizard 1992, www.merckvetmanual.com). For the use in lions, Keet made adjustments to the test used in cattle. Data collected over the years show a sensitivity of 86% and a specificity of 86% of the single intradermal comparative cervical tuberculin test (SICCT) when only regarding the side where PPD B is injected (Keet, personal communication). Only taking the bovine skin test into account increases the sensitivity of the test, what is regarded as wanted for BTB-infection surveillance (in contrast to BTB control that was mentioned earlier).

BCG vaccination for lions

No large experiments have been undertaken with vaccination of lions. Eulenberger describes the single administration of BCG vaccination to 4 to 6 weeks old lion cups in the Leipzig zoological garden together with taking several management measurements. This caused the number of new cases to drop to zero in the vaccinated lions, but what part of this is caused by the vaccination, is unclear (Eulenberger et al. 1992). As far as we are aware of, no other vaccination trials with lions have been tried.

4.3 Bovine tuberculosis in lion populations in other parks

In November 2000, ministers from Mozambique, South Africa and Zimbabwe signed the agreement of the formation of a large park that links the Limpopo National Park in

Mozambique, the Kruger National Park in South Africa and the Gonarezhou National Park, Manjinji Pan Sanctuary and Malipati Safari Area in Zimbabwe. The total surface area of the transfrontier park will be approximately 35.000km^2, and is just the first phase of creating an even bigger conservation area of 100.000 km^2. The formation is still continuing now, with the increased removing of fences that separated the different parks and countries in the past (www.greatlimpopopark.com). The lion population is small in the adjacent parks, and no surveys have been undertaken to investigate the BTB status of the lions there. No reports have been received of diseased lions (Keet, personal communication). With the removal of the fences though, the spread of buffalo, lions and subsequently BTB will be likely.

Tuberculosis might not only be a problem for the lions in the Kruger National Park and adjacent parks, but also for other nature parks in Africa. Cleaveland et al. (2005) performed a study for the existence of BTB in lions in Northern Tanzania and found 4% of the Serengeti lions infected. This percentage is likely to be higher, due to the low sensitivity of the test used (Cleaveland et al. 2005).

Aims and Outline

The rapid spread of tuberculosis (TB), caused by *Mycobacterium bovis (M. bovis)*, amongst the lion population in the Kruger National Park (KNP) raises concerns about the future of these animals, one of the main tourist attractions of the park. The progression and the severity of the infection are different from those in cattle and buffaloes, the latter species being the main reservoir for tuberculosis in the Kruger National Park. Not only does the spread of tuberculosis cause a direct health risk for all species affected, it is also a threat to the tourist industry of the KNP, and may cause international trade-restrictions because of the wildlife-livestock interface.

M. bovis is a member of the Mycobacterium Tuberculosis Complex, and causes tuberculosis in a vast range of hosts, the most well known being cattle. For more than hundred years, researchers have studied possibilities to diagnose the infection or to develop vaccines. Some diagnostic methods or vaccines were more successful than others, but till today, no reliable vaccine has been developed for either humans or cattle, and the many different diagnostic procedures all have their own advantages and disadvantages. Having so many questions unanswered on the human and cattle side, little effort has been put in investigating the infection in lions yet, even though tuberculosis is a major threat to some lion populations, like the one in the KNP.

The aim of the presented investigations was to develop better insight in the development of the immune response of lions infected with *M. bovis*. We focused on three research areas:

1) Diagnostic methods, that were available or needed to be developed
2) T-cell response/cytokine profiles in various stages of the infection
3) LLV/BTB co-infection in lions

For the first focus, various tests were performed: ELISA (antigens MPB70, MPB83, CFP10 and a ESAT-6-CFP10 hybrid), tuberculin skin test and culture. The multi-species IFN-γ test was optimised for lions.

For the second focus, PCR primers were developed for the following cytokines: IFN-γ, TNF-α, IL-4, and IL-10. A reverse transcriptase Real-Time PCR was used to measure the cytokines produced by Th1 and Th2 cells.

Serum samples were tested for the last focus, to determine whether a relation between LLV and BTB infection or disease status could be found.

The immune responses of cattle and human with tuberculosis were used as guidance and for comparison. It is likely that the development of the immune response in lions will follow the same principle, but this has not been proven yet. Different samples of infected/control animals were tested using different diagnostic approaches for sensitivity and specificity. It was expected that, comparing the results, the study could help defining tuberculosis status parameters, as well as offer a clearer view of the relevance of different tests in the diagnostics of bovine tuberculosis in lions, and how the outcome can be used to control the infection. We aimed at identification of a test able to diagnose lions in an early stage of the infectious stage, i.e. early in the shedding phase.

In the first four chapters of this report a general introduction is given regarding *M. bovis*, the immune response to *M. bovis* in cattle as the paradigm of pathogenesis and a description of the current different tests that are available for *M. bovis* diagnostics. Furthermore, the BTB infection in lions is discussed (Chapter 4).

An overview is given of the sampling in the Kruger National Park of two lion populations. Results of tests that were performed in the field lab are presented and discussed (Chapter 5), after which the results and analysis of the iELISAs are discussed that have been performed with the recombinant antigens MPB83, MPB70, CFP10 and DiaSer3 (Chapter 6). A LLV/PLV ELISA has been performed on the samples collected in the KNP and the results are discussed in correlation with BTB in lions (Chapter 7). The multi-species IFN-γ ELISA developed earlier has been optimized for feline species (Chapter 8) and has been used to test the samples collected in the KNP. For four lion cytokines, IFN-γ, TNF-α, IL-4 and IL-10 a reverse transcriptase Real-Time PCR was developed, and the expression of the genes was compared to housekeeping gene GAPDH (Chapter 9). Finally, the sequencing of the IFN-γ gene for the lion and cheetah are described (Chapter 10).

A general discussion follows is given in the eleventh chapter to combine the findings of all the different *M. bovis* diagnostic approaches and to give advice for future BTB research in lions.

Chapter 5

A survey of lions in the Kruger National Park for infection with *M. bovis*

Introduction

Bovine tuberculosis, caused by *Mycobacterium bovis (M. bovis)*, was most likely introduced in South Africa by the first imported European cattle breeds during the 18th and 19th century. It entered the Kruger National Park (KNP) in the '60s, and in 1995, infection of lions (*Panthera leo*) and other wildlife species became evident (Keet et al. 1996). The spread of tuberculosis in the Kruger National Park, has environmental and (agro-)economical implications, besides direct health consequences (de Lisle et al. 2002, Kirkberger, Keet & Wagner 2006).

To monitor the spread of bovine tuberculosis (BTB) under the lion population in the KNP and determine the consequences on the individual and population level, regular screening of the lions is needed. Therefore, Dr. D. Keet, state veterinarian at the KNP, has conducted several lion surveys in the past ten years, where the same study areas were visited every time.

The aim of joining the present survey was to collect and store samples to be used for testing with various diagnostic tests later on, to perform tracheal flushings for Ziehl-Neelsen staining followed by culture and to get a better insight of the prevalence of BTB amongst lions in the KNP and the consequences of the infection. New serum samples were collected to add to those stored from previous surveys. It was the first time that during a survey whole blood cells were collected for cytokine assessment by PCR. It was also important to obtain more information about skin test results together with results of different diagnostic tests, so information could be combined to make suggestions about the development of the infection.

Material and Methods

The study areas

The fieldwork has been conducted in two areas of the KNP: the area between Crocodile Bridge and Lower Sabie in the southern part of the park –where BTB has a high prevalence in the buffalo population-, and the area north of Shingwedzi, in the northern part of the park, where BTB is less prevalent in buffaloes (figure 5.1.). Both areas were visited in several separate sampling periods (see table 5.2.; for an extensive overview see appendix 1) .

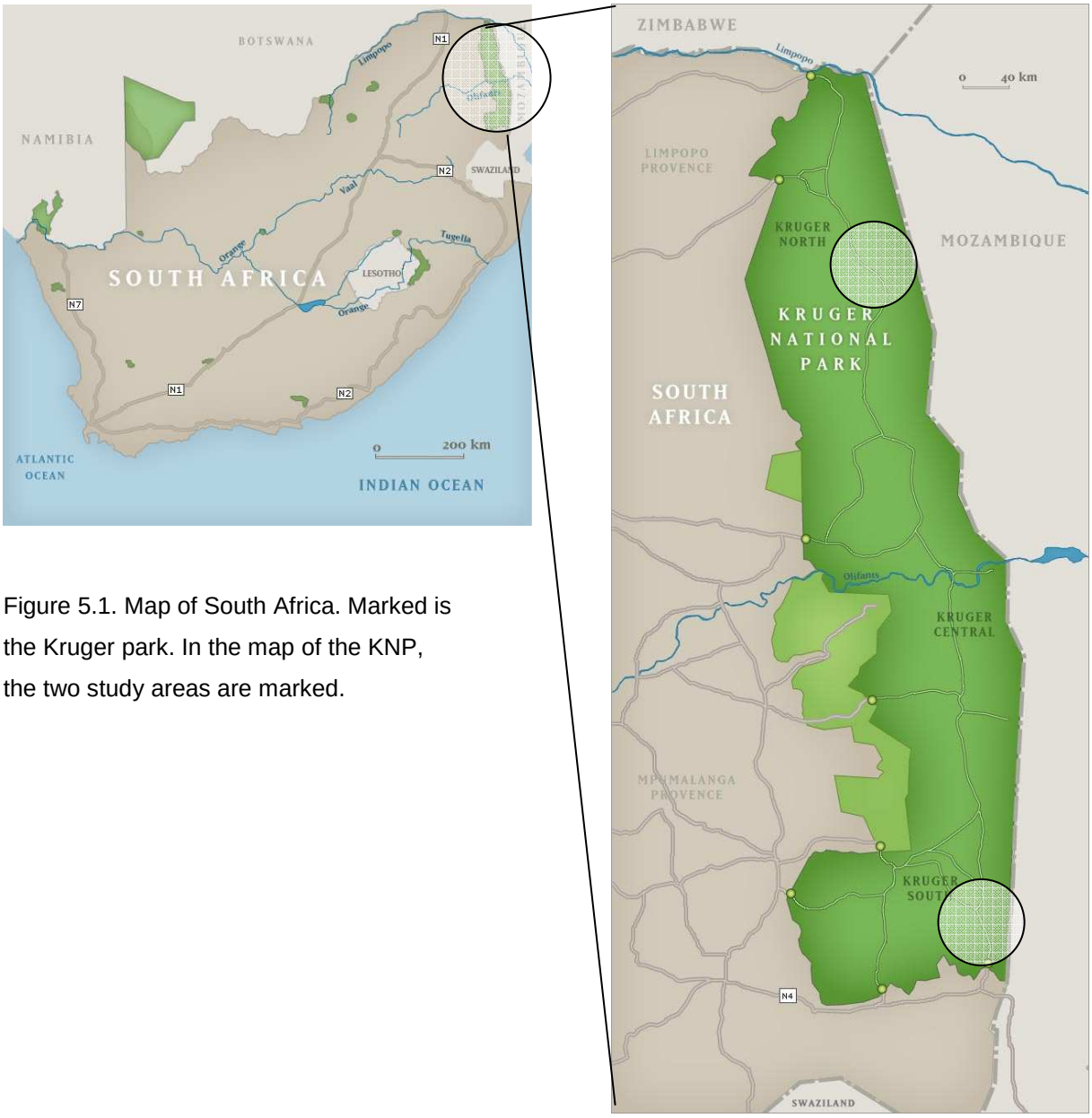

Figure 5.1. Map of South Africa. Marked is the Kruger park. In the map of the KNP, the two study areas are marked.

Sampling

Captures started in the afternoon when lions were called to the capture site by playing sounds of a buffalo calf in distress. Bait, mostly zebra but sometimes buffalo or impala, was positioned +/- 20 m away from the vehicles in such a way that lions were easily darted. Lions were darted with Zoletil® 100 (Tiletamine and Zolazepam mix), using the Dan-Inject® dart gun and Dan-Inject® darts. Darts were aimed at the shoulder or hind-quarters. Subadults received a dosis of 250 mg, adult females 500 mg, and adult males 750 mg. In most cases, the lions were sedated within 15

minutes. Extra doses (`top ups´) were given when necessary for the duration of the sampling.

Check-list for the sampling

* Checking for a microchip and former brands. If there was no evidence of either, an "Identipet" microchip was injected between the shoulder blades/the neck, and the lion was branded with an unobtrusive symbol for identification from a distance
* Data were collected regarding sex, age (determining age was done according to the technique described by Smuts *et al* in 1978 (Smuts, Anderson & Austin 1978)), body condition (scored in a range from 1 to 5, with 1 describing lions in very poor conditions and 5 describing lions in an excellent condition. Lions with clinical signs of BTB –emaciation, elbow hygromas, etc– have lower condition scores.), body measures (total body length, nose to tail, shoulder height, hip height, hind foot from claw to hook, ear length) and weight.
* Lateral photographs of the lions were taken, as well as a frontal photograph of the head
* In the north of the park, a radio transmitter was fitted around the neck of one of the pride members, for easier relocation for the re-capture
* The skin test was performed
* Blood samples were collected in heparin and EDTA tubes (IFN-γ ELISA and cell isolation), as well as clotting tubes (serum collection) from the v. saphena.
* Tracheal flushing was performed on a small number of lions.

After collecting the data, lions were monitored when recovering from the anaesthesia. A state veterinarian stayed with them until they were fully awake.

Samples were kept at temperatures not higher than 20°C (i.e. on hot nights they were kept cool in a cooler box) and were taken to the laboratory in the KNP, where stimulation of the whole blood samples with PPD A and PPD B was started within eight hours, and white blood cells were isolated and stored in RNAlater (a tissue and cell storage reagent that stabilizes and protects cellular RNA). The following day serum was collected and Ziehl-Neelsen staining of the tracheal flushing samples was performed, after which the sample was stored.

Blood samples

Serum blood samples were collected in 10 ml serum tubes. It was left to clot at room temperature for 12-24 hours, and centrifuged at 2500 rpm for ten minutes. Serum was collected and stored at -20℃ until further use .

Heparin blood samples were taken in 10 ml heparinised tubes, and processed in the laboratory in the Kruger National Park within eight hours. The blood was dispensed in 3x1.5 ml aliquots on 24-well plates. One well served as a control (nothing added), one received 30 µl avian PPD and to one 30 µl bovine PPD was added- similar to amounts used in the BOVIGAM® IFN-γ ELISA for cattle (see chapter 8). To check for optimal conditions in one test (27 August) the protocol was slightly adopted and 60 µl of PPD A and PPD B were used in additional wells. Of eight lions from different captures, a second sample has been taken from the animals during the re-capture, to compare the effects of boosting.
At the last sampling period in the northern part of the park, the protocol was adapted: blood was dispensed 1 ml per well, and for both PPD A and PPD B the following amounts were added: 100 µl, 50 µl, 20 µl and 5 µl. 50 µl PBS and a well with nothing extra added served as negative controls. Four wells were stimulated with ESAT-6: 4 µg, 2 µg, 1 µg and 0 µg per well. Blood was incubated as previously: 18-24 hours at 37℃, and supernatant was stored at -20℃ until fur ther use.

An *EDTA* (heparin was used for the first sampling periods in the south) *10 ml blood sample* was taken, and the blood was processed within eight hours. It was centrifuged for 15 minutes at 3200 rpm, after which the buffy coat was collected. 2 ml of erythrocyte cell lysis solution (0.16 M NH_4CL ; 10 mM $KHCO_3$; 0.1 mM Na_2EDTA ; pH 7.4, filter sterilized) were added to the separated buffy coat, and after six minutes the cell lysis was stopped with 25 ml PBS. The cells were centrifuged at 1200 rpm for ten minutes, and the pellet was resuspended in 300-500 ul RNA later, depending on the amount of pellet, and stored at -20℃ until fur ther use.

Skin test

For the skin test, a Modified Comparative Cervical Intradermal test was used, as developed by Dr. D. Keet. On the left and the right side of the neck a rectangular area of approximately 40 mm x 40 mm was shaven with an electrical clipper. During the survey of November 2007, a sharp blade was used. The thickness of the skin was measured with a tuberculosis calliper to the millimetre immediately before injection. A dosis of 6 000 I.U. (0.2 ml) bovine tuberculin (PPD B) (Lelystad) was injected intradermally with a tuberculin syringe and a 23 gauge needle in the centre of the shaved area, on the left hand side of the neck. A dosis of 5 000 I.U. (0.2 ml) of Avian tuberculin (PPD A) (Lelystad) was injected intradermally with a tuberculin syringe and a 23 gauge needle in the centre of the shaved area, on the right hand side of the neck. After 72 hours, the animals were relocated and immobilized again. The thickness of the skin was remeasured with callipers and the swelling was palpated and visually observed. The “severe interpretation” of the skin test is adopted, meaning that an increase of 2.1 mm and more at the bovine injection site is indicative of infection with *M. bovis*, regardless of the avian reaction. Not regarding the side where avian PPD is injected, increases sensitivity of the skin test, but lowers the specificity (de la Rua-Domenech et al. 2006). The avian PPD is still injected however, to be able to determine the difference between the two interpretations, and for possible future use.

Tracheal flushing

Six animals that showed signs of BTB, or that were skin test-positive, were subjected to a tracheal flushing during the first or the second immobilization. A volume of +/- 30 ml was injected deep in the trachea and retrieved immediately, and mostly volumes close to the starting volume were retrieved. It was kept at 20°C or lower (see above) during transportation, and kept in the fridge before further use. The following day the suspension was centrifuged for 10 min at medium speed, and a smear was made from the pellet and coloured with the Ziehl-Neelsen method, described in table 5.1.

Table 5.1. Description of the Ziehl-Neelsen staining, as performed in the Kruger National Park.

Ziehl-Neelsen staining
1. prepare and heat-fix a bacterial smear
2. colour with carbolfuchsin, heat until the dye steams, but take care it doesn´t dry out.
3. rinse with tap water
4. decolourize with acid-alcohol until the smear is light red (+/- 15 sec)
5. rinse with tap water
6. add methylene blue for one minute
7. rinse with water and blow air dry
8. examine under oil immersion objective

The smears were checked for the presence of acid-fast bacteria under the microscope for a minimum of 15 minutes. The remaining pellet was stored in a small volume of PBS in the refrigerator. From October 2007, we changed the storage method by adding glycerol solution. The samples of the tracheal flushings have been cultured at the Onderstepoort Veterinary Institute (OVI).

The Ziehl-Neelsen staining was not possible in the north of the Kruger National Park, since no facilities, nor a microscope were available there to perform the colouring and the microscopy.

Results

Immobilized lions and blood samples

A total of 74 lions has come to the capture sites: 44 in the south of the park and 30 in the north. Samples have been taken of 72 lions; sometimes it was not possible to take all samples of all animals due to circumstances. A complete overview of the animals, capture sites, information about condition etc. can be found in appendix 1 and a short summary can be seen in table 5.3. An overview of the blood samples that have been taken for the various diagnostic tests performed in a later stage can be found in table 5.4.

Table 5.2. Overview of the different captures periods and sites. For an extensive overview –including lions captured at recaptures– see appendix 1.

Date	Capture site	Animals captured
20 August	Crocodile Bridge	8
21 August	Mpanamane	8
21 August	Gomondwane	7
17 September	Powerline Duke Crossing	3
17 September	Nkongoma	6
27 September	Mpanamane	3
15 October	Mhlanganzwane	8
6 November	Nwarihlangary	6
14 November	Boyela	2
20 November	Langtoondam	5
2 June	Nwarihlangari	1
6 June	Stangeni	10
8 June	Stangeni	5

Table 5.3. Short summary of the lion data. The extensive overview in appendix 1 also gives an overview of skin test results with taking PPD A into consideration.

Total number of lions:	**74**
Lions in the south:	44
Lions in the north:	30
Lions with skin test result:	**29**
Lions with positive skin test result:	24; 17 in the south, 7 in the north
Lions with negative skin test result:	5; 1 in the south, 4 in the north

Table 5.4. An overview of the blood samples that have been taken

Serum samples for ELISAs:	**72**
Serum samples in the south:	42
Serum samples in the north:	30
Heparin sample for IFN-γ assay:	**71**
Heparin samples in the south:	42
Heparin samples in the north:	29
EDTA/Heparin sample for RT qPCR:	**62**
EDTA/Heparin samples in the south:	34
EDTA/Heparin samples in the north:	28

Tracheal flushing, Ziehl-Neelsen staining, bacterial culturing

The tracheal flushing was performed on six animals. The pellets of the samples were sent to the OVI for culture after each survey. Cultured samples that showed signs of growth were processed further with PCR techniques to find proof of infection with *M. bovis*. Two lions showed growth of mycobacteria, but PCR showed that these were

environmental mycobacteria. All samples that were cultured, had a negative result for *M. bovis* after 12 weeks. An overview of the six animals, their Ziehl-Neelsen staining, culture and skin test results can be found in table 5.5.

Table 5.5 Overview of the results of the various tests for six lions that had a tracheal flushing performed

Lion number	Tracheal flushing	Ziehl-Neelsen	Culture	Skin test
49297D0A60	Yes	Positive	Negative	Unknown
494A2A7564	Yes	Positive	MOTT	Unknown
*	Yes	Positive	Negative	Positive
4115092830	Yes	Negative	MOTT	Positive
492E456C57	Yes	Not performed	Negative	Positive
492A3D0D17	Yes	Not performed	Negative	Negative

* This was an animal with unknown identity

MOTT= Mycobacteria other than *M. tuberculosis* (=environmental bacteria)

Body condition score (BCS)

The mean of the body condition score of the southern lions was 4.6 (n=38). Two animals scored a BCS=3, one lion scored BCS=3.5 and nine lions scored a BCS=4. The others scored BCS=5.

The mean of the body condition score of the northern lions was 4.8 (n=30). One lion scored BCS=3.5 and four lions scored a BCS=4. The others scored BCS=5.

The mean of the BCS of the skin test positive lions was 4.5 (n=24). The mean of the BCS of the skin test negative lions was 4.8 (n=5).

One of the two lions in the south (lion 456B0D3468) that scored a BCS of 3, had a positive MPB83 and MPB70 ELISA later. See appendix 1 for an overview of the BCS of the other lions.

Discussion

For the present study, only the swelling of the bovine PPD injection was used to classify an animal as skin test positive or negative, as this is the way the skin test is analyzed in the KNP. In the future however, if the prevalence of BTB in the southern and the northern part of the park increases, it would be a possibility to take the

swelling on the avian PPD injection side into account as well, to increase the specificity.
For the northern part of the KNP no case descriptions have been made yet for BTB positive lions. In the present study, seven northern lions tested skin test-positive, though none of them had swelling as large as some of the lions in the south showed (see appendix 1). Five of these animals were immobilized during the survey of November 2007, where a different shaving method was used than the official protocol describes and swelling in these animals could also be caused by inflammation reactions in the damaged skin, that was caused by using a sharp blade instead of an electrical clipper. In retrospective, Dr. D. Keet therefore doubts their positive status, as he also doubts the establishment of the BTB infection in the northern lions in total (personal communication). For the presented study however, animals are classified according to the skin test result and these five lions are therefore classified as skin positive animals in the following chapters.

Lions that had a positive skin test result had a slightly lower BCS than lions with a negative skin test result (resp. 4.5 and 4.8). The number of lions with a negative skin test result (5) was very low, and to show the principle of a lower BCS in infected lions, more negative animals should be included.

It was not possible to process the blood immediately after collection, but this was done within eight hours in the lab. Waters et al. (2007) showed that processing the blood immediately after collection gives the best responses later in the BOVIGAM® test. Responses eight hours after collection were lower than responses of blood processed immediately after collection, but still present. Responses of blood processed eight or 24 hours after collection were comparable. Storage at 4℃ or 22℃ didn't make a difference (Waters et al. 2007).
Reason to change the IFN-γ stimulation protocol was that the initial amounts of PPD A and B used in whole blood culture, didn´t result in responses when performing the IFN-γ assay later between sampling periods. For the last survey in June 2008, additional higher concentrations were tested. These supernatants could not be tested in the IFN-γ ELISA for this study anymore, because difficulties were experienced after that with the ELISA (see chapter 8). When these difficulties are overcome, the

various concentrations of PPD A and PPD B, as well as other stimulants as ESAT-6, should be tested again, to determine to optimum for the IFN-γ ELISA.

The buffy coat that was collected from the EDTA samples was often not a well-defined layer because of the milky consistency of the plasma due to feeding on the carcass prior to darting that caused lipids to circulate in the blood. Especially in the June 2008 survey problems were experienced with not well-defined buffy coats and badly lysed red blood cells, that obscured the storage of the white blood cells in RNAlater. Whether the change from heparin to EDTA tubes may have had an influence remains to be determined.

Preliminary results have already shown that from necropsies from animals showing severe clinical signs of tuberculosis *M. bovis* can be cultured (Keet, personal communication). From tracheal lavages performed during sampling, no mycobacteria were cultured, but the Ziehl-Neelsen staining showed acid-fast bacteria, which should encourage performing more lavages. The storage method for the tracheal flushing samples was changed in October by adding glycerol solution. This was done, because in spite of the fact that acid-fast bacteria had been found during microscopy, culture had not been able to confirm presence of *M. bovis* and we suspected this might be due to the storage method. Though we already improved the storage method by adding glycerol, in retrospect, it would have been even better to store pellets in enriched Middlebrook medium and freeze them immediately, avoiding repeated freeze and thaw cycles. This is a strong recommendation for the future tracheal lavages.

Approval

Dr. D. Keet's project description for the lion captures, and my project proposal, were approved by the Conservation Services Management Committee of the Kruger National Park.

Chapter 6

The development of indirect ELISAs with recombinant *M. bovis* antigens MPB83, MPB70, CFP10 and DiaSer3 for the diagnosis in early stages of bovine tuberculosis in lions

Introduction

The rapid spread of bovine tuberculosis (BTB), caused by *Mycobacterium bovis (M. bovis)*, amongst the lion population in the Kruger National Park (KNP) raises concerns about the future of these animals, one of the main tourist attractions of the park. Tests to detect BTB infection in an early stage are currently based on assessment of cell-mediated immunity (CMI), i.e. the tuberculin skin test, because of the poor sensitivity of bacterial culture and antibody based tests for the early detection of *M. bovis*. The most frequently used diagnostic test based on the humoral response is the Enzyme-linked Immunosorbent Assay (ELISA), so far employing PPD's as antigens. It may complement the tests based on CMI and may be especially helpful in chronically infected animals that gradually loose the capacity to mount CMI and become anergic.

Waters et al., (2006) found that if serological assays using so called early or latency antigens are applied in cattle, antibodies may be detected early after infection (Waters et al. 2006). In the present study, indirect ELISAs (iELISAs) were performed with the recombinant antigens MPB70, MPB83, CFP10 and DiaSer3 (a hybrid of CFP10 and ESAT-6) to study their potential use in (early) diagnosis of BTB in lions. Earlier investigations by students of the University of Utrecht on MPB83 and MPB70 had already given preliminary indications for the possible use of MPB83 and MPB70 (Hoogeveen 2006, Koolen 2007).

Studies in the past reported that sensitivity of an iELISA may increase when using serum samples that have been boosted. Griffin reported in 1994 that ten days after the skin test, the sensitivity of the ELISA increased significantly from 45.7% to 85.3% (Griffin et al. 1994). Lyashchenko et al. (2004) reports the tuberculin skin test boosted IgG, IgG1 and IgG2 antibody response, especially against MPB83 and MPB70 in unvaccinated, BTB positive cattle (Lyashchenko et al. 2004).

The aim of this study was to develop and use a CFP10 and DiaSer3 iELISA for the detection of BTB in lions and compare the test characteristics and test results with preliminary established iELISA's with MPB83 and MPB70. Since the aim of BTB diagnostics in wildlife is to control the infection, it is important to be able to detect animals early in the shedding stage, i.e. not to cull all infected animals, but rather limit

action to animals shedding bacteria. Thus high specificity of tests is important to avoid false positives results.
Once established, these four iELISA's were used to assess the infection status of lions captured during the sampling period August 2007-June 2008. The second serum samples of some animals that were obtained during recaptures were used to investigate the effects of boosting.

Material and methods

Samples

Serum samples from the KNP that have a known BTB status –either classified by skin test and/or culture– collected in the past by Dr. D. Keet and the State Veterinary team of Skukuza, and a batch of lion serum samples from BTB free areas –kindly provided by prof. M. van Vuuren, Onderstepoort- were used to determine the threshold of seropositivity, sensitivity and specificity of the ELISA using the different antigens. In total 225 serum samples were used for this.
The KNP serum samples of Dr. D. Keet consisted of 26 samples (batch four) that were tested positive with histopathology (26/26), culture (20/26) and skin test (26/26). Two other batches consisted of respectively 86 skin test positive samples (batch three) and 65 negative skin test samples (batch one). The serum samples from prof. M. van Vuuren consisted of 45 lion samples, mostly from the Gauteng area (batch two). These samples had been sent in to the serology lab for other purposes and since these 45 lions came from BTB free areas, it was assumed they were BTB negative animals. Later during the study, three new lion sera were added to this batch, making a total of 48 samples that were negative on basis of geographical location.

During the sampling from August 2007-June 2008, new serum samples of 72 lions have been taken, that together form the fifth batch. Eight lions had a second serum sample taken during recaptures. 55 Samples were tested for MPB83. For MPB70, DiaSer3 and CFP10 all 72 samples were tested.

The iELISA

General protocol

Nunc Maxisorb® ELISA plates were coated with the antigen in the tested optimal concentration in 50 µl 0.05 M carbonate/bicarbonate buffer, pH 9.6 (Sigma®) per well and incubated for an hour at 37℃. The plate was wa shed four times with Phosphate-buffered saline (PBS) (0.15 M), Tween-20 (0.05%) (PBS/T) adding 200 µl each time. Subsequently 50 µl per well block buffer with 5% Oxoid® skim milk powder diluted in PBS/T was added and the plate was incubated at room temperature for one hour. The plate was washed as before, and test and control sera were added in the tested optimal dilution with PBS/T, 50 µl per well. After incubation for one hour at room temperature, the plate was washed again as described earlier. Anti-Cat immunoglobulin (Peroxidase-Labeled affinity purified antibody to cat IgG(γ) produced in goat, Kirkegaard & Perry Laboratories®) conjugate was added in the tested optimal dilution (50 µl/well) and the plate was incubated for an hour at room temperature, after which the plate was washed again as before. O-Phenylenediamine dihydrochloride (OPD) substrate (4 mg tablet in 10 ml dH_2O) was added (50 µl per well) and the plate was kept at room temperature in the dark for ten minutes. The colour-changing-reaction was stopped by adding 50 µl of 1 M H_2SO_4 stop solution per well. The plate was read immediately with an ELISA plate reader (EL 808 Ultra Microplate Reader ®, Bio-tek Instruments) at 490 nm and the raw data optical density (OD) values stored.

The specifics for the various iELISA's

MPB83: MPB83 (Rv2873), was kindly provided by Dr Jim McNair of the Veterinary Sciences Division, Belfast, United Kingdom. A protocol for the MPB83 ELISA was developed in an earlier study by Koolen (Koolen 2007). Serum dilutions of 1:10, 1:50, 1:150 and 1:200 have been tested for verification of this protocol, together with conjugate dilutions of 1:2000 and 1:3000. An antigen concentration of 0.55 µg/well was used (50 µl per well).

MPB70: The recMPB70 protein was kindly provided by Dr Jim McNair of the Veterinary Sciences Division, Belfast, United Kingdom. A protocol was available for performing the MPB70 ELISA, developed by Hoogeveen, but it was first optimized

(Hoogeveen 2006). An antigen concentration of 0.5 μg/well was used (50 μl per well). Serum dilutions of 1:50, 1:100, 1:150 and 1:200 and conjugate dilutions of 1:2000 and 1:3000 have been tested for verification of this protocol.

CFP10

The CFP10 protein (Diaser 5 030626, 0500117 batch 1122.1) was kindly provided by the Statens Serum Institute, Copenhagen, Denmark. The CFP10 iELISA was developed for the present study with use of the four batches of lion sera with known infection status. Serum dilutions 1:50, 1:100, 1:150 and 1:200, conjugate dilutions 1:1000 and 1:2000 and antigen concentrations 0.25, 0.50 and 1 μg/well have been tested to determine the most optimal conditions. The protocol can be found in appendix 2.

DiaSer3

The DiaSer3 protein (ESAT-6:CFP10 hybrid, 030618) was kindly provided by the Statens Serum Institute, Copenhagen, Denmark. The DiaSer3 iELISA was developed with use of the four batches of lion sera with a known infection status. Serum dilutions 1:10, 1:50, 1:100, 1:150 and 1:200, conjugate dilutions 1:1000 and 1:2000 and antigen concentrations 0.25, 0.50, 1 and 2 μg/well were tested to determine the most optimal conditions. The protocol can be found in appendix 3.

For all four antigens the five batches have been tested, only for MPB83 the batch of 48 BTB negative lions and the KNP lions from the June 2008 survey could not be tested. The three negative lions that were added extra to the batch of BTB negative lions (based on geographical location) were only tested for CFP10 and DiaSer3.
Part of the work for MPB83 and MPB70 has been done by respectively Koolen and Hoogeveen (Hoogeveen 2006, Koolen 2007).

Analysis of the results

Control samples: The different antigen iELISAs all had control samples that were used as reference standards: these included minimally a strong positive, a weak positive and a negative sample (for all defined with MPB70 and MPB83 ELISA).

Inter- and intra-plate variation: Control samples were added to each plate to serve as extra quality control measurements. For MPB83 and MPB70 single strong positive controls served as a control for inter- and intra plate variation. For CFP10 and DiaSer3 the mean of the positive control sample in triplicate provided a control, so variation within and between plates could be determined.

Plate acceptability: The (mean of the) high positive control sample(s) was used for determination of plate acceptability: a range defined as the mean OD raw values of the high positive control samples of all plates plus and minus a chosen range, for this study 20% of the mean (Jacobson 1998). Plates not within this range were rejected and tests were repeated.

Data expression: Raw data were generated as optical density (OD) values. Several methods exist for the expression of antibody activity (Wright et al. 1993). It was chosen to convert OD readings to sample to positive (S/P) values, using the (mean of the) strong positive control(s). This sample-to-positive (S/P) value also corrects for small plate differences and results in a uniform and continuous scale of 0 to 1 for all ELISAs done.

Gaussian distribution method, cut-off: The Gaussian (normal) distribution method, a common way to express decision criteria, was chosen to determine the cut-off value. The cut-off is defined as the level of antibody activity which determines negative or positive reactor status for a given animal. The chosen cut-off is important, because it determines the diagnostic sensitivity, specificity and the predictive value of the test (Wright et al. 1993). As explained in the introduction, the aim of this test is to achieve a high specificity, so the cut-off –based on the two batches negative lion samples– is set to three standard deviations of the negative reference sera added to the mean of the negatives ($\underline{X}$ + 3 SD). All serum samples above this cut-off were classified as positive. Taking this standardized cut-off also makes comparison of the different ELISAs possible.

Results

MPB83 iELISA

The experiments for verification of the existing protocol confirmed the concentrations used by Koolen were optimal: serum dilution 1:200 and conjugate dilution 1:2000. No extra lion sera could be tested for the validation, and test characteristics as shown by Koolen are used for comparison with the other antigens: cut-off was 0.261 (S/P

value), specificity was 100% (n=65), sensitivity 38.5% (n=26) based on batch four only, 18.6% (n=86) for skin test positive animals (batch three) and for batch three and four together 23.2% (n=112) (Koolen 2007).

Of the fifth batch, the KNP lions, seven lions tested positive in the MPB83 ELISA, of which three were also skin test positive. Three lions had an unknown skin test result and one was skin test negative. For an extensive overview see appendix 1.

MPB70 iELISA

The verification experiments confirmed the existing protocol: serum dilution 1:150 and conjugate dilution 1:2000. To the original database and work performed by Hoogeveen, the batch of 45 negative animals as determined by geographical location of the living areas, was added and additional tests were performed where the original database was not complete (Hoogeveen 2006). The original raw data and new OD-values were transformed into S/P values. The mean was calculated and a cut-off of the mean plus three times the standard deviation was used ($\underline{X}$ + 3 SD).

Cut-off was 0.588 (S/P value), specificity was 99.1% (n=110), sensitivity 26.9% (n=26) based on the fourth batch only, 12.8% (n=86) for skin test positive animals and for animals from the third plus the fourth batch 16.1% (n=112). See table 6.1.

Of the KNP lions, the fifth batch, one lion tested positive in the MPB70 ELISA that was also skin test positive.

Table 6.1. 2x2 table of the MPB70 iELISA results

Sera	**Culture/ SICCT positive**	**SICCT/ location negative**	**Total**
MPB70 positives	18	1	19
MPB70 negatives	94	109	203
Total	112	110	222
	sensitivity: 16.1%	specificity: 99.1%	

DiaSer3 iELISA

After optimisation, the most optimal conditions showed to be an antigen concentration of 0.25 µg/well, a serum dilution of 1:200 and a conjugate dilution of 1:2000. With these values the fifth batch, the KNP lion sera, was tested.

All four batches, in total 225 lion serum samples, have been tested with DiaSer3 antigen in this study for validation of the DiaSer3 iELISA. The cut-off was calculated as ($\underline{X}$ + 3 SD), based on the lions of the first and the second batch and was 0.319 (S/P value). Specificity is 99.1% (n=113). Sensitivity calculated for batch four only is 30.8% (n=26), for batch three 15.1% (n=86) and for animals from batch three plus four 18.8% (n=112) (table 6.2.). The positive predictive value is 95.5% (n=22) and the negative predictive value is 55.2% (n=203) (see table 6.2. and figure 6.1.)

Of the KNP lions, five lions tested positive, of which one was skin test positive. One of these lions was negative and three were unknown.

Table 6.2. 2x2 table of the DiaSer3 iELISA results

Sera	**Culture/ SICCT positive**	**SICCT/ location negative**	**Total**
DiaSer3 positives	21	1	22
DiaSer3 negatives	91	112	203
Total	112	113	225
	sensitivity: 18.8%	specificity: 99.1%	

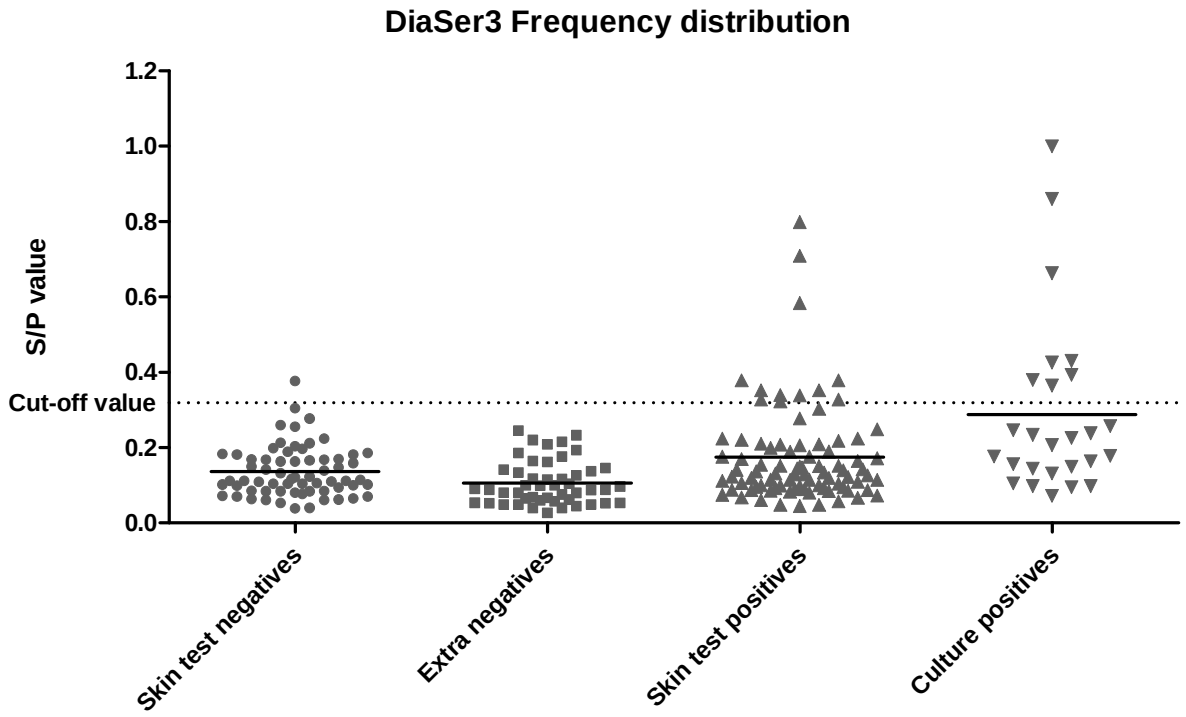

Figure 6.1. Frequency distribution of DiaSer3 for the four different batches of lion serum samples, the cut-off is at $\underline{x}$ + 3 SD (0.319)

CFP10 iELISA

The optimization experiments showed that the optimal conditions were an antigen concentration of 0.5 µg/well, a serum dilution of 1:150 and a conjugate dilution of 1:2000. In total 225 lion sera have been tested (see DiaSer3 above). The mean was calculated and a cut-off of the mean plus three times the standard deviation was used ($\underline{X}$ + 3 SD).

The cut-off was 0.571 (S/P value), specificity was 99.1% (n=110), sensitivity 7.7% (n=26) based on batch four only, 4.7% (n=86) for animals of batch three, and for the third batch plus the fourth 5.4% (n=112) (see table 6.3. and figure 6.2.)

Of the KNP lions no lions tested positive.

Table 6.3. 2x2 table of the CFP10 iELISA results

Sera	***Culture/ SICCT positive***	***SICCT/ location negative***	***Total***
CFP10 positives	*6*	*1*	*7*
CFP10 negatives	*106*	*109*	*215*
Total	*112*	*110*	*222*
	sensitivity: 5.4%	*specificity: 99.1%*	

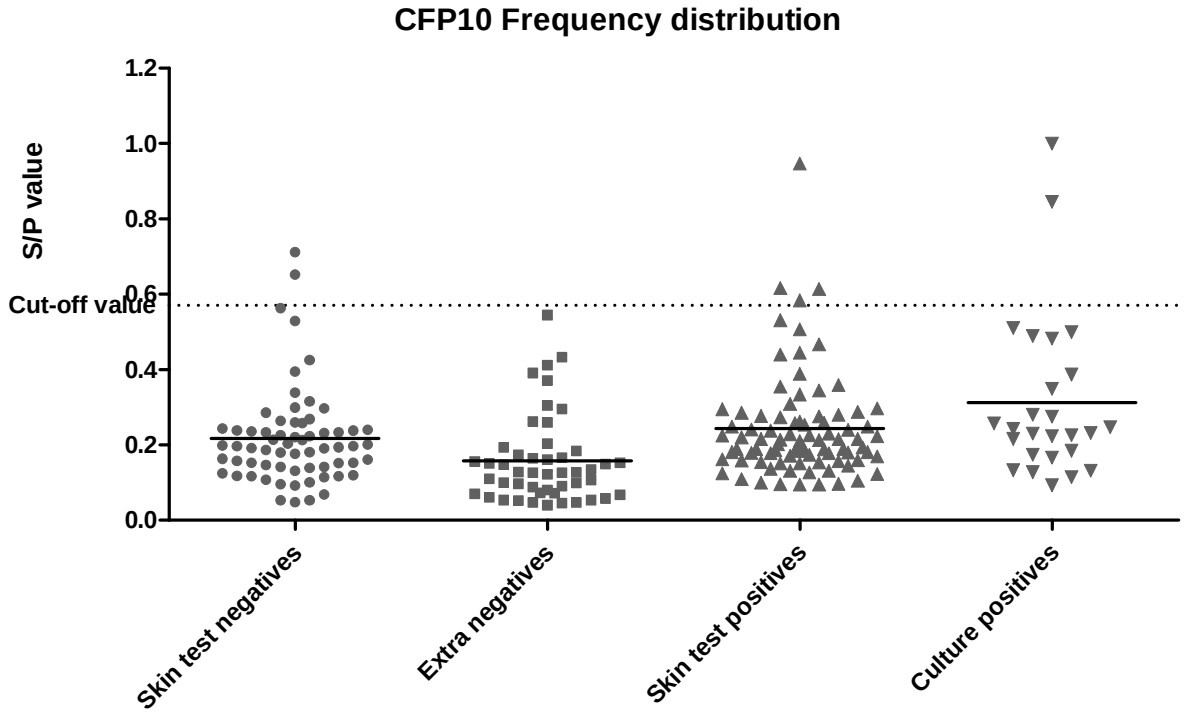

Figure 6.2. Frequency distribution of CFP10 for the four different batches of lion serum samples, the cut-off is at $\underline{x}$ + 3 SD (0.571)

Table 6.4. Overview of the sensitivity and the specificity of the four different antigens.

	MPB83	MPB70	CFP10	DiaSer3
Sensitivity	**23%**	**16%**	**5%**	**18%**
Specificity	**100%**	**99%**	**99%**	**99%**

The second serum samples

Of eight lions a second serum sample has been taken during the sampling when the animals were recaptured. Six of these lions were skin test positive. The S/P values of the boosted samples only showed minimal differences, sometimes higher, sometimes lower.

Table 6.5. Overview of the S/P values of the first and second serum samples of 8 lions. (p) is for skin test positive lions and (n) is for skin test negative lions. (+) Indicates the iELISA is positive for the antigen.

Serum	1st MPB83	2nd MPB83	1st MPB70	2nd MPB70	1st Diaser3	2nd Diaser3	1st CFP10	2nd CFP10
494A185A30 (p)	0,319 (+)	0,409 (+)	0,370	0,377	0,175	0,150	0,219	0,186
494748583B (p)	0,420 (+)	0,299 (+)	0,331	0,445	0,196	0,178	0,234	0,214
494A427E14 (p)	0,108	0,064	0,088	0,067	0,122	0,125	0,090	0,077
4115092830 (p)	0,242	0,195	0,263	0,242	0,227	0,193	0,326	0,335
456B4F4153 (survey June ‘ 08) (p)	-	-	0,126	0,398	0,149	0,300	0,464	0,467
4A5E685451 (n)	-	-	0,130	0,127	0,088	0,135	0,118	0,119
4A6A682D39 (p)	-	-	0,125	0,198	0,109	0,127	0,153	0,236
4A441E5F2A (n)	-	-	0,177	0,183	0,230	0,124	0,207	0,204

Discussion

Development of a diagnostic test for BTB requires that the reference animals are classified correctly as negative or positive for infection with *M. bovis*. According to literature the reference population should be representative for the target population, and should also be large enough to provide the desired statistical power. Jacobson (1998) suggested that these reference populations should include at least 300

known-infected samples and 1,000 known-uninfected samples to compensate for various factors that influence sensitivity and specificity, only to determine initial estimates of diagnostic sensitivity and specificity, respectively. For precise estimates, no less than approximately 1,000 and 5,000 animals should be used respectively (Jacobson 1998). When developing a test for a wildlife species, these large numbers are almost impossible to achieve –especially if the culture is wanted as the gold standard. With the available samples, initial estimates should be made, and when these show potential for future use, validation should continue while meanwhile using the test.

For BTB, the gold standard is the culture. Bacterial-culture is a lengthy process, and sampling is difficult to perform on living animals, which results in few serum samples with a culture-confirmed BTB status –especially negative samples. The skin test is more generally performed on all animals, so more serum samples are available with a BTB status. For that reason, serum samples of animals with a skin test-determined BTB status, have been used for validation of the iELISAs. Only serum samples were used of animals that showed a positive or negative skin test result, i.e. suspect skin test results were excluded from iELISA validation. The risk of misclassification by using samples that have not been classified using the gold standard, is accepted, because otherwise the number of samples that could be used would be too low.

A simple and useful method to accomplish data normalization, and reduce day-to-day variation, is the use of S/P values instead of OD-readings (Jacobson 1998). Figures 6.1. and 6.2. show that a number of serum samples had S/P values that were close to the cut-off values. These samples could be classified incorrectly, which affects the determination of test characteristics. This would better be corrected for when having large sample sizes.

The establishment of a reliable cut-off is essential for a serological test to differentiate between infected and non-infected animals and by choosing a certain cut-off, the sensitivity and specificity can be influenced. The future purpose of the iELISA would be the diagnostics of real positives, i.e. the false-positive ratio should be low, i.e. a high specificity is wanted. Since the sensitivity and specificity are inversely related, often a low sensitivity for serologic tests for BTB is seen. When comparing the

sensitivity and specificity of the four different antigens (table 6.4), MPB83 shows the highest sensitivity with the highest specificity. This was also shown in a study with serum samples of various species, where MPB83 was the most commonly recognized antigen for all species (Lyashchenko et al. 2008). Even though high specificity of the test is the aim, the sensitivity of CFP10 is too low to be used as an antigen on its own. The heterogeneous antibody repertoire of BTB can be used to increase sensitivity and specificity of the iELISA by combining multiple antigens. In our study, test results show that DiaSer3, a hybrid of CFP10 and ESAT-6, gives higher sensitivity than the use of the CFP10 antigen alone (18% compared to 5%, with the same specificity). This could be even more pronounced when cocktails of more antigens would be tested, for example all four antigens that have been used for this study. Other studies show this principle too and rapid tests have been developed, that often combine different antigens (Lyashchenko et al. 2000, Lyashchenko et al. 2008). A disadvantage of antigen cocktails is that sometimes by combining antigens, the absorbance values of the antigens decrease due to competition in binding to the plate, which reduces sensitivity.

Our preliminary results show that no difference in OD reading could be seen between the sera taken before PPD injection and three days after PPD injection (table 6.5.), i.e. no booster reaction was measured. Both Griffin and Lyashchenko et al. have used a longer booster period than the three-day period in our study however, (Griffin et al. 1994, Lyashchenko et al. 2004) and booster reaction may be seen when a longer time period in between is used. Catching the lions ten days after the skin test though, would cause the same disadvantage the skin test has now: another recapture is needed.

In summary, even though the iELISA shows low sensitivity, it has the potential to be useful in BTB diagnostics by complementing CMI and determining anergic animals. Since cattle is tested on a more regular basis, CMI is more useful in that field. In wildlife contrarily, where testing is very infrequent and more animals reach the Th2 stage of the infection i.e. are possible shedders, there is a definite role for serology to play. More animals should be tested that have a defined infection status, and serology results should be correlated to CMI results, age of the lions, and current, non-optimal BTB infection status parameters, in order to estimate the duration of the

infection. To show the principle of a correlation between positive iELISA results and shedding, broncho-alveolar lavages should be implemented on all animals that are necropsied and tracheal lavages should be performed on all animals handled during sampling activities.

Chapter 7

The lentivirus status of two lion populations in a high and low *M. bovis* prevalence area in the Kruger National Park

Introduction

Bovine tuberculosis entered the Kruger National Park (KNP) in the '60s. In 1995 infection of lions (*Panthera leo*) and other wildlife species became evident (Keet et al. 1996). Lions affected with tuberculosis do not show clinical signs for prolonged periods of time, and the onset of overt disease can be very sudden (Eulenberger et al. 1992). Clinical signs include emaciation, depression, alopecia and unilateral ocular lesions (Keet, Michel & Meltzer 2000).

In humans it has been shown that the risk of getting tuberculosis greatly increases in human immunodeficiency virus (HIV) infected people (Raviglione, Snider & Kochi 1995). However, no such correlation has been proven yet for lions with feline immunodeficiency virus (FIV). FIV is a member of the genus *Lentivirus* of the family *Retroviridae*, as is the human member HIV, and has a worldwide distribution in feral and domestic cats. Molecular analysis has shown that some of the lentiviruses from wild felids are genetically different from FIV (Van Vuuren et al. 2003), leading to classifying the immunodeficiency virus in lions as lion lentivirus (LLV). The lentiviruses for domestic cats and non-domestic feline species have shown serological cross-reactivity (Woude, O'Brien & Hoover 1997).

Van Vuuren et al. mentions that the current perception is that LLV has no effect on the lions in the Kruger National Park, and that in LLV-infected lions, lymphocyte blastogenic responses were not affected, and $CD4^+$:$CD8^+$ ratios were within the normal parameters (Van Vuuren, Stylianides & Du Rand 1997). A 30-year study performed by Parker (1999) in the Serengeti showed no evidence that the LLV status influenced lion mortality, nor could any obvious signs of immunodeficiency or clinical pathology in LLV positive animals be observed (Packer et al. 1999). The hypothesis that LLV doesn't cause disease in (free-ranging) lions is questioned by Kennedy-Stoskopf (Kennedy-Stoskopf, Gebhard & English 1994). Clinical signs such as reversal of $CD4^+$:$CD8^+$ lymphocyte ratios, hypergammaglobulinaemia, chronic gingivitis periodic behavioural changes with the development of aggression and the development of retinopathy are described in captive animals. Roelke et al. (2006) found that in free-ranging LLV infected lions there was a dramatic decline in $CD4^+$ subsets, a reduction of the $CD4^+/CD8^+$ ratio, as well as a shift in the $CD8^+$ subsets relative to uninfected lions (Roelke et al. 2006). This could contribute to an increased

susceptibility to other infections, for example tuberculosis. Further research is needed to determine the consequences of LLV infection in captive and free-ranging lions.

In 1992, using 98 lion serum samples collected over a 14-year period (that dated back to 1977) and covered the whole KNP (one from the northern most region, 21 from the central region, 51 from the southern region and the remaining unknown), showed that 83% of the KNP lions was lentivirus-seropositive (Spencer et al. 1992). LLV prevalence seems to be density dependent, and is more prevalent when lion density is high. Lentivirus-positive lions occurred throughout the park, and were not merely confined to the south of the park, as in the case with tuberculosis. Lions that were infected with both LLV and tuberculosis have significantly higher levels of alpha and gamma globulins than lions that are negative for any of the two infections (Keet, personal communication).
By the age of 3 1/2, most lions are infected with tuberculosis or with LLV. By the age of 5, most males are co-infected. Animals with a co-infection tend to develop signs of tuberculosis in three years and tuberculosis lesions are more severe in lions with LLV-TB co-infection. Infection with LLV does not seem to predispose for infection with BTB, nor visa versa (Keet, personal communication).

To gain a better insight in the correlation between LLV and TB in lions in the Kruger National Park, an ELISA was performed on the samples collected during the sampling work (August 2007-June 2008) to determine LLV-status.

Material and methods

The animals

In the present study, serum samples of 72 lions were tested with the LLV/PLV ELISA to confirm LLV status: 42 lions in the south of the KNP, 30 lion in the north.

LLV/PLV ELISA

The protocol was the standard protocol used in the serology laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, for lentivirus diagnosis in wild felids. It was slightly adjusted from the one described by Van Vuuren et al. (2003) (Van Vuuren et al. 2003). The assay uses a puma lentivirus-derived synthetic peptide as coating antigen.

Flat bottomed microtiter plates (immulon 2, Dynatec) were coated with 100 ul PLV peptide antigen diluted in PBS to a concentration of 10 μg/ml. The plates were kept overnight at 4℃ and washed the next day four times with PBS containing 0.05% Tween-20 (PBS-T). Wells were blocked by adding 200 μl of PBS-T to each antigen-coated well and this was left to stand at room temperature for at least an hour. Lion sera were diluted 1:25 in PBS-T and added in duplicate to the wells and incubated for one hour at 37℃. Positive and negative control ser a were also added in duplicate. Plates were washed four times with PBS-T and 100 μl goat anti-cat IgG peroxidase conjugate (Kirkegaard and Perry Laboratories) was added in a concentration of 1:8000. After 1 hour at 37℃ the plates were washed again four times and 100 μl of ABTS substrate {2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)} was added to the wells. The plates were placed in the dark at room temperature and read after 25 minutes at 405 nm.

Analysis of the results

Each plate had a positive and a negative control serum added in duplicate. Raw data were obtained in the form of optical density (OD) readings. The mean of the OD reading of the negative control samples was taken. The cut-off value was double the mean of the negative values. A sample was considered positive if it was equal or higher than the cut-off value.

Results

Of the 72 tested animals, 34 animals had antibody reactive to puma lentivirus.

South: 19 Out of 42 lions that were immobilized in the south yielded positive results. An overview of the results and a comparison with the results of the tuberculin skin test can be found in table 7.1.

Table 7.1. Overview of the test results for lion lentivirus and BTB in the southern KNP lions.

Lion south	BTB +	BTB -	BTB unknown	
LLV +	9	1	9	19
LLV -	8	0	15	23
	17	1	24	42

North: Of the 30 lions that were immobilized in the north, 15 yielded positive results. An overview of the results and a comparison with the results of the tuberculin skin test can be found in table 7.2.

Table 7.2. Overview of the test results for lion lentivirus and BTB in the northern KNP lions.

Lion north	BTB +	BTB -	BTB unknown	
LLV +	4	1	10	15
LLV -	3	3	9	15
	7	4	19	30

Discussion

The present results confirm that LLV is still prevalent in the Kruger National Park, though prevalence measured in this study is 47% (n=72); much lower than the 83% that is mentioned in the literature (Spencer et al. 1992). The south and the north of the park have almost the same prevalences of LLV infection, respectively 45% and 50%. The number of animals tested with a known BTB-infection status is too small now to make definite conclusions about correlation between LLV and BTB. Data collected in the past and to be collected in the future, will be combined with the current findings, so more information becomes available on possible correlations.

Though the current perception is that LLV has no effect on free-ranging lions, observations in captive animals and the current spread of BTB under the KNP lions, ask for the continuation of the monitoring of the lions, to determine if LLV is still non-pathogenic when co-infection with BTB occurs.

Chapter 8

A multi-species IFN-γ assay for diagnosing *Mycobacterium bovis* infection in lions (*Panthera leo*)

Introduction

In 1995, infection of lions in the Kruger National Park (KNP) with *Mycobacterium bovis (M. bovis)* became evident (Keet et al. 1996). Lions affected with bovine tuberculosis (BTB) do not show clinical signs for prolonged periods of time, and the onset of overt disease can be very sudden (Eulenberger et al. 1992). In literature there is a general agreement that cell-mediated immunity (CMI) plays the major role in controlling mycobacterial infections and CMI-directed diagnosis seems to be able to detect infection at an early stage in several species (Pollock et al. 2001). From the various tests available for the diagnosis of bovine tuberculosis, the most widely known is the tuberculin skin test. The skin test is based on a delayed-type hypersensitivity response, i.e. CMI (Welsh et al. 2005). After injection of the PPD, a positive reaction will cause an inflammation, and a swelling can be measured 72 hours later. (see figure 3.2.)

The skin test however is not practical to use for wildlife, because two sedations within a three-day interval are required. Not only is this time-consuming, it causes stress and chances on injury for the animal twice, and it is also not guaranteed that the animals will be re-captured after three days (Waters et al. 2005).

In the IFN-γ test, blood is stimulated and in reaction to avian and bovine PPD or other more specific antigens of *Mycobacterium* spp, sensitized T-cells start producing IFN-γ. The following quantitative measurement of the lymphokine is carried out in a sandwich ELISA (Office International des Epizooties (OIE)). The IFN-γ test is considered at least as sensitive as the skin test and has the advantage that it can detect animals at an earlier stage of the infection than the skin test, where positive results are only seen after one to nine weeks after infection (de la Rua-Domenech et al. 2006). Since the IFN-γ test is based on the cell-mediated immune response, it has the same time limits as the tuberculin skin test, because the cell-mediated immune response fades away. The most important advantage for wildlife of the IFN-γ test compared to the skin test is that only one capture is needed. An additional advantage of the IFN-γ test is that it is more likely to be independent of BCG vaccination when it is based on RD1-specific antigens (Dinnes et al. 2007).

IFN-γ is a cytokine produced significantly by both $CD4^+$ and $CD8^+$ T-cells and has a key role in the CMI and the control of *M. bovis* in infected animals (Pollock et al.

2001). It stimulates the macrophages by up-regulation of the MHC-expression, increasing antigen presentation.

An IFN-γ capture ELISA for cattle has been developed -the officially registered and validated BOVIGAM® test- that utilizes two monoclonal antibodies to bovine IFN-γ, that do not recognize cat or lion IFN-γ (Rothel et al. 1990). For buffalo, the BOVIGAM® test has been modified and has shown to have a high specificity (99.3%) and high sensitivity (84.6%) (Grobler et al. 2002).

Therefore, it is worth investigating the possibilities of using the IFN-γ test for the detection of *M. bovis* infection in lions. Currently, no lion IFN-γ specific antibodies are available. In this study, an already available multi-species anti-IFN-γ test has been used to optimise the ELISA for lion IFN-γ.

Material and methods

The animals

Initially the test was optimised with bovine, equine and domestic cat blood. Blood from lions in the Gauteng province was subsequently used for further optimisation. Lion samples collected during the surveys in the south and in the north of the Kruger National Park were subsequently tested (see chapter 5).

Stimulation of the whole blood

Heparin samples were taken, and within 8 hours these were aliquoted on 24-well tissue culture plates: 1.5 ml per well, three wells per sample. Stimulation of the cells was done as it is performed in the BOVIGAM® test: one well served as a control (nothing added), one received 30 µl avian PPD added and one received 30 µl Bovine PPD (both from the Institute for Animal Science and Health, Lelystad, The Netherlands. Resp. 25.000 I.U/ml and 30.000Ph.Eur. U/ml). Samples were incubated for 18-24 hours at 37℃ without CO_2. After incubation, supernatants were stored at -20℃ until further processing.

In additional experiments during the June 2008 survey, extra PPD volumes and other stimulants were tested on wells with 1 ml of whole blood: 100 µl, 50 µl, 20 µl, 5 µl and 0 µl for both PPD A and PPD B. Four additional wells were incubated with 4 µg, 2 µg,

1 μg and 0 μg ESAT-6 (F1200 ESAT-6, produced in Lactococcus, 1 mg/ml, kindly provided by the Statens Serum Institute, Copenhagen, Denmark).

For the optimisation experiments, whole blood and peripheral blood mononuclear cells (PBMCs) were stimulated with the mitogen Concanavalin A (Con-A; Sigma) in concentrations of 50, 25, 10, 5 and 0 μg/ml and incubated at 37℃ for 18-24 hours in a 5% CO_2 incubator. The contents of the well were centrifuged (10 min 3200 rpm) and supernatants were collected and stored at -20℃ .

The IFN-γ capture ELISA

Morar developed a multi-species IFN-γ capture ELISA assay. The assay involves use of monoclonal antibodies, which were obtained by immunizing mice with recombinant IFN-γ of various species (Morar et al. 2007). Two monoclonals, 1H11 and 1D11, have been used for this study.

The assay designed by Morar was re-evaluated first and optimised for cat blood and after that for lion blood. Minor adjustments were made to the IFN-γ assay protocol of Morar, and in short can be described as follows.

A Microwell™ polysorb ELISA plate (Nunc, C96 446140) was coated with 50 μl/well monoclonal antibodies diluted in 0.15 M PBS at 1 μg/ml. It was put on a shaker for 1-2 hours and left for overnight incubation at 4℃. A fter discarding the overnight coating 100 μl block buffer (2% fat free milk powder in 0.15 M PBS) was added and incubated for 1 hour at 37℃. The block buffer was discarded and the plate was washed five times with tap H_2O. Undiluted supernatants of stimulated target samples were added (50 μl/well). A positive control of recombinant white rhinoceros IFN-γ at a concentration of 5 μg/ml (diluted in 0.15 M PBS) in duplicate and two negative controls (block buffer and 0.15 M PBS, both in duplicate) were added. After an incubation of 1 hour at 37℃, the samples were disc arded and the plate was washed five times with wash buffer (Tap H_2O with 0.1% Tween-20). Polyclonal antibodies to white rhinoceros IFN-γ were added (chicken IgY (700 μg/ml), 1:100 dilution in block buffer+0.1%Tween- 20) 50 μl/well, and incubated for 1 hour at 37℃. After was hing with wash buffer five times, rabbit antibodies specific to chicken IgY H&L conjugate (HRP) (Abcam, ab6753) was added (1:3000 dilution, diluent: block buffer+ 0.1%

Tween 20) and another incubation of 1 hour at 37℃ followed. After washing as described before 50 µl/well substrate (5 mg o-phenylenediamine (OPD; Sigma) tablet diluted in 5 ml citrate buffer with 2.5 µl H_2O_2) was added and the plate was left 30 minutes for incubation at room temperature. 2N H_2S0_4 stop solution was added (50 µl/well) and after ten minutes the optical density (OD) was read at 490 nm.

Analysis of the results

Control samples: All plates had at least one recombinant IFN-γ and two negative control samples –block buffer and 0.15 M PBS–, all in duplicate. These control samples also served to determine variation within and between plates.

Data expression: Raw data was generated as optical density (OD) values.

Results

Optimisation experiments

Optimisation consisted of the following:

- control of the usability of the recombinant rhinoceros IFN-γ (recRhino),
- dilution curves of the various Con-A concentrations,
- a comparison of whole blood and PBMC and
- a comparison of incubation of the whole blood with or without 5% CO_2.

The optimisation experiments were performed with bovine, equine or feline (cat/lion) blood. A representative selection of OD-readings of test results is presented below.

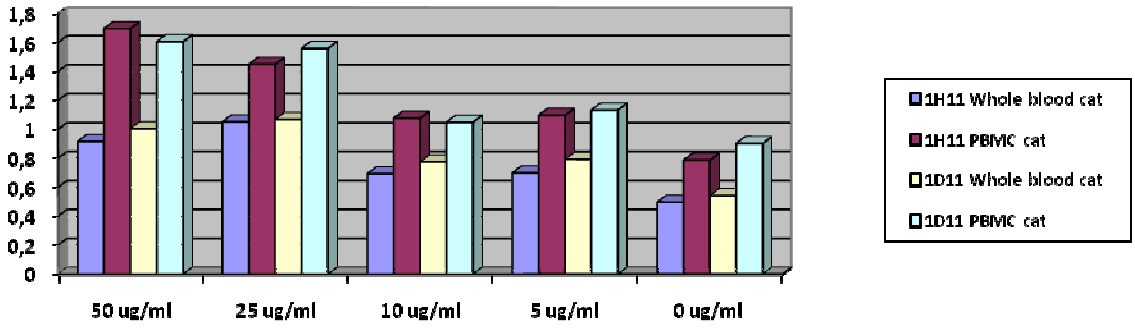

Figure 8.1. Results of the detection of IFN-γ from cat PBMCs and whole blood using two different capture antibodies, stimulated with different concentrations Con-A. The mean values of the positive recRhino control sample and the block buffer negative control for 1H11 were 2,012 and 1,624 respectively and 2.127 and 1,73 for 1D11.

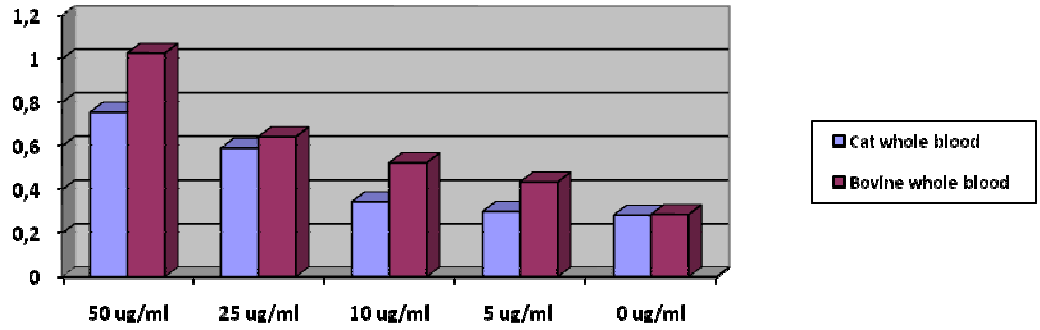

Figure 8.2. Domestic cat and bovine blood tested with monoclonal antibody 1H11, stimulated with different concentrations Con-A. Mean of the recombinant rhino IFN-γ positive control was 1,417 and the mean of the negative block buffer control 0,45.

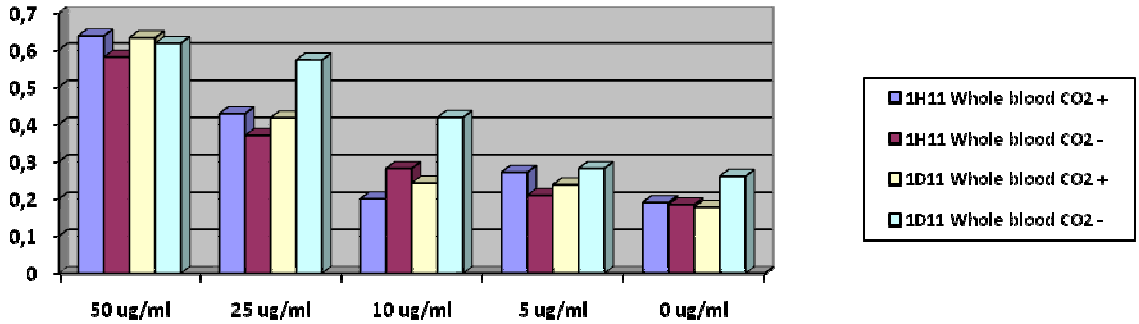

Figure 8.3. OD readings of whole blood of one lion that was tested with two different antibodies and incubated with and without CO_2, stimulated with different concentrations Con-A. Mean of the positive recRhino control sample and the negative milk powder control for 1H11 was 0,798 and 0,182 respectively for 1H11 and 0,806 and 0,235 for 1D11.

Testing with lion control blood

After optimisation of the assay as developed by Morar, the assay was tested on lion control blood. Both whole blood and PBMCs were stimulated with Con-A, for 2 monoclonal capture antibodies 1H11 and 1D11, to test if the assay could detect lion IFN-γ and if a correlation between Con-A stimulation and IFN-γ production could be shown (see figure 8.4.).

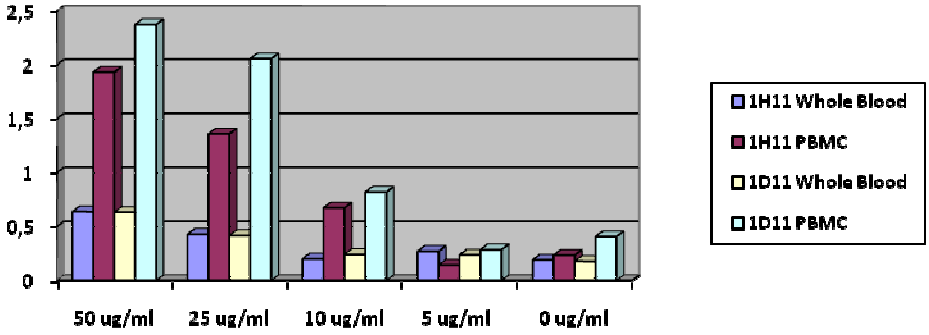

Figure 8.4. OD readings of one lion that was tested with two different antibodies for both whole blood and PBMC, stimulated with different concentrations Con-A. Positive control average and the average of the 2% milk powder negative controls was 0,798 and 0,182 respectively for 1H11 and 0,806 and 0,235 for 1D11.

A general decrease of the OD-readings with decreasing Con-A concentrations can be seen for both whole blood and PBMC for both the monoclonals. The OD-reading and the decrease are more prominent for PBMCs.

Testing with KNP lion blood

Eight lion samples that were collected from lions in the Kruger National Park –of which four lions had a positive skin test result, three had an unknown result (not read) and one was negative– were tested with the monoclonal antibody 1H11 (table 8.1.).

	control	PPD A	PPD B
223 (p)	0.260	0.228	0.241
830 (p)	0.292	0.260	0.284
14E (p)	0.285	0.273	0.257
E14 (p)	0.305	0.223	0.236
363 (?)	0.282	0.260	0.267
833 (?)	0.224	0.216	0.202
A60 (?)	0.238	0.242	0.231
F43 (n)	0.294	0.289	0.285

Table 8.1. Overview of the OD reading of KNP lions in the IFN-γ assay. P=SICCT positive, n= SICCT negative, ?=unknown SICCT status. The mean of Rec. rhino was 0.644, of the 2% MP negative control 0.129 and of the PBS negative control 0.150.

Discussion

For the IFN-γ ELISA reported in literature, PBMCs are often stimulated with mitogen or PPD, since these tend to give more pronounced OD readings in the IFN-γ ELISA. This principle was also shown when optimising the test at the Onderstepoort

Veterinary faculty (figure 8.1. and 8.4.). However, due to restricting lab facilities in the field, only stimulation of whole blood was performed. In the future, more facilities should become available to perform PBMC isolation and stimulation in the field.

Incubation preferably takes place in a 5% CO_2 incubator, but this was not available in the field laboratory. Therefore, whole blood samples were incubated at 37℃ without CO_2. Additional tests at the Onderstepoort Veterinary faculty were performed to determine the difference between these two incubation methods. Preliminary results show a decrease of the OD-readings with a decreasing Con-A concentration for both methods, and differences between the two methods are minimal (figure 8.3.). In additional tests, these differences varied to a small extend, but more animals should be tested in the future to show if there is a significant difference between the two methods and if for field circumstances the CO_2 negative-incubation method can be used without causing significant changes in the subsequent IFN-γ ELISA .

The curves obtained with the lion samples stimulated with Con-A indicated a correlation between the amount of Con-A and OD-reading (see figure 8.3. and 8.4.). The samples taken in the Kruger National Park however, did not show the correlation between mitogen stimulation and OD-reading. No difference was seen between the OD-readings of PPD B, PPD A and the control samples of BTB confirmed animals (table 8.1.). Additional stimulation concentrations were used in the survey of June 2008, but these animals had a negative, unknown or doubtful BTB skin test result, and samples were not tested anymore in the IFN-γ ELISA, due to new developments with the ELISA.

It was noticed that OD-readings in the assay were inconsistent and changing almost every time the assay was performed for both the (recombinant) control samples and the target samples. When testing PBS with different concentrations of Con-A, it was found that Con-A was binding in the assay as well. This could be a reason for the variable results that were obtained.

Concanavalin A (Con-A) is a lectin isolated from jack bean seed (*Canavalia ensiformis*). The binding properties of Con-A to glycopeptides have been recognized for many years (Brewer, Bhattacharyya 1986). Sparbier et al (2006) confirms these glycoprotein binding properties, and successfully competed this binding of Con-A by addition of 500 mM α-methylmannoside (Sparbier, Wenzel & Kostrzewa 2006).

Therefore, α-methylmannoside was added in different concentrations to the sample-binding step in the ELISA. This gave reduction in the OD-reading (data not shown), with which the principle of Con-A binding in the assay was confirmed. It was not possible however, to completely reduce the Con-A binding, because the concentration of α-methylmannoside could not be increased to the predicted optimal concentration. Further research with α-methylmannoside as a competitive binder is needed. It should also be investigated if a different mitogen should be chosen to stimulate the cells/whole blood for control samples.

The stimulation of the whole blood with ESAT-6 will increase the specificity of the test, which has already been shown for cattle (Hope et al. 2005). Two standardized diagnostic kits are on the market now for cattle that use ESAT-6 and CFP10 (a culture filtrate with many similarities to ESAT-6). Specificity is high, and the sensitivity is said to be at least as high as the skin test. Since ESAT-6 is minimally expressed in the BCG vaccination, the advantage of ESAT-6 as a stimulant is that animals vaccinated with BCG will not respond, whereas they do respond to PPD. The high production of IFN-γ after recent stimulation with ESAT-6 might even serve as a prognostic marker in BTB-infected individuals for subsequent development of overt disease, i.e. can serve as an indicator of disease severity (Vordermeier et al. 2002, Andersen et al. 2007).

In conclusion, due to the interference of Con-A that binds in the assay, which was found out only at the end of the year, much of the data collected before could not be used to validate the test. Therefore, with the knowledge of the interference of Con-A, more research needs to be performed before the IFN-γ ELISA can be used in the field. For the present study, multi-species anti IFN-γ has been used. Sequencing of the lion IFN-γ gene that is described in Chapter 10, is the first step made towards the expression of the lion IFN-γ gene as a protein and subsequently the IFN-γ ELISA could be optimised in the future with more specific monoclonal antibodies.

Chapter 9

Development of a real-time qPCR for lion IFN-γ, TNF-α, IL-4 and IL-10 as a tool for immune status assessment during *M. bovis* infection

Introduction

The rapid spread of bovine tuberculosis (BTB), caused by *Mycobacterium bovis (M. bovis)*, amongst the lion population in the Kruger National Park (KNP) raises concerns about the future of these animals, one of the main tourist attractions of the park. Lions affected with tuberculosis do not show clinical signs for prolonged periods of time, and the onset of overt disease can be very sudden (Eulenberger et al. 1992). In the literature there is a general agreement that the cell-mediated immunity (CMI) plays the major role in controlling the infection (Pollock et al. 2001).

The macrophage is the main effector cell for control of mycobacterial infections, but the T-lymphocyte is the major inducer of the protective acquired immune response (Buddle et al. 2002). The functions of macrophages, dendritic cells and T-cells are modulated by cytokines (Widdison et al. 2006). Cytokines are regulatory proteins, secreted by many different cell types of which the most prominent are the leukocytes, and play a central role in the immune system by modulating immune responses (Giulietti et al. 2001). The roles played by cytokines vary widely; some are pro-inflammatory, of which IFN-γ is considered to be critical, activating cells of the immune system to kill mycobacteria and inducing a type I immune response. Others, such as IL-4 and IL-10, are anti-inflammatory, and down-regulate the pro-inflammatory immune response to control tissue damage.

In a mouse model of pulmonary TB by Hernandez-Pando (1996), mRNA levels of IFN-γ and IL-4 were measured and it was shown that chronicity and severity were not necessarily associated with a quantitative decline of Th1 cytokines, but could be due to high levels of Th2 cytokines that reduce the functional efficiency of Th1 cytokines. The authors found that in chronic, advanced murine cases of pulmonary TB, high amounts of IL-4 mRNA were produced (Hernandez-Pando et al. 1996). Thacker et al. (2007) however, doubts the often suggested Th1 to Th2 shift described by Hernandez-Pando that should correlate with increased pathology as infection progresses (Thacker, Palmer & Waters 2007).

Cytokines are often circulating in the blood in low quantities, which makes it difficult to detect them. There are several tests that detect cytokines at the protein level: ELISA, ELISPOT and biological assays (Kabilan et al. 1990), but species-specific immunological reagents are not available for the majority of wildlife species

(Harrington et al. 2007). Detection of cytokines of peripheral blood mononuclear cells (PBMCs) at a mRNA level with reverse transcriptase real time (quantitative) polymerase chain reaction (RT qPCR) allows detection of cytokines that are secreted in amounts too low to detect at protein level (Zimmermann, Mannhalter 1996, Bustin 2000). The real-time Taqman PCR that Leutenegger et al. (1999) described for quantitating feline cytokines requires less than 1 ng of the total RNA per cytokine determination (Leutenegger et al. 1999).

The RT qPCR can be performed in a so-called two-tube system (with separate tubes for the cDNA synthesis and Real-Time PCR), or in a one-tube system (a single reaction). The RT qPCR is based on fluorescence resonance energy transfer (FRET), where a quencher dye silences a reporter dye (Cardullo et al. 1988, Giulietti et al. 2001). This method allows quantification of the PCR product while the PCR is running (Giulietti et al. 2001).

Assessment of cytokine expression of cells in relation to other immune response parameters, might offer insight in immune responses and changes in the immune response during progression of *M. bovis* infection in lions.
For this purpose, four cytokines were chosen: IFN-γ, IL-4, TNF-α and IL-10. A reverse transcriptase Real-Time PCR was developed to measure the cytokine expression in lion white blood cells. In the future, when more parameters are known to diagnose whether a lion is in the Th1 or Th2 stage, the cytokine primers can also be used to determine if there is a shift from Th1 to Th2 dominance in the course of the infection. Understanding the contribution of Th1 and Th2 responses to protective immunity and pathology of *M. bovis* infection might also aid in the development of effective vaccines for lions (Thacker, Palmer & Waters 2007) and contributes to development of new diagnostic methods for *M. bovis* infection in lions.

Material and methods

Material

Cat blood was used initially to test the primers and probes and to optimise the Real-Time PCRs. Subsequently, lion blood was collected from several individual lions in the Gauteng area, to test if the primers also worked on lion blood. Finally, the Kruger National Park lion samples that were obtained during the field work in the north and

the south of the park were tested. Only samples with a known skin test result were used: 22 positive animals and eight negative (one animal had an extra sample collected during the re-capture).

Isolation of the PBMC/white blood cells

At the Onderstepoort Veterinary faculty, EDTA blood was collected of domestic cats and of lions in the Gauteng province. PBMC were isolated by Histopaque gradient centrifugation, washed twice with ice cold medium (RPMI 1640, supplemented with 2mM L-glutamine, 5,000 units Penicillin, and 5,000 ug Streptomycin), stimulated with Con-A and incubated 18-24 hours in a 5% CO_2 incubator at 37℃. The PBMCs were stored in RNAlater at -20℃ until further processin g.

During the sampling in the KNP a different method was used (see chapter 5). This method was also used at the Onderstepoort laboratory to produce positive control samples for the experiment and to calculate PCR efficiency. After collection in either heparin or EDTA 10 ml tubes, the blood was centrifuged for 15 minutes at 3200 rpm. The buffy coat was collected and 2 ml of erythrocyste lysis solution (0.16 M NH_4CL ; 10 mM $KHCO_3$; 0.1 mM Na_2EDTA ; pH 7.4, filter sterilized) was added. The red blood cell lysis was stopped after 6 minutes with 20-25 ml PBS. After a following centrifugation of 10 minutes at 1200 rpm the resulting pellet was stored in 300-500 µl RNAlater, depending on the size of the pellet. Samples were stored at -20℃ until further processing.

RNA extraction

The RNAlater samples produced in the KNP laboratory and at the faculty, were processed at the Onderstepoort Faculty of Veterinary Science. mRNA was extracted using the Qiagen RNeasy mini kit. Due to field circumstances counting of the cells was not possible, so the whole sample was used for the extraction, to maximize the amount of RNA extracted. The samples were mixed with 1.5 ml ice cold PBS, centrifuged for 10 minutes at 11.000 rpm, supernatant was discarded, after which the manufacturer's protocol was followed for the next steps. An extra step with on-clolumn DNase digestion was performed as described by the manufacturer (Qiagen®). The resulting mRNA was eluted in 50 µl of RNase-free water.

cDNA synthesis

The extracted mRNA was transcribed to cDNA, using the TaqMan MultiScribe Reverse Transcription kit (Applied Biostystems). A reverse transcription reaction included 12.5 µl of RNA, 5 µl 10x TaqMan RT Buffer, 11 µl 25 mM Magnesium Chloride, 10 µl deoxyNTPs Mixture, 2.5 µl Random Hexamers, 1 µl RNase Inhibitor, 1.25 µl MultiScribe Reverse Transcriptase (50 U/µl) and RNase-free water to 50 µl. The reverse transcription was performed with the following program: 10 min RT, 50 min at 42℃, 5 min at 95℃. Samples were stored at -20℃.

Real-Time PCR

The TaqMan Universal master mix (Applied Biosystems) was used, together with the StepOne Plus PCR instrument (Applied Biosystems) according to the manufacturer's instructions.

Cytokine primer and probe sequences described by Leutenegger (1999) and Kipar (2001) –who developed the primers and probes for domestic cat cytokines– were used for the PCR of lion cytokines and were purchased from IDT Inc. (table 9.1.).

Table 9.1. Primer and probe sequences for the various cytokines.

CYTOKINE	PRIMER	SEQUENCE 5' → 3'	LENGTH	PROBE	PROBE SEQUENCE 5' → 3'
Feline GAPDH	GADPH.57f	GCCGTGGAATTTGCCGT	82	GAPDH.77p	CTCAACTACATGGTCTA CATGTTCCAGTATGATTCCA
	GAPDH.138r	GCCATCAATGACCCCTTCAT			
IL-4	IL4.143f	GCATGGAGCTGACCGTCAT	81	IL4.169p	TGGCAGCCCCTAAGAA CACAAGTGACAA
	IL4.223r	CGGTTGTGGCTCTGCAGA			
IL-10	IL10.182f	TGCACAGCATATTGTTGACCAG	76	IL10.209p	ACCCAGGTAACCCTTAA GGTCCTCCAGCA
	IL10.257r	ATCTCGGACAAGGCTTGGC			
TNF-α	fTNFα239f	CTTCTCGAACTCCGAGTGACAAG	74	fTNFα266p	TAGCCCATGTAGTAGCAAAC CCCGAAGC
	fTNFα312r	CCACTGGAGTTGCCCTTCA			
IFN-γ	IFN.14f	TGGTGGGTCGCTTTTCGTAG	85	IFN.152p	CATTTTGAAGAACTGGAAAG AGGAGAGTGATAAAACAAT
	IFN.225r	GAAGGAGACAATTTGGCTTTGAA			

The sense and anti-sense primers were always placed in two consecutive exons of the gene. With these primers, short PCR products were selected, in order to achieve primer extension within a few seconds and to avoid the requirement of a separate extension step.

The TaqMan probes were labeled with a reporter (FAM, 6-carboxyfluorescein) at the 5' end and a quencher dye (TAMRA, 6 carboxytetramethyl-rhodamine) at the 3' end. By spanning the junction of the two exons of the primers, the feline-specific TaqMan probes only allowed detection of complementary DNA (cDNA) and no genomic DNA (gDNA), which minimizes gDNA-contamination.

The assay compositions for the 20 µl reactions were as follows: 10 µl TaqMan® Universal PCR Master Mix (2x), 1 µl primer/probe mix (20x) (consisting per 100 µl of 18 µl forward primer (100 µM), 18 µl reverse primer (100 µM), 5 µl probe (100 µM) and 59 µl nuclease free water), 2 µl of cDNA sample and 7 µl of nuclease free water.
The amplification conditions were the same for all cytokines assayed, therefore samples could be combined on the same 96-well plate: 2 min 50℃, 10 min 95℃, 40 cycles of 15s at 95℃ and one minute at 60℃.
Fluorescence was automatically measured during the PCR. Samples were assayed in duplicate in separate wells and the mean was obtained for further calculations. Every run included a no-template sample to check for potential contamination.

Housekeeping gene GAPDH

To reduce experimental variation and perform a reliable qRT-PCR, a correction is needed. This can be achieved by normalization to a housekeeping gene, which is a gene that is ideally expressed at a constant level among different tissues at all times and that is amplified simultaneously with the target cDNA. Vandesompele et al. (2002) recommended the use of three normalization genes, in our assay however, we used only one housekeeping gene because of limited amounts of the samples (Vandesompele et al. 2002).
We have used GAPDH, Glyceraldehyde-3-phosphate dehydrogenase, as a housekeeping gene.

Data analysis

Control samples: Every plate had a negative water control sample in duplicate and a positive lion control sample (of a lion from the Gauteng province) for the four target and the internal control (housekeeping) genes.

Ct: The Threshold Cycle (C_t) is defined as the fractional cycle number where the fluorescence exceeds a threshold (Giulietti et al. 2001) selected to occur during the exponential phase of amplification. If there is more template in the sample, it takes fewer cycles to reach a point where the level of fluorescence is first recorded to be significantly different above the background (Bustin 2000).

To compensate for day-to-day variations, the mean of the thresholds automatically determined by the software of the four plates was used to set a new threshold and the data was reanalyzed.

95% confidence interval: The mean of the Ct's of GAPDH was calculated plus and minus two times the standard deviation (SD) ($\underline{X}$Ct GAPDH +/- 2 SD). GAPDH values that were not within this range were excluded- and so were the cytokine Ct's of these samples.

PCR efficiency: To determine the PCR efficiency, three tenfold dilution steps (1:10, 1:100, 1:1000) of GAPDH, IFN-γ, TNF-α, IL-4 and IL-10 were amplified. The resulting Ct values were plotted against the log of the dilution of the sample, and the regression lines were calculated. The PCR efficiency was calculated as

$E=10[^{-1/slope}]-1$

The amplification rate (AR) was calculated by adding 1 to the PCR efficiency.

Quantification of cytokine transcripts: Calculation of the difference between the target and internal control Ct served to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the reverse transcriptase step. The target Ct values were subtracted of Ct values of the internal control GAPDH:

ΔCt= GAPDH Ct – target Ct

An imaginate ΔCt of 40 was created for samples where no cytokine signal was observed after 40 cycles to give ΔCt values for all four cytokines for all 29 lions. Subsequently, the mean was calculated of the ΔCt's of skin test positive lions and the ΔCt's of the skin test negative lions for all four cytokines.

For the calculation of the difference between the skin test positive group, and the skin test negative group, the mean was taken of all ΔCt's of the different groups, after which the $\Delta\Delta$Ct was calculated.

$\Delta\Delta$Ct= ΔCt mean negatives- ΔCt mean positives

Finally, the amount of target of the skin test positive group, normalized to an internal control and relative to the calibrator, was calculated by ($AR^{\Delta\Delta Ct}$). Thus, all the experimental samples are expressed as an n-fold difference relative to the calibrator (i.e. the negative skin test group).

Results

PCR efficiency

GAPDH had a PCR efficiency of 0,81 and an amplification of 1,81. TNF-α had respectively 0,87 and 1.87. IFN-γ, IL-4 and IL-10 PCR efficiencies could not be calculated, because diluted samples had Ct values beyond detection limits and no regression line could be drawn. For these cytokines, the amplification rate of 2 was used to calculate the relative expression of the cytokines in the different groups.

Cytokine Ct's

The two different values of the cytokine Ct's were in general very minimal. When determining the range of GAPDH that values should be in, two values of skin test negative lions did not fall in the range and the data of these animals were rejected. Two other negative lions had Ct values that were all beyond detection limits except for GAPDH, and a Ct of 40 was used for all the cytokines. Appendix 4 shows the mean Ct results, the ΔCt results and shows skin test and ELISA results. Table 9.2 offers a summary of the subsequent calculations.

Table 9.2. Relative quantification of cytokine transcripts

	IFN-γ	TNFα	IL-4	IL-10
ΔCt SICCT positive (n=19)	7.475	6,486	11,894	8,398
ΔCt SICCT negative (n=6)	9,165	7,734	10,041	8,624
ΔΔCt	1,690	1,248	-1,853	0,226
$AR^{\Delta\Delta Ct}$	3,23	2,18	0,28	1,17

This means that for IFN-γ, in skin test positive lions, expression of IFN-γ is more than 3 times higher than in skin test negative animals.

Discussion

A variety of methods can be used to quantify the cytokine expression of cells, but for samples with low expression of cytokines, reverse-transcription followed by PCR is the method of choice, since it is most sensitive and accurate. Though the reproducibility, and with that the usefulness, of the reverse transcriptase PCR is sometimes doubted, the reverse transcriptase real-time PCR assays are significantly less variable than reverse transcriptase PCR. The small quantities of target molecules means that trivial variations in the early stages of the PCR can greatly influence the final yield of the amplified product and this emphasizes the importance of repetitive testing (Bustin 2000). We used duplicate samples in our study. It would have been better if samples would be tested in triplicate, but this was not possible in our study, because of limited sample volumes.

During the white blood cell isolation in the field laboratory, difficulties were experienced in obtaining good pellets, and often the cell lysis of the erythrocytes was not total. Isolation of white blood cells in the field lab should be further optimised to increase the quality of samples used for cDNA synthesis.

For RT-PCR, there is a general consensus on using a single control gene for normalization. Literature however shows that although housekeeping genes occasionally may be constant in a given cell type or experimental condition, they can vary considerably. No single reference gene is suitable for any given set of conditions and some researchers prefer to use multiple housekeeping genes, arguing that tests would be more reliable then (Vandesompele et al. 2002, Harrington et al. 2007).
One of the most common housekeeping genes is GAPDH, which is an abundant glycolytic enzyme present in most cells (Vandesompele et al. 2002). Criticisms have existed in the past of the use of this enzyme (Giulietti et al. 2001), and Bustin (2000) in her overview gives several examples of situations in which GAPDH is not suitable as an endogenous control –for example when comparing different cell types (Bustin 2000). However, GAPDH has a proven stable expression in leucocytes and has shown to be functional in similar cytokine gene expression experiments that have been performed with cats. (Leutenegger et al. 1999, Vandesompele et al. 2002, Kipar et al. 2006)

The two-tube system, where the cDNA synthesis and Real-Time PCR are separated, has the advantage of generating a stable cDNA pool that can be used for multiple purposes and can be stored for a long time. The disadvantage is that it includes an extra step, which means an added risk of contamination. The use of mRNA-specific primers decreases background priming, whereas the use of random primers maximizes the number of mRNA molecules, so this last one is more favorable when working with small amounts of mRNA (Bustin 2000). Leutenegger showed that for this experiment on cat cytokine gene expression the two tube system was more sensitive than a one tube system (Leutenegger et al. 1999). Therefore, we also used the two-tube system.

PCR efficiencies were obtained for GAPDH and TNF-α and should be obtained for the other cytokines as well, using smaller dilution steps. It would even be better if a large positive control pool would be established, preferably by pooling the positive samples that will be tested, or by use of stimulated samples or plasmids. This control should be added in standard dilutions on every plate to determine PCR efficiency for each separate plate.

The preliminary results of the comparison between skin test positive lions and skin test negative lions, show that a three-fold increase of IFN-γ can be seen in the BTB infected lions (table 9.2.). TNF-α is expressed twice as much in BTB positive animals compared to negative animals. The difference in IL-10 expression is minimal. IL-4 is expressed almost four times lower in BTB positive animals than in BTB negative animals. However, the ratios of IFN-γ, IL-4 and IL-10 might be different than shown in this study, because many samples of the negative skin test lions showed Ct values that could not be determined, i.e. were likely to be higher than 40 (see appendix 4). By estimating the Ct too low, the expression of the cytokine will overestimated. For IFN-γ and IL-10 this means that the difference may even be more pronounced, and for Il-4 this means that instead of being more expressed in skin test negative animals, expression could be higher in skin test positive animals. More samples with a complete Ct set need to be obtained in the future to show this.

In conclusion, we succeeded in developing of a Real-Time qPCR to determine the gene expression of the lion cytokines IFN-γ, TNF-α, IL-4 and IL-10. The RT-PCR

technique for quantification of IFN-γ mRNA levels may be used even as a diagnostic tool for the detection of *M. bovis* lions. This test would have the advantage for wildlife that only one handling event would be necessary, in contrast to the skin test (Harrington et al. 2007). However, the method needs further optimisation and validation –a process that is hindered by the difficult circumstances the samples are collected in the field. For the classification of lions as Th1- or Th2-animals, more information should be available about the infection status of the animals, for which other tests should be developed first or experimental infections should be performed.
A further step could also be determining the cytokine gene expression in lymph nodes, because results from Thacker et al (2007) show that interesting gene expression patterns can be observed in cattle (Thacker, Palmer & Waters 2007).

Chapter 10

Lion (*Panthera leo*) and cheetah (*Acinonyx jubatus*) IFN-γ sequences

Introduction

In 1995, infection of lions in the Kruger National Park (KNP) with bovine tuberculosis (BTB) became evident (Keet et al. 1996). Lions affected with tuberculosis often show no clinical signs for a long period, and the onset of the disease can be very sudden (Eulenberger et al. 1992). IFN-γ is a cytokine produced mainly by lymphocytes and plays an important role in the cell-mediated immunity (CMI) and the control of *M. bovis* in infected animals. IFN-γ is produced by *in vivo* primed T-cells and thus, the production of IFN-γ upon *in vitro* stimulation with *M. bovis* antigens shows prior exposure of the adaptive immune system to *M. bovis.* The sequence of the domestic cat (*Felis catus*) IFN-γ gene was published in 1995 by Argyle et al (Argyle et al. 1995)(accession number NM001009873) and by Schijns et al (Schijns et al. 1995)(accession number X86972). The coding part of the domestic cat IFN-γ gene is 504 base pairs (bp) long. The IFN-γ gene sequence of the lion (*Panthera leo*), or other exotic feline and canine species, has not been sequenced.

The main goal of the work described here was sequence the lion IFN-γ gene. Once the sequence is known, it can be used for a variety of diagnostic and research purposes. For example, the coding sequence of IFN-γ could be expressed and the purified protein could be used to create monoclonal antibodies that could be used in an IFN-γ capture ELISA that would be specific for lion and/or felid species. Recombinant IFN-γ could be used as a positive control in IFN-γ capture ELISA tests.
We also intended to obtain full length cDNA of the IFN-γ gene of more exotic feline species besides lion to make a comparative overview of the genetic relations and distances in the feline group for the IFN-γ gene. Upon expression of the recombinant IFN-γ this may be used for separate research and diagnostic purposes as well.

Material and methods

<u>The animals</u>

Lion blood was collected from three lions in the Gauteng province, SA.
Lion 1 was a subadult male, kept in a tourist park in Gauteng province.
Lion 2 was a captive adult white lion.
Lion 3 was a privately kept male lion, 5.5 months old.
The cheetah blood was collected from a privately kept, wild caught adult male cheetah in the Gauteng province, SA.

PBMC isolation

Blood was collected in EDTA tubes and PBMCs were isolated by density gradient centrifugation with Histopaque (1.077 density, Sigma). The mononuclear cells were collected from the interphase, washed twice in ice cold PBS and diluted in culture medium (RPMI 1640, supplemented with 2mM L-glutamine, 5,000 units Penicillin, and 5,000 µg Streptomycin). They were either stored directly in RNAlater at -20℃ until further use (lion 1) or stimulated with 10 µg Concanavalin A (Con-A)/ml in a 24-well tissue culture plate in a CO_2 incubator at 37℃ for 18-26 hours (lion 2, lion 3 and cheetah) at a cell concentration of 1 million cells/ml. After incubation, the cells were stored in RNAlater at -20℃.

RNA extraction and cDNA synthesis

mRNA was extracted from the samples using the RNeasy® mini kit (Qiagen) according to the protocol of the manufacturer. The mRNA was immediately used in a reverse transcriptase reaction, using the TaqMan ® Reverse Transcription Reagents (Applied Biosystems): 2.0 µl 10x TaqMan RT buffer, 4.4 µl 25mM Magnesium Chloride, 4.0 µl deoxyNTPs Mixture, 1.0 µl Random Hexamers, 0.4 µl RNase Inhibitor, 0.50 µl MultiScribe Reverse Transcriptase (50U/µL) and 5 µl (lion 2 and 3) or 7.7 µl (lion 1 and cheetah) mRNA. RNase free H_2O was added to create 20 µl reactions. The resulting cDNA was stored at -20℃ u ntil further use.

Primer development, PCR, gel electrophoresis and purification

The primers used for the full length lion and cheetah IFN-γ PCR reactions were developed using the known mRNA sequences of the domestic cat (*Felis catus)* and the dog (*Canis lupus familiaris*) (NCBI cat: NM_001009873, D30619, AY878359, X86972; NCBI dog: NM_001003174) since it was expected that these were closest to lion and the other species of felids. Primers were obtained from IDT Inc. (Table 10.1.).

5´ end	GGC CTA ACT CTC YGA AAC GAT G	Tm= 56,5°C
3´ end	CAA ATA TTG CAG GCA GGA YRA CC	Tm= 56,6°C

Table 10.1. IFN-γ primers

Per sample 0.5 µl cDNA was used for the PCR. The PCR conditions were 1 cycle of 94℃ for 5 minutes, 35 cycles of 94℃ for 30 second s, 53℃ for 45 seconds and 72℃ for 30 seconds and a final cycle of 72℃ for 7 minu tes.
The resulting amplicons were run on a gel to confirm products of the expected sizes and the positive samples were subsequently purified with the MinElute PCR purification kit (Qiagen), following the manufacturer´s protocol.

Cloning

DNA was quantitated with a spectrophotometer using the Warburg and Christian method (BECKMAN COULTER™ DU®530 LifeScience UV/Vis Spectrophotometer, Single cell module) (260 nm) and resulting readings were used to calculate the correct amounts of DNA to be ligated in a pGEM®-T vector according to the manufacturer's instructions (Promega Corp, Madison, WI, USA). The ligations were left overnight at 4℃. The pGEM ®-T vector with ligated DNA was inserted in JM109 High Efficiency Competent Cells that were grown overnight and plated out onto duplicate LB/Ampicillin/IPTG/X-Gal plates. White colonies were picked and grown overnight in LB medium. An IFN-γ PCR was run with 0.5 µl of the bacteria suspension in LB medium. Suspensions with inserts of the correct size were centrifuged (1 min 10.000 rpm) plasmids were extracted using the High Pure Plasmid Isolation Kit (Roche Applied Science) following the manufacturer's protocol. These plasmid preparations (Lion 1: four preparations, Lion 2: one preparation, Lion 3: one preparation, Cheetah: five preparations) were sent in for sequencing on both strands using the standard pGEM®-T SP-6 and T-7 primers (Inqaba Biotec, South Africa).

Data analyis

With Chromas 2 the baseplot that was obtained with sequencing was checked for misreadings. Subsequently, the two sequences were merged using both strands with readings in opposite directions. With a BLAST search the sequencing of IFN-γ gene was confirmed and subsequently, the merged sequences were aligned in Clustal W (http://align.genome.jp/) with most similar sequences.

Results

We obtained one full length IFN-γ sequence of each of the three lions, and of two of the cheetah sequences –the other sequences were rejected because extra stop

codons were included (see appendix 5 for the merged sequences). The sequences were confirmed as IFN-γ by BLAST searches, with coding regions of 504 bp long. Local alignment was performed with Clustal W with the domestic cat sequence that showed the greatest homology (X86972.1) (Figure 10.1. and 10.2). Subsequently, the obtained sequences were compared with the NCBI BLAST program with the two most similar sequences of domestic cat (NCBI: X86972.1 and NM_001009873.1) and the sequence of dog (NCBI: NM_001003174.1) (Table 10.2.).

Table 10.2. Overview of the homology (in percentages) between the obtained sequences and two sequences of the domestic cat (X86972.1 and NM_001009873.1) and a dog sequence (NM_001003174.1). In brackets is the number of different nucleotides.

Species	Cat 1 (X86972.1)	Cat 2 (NM_001009873.1)	Dog (NM_001003174.1)
Lion1	98 (6)	98 (7)	89 (52)
Lion2	98 (8)	98 (9)	89 (54)
Lion3	98 (6)	98 (7)	89 (52)
Cheetah clone 1	99 (5)	98 (6)	89 (53)
Cheetah clone 2	99 (4)	99 (5)	89 (52)

Figure 10.1. Clustal Alignment of IFN-γ cDNA nucleotide sequences of lion 1, lion 2, lion 3, two cheetah clones and domestic cat (accession number X86972.1).

```
Cheetah clone 1             ATGAATTACACAAGTTTTATTTTCGCTTTTCAGCTTTGCATAATTTTGTG
Cheetah clone 2             ATGAATTACACAAGTTTTATTTTCGCTTTTCAGCTTTGCATAATTTTGTG
Lion 1                      ATGAATTACACAAGTTTTATTTTCGCTTTTCAGCTTTGCATAATTTTGTG
Lion 3                      ATGAATTACACAAGTTTTATTTTCGCTTTTCAGCTTTGCATAATTTTGTG
Lion 2                      ATGAATTACACAAGTTTTATTTTCGCTTTTCAGCTTTGCATAATTTTGTG
Domestic Cat X86972.1       ATGAATTACACAAGTTTTATTTTCGCTTTCCAGCTTTGCATAATTTTGTG
                            ***************************** ********************

Cheetah clone 1             TTCTTCTGGTTATTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
Cheetah clone 2             TTCTTCTGGTTATTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
Lion 1                      TTCTTCTGGTTGTTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
Lion 3                      TTCTTCTGGTTGTTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
Lion 2                      TTCTTCTGGTTGTTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
Domestic Cat X86972.1       TTCTTCTGGTTATTACTGTCAGGCCATGTTTTTTAAAGAAATAGAAGAGC
                            *********** ************************************

Cheetah clone 1             TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
Cheetah clone 2             TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
Lion 1                      TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
Lion 3                      TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
Lion 2                      TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
Domestic Cat X86972.1       TAAAGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGATGGTGGGTCG
                            ************************************************

Cheetah clone 1             CTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
Cheetah clone 2             CTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
Lion 1                      CTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
Lion 3                      CTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
Lion 2                      CTTTTCGTAGACATTTCAAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
Domestic Cat X86972.1       CTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
                            ***************  ********************************

Cheetah clone 1             AATTCAAAGCCAAATTGTCTCCTTCTACTTGAAAATGTTTGAAAACCTGA
Cheetah clone 2             AATTCAAAGCCAAATTGTCTCCTTCTACTTGAAAATGTTTGAAAACCTGA
Lion 1                      AATTCAAAGCCAAATTGTCTCCTTCTACTTGAAAATGTTTGAAAACCTGA
Lion 3                      AATTCAAAGCCAAATTGTCTCCTTCTACTTGAAAATGTTTGAAAACCTGA
Lion 2                      AATTCAAAGCCAAATTGTCTCCTTCTACTTGAAAATGTTTGAAAACCTGA
Domestic Cat X86972.1       AATTCAAAGCCAAATTGTCTCCTTCTACCTGAAAATGTTTGAAAACCTGA
                            ***************************** ********************

Cheetah clone 1             AAGATGATGACCAGCGCATTCAAAGGAGCATGGACACCATCAGGGAAGAT
Cheetah clone 2             AAGATGATGACCAGCGCATTCAAAGGAGCATGGACACCATCAAGGAAGAT
Lion 1                      AAGATGATGACCAGCGCATTCAAAGGAACATGGACACCATCAAGGAAGAC
Lion 3                      AAGATGATGACCAGCGCATTCAAAGGAACATGGACACCATCAAGGAAGAC
Lion 2                      AAGATGATGACCAGCGCATTCAAAGGAACATGGACACCATCAAGGAAGAC
Domestic Cat X86972.1       AAGATGATGACCAGCGCATTCAAAGGAGCATGGACACCATCAAGGAAGAC
                            *************************** ************** ******

Cheetah clone 1             ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
Cheetah clone 2             ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
Lion 1                      ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
Lion 3                      ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
Lion 2                      ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
Domestic Cat X86972.1       ATGCTTGATAAGTTGTTAAATACCAGCTCCAGTAAACGGGATGACTTCCT
                            ************************************************

Cheetah clone 1             CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
Cheetah clone 2             CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
Lion 1                      CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
Lion 3                      CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
Lion 2                      CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
Domestic Cat X86972.1       CAAGCTGATTCAAATCCCTGTGAATGATCTGCAGGTCCAGCGCAAAGCAA
                            ************************************************
```

```
Cheetah clone 1             TAAATGAACTCTTCAAAGTGATGAATGATCTCTCACCAAGATCTAACCTG
Cheetah clone 2             TAAATGAACTCTTCAAAGTGATGAATGATCTCTCACCAAGATCTAACCTG
Lion 1                      TAAATGAACTCTTCAAAGTGATGAACGATCTCTCACCAAGATCTAACCTG
Lion 3                      TAAATGAACTCTTCAAAGTGATGAACGATCTCTCACCAAGATCTAACCTG
Lion 2                      TAAATGAACTCTTCAAAGTGATGAACGATCTCTCACCAAGATCTAACCTG
Domestic Cat X86972.1       TAAATGAACTCTTCAAAGTGATGAATGATCTCTCACCAAGATCTAACCTG
                            ************************ ************************


Cheetah clone 1             AGGAAGCGGAAAAGGAGTCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
Cheetah clone 2             AGGAAGCGGAAAAGGAGTCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
Lion 1                      AGGAAGCGGAAAAGGAGTCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
Lion 3                      AGGAAGCGGAAAAGGAGTCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
Lion 2                      AGGAAGCGGAAAAGGAGTCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
Domestic Cat X86972.1       AGGAAGCGGAAAAGGAGCCAGAATCTGTTTCGAGGCCGTAGAGCATCGAA
                            ***************** ********************************

Cheetah clone 1             ATAA
Cheetah clone 2             ATAA
Lion 1                      ATAA
Lion 3                      ATAA
Lion 2                      ATAA
Domestic Cat X86972.1       ATAA
                            ****
```

Figure 10.2. Deduced amino amino acids sequences of lion 1, lion 2, lion 3, two cheetah clones and domestic cat (accession number X86972.1).

```
Cheetah clone 1       MNYTSFIFAFQLCIILCSSGYYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Domestic cat          MNYTSFIFAFQLCIILCSSGYYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Cheetah clone 2       MNYTSFIFAFQLCIILCSSGYYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Lion 1                MNYTSFIFAFQLCIILCSSGCYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Lion 2                MNYTSFIFAFQLCIILCSSGCYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDISKNWK
Lion 3                MNYTSFIFAFQLCIILCSSGCYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
                      ****************** ******************************** ****

Cheetah clone 1       EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRSMDTIREDMLDKLLNTSSSKRDDFLKLI
Domestic cat          EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRSMDTIKEDMLDKLLNTSSSKRDDFLKLI
Cheetah clone 2       EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRSMDTIKEDMLDKLLNTSSSKRDDFLKLI
Lion 1                EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRNMDTIKEDMLDKLLNTSSSKRDDFLKLI
Lion 2                EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRNMDTIKEDMLDKLLNTSSSKRDDFLKLI
Lion 3                EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRNMDTIKEDMLDKLLNTSSSKRDDFLKLI
                      ******************************.****:**********************

Cheetah clone 1       QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Domestic cat          QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Cheetah clone 2       QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Lion 1                QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Lion 2                QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Lion 3                QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
                      *******************************************
```

The alignment of the three lions, the two clones of the cheetah and the domestic cat sequence (accession number X86972.1) showed ten nucleotides difference in the sequences that resulted in four differences in the amino acids:

- First AA difference: cheetahs+cat have Tyrosine (Y) instead of cysteine (C) that the lions have. Both are polar AA.

- Second AA difference (in the lions): 1 lion has serine (S) instead of leucine (L), which is a polar (hydrophylic) AA instead of a nonpolar (hydrophobic) AA

- Third AA difference: cheetahs+cat have serine (S) instead of asparagines (N), both polar AA.

- Fourth AA difference: 1 cheetah has arginine (R) instead of lysine (K), which is considered a conservative mutation.

Table 10.3. Overview of homology between Lion 1, Lion 2, Lion 3 and the two cheetah clones on AA level.

Species	Lion1	Lion2	Lion3	Cheetah clone 1	Cheetah clone 2	Domestic cat X86972.1
Lion1	-	99	100	98	98	98
Lion2	-	-	99	97	98	98
Lion3	-	-	-	98	98	98
Cheetah clone 1	-	-	-	-	99	99
Cheetah clone 2	-	-	-	-	-	100

Discussion and conclusion

The IFN-γ gene sequence in the studied species is well conserved, and part of the observed differences could be due to PCR artefacts. For two lions the IFN-γ gene sequence is 100% equal, however, the third lion shows a difference of 2 nucleotides, resulting in one different amino acid with different characteristics. This change is interesting, since this lion was a so-called “white lion”. These are not distinct subspecies, but due to a recessive gene they have a lighter, cream colour of the coat. It can be suspected that the different amino acid in the white lion could be a genetic difference that could be characteristic for white lions in general or a result of inbreeding. More white and tawny coloured (the “normal” colour) lions should be tested for their IFN-γ gene sequence to get more information about this, and to see whether polymorphism with eventual functional consequences is present. For this study, it was chosen only to use samples that were processed in optimal circumstances (i.e. field samples were excluded), but these samples are difficult to obtain. It remains to be established whether the minor difference in AA sequence in IFN-γ has consequences for the adaptive immune response -to BTB or maybe other diseases threatening the lion population.

Unfortunately, no other exotic feline IFN-γ sequences were obtained. This will be a valuable aim for the future, so the proposed comparative overview of the genetic relations and distances in the feline group for the IFN-γ gene can be made. Adding sequences of exotic canines in this comparative overview, is likely to show there is a closer relationship between cat, lion and cheetah, than between these species and canine species, as already shown in BLAST results of nucleotide and AA IFN-γ sequences of cat, lion, cheetah and dog.
The high homology between domestic cat, lion and cheetah gives reason to believe that a lion-specific IFN-γ ELISA could possibly also be used for other feline species.

The now obtained lion IFN-γ sequence can be used for continuous research work on the development of a species specific IFN-γ ELISA, and for the necessary production of recombinant IFN-γ. With this, more specific diagnostic tests for BTB can be developed.

Chapter 11

Summarizing discussion

Bovine tuberculosis, caused by infection with *M. bovis*, in the Kruger National Park is a major problem without a solution at present. Since the infection seems to have been established in a multitude of species –buffalo, kudu, lion, and maybe many more- eradication becomes very difficult, as the infection perpetuates in these maintenance hosts (Keet et al. 1996).

The OIE aims for useful diagnostic tests following the 'Fit for purpose'-concept. This means that the purpose of a test, which can be different per site, species and disease, must be determined first. For wildlife species culling is no reasonable management option, because culling does not meet with ecological or ethical acceptance. Wildlife management in general often aims at controlling infections. This means that animals should be identified early in the infectious stage, before or early after onset of shedding the bacteria.

The skin test based on assessment of cell-mediated immunity is generally considered to be the earliest immune-responsiveness after infection and is used most frequently for diagnosis of BTB in wildlife at present. Unfortunately, it is very impractical because a recapture after three days is necessary, which is a time-consuming and costly procedure. The importance of the recapture was experienced during the sampling period of this study, where many samples couldn't be classified by skin test because the lions could not be recaptured. This resulted in a limited number of samples that could be used later for the development and validation of diagnostic tests.

In view of these difficulties, it is important to develop other tests that only need one capture-moment. The IFN-γ ELISA is a promising alternative to measure CMI, also indentified by the skin test. Diagnosis of bovine tuberculosis in cattle is performed worldwide, using a commercially available bovine IFN-γ ELISA. A multi-species IFN-γ ELISA developed by Morar et al. was tested in our studies, but has shown to be inconclusive and needs optimization. For diagnosis of BTB in lions, a species-specific test might be a better solution for the future. For that reason we started cloning and sequencing of the lion IFN-γ gene, the first step towards the expression of the recombinant cytokine, which may be used in the future to produce monoclonal antibodies and to produce a lion-specific IFN-γ ELISA. Similarly, the cheetah IFN-γ was cloned and sequenced. Alignment of nucleotide and amino acid sequences of these two species and that of domestic cats showed high homology between these

species, thus it is highly likely that an assay once it is established may be used for several feline species. Recently, a feline IFN-γ ELISPOT has been developed, that showed the potential to be used to diagnose *M. bovis* infected cats (Rhodes et al. 2000). Future research needs to be performed to investigate the use of this test for exotic species.

Serologic tests could be used as complementary tests next to CMI tests, especially since in other species it is shown that detection of antibodies indicates the stage of disease in which shedding of bacteria is likely to occur. The current serologic tests for cattle and several wildlife species still suffer from low sensitivity and specificity. In the present study we used the *M. bovis* recombinant antigens MPB83, MPB70, DiaSer3 and CFP10, considered to be so called latency antigen, that are produced in the latent phase early after infection, in the indirect ELISA. With a high specificity, the four antigens showed sensitivities ranging from 23% to 5% -MPB83 gave the highest sensitivity and CFP10 the lowest. Our results show that the antigens that were tested for this study are not sensitive enough to be used alone, but combinations may be used in the future to improve sensitivity.

Lyashchenko et al. (2008) produced a rapid test (Chembio Diagnostic Systems) with a cocktail of selected *M. bovis* antigens including ESAT-6, CFP10 and MPB83 for several wildlife species –including white tailed deer, wild boar and possums- and the test based on MultiAntigen Print ImmunoAssay (MAPIA) to further define specificity of positive samples. Combination of recombinant antigens resulted in high specificity (85.7 to 98.9% for different species) and sensitivities from 44.7 to 76.6% for the different species. This confirms our results, where DiaSer3 had a higher sensitivity than CFP10.

Welsh et al. (2005) described that strong IgG1 humoral responses in BTB infected cattle correlate with a more widespread pattern of tuberculosis. This association has been described by Lyashchenko et al. (2008), whose rapid test was most effective at detecting animals with severe disseminated BTB (Lyashchenko et al. 2008), i.e. a positive association was shown between antibody responses and advanced disease. This could confirm the suspected correlation between antibody responses and shedding.

Understanding the contribution of Th1 and Th2 responses to protective immunity and pathology of *M. bovis* infection may contribute to development of new diagnostic methods for *M. bovis* infection in lions. Assessment of cytokine expression of cells in relation to other immune response parameters, might offer insight in immune responses and changes in the immune response during progression of *M. bovis* infection in lions. Therefore, development of relevant cytokine specific RT qPCR was started, based on homology of cytokine genes with those of domestic cats. Proof of principal was obtained for lion cytokines IFN-γ, IL-4, TNF-α and IL-10, the relevant cytokines to assess reactivity of the various types of T-helper cells. In the present study only a limited number of samples of 29 lions could be tested. Preliminary results show that for IFN-γ, TNF-α, and IL-10 increase of gene expression was seen in skin positive animals, of which the most pronounced was the increase of gene expression for the IFN-γ gene. IL-4 was less expressed in skin test positive animals, but this result might be faulty due to many Ct's that were beyond detection level. Before the RT qPCR can be more optimised and validated, the procedures during field surveys need further optimization,. Determination of cytokine gene expression in additional blood samples, lymph nodes and tonsils may be performed to see whether interesting patterns as preliminary of cattle infected with *M. bovis* may be mimicked (Thacker, Palmer & Waters 2007).

Though improvements in diagnostic tests for tuberculosis are likely in the mid term, it will not be a final solution for the problem and regular sampling of the animals will always remain a time-consuming and costly management tool. Vaccination doesn't have the economical, ethical and practical issues that culling of animals has (de Lisle et al. 2002, Cross, Buddle & Aldwell 2007), though costs of vaccination are still high. The recent progress that is made in development of live bait vaccines for various infectious diseases, makes live bait vaccination a viable option. Besides finding a suitable vaccine for lions, vaccine trials should be continued for buffalo and kudu, to decrease the BTB prevalence in these maintenance species, both main prey species of lions and important infection routes.

Since antibody responses and shedding seem to be correlated in various species, and animals that are shedders of *M. bovis* are likely to show antibody titers that can be detected by serologic tests, this needs to be verified for lions. To prove this

association for lions too, more research on the correlation between immune response and pathogenesis needs to be done and further necropsies and tracheal flushings should be performed. Showing this association may give more insight in the disease progression and possibilities of the assessment of tracheal flushings and may offer management tools for tuberculosis in lions in the KNP. Subsequently, information of necropsies, serology and CMI testing should be combined. Our study showed for half of the tracheal flushings that acid-fast bacteria could be found with microscopy. No bacteria could be cultured however, but this might have been due to the storage methods.

Since it was shown that an average of almost 50% of the KNP lions is infected with LLV, more research needs to be performed on the effects of LLV on the lion health, as well as on the correlation between LLV and BTB, though our preliminary results show no correlation. The prevalence of LLV infection in lions in the north and the south of the KNP is almost equal, and the prevalence of BTB in lentivirus-infected lions is also similar to the prevalence of BTB in non-lentivirus-infected lions. A correlation may be found if larger data sets become available.

In conclusion, BTB diagnosis and development of new diagnostic methods for lions has proven to be difficult. Very little research has been performed yet on lion BTB diagnostics and the present project was the first to combine different diagnostic methods. Low sample sizes and to a certain extend samples of variable quality due to field conditions, used to perform the various tests –some of which still need further development or have low sensitivity– complicated analysis of test outcomes and interpretation of results in view of progression of BTB in lions. The number of animals that was recaptured during the surveys to read the skin test –of crucial importance since it is still considered the gold standard next to culture– was relatively low. Without an easy, sensitive and specific test it is difficult to determine *M. bovis* infection status in lions, which makes it difficult to validate new diagnostic tests. This emphasizes the need for development of a useful diagnostic test, like the IFN-γ assay for *M. bovis* infection even more.

This study has made some first steps in the development of new diagnostic tests and has helped making promising progress in others. It has shown the pro’s and con’s of the various techniques, and on basis of this report, decisions may be made to focus

future research on lion BTB diagnostics, that will ultimately result in one (or more) test(s) applicable under field conditions.

Abstract

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis (M. bovis)*, was most likely introduced in South Africa by the first imported European cattle breeds during the 18th and 19th century. The rapid spread of BTB amongst the lion population in the Kruger National Park (KNP) raises concerns about the future of these animals, one of the main tourist attractions of the park. The main goal of the presented study was to develop better insight in the development of the immune response of lions infected with *M. bovis*.

During a field survey in the Kruger National Park, the tuberculin skin test, based on responsiveness of cell-mediated immunity in the early stages of infection, was performed to determine disease status of the lions. However, the reading of the skin test during recapture could only be performed for 29 lions, confirming the need of a test that does not need recapture of the animals. Of these 29 animals, 23 animals were classified as positive animals. To find proof of lions shedding *M. bovis* six tracheal flushings were performed, smears of the flushings were coloured with Ziehl-Neelsen, checked for mycobacteria with microscopy and subsequently cultured. In three smears, acid-fast bacteria were found, an indication of presence of *M. bovis*, but this could not be confirmed by culture.

A multi-species IFN-γ ELISA was optimized for lions, but the ELISA could not be completed due to difficulties that were experienced in the process of optimization of the test. Sequencing of the lion IFN-γ gene has been performed, which may be a first step in development of a lion-specific IFN-γ ELISA. The similarity between the sequences of the lion, cheetah and domestic cat suggests that this IFN-γ ELISA may also be used as a feline-specific IFN-γ ELISA.

To determine BTB infection status in lions in later stages of the disease, indirect ELISA's were developed for the recombinant antigens CFP10 and DiaSer3. Test characteristics of these two iELISA's and the MPB70 and MPB83 iELISA's, showed that, with a high specificity, the iELISA's for these four antigens have low sensitivities –a difficulty of serology-based tests that is generally acknowledged and that might be avoided by using multiple recombinant antigens together. The four iELISA's where subsequently used to test the KNP lion serum samples collected during the field surveys, classifying a low number of samples as positive.

With the development of a Real-Time reverse transcriptase PCR for lion cytokines IFN-γ, TNF-α, IL-4, and IL-10 we introduced new methods for BTB diagnostics in

lions. This RT qPCR has been used on 29 lions of the KNP with confirmed skin test results. For IFN-γ, TNF-α, and IL-10 increases of gene expression were seen in skin positive animals, of which the most pronounced was the increase of gene expression for the IFN-γ gene. IL-4 was less expressed in skin test positive animals than in skin test negative animals. In four out of six negative skin test lions the Ct for IL-4 was beyond detection level however. If more samples and data become available in the future, the test can be validated and the technique could be used as well to classify stage of disease, i.e. Th1 or Th2 stage animals.

With the data of this study, no correlation can be shown between LLV and BTB infection. An average of almost 50% of the KNP lions is infected with LLV, but the prevalence of LLV infection in lions in the north and the south of the KNP is almost equal, and the prevalence of BTB in lentivirus-infected lions is also similar to the prevalence of BTB in non-lentivirus-infected lions.

Low sample sizes and to a certain extend variable sample quality due to field conditions, of the samples used to perform the various tests –some of which need further development still or have low sensitivity– complicates analysis of test outcomes and interpretation of results in view of progression of BTB in lions. Without an easy, sensitive and specific test it is difficult to determine *M. bovis* infection status in lions, which makes it difficult to validate new diagnostic tests, and the preliminary results of the various diagnostic tests in this study could change if larger data sets become available. This emphasizes the need for development of a useful diagnostic test for *M. bovis* infection in lions and with this study, progress to achieve that goal has been made.

Acknowledgements

Without the chance to perform an Excellent Track research, this project would not have taken place and I would like to start to thank the CIDD/CHPD and the Faculty of Veterinary Science for that. For the financial support of this project, I would like to thank both the Utrecht University, Department of Infectious Diseases and Immunology and the University of Pretoria, Department of Tropical Veterinary Diseases, Onderstepoort. A scholarship was received from the “Stichting Jubileumfonds” and a “Trajectumbeurs” was received from the University of Utrecht.
The Veterinary Sciences Division, Belfast, United Kingdom and the Statens Serum Institute, Copenhagen, Denmark provided both two antigens that were used in this study for development of iELISA’s.

My supervisors have been a great support, particularly Victor Rutten, who spent many hours answering all my e-mails. Many thanks go also out to Jacques Godfroid, Hans Heesterbeek and Dewald Keet.

I would also like to thank the people at the Division of State Veterinary Services of Skukuza from the Kruger National Park: At Dekker, Schalk van Dyk, Johan Oosthuizen, Dewald Keet and Kenneth Muchocho, who helped collecting samples during the survey. It was nice, but hard work and I am very grateful for the wonderful learning experience.
I am also very thankful for all the help of Sr. Marizelle de Cock from the Onderstepoort Veterinary Animal Hospital, who has collected cat blood for me many times. Without her, it would have been much more difficult to perform all the tests that were necessary. The Onderstepoort Veterinary Animal Hospital provided equine and bovine horse on several occasions.
Many others have helped me in the search of lion/exotic cat blood: Dr. Claire Speedy, Dr. Peter Caldwell, prof. Leon Ventor and the exotic animal clinic Onderstepoort.

Naturally, many people working at the Department of Veterinary Tropical Diseases supported me with my research, for which I am very grateful: Head of the Department Prof. Koos Coetzer, Darshana Morar, Dr. Paula Allsopp, Dr. Marinda Oosthuizen, Dr. Melvyn Quan, Dr. Jannie Crafford, prof. Moritz van Vuuren and Sandra Prinsloo. It would have been impossible to have done all the work without them.

From Utrecht University, Ildiko has been a great support, and Tawee and Peter have both performed a lot of work on the IFN-γ ELISA in Utrecht. Louis Penning has helped me with analyzing RT qPCR data.

My fellow post-grad students at Onderstepoort were great: always ready to answer questions, or to listen to complaints if things had gone wrong. In a year away from home, they became my surrogate family and good friends.
My house mates at Flemming house fulfilled the same roles, joining me on many trips through South Africa to discover this beautiful country. My car only broke down a couple of times - I thank Ian and JJ for that.

Last but not least I would like to thank my family: my sisters, but especially my parents, who have supported me immensely during this year, as they have always done during my life.

References

Aldwell, F.E., Keen, D.L., Parlane, N.A., Skinner, M.A., de Lisle, G.W. & Buddle, B.M. 2003, "Oral vaccination with Mycobacterium bovis BCG in a lipid formulation induces resistance to pulmonary tuberculosis in brushtail possums", *Vaccine,* vol. 22, no. 1, pp. 70-76.

Andersen, P., Munk, M., Pollock, J. & Doherty, T. 2000, "Specific immune-based diagnosis of tuberculosis", *The Lancet,* vol. 356, no. 9235, pp. 1099-1104.

Andersen, P., Doherty, T.M., Pai, M. & Weldingh, K. 2007, "The prognosis of latent tuberculosis: can disease be predicted?", *Trends in Molecular Medicine,* vol. 13, no. 5, pp. 175-182.

Argyle, D.J., Smith, K., McBride, K., Fulton, R. & Onions, D.E. 1995, "Nucleotide and predicted peptide sequence of feline interferon-gamma (IFN-γ)", *DNA Sequence-The Journal of Sequencing and Mapping,* vol. 5, pp. 169-171.

Brewer, C.F. & Bhattacharyya, L. 1986, "Specificity of concanavalin A binding to asparagine-linked glycopeptides. A nuclear magnetic relaxation dispersion study", *The Journal of biological chemistry,* vol. 261, no. 16, pp. 7306-7310.

Buddle, B.M., Skinner, M.A., Wedlock, D.N., Collins, D.M. & de Lisle, G.W. 2002, "New generation vaccines and delivery systems for control of bovine tuberculosis in cattle and wildlife", *Veterinary immunology and immunopathology,* vol. 87, no. 3-4, pp. 177-185.

Buddle, B.M., Wedlock, D.N. & Denis, M. 2006, "Progress in the development of tuberculosis vaccines for cattle and wildlife", *Veterinary microbiology,* vol. 112, no. 2-4, pp. 191-200.

Buddle, B.M., Ryan, T.J., Pollock, J.M., Andersen, P. & de Lisle, G.W. 2001, "Use of ESAT-6 in the interferon-γ test for diagnosis of bovine tuberculosis following skin testing", *Veterinary Microbiology,* vol. 80, no. 1, pp. 37-46.

Bustin, S.A. 2000, "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays", *Journal of Molecular Endocrinology,* vol. 25, no. 2, pp. 169-193.

Cardullo, R.A., Agrawal, S., Flores, C., Zamecnik, P.C. & Wolf, D.E. 1988, "Detection of Nucleic Acid Hybridization by Nonradiative Fluorescence Resonance Energy Transfer", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 85, no. 23, pp. 8790-8794.

Caron, A., Cross, P.C. & Toit, J.T.d. 2003, "Ecological Implications of Bovine Tuberculosis in African Buffalo Herds", *Ecological Applications,* vol. 13, no. 5, pp. 1338-1345.

Cheeseman, C.L., Wilesmith, J.W. & Stuart, F.A. 1989, "Tuberculosis: The Disease and Its Epidemiology in the Badger, a Review", *Epidemiology and infection,* vol. 103, no. 1, pp. 113-125.

Cleaveland, S., Mlengeya, T., Kazwala, R.R., Michel, A., Kaare, M.T., Jones, S.L., Eblate, E., Shirima, G.M. & Packer, C. 2005, "Tuberculosis in Tanzanian Wildlife", *Journal of Wildlife Diseases,* vol. 41, no. 2, pp. 446-453.

Clifton-Hadley, R.S., Wilesmith, J.W. & Stuart, F.A. 1993, "Mycobacterium bovis in the European Badger (Meles meles): Epidemiological Findings in Tuberculous Badgers from a Naturally Infected Population", *Epidemiology and infection,* vol. 111, no. 1, pp. 9-19.

Coetzer, J.A.W. & Tustin, R.C. 2004, "Chapter 196 Tuberculosis" in *Infectious diseases of livestock*, 2nd edn, Oxford Southern Africa, pp. 1965-1993.

Corner, L.A.L., Norton, S., Buddle, B.M. & Morris, R.S. 2002, "The efficacy of bacille Calmette-Guerin vaccine in wild brushtail possums (Trichosurus vulpecula)", *Research in veterinary science,* vol. 73, no. 2, pp. 145-152.

Cousins, D.V. & Florisson, N. 2005, "A review of tests available for use in the diagnosis of tuberculosis in non-bovine species", *Revue scientifique et technique (International Office of Epizootics),* vol. 24, no. 3, pp. 1039-1059.

Cross, M.L., Buddle, B.M. & Aldwell, F.E. 2007, "The potential of oral vaccines for disease control in wildlife species", *Veterinary journal (London, England : 1997),* vol. 174, no. 3, pp. 472-480.

De Klerk, L.M., Michel, A.L., Bengis, R.G., Kriek, N.P.J. & Godfroid, J. 2008, *BCG Vaccination failed to protect yearling African buffaloes (Syncerus caffer) against experimental challenge with Mycobacterium bovis*, University of Utrecht.

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H. & Clifton-Hadley, R.S. 2006, "Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques", *Research in veterinary science,* vol. 81, no. 2, pp. 190-210.

de Lisle, G.W., Bengis, R.G., Schmitt, S.M. & O'Brien, D.J. 2002, "Tuberculosis in free-ranging wildlife: detection, diagnosis and management", *Revue scientifique et technique (International Office of Epizootics),* vol. 21, no. 2, pp. 317-334.

Demissie, A., Leyten, E.M., Abebe, M., Wassie, L., Aseffa, A., Abate, G., Fletcher, H., Owiafe, P., Hill, P.C., Brookes, R., Rook, G., Zumla, A., Arend, S.M., Klein, M., Ottenhoff, T.H., Andersen, P., Doherty, T.M. & the VACSEL Study Group 2006, "Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with Mycobacterium tuberculosis", *Clinical and vaccine immunology : CVI,* vol. 13, no. 2, pp. 179-186.

Dinnes, J., Deeks, J., Kunst, H., Gibson, A., Cummins, E., Waugh, N., Drobniewski, F. & Lalvani, A. 2007, "A systematic review of rapid diagnostic tests for the detection of tuberculosis infection", *Health technology assessment (Winchester, England),* vol. 11, no. 3.

Dorrington, R., Bourne, D., Bradshaw, D., Laubscher, R. & Timaeus, I.M. 2001, "The impact of HIV/AIDS on adult mortality in South Africa", *Technical Report of the Burden of Disease Research Unit of the Medical Research Council, South Africa.*

Eulenberger, K., Elze, K., Schuppel, K.F., Seifert, S., Ippen, R. & Schroder, H.D. 1992, "Tuberkulose und ihre Bekampfung bei Primaten und Feliden im Leipziger Zoologischen Garten von 1951-1990", *Erkrankungen der Zootiere. Verhandlungsbericht des 34. Internationalen Symposiums uber die Erkrankungen der Zoo- und Wildtiere, Santander, Spain 1992,* , pp. 7-15.

Farrar, M.A. & Schreiber, R.D. 1993, "The molecular cell biology of interferon-gamma and its receptor", *Annual Review of Immunology,* vol. 11, pp. 571-611.

Finkelman, F.D., Holmes, J., Katona, I.M., Urban, J.F.,Jr, Beckmann, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R. & Paul, W.E. 1990, "Lymphokine control of in vivo immunoglobulin isotype selection", *Annual Review of Immunology,* vol. 8, pp. 303-333.

Funston, P. 1998, "Predator-prey relationships between lions and large ungulates in the Kruger National Park", *Ph.D. Thesis. University of Pretoria, South Africa,* .

Gajewski, T.F., Schell, S.R., Nau, G. & Fitch, F.W. 1989, "Regulation of T-cell activation: differences among T-cell subsets", *Immunological reviews,* vol. 111, pp. 79-110.

Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V. & Hewinson, R.G. 2003, "The complete genome sequence of Mycobacterium bovis", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 13, pp. 7877-7882.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. & Mathieu, C. 2001, "An overview of real-time quantitative PCR: applications to quantify cytokine gene expression", *Methods,* vol. 25, no. 4, pp. 386-401.

Glatman-Freedman, A. & Casadevall, A. 1998, "Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis", *Clinical microbiology reviews,* vol. 11, no. 3, pp. 514-532.

Gormley, E., Doyle, M.B., Fitzsimons, T., McGill, K. & Collins, J.D. 2006, "Diagnosis of Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam) assay", *Veterinary microbiology,* vol. 112, no. 2-4, pp. 171-179.

Griffin, J.F.T., Cross, J.P., Chinn, D.N., Rodgers, C.R. & Buchan, G.S. 1994, "Diagnosis of tuberculosis due to Mycobacterium bovis in New Zealand red deer (Cervus elaphus) using a composite blood test and antibody assays", *New Zealand veterinary journal,* vol. 42, no. 5, pp. 173-179.

Grobler, D.G., Michel, A.L., De Klerk, L.M. & Bengis, R.G. 2002, "The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes

(Syncerus caffer) in the Kruger National Park", *The Onderstepoort journal of veterinary research,* vol. 69, no. 3, pp. 221-227.

Harboe, M., Oettinger, T., Wiker, H.G., Rosenkrands, I. & Andersen, P. 1996, "Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG", *Infection and immunity,* vol. 64, no. 1, pp. 16-22.

Harrington, N.P., Surujballi, O.P., Waters, W.R. & Prescott, J.F. 2007, "Development and evaluation of a real-time reverse transcription-PCR assay for quantification of gamma interferon mRNA to diagnose tuberculosis in multiple animal species", *Clinical and vaccine immunology : CVI,* vol. 14, no. 12, pp. 1563-1571.

Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J.M. & Madrid, M.V. 1996, "Correlation between the kinetics of Th1/Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis", *Immunology,* vol. 89, no. 1, pp. 26-33.

Holland, P.M., Abramson, R.D., Watson, R. & Gelfand, D.H. 1991, "Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 88, no. 16, pp. 7276-7280.

Hoogeveen, G.M. 2006, Improved diagnostics and control strategies of bovine tuberculosis in livestock and wildlife. Recombinant antigen MPB70 ELISA, *Research project of Utrecht University*, Utrecht.

Hope, J.C., Thom, M.L., Villarreal-Ramos, B., Vordermeier, H.M., Hewinson, R.G. & Howard, C.J. 2005, "Exposure to Mycobacterium avium induces low-level protection from Mycobacterium bovis infection but compromises diagnosis of disease in cattle", *Clinical and experimental immunology,* vol. 141, no. 3, pp. 432-439.

Howard, A.D. & Zwilling, B.S. 1999, "Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines", *Clinical and experimental immunology,* vol. 115, no. 3, pp. 428-434.

Howard, A.D. & Zwilling, B.S. 1998, "Cytokine production by CD4 and CD8 T cells during the growth of Mycobacterium tuberculosis in mice", *Clinical and experimental immunology,* vol. 113, no. 3, pp. 443-449.

Howard, M. & O'Garra, A. 1992, "Biological properties of interleukin 10", *Immunology today,* vol. 13, no. 6, pp. 198-200.

Jacobson, R.H. 1998, "Validation of serological assays for diagnosis of infectious diseases", *Revue scientifique et technique (International Office of Epizootics),* vol. 17, no. 2, pp. 469-486.

Kabilan, L., Andersson, G., Lolli, F., Ekre, H.P., Olsson, T. & Troye-Blomberg, M. 1990, "Detection of intracellular expression and secretion of interferon-gamma at

the single-cell level after activation of human T cells with tetanus toxoid in vitro", *European journal of immunology,* vol. 20, no. 5, pp. 1085-1089.

Keet, D.F., Kriek, N.P.J., Penrith, M.-. & Michel, A. 1997, "Tuberculosis in lions and cheetahs", *Proceedings of a Symposium on Lions and Leopards as Game Ranch Animals, Onderstepoort, October 1997,* , pp. 151-156.

Keet, D.F., Kriek, N.P., Penrith, M.L., Michel, A. & Huchzermeyer, H. 1996, "Tuberculosis in buffaloes (Syncerus caffer) in the Kruger National Park: spread of the disease to other species", *The Onderstepoort journal of veterinary research,* vol. 63, no. 3, pp. 239-244.

Keet, D.F., Michel, A. & Meltzer, D.G.A. 2000, "Tuberculosis in free-ranging lions (Panthera leo) in the Kruger National Park", *Proceedings of the South African Veterinary Association Biennial Congress, Durban, Kwazulu-Natal, 20-22 September 2000,* pp. 232-241.

Kennedy-Stoskopf, S., Gebhard, D.H. & English, R.V. 1994, "Clinical implications of feline immunodeficiency virus infection in African lions (Panthera leo): preliminary findings", *Proceedings of the American Association of Zoo Veterinarians and Association of Reptilian and Amphibian Veterinarians Annual conference, Pittsburgh, 22-27 October 1994,* pp. 345-346.

Kipar, A., Meli, M.L., Failing, K., Euler, T., Gomes-Keller, M.A., Schwartz, D., Lutz, H. & Reinacher, M. 2006, "Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection", *Veterinary immunology and immunopathology,* vol. 112, no. 3-4, pp. 141-155.

Kirkberger, R.M., Keet, D.F. & 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,* vol. 47, no. 2, pp. 145-152.

Koo, H.C., Park, Y.H., Ahn, J., Waters, W.R., Palmer, M.V., Hamilton, M.J., Barrington, G., Mosaad, A.A., Park, K.T., Jung, W.K., Hwang, I.Y., Cho, S.N., Shin, S.J. & Davis, W.C. 2005, "Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme-linked immunosorbent, immunochromatographic, and latex bead agglutination assays for serodiagnosis of bovine tuberculosis", *Journal of clinical microbiology,* vol. 43, no. 9, pp. 4498-4506.

Koolen, E.C. 2007, The potential of a MPB83 specific ELISA for diagnoses of bovine tuberculosis in cattle, African buffalo (Syncerus caffer) and lion (Panthera leo), *Research project of Utrecht University*, Utrecht.

Leutenegger, C.M., Mislin, C.N., Sigrist, B., Ehrengruber, M.U., Hofmann-Lehmann, R. & Lutz, H. 1999, "Quantitative real-time PCR for the measurement of feline cytokine mRNA", *Veterinary immunology and immunopathology,* vol. 71, no. 3-4, pp. 291-305.

Lightbody, K.A., Skuce, R.A., Neill, S.D. & Pollock, J.M. 1998, "Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with

potential to confirm disease status", *The Veterinary record,* vol. 142, no. 12, pp. 295-300.

Lloyd-Smith, J.O., Cross, P.C., Briggs, C.J., Daugherty, M., Getz, W.M., Latto, J., Sanchez, M.S., Smith, A.B. & Swei, A. 2005, "Should we expect population thresholds for wildlife disease?", *Trends in ecology & evolution (Personal edition),* vol. 20, no. 9, pp. 511-519.

Lyashchenko, K., Whelan, A.O., Greenwald, R., Pollock, J.M., Andersen, P., Hewinson, R.G. & Vordermeier, H.M. 2004, "Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with Mycobacterium bovis BCG and infected with M. bovis", *Infection and immunity,* vol. 72, no. 5, pp. 2462-2467.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Chambers, M.A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B.M., Jackson, R., O'Brien, D.J., Schmitt, S., Palmer, M.V., Delahay, R.J. & Waters, W.R. 2008, "Animal-side serologic assay for rapid detection of Mycobacterium bovis infection in multiple species of free-ranging wildlife", *Veterinary microbiology.*

Lyashchenko, K.P., Singh, M., Colangeli, R. & Gennaro, M.L. 2000, "A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases", *Journal of immunological methods,* vol. 242, no. 1-2, pp. 91-100.

Mahairas, G.G., Sabo, P.J., Hickey, M.J., Singh, D.C. & Stover, C.K. 1996, "Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis", *Journal of Bacteriology,* vol. 178, no. 5, pp. 1274-1282.

Maue, A.C., Waters, W.R., Palmer, M.V., Whipple, D.L., Minion, F.C., Brown, W.C. & Estes, D.M. 2004, "CD80 and CD86, but not CD154, augment DNA vaccine-induced protection in experimental bovine tuberculosis", *Vaccine,* vol. 23, no. 6, pp. 769-779.

Michel, A.L. 2002, "Implications of tuberculosis in African wildlife and livestock", *Annals of the New York Academy of Sciences,* vol. 969, pp. 251-255.

Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., Klerk, L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P. & Godfroid, J. 2006, "Wildlife tuberculosis in South African conservation areas: implications and challenges", *Veterinary microbiology,* vol. 112, no. 2-4, pp. 91-100.

Mishra, A., Singhal, A., Chauhan, D.S., Katoch, V.M., Srivastava, K., Thakral, S.S., Bharadwaj, S.S., Sreenivas, V. & Prasad, H.K. 2005, "Direct detection and identification of Mycobacterium tuberculosis and Mycobacterium bovis in bovine samples by a novel nested PCR assay: correlation with conventional techniques", *Journal of clinical microbiology,* vol. 43, no. 11, pp. 5670-5678.

Monaghan, M.L., Doherty, M.L., Collins, J.D., Kazda, J.F. & Quinn, P.J. 1994, "The tuberculin test", *Veterinary microbiology,* vol. 40, no. 1-2, pp. 111-124.

Mond, J.J., Carman, J., Sarma, C., Ohara, J. & Finkelman, F.D. 1986, "Interferon-gamma suppresses B cell stimulation factor (BSF-1) induction of class II MHC

determinants on B cells", *Journal of immunology (Baltimore, Md.: 1950),* vol. 137, no. 11, pp. 3534-3537.

Morar, D., Tijhaar, E., Negrea, A., Hendriks, J., van Haarlem, D., Godfroid, J., Michel, A.L. & Rutten, V.P.M.G. 2007, "Cloning, sequencing and expression of white rhinoceros (Ceratotherium simum) interferon-gamma (IFN-gamma) and the production of rhinoceros IFN-gamma specific antibodies", *Veterinary immunology and immunopathology,* vol. 115, no. 1-2, pp. 146-154.

Morris, R.S., Pfeiffer, D.U. & Jackson, R. 1994, "The epidemiology of Mycobacterium bovis infections", *Veterinary microbiology,* vol. 40, no. 1-2, pp. 153-177.

Nol, P., Palmer, M.V., Waters, W.R., Aldwell, F.E., Buddle, B.M., Triantis, J.M., Linke, L.M., Phillips, G.E., Thacker, T.C., Rhyan, J.C., Dunbar, M.R. & Salman, M.D. 2008, "Efficacy of oral and parenteral routes of mycobacterium bovis bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (Odocoileus virginianus): a feasibility study", *Journal of wildlife diseases,* vol. 44, no. 2, pp. 247-259.

Noordhoek, G.T., Kolk, A.H., Bjune, G., Catty, D., Dale, J.W., Fine, P.E., Godfrey-Faussett, P., Cho, S.N., Shinnick, T., Svenson, S.B., Wilson, S. & Embden, J.D.A.v. 1994, "Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories", *Journal of clinical microbiology,* vol. 32, no. 2, pp. 277-284.

Office International des Epizooties (OIE) 2008, *Manual of diagnostic tests and vaccines for terrestrial animals*.

Packer, C., Altizer, S., Appel, M., Brown, E., Martenson, J., O'Brien, S.J., Roelke-Parker, M., Hofmann-Lehmann, R. & Lutz, H. 1999, "Viruses of the Serengeti: patterns of infection and mortality in African lions", *Journal of Animal Ecology,* vol. 68, pp. 1161-1178.

Pollock, J.M., McNair, J., Bassett, H., Cassidy, J.P., Costello, E., Aggerbeck, H., Rosenkrands, I. & Andersen, P. 2003, "Specific delayed-type hypersensitivity responses to ESAT-6 identify tuberculosis-infected cattle", *Journal of clinical microbiology,* vol. 41, no. 5, pp. 1856-1860.

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", *Tuberculosis (Edinburgh, Scotland),* vol. 81, no. 1-2, pp. 103-107.

Pollock, J.M. & Neill, S.D. 2002, "Mycobacterium bovis infection and tuberculosis in cattle", *Veterinary journal,* vol. 163, no. 2, pp. 115-127.

Pollock, J.M., Rodgers, J.D., Welsh, M.D. & McNair, J. 2006, "Pathogenesis of bovine tuberculosis: the role of experimental models of infection", *Veterinary microbiology,* vol. 112, no. 2-4, pp. 141-150.

Raviglione, M.C., Snider, D.E.,Jr & Kochi, A. 1995, "Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic", *JAMA : the journal of the American Medical Association,* vol. 273, no. 3, pp. 220-226.

Rhodes, S.G., Palmer, N., Graham, S.P., Bianco, A.E., Hewinson, R.G. & Vordermeier, H.M. 2000, "Distinct response kinetics of gamma interferon and interleukin-4 in bovine tuberculosis", *Infection and immunity,* vol. 68, no. 9, pp. 5393-5400.

Rodwell, T.C., Kriek, N.P., Bengis, R.G., Whyte, I.J., Viljoen, P.C., de Vos, V. & Boyce, W.M. 2001, "Prevalence of bovine tuberculosis in African buffalo at Kruger National Park", *Journal of wildlife diseases,* vol. 37, no. 2, pp. 258-264.

Roelke, M.E., Pecon-Slattery, J., Taylor, S., Citino, S., Brown, E., Packer, C., Vandewoude, S. & O'Brien, S.J. 2006, "T-lymphocyte profiles in FIV-infected wild lions and pumas reveal CD4 depletion", *Journal of wildlife diseases,* vol. 42, no. 2, pp. 234-248.

Rothel, J.S., Jones, S.L., Corner, L.A., Cox, J.C. & Wood, P.R. 1990, "A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle", *Australian Veterinary Journal,* vol. 67, no. 4, pp. 134-137.

Scanga, C.A., Mohan, V.P., Yu, K., Joseph, H., Tanaka, K., Chan, J. & Flynn, J.L. 2000, "Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2", *The Journal of experimental medicine,* vol. 192, no. 3, pp. 347-358.

Schijns, V.E.C.J., Wierda, C.M.H., Vahlenkamp, T.W., Horzinek, M.C. & de Groot, R.J. 1995, "Molecular cloning and expression of cat interferon-gamma", *Immunogenetics,* vol. 42, pp. 440-441.

Seah, G.T., Scott, G.M. & Rook, G.A.W. 2000, "Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis", *The Journal of infectious diseases,* vol. 181, no. 1, pp. 385-389.

Smuts, G.L., Anderson, J.L. & Austin, J.C. 1978, "Age determination of the African Lion (Panthera leo)", *Journal of Zoology, London,* vol. 185, pp. 115-146.

Snapper, C.M. & Paul, W.E. 1987, "Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production", *Science,* vol. 236, no. 4804, pp. 944-947.

Sparbier, K., Wenzel, T. & Kostrzewa, M. 2006, "Exploring the binding profiles of ConA, boronic acid and WGA by MALDI-TOF/TOF MS and magnetic particles", *Journal of chromatography.B, Analytical technologies in the biomedical and life sciences,* vol. 840, no. 1, pp. 29-36.

Spencer, J.A., Van Dijk, A.A., Horzinek, M.C., Egberink, H.F., Bengis, R.G., Keet, D.F., Morikawa, S. & Bishop, D.H. 1992, "Incidence of feline immunodeficiency virus reactive antibodies in free-ranging lions of the Kruger National Park and the Etosha National Park in southern Africa detected by recombinant FIV p24 antigen", *The Onderstepoort journal of veterinary research,* vol. 59, no. 4, pp. 315-322.

Tanner, M. & Michel, A.L. 1999, "Investigation of the viability of M. bovis under different environmental conditions in the Kruger National Park", *The Onderstepoort journal of veterinary research,* vol. 66, pp. 185-190.

Thacker, T.C., Palmer, M.V. & Waters, W.R. 2007, "Associations between cytokine gene expression and pathology in Mycobacterium bovis infected cattle", *Veterinary immunology and immunopathology,* vol. 119, no. 3-4, pp. 204-213.

Thorel, M.F. 1994, "Mycobacterium bovis in zoo animals", *Paper presented at the meeting of the Committee "Tuberculosis in Animals" of the International Union Against Tuberculosis and Lung Disease at Mainz, June 1994*.

Tizard, I.R. 1992, in *Veterinary Immunology- An introduction*, 6th edn, WB Saunders Company, Philadelphia, pp. 376.

van Pinxteren, L.A.H., Cassidy, J.P., Smedegaard, B.H., Agger, E.M. & Andersen, P. 2000, "Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells", *European journal of immunology,* vol. 30, no. 12, pp. 3689-3698.

Van Vuuren, M., Stylianides, E. & Du Rand, A. 1997, "The prevalence of viral infections in lions and leopards in Southern Africa", *Proceedings of a Symposium on Lions and Leopards as Game Ranch Animals, Onderstepoort, October 1997,* pp. 168-173.

Van Vuuren, M., Stylianides, E., Kania, S.A., Zuckerman, E.E. & Hardy Jr, W.D. 2003, "Evaluation of an indirect enzyme-linked immunosorbent assay for the detection of feline lentivirus-reactive antibodies in wild felids, employing a puma lentivirus-derived synthetic peptide antigen", *The Onderstepoort Journal of Veterinary Research,* vol. 70, pp. 1-6.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002, "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes", *Genome biology,* vol. 3, no. 7, pp. 34.1-34.11.

Vordermeier, H.M., Chambers, M.A., Cockle, P.J., Whelan, A.O., Simmons, J. & Hewinson, R.G. 2002, "Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following Mycobacterium bovis BCG vaccination against experimental bovine tuberculosis", *Infection and immunity,* vol. 70, no. 6, pp. 3026-3032.

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N. & Hewinson, R.G. 2001, "Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle", *Clinical and diagnostic laboratory immunology,* vol. 8, no. 3, pp. 571-578.

Waters, W.R., Nonnecke, B.J., Olsen, S.C. & Palmer, M.V. 2007, "Effects of pre-culture holding time and temperature on interferon-gamma responses in whole blood cultures from Mycobacterium bovis-infected cattle", *Veterinary microbiology,* vol. 119, no. 2-4, pp. 277-282.

Waters, W.R., Palmer, M.V., Bannantine, J.P., Greenwald, R., Esfandiari, J., Andersen, P., McNair, J., Pollock, J.M. & Lyashchenko, K.P. 2005, "Antibody responses in reindeer (Rangifer tarandus) infected with Mycobacterium bovis", *Clinical and diagnostic laboratory immunology,* vol. 12, no. 6, pp. 727-735.

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., Andersen, P. & Lyashchenko, K.P. 2006, "Early antibody responses to experimental Mycobacterium bovis infection of cattle", *Clinical and vaccine immunology,* vol. 13, no. 6, pp. 648-654.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G. & Pollock, J.M. 2005, "Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis", *Immunology,* vol. 114, no. 1, pp. 101-111.

Widdison, S., Schreuder, L.J., Villarreal-Ramos, B., Howard, C.J., Watson, M. & Coffey, T.J. 2006, "Cytokine expression profiles of bovine lymph nodes: effects of Mycobacterium bovis infection and bacille Calmette-Guerin vaccination", *Clinical and experimental immunology,* vol. 144, no. 2, pp. 281-289.

Wiker, H.G., Lyashchenko, K.P., Aksoy, A.M., Lightbody, K.A., Pollock, J.M., Komissarenko, S.V., Bobrovnik, S.O., Kolesnikova, I.N., Mykhalsky, L.O., Gennaro, M.L. & Harboe, M. 1998, "Immunochemical characterization of the MPB70/80 and MPB83 proteins of Mycobacterium bovis", *Infection and immunity,* vol. 66, no. 4, pp. 1445-1452.

Willemse, A. & Beijer, E.G. 1979, "Bovine tuberculose bij een kat", *Tijdschrift voor diergeneeskunde,* vol. 104, no. 18, pp. 717-721.

World Health Organization 2008, *Global tuberculosis control: surveillance, planning, financing: WHO report 2008*, Switzerland.

Woude, S.V., O'Brien, S.J. & Hoover, E.A. 1997, "Infectivity of lion and puma lentiviruses for domestic cats", *Journal of General Virology,* vol. 78, pp. 795-800.

Wright, P.F., Nilsson, E., Van Rooij, E.M.A., Lelenta, M. & Jeggo, M.H. 1993, "Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis", *Revue scientifique et technique (International Office of Epizootics),* vol. 12, no. 2, pp. 435-450.

www.greatlimpopopark.com, consulted May 2008

www.merckvetmanual.com, consulted August 2008

Zimmermann, K. & Mannhalter, J.W. 1996, "Technical aspects of quantitative competitive PCR", *BioTechniques,* vol. 21, no. 2, pp. 268-72, 274-9.

List of Appendices

Appendix 1. Overview of the Kruger National Park lions that were sampled

- Age-column: m=months
- Sex: M=Male, F=Female
- BCS = Body condition Score
- PPD B: the difference between the first and the second skin reading on the side that had PPD B injected
- PPD A: the difference between the first and the second skin reading on the side that had PPD A injected
- TB I: the BTB status when both the swelling caused by PPB and PPD A are taken into consideration
- TB II: the BTB status when only the swelling caused by PPB is taken into consideration
- ELISA: the iELISA that showed a positive result is mentioned

Appendix 2. CFP10 iELISA protocol

Appendix 3. DiaSer3 iELISA protocol

Appendix 4. Overview RT qPCR results

- TB II: the BTB status when only the swelling caused by PPB is taken into consideration
- * Are mean values of the various ΔCt values
- ** These samples were rejected, because the GAPDH value did not fall in the range of 19.881-34.588.
- If an iELISA was positive, the antigen is given in the “extra” column.

Appendix 5. Merged sequences

- The start and stop codon are marked in grey and the primers are underlined.

Appendix 1: Overview of the Kruger National Park lions that were sampled

Date	Locality	Age (y)	Sex	BCS	Weight	ID Number	PPDB	PPDA	TB I	TB II	FIV	ELISA+	Extra
20-aug	Crocodile Bridge	3	M	5	179	451F726223	7.9	3,5	pos	pos	pos	neg	
20-aug	Crocodile Bridge	3	F	4	123	454A696521	9.7	1,8	pos	pos	neg	neg	
20-aug	Crocodile Bridge	1	F	4	71	4536256D07	6.0	2,3	pos	pos	neg	neg	
20-aug	Crocodile Bridge	4	F	4	129	4542767511	10.7	1,9	pos	pos	pos	neg	
20-aug	Crocodile Bridge	6	F	3	130,5	456B0D3468	15.7	3	pos	pos	neg	MPB70,83	
20-aug	Crocodile Bridge	1,5	F	5	102	456B2A755A	6.8	7	neg	pos	neg	neg	
20-aug	Crocodile Bridge	1,5	F	5	95	456E016973					neg	neg	
20-aug	Crocodile Bridge	1,5	M	5	105	456A62761F	9.2	5,6	pos	pos	neg	neg	
21-aug	Mpanamane	young	M	ND	79	49354D1067					neg	neg	
21-aug	Mpanamane		F	5	120	434C656B6B					pos	neg	
21-aug	Mpanamane	1	M	5	69	494A36140A					neg	neg	
21-aug	Mpanamane	1	F	5	67	492E73601B					neg	neg	
21-aug	Mpanamane	1	M	5	81	4959717807					neg	neg	
21-aug	Mpanamane	1	M	5	87	492A10157F					neg	neg	
21-aug	Mpanamane	1	M	5	85,5	49301C174B					neg	MPB83	
21-aug	Mpanamane	10	F	5	117	434E630268	0	0,8	neg	neg	pos	neg	
21-aug	Gomondwane	4	F	4	122	44155D142B	4.0	4,6	neg	pos	pos	neg	
21-aug	Gomondwane	8	F	5	160	442E1B4D0F					neg	neg	
21-aug	Gomondwane	20 m	M	4	126	_49482E0022	7.2	4,5	pos	pos	pos	DiaSer3	
21-aug	Gomondwane	9 or 10	F	4	140	7F7D3D2B0F	6.7	5,7	pos	pos	pos	neg	
21-aug	Gomondwane	7	M	5	189	441547114E	9.5	5,3	pos	pos	pos	neg	
21-aug	Gomondwane	9	F	5	142	494748583B	17.3	1,8	pos	pos	pos	MPB83	
21-aug	Gomondwane	10+	F	3,5		456A67644D							
23-aug	Crocodile Bridge	18 m	M	5		492A5E5824							
17-sep	Powerline Duke Cr.	1,5	F	4	97	49492B451E					neg	neg	
17-sep	Powerline Duke Cr.	8	F	3	132	4932190F1C					pos	neg	
17-sep	Powerline Duke Cr.	3	M	4	121	49494C5264					neg	neg	
17-sep	Nkongoma	4	F	5	135,5	494A2A7564					pos	DiaSer3	TF+
17-sep	Nkongoma	8	M	4	148	434D092D75	>5	Not done		pos	pos	neg	
17-sep	Nkongoma	1,5	F	5	86,5	49302A521B					neg	MPB83	
17-sep	Nkongoma	5	F	5	116	4948211C28					pos	neg	
17-sep	Nkongoma	3	M	5	165	492D150E07					neg	neg	
17-sep	Nkongoma	2	M	5	127,5	494A1B6278					pos	neg	
27-sep	Mpanamane	4	F			49297D0A60					pos	neg	TF+
27-sep	Mpanamane	4	M			4958633932					neg	neg	

Appendix 1: Overview of the Kruger National Park lions that were sampled

Date	Locality	Age (y)	Sex	BCS	Weight	ID Number	PPDB	PPDA	TB I	TB II	FIV	ELISA+	Extra
27-sep	Mpanamane	8	F			442D287135					pos	MPB83	
15-okt	Mhlanganzwane	9	F	5	142,5	4115092830	7,6	0,8	pos	pos	pos	neg	TF
15-okt	Mhlanganzwane	18 m	F	5	90	4930754363					neg	neg	
15-okt	Mhlanganzwane	3	F	5	120	492A562508					neg	neg	
15-okt	Mhlanganzwane	18 m	M	5	140	494A185A30	5	3,4	neg	pos	neg	MPB83	
15-okt	Mhlanganzwane	18 m	F	5	105	494A427E14	7,1	3,2	pos	pos	neg	neg	
15-okt	Mhlanganzwane	7	F	5	146	441550741E	8,4	1,3	pos	pos	neg	neg	
15-okt	Mhlanganzwane		F			441F321408					pos	neg	
15-okt	Mhlanganzwane		F			4415633833					pos	neg	
6-nov	Nwarihlangary	1	F	4	68	494A123F43	1,9	2,6	neg	neg	pos	neg	
6-nov	Nwarihlangary	10 m	F	4	127,5	4949447313	3,3	2,2	neg	pos	pos	neg	
6-nov	Nwarihlangary	14 m	M	4	83	492E456C57	2,8	4	neg	pos	neg	neg	TF
6-nov	Nwarihlangary	9	F	5	120	4949226F2C					pos	neg	
6-nov	Nwarihlangary	4	F	5	80	492A3D0D17	0,5	0,4	neg	neg	neg	MPB83, DiaSer3	TF
6-nov	Nwarihlangary	14 m	M	4	66	492F38266D	2,7	4,9	neg	pos	pos	neg	
14-nov	Boyela	5	M	5	195	4959570039	3	1,2	neg	pos	neg	neg	
14-nov	Boyela	7	F	5	119	49495E034B	3	4,2	neg	pos	pos	neg	
20-nov	Langtoondam	5	M	5	180	456B4F4153					pos	neg	
20-nov	Langtoondam	4	M	5	167	44666A567					pos	neg	
20-nov	Langtoondam	10+	M	3,5	156	434C4B6A63					pos	neg	
20-nov	Langtoondam	7	F	5	122	456B07B2902					neg	neg	
20-nov	Langtoondam	7	F	5	92	43277C734F					pos	neg	
2-jun	Nwarihlangari	4 or 5	M	5	155	456B4F4153	2,7	3,2	neg	pos	pos	neg	
6-jun	Stangeni	8 m	F	5	93,5	4A610A716A					neg	neg	
6-jun	Stangeni	8 m	F	5	85	4A6A303478					neg	neg	
6-jun	Stangeni	2	M	5		4A68590F16					neg	neg	
6-jun	Stangeni	1	F	5		4A5E685451	1,1	2,5	neg	neg	neg	neg	
6-jun	Stangeni	1	F	5		4A6A491038					neg	neg	
6-jun	Stangeni	1	F	5		4A44026512					neg	neg	
6-jun	Stangeni	8 m	M	5		4A441E5F2A	1,1	1,3	neg	neg	neg	neg	
6-jun	Stangeni	7 or 8	F	5		456A4D023E					neg	neg	
6-jun	Stangeni	1	M	5		4A6A682D39	2,2	4,3	neg	pos	neg	neg	
6-jun	Stangeni	18 m	M	5		4A5F1E4D5F					neg	neg	
7-jun	Stangeni			5		44666A5267					pos	neg	
8-jun	Stangeni		F	5		434E53772E					pos	neg	

Appendix 1: Overview of the Kruger National Park lions that were sampled

Date	Locality	Age (y)	Sex	BCS	Weight	ID Number	PPDB	PPDA	TB I	TB II	FIV	ELISA+	Extra
8-jun	Stangeni		F	5		4327566122					pos	DiaSer3	
8-jun	Stangeni	15 m	M	5		4A5A777B01					neg	neg	
8-jun	Stangeni		F	5		432757363C					pos	DiaSer3	
8-jun	Stangeni	17	F	5		434C744B10					pos	neg	

Appendix 2: CFP10 iELISA protocol

Solutions

* protein dilution	Reconstitution of freeze dried protein CFP10 in ddH2O in a concentration of 1 mg/ml
* coating solution	Protein dilution diluted in carbonate-bicarbonate Concentration 10 μg/ml (=0.5 μg/well)
* wash buffer	PBS/T (PBS 0.15 M, 0.05% Tween-20)
* blocking buffer	PBS/T, 5% milk powder (Okoid LTD LP0031 skim milk powder)
* serum dilution	1:150 dilution
* conjugate dilution	Anti-Cat immunoglobulin (Peroxidase-Labeled affinity purified antibody to cat IgG(γ) produced in goat, Kirkegaard & Perry Laboratories®) conjugate 1:2000 dilution
* substrate	O-Phenylenediamine dihydrochloride (OPD) 4 mg tablets 1 tablet + 10 ml distilled water + 5 μl H2O2 ! Wear gloves, because this solution is carcinogenic ! Keep the solution in the dark as much as possible
* stop solution	1 M H_2SO_4

Material

* plate	Immunoplate Maxisorp ®ELISA plate Nunc Copenhagen, Denmark
* reader	EL808 Ultra Microplate Reader® Biotek Instruments, inc.

Method

1) **Coating of the plate with antigens**
   Make a coating solution of 10 μg/ml (=0.5 μg/well) of CFP10 antigen diluted in carbonate-bicarbonate
   Add 50 μl coating solution in each well
   Cover the plate(s) and incubate the plate(s) one hour at 37℃
2) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
3) **Blocking of the plate**
   Add 50 μl blocking buffer in each well
   Cover the plate(s) and incubate the plate(s) for one hour at room temperature (20℃)
4) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
5) **Adding the sera**
   Add 50 μl sera diluted 1:150 to each well
   Cover the plate(s) and incubate the plate(s) for 1 hour at room temperature (20℃)
6) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
7) **Adding the conjugate**
   Add 50 μl conjugate diluted 1:2000 to each well
   Cover the plate(s) and incubate the plate(s) for 1 hour at room temperature (20℃)
8) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
9) **Adding the substrate**
   Add 50 μl of the substrate to each well
   Cover the plate(s) and incubate for 10 minutes in the dark at room temperature (20℃)
10) **Stopping the color development**
   Add 50 μl of stopping solution to each well
11) **Reading the plate**
   Wipe the bottom of the plate
   Read the plate immediately after stopping the reaction
   Use wavelength 490 nm

Appendix 3: DiaSer3 iELISA protocol

Solutions

* protein dilution	Reconstitution of freeze dried protein DiaSer3 in ddH2O in a concentration of 2.1 mg/ml
* coating solution	Protein dilution diluted in carbonate-bicarbonate Concentration 5 μg/ml (=0.25 μg/well)
* wash buffer	PBS/T (PBS 0.15 M, 0.05% Tween-20)
* blocking buffer	PBS/T, 5% milk powder (Okoid LTD LP0031 skim milk powder)
* serum dilution	1:200 dilution
* conjugate dilution	Anti-Cat immunoglobulin (Peroxidase-Labeled affinity purified antibody to cat IgG(γ) produced in goat, Kirkegaard & Perry Laboratories®) conjugate 1:2000 dilution
* substrate	O-Phenylenediamine dihydrochloride (OPD) 4 mg tablets 1 tablet + 10 ml distilled water + 5 μl H2O2 ! Wear gloves, because this solution is carcinogenic ! Keep the solution in the dark as much as possible
* stop solution	1 M H_2SO_4

Material

* plate	Immunoplate Maxisorp ®ELISA plate Nunc Copenhagen, Denmark
* reader	EL808 Ultra Microplate Reader® Biotek Instruments, inc.

Method

1) **Coating of the plate with antigens**
   Make a coating solution of 5 μg/ml (=0.25 μg/well) of DiaSer3 antigen diluted in carbonate-bicarbonate
   Add 50 μl coating solution in each well
   Cover the plate(s) and incubate the plate(s) one hour at 37℃
2) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
3) **Blocking of the plate**
   Add 50 μl blocking buffer in each well
   Cover the plate(s) and incubate the plate(s) for one hour at room temperature (20℃)
4) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
5) **Adding the sera**
   Add 50 μl sera diluted 1:200 to each well
   Cover the plate(s) and incubate the plate(s) for 1 hour at room temperature (20℃)
6) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
7) **Adding the conjugate**
   Add 50 μl conjugate diluted 1:2000 to each well
   Cover the plate(s) and incubate the plate(s) for 1 hour at room temperature (20℃)
8) **Washing of the plate**
   Wash 4 times with was buffer with multichannel pipette, add 200 μl in each well each time
9) **Adding the substrate**
   Add 50 μl of the substrate to each well
   Cover the plate(s) and incubate for 10 minutes in the dark at room temperature (20℃)
10) **Stopping the color development**
   Add 50 μl of stopping solution to each well
11) **Reading the plate**
   Wipe the bottom of the plate
   Read the plate immediately after stopping the reaction
   Use wavelength 490 nm

Appendix 4: Overview RT qPCR results

ID Number	TB II	GAPDH	IFNγ	TNFα	IL-4	IL-10		Δ Ct IFNγ	Δ Ct TNFα	ΔCt IL-4	ΔCt IL-10	Extra
451F726223	pos	25,441	30,454	30,232	36,207	31,326		5,013	4,790	10,766	5,885	
454A696521	pos	23,755	29,870	29,725	35,370	32,445		6,115	5,970	11,616	8,691	
4536256D07	pos	25,358	31,829	32,991	39,312	35,887		6,471	7,632	13,953	10,529	
4542767511	pos	24,501	29,733	30,137	35,415	31,459		5,233	5,636	10,914	6,958	
456B0D3468	pos	23,582	28,962	29,618	35,457	31,057		5,380	6,036	11,875	7,475	MPB70, 83
456B2A755A	pos	29,966	37,513	36,980	40,000	36,857		7,547	7,014	10,034	6,891	
456A62761F	pos	25,110	31,351	30,584	36,690	32,534		6,241	5,474	11,581	7,424	
441547114E	pos	24,870	32,769	30,846	37,624	35,042		7,899	5,976	12,754	10,172	
494748583B	pos	30,633	37,315	36,098	40,000	40,000		6,681	5,465	9,367	9,367	MPB83
434D092D75	pos	27,967	35,933	34,475	40,000	36,896		7,966	6,508	12,033	8,929	
4115092830	pos	25,458	32,848	32,519	35,868	33,208		7,390	7,061	10,410	7,750	
494A185A30	pos	26,886	35,506	32,387	39,967	36,370		8,620	5,501	13,081	9,484	MPB83
494A427E14	pos	25,501	33,595	31,360	40,000	34,220		8,094	5,859	14,499	8,719	
441550741E	pos	26,137	35,556	34,530	40,000	36,726		9,419	8,393	13,863	10,589	
4949447313	pos	23,940	33,028	31,864	36,891	31,308		9,087	7,924	12,951	7,368	
492E456C57	pos	25,783	34,724	31,758	37,548	32,348		8,942	5,975	11,765	6,565	
492F38266D	pos	24,745	33,291	30,339	34,667	31,790		8,545	5,593	9,922	7,045	
4959570039	pos	23,661	33,140	31,825	37,158	35,137		9,479	8,164	13,497	11,476	
49495E034B	pos	28,896	36,806	37,158	40,000	37,133		7,910	8,262	11,104	8,237	
								7,475	**6,486**	**11,894**	**8,398**	*
434E630268	neg	35,391	40,000	40,000	40,000	40,000						**
494A123F43	neg	24,589	34,512	30,171	36,042	30,603		9,924	5,582	11,454	6,014	
492A3D0D17	neg	24,103	32,265	29,785	34,470	31,409		8,161	5,682	10,367	7,306	MPB83,DiaSer3
456B4F4153	neg	29,105	38,481	37,498	40,000	40,000		9,376	8,393	10,895	10,895	
4A5E685451	neg	29,273	40,000	40,000	40,000	40,000		10,727	10,727	10,727	10,727	
4A441E5F2A	neg	29,887	40,000	40,000	40,000	40,000		10,113	10,113	10,113	10,113	
4A6A682D39	neg	33,308	40,000	39,219	40,000	40,000		6,692	5,910	6,692	6,692	
2nd 456B4F4153	neg	37,485	40,000	38,591	40,000	40,000						**
								9,165	**7,734**	**10,041**	**8,624**	*
							ΔΔCt	1,690	1,248	-1,853	0,226	

Appendix 5: Merged sequences

The start and stop codon are marked in grey and the primers are underlined.

Lion 1, male, adult

GGCCTAACTCTCTGAAACGATGAATTACACAAGTTTTATTTTCGCTTTTC
AGCTTTGCATAATTTTGTGTTCTTCTGGTTGTTACTGTCAGGCCATGTTT
TTTAAAGAAATAGAAGAGCTAAAGGGATATTTTAATGCAAGTAATCCAGA
TGTAGCAGATGGTGGGTCGCTTTTCGTAGACATTTTGAAGAACTGGAAAG
AGGAGAGTGATAAAACAATAATTCAAAGCCAAATTGTCTCCTTCTACTTG
AAAATGTTTGAAAACCTGAAAGATGATGACCAGCGCATTCAAAGGAACAT
GGACACCATCAAGGAAGACATGCTTGATAAGTTGTTAAATACCAGCTCCA
GTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAACGATCT
CTCACCAAGATCTAACCTGAGGAAGCGGAAAAGGAGTCAGAATCTGTTTC
GAGGCCGTAGAGCATCGAAATAATGGTCGTCCTGCCTGCAATATTTG

Lion 2, adult white lion

GGCCTAACTCTCTGAAACGATGAATTACACAAGTTTTATTTTCGCTTTTC
AGCTTTGCATAATTTTGTGTTCTTCTGGTTGTTACTGTCAGGCCATGTTT
TTTAAAGAAATAGAAGAGCTAAAGGGATATTTTAATGCAAGTAATCCAGA
TGTAGCAGATGGTGGGTCGCTTTTCGTAGACATTTCAAAGAACTGGAAAG
AGGAGAGTGATAAAACAATAATTCAAAGCCAAATTGTCTCCTTCTACTTG
AAAATGTTTGAAAACCTGAAAGATGATGACCAGCGCATTCAAAGGAACAT
GGACACCATCAAGGAAGACATGCTTGATAAGTTGTTAAATACCAGCTCCA
GTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAACGATCT
CTCACCAAGATCTAACCTGAGGAAGCGGAAAAGGAGTCAGAATCTGTTTC
GAGGCCGTAGAGCATCGAAATAATGGTCGTCCTGCCTGCAATATTTG

Lion 3, 5 1/2 months old male cub

GGCCTAACTCTCTGAAACGATGAATTACACAAGTTTTATTTTCGCTTTTC
AGCTTTGCATAATTTTGTGTTCTTCTGGTTGTTACTGTCAGGCCATGTTT
TTTAAAGAAATAGAAGAGCTAAAGGGATATTTTAATGCAAGTAATCCAGA
TGTAGCAGATGGTGGGTCGCTTTTCGTAGACATTTTGAAGAACTGGAAAG
AGGAGAGTGATAAAACAATAATTCAAAGCCAAATTGTCTCCTTCTACTTG
AAAATGTTTGAAAACCTGAAAGATGATGACCAGCGCATTCAAAGGAACAT
GGACACCATCAAGGAAGACATGCTTGATAAGTTGTTAAATACCAGCTCCA
GTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAACGATCT
CTCACCAAGATCTAACCTGAGGAAGCGGAAAAGGAGTCAGAATCTGTTTC
GAGGCCGTAGAGCATCGAAATAATGGTCATCCTGCCTGCAATATTTG

Cheetah clone 1

GGCCTAACTCTCTGAAACGATGAATTACACAAGTTTTATTTTCGCTTTTC
AGCTTTGCATAATTTTGTGTTCTTCTGGTTATTACTGTCAGGCCATGTTT
TTTAAAGAAATAGAAGAGCTAAAGGGATATTTTAATGCAAGTAATCCAGA
TGTAGCAGATGGTGGGTCGCTTTTCGTAGACATTTTGAAGAACTGGAAAG
AGGAGAGTGATAAAACAATAATTCAAAGCCAAATTGTCTCCTTCTACTTG
AAAATGTTTGAAAACCTGAAAGATGATGACCAGCGCATTCAAAGGAGCAT
GGACACCATCAGGGAAGATATGCTTGATAAGTTGTTAAATACCAGCTCCA
GTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAATGATCT
CTCACCAAGATCTAACCTGAGGAAGCGGAAAAGGAGTCAGAATCTGTTTC
GAGGCCGTAGAGCATCGAAATAATGGTCATCCTGCCTGCAATATTTG

Cheetah clone 2

GGCCTAACTCTCTGAACGATGAATTACACAAGTTTTATTTTCGCTTTTCA
GCTTTGCATAATTTTGTGTTCTTCTGGTTATTACTGTCAGGCCATGTTTT
TTAAAGAAATAGAAGAGCTAAAGGGATATTTTAATGCAAGTAATCCAGAT
GTAGCAGATGGTGGGTCGCTTTTCGTAGACATTTTGAAGAACTGGAAAGA
GGAGAGTGATAAAACAATAATTCAAAGCCAAATTGTCTCCTTCTACTTGA
AAATGTTTGAAAACCTGAAAGATGATGACCAGCGCATTCAAAGGAGCATG
GACACCATCAAGGAAGATATGCTTGATAAGTTGTTAAATACCAGCTCCAG
TAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTGC
AGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAATGATCTC
TCACCAAGATCTAACCTGAGGAAGCGGAAAAGGAGTCAGAATCTGTTTCG
AGGCCGTAGAGCATCGAAATAATGGTTATCCTGCCTGCAATATTTG