Tuberculosis in African lions

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Dissertation Utrecht University, Faculty of Veterinary Medicine
ISBN: 978-90-393-5914-3
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Cover image: Ed Geis
Layout: Miriam Maas
Printed by Gildeprint Drukkerijen - Enschede

This research was partially financially supported by the Netherlands Organisation for Scientific Research (NWO/ZonMw grant 918.56.620) and the Prins Bernhard Cultuurfonds on behalf of the Niemans-Schootemeijer Fonds.

Tuberculosis in African lions

Tuberculose in Afrikaanse leeuwen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
dinsdag 5 maart 2013 des middags te 2.30 uur

door

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geboren op 9 maart 1985 te Oosterhout

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Chapter 1

General introduction

Mycobacterium bovis (*M. bovis*) is the causative agent of bovine tuberculosis (BTB) in domestic and wild animals, as well as in humans. Although the risk of *M. bovis* infection in many developed countries has been drastically reduced by national and international BTB control programs, the global economic impact of *M. bovis* is considerable, and it still constitutes a major zoonotic risk in developing countries. The presence of *M. bovis* in wildlife creates a complicated wildlife-livestock-human interface, and may threaten the conservation of vulnerable wildlife species like the lion.

Mycobacteria

Mycobacteria are aerobic, non-motile, non-spore forming, straight or slightly curved rods that are 1.5 to 4.0 μm long and 0.3 to 0.5 μm wide [1]. Most mycobacteria are non-harmful, but pathogenic ones like *M. tuberculosis* and *M. bovis*, can cause serious health problems and even death as a consequence of tuberculosis. Both *M. tuberculosis* and *M. bovis* are members of the Mycobacterium Tuberculosis Complex (MTBC), which comprises a variety of closely related mycobacteria [2]. *M. tuberculosis* infection is known to cause human tuberculosis, but also other species like elephants [3] or non-human primates [4] may be affected. In 2010, human tuberculosis caused 1.45 million deaths and 8.8 million incident cases [5].

The genome sequence of *M. bovis* is >99.95 % identical to that of *M. tuberculosis* at the nucleotide level, but deletions have caused a reduced genome size (<1.5%) [6]. *M. bovis* has an almost global distribution (Figure 1). Its major host is cattle, but *M. bovis* has a broad host range and is the principal causative agent of bovine tuberculosis (BTB) in domestic and wild mammals [7], as well as in humans [8]. The economic impact of *M. bovis* is considerable, e.g. the USA spend yearly almost $10 million USD on BTB related activities [9]. The zoonotic risk of *M. bovis* in the developed world is considered of minor importance through the test-and-slaughter policies and consumption of pasteurized milk. However, BTB has been qualified as a neglected zoonosis by the WHO in conjunction with the FAO and OIE, with special reference to developing countries [10]. Studies concerning the relative contributions of the two mycobacteria to tuberculosis cases illustrate this inequality between the developed and developing world: in the USA 1.4% of the TB cases were caused by *M. bovis* [11] as compared to 5% of sputum samples that were *M. bovis* positive in a Nigerian study [11,12]. However, these studies are complicated by the fact that tuberculosis in humans due to *M. bovis* is both clinically and pathologically indistinguishable from cases caused by *M. tuberculosis* [13] and humans are as susceptible to *M. bovis* infection as they are to *M. tuberculosis* infection [1].

Bovine tuberculosis in wildlife

Mycobacterium bovis can infect a wide range of mammalian species, including many wildlife species [7]. The presence of *M. bovis* in wildlife creates a complicated wildlife-livestock-human interface and wildlife maintenance hosts, as potential sources of infection for livestock, hamper national control and eradication programs. *M. bovis* may also spillover to other wildlife species, creating conservation issues for endangered species, as well as involving new potential maintenance hosts in the epidemiology of *M. bovis* infection [15].

Control or elimination of BTB is increasingly difficult when multiple (wildlife) species are involved and identifying which species are the principal drivers of BTB persistence is essential [16]. Different scenarios of involvement of maintenance in wildlife hosts exist, ranging from the simplest scenario where BTB is maintained only by cattle or other bovid species (e.g. water buffalo in Australia) to single, non-bovid wildlife hosts (e.g. badgers in the UK or white-tailed deer in the USA), to a small number of mammal species (e.g. wild boar and red deer in Spain, and possums, red deer and ferrets New Zealand) to a complicated web of multiple wildlife hosts

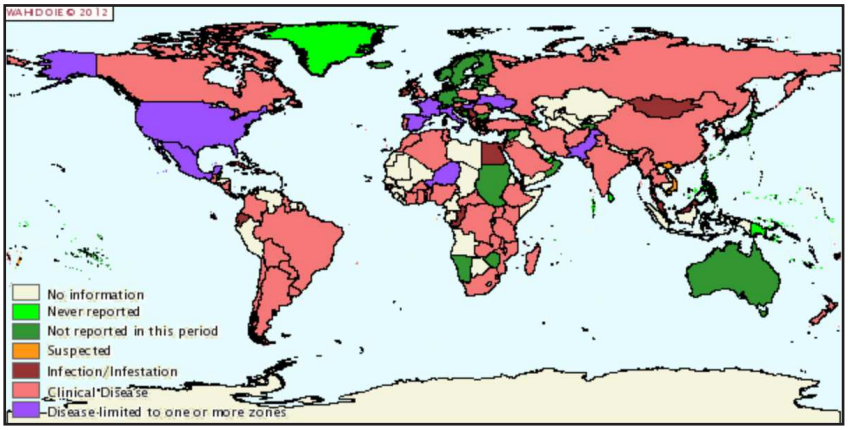

Figure 1. Bovine tuberculosis distribution map, period January-June 2011 [14].

(e.g. Kruger National Park, South Africa) [9,15-22].

The immune response in course of time after M. bovis infection- humans and cattle as an example

When animals (or humans) are exposed to *M. bovis* the infection may develop in several ways. Depending on the initial challenge dose of *M. bovis* and the development of anti-mycobacterial immune responses (influenced by other factors like nutritional status and stress) [23], bacteria may be cleared, the infection may go into a dormant state (latent infection) or (eventually) active tuberculosis may develop [24].

Immune responsiveness in tuberculosis is aimed at containment of the spread of bacteria [7]. After deposition of bacilli on the respiratory surface, phagocytosis is the initial step in innate (non-specific) reactivity followed by induction of a cell-mediated immune response (CMI) and only in later stages of infection the humoral immune response develops, hence antibody titers increase [25]. Cell-mediated immunity (CMI) is thought to play the major role in controlling the infection [26,27].

The macrophage, that "hosts" the bacteria, is the main effector cell for control of mycobacterial infections, but in addition T-lymphocytes are major players in the protective acquired immune response [28]. Two major subsets of T-lymphocytes exist: cytotoxic T-cells, which express the CD8 marker and are known to mediate lysis of autologous cells infected by intracellular pathogens, thus helping in controlling of the infection, and T-helper cells, expressing the surface protein CD4 [29]. At least four major subsets of CD4 T cells are described in humans: Th (helper) 1, Th2, Th17 and Treg (regulatory) cells [30]. Th1 cells produce IFN-γ, IL-2, and TNF-α and are considered essential in the immune response to mycobacteria and for the containment of the mycobacterial infection. They are associated with macrophage activity and T cell proliferation [28,31]. Th2 cells produce IL-4, IL-5, IL-9, IL-10, support humoral immune responsiveness and act as regulatory cells [32]. A dominant type 2 immunity is related to active infection [29]. Although functions of T cell subsets show plasticity depending on the species, the conventional definition of a Th1 or Th2 cell depends strictly on the secretion of IFN-γ or IL-4: Th1 cells secrete IFN-γ but do not secrete IL-4, whereas Th2 cells secrete IL-4 but not IFN-γ [29]. Th17 cells, producing IL-17 and IL-22, have only been described recently. IL-17 is a potent inflam-

matory cytokine in the primary phase of infection. During the chronic phase, there is a balance between Th1 and Th17 to promote anti-mycobacterial immunity and to control bacterial growth and limit tissue damage [33]. Regulatory T cells can produce the anti-inflammatory cytokine IL-10 and are emerging as important contributors to *M. tuberculosis* immunity, regulating and balancing T cell and immune reactivity [31].
Though the pivotal role of IFN-γ is undoubted, it is not a good correlate of protection in humans infected with *M. tuberculosis* [31,34]. Recent research therefore also focuses on other subsets than Th1 and Th2, but the mechanisms of protection and pathology associated with Tregs and Th17 cells still need to be further elucidated [33,35]. Immune responsiveness in tuberculosis may also be influenced by Type I IFN (IFN-α and IFN-β) and natural killer (NK) cells, which are usually associated with immune responsiveness against viral infections. In addition, natural killer T (NKT) cells, invariant NKT (iNKT) cells and the recently identified mucosal-associated invariant T (MAIT) lymphocytes may be of importance, but their roles need to be established [31,33]. Inevitably, most research focuses on CMI, but naturally the humoral response may also contribute to infection control, of which the synergy and mutual interdependence with CMI needs to be studied further [36].

Knowledge on the immune responsiveness may be used to improve Bacillus Calmette-Guérin (BCG) vaccination and to generate more effective vaccination strategies. CD4 memory T cells may play diverse roles in protection, from production of effector cytokines early in the response, to enhancement of B cell and CD8 T cell responses, the latter directly killing infected cells [37]. CD4 memory T cells are divided into two subsets, effector memory T cells and central memory T cells. Effector memory T cells lack lymph-node-homing receptors, but express receptors that enable them to move into inflamed tissue where they can exert immediate effector functions. Central memory T cells are antigen-experienced T cells that are thought to be long-lived and express receptors for homing to secondary lymphoid organs and can serve as the precursor for effector T cells in recall responses [30]. It is hypothesized that BCG vaccination is a poor inducer of central memory T cells which may be the reason why vaccination appears to lose efficacy in children over a period of several years [38].

Pathogenesis of M. bovis infection- humans and cattle as an example
Tuberculosis is primarily an infection of the respiratory tract, where mycobacteria use macrophages as primary host cells for intracellular replication [25]. Aerosols constitute the most important transmission mode for humans and cattle [23]. The efficiency of this transmission depends on the size and consistency of the aerosolized droplets, fine aerosol suspensions of low viscosity being the most effective for delivering mycobacterial content, although only a very small fraction of those droplets contains viable bacilli. In experimental cattle models, the challenge dose was positively associated with the proportion of animals that became diseased and with the severity of the disease [23]. Infection with *M. bovis* results in the formation of granulomas within the lungs and lymph nodes, comprised of a core of infected and killed macrophages surrounded and infiltrated by T-lymphocytes. The granulomas act to control and restrict the spread of infection, but may also result in significant tissue damage [39]. Potential aerosol transmission is facilitated by destruction of lung tissue and macroscopic open spaces containing mycobacteria and connecting to large airways [30].

Diagnostic options
Infection of cattle with *M. bovis* is often chronic and can remain subclinical for long periods [40]. Early after infection, CMI assays, as well as those measuring the humoral response, will

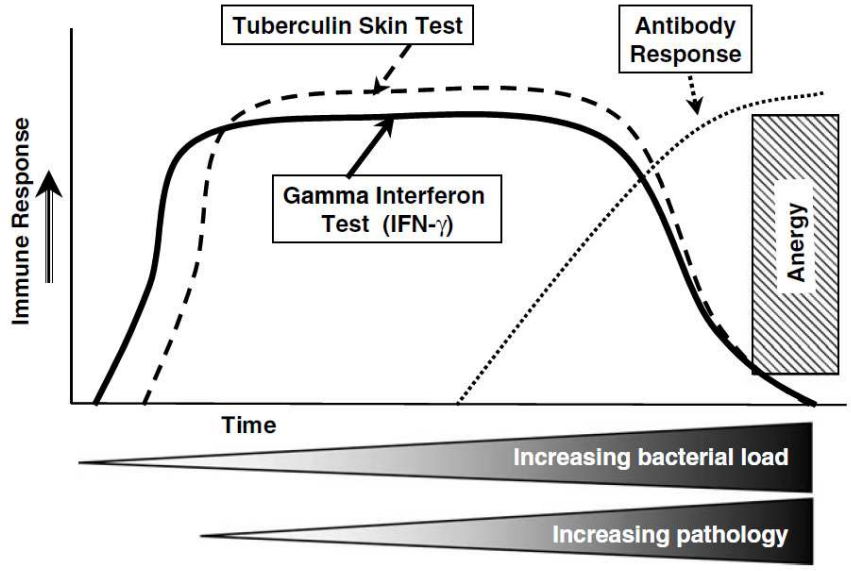

Figure 2. Immune responses, bacterial load and pathology in cattle in the course of *M. bovis* infection ©[40].

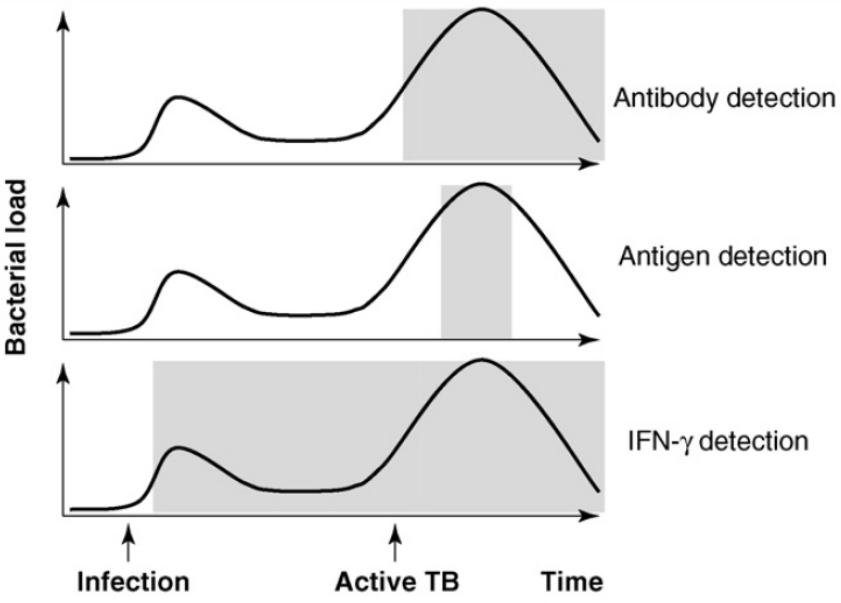

Figure 3. Schematic representation of the immune responses during the course of human TB infection. The shaded areas illustrate detection of responses in course of time using the given test ©[42].

be negative. When CMI develops, usually within weeks, it can be measured with the tuberculin skin test and the IFN-γ assay. When CMI declines, responses assessed in the tests will diminish as well and may become negative. A temporal decline of CMI may also be induced by stress, e.g. in animals that have just given birth [23]. As CMI declines, gradually the humoral response develops, and specific antibodies appear (Figure 2). These can be detected with serologic tests like the Enzyme-Linked Immunosorbent Assay (ELISA). The animal is likely to start shedding bacteria and to show increasing clinical symptoms of tuberculosis [41]. Infection kinetics studied in humans infected with *M. tuberculosis* show that the IFN-γ response is maintained throughout the course of infection, though it can wane in individuals developing very severe TB (Figure 3) [42].

Mycobacterium bovis infection of free-ranging lions

After the first discovery of *M. bovis* in free-ranging lions in the Kruger National Park (KNP) in 1995 [43], *M. bovis* infections were also confirmed in other free-ranging lion populations, like in the Hluhluwe-iMfolozi park in South Africa [44]. In Northern Tanzania, 4% of lions in the Serengeti tested positive in an ELISA. Although infection was not confirmed with a bacterial culture, the results indicated the presence of *M. bovis* in this population, and the prevalence is likely to be even higher, due to the low sensitivity of the test [45]. It is possible that other African wildlife parks also suffer from BTB in their (buffalo and) lion population, taking into account that many African countries suffer from *M. bovis* infection in their cattle and the ease of contact between cattle and wildlife species like buffaloes. Furthermore, lions from the KNP have been translocated to other wildlife reserves all over the African continent (D.F. Keet, personal communication). If lions have maintenance potential, these translocations may have facilitated the spread of *M. bovis* amongst lion populations.

Lions are listed as a vulnerable species on the IUCN red list and the populations of the KNP and the Serengeti are two of the few remaining strongholds of lions that are considered to be genetically viable [46](IUCN website, assessed 13.10.2012). The introduction of *M. bovis* in these populations has direct health consequences for the lions and could thus be a potential conservation issue. It may also affect prey populations and alter interspecific competition with other predators [47,48]. Finally, it causes an economic threat as lions are a major tourist attraction [7,49].

The major part of current knowledge about *M. bovis* in African wildlife is obtained in studies in the KNP after the official discovery in native, free-ranging African buffaloes (Syncerus caffer) in 1990 [50]. In the KNP, *M. bovis* was initially transmitted from livestock to buffaloes at a single entrance point in the south of the park, and spread progressively northwards- a process that still continues [51]. The regional prevalence of the infection in buffaloes in 1998 was 38.2% in the southern region and 1.5% in the northern district [52]. In 2007, the estimated prevalences of infected buffaloes were 30-40% in the south of the park, 15-24% in the central area, 10-16% in the northern area and <10% in the far north of the park (de Klerk, personal communication). In buffaloes, a correlation between increasing BTB herd prevalence and a decrease in overall body score was found [53]. Weak prey animals, hence buffaloes worst affected by the disease, have been proven to be a likely target for lion predation [53,54], resulting in frequent exposure of lions to *M. bovis*. Buffaloes are considered to be one of the four preferential prey species of lions, especially in dry seasons, and lions in the KNP most likely became infected by predation on infected buffaloes [52,55]. Genetic typing confirmed spillover of *M. bovis* from buffaloes to lions in the KNP [51]. Since the prevalence of *M. bovis* amongst buffaloes is still increasing, and likewise that in lions, it is very likely that the majority of the KNP lions will become infected in the future [52].

Clinical signs and pathology of BTB in lions

Adult lions are more likely to be infected than sub adults and cubs [52]. No precise data are available on the life expectancy once lions are infected. The immunopathogenesis of tuberculosis in lions is unclear, and only few studies have been published on the clinical sings and pathology of (free-ranging and captive) BTB positive lions.

Generally, loss of body condition and poor skin and coat quality are the first signs of advanced infection (D.F. Keet, personal communication) [52,56,57]. These non-specific signs may be accompanied by elbow hygromas: firm but fluctuating swellings behind the elbows that can become quite large (10x10 cm), which, in *M. bovis*-infected ecosystems and especially in older lions, are often indicative for BTB (D.F. Keet, personal communication).

Post mortem macroscopic pathological lesions are difficult to discern and vary from those described in ungulates and non-human primates. Contrary to the situation in omnivores and herbivores, no abscessation, caseation or mineralization is present [7]. Pulmonary lesions in lions are diverse and are usually seen in more advanced cases. Granulomas are found and are the only pathognomonic macroscopic lesions [52]. The lungs may show signs of bronchiectasis and numerous fibrous but fairly thin-walled cavities with small quantities of an opaque, greyish-white mucoid exsudate, that in most cases contains large numbers of acid fast bacteria (D.F. Keet, personal communication). Histopathology may show granulomatous pneumonia, but without necrosis in the inflammatory reaction [52]. The multinucleated giant cells that are prominent in the granulomatous reactions of buffaloes, kudus and baboons, are rare in lions (D.F. Keet, personal communication).

Hyperplastic lymph nodes are often seen in other species [58], but in advanced BTB cases in lions these are more often atrophied, rather than hyperplastic, and often no lesions can be identified macroscopically, hence mycobacterial culture and histology are essential for diagnosis of BTB from lymph nodes. No macroscopic intestinal lesions are found, although draining lymph nodes of the intestinal tract may show lesions (D.F. Keet, personal communication). Osseus lesions were found in almost half of the tuberculosis positive lions, suspected to be caused by *M. bovis* [49]. Other than these, a variety of organs may be infected occasionally due to haematogenous and lymphatic spread, including eyes, liver and kidneys [52,59](D.F. Keet, personal communication).

Transmission routes of M. bovis in lions

To determine the role of lions in the persistence of *M. bovis*, which may aid in management measures, they need to be classified as either maintenance or spillover hosts. As a definition, in a maintenance host the infection can persist by intraspecies transmission. In a spillover host the infection cannot persist indefinitely without re-infection coming from other species [21]. Both maintenance and spillover hosts can be a source of infection for other species and can thus cause spill-back to the reservoir/original source of BTB [21]. Determining maintenance host potential can be challenging, because this is influenced by different factors like location of lesions and may change under certain conditions, e.g. population density [21,60,61].

Lions have mainly been classified as spillover hosts [7,15,62]. However, it has also been suggested they have maintenance potential [43,63]. The high prevalence of BTB in lions compared to buffaloes is not necessarily an indication for maintenance potential, but could merely be a result of frequent exposure to infected prey animals [21].

Three likely infection routes exist for lions: oral, respiratory and percutaneuous. In many animal hosts, the route of transmission of *M. bovis* can be deducted by the pattern of lesions observed

at necropsy [64]. Lesions restricted to the thoracic cavity indicate aerosol transmission, while lesions in mesenteric lymph nodes are thought to indicate oral transmission [23,56]. However, doses of infection may be of influence on the location of the lesions and thus results should be interpreted with caution [65].
Oral infection of free-ranging lions takes place when feeding on infected prey species [52]. Feeding of infected meat has also resulted in *M. bovis* infection in captive lions [7,56,57]. The existence of an oral transmission route was confirmed by culture of *M. bovis* from draining lymph nodes of the intestinal tract (D.F. Keet, personal communication).
Compared to oral infection, fewer mycobacteria are thought to be required to establish infection through the respiratory route [66]. During suffocation of an infected buffalo, as part of the hunt, a lion is prone to inhale a large number of mycobacteria, promoting aerosol infection. Spreading of the bacteria by aerosols by heavy breathing and growling can also occur when lions are in close proximity to each other when feeding together on the carcass [59]. The fact that BTB ultimately results in pulmonary involvement renders lions infectious to other pride members, which contributes to their maintenance potential (D.F. Keet, personal communication). Indications for aerosol transmission, i.e. lung lesions, were also found in orally infected zoo lions [56].
Transmission amongst lions is most likely predominantly via the aerosol route, but may also occur percutaneously (by biting), through the presence of exhaled or ingested free mycobacteria in the mouth of an infected individual. Biting occurs during intraspecies aggression [52,59] and has also been established as an important infection route in badgers [67,68].

The youngest lion cub showing a positive response in the tuberculin skin test was five months old, in addition a ten months old cub in poor condition showed signs of advanced tuberculosis (supplement [69])[52]. Though young lions may start feeding on carcasses from three months of age onwards, these cases may suggest additional infection routes. A congenital route of infection was suggested after finding tuberculous lesions in the uteri of three domestic cats [70]. Another possibility is lactogenic transmission and *M. bovis* has in fact been isolated from the mammary lymph nodes of three lionesses [52]. This transmission route through mammary gland infection hasn't been proven yet for lions, but transmission of *M. bovis* to humans via cow milk used to be a common infection route before pasteurization of milk was implemented [71]. Historically, tuberculosis in domestic cats resulted from ingesting infected cow milk, leading to gastrointestinal lesions (cited in [72]).

The diversity of the pulmonary lesions found in lions, may result from two possible routes of pulmonary infection: haematogenous and inhalation. Granulomatous interstitial pneumonia, causing lesions in the dorso-caudal lobes, is associated with haematogenous spread, whereas bronchopneumonia, causing lesions in the ventral aspects of the cranial lobes, is associated with infection through inhalation. Based on the type and position of lesions found in lions, it is estimated that about 40% of pulmonary infections were of aerosol origin and thus respiratory infections may be more important than was previously considered (D.F. Keet, personal communication). However, the intermittent and concurrent occurrence of intestinal, aerosol and percutaneous infections makes it difficult to really quantify the various infections routes.

Diagnostic methods for BTB in wildlife and in particular lions
The occurrence of *M. bovis* in free-ranging lions has urged the need for new diagnostic methods for this species. The OIE aims for useful diagnostic tests following the 'Fit for purpose'-concept, meaning that the purpose of a test, which can be different per geographical site, species and disease, must be determined first [73]. Often this implies an emphasis on either test sensitivity

(the proportion of infected animals detected as positive in the diagnostic assay) or test specificity (the proportion of non-infected animals detected as negative in the diagnostic assay), as well as a choice between diagnosing infection in early or late stages of the infection. Single blood-based tests are appealing for use in wildlife, since only one manipulation is necessary, which reduces stress for the animal and reduces chances of injuries [74].
Like in other species, diagnosis of *M. bovis* in lions is complicated by the chronic nature of the infection and initially clinical signs are non-existent and lions are found to be in good condition [7]. Apart from observation of clinical signs, the only available ante mortem diagnostic approaches for lions are skin testing (SICT: Single Intradermal Cervical Test) [75] and serology. The SICT, currently used for diagnosis of BTB has a sensitivity of 86.5% and a specificity of 81% [75]. As it assesses cell-mediated immunity (CMI), it detects early stages of infection, but it has as a disadvantage that animals need to be recaptured after three days, which is time and money consuming, stressful for the animals and difficult to implement as often lions cannot be recaptured. In vitro cell-based (CMI) 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 conditions that might not be achievable in the field. Although serological assays have advantages in terms of logistics, lower costs, and ease of application, only recently have new diagnostic assays been developed for several wildlife species that seem to have adequate sensitivity and specificity suitable for routine diagnostic use [76]. Serology is appealing for free-ranging lions and preliminary studies with new serologic assays have shown diagnostic potential [77]. Considerations for the choice of diagnostic methods and current developments in the diagnostic methods for wildlife are further discussed in detail in chapters 2 and 4.

FIV_{ple} in lions

FIV_{ple} is an endemic disease in many African lion populations, including those in the KNP and the Serengeti [78-81]. The long-held paradigm was that FIV_{ple} was not associated with pathology, nor reduced lifespan or fecundity, suggesting an old co-evolved host-pathogen symbiosis (or commensalism) [82-86]. In 2006, however, an increase was found in the CD4+/CD8+ T-cell subset ratio in FIV_{ple}-infected lions as compared to non-infected lions [81], and in 2009, in several FIV_{ple}-infected lions evidence was found of lymphoid depletion. Relative to uninfected lions, FIV_{ple}-infected lions displayed a significant elevation in the prevalence of AIDS-defining conditions: lymphadenopathy, gingivitis, tongue papillomas, dehydration, and poor coat condition, as well as abnormal red blood cell parameters, depressed serum albumin, and elevated liver enzymes and gamma globulin [87]. The reason for these contradicting findings, may be the difference in pathogenicity of the various FIV_{ple} strains [88,89].

OUTLINE OF THIS THESIS

A lack of knowledge of the (immune)pathogenesis of bovine tuberculosis in lions hampers development of control and eradication strategies. Accurate and practical assays for (early) diagnosis are urgently needed. Furthermore, it is unclear whether *M. bovis* is a threat to the lion population, especially taking into account the endemic FIV_{ple} infection. This thesis aims at contributing to the current knowledge on *M. bovis* infection in lions by development and assessment of diagnostic assays, by assessment of aspects of immunopathogenesis and by investigation of the impact of FIV_{ple}-*M. bovis* co-infection.

Diagnosis of *M. bovis*-infected wildlife populations or individual animals is a challenge. Chapter 2 reviews the currently available diagnostic assays used for BTB testing of wildlife species in general, from a 'fit-for-purpose' perspective.

For lions, the only diagnostic method that is currently validated is the tuberculin skin test, which has, however, the severe limitation that a second immobilisation is necessary. Therefore, one of the aims of this study was to develop an IFN-γ assay for lions, an assay based on CMI, as a contribution to the diagnostic spectrum for *M. bovis* infection in lions. Chapter 3 describes the sequencing of the lion and cheetah IFN-γ genes: the basis for the production of recombinant lion IFN-γ and lion IFN-γ specific antibodies, as part of the development of a lion-specific IFN-γ assay, described in chapter 4b. In that chapter, an optimised procedure and proof of recognition of native lion IFN-γ will be provided. Also, a lion-adapted feline IFN-γ assay was assessed for the use in lions (chapter 4a).

To provide tools for the investigation of the immunopathogenesis of *M. bovis* infection in lions, the development of reverse transcriptase real-time PCRs for four cytokines involved in the immune response induced by *M. bovis* and their use in a small pilot study is described in chapter 5. The ability to assess the expression of these cytokines may help to describe the development of immune responsiveness in *M. bovis*-infected lions, and, used as diagnostic assays, these PCR's may facilitate diagnosis.

When determining the risk of *M. bovis* for the lion population, potential co-infection with other pathogens needs to be considered. In chapter 6 the potential effect of co-infection of lions with *M. bovis* and FIV_{ple} is investigated, considering the increased disease burden in humans co-infected with *M. tuberculosis* and HIV.

Reference intervals for hematological and serum chemistry values are scarce for free-ranging lions. Therefore, the large data set that is used for the analysis in chapter 6, was also used to determine reference ranges for several blood parameter values reported in chapter 7.

A general discussion of the different aspects of *M. bovis* infection in lions studied and recommendations for continuing research are presented in chapter 8

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Chapter 2

Facts and dilemmas in diagnosis of tuberculosis in wildlife

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Accepted for publication:
Comparative Immunology, Microbiology & Infectious Diseases

ABSTRACT

Mycobacterium bovis, causing bovine tuberculosis (BTB), has been recognized as a global threat at the wildlife-livestock-human interface, a clear "One Health" issue. Several wildlife species have been identified as maintenance hosts. Spillover of infection from these species to livestock or other wildlife species may have economic and conservation implications and infection of humans causes public health concerns, especially in developing countries. Most BTB management strategies rely on BTB testing, which can be performed for a range of purposes, from disease surveillance to diagnosing individual infected animals. New diagnostic assays are being developed for selected wildlife species. This review investigates the most frequent objectives and associated requirements for testing wildlife for tuberculosis at the level of individual animals as well as small and large populations. By aligning those with the available (immunological) ante mortem diagnostic assays, the practical challenges and limitations wildlife managers and researchers are currently faced with are highlighted.

INTRODUCTION

Animal health and public health are inextricably intertwined and recognition of this crucial interdependence has led to the multi-disciplinary concept of "One World, One Health, One Medicine". Pathogens that are transmitted between wildlife, livestock and humans represent major challenges for human and animal health, the economic sustainability of agriculture, and the conservation of wildlife [1].

In this context, bovine tuberculosis (BTB), caused by *Mycobacterium bovis* (*M. bovis*), is a relevant disease threat, impacting on the human-livestock-wildlife interface globally [2]. *Mycobacterium bovis* is a member of the *Mycobacterium tuberculosis* complex, which also contains other pathogenic mycobacteria like *M. tuberculosis* [3]. Wildlife species are potential reservoirs of *M. bovis* for domestic animals and humans [4], which may hamper national control and eradication programs that are in place in many (developed) countries [5-7]. *Mycobacterium bovis* infections have been described in both free-ranging [8] and captive wildlife species [9,10] in various regions of the world. Some of these may act as maintenance hosts, while infection in others is incidental. Animal populations now known to be maintenance host include Eurasian badger (*Meles meles*, United Kingdom) [11,12], African buffalo (*Syncerus caffer*) [13], brushtail possum (Trichosurus vulpecula, New Zealand) [14], white-tailed deer (*Odocoileus virginianus*, United States) [15] and European wild boar (*Sus scrofa*, Spain)[16]. The spectrum of potential spillover hosts of *M. bovis* is extensive and appears to include a wide range of mammalian species, e.g. gorillas (*Gorilla gorilla gorilla*) [17], lynx (*Lynx pardinus*) [18], rhinoceros (*Diceros bicornis minor* and *Ceratotherium simum simum*) [19,20], cheetah (*Acinonyx jubatus*) [21] and lion (*Panthera leo*) [22]. Since it became known that wildlife can act as reservoirs for *M. bovis* the need for BTB control strategies in these species has been emphasized in a number of countries[8].

In view of the "One Health" concept, the extent of contact and interaction of wildlife reservoirs with domestic animals and humans is one of the main risk factors of infection [23]. Direct wildlife-to-human transmission of *M. bovis* is mainly limited to consumers and processors of (raw) infected wildlife products [24,25], and to keepers of captive wildlife [20]. The indirect BTB transmission from wildlife to humans through livestock is more likely to occur: in developed countries 0-2% of the tuberculosis cases in humans are caused by BTB [26]; in developing countries these percentages may be much higher and BTB still constitutes a major zoonotic risk there [2,27]. The human-to-wildlife transmission is considered a high risk for especially captive wildlife, that is exposed to human pathogens transmitted by their owners [28] or handlers and the public, e.g. in zoological collections [29-32], wildlife rehabilitation and primate research centers [33] as well as culturally based Asian elephant-human interactions [34] and the transmission can even occur to free-ranging wildlife as was shown in meerkats *(Suricata suricatta)* in South Africa [35]. In addition, spillover of BTB from wildlife reservoirs to isolated, small wildlife populations like the Iberian lynx or the black rhinoceros (*Diceros bicornis minor*), may be reason for concern regarding species conservation [18,36]: it not only causes a potential mortality risk, but may also limit translocation movements [19].

THE ROLE OF DIAGNOSTIC TESTS IN THE MANAGEMENT OF BOVINE TUBERCULOSIS IN WILDLIFE

One of the pivotal issues of managing wildlife BTB is the availability of diagnostic assays, which is often limited to those developed for domestic animals and humans. On a daily basis, wildlife species are tested for BTB for various purposes, often with diagnostic assays that are

accepted due to a lack of a better alternative [37]. However, because of the recognition of the role of particular wildlife species like badgers, possums and white-tailed deer in the maintenance of the *M. bovis* infection, as well as its spillover into additional species, an increasing number of studies has been dedicated to the development of diagnostic assays for specific wildlife species, resulting in assay prototypes and partially or fully validated surveillance can be conducted. This is a more "snapshot-like" approach focused on a particular pathogen in a specified wildlife population which is classified as healthy, but is considered at risk of exposure to this pathogen from an identified source e.g. screening a wildlife population when positive cases in nearby cattle have been found [46]. Diagnosis of BTB in a deer based on gross lesions (general surveillance) resulted in subsequent targeted sampling of the white-tailed deer population in 1994 in northeastern Michigan (USA), including necropsies and additional culturing [47]. This confirmed infection in the population at a prevalence of nearly 5% and a BTB management program has been implemented since then for this wildlife reservoir [15].

BTB surveillance of cattle and wildlife is ongoing in many developed countries that seek to maintain a BTB free status, especially those that have established wildlife reservoirs, as early detection of infection may prevent extensive spread through the population. In this situation, diagnostic assays are used as tools to determine whether *M. bovis* is present in a certain area or population, rather than to determine exact prevalence. Surveillance is often passive under these circumstances [48] and involves the help of State and Federal wildlife agencies and hunters [46,49], like in case of the discovery of the first BTB diseased possum in New Zealand [50]. Surveillance generates a continuous flow of potentially large sample sets to the laboratory and requires logistics (i.e. technical simplicity, stability of the test under user conditions, automation) that are easy and robust and are not compromised by long transport times of samples. While the emphasis is on affordability, because of the high throughput, short turn-around times are of less importance [47]. As far as surveillance relies on direct detection methods (necropsies/meat inspection/culturing/histopathology) [51] and serology, it can be used for many wildlife species, provided cross-reactive reagents are available for the latter [52].

Monitoring

According to Artois et al., monitoring is restricted to known infected populations, and consists of the systematic recording of epidemiological data, with no other specific purpose than detecting spatial and temporal trends [48]. For *M. bovis*, monitoring may assess the spread, interspecies transmission or the effect of management interventions in wildlife populations, like the culling of badgers [1] or vaccination of possums and badgers [53]. The latter necessitates the use of tests that can differentiate between vaccinated and infected animals (DIVA).

Monitoring targets specific infected populations and diagnostic methods are needed that have been validated for that species. It is often executed on a smaller scale than surveillance, e.g. wildlife parks instead of countries, but with a more intensive, active approach, like BTB testing of buffaloes using the IFN-γ assay and the tuberculin skin test in South African conservation areas [4]. It does not usually include passive surveillance, or passive surveillance plays only a minor role. In general, monitoring is more invasive and expensive than surveillance, with a larger number of animals needing to be tested in a shorter time. This makes monitoring more sensitive to the cost of the test, compared to surveillance, while time constraints are of less importance.

Though diagnostic methods used can identify *M. bovis* infected individuals, both surveillance and monitoring are not applied to diagnose *M. bovis* infection in a specific, identified individual,

but are rather used as a screening tool to determine whether the infectious agent is present in a defined population and area and if so, whether changes occur in prevalence or distribution of the agent in the studied areas and periods of time.

Diagnosis of M. bovis infection at individual animal level/in small populations

In contrast with surveillance and monitoring, when testing individual animals and small populations for BTB, the assays have a short-term, true diagnostic application, aiming at confirmation of the *M. bovis* infection status. This includes diagnosis and tracing back of mycobacterial infections in free-ranging and captive wildlife populations including zoological collections, for example after confirmation of an initial case in a pot-bellied pig (*Sus scrofa vittatus*) in a wildlife park [54]. Trace back of *M. bovis* infection of farmed fallow deer (*Dama Dama*) in Sweden was complicated due to the lack of individual identification [51]. Assessment of the human health risk as a result of close contact with infected wildlife, as described for various captive wildlife species, including elephants, in zoological collections [30,31,55] or for an infected marmoset [28] may be part of these analyses. Diagnosis of BTB in small numbers of individuals has far-reaching implications on the BTB infection status of the respective population and may lead up to control measures like regulatory quarantine or culling, and is therefore traditionally performed with or followed by an accepted gold standard test, i.e. culture. However, since culture is, in most cases, performed most reliably on post mortem tissue samples, there may be a difficult trade-off between loss of a valuable individual, in the context of species conservation, and the consequences of maintaining a potentially BTB infected shedder in the collection. Management choices may differ, as exemplified by BTB infection in Bactrian camels [56] and Asian elephants in zoological collections [57], where respectively culling and medical treatment were chosen.

Diagnosing BTB at the small scale level may benefit from clear classification of the clinical disease status, distinguishing between early infections (pre-clinical) and active disease. This may provide valuable information with regard to the type of control strategy required [58]. Early detection methods may minimize the spread and the effects of an outbreak. However, a diagnostic assay or testing strategy that could detect the (probably small) proportion of highly infectious individuals, i.e. the shedders, can be useful for wildlife species of high value [59], chronically infected populations, as well as wildlife populations with unknown BTB status [60].

Certification of BTB-free status of animals

Certification of freedom from BTB, like other infectious diseases, is an official requirement for the safe trade of game meat for human consumption (EU Directive 2003/99/EC)[61,62], as well as for wildlife translocations, e.g. between zoological collections, game parks and game farms [63]. This certification is based on risk assessment involving individual animal and population data relating to the BTB infection status of the particular environment and based on historical or preferably on surveillance data, as well as to a very large extent on diagnostic test results [64]. Since the latter depend on the availability of analytical and diagnostic performance data of the test used, the accuracy of these risk assessments would strongly increase with validated diagnostic methods for specific wildlife species.

No official international protocol for BTB-free certification for wildlife exportation and translocations purposes between zoological collections exists in Europe nor in the USA, and the requirements can differ between countries and states [9]. In Southern Africa, where African buffaloes are a known reservoir for BTB, foot-and mouth disease, theileriosis and brucellosis, strict control measures are in place which require negative test results for all four disease agents

prior to movement [65,66]. Due to the close phylogenetic relationship to domestic cattle, applicable ante mortem tests such as the tuberculin skin test are accepted although not validated. More ideally and to comply with importation regulations, the assay should be animal species-specific, have a high sensitivity and a very high specificity and cover a large part of the immune response and test results should be available within a short space of time to avoid long quarantine periods.
If wildlife species are used for meat consumption, the risk assessment for certification will be aided by the use of post mortem examination, whereby the disease free status of the animals must be ensured whenever the meat is destined for the international market. Meat inspection is currently the only method suitable for large-scale screening in slaughtered deer, with final confirmation by culturing [64]. However, as has been shown in wild boar, this may actually not be a very sensitive method [67].

Research purposes
Though not directly related to the management of BTB, diagnostic tests providing baseline data also play a crucial role in research in the context of disease ecology, epidemiology, vaccine efficacy or intervention strategies. For example, *M. bovis* infection was measured with an IFN-γ ELISA in an epidemiologic study on micro- and macroparasites in buffalo [68] and immunopathogenesis in experimentally infected badgers was assessed with various cell-mediated and serological assays [69] The diagnostic assays can also be used to study the organism, e.g. characterization of (spread of) different strains using mostly DNA fingerprinting and PCR techniques [54,70]. Furthermore, experimental infection studies are also conducted to evaluate the performance of a newly developed test or to validate an existing diagnostic assay, used in domestic animals, for a wildlife species, of which examples can be found in Table 1. The success and feasibility of these studies depends upon the availability of a suitable test for the wildlife species of interest.

Diagnostic methods in these research settings can be practically as well as financially more demanding, as it often concerns only small populations/study groups for pre-defined periods. They need to be animal species-specific, and often several tests are combined [71].

DIAGNOSTIC ASSAYS THAT ARE AVAILABLE
A literature review was performed focusing on the search terms: bovine tuberculosis, wildlife (and more specific search terms as deer, buffalo etc), diagnostic assays (and more specific search terms as IFN-γ assay, serology, culture etc), and in addition considering cited and citing articles. Diagnostic methods for non-bovine (wildlife) species have been reviewed in 2005 [37] and 2009 [38] as well, and therefore the emphasis of this review is on studies after 2005. Most of the new developments regarding diagnostic methods for *M. bovis* infection are based on indirect detection, aiming at the assessment of immune response parameters. Table 1 provides an overview of the different studies that have been published on the immunological diagnostic methods for *M. bovis (*and *M. tuberculosis*) infections in wildlife species.

Direct assays
Diagnostic assays may be based on the direct detection of the infectious agent. Since this is independent of the host species, they are generally well established for the different *Mycobacteria* spp.. Direct identification of the agent may be by microscopic demonstration of acid-fast bacilli in various samples, bacterial culture and the polymerase chain reaction (PCR), whereby the latter is also useful for differentiation between *Mycobacterium* spp. [43] as was used e.g. in elk [72] and lynx [18]. Direct microscopic smear examination is a fast, inexpensive method and

can provide a presumptive diagnosis, especially when clinical signs and lesions are present. Its sensitivity has been reported to be variable depending on the wildlife species and severity of infection and ranged from 55.6% in wild boar [67] to 90% in white-tailed deer [73], while in lions there was an apparent absence of acid-fast bacilli in culture positive organs [22]. On the other hand, in combination with gross pathological examination, direct microscopic smear examination was found highly sensitive (95%) in infected wild boar and recommended as a useful tool for surveys and game meat inspection schemes [67].

Gross pathology observed post mortem in tissues, organs and carcasses due to *M. tuberculosis* complex infection is based on the typical granulomatous appearance of tuberculous lesions in most wildlife species. The sensitivity of gross pathology is higher in advanced stages of the diseases, but has been reported to differ from 63% in African buffalo [74], 75% in white-tailed deer [75] to 93% in elk [76]. In contrast, *M. bovis* infection is pre-clinical in most Eurasian badgers and hence gross pathological examination is generally insensitive [77]. Likewise, culture positive hyena either showed no visible lesions or lesions were limited to mesenteric lymph nodes and are easily overlooked (Bengis, pers. comm.)

Culture of mycobacteria can be performed using different culture systems and different decontamination protocols have been described (e.g. [78,79]). It is still recognized as the gold standard for diagnosis of mycobacterial infections, and is as such used for comparison when validating new assays. *M.bovis* isolates can be used for subsequent DNA typing, which is a valuable contribution to assess the epidemiology of BTB [80]. Culture has a high specificity throughout different studies and its sensitivity is generally estimated rather high, especially in the presence of lesions in the culture tissues, e.g. in white-tailed deer [73]. However, if sample types with no proven involvement in the BTB pathogenesis are chosen, a lower sensitivity must be expected, e.g. in tracheal washes in meerkats [81]. Additionally, it is complicated by intermittent shedding of bacteria, as confirmed in cattle [82] and badgers [83]. A further disadvantage is that culture requires up to 6-12 weeks before a result is obtained [43,79].

PCR has been the most studied of the different molecular techniques that exist for the species identification of *Mycobacterium* [43]. It is widely used in all wildlife species for differentiation of mycobacteria of the *M. tuberculosis* complex from non-tuberculous mycobacteria, as well as for more specific differentiation of *M. bovis* from other members of the *M. tuberculosis* complex [52,72,84,85]{{151;354;386}}. It can be performed after culture or directly in the suspect samples [34], but the latter approach demands a sufficiently high bacterial load, as obviously it is influenced by irregular shedding of the bacteria [83]. Up till now, it produced variable and less than satisfactory results for the use in animals, particularly in specimens containing low number of bacilli [81]. To improve the reliability of PCR as a diagnostic method, standardization of the technique will be required [43].

For human TB diagnostics, culture and/or PCR is often performed on sputum samples [43]. This approach is a routine procedure for elephants (trunk washes) [57] and has also been used for tracheal washings of possums [86] and meerkats [81], though with limited sensitivity. In most wildlife species however, culturing, with possible subsequent PCR, has mainly been used as post mortem method, starting from samples from affected lymph nodes and other tissues (e.g. [87]). Culture and PCR have not been included in Table 1, that focuses on ante mortem methods.

Indirect assays

Immune response

Tuberculosis is primarily an infection of the respiratory tract, where the agent uses the macrophage as primary host cell for intracellular replication [88], though for carnivores and omnivores, the primary infection route may also be via the gastro-intestinal tract i.c. the head and mesenteric lymph nodes. Early after infection, innate protective responsiveness and cell-mediated immune responses (CMI) are activated and are generally believed to play a major role in controlling the infection [89]. As the disease progresses, sometimes after prolonged pre-clinical periods, the humoral response is activated and antibody titers increase [88]. The time frame of this shift from CMI to the humoral response depends on many factors, like host species and immunity, initial infection dose, re-infection etc. The measurement of either or both stages of the immune response can be used for immunological diagnosis of *M. bovis* infection and new developments in diagnosis of *M. bovis* infection in wildlife are reviewed below for CMI and the humoral response separately.

Cell mediated immunity

Activation of lymphocytes that produce Th1 type cytokines, the most prominent of which is IFN-γ, is of major importance as a defence mechanism of the body against *M. bovis* infection. Activating these lymphocytes can result in a delayed type hypersensitivity reaction in vivo [90], including production of cytokines, that can be mimicked in stimulated blood cultures in vitro. Based on this knowledge, CMI related diagnostic tests have been developed, that in general have the advantage to become positive in an early stage of the disease, but that may fade away when BTB progresses [91,92].

Tuberculin skin test

The in vivo measurement of a delayed type hypersensitivity induced by Purified Protein Derivate (PPD), i.e. the tuberculin skin test, has long been the standard diagnostic test for tuberculosis in human and cattle [90]. PPDB is produced from heat processed *M. bovis* cultures, whereas PPDA, used as a control for exposure to environmental, non-tuberculous mycobacteria, is *M. avium* subsp. *avium derived* [92]. For BTB diagnosis in cattle, the tuberculin skin test is still the test of choice as prescribed by the OIE, with the IFN-γ assay as alternative test [43]. The skin test has been used as an individual animal test in support of eradicating BTB from infected herds in many countries worldwide [93]. However, depending on test interpretation, stage and severity of disease, BTB prevalence, cross-reacting organisms and other factors, sensitivity and specificity are highly variable and estimates in cattle range from respectively 52-100% and 75.5-100% (reviewed in [92,94]). In its original fashion, using PPD's as stimulants, it is unable to differentiate between infected or BCG vaccinated animals [53]. Though its applicability has been evaluated for wildlife species like elk [95], lions [22] and deer [96], the tuberculin skin test has not been validated and standardized for many exotic species [97]. The tuberculin skin test has severe limitations when applied to free-ranging wildlife [8]: i.e. optimal tuberculin doses are often unknown, recapture of animals after 72 hours is at the least impractical if not impossible [38], exposure to environmental mycobacteria may cause high background values [84,92] and in species belonging to the pachyderms the nature of the skin renders the intradermal test impractical [19].

IFN-γ release assays

The IFN-γ release assays are in vitro assays, based on Th1 cell reactivity like in the intradermal skin test. Whole blood or isolated peripheral blood mononuclear cells (PBMCs) are stimulated in

the presence or absence of mycobacterial antigens (avian or bovine PPD or other more specific antigens of *Mycobacterium* spp), that induce previously sensitized T-cells to produce IFN-γ. Quantification of this IFN-γ is performed in a sandwich ELISA or ELISPOT. Interpretation criteria for positive reactors may be adjusted, depending on the test purpose and disease prevalence [98]). For cattle, the IFN-γ ELISA (Bovigam) is approved under EU directive 64/432 EEC annex B as ancillary test for BTB diagnosis and studies in cattle have shown that the sensitivity of the bovine IFN-γ assay varied between 73.0% and 100%, and the specificity between 85.0–99.6% (reviewed in [92]). For selected bovid wildlife species, the Bovigam has been provisionally validated [39]. New commercial whole blood IFN-γ assays are also available for other wildlife species, of which PRIMAGAM (Prionics) received provisional USDA licensure for use in nonhuman primates [58]. Modification of a human TB assay was recently reported to show promising results when used in African buffalo [99] As further illustrated in Table 1, IFN-γ assays are still being developed of which some results are promising [33], though not always confirmed in other studies [100].

The ELISPOT is a relatively new IFN-γ release assay, which was used for example to determine BCG vaccination efficacy in badgers [101]. The ELISPOT requires the isolation of a known number of PBMC and measures their IFN-γ production after antigen stimulation by counting the "spots" of captured IFN-γ, representing cells producing it [102,103]. In a study in humans, ELISPOT had a higher sensitivity compared to the IFN-γ ELISA, but its results showed a more rapid decline in sensitivity than the IFN-γ ELISA in case of delays in sample processing [104].

The identification of immunodominant proteins unique to the *M. tuberculosis* complex, for example ESAT6 and CFP10, as stimulatory antigens in the IFN-γ assay may increase test specificity [105], though sensitivity may be less than with PPD's alone, as was shown in badgers [106]. ESAT6 and CFP10 have also shown potential to circumvent cross-reactivity that could lead to misclassification [107], which was noticed for example in cattle co-infected with other mycobacteria, when PPD's were used [108]. Potentially these proteins, or other newly discovered immunodominant proteins [109-111], are able to differentiate infected from vaccinated animals (DIVA), hence can also be used in Bacillus Calmette-Guérin (BCG) vaccinated populations [105,112-114].

Like the skin test, the IFN-γ release assay has time limits because of decreasing CMI responsiveness during progression of disease, as was also shown in deer [71]. Its test result is known to be influenced by the time lapse between collection of samples and their processing [115,116], though samples may be potentiated by addition of IL-12 [117]. IFN-γ release assays are laboratory based techniques [92], but the development of `in tube` or `in plate` techniques may increase the ease of use [93]. The assay can be performed with either whole blood or PBMC. However, the effort of PBMC isolation may not be necessary as results of the assay with whole blood or PBMC were found to be comparable in humans [104]. The Bovigam assay used in cattle is performed using whole blood [118].

In humans and cattle, antigen-specific IFN-γ responsiveness decreased over time during anti-tuberculosis treatment [119-121], showing the potential of the IFN-γ release assay as a monitoring method for treatment. However, this relation between treatment and IFN-γ decline was not found in other human studies [122,123].

Table 1. Summary of the diagnostic tests that have been employed in wildlife. The sensitivity and specificity of diagnostic tests depend on multiple factors, including test populations and test interpretation. This table serves as a general overview of the different studies performed and gives the published estimates of sensitivity and specificity, but readers are referred to the original papers for a more detailed interpretation of these estimates. NE= not estimated, Se= sensitivity, Sp= specificity, SICCT= single intradermal comparative tuberculin test, DPP= Dual path platform, LPA = lymphocyte proliferation assay, AB= antibodies, PPDB = Bovine Purified Protein Derivate, PPDA = Avian Purified Protein Derivate

Species	Test	Se[a,b] of test	Sp[a] of test	Number of animals tested	Infection: natural (N)/ experi-mental (E)	Details	References
Badger (*Meles* meles)	IFN-γ assay	80.9%	93.6%	235	N	Whole blood; monoclonal AB; PPDB-PPDA comparison	[106]
	Brock ELISA	48.9%	93.6%	235	N		
	RT qPCR	70.6%	90.7%	247		PPDB- PPDA comparison; specific antigens	[124]
	Rapid test	50.7%	93.1%	1532	N		[140]
	Brock STAT-PAK[c]	49.2%	93.1%	1464	N	Higher Se for animals with severe tuberculosis	[59]
	Brock ELISA	68%	NE	128	N	MPB83 antigen	[156]
	MAPIA	48.7%	88.0%	178	N		[138]
	Rapid test	52.6%	95.0%	178	N		
	Brock ELISA	47.4%	89.0%	178	N	MPB83 antigen	
Possum (*Trichosurus Vulpecula*)	Rapid test	44.7%	85.7	129	N		[140]
Fallow deer (*Dama dama*)	CervidTB STAT-PAK	80.1	NE	134	N		[167]
	CervidTB STAT-PAK	91%	91%	139	N		[139]
	Dual path platform VetTB test	91%	99%	139	N		
Red deer (*Cervus elaphus elaphus*)	RT qPCR	78.6%	97.5%	15	E	Red deer x elk hybrids	[71]
	IFN-γ ELISA (Cervigam)	70%	100%	15	E	Red deer x elk hybrids	
	LPA	65.7%	92.5%	15	E	Red deer x elk hybrids	

Species	Test	Se[a,b] of test	Sp[a] of test	Number of animals tested	Infection: natural (N)/ experi-mental (E)	Details	References
	CervidTB STAT-PAK	86.5%	83.8%	157	N+E	Lower Se for only natural infected deer	[149]
	DPP VetTB test	84.6%	91.4%	157	N+E	Lower Se for only natural infected deer	
Elk (*Cervus elaphus nelsoni*)	Intradermal tuberculin test	88%	69%	60	N	Single cervical test	[95]
	CervidTB STAT-PAK	82%	93%	175	N		[139]
	Dual path platform VetTB test	79%	98%	175	N		
Reindeer (*Rangifer tarandus*)	IFN-γ ELISA (Cervigam)	NE	90%	51	N	PPDB–PPDA comparison	[180]
	ELISA	100%	50%	15	E	lipoarabinomannan (LAM)-enriched mycobacterial antigen from *M. bovis* strain 95-1315	[147]
	Immunoblot	90.9%	50%	15	E	Antigen: whole-cell sonicate (WCS) of *M. bovis* strain 95-1315	
	MAPIA	100%	85%	34	E	Including MPB83	
White-tailed deer (*Odocoileus virginianus*)	Rapid test	75%	98.9%	463	N+E		[140]
	CervidTB STAT-PAK	56%	98.9%	556	N	Whole blood	[137]
	CervidTB STAT-PAK	54.5%	98.1%	746	N	Serum	
	MAPIA	68.2%	97.1%	749	N		
	Immunoblot	46.2%	92.5%	346	N	*M.bovis* whole-cell sonicate	
		55%	99.3%	691	N	MPB83 antigen	

Species	Test	Se[a,b] of test	Sp[a] of test	Number of animals tested	Infection: natural (N)/ experi-mental (E)	Details	References
	ELISA	66.7%	95.1%	341	N	Lipoarabinomannan-enriched antigen from *M. bovis* strain 95-1315; ΔOD≥0.25	
	ELISA	58.3%	97.3%	341	N	Lipoarabinomannan-enriched antigen from *M. bovis* strain 95-1315; ΔOD≥0.3	
Multiple cervid species	CervidTB STAT-PAK	85.7%	94.8%	432	N	Roe deer, fallow deer, red deer	[181]
	FPA	81%	80%	31	N	For Se: positive or suspect result. Two FPA suspect results were culture negative, but histopathology suspect	[155]
Bison (*Bison bison athabascae*)	FPA	67%	34%	56	N		[142]
	MAPIA	92%	97%	82	N		
	Rapid test	67%	99%	82	N		
Buffalo (*Syncerus caffer*)	Modified QuantiFERON-TB Gold (In-Tube)	98%	96%	174	N	Compared to the SICCT; Cut-off value IFN-γ optical density difference antigen-mitogen≥ 66 pg/ml	[99]
	IFNg ELISA	92.6%	68.3%	493	N	Standard test interpretation	[39]
		93.9%	85.4%	493	N	Optimal overall test validity	
	Rapid test BovidTB STAT-PAK	33%	90%	200	N	Inclusion of suspect reactions increased Se, but lowered Sp	[60]
	Rapid test Anigen	23%	94%	200	N	Inclusion of suspect reactions increased Se, but lowered Sp	
Wild boar (*Sus scrofa*)	Rapid test	76.6%	97.3%	177	N	Se higher in animals with lesions	[140]
	ELISA	72.6%	96.4%	185	N	Se+Sp dependent on cut-of value; PPDB antigen	[171]

Species	Test	Se[a,b] of test	Sp[a] of test	Number of animals tested	Infection: natural (N)/ experi-mental (E)	Details	References
	ELISA	79.2%	100%	200	N	PPDB	[182]
	DPP TB	89.6%	90.4%	200	N		
Elephant, African (*Loxodonta africana*) and Asian (*Elephas maximus*)	ElephantTB STAT-PAK	100%	95.2%	173	N	*M. tuberculosis*	[143]
	MAPIA	100%	100%	173	N	*M. tuberculosis*	
	DPP VetTB	100%	100%	173	N	*M. tuberculosis*	
Lion (*Panthera leo*)	Intradermal tuberculin test	86.5%	81.3%	84	N	Single Intradermal Cervical Test	[22]
	ELISA	46.1%	NE	26	N	MPB70 antigen	As referred to in [22]
Meerkat (*Suricata suricatta*)	Rapid test	43%	85%	110	N	Bayesian estimates	[81]
	MAPIA	90%	48%	110	N	Bayesian estimates	
Alpaca (*Lama pacos*)	Rapid test	71%	98%	156	N	*M. bovis* or *M. microti*	[183]
	Dual-path platform	74%	98%	156	N		
Llama (Lama glama)	Rapid test	77%	94%	175	N		[183]
	Dual-path platform	77%	98%	175	N		
Dromedary (*Camelus dromedarius*)	StatPak, MAPIA				N	Both tests correctly identified 3 culture positive dromedary	[184]
Multiple camelid species	Rapid test	63%	90%	87	N	Alpaca, llama. *M. microti* infection	[141]
	MAPIA	88%	97%	87	N	Alpaca, llama. *M. microti* infection	
Chacma Baboon (*Papio ursinus*)	IFN-γ assay	100% (2/2)	100%	51	N	QuantiFERON-TB Gold system; in-tube tb antigen stimulation; Sp partly based on TST; *M. tuberculosis*	[185]

Species	Test	Se[a,b] of test	Sp[a] of test	Number of animals tested	Infection: natural (N)/ experi-mental (E)	Details	References
Rhesus monkey (*Macaca mulatta*)	PrimaTB STAT-PAK	89.7%	98.6%	243	E	5 animals used as pre-inoculation control group; *M. tuberculosis*	[128]
Cynomolgus monkey (*Macaca fascicularis*)	PrimaTB STAT-PAK	93.8%	100%	46	E	*M. tuberculosis*	
African Green monkey (*Cercopithecus aethiops sabaeus*)	PrimaTB STAT-PAK	80%	99.2%	133	E	*M. tuberculosis*	
Various monkey species	IFN-γ assay (PRIMAGAM)	100%	100%	343	E	225 Rhesus, 82 cynomolgus, 19 chimpanzees, 17 new world monkeys; *M. tuberculosis*	[33]
						cynomolgus and rhesus monkeys; for cynomol-gus macaques different cut-of levels for the PRIMAGAM test should be considered; *M. bovis*	
Various monkey species	IFN-γ assay (PRIMAGAM)	68%	97%	64	N		[100]

[a] Sensitivity and specificity of tests are calculated by comparing with mycobacterial culture results, unless stated otherwise.
[b] Some studies have used only small numbers of confirmed positive animals.
[c] STAT-PAK tests are also known as lateral-flow immunochromatographic tests or rapid tests

qRT-PCR

The quantitative real time polymerase chain reaction (qPCR) amplifies and simultaneously quantifies one or more specific sequences in a DNA sample, by measuring (incorporated) fluorescent signals. It is often combined with reverse transcription (qRT-PCR) to quantify mRNA, as is the case when assessing the production of IFN-γ, and optionally other cytokines. This IFN-γ mRNA presence was shown to correlate well with IFN-γ protein production in deer, as shown in Table 1 [71].

The qRT-PCR may be adapted for the use in closely related species by designing, testing and optimising consensus sequences for primers. It can also be easily applied to non-related species if IFN-γ sequence information is available [71,124]. The qRT-PCR is a laboratory based technique and compared to other assays in this review, relatively advanced technical equipment is needed. Its sensitivity can be affected by delays in the processing of blood, causing significant, selective changes in cytokine mRNA expression, as was shown in humans [104].

Serology

Serological tests distinguish between animals that do and do not have circulating antibodies against *M. bovis*. A positive test result may indicate: 1) present infection; 2) immunity to a previous infection; 3) cross-reaction with a shared antigen from other infection agents; 4) the presence of maternal antibodies or 5) antibodies present as a result of vaccination (e.g. with BCG). A negative test result may indicate: 1) the individual is not infected and was never before; 2) the infection is recent and detectable antibody responses have not yet developed; 3) priming has taken place in the past but antibodies are not present anymore in detectable quantities or 4) the host is or was infected, but was not capable of producing antibodies against the infection [125].

Animals with progressive disease tend to gradually lose the capacity to mount CMI and develop serological responses. Compared to cattle, disease progression will be more often encountered in infected free-ranging wildlife species due to the absence of routine testing and test-and-slaughter policy. For these animals, antibody-based diagnosis may be of greater importance [89]. However, when ELISA based tests are used to obtain a TB free population, in course of time, with fewer animals in an advanced stage of infection, the sensitivity will decrease [126].

Serological assays have advantages in terms of logistics, due to stability of antibodies during transport, storage and handling, which reduces the costs and increases the ease of application. Their results, however, may also vary depending on the state of the samples: samples that were hemolyzed or lipemic had a statistically significant reduction in sensitivity, but not in specificity, compared to "normal" samples in the BrockTB STAT-PAK Assay [59]. In the past, their sensitivity used to be too low, but the use of early antigens for recognition and the development of new techniques has improved sensitivity [127] to an extent that serological responses in non-human primates and in cervids were found positive starting from 4 weeks post experimental infection [128,129].

3.2.2.1 ELISA and immunochromatographic tests

Enzyme-linked immunosorbent assays (ELISAs) were initially developed with PPD's as antigens, but suffered from low specificity due to cross-reactivity [130]. For cattle, the specificity was improved by the use of more specific antigens (MPB83, MPB70, etc) [131-133]. The MPB70 ELISA, in particular, has been used in various studies to determine the prevalence of *M. bovis* in wildlife populations [52,134], but the value of the results of these studies may be limited, as the

ELISA was not validated for most of these wildlife species.
To improve sensitivity of serological assays, new techniques have been developed, amongst which is the lateral-flow immunochromatographic test, an animal-side test that uses a cocktail of a limited number of antigens [135]. To date, this technique is commercially available for elephants, several deer and camelid species, badgers, and non-human primates (known as *STAT-PAK*, Chembio Diagnostic Systems, Inc., Medford, NY, USA). Results of this assay in studies with various wildlife species are listed in Table 1, and show that study outcomes vary between the species, which may be explained by different immune responses between species. In the USA, this test format is licensed by the USDA for elephants and nonhuman primates, and in the United Kingdom for badgers [43].

Another confirmative serological technique is multi-antigen print immunoassay (MAPIA), which can be used to determine the species-specific, immunological profile to *M. bovis* infection. This specialized technique is based on immobilization of a number of individual antigens onto nitrocellulose membranes by semi-automated microspraying, followed by standard chromogenic immunodevelopment [136]. It has aided in improvement of the various lateral-flow immunochromatographic tests and in the design of the dual-path platform assay (DPP). The DPP is a next generation immunochromatographic test, which makes use of a dual-flow pathway. As shown in Table 1, it has been tested for various wildlife species in comparison to the lateral-flow immunochromatographic test, and in general, it gave equal or slightly higher specificity [81,137,138].

Variation between and within species in the recognition of antigens has been found [139,140], as well as different affinities of antibodies to test reagents. MPB83 is an early antigen that, in general, is best recognized in species like Eurasian badgers, white-tailed deer, brushtail possums, cervids and new world camelids [69,129,140,141]. Likewise, ESAT6 and CFP10 are well recognized [97,139,142-144]. Combining antigens for the detection of *M. bovis* specific antibodies in wells of ELISA plates may increase sensitivity of the assay, without significantly affecting the specificity [145], however total serologic response may be lower than those calculated by the sum of the activities of their components [136].

There may also be potential for the serological techniques to discriminate between *M. bovis*-infected and BCG-vaccinated individuals [138,146], or to monitor antibody responses during treatment [97,135]. Antibody responses to *M. bovis* infection in, for example, wild boar were positively associated with advanced disease, i.e. with the presence of extensive gross lesions [140]. Similar observations have been previously made for other host species, e.g. badgers and reindeer [69,147]. Given that individuals with advanced disease are also more likely to be excreting [148], these animals may be important targets for control programs.

The lateral-flow immunochromatographic test did not show cross-reaction in small numbers of wild boar exposed to *M. avium* infection or deer exposed to *Mycobacterium avium* subspecies *paratuberculosis*, supporting the high specificity of the assay [140]. However, this was contradicted in a study in farmed red deer, which showed that vaccination against paratuberculosis and subsequent skin testing adversely affected the specificity of the CervidTB STAT-PAK and DPP VetTB test, as did natural infection with *M. avium* subsp. *paratuberculosis* for the specificity of the CervidTB STAT-PAK test [149].

Other methods

The multiplex chemiluminescence immunoassay is a new diagnostic assay that can detect antibodies specific to *M. bovis* [150]. This assay employs up to 25 individual mycobacterial antigens that are printed in small dots in a single well in a 96-well plate array format and could thus be useful for large-scale testing. Individual serum samples are added to each well. The chemiluminescent signal is captured by digital imaging. This assay has shown to have a sensitivity of 93.1% and a specificity of 98.4% in cattle, also detecting antibodies as early as 2 weeks post infection [150], though under field conditions, slightly lower sensitivity and specificity were achieved, depending on the cut-off level [126]. It has also shown to have high sensitivity (98.3%) and specificity (100%) in detecting *M. bovis* infection in goats (n=180)[151]. Other assays based on similar principals involve a single antigen chemiluminescence assay with magnetic iron beads (SeraLyte-*Mbv* ™ [152]) and the multiplex microbead immunoassay, based on Luminex technology, that was developed in an experimental nonhuman primate model and showed potential for clinical use [153].

The fluorescence polarization assay (FPA) uses a tracer (the target antigen or part of it) with a fluorescent molecule bound to it, to detect antibody in serum [154]. It has recently been tested in elk and red deer [155]. Though the OIE previously classified this as a test of scientific value only, due to costs and logistic demands [43], the development of a portable fluorescence polarization analyzer facilitates testing performed in the field and the test procedure has now become simple and rapid [155].

Western blotting is an established technique and uses gel electrophoresis to detect proteins. When it was compared with the Brock ELISA, a serological test for diagnosis of BTB in badgers, it was less sensitive than the ELISA (68% *versus* 57%, same group of badgers, no specificity was measured) [156]. Western blotting was also used in other studies as a proof of principle method to show that real antibodies were measured with MAPIA / ELISA [147] or the lateral-flow immunochromatographic test [129].

Other BTB diagnostic methods, which are more laboratory based methods, include the lymphocyte transformation assay [69,71] and the measurement of production of nitric oxide (NO) or Tumor Necrosis Factor (TNF) α to measure macrophage activity [157]. The production of NO in response to antigen specific stimulation of PBMC of *M. bovis* infected white-tailed deer gave promising results [158], but additional studies to measure NO responses of more species, or follow-up studies, are lacking.

New non-immunological methods are also being developed, including a volatile compound-sensing system, that was tested for badgers and cattle. This 'electronic nose' used serum samples and was able to discriminate experimentally infected animals from controls as early as 3 weeks after infection with *M. bovis* [159]. A similar serological technology, "selected ion flow tube mass spectrometry", was combined with multivariate data analysis for the diagnosis of *M. bovis* in badgers, which showed 88% true positives, but only 62% true negatives [160].

DIAGNOSTIC APPROACHES TO IMPROVE ASSAY FITNESS FOR PURPOSE

Currently, no single test is applicable for all species and/or test purposes. Part of the BTB test requirements may still be covered by applying various diagnostic approaches to the available, imperfect diagnostic methods. Several of these approaches that could improve the fitness of diagnostic assays are discussed here and should be considered in particular management

situations.

Sensitivity (Se) versus specificity (Sp)
A balance exists between the Se of a test, i.e. the probability of correctly identifying an infected animal, and the Sp, i.e. the probability of correctly identifying a non-infected animal [161]. This balance can be shifted by adjusting the cut-off value of the diagnostic assay to optimise the test outcome for the desired test characteristics. Adjusting the balance with the use of two different interpretation schemes for infected versus uninfected populations was shown to add value to the use of both the tuberculin skin test in domestic cattle [162] and the IFN-γ assay in buffalo [39].

Herd-level test characteristics may differ from individual-level test characteristics, because the unit of inference is different [163]. For example, in general detection of *M. bovis* infection on herd level is deemed more easy, since in theory only one animal needs to be diagnosed with *M. bovis* infection to classify the entire herd positive for BTB, a method that is used in farmed deer in Sweden [51]. Therefore, a test with imperfect Se could give reasonable results when used on herd/population level [163]. Additional methods exist to compensate for a lack in Se, like increasing the sample number in surveillance and monitoring exercises. Depending on definitions of positive results, the use of defined mycobacterial antigens in the IFN-γ assay, for example ESAT6 and CFP10, additional to PPDA and PPDB, may be used to increase Sp (i.e. an animal is regarded positive when both are positive) or Se (i.e. an animal is regarded positive when either one is positive) [105,133,164].

Combination of tests
Combining different testing methods is another strategy to improve the Se or Sp, though at the expense of higher costs. Parallel testing of multiple tests for individual diagnosis results in maximal Se, at the cost of lower Sp, which can be used when disease prevalence is high. On the other hand, to increase Sp serial testing can be used in (individual animals in) herds where negative results are expected, even though it will lower the Se. Another strategy is to use an inexpensive screening test with a high Se and moderate Sp, with subsequent retesting of positive animals with a more expensive ancillary test which is both sensitive and specific.

How the Se and Sp are influenced by the parallel or serial combination of tests, also depends on the conditional dependence between the tests, i.e. if they measure the same biological processes [163,165]. With the combination of CMI and serology-based tests, a larger window of the immune response can be covered, resulting in the detection of a higher percentage of infected animals in cattle [166], non-human primates [128] and fallow deer [167]. However, such combinations improved the Se of detection only marginally in badgers [106] and red deer [149]. Combinations of two CMI based tests or two serology-based assays have also been tested. Using PRIMAGAM (primate IFN-γ assay) and the skin test in parallel increased the overall Se of screening to 100% [100]. The parallel use of lateral-flow immunochromatographic test and MAPIA in wild meerkats produced estimates of Se and Sp that were considered high enough to be useful for BTB diagnosis, where the use of either test alone was not [81].

Serial testing, can influence the outcome of the second test: performing a serologic assay 2-8 weeks after tuberculin testing of infected cervids resulted in improved results due to an anamnestic rise in antibodies specific for *M. bovis* [129,147]. It may also influence the IFN-γ assay, though literature is contrasting in this field, as reviewed in [115]. Serial testing of (free-ranging) wildlife is difficult, since it necessitates multiple captures.

Targeted animal sampling

Specific species can be targeted for sampling to detect *M. bovis* infection, for example for surveillance or monitoring. These species could be scavenger species like coyotes, that could serve as sentinel species for *M. bovis* presence [168]. Also, animals that are highly susceptible for *M. bovis* (bovine/deer species) could be targets. Targeted sampling may also involve only sampling a specific (fraction of a) population that is more likely to be infected, increasing the herd Se of diagnostic methods, as the prevalence of that target population is higher [163]. An example of this type of targeted sampling is the culture of samples from only those animals during routine cropping, e.g. hunting, that show lesions during necropsies.

CONSIDERATIONS FOR FUTURE TEST DEVELOPMENT AND APPLICATIONS

Even with the application of the various approaches to the available diagnostic assays, there are still numerous situations where assays do not meet the requirements for BTB management, especially when testing individual animals or small populations [9] testing for BTB-free certification [64]. New and modified diagnostic methods continue to be developed and validated, a process that could be aided by the following considerations.

Multi-species tests

Multi-species tests could offer a solution to testing those wildlife species for which development of a species specific assay would not be economically viable, e.g. many captive wildlife species in zoological collections. Multi-species wildlife tests can potentially arise from existing assays for domestic animals [39,169] or humans [99]. However, these tests cannot be assumed to be equally sensitive or specific in their wildlife counterparts [36,48] and test validation is crucial for each (group of) species and sample type [67].

Direct tests like culture, PCR or the direct immunofluorescense assay can be used as multi-species test and though test characteristics per se are similar for most species [36], the applicability may differ across species, as the bacterial load in lesions and the extent of lesions may differ between species [22,73,170] and shedding of mycobacteria is irregular [82,83].

Indirect immunological methods that use cross-reactive reagents have also been used as multi-species test, though with varying success [36]. For example, the Bovigam IFN-γ assay for cattle, can be used in similar species like buffaloes [39], and the Primagam IFN-γ assay in various non-human primates [33], though validation for each species remains essential as was shown for cynomolgus macaques [100], for which lower test sensitivities were found. Serological assays like ELISAs [171] or MAPIA [127] also offer multi-species potential, for example by using protein A or G as secondary antibody, binding immunoglobulins across species [172]. The lateral-flow immunochromatographic tests have already shown to be useful across different species, though for some species the composition of the different antigens that are being used, should be optimized [140].

Validation challenges

After initial assay development, including optimization and calibration of the assay to standard reagents, the first stage of assay validation constitutes the determination of the analytical Se and Sp and of the test repeatability. This involves a small panel of coded control samples and should preferably be performed in multiple laboratories. The second stage involves assessment of the diagnostic performance, measured against a gold standard test, and uses field samples [173].

Practically, acquiring the OIE recommended numbers of known positive and negative controls for validation [173,174] will be difficult for many wildlife species [48], and initially the necessary sample size may not be achievable. However, over time, collecting data should result in large enough sample numbers to estimate diagnostic Se and Sp. Collection of data may be aided by following standard protocols for testing, e.g. the Standard for Reporting of Diagnostic Accuracy (STARD), which can help improve quality of the methods [44,175]. However, challenges like a validated ante mortem diagnosis in rare species/zoo animals still exist and may not be solved soon.

Estimates of diagnostic Se and Sp should be made in populations that are as close as possible to the population in which the diagnostic tests will be used, as was stipulated by the Office International des Epizooties [37]. Test characteristics and the outcome of the study are influenced by the type of study (natural *versus* experimental infection, different gold standards), as well as confounding factors such as co-infection need to be taken into account when possible [149] .

Gold standard versus latent class models

The gold standard diagnostic method for tuberculosis is the mycobacterial culture. However, this direct assay has several disadvantages, like long processing time and variable sensitivity across species, and it is therefore an imperfect gold standard. If the gold standard test itself does not have 100% Se and 100% Sp, errors will arise in defining Se and Sp of new diagnostic assays [44]. The sensitivities of new diagnostic tests should therefore rather be described as 'relative sensitivities', since they are estimated by comparison to the imperfect gold standard of culture.

Because an ideal gold standard is not available, new methods have been developed to avoid the need of the gold standard for the validation of diagnostic assays. A very useful method is the use of latent class models. These can use either frequentist (maximum likelihood estimation) or Bayesian modeling strategies to estimate the operating characteristics of two or more diagnostic tests where true disease status is not known [176-178]. Studies using this method are increasingly published, for cattle [126,179] as well as as for wildlife [81]. A disadvantage of Bayesian modeling is the use of prior assumptions, including independence of different tests, which complicates analyses of diagnostic tests that are based on the same stage of the immune response (i.e. the tuberculin skin test and the IFN-γ assay).

CONCLUSION

This review investigated the available (ante mortem) diagnostic assays for detection of *M. bovis* infection. CMI based tests like the IFN-γ assay still form the major basis of (B)TB testing in cattle and humans and are still regarded as the earliest detectors of positive animals, but their use in wildlife species is complicated by their species-specific set-up. In general, they offer reasonably high test sensitivities in the wildlife species they have been developed for (Table 1). New specific immunodominant antigens offer potential to increase specificity and avoid cross-reactions with other mycobacteria and BCG vaccination. Serological tests have greatly improved their sensitivity, though still for some species the low sensitivity and the need for species specific antibodies remains problematic, as shown in Table 1. Their ease of use as animals-side tests may offer great potential for BTB detection in (free-ranging) wildlife.

Diagnostic testing of wildlife species for *M. bovis* can be necessary for various purposes, from surveillance on population level, to certification of BTB-free status for individual animals or the confirmatory diagnosis in suspected cases of the disease. Given the complex background

of pathogenesis and host immune responses to *M. bovis* infection, it is not likely that in the near future a single assay with perfect diagnostic performance will become available for BTB management. Therefore, a test or combination of tests and diagnostic approaches need to be chosen that are most "fit for purpose". The fit for purpose principal includes the crucial balance between sensitivity and specificity and specification of the species, as well as availability, ease of use, logistical demands, reproducibility and the costs of the diagnostic assays [149]. A cost-effective test therefore does not generally mean the cheapest test [93] and fitness may differ between geographical locations and between developed and developing countries [2]. Important shortcomings remain present, and for some species or testing purposes, it is unlikely that a good diagnostic assay will be available soon.

ACKNOWLEDGEMENTS

The work of MM was supported by the Netherlands Organisation for Scientific Research (NWO/ZonMw grant 918.56.620) and the Prins Bernhard Cultuurfonds on behalf of the Niemans-Schootemeijer Fonds.

CONFLICT OF INTEREST

None

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Chapter 3

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

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Veterinary Immunology and Immunopathology (2010) 134, 296-298

ABSTRACT

Cloning and sequencing of the full length lion and cheetah interferon-gamma (IFN-γ) transcript will enable the expression of the recombinant cytokine, to be used for production of monoclonal antibodies and to set up lion and cheetah-specific IFN-γ ELISAs. These are relevant in blood-based diagnosis of bovine tuberculosis, an important threat to lions in the Kruger National Park. Alignment of nucleotide and amino acid sequences of lion and cheetah and that of domestic cats showed homologies of 97 - 100%.

In 1995, spread of *Mycobacterium bovis* (*M. bovis*), the causative agent of bovine tuberculosis in African buffaloes, to lions (*Panthera leo*) became evident in the Kruger National Park. This showed to have direct health consequences for lions [1,2], besides potentially economical implications by impact on ecotourism [3]. Similar to the pathology in lions, *M. bovis* infection of cheetah, with pulmonary lesions as the primary lesions, has been described [2,4]. Detection of a pathogen-specific immune response in blood based assays may be used for early and reliable diagnosis of tuberculosis. Interferon-gamma (IFN-γ), a cytokine produced by lymphocytes, plays an important role in the initial cell-mediated immunity that is thought to control *M. bovis* in infected animals [5].
Production of IFN-γ in whole blood assays upon stimulation with *M. bovis* antigens indicates prior exposure of the adaptive immune system to the bacterium. Currently commercially available IFN-γ tests are used for diagnosis of infection with *M. bovis* in cattle (Bovigam) and *M. tuberculosis* in humans (Quantiferon) [6,7].

To enable production of a lion IFN-γ assay, cloning and sequencing of its IFN-γ cDNA was initiated. Full nucleotide sequences may serve the design of primers for RT qPCR, whereas expression of the protein will enable the generation of (monoclonal) antibodies to be used in a lion-specific IFN-γ ELISA and to assess potential cross-reactivity of feline specific antibodies that have been used in recently developed domestic cat IFN-γ assays [8]. In addition the cheetah *(Acinonyx jubatus)* IFN-γ cDNA was sequenced to provide a comparative overview of genetic relations in the feline group.

Heparinised lion blood was collected from three captive lions in the Gauteng province, South Africa. Lion 1 was a subadult male, lion 2 was an adult white lion and lion 3 was a young male lion, 5.5 months old. The cheetah blood was collected from a privately kept, wild caught adult male cheetah in the Gauteng province.

PBMC were isolated by density gradient centrifugation on Histopaque (1.077 density, Sigma), washed twice in ice cold PBS and stored in RNAlater (QIAGEN) at -20°C until further use, or resuspended at a concentration of 1 million cells/ml in culture medium (RPMI 1640, supplemented with 2mM L-glutamine, 5000 units penicillin, and 5000 µg streptomycin) and stimulated with 10 µg ConA/ml in a 24-well tissue culture plate in a CO_2 incubator at 37°C for 18-26 hours and subsequently stored in RNAlater at -20°C. mRNA was extracted from the samples using the RNeasy® mini kit (QIAGEN) according to the protocol of the manufacturer and immediately used in a reverse transcriptase reaction, using the TaqMan® Reverse Transcription Reagents (Applied Biosystems).

The sequence of the domestic cat (*Felis catus*) IFN-γ gene was published by [9] (GenBank accession number NM001009873) and by [10] (GenBank accession number X86972). The coding part of the domestic cat IFN-γ gene is 504 base pairs (bp) long. Primers for full length lion and cheetah IFN-γ PCR reactions were designed using the consensus of aligned IFN-γ 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). Forward (GGC CTA ACT CTC YGA AAC GAT G) and reverse (CAA ATA TTG CAG GCA GGA YRA CC) primers were synthesized commercially (IDT Inc.) and used under the following PCR conditions: 1 cycle of 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and a final cycle of 72°C for 7 minutes, using TaKaRa Ex Taq (TAKARA BIO INC.). After gel electrophoresis, PCR products of the correct size were purified with the MinElute PCR

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                                1-60
Cheetah clone 1                 MNYTSFIFAFQLCIILCSSGYYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Cheetah clone 2& Domestic cat   MNYTSFIFAFQLCIILCSSGYYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Lion 1& Lion 3                  MNYTSFIFAFQLCIILCSSGCYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDILKNWK
Lion 2                          MNYTSFIFAFQLCIILCSSGCYCQAMFFKEIEELKGYFNASNPDVADGGSLFVDISKNWK
                                ********************* ********************************** ****

                                61-120
Cheetah clone 1                 EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRSMDTIREDMLDKLLNTSSSKRDDFLKLI
Cheetah clone 2& Domestic cat   EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRSMDTIKEDMLDKLLNTSSSKRDDFLKLI
Lion 1& Lion 3                  EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRNMDTIKEDMLDKLLNTSSSKRDDFLKLI
Lion 2                          EESDKTIIQSQIVSFYLKMFENLKDDDQRIQRNMDTIKEDMLDKLLNTSSSKRDDFLKLI
                                *******************************.****:**********************

                                121-168
Cheetah clone 1                 QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Cheetah clone 2& Domestic cat   QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Lion 1& Lion 3                  QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
Lion 2                          QIPVNDLQVQRKAINELFKVMNDLSPRSNLRKRKRSQNLFRGRRASK-
                                ********************************************
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Figure 1. Predicted amino acids sequences of lion 1, lion 2, lion 3, cheetah clone 1 and 2 (GenBank accession numbers respectively FJ712034 (similar for lion 3), FJ712037, FJ712035, FJ712036) and domestic cat (GenBank accession number X86972.1). Differences in amino acid sequence are highlighted. The predicted N-glycosylation sites (positions 39 and 107) are marked in bold.

purification kit (QIAGEN) and ligated overnight at 4°C into pGEM®-T easy vector according to the manufacturer's instructions (Promega Corp, Madison, WI, USA) and subsequently used to transform JM109 High Efficiency Competent Cells. Individual transformants grown overnight in LB/ampicillin broth were purified (High Pure Plasmid Isolation Kit, Roche Applied Science) and sequenced on both strands using the standard pGEM®-T SP-6 and T-7 primers (Inqaba Biotec, South Africa). Chromas 2 was used to check the chromatogram obtained for misreadings. Sequence assembly was carried out manually and BLAST searches identified the sequences as novel IFN-γ sequences. Signal peptides and glycosylation sites were predicted using signalP and NetNGlyc from the Center for Biological Sequence Analysis.

Full-length IFN-γ cDNA sequences were obtained from clones of each of the three lions, and from two clones of the cheetah and were subsequently submitted to GenBank. The obtained sequences were aligned with the sequences of domestic cat (NCBI: X86972.1) and dog (NCBI: NM_001003174.1) using ClustalW.

The cDNA sequences obtained from lion 1 and lion 3 (GenBank accession number FJ712034) were identical. Lion 2, the white lion, showed a difference of two nucleotides with the cDNA sequences of the other two lions –a homology of 99%-, resulting in one different amino acid. All lion nucleotide sequences showed a homology of 98% with the sequence of the domestic cat.

The two cheetah clone sequences showed one nucleotide difference (99% homology), resulting in one different amino acid. The IFN-γ sequence from cheetah clone 2 (GenBank accession number FJ712036) was identical to that of the domestic cat. The homologies between the two cDNA sequences of the cheetah and that of the domestic cat were both 99%.

The largest difference (seven nucleotides, resulting in four different amino acids) between the feline sequences was between the cDNA sequences of lion 2 (GenBank accession number FJ712037) and cheetah clone 1 (GenBank accession number FJ712035), with a homology of

97%. The homologies of cDNA sequences of the three lions, and those derived from the two cheetah clones with the dog IFN-γ sequences were 89% each.

The alignment of the amino acid sequences from the three lions, the two clones of the cheetah and the domestic cat sequence (GenBank accession number X86972.1) are shown in figure 1. In conclusion, the IFN-γ gene sequence between the species studied is well conserved. Two N-glycosylation sites were predicted in all cheetah and lion IFN-γ sequences by the signaIP software: N39 and N107, which are homologous to the human N-glycosylation sites N48 and N120 (GenBank accession number NM_000619).

The IFN-γ sequences of two different lions were 100% identical, making it very unlikely that these sequences contain PCR artefacts. The high homology between IFN-γ molecules of domestic cat, lion and cheetah, most likely indicates a very similar conformational make up and gives reason to believe that a cat or lion-specific IFN-γ ELISA may potentially be used for other feline species. Investigation of cross-reactivity will clarify whether existing tests may be used throughout feline species The presented lion and cheetah IFN-γ may be the basis to develop species-specific IFN-γ ELISAs.

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Chapter 4a

Detection of native lion interferon-gamma in whole blood cultures using a feline interferon-gamma assay

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Submitted

ABSTRACT

The ongoing spread of bovine tuberculosis (BTB) poses a health risk to the lion population of the Kruger National Park (KNP) and potentially other African lion populations. To assess the prevalence and progression of the disease within the lion population, a diagnostic test is needed that is able to identify *Mycobacterium bovis* (*M. bovis)* infected animals at an early stage of the disease. An existing feline interferon-gamma (IFN-γ) assay was modified for the detection of lion IFN-γ. Using eukaryotic recombinant lion IFN-γ, the detection limit of the assay was shown to be as low as 3 ng/ml, and was shown to detect native lion IFN-γ in supernatants of lion whole blood cultures stimulated with the mitogen PMA/CaI. Moreover, as anticipated, supernatants of mycobacterial antigen stimulated whole blood cultures from 10 animals from BTB-free private game farms in South Africa, did not show production of IFN-γ. The IFN-γ assay still needs to be validated using whole blood cultures of known positive and negative lions, but results obtained so far are promising for the potential application of the IFN-γ assay towards early diagnosis of *M. bovis* infection in lions.

INTRODUCTION

Mycobacterium bovis (*M. bovis*), the causative agent of bovine tuberculosis (BTB), was first diagnosed in free-ranging lions (*Panthera leo*) in South Africa in 1995 [1]. Bovine tuberculosis in lions is a chronic, debilitating disease [2,3], that can affect multiple organ systems [1,4]. Clinical signs frequently observed in affected lions are emaciation, alopecia and elbow hygromas [4]. Today, the prevalence of BTB among the lion population in the southern part of the Kruger National Park (KNP) is most likely as high as 72% [5] and the prevalence in the northern part is still increasing [6]. Not only does BTB affect the health of lions due to loss of body condition, it may also have economical implications for the tourism industry [7] and potential decreases of the lion population may affect the ecosystem by changed predator-prey equilibrium [8].

The only validated test that is currently available for the diagnosis of BTB in lions is the Single Intradermal Cervical Test (SICT) [9]. However, this method has practical constraints in case of free-ranging lions, such as cost and effort of capture and re-capture. Therefore, a diagnostic test requiring a single capture would be preferential. The IFN-γ assay is generally used as an ancillary test to the tuberculin skin test for diagnosis of *M. bovis* infection in ruminants [10], humans [11] and several wildlife species [12] for example buffaloes [13]. Similar to the tuberculin skin test, the IFN-γ assay assesses cell mediated immunity, accepted to be the initial responsiveness after *M. bovis* infection. It measures the production of IFN-γ by sensitized T-cells in response to mycobacterial antigen stimulation and is reported to detect infection at an earlier stage than the skin test [14]. We were prompted to assess a feline IFN-γ assay that was developed for the diagnosis of BTB in domestic cats, that also showed to detect native IFN-γ of a healthy Siberian tiger (*Panthera tigris altaicaI)* [15,16]. Furthermore, the sequence of IFN-γ of domestic cats and lions showed a high degree of homology (97-100%) [17]. To assess this IFN-γ assay, cross-reactivity of the assay with eukaryotic recombinant lion IFN-γ (rLIFN-γ) was confirmed and was used to optimize conditions for the detection of native lion IFN-γ. The resulting modified feline IFN-γ assay is sensitive and has potential for use in the diagnosis of *M. bovis* infection in lions.

MATERIALS AND METHODS

Determining optimal whole blood stimulation conditions

Animals: Heparinised blood was collected of 10 adult lions from two different private game farms in the Gauteng province, South Africa. Both game farms had no known history of BTB. Blood samples were kept at ambient temperature and processed within 10 hours.

Whole blood culture: Whole blood samples (1 ml) were incubated in eppendorf tubes with mitogen, phorbol 12-myristate 13-acetate and Calcium Ionophore (PMA/CaI) (respectively 100 ng/ml and 2 µg/ml) (Sigma-Aldrich Chemie B.V. the Netherlands) and pokeweed mitogen (PWM) (10 µg/ml) (Sigma-Aldrich Chemie B.V. the Netherlands), as positive controls; mycobacterial antigens, bovine purified protein derivative (PPDB) (20 µg/ml; Lelystad, the Netherlands), avian PPD (20 µg/ml; Lelystad, the Netherlands) and CFP10/ESAT6 (10 µg/ml; Staten Serum Institute, Kopenhagen, Denmark); and finally a negative control with medium only. All were diluted in 100 µl of medium (RPMI1640 containing 10% FCS). Whole blood cultures were incubated at 37°C and supernatants were harvested after 24, 48, 72 and 96 hours by centrifuging the tubes for 10 min at 1500 X g. All samples were stored at -80°C.

Final feline IFN-γ assay protocol

The feline IFN-γ assay using commercially available polyclonal feline IFN-γ specific antibodies [15] was modified as follows: The supernatants of whole blood cultures were incubated for 10 min with an equal amount of block buffer (4% BSA in PBS) containing 10% goat serum (GS) before adding them to the plates coated with capture antibody. In addition, eukaryotic recombinant lion

IFN-γ (rLIFN-γ) (U-Protein Express BV, The Netherlands) [18], and prokaryotic recombinant feline IFN-γ (rFeIFNg) (R&D systems, United Kingdom), were now both used to produce standard concentration curves. Plates were read at $OD_{492\ nm}$.

Data Analysis

In the assay, modified as described above, samples were assayed in duplicate and results were expressed as the average $OD_{492\ nm}$ value. The cut-off detection level was defined as twice the average $OD_{492\ nm}$ value of the medium only samples of the animal.

RESULTS AND DISCUSSION

As described (section final feline IFN-γ assay protocol), modifications to the feline IFN-γ assay [15] using eukaryotic rLIFN-γ, resulted in optimal conditions for the measurement of lion IFN-γ. Initially, supernatants of medium only lion whole blood cultures showed high $OD_{492\ nm}$ values when tested in the feline IFN-γ assay. High $OD_{492\ nm}$ values were found in 5 out of 10 animals. Such a high incidence of false positive results has been mentioned in human and canine immunoassays as well, [19] and it is believed that heterophile antibodies present in the serum of this species were responsible for this interference [20]. The interfering signal was successfully reduced by incubation of the lion whole blood supernatants with an equal amount of 10% GS in block buffer for 10 min, before adding the mixture to the ELISA plate. This incubation did not compromise the detection limit of rLIFN-γ (results not shown).

Background levels of the assay were defined as twice the average $OD_{492\ nm}$ value of medium only whole blood cultures used as negative controls in the same assay. As a result the lower detection level, defined using eukaryotic rLIFN-γ as a standard, was 3 ng/ml for rLIFN-γ. This was comparable to the detection limit determined for rFeIFN-γ (1 ng/ml) (results not shown). The detection level of 3 ng/ml for rLIFN-γ is in the range of quantities of IFN-γ produced in mycobacterial antigen stimulated whole blood cultures of other species, e.g. cats (1.2 ng/ml) [15].

Whole blood cultures from 10 lions from two BTB-free farms were used to determine optimal stimulation conditions, regarding the mitogen used and the incubation period (Figure 1), hence positive controls for further testing. High IFN-γ levels were reached in PMA/CaI as a positive control at an optimal culture duration of 72 hours (OD_{492} mean 0.75; standard deviation 0.20). PWM did not show to be suitable as a mitogen for lion whole blood culture (OD_{492} mean 0.08 72 hours; standard deviation 0.02). These findings are in line with those found for domestic cats in the original protocol [15].

As a first step to gather data for validation of the diagnostic specificity of the assay in BTB diagnosis, additional to the mitogen as positive control stimulant, the lion whole blood cultures were stimulated with mycobacterial antigens PPDA, PPDB and CFP10/ESAT6 (Figure 2). As anticipated, this stimulation did not result in IFN-γ production, since these lions originated from BTB-free private game farms. As a next step in validation of the diagnostic sensitivity of this assay should be determined in confirmed BTB infected lions.

In conclusion, this study reports the successful detection of native lion IFN-γ in whole blood cultures stimulated with PMA/CaI in a modified feline IFN-γ assay. Although the assay has not been validated yet and further improvement of the sensitivity may be needed depending on the preferred test characteristics, results are promising for application of this IFN-γ assay towards the early diagnosis of *M. bovis* infection in lions. It may be a valuable tool in future investigation of the immunopathogenesis, prevalence and, impact of *M. bovis* infection in the

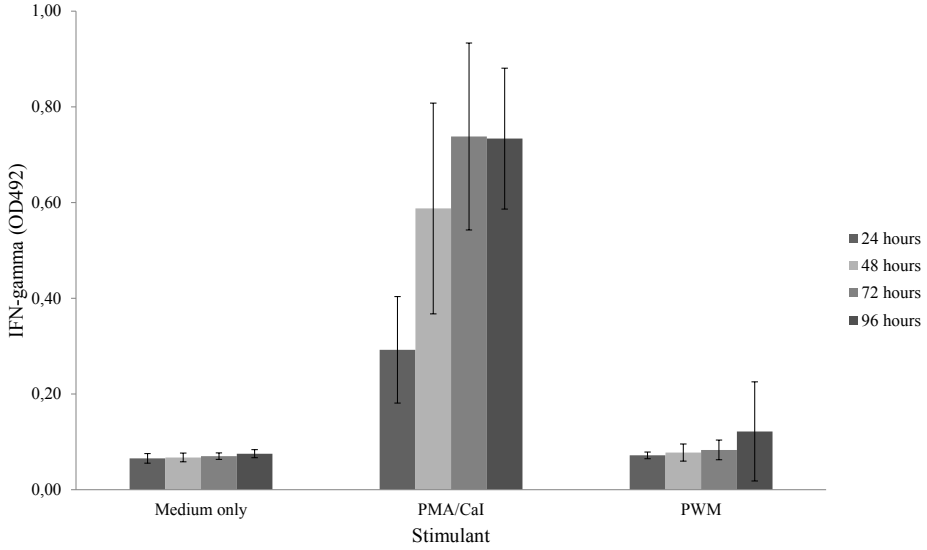

Figure 1. Mean IFN-γ levels ($OD_{492\ nm}$) of whole blood cultures of 10 lions from BTB-free areas stimulated with different mitogens (medium only, PMA/CaI, PWM). Error bars indicate standard deviation. The mean $OD_{492\ nm}$ value of the samples stimulated with PMA/Ca differed significantly (two-tailed paired T-test; P value < 0.0001) from the negative sample with an optimum stimulation period of 72 hours. The mean $OD_{492\ nm}$ value of samples stimulated with PWM did not differ significantly (two-tailed paired T-test; P-value = 0.1018) from the negative samples.

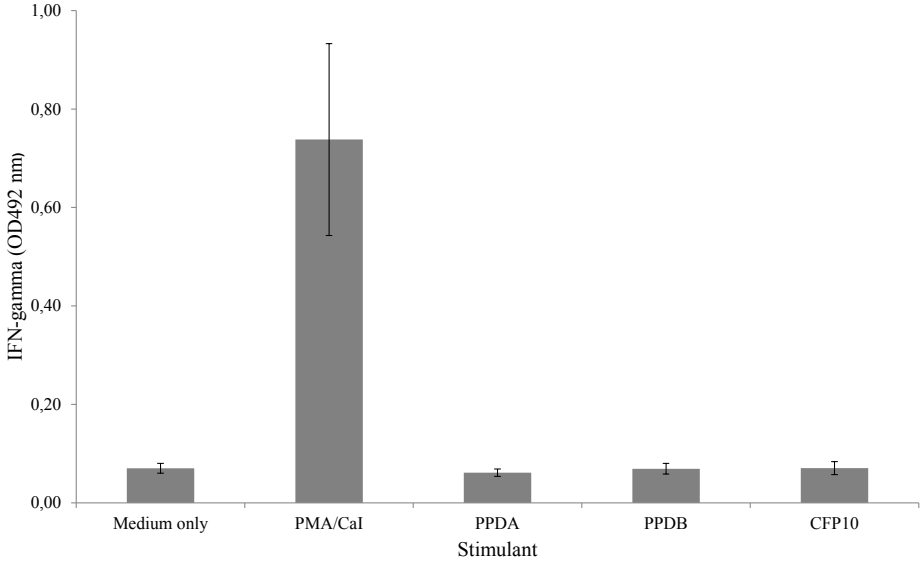

Figure 2. Mean IFN-γ levels (OD_{492}) of whole blood cultures of 10 lions from BTB-free farms stimulated with mycobacterial antigens and incubated for 72 hours. Error bars indicate standard deviation. IFN-γ levels in PMA/CaI stimulated cultures ($OD_{492\ nm}$ mean 0.74; SD 0.20) differ significantly from those in the unstimulated cultures ($OD_{492\ nm}$ mean 0.07; SD 0.01). Stimulation of whole blood cultures with mycobacterial antigens did not result in measurable IFN-γ levels; PPDA (OD_{492} mean 0.06; SD 0.007), PPDB (OD_{492} mean 0.07; SD 0.011) or CFP10 (OD_{492} mean 0.07; SD 0.013).

KNP lion population.

ACKNOWLEDGEMENTS

Part of the work of Darshana Morar was financially supported by the ITM Institutional Collaboration FA3. We thank private game farms “Ukutula Lion Park” (SA) and the “Kingdom Wildlife Sanctuary” (SA) for providing us with lion samples, as well as Shelley Rhodes for her advice regarding the IFN-γ assay.

CONFLICTS OF INTERESTS

None

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Chapter 4b

Development of a lion-specific interferon-gamma assay

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Veterinary Immunology and Immunopathology (2012) 149, 292-297

ABSTRACT

The ongoing spread of bovine tuberculosis (BTB) in African free-ranging lion populations, for example in the Kruger National Park, raises the need for diagnostic assays for BTB in lions. These, in addition, would be highly relevant for zoological gardens worldwide that want to determine the BTB status of their lions, e.g. for translocations. The present study concerns the development of a lion-specific IFN-γ assay, following the production and characterization of monoclonal antibodies specific for lion interferon-gamma (IFN-γ). Recombinant lion IFN-γ (rLIFN-γ) was produced in mammalian cells and used to immunize mice to establish hybridoma cell lines producing monoclonal antibodies. These were used to develop a sensitive, lion IFN-γ-specific capture ELISA, able to detect rLIFN-γ to the level of 160 pg/ml. Recognition of native lion IFN-γ was shown in an initial assessment of supernatants of mitogen stimulated whole blood cultures of 11 known BTB-negative lions. In conclusion, the capture ELISA shows potential as a diagnostic assay for bovine tuberculosis in lions. Preliminary results also indicate the possible use of the test for other (feline) species.

INTRODUCTION

Lions (*Panthera leo*) are susceptible to *Mycobacterium bovis* (*M. bovis)* infection, resulting in bovine tuberculosis (BTB). This chronic, debilitating disease [1,2] can affect multiple organs, particularly the lungs [3-5], and may ultimately lead to death of the infected animal [6]. Cases of lion BTB have been described in zoological collections [7,8] as well as in free-ranging lion populations [3,9,10], where *M. bovis* prevalence may be as high as 79%[11]. Testing of lions for BTB may thus be required for disease surveillance, in case of translocations between zoological collections or wildlife parks or for research purposes. Currently, the only validated method for diagnosis of BTB in lions is the tuberculin skin test [6]. This requires a recapture after 72 hours, which is labour intensive as well as impractical under free-ranging conditions.

To replace the tuberculin skin test, other diagnostic methods are being investigated, including the interferon-gamma (IFN-γ) release assay, which is already complementing the tuberculin skin test in humans and cattle [12,13]. This assay is based on TB antigen specific stimulation of cell-mediated immunity (CMI) and measures production of IFN-γ, a cytokine recognized to play a key role in anti-mycobacterial immunity [14], by sensitized T-cells. Assays assessing CMI are generally believed to detect *M. bovis* infection at an earlier stage than serologic assays [14]. When comparing the IFN-γ release assay with the tuberculin skin test (both cell-mediated responses), the advantages of the IFN-γ assay are its increased sensitivity, detection of infection in an earlier stage, the possibility of more rapid repeat testing, no need for a second capture of the animal and more objective test procedures and interpretation [12,15,16]. Also, the interpretation criteria for positive reactors can be adjusted easily, thereby customizing the sensitivity and specificity of the assay according to need.

The IFN-γ release assay has been developed for an increasing number of wildlife species, for example primates [17], buffalo [18] and deer [19], however it is not available for lions. The aim of this study was therefore to develop a lion-specific IFN-γ assay. For this purpose, recombinant lion IFN-γ (rLIFN-γ), produced in mammalian cells, was used to immunize mice for production of monoclonal antibodies (mAb). These were used to develop a capture ELISA that had a lower detection level of 160 pg/ml rLIFN-γ and was able to detect native IFN-γ in supernatants of mitogen stimulated whole blood cultures of lions as well as other (feline) species.

MATERIAL AND METHODS

Preparation of recombinant lion IFN-γ (rLIFN-γ)

Recombinant lion IFN-γ was produced by U-Protein Express BV, Utrecht The Netherlands. The gene encoding rLIFN-γ [20] was synthesized by GeneArt and cloned into a pUPE expression plasmid. Expression vectors were transiently transfected to HEK293 EBNA cells as described by Durocher et al. [21]. Expression media were harvested six days post-transfection.

Monoclonal antibody production and tandem selection

Mouse immunization: Two female BALB/c mice, 8 weeks old (Charles River, Someren, The Netherlands) were immunized intraperitoneally with 50 µg rLIFN-γ in 50 ul sterile PBS and 50 ul Specol (Prionics, The Netherlands) on days 0, 21 and 42. On days 63 and 64, rLIFN-γ in PBS was injected intravenously without Specol and on day 67 the mice were sacrificed. Seroconversion was tested with serum of days 0 (pre-immune), 28 and 49 in an indirect ELISA as described below. Approval for this procedure was obtained from the Animal Care and Ethics Committee (AEC) of Utrecht University, according to the Dutch Experiments on Animals Act (license number DEC2010.II.07.119).

Cell fusion and cloning: Spleen cells of the immunized mice were fused with Sp2/0 myeloma cells and the resulting hybridomas were subsequently cultured in hypoxanthine aminopterin thymidine (HAT) selection medium for hybridomas. After 2 weeks, hybridoma supernatants were screened with the ELISA described below. Hybridomas producing antibodies specific for rLIFN-γ were selected and single cell clones were created with a BD Influx Cell Sorter (BD Biosciences) and then grown to produce mAbs.
Isotyping, purification and biotinylation of mAbs: Isotyping of four mAbs was performed using the Beadlyte Mouse Immunoglobulin Isotyping Kit (Millipore, The Netherlands) using the Luminex 100™ System according to the manufacturer's instructions. Subsequently, mAbs were affinity-purified using a GammaBindTM Plus SepharoseTM column (GammaBind G, Type 3, Mr 15 000; GE Healthcare) and 1 mg of the mAbs was biotinylated with Biotin-ε-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS) according to the manufacturer's instructions (Roche Diagnostics, the Netherlands).
Indirect ELISA: Indirect ELISAs were performed to test seroconversion of the mice, as well as for selection of hybridomas. The standard protocol of the ELISAs was as follows: ELISA plates (Costar, Corning Incorporated, USA) were coated with rLIFN-γ (5 µg/ml, 100 ul/well) for 1 hour on a shaker. Antigen was discarded and 200 ul/well block buffer (Roche Diagnostics, The Netherlands) was added and the plate was placed on a shaker for 30 minutes. Block buffer was discarded and serum/supernatant was added (100 ul/well) and incubated for 1 hour on a shaker. Plates were washed 3 times with PBS-Tween20 0.1%. Conjugate was added 100 ul/well: to test for seroconversion initially rabbit-anti-mouse Ig-horseradish peroxidase (HRP) antibodies were added 1:2000 (Dako, Belgium); goat-anti-mouse IgG-HRP 1:6000 (Southern Biotech, Alabama, USA) was used for screening of hybridomas, while already selecting for mAbs with IgG isotypes. Plates were washed as described above including a final wash of tap water and 100 ul of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Roche Diagnostics, The Netherlands) was added to each well. Plates were read within 30 minutes. All steps were performed at room temperature.
After purification and biotinylation, indirect ELISAs were performed to determine affinity of the (biotinylated) mAbs to rLIFN-γ. For this, rLIFN-γ was coated at 2 µg/ml (50ul) and (biotinylated) mAbs were diluted in 4-fold dilutions from 10 to 0.0024 µg/ml.
Capture ELISA: Finally, tandems of the mAbs were tested to determine the best mAb combination. In short, the non-biotinylated mAbs were coated at 5 µg/ml (50ul), rLIFN-γ was added at 1 µg/ml at first and later in two-fold dilutions from 200 to 0.2 ng/ml (50ul), biotinylated mAbs were added at 1 µg/ml (50ul) and streptavidin-HRP (Biosource, Belgium) 1:2000 was used as conjugate; other ELISA procedures were as described above.
With the four best mAb combinations for detection of rLIFN-γ, native lion IFN-γ was tested in the capture ELISA, and of the four tandems tested, the most optimal mAb combination was selected. As a negative control protein, recombinant IFN-γ of white rhino was used.
Dot blot assay: To determine applicability of the mAbs for other diagnostic purposes, it was investigated whether epitopes recognized by the mAbs were linear or conformational. The rLIFN-γ was heated to 100°C for 10 minutes in a mixture with laemmli buffer and PBS (1:3). Heated rLIFN-γ and non-heated rLIFN-γ in PBS only were spotted onto Whatman Protran nitrocellulose membranes (Sigma Aldrich, the Netherlands). After blocking with block buffer (Roche Diagnostics, The Netherlands), the membranes were incubated in 1 µg/ml dilutions of the two mAbs in PBS, followed by washing (3x for 10 minutes with PBS-Tween 20 0.05%), and incubated with goat-anti-mouse-AP (Southern biotech, Alabama, USA) 1:2000, in PBS-Tween 20 0.05%. After washing, nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) was added (Roche Diagnostics, The Netherlands) for color development. A negative

control without addition of a mAb was run simultaneously.
Western blot assay: To confirm binding of fragments of the correct size, rLIFN-γ was mixed with laemmli buffer, heated, resolved on a SDS polyacrylamide gel and subsequently electroblotted to a Whatman Protran nitrocellulose membrane (Sigma Aldrich, The Netherlands). Using the SNAP i.d. (Millipore, USA) according to manufacturer's instructions, the blot was blocked (Roche Diagnostics, The Netherlands), biotinylated Li2B7.2G7 was added (0.67 μg/ml, 1.5 ml) and streptavidin-HRP80 was added (1:20000, 1.5 ml) with washing steps in between with 0.05%Tween-20 in PBS. Finally, detection was carried out using Luminata Crescendo Western HRP (2 ml) (Millipore, USA) and Amersham hyperfilms (GE healthcare).

Final capture ELISA protocol for IFN-γ detection
A final protocol for a capture ELISA was developed using the most optimal mAb tandem. ELISA plates (Greiner Microlon, extra high binding) were coated for 1 hr with mAb Li2B7.2G7 in PBS at a concentration of 2 μg/ml (50 ul/well). Subsequently, they were blocked with 1.3% universal casein diluent (SDT, Germany) (75 ul/well). After 1 hour, the wells were emptied and washed four times with PBS-Tween 0.05% and supernatants of stimulated whole blood were added in duplicate, diluted 1:1 in the 1.3% casein buffer, with bovine serum added to a final concentration of 10%. Standard concentration curves were made by adding two-fold dilutions of rLIFN-γ in casein/PBS. After 2 hours, plates were washed as described previously and biotinylated detecting mAb Li7A9B4 (50 ul) was added 1:10 000 diluted in 0.43% casein buffer in PBS with bovine serum at a final concentration of 10%. After incubation, plates were washed five times as described above and 50 ul 1:20 000 streptavidin-HRP80 (SDT, Germany) in 0.43% casein/PBS was added. After incubation for 30 minutes, plates were washed eight times as described and es(HS)TMB reagent (SDT, Germany) (50 ul/well) was added. Color reaction was stopped after 10 minutes by adding H_2SO_4 (2 M; 50 ul/well) and plates were read at OD_{450}.

Whole blood stimulation of multiple (feline) species
Animals: Whole blood samples were collected from 3 African lions, 1 Asiatic lion, 1 Amur panther, 3 tigers, 2 domestic cats and 1 maned wolf (all adult animals) at opportunistic sampling moments when animals were immobilized for other purposes. These animals were experimental animals or came from zoological and private collections and were assumed to be BTB-free. Approval of this procedure was obtained from the AEC, according to the Dutch Experiments on Animals Act (license number DEC 2007.II.06.152)
Whole blood stimulation: Heparinised whole blood samples were collected (10 ml, Vacutainer) and tested within 8 hours. Aliquots were incubated at 37°C and in 5% CO_2 for 48 and 72 hours with various mitogen concentrations (see Table 1). In an initial experiment screening for optimal stimulation conditions of lion whole blood, a blood sample of one lion was used and tested at the following mitogen concentrations: phorbol 12-myristate 13-acetate (PMA)/ionomycine (3 conditions, respectively PMA 100, 100 or 50ng/ml and ionomycine 2,1,1 μg/ml), pokeweed mitogen (PWM) (10 and 5 μg/ml), phytohaemagglutinin-leucoagglutinin (PHA-L) (10 and 5 μg/ml), Concanavalin A (ConA) (10 and 5 μg/ml) and medium alone (negative control-nill)- all diluted in RPMI medium to a total volume of 100 ul. This experiment clearly showed superiority of PMA/ionomycine as a mitogen (Maas, unpublished data), which was in agreement with a protocol described for the stimulation of PBMCs of domestic cats [22]. Therefore, subsequent samples were only stimulated with the three concentrations of PMA/ionomycine and with medium alone (nil control) for 48 and/or 72 hours. After incubation, blood samples were centrifuged at 1500 X g for 10 minutes and the supernatant was harvested and stored at −20°C until further use in the IFN-γ capture ELISA as described above.

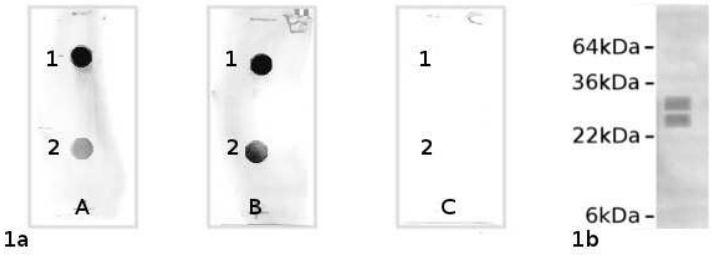

Figure 1. Analysis of the binding characteristics of the mAbs. (a) Dot blot analysis of rLIFN-γ with mAb Li7A9B4 (A), mAb Li2B7.2G7 (B) and a negative control (C) without antibody. Both non-denatured rLIFN-γ (1) and denatured rLIFN-γ (2) were recognized by the mAbs, which indicates rLIFN-γ is recognized conformationally and non-conformationally. (b) Western blot of rLIFN-γ with biotinylated mAb Li2B7.2G7. The double band that is visible is due to posttranslational modification.

Initial assessment of the specificity of the IFN-γ capture ELISA with 11 lions from BTB-free areas

Heparinised whole blood samples (10 ml, Vacutainer) were collected from 11 lions from game farms with a BTB-free history and were stimulated for 72 hours with PMA/ionomycine (respectively 100 ng and 2 µg/ml), purified protein derivatives (PPD)A (20 µg/ml; Lelystad, The Netherlands), PPDB (20 µg/ml; Lelystad, The Netherlands), CFP10/ESAT6 (10 µg/ml; Statens Serum Institute, Copenhagen) and medium (negative control). After incubation, blood samples were centrifuged at 1500 X g for 10 minutes and the supernatant was harvested and stored at −20°C until further use in the IFN-γ capture ELISA as described above.

Data analysis: Samples were assayed in duplicate and results were expressed as the averaged OD_{450} value. The cut-off level was calculated as twice the average OD value of the nil control samples of the animal.

RESULTS AND DISCUSSION

Serum titers of the immunized mice reached levels of 1:160 000 at the moment of sacrifice (day 67). In total 31 hybridomas were obtained that produced IFN-γ specific antibodies. After single cell cloning, expansion and additional screening, four hybridomas were selected, based on strong reactivity with rLIFN-γ and growth capabilities. Isotypes of the mAbs produced were all IgG1-κ. Capture ELISAs performed to test different tandems of these four antibodies identified mAb Li2B7.2G7 as capture Ab and mAb Li7A9B4 as detection Ab as the optimal combination to detect rLIFN-γ and native lion IFN-γ. In a dot blot assay it was shown that rLIFN-γ is recognized conformationally and non-conformationally (Figure 1a). This may be of relevance for use of these antibodies in other diagnostic assays. The western blot confirmed binding of fragments of the correct size (Figure 1b). Since no false positive reactions occurred with white rhino recombinant IFN-γ (78% homologous to rLIFN-γ), the antibodies should be considered specific for lion IFN-γ.

The final optimal conditions of the IFN-γ specific capture ELISA using the mAbs Li2B7.2G7 and Li7A9B4 have been described in Section 2.3. A typical rLIFN-γ titration curve is shown in Figure 2. The lower detection level of the assay, defined as twice the average OD_{450} value of the negative samples, was 160 pg/ml. This detection limit is comparable with detection limits described for humans (275 pg/ml and 292 pg/ml) and cats (300 pg/ml and 50 pg/ml), hence sensitivity of the ELISA is sufficient to detect IFN-γ levels elicited by antigen stimulation

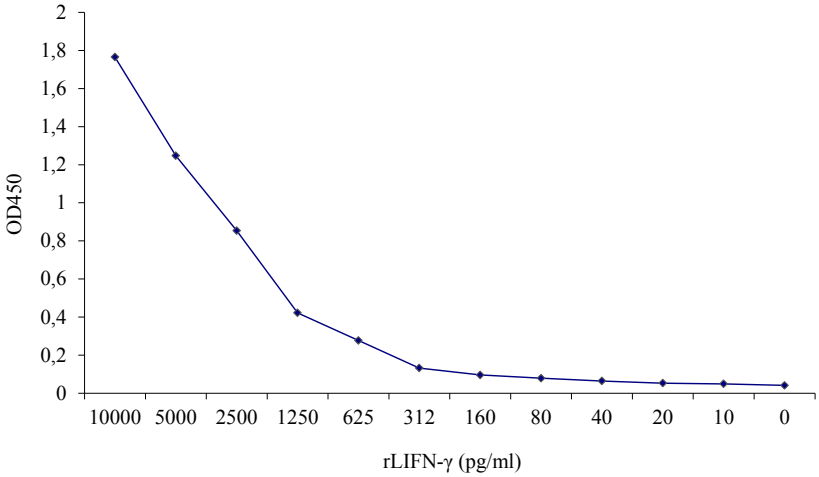

Figure 2. Typical titration curve of rLIFN-γ with measurement of OD at 450 nm. Two-fold dilutions were titrated in the ELISA. The lower detection limit of the assay was 160 pg/ml rLIFN- γ, based on twice the average OD_{450} value of the negative samples.

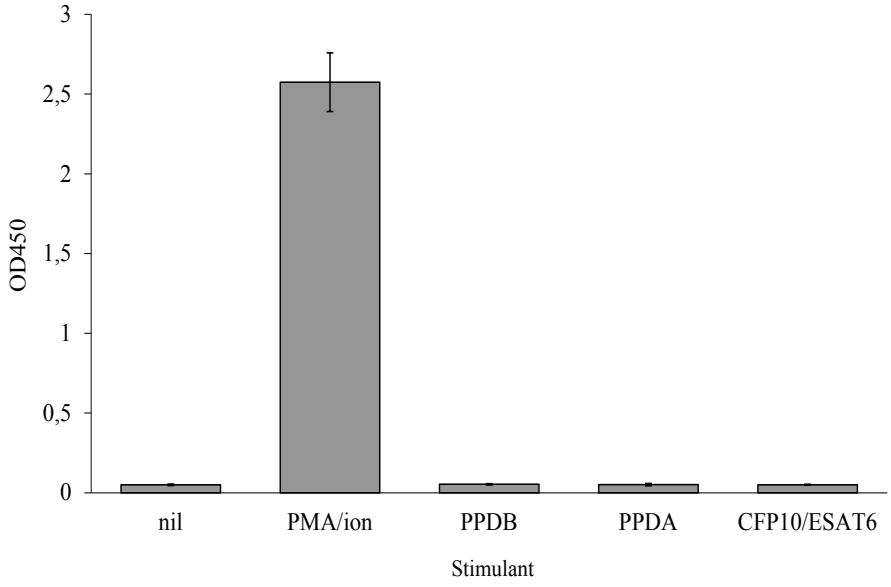

Figure 3. Mean OD_{450} values of whole blood samples of 11 lions from BTB-free areas. Error bars indicate standard deviations. The mean OD_{450} value of the samples stimulated with PMA/ionomycine differed significantly from the nil sample and is comparable with an IFN-γ concentration of >10 ng/ml. The mean OD_{450} value of samples stimulated with PPDB, PPDA and CFP10/ESAT6 did not differ significantly from the nil samples.

[22,23]. Supernatants of whole blood samples of 11 lions from BTB free areas all showed high levels of IFN-γ when PMA/ionomycine was used for stimulation (OD_{450} mean: 2.57, which is comparable with >10 ng/ml IFN-γ; standard deviation: 0.18). In case of stimulation with PPDB, PPDA and CFP10/ESAT6, the obvious *M. bovis* related antigens, no IFN-γ response was found (Figure 3) [16].

Table 1. Mean OD_{450} values, obtained from different experiments with comparable standard curves. Stimulation of whole blood samples was 72 hours, unless indicated with *: in that case samples were stimulated 48 hours. For the maned wolf, stimulation with PMA/ionomycine with the concentrations of 50 ng and 1µg/ml resulted in the highest response.

	-ve (medium)	PMA/ion (resp. 100 ng and 2 ug/ ml)
Asiatic lion*	0.134	2.713
African lion 1*	0.094	2.720
African lion 2	0.050	2.274
African lion 3	0.050	0.796
Amur panther*	0.054	2.719
Tiger S	0.055	2.524
Tiger O	0.051	2.555
Tiger R	0.056	2.837
Cat V	0.053	2.647
Cat O	0.061	2.611
Maned wolf	0.046	1.655

Cross-reactivity of the selected mAbs with IFN-γ of other feline species and potentially other species, was also tested. Tigers have been reported to be susceptible for *M. bovis* [24], as well domestic cats [23]. As shown in Table 1, the assay was able to detect native IFN-γ as elicited by PMA/ionomycine stimulation of whole blood from an Asiatic lion, an Amur panther, domestic cats, tigers and a maned wolf. Amongst the feline species the IFN-γ concentrations were all above 10 ng/ml, except for lion 3, for which no explanation could be found as no further data for this animal were available. Although the assay needs to be optimized for these species, it has potential to contribute to the diagnosis of *M. bovis* infection in these species. It would be worthwhile to assess whether the assay may also be useful for other feline species like the highly endangered Iberian lynx, since cases of BTB have been described amongst free-ranging individuals of this species [25]. Surprisingly, native IFN-γ of the maned wolf was detected as well, even though canid species have a lower genetic homology (dog IFN-γ is 89% homologous with lion IFN-γ [20]).

The development of a sensitive lion-specific IFN-γ release assay that detects native lion IFN-γ in supernatants of mitogen stimulated whole blood offers potential for a new diagnostic method for *M. bovis* infection in lions. The use of whole blood for the assay instead of isolated PBMCs, which was shown to be almost equally sensitive in a human study [26], should facilitate its use in the field. Eukaryotic rLIFN-γ is likely to play a pivotal role in future assay development as a positive control. As a next step, the assay should be standardized and validated with larger numbers of BTB negative and positive lions.

ACKNOWLEDGEMENTS

The work of MM was supported by the Netherlands Organisation for Scientific Research (NWO/ZonMw grant 918.56.620) and the Prins Bernhard Cultuurfonds on behalf of the Niemans-Schootemeijer Fonds. We thank “the Kingdom” and “Ukutula” for the lion samples, as well as the zoological gardens in Pretoria (SA), Rotterdam (NL) en Beekse Bergen (NL) for the various other animal samples.

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Chapter 5

IFN-γ, TNF-α, IL-4 and IL-10 reverse transcriptase real-time PCR's as potential tools to assess immune responsiveness of lions infected with *Mycobacterium bovis*

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Ongoing work

ABSTRACT

Bovine tuberculosis spreads rapidly through the Kruger National Park lion population and potentially other African lion populations. Knowledge of immune responsiveness after *Mycobacterium bovis* infection may facilitate development of vaccines and diagnostic assays for lions. Since in several species immune responses in course of infection with *Mycobacterium bovis* were characterized by differential cytokine expression, reverse transcriptase real-time PCR's for lion cytokines IFN-γ, TNF-α, IL-4 and IL-10 were developed. These assays were tested in a pilot study consisting of 26 lions with either tuberculin skin test positive (n=19) or negative (n=7) statuses. Cytokine profiles in blood tended to differ between skin test positive lions and skin test negative lions and need to be confirmed and extended in larger study groups and longitudinal follow up.

INTRODUCTION

Mycobacterium bovis (M. bovis), causing bovine tuberculosis (BTB), has been found in the lion populations of the Kruger National Park (KNP) and the Hluhluwe-Imfolozi Park (HIP) in South Africa, and is likely to be present in the Serengeti lion population [1-3]. The ultimate effect of the infection is currently unknown, but a rapid spread like that ongoing in the KNP raises concerns about the conservation of its lion population.

Based on studies in humans and other species, it is generally agreed that cell-mediated immunity (CMI), involving T-lymphocytes as well as macrophages, plays a major role in controlling *M. bovis* infection [4]. The T-lymphocyte (T cell) is the major player in the protective acquired immune response [5]. T-lymphocytes are divided into at least four different subpopulations, based on differential cytokine profiles: Th1, Th2, regulatory T cells and Th17 cells [6]. Th1 cells produce IFN-γ, interleukin (IL-)2, and TNF-α and are considered essential in the immune response to mycobacteria and for the containment of the mycobacterial infection. They are associated with macrophage activity and T cell proliferation [5,7]. Th2 cells produce IL-4, IL-5, IL-9, IL-10, support humoral immune responsiveness and act as regulatory cells [8]. Th17 cells produce, amongst other cytokines, IL-17, which is a potent inflammatory cytokine in the primary phase of infection. During the chronic phase, there is a balance between Th1 and Th17 to promote anti-mycobacterial immunity and to control bacterial growth and limit tissue damage [9]. Regulatory T cells can produce the anti-inflammatory cytokine IL-10 and are emerging as important contributors to *M. tuberculosis* immunity, regulating and balancing immune reactivity [7].
Cytokines like IFN-γ, TNF-α, IL-4 and IL-10 play a central role in the immune system by widely varying roles in modulation of immune responses [10]. IFN-γ is a pivotal pro-inflammatory cytokine that is largely produced by Th1 cells and that activates cells of the immune system to kill mycobacteria and induces a type 1 immune response. TNF-α is also considered essential for the control of *M. tuberculosis*, acting together with IFN-γ to activate macrophages, but an excess of TNF-α can also lead to tissue damage [7]. IL-4 is a typical anti-inflammatory cytokine that is mainly produced by Th2 cells. IL-4 down-regulates the pro-inflammatory immune response to control tissue damage and is associated with the humoral response [7,11]. IL-10 is a cytokine that was at first thought to be only produced by Th2 cells, but nowadays it is known that IL-10 is expressed by many cells of the adaptive immune system, including Th1, Th2, regulatory T cells and TH17 cell subsets, and expression levels may differ according to the strength of the stimulus. It has a central role in infection by limiting the immune response and thereby preventing damage to the host [12]. In humans, it has been suggested that the ratio between IFN-γ and IL-10 may be used to predict disease severity [13].

Most research regarding the role of cytokines in immune responsiveness to *M. bovis* has been performed in mouse models and humans [6]. Little is clear about the development of immunity in course of *M. bovis* infection in lions. Extended knowledge of the cytokine expression in relation to other immune response parameters and clinical symptoms may offer insight in the development of disease in lions, beneficial to development of effective vaccines [14] and new diagnostic methods. Since cytokine release is localized and not readily measurable in whole blood when measuring cytokines as such, we developed reverse transcriptase real-time PCR's (qRT-PCR) to measure expression of four cytokines in lion white blood cells: IFN-γ, IL-4, TNF-α and IL-10, representing differential immune responsiveness in course of time. These assays were tested in a pilot study of 26 lions, either skin test positive (n=19) or skin test negative (n=7). Cytokine profiles of skin test positive lions tended to differ from those of skin test negative lions.

MATERIAL AND METHODS

The assay was initially developed using blood samples from domestic cats. Subsequently, blood of two lions from BTB-free areas was used to test the primers and probes and to optimise the qRT-PCR. Samples from a third lion from a BTB-free area were processed similarly to the field samples and were used to account for plate variability when testing the field samples.

Animals

Blood of four domestic cats was collected in EDTA vacutainer tubes during opportunistic sampling moments. Lion blood was collected in EDTA vacutainer tubes at *ad hoc* sampling moments from three lions in BTB-free areas. Blood samples (EDTA/heparin vacutainer tubes, depending on availability) were taken from 26 lions from the KNP, immobilized as part of a large BTB survey described elsewhere [3], that had also been subjected to a tuberculin skin test [15]. Nineteen lions were found to be skin test positive and seven skin test negative.

Isolation of the PBMC or white blood cells

Peripheral blood mononuclear cells (PBMCs) of the domestic cat samples and from the two lions 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) and stored in RNAlater at -20°C until further processing.

To obtain white blood cells, KNP field blood samples were centrifuged for 15 minutes at 3200 rpm. The buffy coat was collected and 2 ml of erythrocyte 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 10 minutes centrifugation at 1200 rpm, the resulting pellet was suspended in 300-500 µl RNAlater, depending on the size of the pellet. Samples were stored at -20°C until further processing.

One lion sample from a BTB-free area, used as a reference sample to account for plate variability, was treated as described for the KNP samples.

RNA extraction

mRNA was extracted from the stored samples using the RNeasy mini kit (Qiagen®). The samples were mixed with 1.5 ml ice cold PBS and centrifuged for 10 minutes at 11 000 rpm The supernatant was discarded, after which the manufacturer's protocol was followed, including an extra step with on-column DNase digestion. 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°C, 5 min at 95°C. The cDNA extracted was stored at -20°C.

qRT-PCR

Samples were processed with the StepOne Plus PCR (Applied Biosystems) reagents according to the manufacturer's instructions, using the TaqMan Universal master mix (Applied Biosystems) and cytokine primers and probes that had been described for domestic cats [16,17](Table 1).

Table 1. Primer and probe sequences for the (feline) control and cytokine genes GAPDH, IFN-γ, TNF-α, IL-4 and IL-10 (produced at IDT Inc.).

Cytokine	Primer	Sequence 5' to 3'	Length	Probe	Probe sequence 5' to 3'
GAPDH	GADPH.57f	GCCGTGGAAT TTGCCGT	82	GAPDH.77p	CTCAACTACATGGTCTA CATGTTCCAGTATGATTCCA
	GAPDH.138r	GCCATCAATGA CCCCTTCAT			
IL-4	IL4.143f	GCATGGAGCTG ACCGTCAT	81	IL4.169p	TGGCAGCCCCTAAGAA CACAAGTGACAA
	IL4.223r	CGGTTGTGGCT CTGCAGA			
IL-10	IL10.182f	TGCACAGCATA TTGTTGACCAG	76	IL10.209p	ACCCAGGTAACCCTTAA GGTCCTCCAGCA
	IL10.257r	ATCTCGGACAA GGCTTGGC			
TNF-α	fTNFα239f	CTTCTCGAACTC CGAGTGACAAG	74	fTNFα266p	TAGCCCATGTAGTAGCA AACCCCGAAGC
	fTNFα312r	CCACTGGAGTT GCCCTTCA			
IFN-γ	IFN.14f	TGGTGGGTCGC TTTTCGTAG	85	IFN.152p	CATTTTGAAGAACTGGAAAG AGGAGAGTGATAAAACAAT
	IFN.225r	GAAGGAGACAA TTTGGCTTTGAA			

The sense and anti-sense primers were always located in two consecutive exons of the gene. With these primers, short PCR products were selected, in order to achieve fast primer extension. The TaqMan probes were labeled with a reporter dye (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 by the primers, the feline-specific TaqMan probes only allowed detection of complementary DNA (cDNA) and no genomic DNA (gDNA), which minimized gDNA-contamination.

The assay compositions for the 20 µl reactions were as follows: 10 µl TaqMan® Universal PCR Master Mix, 1 µl primer/probe mix (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: 2 min 50°C, 10 min 95°C, 40 cycles of 15s at 95°C and one minute at 60°C. Samples were assayed in duplicate in separate wells on 96-well plates and the mean was obtained for further calculations.

Reference gene: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize gene expression to reduce experimental variation.
Control samples: To control for potential contamination, every run included a no-template, negative water control in duplicate. To check for plate variability, a control sample from a lion from a BTB-free area, was included in every run for the four target genes and the reference gene.
Ct: The threshold cycle (Ct) is defined as the cycle number where the fluorescence signal measured exceeds a (pre-defined) threshold during the exponential phase of amplification. To limit the number of missing values, a Ct of 40 was used as a result for qPCR of samples that did not show a cytokine signal after 40 cycles. All samples were run in duplicate and the mean

of these values was used for further calculations. 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 with which data were corrected. These corrected data were subsequently analyzed, using Microsoft Excel (2003).

Data analysis

Results were analyzed using the comparative Ct method [18,19]. This method makes several assumptions, which include an efficiency of the PCRs close to 2, and that the PCR efficiency is similar between the target genes and the internal control gene. The following equations were used:

$$\Delta Ct = Ct \text{ gene of interest} - Ct \text{ GAPDH} \quad [1]$$

Equation 1 was used to calculate the difference between the Ct of the target and the Ct of the internal control (GAPDH) to normalize for differences in the amount of total nucleic acid added to each reaction.

$$2^{-\Delta Ct} = 2^{-(Ct \text{ gene of interest} - Ct \text{ GAPDH})} \quad [2]$$

Equation 2 was used to calculate the amount of target, corrected for its exponential multiplication (without a unit).

$$\text{Difference in expression (fold change)} = \text{mean } 2^{-\Delta Ct} \text{ positive lions} / \text{mean } 2^{-\Delta Ct} \text{ negative lions} \quad [3]$$

The relative ratio of gene expression between the skin test positive and skin test negative lions was calculated with equation 3.

The results of $2^{-\Delta Ct}$ were tested with the Grubbs' test for outliers with GraphPad Software.

RESULTS

Day to day plate variation was small (results not shown) and the corrected threshold influenced the cytokine Ct's only minimally. Appendix 1 shows the mean Ct, the ΔCt and $2^{-\Delta Ct}$ results of the corrected values. Tables 2 and 3 show the results of subsequent calculations. Lion 434E630268 was identified as an outlier by the Grubbs' test and was excluded for the calculations of the mean, standard deviation and the differences in expression between skin test positive and negative lions.

In Table 2, a higher expression of IFN-γ is shown in skin test positive lions, compared to skin test negative lions. Table 3 shows that in skin test positive lions, expression of IFN-γ is more than three times higher than in skin test negative animals. TNF-α is expressed 1.5 times more in skin test positive animals compared to skin test negative animals. IL-4 is expressed more than five times lower in skin test positive animals compared to skin test negative animals. Expression of IL-10 is about 1.2 times lower in skin test positive animals than in skin test negative animals. However, none of these findings is significant (based on $p < 0.05$).

Table 2. Mean ΔCt for skin test positive lions (n=19) and skin test negative lions (n=6).

		IFN-γ	TNF-α	IL-4	IL-10
ΔCt positive lions	Mean	7.55	6.49	11.91	8.55
ΔCt negative lions	Mean	9.75	8.01	10.04	8.62

Table 3. Relative quantification of cytokine transcription for IFN-γ, TNF-α, IL-4 and IL-10 of skin test positive lions (n=19) as compared to skin test negative lions (n=6).

	IFN-γ	TNF-α	IL-4	IL-10
fold change	3.29	1.53	0.19	0.81
T-test P value	0.14	0.26	0.05	0.64

DISCUSSION

This pilot study shows the potential of the use of qRT-PCR's for relevant cytokines as potential markers for stages of *M. bovis* infection in lions. The primers that were initially developed for domestic cats could also detect lion cytokine cDNA. Comparison between skin test positive lions and skin test negative lions shows a more than three-fold increase of IFN-γ in the *M. bovis* infected lions compared to un-infected animals (Table 3). Also TNF-α expression tends to be higher in skin test positive lions. Although the differences measured were not statistically significant, they are in line with skin test reactivity and its potential Th1 bias. IL-4, the cytokine typifying Th2 responses, was expressed to a lesser extent in skin test positive lions. IL-10 differences between the two groups were only small, which is not surprising, seen the variety of cells that the cytokine can originate from [12].

Statistical analyses of the cytokine expression data were complicated by several factors and the results of this pilot study should therefore be interpreted with caution. Due to the minimal amounts of mRNA measured in the qRT-PCR trivial variations in the early stages of the qRT-PCR procedure can greatly influence the final yield of the amplified product [20]. Especially assessment of cytokines expressed at low levels, like IL-4, may be influenced by e.g. time delays in blood processing, as shown in human TB studies [21]. Interpretation of statistical analyses of small groups, like in our study, is difficult, especially because relatively many lions had immeasurable Ct values (see Appendix 1), particularly lions with a negative skin test. The cut-off value of 40 that was chosen is likely to be an underestimation of the Ct, i.e. an overestimation of the expression of the cytokine, hence differences could be larger in reality. Mean $2^{-\Delta Ct}$ values (Appendix 2) showed large standard deviations, which may be ascribed to different stages of infection of the lions in the skin test positive group, resulting in various cytokine expression patterns. To define these stages, markers are not available as yet. Age and sex have not been taken into account in these analyses due to the limited number of animals, though sex-related differences in cytokine expression have been shown e.g. in human rhinoviruses infection [22]. Obviously, cytokine expression analyses of lions in general may also be complicated by other infections, e.g. FIV_{ple} infection or parasitic infections, known to elicit Th2 responses. To compensate for these complicating factors, larger numbers of lions will need to be tested in randomized groups, preferentially in course of time.

The comparative Ct method assumes that the amplification efficiencies of the PCRs are the same for all cytokines. Though there are no strict rules as to what makes the efficiency of genes "similar enough" to use the comparative Ct method, a rough guide is that the efficiencies should be within 10% of 2 (1.8x to 2.2x) [18]. In the current study, PCR efficiency of GAPDH was 1.81 and PCR efficiency of TNF-α was 1.87. PCR efficiencies for the other cytokines remain to be determined.

Only GAPDH, an abundant glycolytic enzyme present in most cells [23] that is expressed stably in leukocytes, was used as reference gene. Although widely used [16,23,24], criticism exists about the use of this enzyme [10,20]. Therefore, in future studies, the use of multiple (>3) reference genes is recommended [23,25]. Suggestions for new reference genes may come from

research in domestic cats [26,27].
Finally, since sample volumes were a limiting factor in this study, duplicates were tested, though triplicates would be preferred. To increase the starting amounts and quality of mRNA, the method of PBMC isolation in the field needs to be improved, e.g. by using other blood collection systems, like PAXgene tubes [21].

The cytokine qRT-PCRs have been suggested to be useful as diagnostic methods for the detection of *M. bovis* infection in deer [25] and this could also be true for lions, as an indication was found that IFN-γ is more expressed in skin test positive lions compared to skin test negative lions. However, currently the qRT-PCR is logistically more demanding than other diagnostic methods like the IFN-γ assay [28] or serologic tests [29]. Therefore, we suggest that the main role of the qRT-PCR for relevant cytokines will be in the longitudinal assessment of precise immunopathogenesis of bovine tuberculosis in lions, e.g. after experimental infection.

This pilot study has shown that qRT-PCR has potential to be used to determine the gene expression of the lion cytokines IFN-γ, TNF-α, IL-4 and IL-10. However, the results of the qRT-PCR's in this small study of 26 lions are not clear-cut, hence not conclusive yet. For the classification of lion immune responsiveness in course of disease progression, assessment of cytokine expression longitudinally will be a valuable contribution.

ACKNOWLEDGEMENTS
We would like to acknowledge Dr. Marinda Oosthuizen, Ildiko van Rhijn and Bas Brinkhof for their advice regarding the practical work and Jan van den Broek, Louis Penning and Prof. Mirjam Nielen for their advice on the statistical analysis and for critically reading the manuscript.

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Appendix 1. Cytokine expression in white blood cells of skin test positive (n=19) and skin test negative (n=7) lions, as measured by qRT-PCR and expressed as Ct, ΔCt and $2^{-\Delta Ct}$.

	Ct					ΔCt				$2^{-\Delta Ct}$			
Skin test positive lions (n=19)													
ID Number	**GAPDH**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**
451F726223	25.441	30.454	30.232	36.207	31.326	5.013	4.790	10.766	5.885	0.031	0.036	0.001	0.017
454A696521	23.755	29.870	29.725	35.370	32.445	6.115	5.970	11.616	8.691	0.014	0.016	0.000	0.002
4536256D07	25.358	31.829	32.991	39.656	35.887	6.471	7.632	14.298	10.529	0.011	0.005	0.000	0.001
4542767511	24.501	29.733	30.137	35.415	31.459	5.233	5.636	10.914	6.958	0.027	0.020	0.001	0.008
456B0D3468	23.582	28.962	29.618	35.457	31.057	5.380	6.036	11.875	7.475	0.024	0.015	0.000	0.006
456B2A755A	29.966	37.513	36.980	40.000	36.857	7.547	7.014	10.034	6.891	0.005	0.008	0.001	0.008
456A62761F	25.110	31.351	30.584	36.690	32.534	6.241	5.474	11.581	7.424	0.013	0.022	0.000	0.006
441547114E	24.870	32.769	30.846	37.624	35.042	7.899	5.976	12.754	10.172	0.004	0.016	0.000	0.001
494748583B	30.633	38.657	36.098	40.000	40.000	8.024	5.465	9.367	9.367	0.004	0.023	0.002	0.002
434D092D75	27.967	35.933	34.475	40.000	38.448	7.966	6.508	12.033	10.481	0.004	0.011	0.000	0.001
4115092830	25.458	32.848	32.519	35.868	33.208	7.390	7.061	10.410	7.750	0.006	0.007	0.001	0.005
494A185A30	26.886	35.506	32.387	39.984	36.370	8.620	5.501	13.098	9.484	0.003	0.022	0.000	0.001
494A427E14	25.501	33.595	31.360	40.000	34.220	8.094	5.859	14.499	8.719	0.004	0.017	0.000	0.002
441550741E	26.137	35.556	34.530	40.000	36.726	9.419	8.393	13.863	10.589	0.001	0.003	0.000	0.001
4949447313	23.940	33.028	31.864	36.891	31.308	9.087	7.924	12.951	7.368	0.002	0.004	0.000	0.006
492E456C57	25.783	34.724	31.758	37.548	32.348	8.942	5.975	11.765	6.565	0.002	0.016	0.000	0.011
492F38266D	24.745	33.291	30.339	34.667	31.790	8.545	5.593	9.922	7.045	0.003	0.021	0.001	0.008
4959570039	23.661	33.140	31.825	37.158	35.137	9.479	8.164	13.497	11.476	0.001	0.003	0.000	0.000
49495E034B	28.896	36.806	37.158	40.000	38.566	7.910	8.262	11.104	9.671	0.004	0.003	0.000	0.001

	Ct					ΔCt				$2^{-\Delta Ct}$			
Skin test negative lions (n=7)													
ID Number	**GAPDH**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**	**IFN-γ**	**TNF-α**	**IL-4**	**IL-10**
434E630268[1]	37.695	40.000	40.000	40.000	40.000	2.305	2.305	2.305	2.305	0.202	0.202	0.202	0.202
494A123F43	24.589	37.256	30.171	36.042	30.603	12.668	5.582	11.454	6.014	0.000	0.021	0.000	0.015
492A3D0D17	24.103	32.265	29.785	34.470	31.409	8.161	5.682	10.367	7.306	0.003	0.019	0.001	0.006
456B4F4153	29.105	39.241	38.749	40.000	40.000	10.135	9.644	10.895	10.895	0.001	0.001	0.001	0.001
4A5E685451	29.273	40.000	40.000	40.000	40.000	10.727	10.727	10.727	10.727	0.001	0.001	0.001	0.001
4A441E5F2A	29.887	40.000	40.000	40.000	40.000	10.113	10.113	10.113	10.113	0.001	0.001	0.001	0.001
4A6A682D39	33.308	40.000	39.609	40.000	40.000	6.692	6.301	6.692	6.692	0.010	0.013	0.010	0.010

[1] lion 434E630268 was an outlier and its results were not included in analyses.

Appendix 2. Means ($x10^{-3}$) and standard deviations (SD, $x10^{-3}$) of cytokine production expressed as $2^{-\Delta Ct}$ in white blood cells of skin test positive (n=19) and skin test negative (n=6) lions.

	Positive lions $2^{-\Delta Ct}$ mean $x10^{-3}$(SD $x10^{-3}$)	**Negative lions $2^{-\Delta Ct}$ mean $x10^{-3}$(SD $x10^{-3}$)**
IFN-γ	8.61 (9.17)	2.62 (3.65)
TNF-α	14.18 (8.89)	9.30 (9.59)
IL-4	0.41 (0.40)	2.13 (3.70)
IL-10	4.52 (4.39)	5.58 (6.12)

Chapter 6

Assessing the impact of feline immunodeficiency virus and bovine tuberculosis co-infection in African lions

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Proceedings of the Royal Society B: Biological Sciences (2012) 279, 4206-4214

ABSTRACT

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a disease that was introduced relatively recently into the Kruger National Park (KNP) lion population. Feline immunodeficiency virus (FIV_{ple}) is thought to have been endemic in lions for a much longer time. In humans, co-infection between *M. tuberculosis* and human immunodeficiency virus increases disease burden. If BTB were to reach high levels of prevalence in lions, and if similar worsening effects would exist between FIV_{ple} and BTB as for their human equivalents, this could pose a lion conservation problem. We collected data on lions in KNP from 1993-2008 for spatio-temporal analysis of both FIV_{ple} and BTB, and to assess whether a similar relation between the two diseases exists in lions. We found that BTB prevalence in the south was higher than in the north (72 versus 19% over the total study period) and increased over time in the northern part of the KNP (0 - 41%). No significant spatio-temporal differences were seen for FIV_{ple} in the study period, in agreement with the presumed endemic state of the infection. Both infections affected haematology and blood chemistry values, FIV_{ple} in a more pronounced way than BTB. The effect of co-infection on these values, however, was always less than additive. Though a large proportion (31%) of the lions was co-infected with FIV_{ple} and *Mycobacterium bovis*, there was no evidence for a synergistic relation as in their human counterparts. Whether this results from different immunopathogeneses remains to be determined.

INTRODUCTION

Both feline immunodeficiency virus (FIV_{ple}) and *Mycobacterium bovis (M. bovis),* causing bovine tuberculosis (BTB), are found in the lion (*Panthera leo*) population in the Kruger National Park (KNP), South Africa. FIV_{ple} is an endemic pathogen in many lion populations in eastern and southern Africa [1-5], and its presence may even date back as far as the species divergence of the genus *Panthera* [6,7]. Differences have been found recently in the $CD4^+/CD8^+$ T-cell subset [4] and the prevalence of AIDS-defining conditions [8] in FIV_{ple}-infected lions compared to non-infected lions, contradicting studies that did not find pathologic effects associated with FIV_{ple} infection [7,9-11]. This may also depend on differences in pathogenicity between FIV_{ple} subtypes [12]. Common haematological and blood chemistry changes that are found in FIV infected domestic cats are lymphopoenia, leucopoenia, neutropoenia, anaemia, hyperproteinaemia and hyperglobulinaemia [13-17]. In lions, FIV_{ple} is associated with dehydration and abnormal red blood cell parameters, e.g. anaemia, depressed serum albumin and elevated liver enzymes, total protein, globulin and gamma globulin [8].

Mycobacterium bovis was introduced in the southeast corner of the KNP in the 1960s [18], spreading from infected cattle to buffaloes. The first case of lion BTB was found in 1995, probably resulting from consumption of infected buffalo carcasses [19]. A prevalence of almost 80% of the lion population in the south of the KNP was reported in 2000 [20]. Limited information is available about the effect of (B)TB on haematologic and blood chemistry values, but in humans with minimal active tuberculosis a significant rise in the gamma globulin fraction with a corresponding decrease in albumin was found. In far advanced cases, all globulins were increased, but the mean total protein did not differ from the normal value [21]. In a report of a BTB infected lion, leukocytosis, monocytosis, anaemia, neutrophilia, hypoalbunemia and hyperglobulinemia were found [22].

In humans, one of the most well-known pathogen-pathogen interactions is that between human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis (M. tuberculosis)*. HIV is the strongest known risk factor for TB, affecting on the immunity by T-cell depletion [23]. On the other hand, TB can accelerate the progression of HIV [24]. These synergistic interactions magnify the burden of disease of both infections [23,25,26]. In animals, pathogen-pathogen interactions are also described, for example between *Babesia* and canine distemper virus in lions [27] or between various infectious agents in voles [28]. Interactions may be synergistic or antagonistic to one or both of the infectious agents [29], which may be explained by a variety of mechanisms influencing host susceptibility, pathogenicity or infectiousness in both positive and negative ways [28-31].

Lions are listed as a vulnerable species by the International Union for Conservation of Nature (http://www.iucnredlist.org/apps/redlist/details/15951/0) and the relatively recent introduction of *M. bovis* in a population where FIV_{ple} is highly prevalent, could be an even more serious threat to lion conservation if the two infections would enhance each other's effects as their counterparts do in humans. Although it has been reported that 53% of the lions in the southern half of KNP are co-infected with FIV_{ple} and *M. bovis*[32], little is known about the effects of their interaction. Previous literature on FIV_{ple} and *M. bovis* infection in lions in the KNP is scarce and has often only been anecdotal, using small groups of animals. We collected data from 1993-2008, which resulted in a unique dataset of 669 lions, that was used
(i) to assess what variables (area, period, age, sex, body condition) are related with FIV_{ple} and BTB infection in lions, studied by a multivariable logistic regression model, and

Table 1. Definitions of the different body condition scores.

Body condition score	Definition
5. Excellent	Hindquarters well rounded and no ribs showing; general appearance in relation to posture and coat sheen excellent.
4. Good	Hindquarters rounded, but ribs showing slightly.
3. Fair	Hindquarters angular in appearance and ribs well defined.
2. Poor	Pelvic bones and pelvic-femoral joint prominent and ribs protruding. Tail root is sunken in. The dorsal spinae of the vertebrae becomes apparent.
1. Very poor	Skeletal details clearly visible and general appearance, posture and coat condition deteriorated. The dorsal and lateral processes of the vertebrae clearly visible.

(ii) to assess the pathogen-pathogen interaction of FIV_{ple} and BTB in lions.
For the latter, we used a sub-group of 205 lions that had been subjected to diagnostic tests for both infections. Body condition and haematological and blood chemistry values, which were deemed relevant based on literature [17,22,33], were used as dependent variables in general linear models to assess this potential interaction.

MATERIAL AND METHODS

The Kruger National Park

The KNP is a partly fenced, wooded savannah covering about 20 000 km^2. The total KNP lion population is estimated to be about 1600-1700 [34]. For the purpose of this analysis, the KNP was divided into three regions based on the prevalence of *M. bovis* in buffaloes, namely High, Medium and Low Prevalence Zones (HPZ, MPZ, LPZ; roughly corresponding to the southern, central and northern part of the park), separated by the Sabie River (south-central) and the Olifants River (central-north). Prevalences of *M. bovis* in buffaloes in 1998 were, respectively, 38.2%, 16.0% and 1.5% [35]. Data obtained from lions from adjacent game reserves with open access to the KNP were included in the analyses, according their locations.

BTB in the KNP is not controlled. This makes the ecosystem unique, as many other ecosystems with *M. bovis* presence have a test-and-removal [36,37] or culling strategy [37].

The animals

Most lions were captured with call-up stations in designated areas in the southern, central and northern part of the park, which were known to have lions, based on ranger information. These stations were randomly distributed as much as logistic considerations allowed (Figure 1a). About 25% of the study lions were brought to the Veterinary Station as emaciated or problem lions.

Before handling, all lions were immobilized with a combination of tiletamine and zolazepam (Zoletil 100, Virbac). Venous blood samples were obtained from the medial saphenous vein as soon as possible after anaesthesia in heparin, EDTA and serum Vacutainer tubes, which were kept at ambient temperature and were processed within preferably 8, but maximum 24 h. Serum was collected and stored at -20°C. All lions in this study were aged by examining dental attrition according to Smuts et al. [38]. Body condition score (BCS) was assessed according to criteria that were determined beforehand and ranged from 5 (excellent) to 1 (very poor; Table 1). Lions were micro-chipped and were given a unique brand so the animals could be recognized at future captures. Owing to the higher lion density in the south (compared with the north) as well as the

Table 2. An overview of the lion data set (n=669) and the number of lions that were available for the different analyses. A difference has been made for lions either captured at a call-up station (cal), lions that were brought to the veterinary station (vet) or lions with an unknown capture method (unk). Ht= Haematocrit; WBC= White blood cell count; TSP= Total serum protein; Alb= Albumin; Glob= Globulin; A/G ratio= Albumin/Globulin ratio; gamma glob= gamma globulin.

	Number of lions	
	Total (cal.; vet.; unk)	**With SICT and FIV_{ple} result (cal.; vet.)**
SICT result	240 (191; 49; 0)	205 (165; 40)
FIV_{ple} result	561 (415; 137; 9)	205 (165; 40)
Haematology (Ht, WBC)	Ht:435 (320; 105; 10) WBC: 375 (260; 105; 10)	Ht: 164 (124; 40) WBC: 163 (123; 40)
Blood chemistry (TSP, Alb, Glob, A/G ratio, gamma glob)	All: 500 (358; 133; 9)	All: 172 (132; 40)

location of the veterinary staff headquarters in the south of KNP, almost twice as many study lions originated from the south, compared with the central and northern areas.

Sample collection

(i) Bovine tuberculosis status: The BTB status of individual lions was determined by performing the Single Intradermal Cervical Test (SICT/skin test) as described by Keet et al. [32]. A lion was considered BTB positive when 3 days after intradermal administration of bovine tuberculin, the skin swelling was 2 mm or larger, irrespective of the response to the avian tuberculin. The SICT has a sensitivity and specificity of, respectively, 86.5% and 81%. The SICT appears not to be influenced by FIV_{ple}, in contrast to the tuberculin skin test in humans, which is affected by HIV infection [32,39].

(ii) Feline immunodeficiency virus status: Serum samples were tested for FIV_{ple}-specific antibodies at the Department of the Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, using a protocol described by van Vuuren *et al*. [40]. The sensitivity of the enzyme-linked immunosorbent assay, when using the Western blot as the gold standard, is 78.6% and the specificity 100% [40].

(iii) Haematology: Haematology analysis was performed in the KNP with a Coulter AcT diff analyzer (Beckman Coulter).

(iv) Blood chemistry: Blood chemistry analysis was conducted with a NExCT/VetEX (Bayer Health) at The Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria.

The dataset

From 1993-2008, a large dataset has been established, consisting of 669 lions from the KNP and adjacent game reserves. Descriptions of the dataset can be found in the supplementary material Tables and Figures S1 - S5. Small, specific subsets of this extensive dataset have been used in various studies in the past [1,5,20,32,41]. Not all information was available for each animal, and Table 2 gives an overview of the cross-sectional data that has been used for the analyses in this study.

Data analysis

As age and BCS are subjective characteristics and age is increasingly difficult to determine in the older age classes, these nominal variables were recoded to binary variables to facilitate the modelling, resulting in the following variables: age (binary, less than or equal to 36 months or

more than 36 months, reference level: less than or equal to 36 months), BCS (binary, BCS 1,2,3 and BCS 4,5, reference level: BCS 4,5), sex (binary, reference level: female), area (nominal, three levels (HPZ, MPZ and LPZ), reference level: MPZ) and period (nominal, three levels (1 : 1993-1998; 2 : 1999-2002 and 3 : 2003-2008), reference level: 1).

To assess which variables potentially influenced FIV_{ple} and BTB infection (both binary, reference level: negative), the following multivariable logistic regression models were used:

$$FIV = \mu + age + BCS + sex + area + period + e \quad [1a]$$
$$BTB = \mu + age + BCS + sex + area + period + e \quad [1b]$$

where μ represents the intercept, e residual error and where the other variables are coded as mentioned earlier. The e^{β} was used to calculate the odds ratio [42].

A total of 205 lions that had been subjected to both FIV_{ple} and BTB-specific diagnostic tests were used to study the potential synergistic effects of the infectious agents on the body condition and seven blood parameters. To study the effects of FIV_{ple} and *M. bovis* and their interaction on BCS, the following multivariable logistic regression model was used:

$$BCS = \mu + FIV + BTB + FIV \times BTB + e \quad [2]$$

where μ represents the intercept, e residual error and BCS, FIV and BTB are binary variables coded as defined earlier.

Blood parameters were selected that were deemed relevant in the literature for either or both of the infections: haematocrit (Ht), white blood cell count (WBC), total serum protein (TSP), albumin (ALB), globulin, gamma globulin and albumin:globulin (A:G) ratio [17,22,33,43]. To assess the effects of FIV_{ple} and *M. bovis* and their interaction, as well as other potentially

Table 3. Results of the two final logistic regression models with dependent variables FIV_{ple} and BTB. Odds ratios are given with their 95% confidence interval between brackets. NS = these variables were not statistically significant in the model (based on the AIC) and were not included in the final model.

	FIV_{ple}	**BTB**
Sex (M)	1.5 (1.0 to 2.2)	NS
Age (>36 months)	3.5 (2.4 to 5.1)	NS
BCS (1,2,3)	1.7 (1.1 to 2.6)	NS
Area		
LPZ/north	NS	0.1 (0.0 to 0.3)
MPZ/central	NS	1
HPZ/south	NS	2.2 (1.2 to 4.3)
Period		
Period 1 (1993-1998)	NS	1
Period 2 (1999-2002)	NS	0.8 (0.4 to 1.6)
Period 3 (2003-2008)	NS	3.4 (1.2 to 11.1)

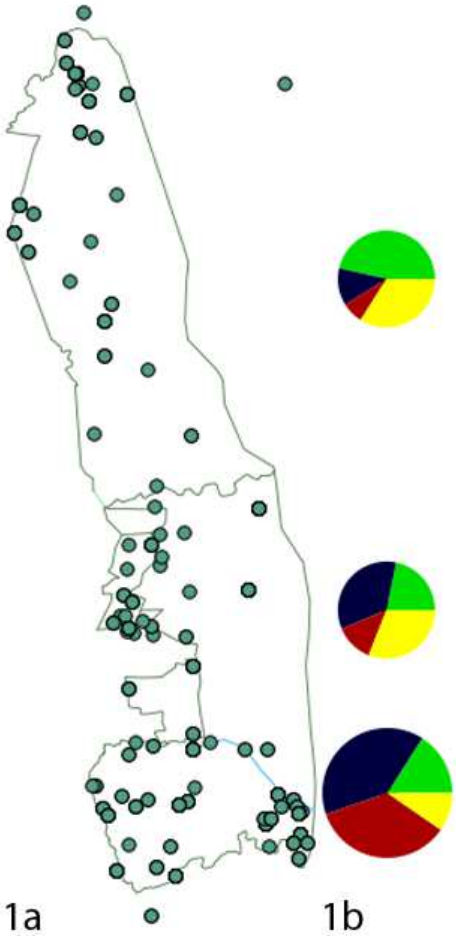

Figure 1a. Capture locations of lions, covering 93% of the lions from the data set, of which exact locations were known. Multiple lions may have been captured at one location. Locations outside KNP indicate escaped lions.
Figure 1b. Pie charts presenting the numbers of lions in the four co-infection groups (n=205) in the three different areas of the KNP. Size indicates the number of lions captured. $FIV_{ple}^{+}BTB^{+}$= blue, $FIV_{ple}^{-}BTB^{+}$= red, $FIV_{ple}^{+}BTB^{-}$= green and $FIV_{ple}^{-}BTB^{-}$= yellow.

influencing variables on these parameters, the following full general linear regression model was used:

$$\text{blood parameter} = \mu + FIV + BTB + FIVxBTB + age + BCS + sex + area + e \quad [3]$$

with variables as defined earlier.

The Akaike Information Criterion (AIC) was used to rank the models [44], following backward stepwise selection. Models with smaller values of the raw AIC values were preferred for each step, unless the difference between the two smallest models was less than 2. In those situations, the principle of Occam's razor was used to select the "simplest" model with the least parameters,

Table 4. Final multivariable models for seven haematologic and blood chemistry values. Beta coefficients are given with their 95% confidence interval between brackets. To determine the effect of interaction, all values of a blood parameter should be added, for example for globulin: 9.3+7.5-6.6= 10.2. NS = these variables were not statistically significant (based on the AIC) and were not included in the final model.

		Dependent variables						
Explanatory variables		**Ht (%)**	**WBC (*10^9/L)**	**TSP (g/L)**	**Alb (g/L)**	**Glob (g/L)**	**Gamma glob (g/L)**	**A:G ratio**
N		164	163	172	172	172	172	172
Observed mean values in population		34.3	18.6	84.0	28.3	55.7	24.9	0.5
FIV		-2.0 (-3.5 to -0.6)	NS	6.9 (3.1 to 10.6)	-2.4 (-3.5 to -1.2)	9.3 (5.7 to 12.9)	NS	-0.1 (-0.2 to -0.1)
BTB		NS	NS	7.8 (3.3 to 12.3)	NS	7.5 (3.3 to 11.8)	NS	-0.1 (-0.2 to 0.0)
FIVxBTB		NS	NS	-6.7 (-12.2 to -1.1)	NS	-6.6 (-11.9 to -1.3)	NS	0.1 (0.0 to 0.2)
Age		1.7 (0.2 to 3.2)	2.6 (0.7 to 4.5)	NS	NS	NS	3.5 (1.9 to 5.0)	NS
BCS		-6.9 (-8.5 to -5.3)	4.8 (2.8 to 6.9)	-4.8 (-8.1 to -1.5)	-6.7 (-8.1 to -5.4)	NS	1,9 (0.2 to 3.7)	-0.1 (-0.2 to -0.1)
Sex		1.8 (0.4 to 3.2)	NS	NS	NS	NS	NS	NS
TB area	HPZ/south	-3.3 (-4.9 to -1.8)	NS	-3.8 (-7.1 to -0.5)	-0.4 (-1.8 to 0.9)	-3.3 (-6.4 to -0.2)	NS	NS
	MPZ/central (reference)							
	LPZ/north	2.0 (0.0 to 3.9)	NS	4.1 (0.3 to 7.9)	2.3 (0.7 to 3.8)	1.4 (-2.2 to 5.0)	NS	NS
Intercept		36.1	15.6	80.0	30.8	49.8	22.2	0.7

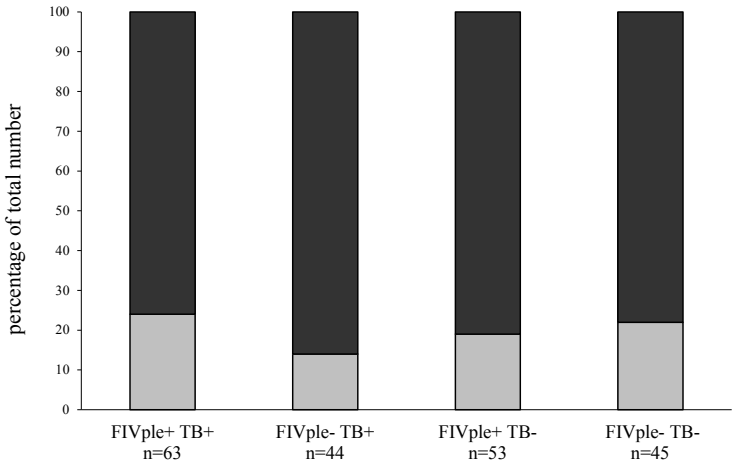

Figure 2. Lions (n=205) grouped according to their FIV_{ple} and BTB status and their BCS. Light grey = lions with a low BCS (BCS 1,2,3). Dark grey = lions with a high BCS (BCS 4,5).

i.e. with the highest information gain [45,46], following standard statistical methods [42]. All models were checked for normality and homoscedasticity. For data analysis R v. 2.15.0 was used (including packages Hmisc and modeest)[47].

RESULTS

The models assessing the dependency of FIV_{ple} and BTB on the variables area, period, age, sex and BCS, showed that FIV_{ple} positivity was significantly related to sex (males were more likely to be FIV_{ple} positive), higher age and lower BCS. *Mycobacterium bovis* infection was related with to the area (lions were more likely to be infected in the south of the park) and the period (lions were more likely to be infected in the last time period; Table 3). Additional information can be found in the supplementary material (Tables and Figures S7 - S10).

The 205 lions with test results for both FIV_{ple} and BTB were divided into four groups: $FIV_{ple}^{+}BTB^{+}$, $FIV_{ple}^{-}BTB^{+}$, $FIV_{ple}^{+}BTB^{-}$ and $FIV_{ple}^{-}BTB^{-}$. No significant differences were found between the observed group sizes and the expected group sizes based on the FIV_{ple} and BTB prevalences (see the supplementary material, Table S7a); neither for the total KNP, nor for the three areas separately (Figure 1b, for details, see the supplementary material, Table S6). This may indicate that there is no significant relationship between the pathogens, but may also result from a balance between an increased incidence and an increased mortality caused by co-infection of FIV_{ple} and BTB, and should thus be interpreted with caution. When assessing the effects of FIV_{ple} and BTB on the BCS, the co-infected lions had a slightly higher percentage of lions with a low BCS (Figure 2) compared with the other groups, but this was not statistically significant (the logistic regression model confirmed this finding; equation 2).

Linear regression models to determine associations with FIV_{ple} and/or *M. bovis* infection showed that in co-infected animals there is a statistically significant antagonistic interaction between FIV_{ple} and *M. bovis* for three of the seven blood parameters, resulting in less deviation than expected from the sum of the individual effects (Table 4). Effects of FIV_{ple} were more pronounced, except for the hyperproteinaemia. Neither FIV_{ple} nor BTB, nor their interaction had a significant influence on the white blood cell count or the gamma globulins. Comparisons with

reference values from zoo lions can be found in the supplementary material, Table S11.

DISCUSSION

Infectious diseases are an important issue in conservation, having the power to dramatically influence the dynamics of wildlife species and populations [27,48,49], especially in dwindling populations [50,51]. Because it is difficult to determine their effect, it is common to deal with each infectious disease as a separate entity caused by a single pathogen [31]. However, in nature, multiple pathogens are often encountered simultaneously by individual hosts, which can lead to additive, antagonistic, or synergistic effects on hosts and pathogens [52]. It was expected that co-infection with FIV_{ple} and *M. bovis* in lions would have a synergistic effect, similar to the human counterparts [25]. However, although there was an indication that co-infected lions more often had a low BCS, this was not statistically significant (Figure 2). FIV_{ple} alone was significantly correlated with a lower BCS. However, though *M. bovis*-infected lions were noted to often have a 'scruffy and unthrifty look' (D.F. Keet 2008, personal observations), BTB was not significantly correlated with BCS. This was surprising as pathologic lesions have been described in *M. bovis*-infected lions with a poor condition [19,20]. In a study of BTB in buffaloes, it was found that 70% of the infected animals examined post mortem had only a mild infection and were unlikely to have shown symptoms while alive [53]. Our dataset contained several lions with necropsy results, but unfortunately their number was too small to allow statistically significant conclusions. Therefore, whether there are patterns in the clinical signs in lions, like in buffaloes, remains to be seen.

Effects of both FIV_{ple} and *M. bovis*-infection on the various blood parameters may indicate chronic disease, such as anaemia [43,54], but could also be exaggerated by the effect of age, for example, the hyperglobulinaemia, as older lions are more likely to be FIV_{ple} or BTB positive, and in general show an increase in globulins [54]. The values might be slightly biased by the delay between sampling and analysis and possible temperature differences during this delay, although the majority of the blood samples was collected at night in the winter season. For all blood parameter values, the interaction of the infections was shown to be less than additive. Explanations for this may be that the immune response already reaches maximum capacity for one infection, or that the body is able to keep the various parameters between homeostatic limits. Although not specific for either infection, the direction of changes in blood parameter values was comparable to that observed in previous studies on FIV in cats [13-17] and FIV_{ple} in lions [8], and to results from the less abundant literature on (B)TB [21,22]. Anaemia and hypoalbuminaemia are associated with progression to AIDS and death in human pre-AIDS patients [55,56]. In contrast to the decrease of $CD4^+$ and $CD8^+$ T-cell counts observed in HIV-infected humans, previously also reported for FIV_{ple} in lions [4], no decrease of white blood cells was seen in FIV_{ple}-positive lions in the present study. White blood cells were not further typed, which would be needed to determine possible changes in the numbers of the different cell types.

Mycobacterium bovis prevalence was significantly different between the three areas in the KNP, coinciding with observations on *M. bovis* prevalence in the buffaloes [57], one of the four preferential prey species of lions in the KNP [58]. Surprisingly, this did not result in an age distribution skewed to the younger ages in the HPZ compared to the LPZ (see the supplementary material, Table and Figure S5). Also, the prevalence of *M. bovis*-infected animals in the northern part of the KNP increased significantly with time from 0% to 41% (Fisher's exact test, two tailed, $p = 0.014$; supplementary material, Table S7c). These findings suggest that *M. bovis* infection in lions is caused by an external source of infection, i.e. that lions are spillover hosts, in agreement

with [59,60]. This contradicts suggestions from the past about their role as a maintenance host [19,61]. FIV_{ple}, on the other hand, shows no significant relationship with the external factors measured, supporting the intraspecies transmission route.

It was expected that, like HIV and *M. tuberculosis* in humans, a synergy between FIV_{ple} and *M. bovis* was to be found in lions, but this large dataset shows no proof for detrimental synergy in the tested parameters. There may be various reasons for not finding a similar relationship of FIV_{ple} and *M. bovis* like in humans.

- The immunopathological characteristics of BTB can vary in different species [62] and macroscopic lesions in *M. bovis* positive lions have been found to be very different from those in ungulates and non-human primates [20]. This may mirror a difference in susceptibility to infection, but the knowledge on immunopathogenesis is still very limited in lions. Differences in immune response in feline and simian species have also been noted for immunodeficiency viruses, related with specific virus-host co-adaptation and viral load [9,12,63]. One reason for a difference in immunopathogenesis may be that lions have co-evolved with the endemic disease FIV_{ple}, whereas *M. bovis* is a recently introduced pathogen, in contrast to the situation in humans, where *M. tuberculosis* has been in the population for many centuries, and HIV/AIDS was introduced relatively recently [63].
- It remains possible that even our extensive dataset was not suitable to detect a pathogen-pathogen interaction. In literature on the HIV-TB interaction, emphasis has been laid on the changes in $CD4^+/CD8^+$ T-cell counts, pathology and the collection of longitudinal data [25,64,65]. The present dataset was not collected for the purpose of assessing FIV_{ple}- *M. bovis* interaction and therefore lacks results on these important parameters. This precludes determining the directionality of any interactions, while order of infection can be crucial in the outcome of the pathogen-pathogen interaction [66]. Also, stage of infection and time of infection for both BTB and FIV_{ple} were not known, but may affect, for example, blood parameter values such as WBC, and this could thus be a potential source of error. Selection pressures that were not determined in this study, for example, prey availability, could also be confounding the pathogen-pathogen interaction [34].
- Although the use of call-up stations is accepted for the non-lethal capture of wildlife [67], our sampling method is likely not to have been truly random. For example, relatively few young animals have been captured (supplementary material, Table S3). These are likely to be more cautious approaching a call-up station. This may also count for animals in a bad condition, since fewer animals than expected were captured at call-up stations in poor conditions Lions that were brought to the veterinary station were collected with different effort over the three areas. They had a lower mean BCS and higher mean age compared with lions captured at call-up stations, but the prevalence of FIV_{ple} and *M. bovis* infection for the lions (respectively, 63% and 47%) brought to the veterinary station were not statistically different (χ^2-tested, *p*-value, respectively, 0.40 and 0.82) from prevalence found for the lions sampled at call-up stations (respectively, 61% and 55%), and either including or excluding the emaciated lions had little influence on the various statistics (results not shown); therefore, they were included to increase the power of the analyses. Although we tried to control for bias as much as possible, field datasets like these may include unmeasured biases, and results should be interpreted with caution.

Finally, we remark that the pathogen-pathogen interaction may become more important when the lions are under additional stress, for example, owing to high parasite load or bad nutritional status when there is a low prey density [30,49,66,68]. This complexity of disease in general and especially the interaction of pathogens, necessitates an extensive, long-term research programme

requiring large sample sizes from the host population [31]. In future studies, besides $CD4^+/CD8^+$ T-cell counts, macro parasite infestation [69,70], and social interaction networks to assess the infectiousness of individual animals [71] could be valuable inclusions.

With the tested parameters, no evidence was found that FIV_{ple} or *M. bovis*, or a co-infection of these, is currently causing a serious conservation threat to KNP lions. However, a significant spatio-temporal increase of BTB was found, which may impact on lion health, as previous studies have related BTB to diverse pathological lesions [72]. In buffaloes, the population growth rate was negatively affected by BTB without altering the population age distribution significantly, thus reducing the resilience of the population to disturbances [53], which may also apply to lions. With the recent creation of the Greater Limpopo Transfrontier Conservation Area and the knowledge that the co-infection of FIV_{ple} and *M. bovis* is probably also present in lions in other African parks [7,73], it should be closely monitored how the KNP and other co-infected lion populations respond to a severe environmental perturbation compared with populations that are infected with only one or neither of these two agents.

This project was approved by the South African National Parks conservation and ethics committees as well as the Animal Use & Care Committee of the University of Pretoria.

ACKNOWLEDGEMENTS

This research was predominantly funded by the South African Veterinary Foundation and the Directorate of Veterinary Services, National Department of Agriculture, Forestry and Fisheries, South Africa. D.F. Keet was the recipient of a doctoral bursary awarded by the University of Pretoria. The work of M. Maas was supported by The Netherlands Organisation for Scientific Research (NWO/ZonMw grant 918.56.620). We are grateful to the field staff of the Directorate of Veterinary Services, Messrs. S. van Dyk, E.H. Dekker, T.K. Muchocho and J. Oosthuizen and Dr. R. Bengis whom assisted with technical execution of the project, as well as the physical assistance of Tradesman Aides: Messrs C. Masinga, E. Ubisi, K. Nkuna, E. Munyamela and P. Baloyi in the management of the lion holding facilities, and assistance with necropsies; the ranger staff of SANParks (KNP) for reporting and assisting with the capture of some of the study lions; the Bacteriology Section of the ARC-Onderstepoort Veterinary Research Institute for mycobacterial culture. Dr. W. Botha and Dr. L. du Plessis from Golden Vetpath for histological examinations of referred cases. We also acknowledge Prof. N.P.J. Kriek, Mrs. A. Lourens, H. Vernooij and J. van den Broek for valuable advice, as well as the reviewers for their thorough reviews and useful comments on the manuscript.

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SUPPLEMENT 1: DESCRIPTION OF THE WHOLE DATA SET

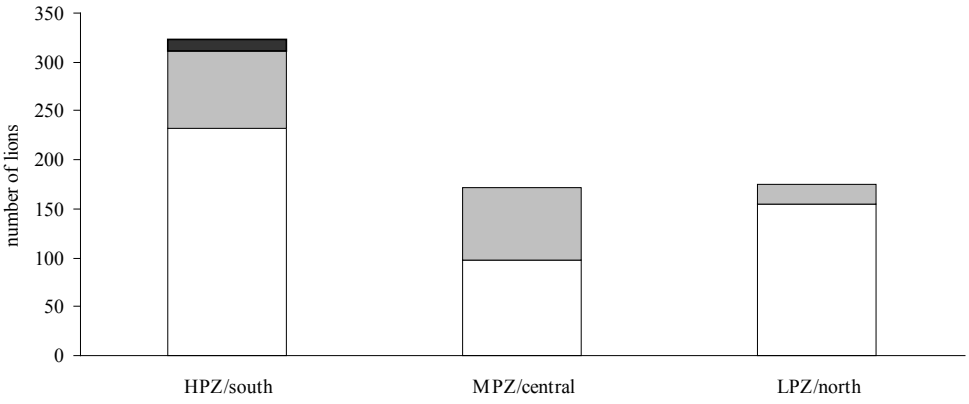
To give insight in the relative proportions of the lions that were either captured at call-up stations or had been brought to the veterinary station, the data set was described with different subdivisions in the tables and graphs, regardless of FIV_{ple} and BTB status. Results are listed in Tables S1 to S4 and Figures S1 to S4. Table S5 and Figure S5 describe the age distribution in the different areas.

Supplementary table and figure S1: area

(a) Numbers of study lions in the three parts of the Kruger National Park (KNP) and adjacent game reserves. HPZ= high prevalence zone. MPZ= medium prevalence zone. LPZ= low prevalence zone.

	Area (n=669)			
	HPZ/south	**MPZ/central**	**LPZ/north**	**Total**
Call-up station	233	97	154	484
Veterinary station	79	74	21	174
Unknown	11	0	0	11
Total	323	171	175	669

(a)



(b)

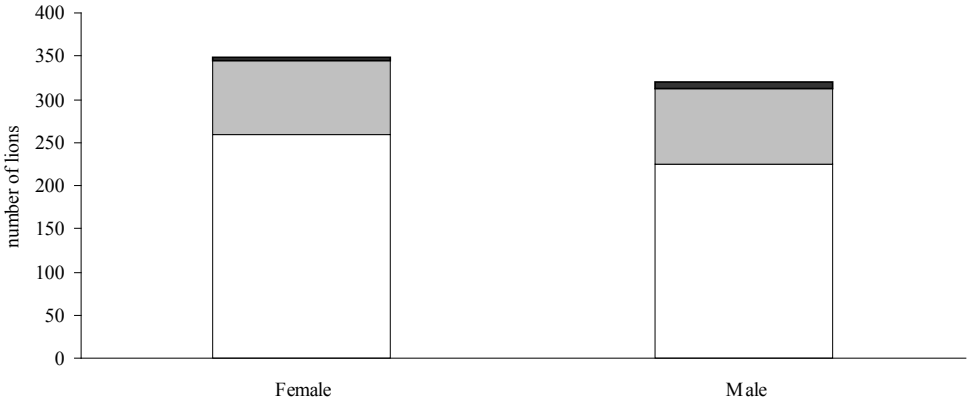
(b) Numbers of study lions in the three parts of the KNP and from a private game reserve (n=669). White bars= lions captured at a call-up station. Grey bars= lions brought to the veterinary station. Black bars= Unknown background.

Supplementary table and figure S2: sex

(a) Description of the sex of the lions in the data set.

	Sex (n=669)	
	Female	**Male**
Call-up station	260	224
Veterinary station	85	89
Unknown	4	7
Total	349	320

(a)



(b)

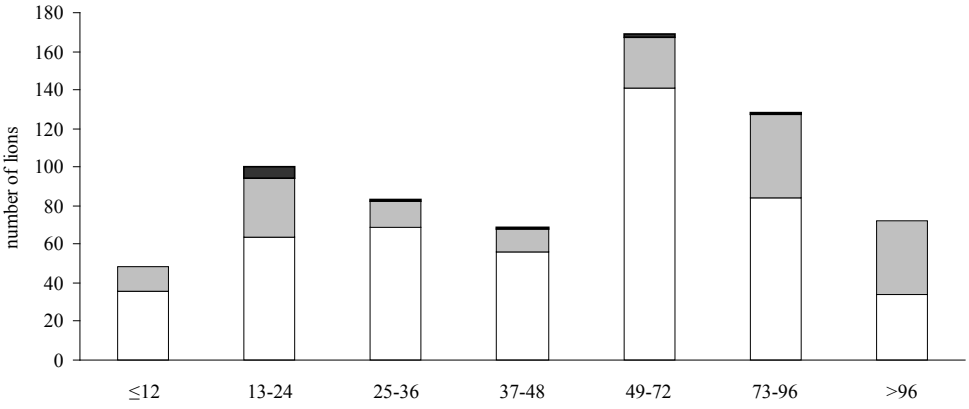
(b) Relative proportion of males and females in the data set (n=669). White bars= lions captured at a call-up station. Grey bars= lions brought to the veterinary station. Black bars= Unknown background.

Supplementary table and figure S3: age

(a) Numbers of lions per age class in the data set, with the age in months. A one-month interval was chosen for ages <36 months, a two-month interval was chosen for the higher ages, as age becomes increasingly difficult to estimate.

	Age classes (n=669)						
	≤12	**13-24**	**25-36**	**37-48**	**49-72**	**73-96**	**>96**
Call-up station	36	64	69	56	141	84	34
Veterinary station	12	30	13	12	26	43	38
Unknown	0	6	1	1	2	1	0
Total	48	100	83	69	169	128	72

(a)



(b)

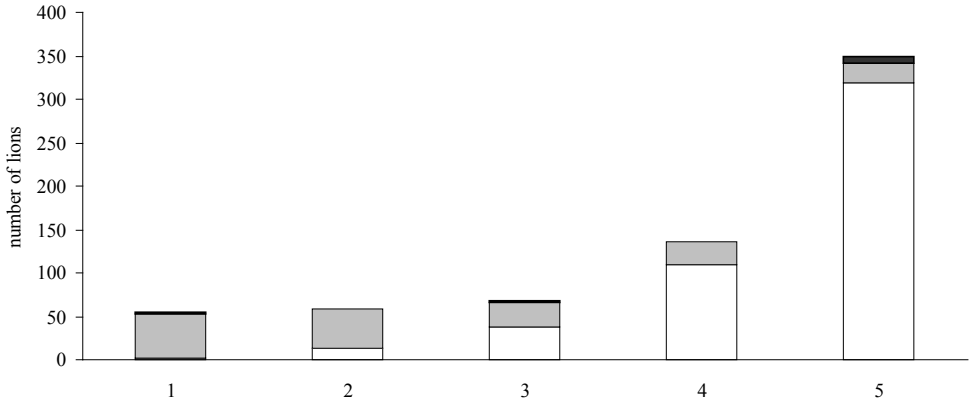
(b) Number of lions per age class, age in months (n=669). White bars= lions captured at a call-up station. Grey bars= lions brought to the veterinary station. Black bars= Unknown background.

Supplementary table and figure S4: BCS

(a) Description of the numbers of lions per body condition score (BCS) in the data set. BCS ranked from 1 (very poor) to 5 (excellent).

	Body condition score (n=666)				
	1	**2**	**3**	**4**	**5**
Call-up station	2	13	37	110	319
Veterinary station	51	46	29	25	23
Unknown	2	0	1	1	7
Total	55	59	67	136	349

(a)



(b)

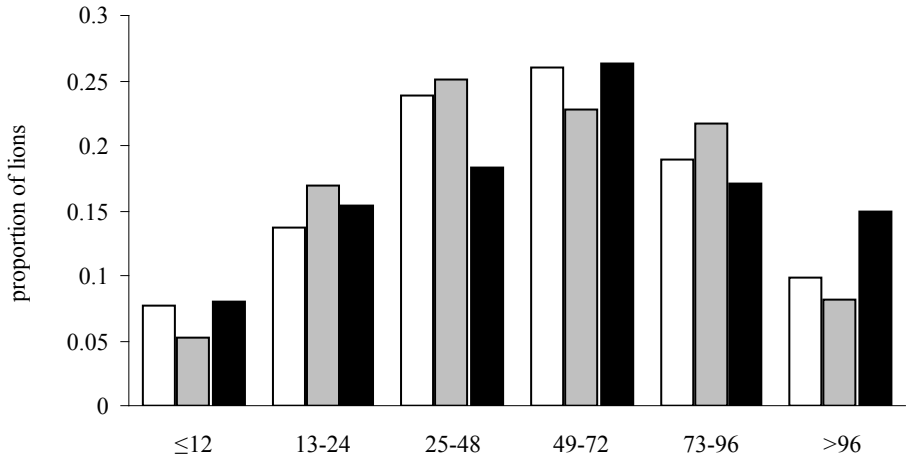
(b) Number of study lions per body condition score. BCS ranked from 1 (very poor) to 5 (excellent) (n=666). White bars= lions captured at a call-up station. Grey bars= lions brought to the veterinary station. Black bars= Unknown background.

Supplementary table and figure S5: Population age distribution per KNP area

(a) Number of study lions in the three different areas in the Kruger National Park divided in six age classes and the mean and median values per area.

	Area (n=669)		
	HPZ/south	**MPZ/central**	**LPZ/north**
≤12	25	9	14
13-24	44	29	27
25-48	77	43	32
49-72	84	39	46
73-96	61	37	30
>96	32	14	26
n total	323	171	175
Mean	58.2	58.6	63.5
Median	60	56	66

(a)



(b)

(b) Proportions of study lions per age class per area. White bars= HPZ/south, grey bars= MPZ/central, black bars= LPZ/north. Visual inspection shows similar population age distributions for the three areas.

SUPPLEMENT 2: DESCRIPTION OF THE DATA SET FOR LIONS WITH AN FIV_{PLE} OR BTB RESULT

All study lions with a test result for FIV_{ple} and/or BTB, were used for descriptive analysis of FIV_{ple} and BTB in the Kruger National Park regarding area, period, sex, age and BCS. Temporal changes in the areas were assessed for three periods, 1993 to 1998, 1999 to 2002 and 2003 to 2008 (resp. 152, 337, 157 lions; 25 lions had an unknown capture date), based on attaining comparable numbers of observations.

Supplementary table S6: number of lions with FIV_{ple}, BTB, with both infections or no infections

Number of lions with FIVple, BTB, with both infections or no infections per area.

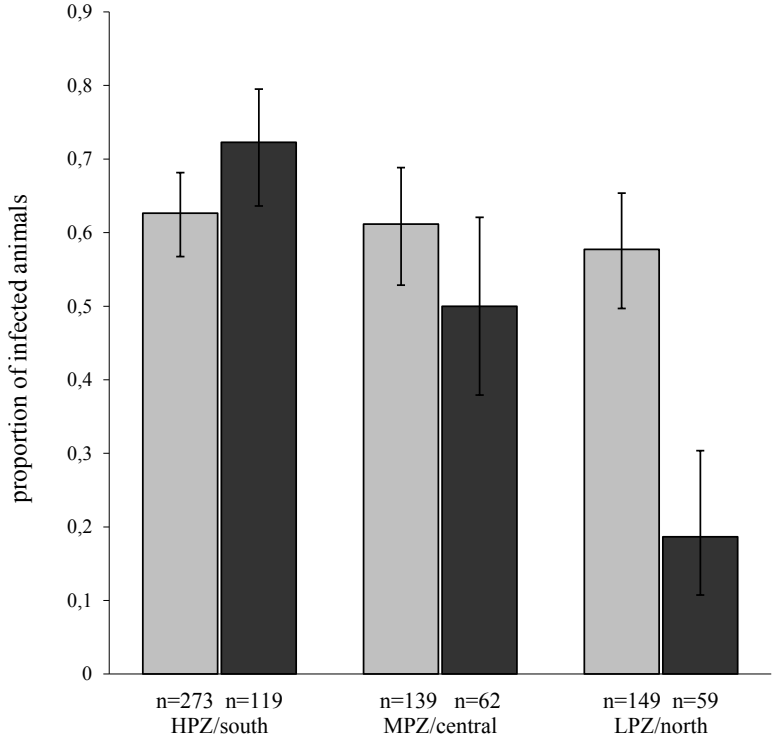
	BTB pos		BTB neg		Total
FIV_{ple} pos					116 (57%)
HPZ/south	63	37	53	15	
MPZ/central		19		12	
LPZ/north		7		26	
FIV_{ple} neg					89 (43%)
HPZ/south	44	33	45	9	
MPZ/central		7		17	
LPZ/north		4		19	
Total	107 (52%)		98 (48%)		205

Supplementary tables and figure S7: area

(a) The proportions of FIV_{ple} and BTB positive study lions over the total study period in the different parts of the KNP and their 95% confidence intervals (95% CI). The prevalence of FIV_{ple} in the three areas in the KNP found in this study compares well to smaller studies that were executed in the past [1,2].

	FIV_{ple} (n=561)			**BTB (n=240)**		
	n	**FIV_{ple} pos (proportion)**	**95% CI**	**n**	**BTB pos (proportion)**	**95% CI**
HPZ/south	273	0.63	0.57–0.68	119	0.72	0.64–0.80
MPZ/central	139	0.61	0.53–0.69	62	0.50	0.38–0.62
LPZ/north	149	0.58	0.50–0.65	59	0.19	0.11–0.30

(a)



(b)

(b) Proportions of FIV_{ple} and BTB infected study lions in the three different KNP areas over the total study period. Grey bars= FIV_{ple} positive lions. Black bars= BTB positive lions. Line segments indicate the 95% confidence interval.

(c) Proportions of FIV_{ple} and BTB positive study lions in the HPZ and the LPZ in three time periods. The central area was not assessed, because for the last time period, data of only three lions could be collected.

	HPZ/south		LPZ/north	
	FIV_{ple}	**BTB**	**FIV_{ple}**	**BTB**
Period 1993-1998	0.63 (n=64)	0.77 (n=31)	0.48 (n=21)	0 (n=11)
Period 1999-2002	0.66 (n=108)	0.66 (n=69)	0.64 (n=76)	0.13 (n=31)
Period 2003-2008	0.62 (n=81)	0.84 (n=19)	0.52 (n=52)	0.41 (n=17)

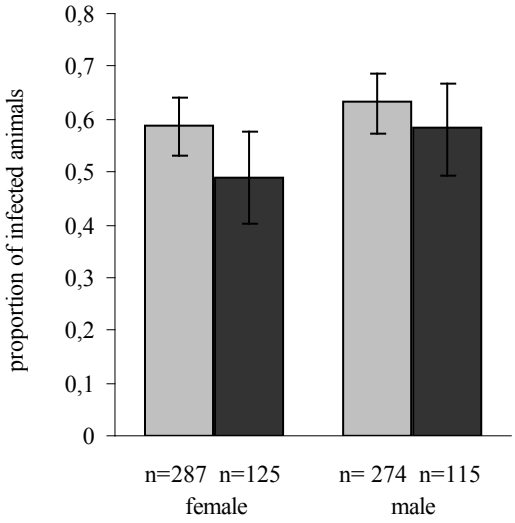
(c)

Supplementary table and figure S8: sex

(a) Proportions of FIV_{ple} and BTB positive study lions for the different sexes. No significant differences were found between males and females for either of the infections using the chi-square test.

	FIV_{ple} (n=561)			BTB (n=240)		
	n	**FIV_{ple} pos (proportion)**	**95% CI**	**n**	**BTB pos (proportion)**	**95% CI**
Female	287	0.59	0.53–0.64	125	0.49	0.40–0.57
Male	274	0.63	0.57–0.68	115	0.58	0.49–0.67

(a)



(b)

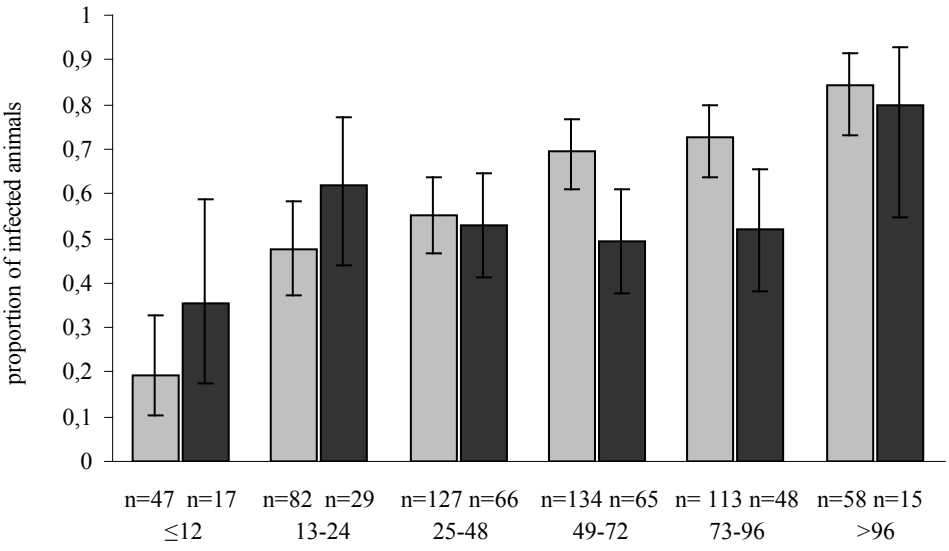
(b) Proportions of FIV_{ple} and BTB infected study lions per sex. Grey bars= FIV_{ple} positive lions, black bars= BTB positive lions. Line segments indicate the 95% confidence interval.

Supplementary table and figure S9: age

(a) Proportions of FIV_{ple} and BTB study positive lions in the different age classes. The youngest lions that were FIV_{ple} positive were 5 months old (4 cases). The two youngest BTB cases were 5 and 7 months old.

	FIV_{ple} (n= 561)			BTB (n=240)		
	n	**FIV_{ple} pos (proportion)**	**95% CI**	**n**	**BTB pos (proportion)**	**95% CI**
≤12	47	0.19	0.10–0.33	17	0.35	0.17–0.59
13-24	82	0.48	0.37–0.58	29	0.62	0.44–0.77
25-48	127	0.55	0.46–0.63	66	0.53	0.41–0.65
49-72	134	0.69	0.61–0.77	65	0.49	0.37–0.61
73-96	113	0.73	0.64–0.80	48	0.52	0.38–0.66
>96	58	0.84	0.73–0.92	15	0.80	0.55–0.93

(a)



(b)

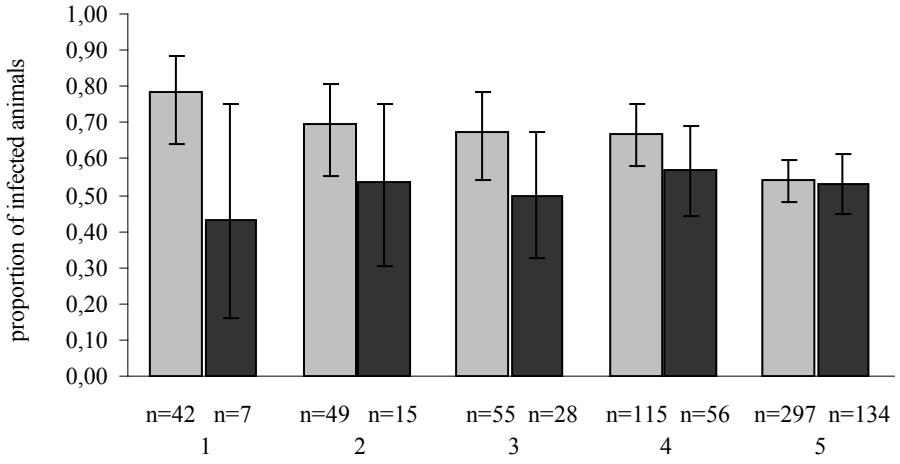
(b) Proportions of FIV_{ple} and BTB infected study lions per age class. A general increase with age of the prevalences of FIV_{ple} and *M. bovis* was found. Grey bars= FIV_{ple} positive lions, black bars= BTB positive lions. Line segments indicate the 95% confidence interval. Little information exists about the course of infection of FIV_{ple} and *M. bovis* in a lion, and the common belief is that both infections are life long in lions. Thus, the prevalence of both infections shows an increase with age.

Supplementary table and figure S10: BCS

(a) Proportions of FIV_{ple} and BTB positive study lions in the different BCS classes.

	FIV_{ple} (n=558)			BTB (n=240)		
	n	**FIV_{ple} pos (proportion)**	**95% CI**	**n**	**BTB pos (proportion)**	**95% CI**
BCS 1	42	0.79	0.64–0.88	7	0.43	0.16–0.75
BCS 2	49	0.69	0.55–0.80	15	0.53	0.30–0.75
BCS 3	55	0.67	0.54–0.78	28	0.50	0.33–0.67
BCS 4	115	0.67	0.58–0.75	56	0.57	0.44–0.69
BCS 5	297	0.54	0.48–0.59	134	0.53	0.45–0.61

(a)



(b)

(b) Proportion of FIV_{ple} and BTB infected study lions per BCS class. Grey bars= FIV_{ple} positive lions. Black bars= BTB positive lions. Line segments indicate the 95% confidence interval.

Supplementary table S11

Comparison of the calculated lion blood parameter values from the data set with the values of the International Species Information System (ISIS) for lions [3]. These values have been collected using zoo lions worldwide and the means and standard deviations have been given. A difference has been made between lions younger than 3 years old, and lions older than 3 years. Sample size is the number of samples, which comes from the same or from a smaller number of individuals lions (indicated as "animals"). The proportion of lions used for the calculations of the mean values for lions infected with FIV_{ple}, BTB or both, was comparable with overall infection rates. NA=Not available.

	Dependent variables						
Explanatory variables	**Ht (%)**	**WBC (*10^9/L)**	**TSP (g/L)**	**Alb (g/L)**	**Glob (g/L)**	**Gamma glob (g/L)**	**A:G ratio**
Data set							
Mean total	34.3	18.6	84.0	28.3	55.7	24.9	0.5
Animals (n)	164	163	172	172	172	172	172
Mean FIV^+	33.0	19.6	85.5	25.7	59.7	25.9	0.4
Mean BTB^+	33.7	19.4	84.6	27.7	56.9	26.0	0.5
Mean FIV^+ and BTB^+	32.9	19.1	84.7	26.5	58.2	26.0	0.5
ISIS values							
Age <3							
Mean	35.9	12.4	67	34	33	NA	NA
St. Deviation	5.2	4.8	7	5	7	NA	NA
Sample size (n)	135	131	105	101	98	NA	NA
Animals (n)	90	91	71	69	67	NA	NA
Age >3							
Mean	39.4	13.6	75	33	42	28	NA
St. Deviation	5	4.2	6	4	7	10	NA
Sample size (n)	538	514	481	424	417	6	NA
Animals (n)	248	241	215	193	186	6	NA

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Chapter 7

Hematologic and serum chemistry reference intervals for free-ranging lions (*Panthera leo*)

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Accepted for publication:
Research in Veterinary Science

ABSTRACT

Hematologic and serum chemistry values are used by veterinarians and wildlife researchers to assess health status and to identify abnormally high or low levels of a particular blood parameter in a target species. For free-ranging lions (*Panthera leo*), information about these values is scarce. In this study, 7 hematologic and 11 serum biochemistry values were evaluated from 485 lions from the Kruger National Park, South Africa. Significant differences between sexes and sub-adult (≤36 months) and adult (>36 months) lions were found for most of the blood parameters and separate reference intervals were made for those values. The obtained reference intervals include the means of the various blood parameters values measured in captive lions, except for alkaline phosphatase in the subadult group. These reference intervals can be utilized for free-ranging lions, and may likely also be used as reference intervals for captive lions.

Blood parameter values can be used by veterinarians or researchers for the assessment of the health of animals or a population. These blood parameter values may vary between free-ranging and captive species, which was shown for e.g. African buffalo [1]. Information on hematologic and blood chemistry values is available for captive lions (*Panthera leo*) [2,3], but is scarce for free-ranging lions. Therefore, the aim of this study was to establish reference values for free-ranging lions for commonly used hematologic and blood chemistry values, adjusted for sex and age, and to compare these with values found for captive lions.

Lion blood samples were collected from 485 free-ranging lions from 1993-2008 in the Kruger National Park, South Africa. The complete lion data set is described elsewhere [4]. The selection of the lions used for the current study was based on the body condition, only including clinically healthy lions with a good body condition score (scored 4/5 and 5/5, where 5 represents an excellent body condition).

Lions were immobilized with a combination of tiletamine and zolazepam (Zoletil® 100, Virbac) and venous blood samples were obtained from the medial saphenous vein as soon as possible, but usually within 30-60 minutes after anaesthesia, in EDTA and serum Vacutainer® tubes. Blood tubes were kept at ambient temperature and were processed within preferably 8, but maximum 24 hours. Lions were aged by examining dental attrition according to Smuts et al. [5]. Hematology analysis of EDTA samples was performed in the the Kruger National Park within 12 hours with a Coulter AcT diff analyzer (Beckman Coulter). Samples for serum chemistry were frozen at -20°C until analysis. This serum chemistry analysis was conducted with a NExCT/VetEX (Bayer Health) at The Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria.

The exact numbers of lions that were used for each individual blood parameter are listed in Table 1 and Table 2. Seven hematological parameters were measured (hemoglobin (Hgb), haematocrit (Hct), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC)) and 11 serum chemistry parameters (cholesterol, total protein, albumin, globulin, albumin:globulin ratio (AG ratio), alpha globulins, beta globulins, gamma globulins, blood urea nitrogen (BUN), alkaline phosphatase).

General linear models with the blood parameter as dependent variable were used to determine the effect of explanatory values sex (binary; M or F) and age (binary; ≤36 months or >36 months). The residuals of these models were checked visually in Q-Q plots to assess normality of the blood parameter values. When residuals were not normally distributed, the variable was log-transformed and model residuals were checked again in a Q-Q plot.

When one or both of the explanatory values had a significant influence ($p<0.05$), the mean, standard deviation (SD) and reference interval (mean±1.96 SD) were calculated separately for the different groups. If not, all lions were grouped together and one mean, SD and reference interval were given. For normally distributed data the mean, SD and the reference interval are reported. For log-transformed data, the mean and the reference interval are reported in the original scale. For the statistical analyses R version 2.15 was used [6]. Values were compared with the values for captive lions (mean, SD, minimal and maximal value) of the International Species Information System (ISIS), which maintains an electronic database of animals held in zoological institutions and to which member institutions provide health and genetic data [3].

Age of the lions in the database ranged from 3 to 144 months old (≤ 36 months: 172 lions, > 36 months: 313 lions). The following parameters were normally distributed only after log-transformation: albumin, AG ratio and beta globulins. Estimated reference intervals for all

Table 1. Reference interval for seven hematologic parameters of free-ranging lions. For the presentation of the ISIS values the same groups were evaluated as for the free-ranging lions from the study.

		Free-ranging lions				ISIS/ captive lions				
Parameter	**Group**	**n**	**Mean**	**SD**	**Reference interval**	**n**	**Mean**	**SD**	**Minimal value**	**Maximal value**
Hgb (g/dL)	All	324	11.8	1.47	8.9 - 14.6	766	13	2.0	4.9	23
Hct (%)	All	324	35.5	4.43	26.8 - 44.1	890	39.1	5.4	24.8	54.0
RBC (x10^{6}cells/µl)	M, ≤36	44	6.7	0.81	5.1 – 8.3	68	7.47	1.32	3.8	11.7
	F, ≤36	41	6.7	0.89	5.0 – 8.4	93	7.06	1.11	3.8	9.98
	M, >36	69	7.2	0.78	5.7 – 8.7	222	8.28	1.17	5.1	14.0
	F, >36	104	6.8	0.83	5.2 – 8.5	349	7.96	1.07	5.22	11.0
MCV (fL)	M	113	51.3	2.4	46.6 – 55.9	285	49.9	5.1	29.9	76.0
	F	145	52.3	2.2	48.0 – 56.7	441	50.0	4.1	21.4	64.0
MCH (pg/cell)	M	113	16.7	0.94	14.8 – 18.5	266	16.5	1.5	11.2	27.2
	F	145	17.3	0.94	15.5 – 19.1	432	16.6	1.4	7.2	22.0
MCHC (g/dL)	M	113	32.6	1.51	29.6 – 35.5	280	33.0	3.0	20.4	42.8
	F	145	33.1	1.35	30.4 – 35.7	469	33.2	2.9	20.5	49.7
WBC (x10^{3}cells/µl)	≤36	87	16.4	4.69	7.2 – 25.6	176	12.15	4.64	4.73	31.2
	>36	183	19.0	4.73	9.7 – 28.2	683	13.38	4.08	5.5	28.2

Table 2. Reference intervals for eleven serum chemistry parameters of free-ranging lions. For the presentation of the ISIS values the same groups were evaluated as for the free-ranging lions from the study. NA= not applicable. Since these values were log-transformed, the SD is not given, as this value cannot be log-transformed. - = not available.

		Free-ranging lions				ISIS/ captive lions				
analyte	**Group**	**n**	**Mean**	**SD**	**Reference interval**	**n**	**Mean**	**SD**	**Minimal value**	**Maximal value**
Cholesterol (mmol/L)	M, ≤36	69	3.4	0.82	1.8 – 5.0	56	4.6	1.14	2.33	6.63
	F, ≤36	49	3.4	1.30	0.9 – 6.0	88	4.9	1.17	2.36	7.64
	M, >36	104	3.0	0.77	1.5 – 4.6	201	3.9	0.91	1.50	6.48
	F, >36	138	3.4	0.86	1.7 – 5.1	343	4.6	1.17	1.43	8.78
Total protein (g/L)	All	360	85.4	9.58	66.7 – 104.2	750	74	7	53	97
Albumin (g/L)	All	360	28.0	NA	20.0 - 39.0	692	33	5	19	56
Globulin (g/L)	≤36	118	55.2	10.85	33.9 – 76.5	146	34	8	19	57
	>36	242	58.0	8.73	40.9 – 75.1	530	43	6	26	63
A:G ratio	≤36	118	0.52	NA	0.31 - 0.89	-	-	-	-	-
	>36	242	0.48	NA	0.29 – 0.81	-	-	-	-	-
Alpha glob (mg/L)	All	360	15.5	2.57	10.5 – 20.5	4	13 [1]	-	-	-
Beta glob (mg/L)	All	360	10.2	NA	5.5 - 18.8	5	8	2	5	10
Gamma glob (g/L)	≤36	118	22.6	5.61	11.6 – 33.6	-	-	-	-	-
	>36	242	25.5	5.57	14.6 – 36.4	7	29	10	19	41
BUN (mmol/L)	All	360	17.9	7.30	3.6 - 32.2	811	11.4	3.21	4.28	29.27
Creatinine (µmol/L)	≤36	118	145.2	44.29	58.4 – 232.0	165	159	62	53.0	327
	>36	242	182	52.71	78.7 – 285.3	620	239	53	0	424
Alkaline phosphatase (IU/L)	≤36	118	36.2	22.88	0 – 81.0	163	105	61	16	355
	>36	242	12.7	8.84	0 – 30.0	595	19	14	0	89

[1] Four lions had values for both alpha 1 and alpha 2 in the ISIS database. These were expected to be the same lions, and the total alpha value is thus given; SD for these lions cannot be given for this.

hematological and serum chemistry parameters are shown in respectively Table 1 and Table 2.
No explicit differences were found comparing the found results with the values of captive lions, and except for alkaline phosphatase (subadult group), the reference intervals all included the mean values found for captive lions. Text books about exotic animal medicine are often based on these values of captive animals [7]. However, no information about health status is available for the ISIS values and interpretation is complicated by the fact that they may include multiple samples per animals. Furthermore, normality of these data is not reported, thus it is unclear if the mean and standard deviation can be used to establish reference intervals.
The lions used for this study were all in a clinically healthy state, residuals of the models were normally distributed (for albumin, AG ratio and beta globulins after log-transformation) and thus reference intervals could be calculated. It could therefore be argued that these reference intervals are a better representation of blood parameter values for lions than those collected from the captive lions. However, also these reference intervals have their limitations:
Part of these lions have been used in another study that showed that diseases (viz. feline immunodeficiency virus and bovine tuberculosis (BTB)) may account for significant differences in blood value parameters [4]. For the lions in this study, underlying diseases were not accounted for, for several reasons. First, though information about the BTB disease status was known, as well as the status for several viral infections, nothing was known about e.g. parasitic infections. We therefore thought it was incorrect to classify animals negative for the tested infections as "healthy". Also, making general linear models with infectious diseases as explanatory variables would reduce the number of lions with complete datasets available drastically, thus decreasing the reliability of the reference intervals. Furthermore, as often the infection status for all these infectious diseases is unknown for individual lions, it is debatable what advantage this would have for veterinarians or researchers. Therefore, we only included lions in a clinically healthy state and disregarded by definition incomplete disease status information.
Blood samples were collected under field circumstances, and no standardized protocol was used for the transport to the laboratory. However, as most lions were captured at night in the winter season, temperatures during transport should have been within a reasonable range, and the large number of samples should compensate for this.
This dataset contained only lions from the Kruger National Park in South Africa. Lions from other locations may have somewhat different blood parameter values, but these differences are expected to be only small for clinically healthy lions.

We conclude that these reference intervals can be used by veterinarians and wildlife researchers to assess the health status of free-ranging lions, and we propose they can also be used for captive lions as they are arguably more informative than the currently available mean, SD, minimal and maximal values.

ACKNOWLEDGEMENTS

This research was part of a larger study that was predominantly funded by the South African Veterinary Foundation and the Directorate of Veterinary Services, National Department of Agriculture, Forestry and Fisheries, South Africa. D.F. Keet was the recipient of a doctoral bursary awarded by the University of Pretoria. The work of M. Maas was supported by the Netherlands Organisation for Scientific Research (NWO/ZonMw grant 918.56.620). This project was approved by the South African National Parks conservation and ethics committees as well as the Animal Use & Care Committee of the University of Pretoria.

We acknowledge the many people that have helped with the collection of the samples and Jan van den Broek for statistical advice.

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Chapter 8

General discussion

The general aim of this thesis was to contribute to the knowledge of bovine tuberculosis (BTB) in lions by addressing the following objectives 1) development of an accurate and practical diagnostic assay to be used in monitoring and surveillance; 2) development of new tools to assess the immunopathogenesis of BTB in lions; 3) evaluation of the potential risk of co-infection with FIV_{ple}. The main findings and their implications for the control of BTB in lion populations will be discussed in this chapter and recommendations for future research will be given.

Diagnostic challenges

The discovery of *M. bovis* infection in free-ranging lions in various African wildlife parks has raised concerns among wildlife park managers and conservationists, since BTB could pose a potential threat to the conservation of lions. Monitoring is needed to gain insight in the severity of this threat, but is complicated by the lack of a validated, practical diagnostic test for BTB in lions. Currently, the only available validated ante-mortem test for lions is the tuberculin skin test [1]. However, the fact that animals need to be recaptured is a practical limitation, e.g. in the 2007-2008 survey in the Kruger National Park less than 50% of the lions could be immobilized a second time. A new diagnostic assay without the need to recapture would therefore be an important step forward in lion BTB research.

In chapter 2 an update was given on current diagnostic methods for *M. bovis* infection in different wildlife species, based on the OIE's 'Fit for purpose'-concept for diagnostic tests. Following this concept for free-ranging lions, a diagnostic assay needs to be available that can be used for non-lethal sampling in order to perform surveillance and monitoring of the spread of disease. As explained above, a test that only requires one capture would be preferred.

We aimed at developing a diagnostic assay based on the measurement of IFN-γ, as this is a validated procedure for other species like humans and cattle [2,3]. An adapted feline IFN-γ ELISA detected native lion IFN-γ (chapter 4a), but the detection levels were not satisfactory. Therefore, we focused on a lion-specific IFN-γ ELISA, which showed to have superior detection levels (chapter 4b). It detected native lion IFN-γ in supernatants of mitogen-stimulated whole blood and showed no positive results for supernatants of antigen-stimulated whole blood of lions from a BTB-free area. These promising results could not be substantiated, because blood from BTB positive lions was not available at the final stage of our studies, so an indication of the sensitivity of the assay could not be obtained. The lion-specific IFN-γ assay also detected native IFN-γ from other feline species, and even of a canine species. This suggests that apart from lions it may be used for other (big) cats in future. This would be especially relevant for the Iberian lynx (*Lynx pardinus*), as this is a critically endangered species [4] in which multiple cases of BTB have been found in free-ranging animals. As the lion and lynx are closely related within the *Panthera* group [5,6], it is highly likely that the assay will also detect Iberian lynx IFN-γ.

Development of new diagnostic assays involves various stages before an assay is fully validated [7]. The lion-specific IFN-γ assay has covered a large part of the first, analytical stage, but to determine diagnostic sensitivity and specificity, which is done in the subsequent stage, large numbers of animals are required. Obtaining these large numbers is very challenging when working with wildlife species. This was recognized by the OIE, and "provisional acceptance" of an assay has been proposed after finishing part of the second stage, which enables assays to be used in local settings in which the provisional validation has been conducted, while being further validated [8]. However, until larger numbers of lions are tested, preliminary studies with small numbers will result in large confidence intervals and high degrees of uncertainty.

Another challenge of the validation of new diagnostic assays is the lack of an ante-mortem

golden standard, which complicates confirmation of test results. Longitudinal studies may aid in interpretation of results, especially if they include post-mortem examinations. Experimental infection of lions may be considered, but potential differences in immune responsiveness compared to naturally infected animals need to be taken into account in that case. Another possibility to circumvent the lack of an ante-mortem golden standard is the use of multiple diagnostic assays, combined with clinical observations, for Bayesian statistical analysis. As was discussed in chapter 2, serologic tests could be used as such complementary tests. Although for human use, serologic assays have not yet reached a satisfactory level of sensitivity and specificity and the WHO recommends that commercial serological tests should not be used for the diagnosis of pulmonary and extrapulmonary TB [9], for several wildlife species the serologic assays show promising diagnostic potential [10]. An animal-side serologic assay for lions has been tested recently in a preliminary study of 14 lions and detected 70% of the culture-positive animals. When combined with skin testing results, this increased to 91% [11]. For badgers and wild boar it is suggested that detection of antibodies indicates the stage of disease in which shedding of bacteria is likely to occur [10,12]. Others suggest that antibody responses are associated with antigen burden rather than the pathology [13].

Finally, new diagnostic methods and approaches developed for humans and other species may offer possibilities for diagnosis of BTB in lions. Diagnostic methods for TB in humans are still being developed or improved as exemplified by the Xpert MTB/RIF (based on real-time PCR), liquid culture and rapid speciation, molecular line probe assays and light-emitting diode fluorescence microscopy [9]. In non-human primates the potential of an ELISA for detection of *M. tuberculosis* antigens in urine or antibodies to *M. tuberculosis* in fecal samples is being assessed [14].

The immunopathogenesis

Though the immunopathogenesis for *M. tuberculosis* infection in humans and *M. bovis* infection in cattle has been resolved to a certain extent, little is known about immune responsiveness in *M. bovis* infected lions, and which factors are of influence, e.g. transmission modes (oral vs aerosol) and doses, co-infection, pregnancy, environmental circumstances etc. Knowledge of immune responses is essential in diagnostic assay development, interpretation of test results and for the development of new or improved vaccines or vaccination strategies [15].

To assess immune responsiveness in *M. bovis* infected lions, a pilot study was performed that included the development of reverse transcriptase qPCRs and subsequent their application in 26 lions, described in chapter 5. This pilot study showed differences in cytokine expression between skin test positive and negative lions, supporting the potential use of these PCRs in the assessment of the immunopathogenesis. The difference in expression of IFN-γ between the skin test positive and negative lions observed, supports a diagnostic potential, as was also shown in other species, e.g. deer [16]. Even though other assays may be logistically more appealing as diagnostic assays, the addition of reverse transcriptase qPCRs to the above suggested Bayesian analysis approach may be very useful.

Correlating clinical, laboratory diagnostic and/or macro- and microscopic pathological parameters to cytokine expression will add to the value of reverse transcriptase qPCR tests and correlation of certain cytokine profiles to shedding, would offer tools for managing *M. bovis* infection in lions. Including necropsy results is especially valuable if macroscopic lesions are present and mycobacteria can be cultured. However, attention should be paid to the possibility of false-negative necropsy results since in other species like badgers and buffaloes, pathology is

complicated by "no-visible-lesions" animals, in which no macroscopic lesions are found [17,18].

Bovine tuberculosis: a threat to the lion population?

In addition to the well-described effect of co-infection of HIV and *M. tuberculosis* in humans, negative effects of co-infection have been found for *M. tuberculosis* and helminth infections in mice [19], resulting in enhanced intracellular persistence of *M. tuberculosis,* and for *M. bovis* and Porcine Circovirus Type 2 infection in wild boar [20], resulting in increased severity of lesions. However, assessment of the effect of co-infection of *M. bovis* and FIV_{ple} in lions (chapter 6) did not show proof of a synergistic relationship. For a number of lions (n=20) included in our studies, post-mortem gross pathology and histopathology data were available, as well as test results for both FIV_{ple} and *M. bovis* infection. When assessing numbers, types and severity of lesions in organs and lymph nodes of these 20 lions, also no statistically significant differences were found in either single infected (FIV_{ple} or *M. bovis*) or co-infected lions compared to non-infected lions (results not shown). Possible explanations for this discrepancy between co-infection of *M. tuberculosis* and HIV in humans and *M. bovis* and FIV_{ple} in lions have been discussed in chapter 6.

Surprisingly, no correlation was found between BTB and body condition. Other studies also suggested that, although BTB compromises health of individual lions, the lion population is apparently not severely affected at present [21]. In this light, it is interesting to know that in humans there is an apparent "resistance" of some individuals to mycobacterial infection. Whether resistance, or the severity of disease, is driven by genetic variation in both the pathogen and the host, needs to be determined [22]. Also in boar the heterogeneity of immune responsiveness was assessed and some suggestions for genes involved have already been made, e.g. complement component 3 (C3) and methylmalonyl coenzyme A mutase (MUT), although the mechanisms remain unknown [15]. Potential variations in susceptibility of lions to *M. bovis* infection and consequent selection advantages, may offer a future perspective of a lion population in which *M. bovis* infection may only have a minor influence. However, at the moment it is still unsure what the long-term disease effects are for the lion population and the ecosystem, especially in extreme situations like drought, thus a *laissez-fare* approach is not justified [21].

Implications of findings and the way forward

Mycobacterium bovis has established itself in a multitude of species: buffalo, kudu, lion, and maybe more, in various wildlife parks in Africa, severely complicating eradication of *M. bovis* [23,24]. Control of *M. bovis* infection is a more realistic goal and could be approached in various ways: (1) reducing host (or infected host) density, (2) reducing the reproductive rate of the pathogen, or (3) manipulating the environment to reduce the contact between diseased and susceptible animals [25]. This last approach is not feasible for reduction of transmission from buffaloes to lions, but to reduce contact between buffaloes and cattle, and hence transmission of the infection to cattle, fencing of game parks will be an important component.

Reducing infected host density by culling of *M. bovis* positive animals or herds, which is the approach in most countries for cattle, is often not a reasonable management option for wildlife, because it does not meet with ecological or ethical acceptance. However, in several situations culling has been tried as a management option, with varying success. In Australia, *M. bovis* was eradicated by culling infected cattle and water buffalo (the reservoir species) [26]. Non-selective culling of possums in New Zealand to reduce the possum population long-term, together with other management strategies, resulted in a major reduction from 2.4% in 1993 to 0.35% in 2004 in the (herd) point prevalence and in the incidence of BTB in possums [27]. In Michigan in the USA,

where infected white-tailed deer have been found to infect livestock, management strategies to decrease the deer population densities to biological carrying capacity and by implementation of restrictions on supplemental feeding and baiting have decreased the prevalence of tuberculosis in the core area by >60% [28]. However, large-scale culling experiments of infected badgers in the United Kingdom have had complex epidemiological outcomes and in general have been unsuccessful so far [29]. In Hluhluwi-Imfolozi park in South Africa, the buffalo population is tested yearly, followed by selective removal of buffaloes that tested positive in the IFN-γ assay from the population. By controlling the infection in buffaloes, spillover to other species like lions is reduced [23]. In the Kruger National Park, selective culling is not considered as an option, as the number of buffaloes is too large to test all animals. Without reduction of the constant supply of *M. bovis* from the buffaloes, or other prey species, culling infected lions would not be a realistic management option, especially since they are considered a vulnerable species [30].

Reducing the reproductive rate of the pathogen can be achieved by either antibiotic treatment of infected individuals, or by vaccination. Large-scale treatment may be an option for captive wildlife, but for free-ranging animals it is not since the antibiotics need to be given for an extended period. Vaccination doesn't have the economical, ethical and practical issues that culling and treatment of animals have [31,32], and may offer a more long-term solution. In general, vaccination is likely to be the most efficient control method in populations where host birth, host death and disease transmission rates are relatively low [33]. To date, the only available vaccine is BCG, an attenuated strain of *M. bovis*. Also other vaccination strategies are being tested in various species, e.g. combinations of BCG with different adjuvants, virus vectors or DNA vaccines (reviewed in [34]), as well as oral vaccination of wild boar with a new heat-killed *M. bovis* vaccine, which gave promising results [15].
Vaccination of cattle and humans with BCG is associated with variable efficacy, but this does not preclude its use for wildlife, since the key attribute for wildlife would not be to prevent infection, but to reduce the risk of spread of *M. bovis* to other wildlife and cattle [34,35]. Oral-delivered BCG vaccination is considered in several countries for a diversity of wildlife species [32]. However, before vaccination can be a viable management measure, diagnostic methods may need to be adjusted, as some diagnostic assays (e.g. tuberculin skin test) cannot distinguish between *M. bovis* infection and BCG vaccination. Complementary diagnostic methods are being developed that are able to differentiate between vaccinated animals and those infected with *M. bovis* [36].
The ideal species for vaccination in Africa would be the buffalo, the reservoir species in many areas. However, studies of vaccination of buffaloes in South Africa have not given significant positive results up till now [37,38]. Therefore, to reduce negative impacts of *M. bovis* infection on the lion population, vaccination of lions, provided an effective and safe vaccine is available, could be an intermediate approach until an effective buffalo vaccine is available. Except for a small trial with a group of zoo lions that was vaccinated with BCG, of which interpretation of results was complicated by simultaneous management actions [39], no vaccine trials have been initiated in lions yet.

Effects of wildlife BTB and its control should be monitored as it inevitably interferes with wildlife ecology, with often complex outcomes [33]. Disease effects of *M. bovis* infection on the lion population could have ecological implications for other animal populations, both of prey species and of competitive predator species [40,41]. Ideally, monitoring of *M. bovis* in the lion population should therefore be integrated with other research data, e.g. on host abundance and distribution [42].

Although major effects of BTB on the lion population are not apparent at the moment, improvement of our knowledge about BTB in lions remains important. Lions are listed as vulnerable on the IUCN red list [30,43], and infectious diseases have been known to be the cause of decimation or extinction in the past [30,44,45]. Currently, *M. bovis* has been found in two of the largest lion populations in Africa, but it may be present in many other wildlife parks, either because of the presence of infected prey species like the buffaloes in Mikumi-Selous reserve (Tanzania) and the Okavango Delta (Botswana), or because of translocation of potentially *M. bovis* infected lions in the past (Keet, personal communication)[46,47]. Future translocation activities of lions from the Kruger National Park should include thorough assessment of the risk of disease spread [48]. Besides conservation and ecological arguments, there are also economical arguments to improve our knowledge on BTB in lions. Lions are a main tourist attraction and a decline of the lion population could also result in decreased income for many African countries that heavily rely on this source of income. Furthermore, national control programmes aiming at a BTB-free status are complicated by the presence of a BTB wildlife reservoir.

In conclusion, this thesis describes several steps forward regarding knowledge of BTB in lions, including a new diagnostic method, the lion-specific IFN-γ assay. Validation of new diagnostic tests remains challenging and may require additional approaches, combining data of different disease markers like in Bayesian analysis. Monitoring disease development and testing multiple diagnostic assays longitudinally is likely to result in a good understanding of diagnostic possibilities for the different stages of *M. bovis* infection. Remaining gaps in our knowledge regarding immunopathogenesis of lions may be filled by experimental infection studies, which may also help to determine the maintenance potential of lions. Combining these data with epidemiological and ecological studies may offer tools for conservationists and wildlife park managers to control *M. bovis* in lion populations.

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Nederlandse samenvatting

Tuberculose in Afrikaanse leeuwen

Rundertuberculose (boviene tuberculose, BTB) wordt veroorzaakt door de bacterie *Mycobacterium bovis* (*M. bovis*). De bacterie maakt deel uit van het Mycobacterium Tuberculosis Complex (MTBC), een verzameling van pathogene mycobacteriën die tuberculose veroorzaken in verschillende diersoorten. In veel ontwikkelde landen is het risico op *M. bovis* infectie erg klein door nationale controleprogramma's, maar in ontwikkelingslanden is het nog steeds een belangrijke zoönose en heeft het door o.a. handelsrestricties een grote economische impact. *M. bovis* kan naast runderen, die de belangrijkste gastheer zijn, zowel gedomesticeerde als wilde dieren infecteren. De aanwezigheid van *M. bovis* in wilde dieren bemoeilijkt het verkrijgen van een nationale vrij-status, omdat overdracht (terug) naar runderen mogelijk is. Voorbeelden van wilde dieren die een *M. bovis* reservoir vormen, zijn wilde zwijnen in Spanje, dassen in het Verenigd Koninkrijk en opossums in Nieuw Zeeland. Wanneer meerdere wilde diersoorten betrokken zijn als reservoir of als accidentele gastheer (spillover), zoals het geval is in het Kruger National Park (KNP) in Zuid-Afrika, wordt interventie en/of eradicatie nog gecompliceerder. Spillover van de bacterie vanuit een reservoirspecies kan ook een risico zijn voor bedreigde of kwetsbare diersoorten, zoals de Iberische lynx of de Afrikaanse leeuw.

Het eerste geval van *M. bovis* infectie bij leeuwen in het KNP werd in 1995 gediagnosticeerd. Ook in de Serengeti (Tanzania) en het Hluhluwe-iMfolozi park (Zuid-Afrika) zijn *M. bovis* geïnfecteerde leeuwen gevonden. Leeuwen zijn een kwetsbare soort volgens de *IUCN red list*. Infectie met *M. bovis* heeft mogelijk gevolgen voor het behoud van deze diersoort en (negatieve) consequenties voor het betreffende ecosysteem en het toerisme. In het KNP zijn de leeuwen geïnfecteerd geraakt door het eten van *M. bovis*-geïnfecteerde buffels. Aangezien in dit park de prevalentie van *M. bovis* bij buffels nog steeds stijgt, is het waarschijnlijk dat in de toekomst het merendeel van de leeuwen uiteindelijk ook geïnfecteerd zal zijn.

Tuberculose is primair een luchtweginfectie en de belangrijkste transmissieroute bij mensen en runderen is via aerosolen. In de longen en lymfeknopen worden granulomen gevormd. Deze granulomen zorgen voor een beperking van de verspreiding van de infectie, maar kunnen ook (veel) weefselschade veroorzaken. De klinische verschijnselen van BTB bij leeuwen zijn in het algemeen niet-specifieke verschijnselen, zoals een verlies van lichaamsconditie en een slechte vacht. Bij een deel van de dieren worden zogenaamde "ellebooghygromen" gevonden: stevige, fluctuerende zwellingen op de caudale zijde van de ellebogen. De longlesies die bij necropsie van vergevorderd zieke dieren worden gevonden, zijn divers en verschillen van lesies bij primaten en hoefdieren: er wordt geen abcesvorming, verkazing of mineralisatie waargenomen, maar wel bronchiëctasie en fibreuze, dunwandige holtes gevuld met exsudaat. Daarnaast kunnen afwijkingen worden gevonden in lymfeknopen, botten, ogen, lever en nieren. De lesies zijn onder meer afhankelijk van de verspreiding van de bacteriën en de transmissieroute. De belangrijkste transmissieroutes voor leeuwen zijn oraal, respiratoir en percutaan.

Diagnostiek van tuberculose

Momenteel is alleen de tuberculine huidtest gevalideerd als *ante-mortem* test om *M. bovis* infectie bij leeuwen vast te stellen. Deze heeft echter als nadeel dat leeuwen drie dagen na de initiële immobilisatie opnieuw moeten worden gevangen om een eventuele huidzwelling te meten. Daarom wordt er naar een betere diagnostische methode gezocht, net zoals bij veel andere wilde diersoorten. De laatste ontwikkelingen op dit gebied zijn beschreven in hoofdstuk 2, waarin een overzicht is gegeven van verschillende testen die voor wilde diersoorten beschikbaar zijn. Hierbij is rekening gehouden met het "fit-for-purpose" concept van de OIE, waarbij een test

geschikt moet zijn voor het doel waarvoor de test wordt gebruikt. Voor wilde dieren kan dus een andere test geschikter zijn dan bijvoorbeeld voor dierentuindieren of gedomesticeerde dieren, en voor surveillance kunnen andere testkarakteristieken prioriteit hebben dan voor diagnose van het individuele dier. Testkarakteristieken als de sensitiviteit en specificiteit, maar ook praktische toepasbaarheid spelen hierbij een belangrijke rol.
Voor verschillende diersoorten, waaronder runderen en mensen, is de interferon-gamma (IFN-γ) assay ontwikkeld, die gebaseerd is op dezelfde (vroege) immuunrespons als de tuberculinetest. Een van de doelen van dit promotieonderzoek was het ontwikkelen van een IFN-γ assay voor leeuwen. In eerste instantie werd een IFN-γ assay die ontwikkeld was voor katten gemodificeerd en aangetoond werd dat deze natief leeuwen IFN-γ kon herkennen in het supernatant van volbloed dat gestimuleerd was met mitogenen (beschreven in hoofdstuk 4a). Daarnaast werd bij leeuwen uit een BTB-negatieve omgeving geen productie van IFN-γ gemeten na stimulatie met mycobacteriële antigenen. De detectielimiet van 3 ng/ml zou echter in de praktijk een te lage testsensitiviteit kunnen betekenen. Een leeuwspecifieke IFN-γ assay werd verwacht sensitiever te zijn. Het kloneren en sequencen van het leeuwen IFN-γ gen, beschreven in hoofdstuk 3, was de eerste stap in de ontwikkeling van een leeuwspecifieke IFN-γ assay. Een vergelijking met de sequentie van het cheetah IFN-γ gen en het katten IFN-γ gen, liet een homologie van 97-100% tussen de IFN-γ genen van de verschillende katachtigen zien.
Voor de productie van monoclonale antilichamen werden muizen geïmmuniseerd met recombinant leeuwen IFN-γ geproduceerd in een eukaryoot expressiesysteem. Deze monoclonale antilichamen zijn gebruikt in de ontwikkeling van een leeuwspecifieke IFN-γ assay, beschreven in hoofdstuk 4b. Deze ELISA heeft een detectielimiet van 160 pg/ml en herkende natief leeuwen IFN-γ in supernatanten van volbloed dat gestimuleerd was met mitogenen. Antigeenstimulatie van volbloed van leeuwen uit een *M. bovis*-negatieve omgeving leverde geen vals-positieve reacties op. Grote groepen *M. bovis*-geïnfecteerde leeuwen en *M. bovis*-negatieve leeuwen zullen moeten worden getest om de sensitiviteit en specificiteit van de test te kunnen bepalen. Herkenning van natief IFN-γ van mitogeen-gestimuleerd volbloed van tijgers is een indicatie dat deze test ook voor andere katachtigen kan worden gebruikt, maar dit dient verder bestudeerd te worden. Door het gebruik van MTBC-specifieke antigenen, waardoor gedifferentieerd kan worden tussen geïnfecteerde en gevaccineerde dieren, zou deze test in de toekomst ook kunnen worden gebruikt als er een vaccin beschikbaar komt voor leeuwen. Zeker indien de IFN-γ assay wordt gecombineerd met testen die een ander spectrum van de immuunrespons meten (bijv. serologische testen), biedt deze test veelbelovende mogelijkheden voor de diagnostiek van *M. bovis* infectie in leeuwen.

De immuunrespons

Als een dier blootgesteld wordt aan *M. bovis* kan het immuunsysteem de bacterie opruimen of kan er sprake zijn van een latente of (uiteindelijk) een actieve infectie. De cel-gemedieerde immuniteit, die geacht wordt de infectie zoveel mogelijk in te perken, speelt hierbij een essentiële rol. Belangrijke cellen zijn hierbij de macrofagen, die doelwit zijn van de bacterie, en de T-lymfocyten. Er bestaan verschillende subtypes van T-lymfocyten, zoals Th (T helper) 1, Th2, Th17 and Treg (T regulatoire) cellen, die een rol spelen in de opbouw en de uitkomst van de immuunrespons. Deze cellen produceren cytokines die de immuunrespons reguleren en waarvan de belangrijkste IFN-γ is. Later in het verloop van de infectie wordt de humorale (antilichaam) respons steeds belangrijker.
Kennis van het verloop van de immuunreactie na infectie kan worden gebruikt bij de ontwikkeling van vaccins en diagnostische testen voor leeuwen. In hoofdstuk 5 wordt de optimalisatie en het gebruik beschreven van de real-time PCR ter bepaling van vier cyokines die van belang

zijn in de immuunrespons tegen *M. bovis:* IFN-γ, IL-4, TNF-α and IL-10. Deze voorstudie laat zien dat de ontwikkelde real-time PCR's te gebruiken zijn voor leeuwen, maar grotere aantallen leeuwen zijn nodig om conclusies te kunnen trekken over eventuele correlaties tussen de relatieve verhoudingen van de cytokines en het infectiestadium, of tussen *M. bovis*-positieve en -negatieve leeuwen.

Co-infectie met FIV$_{ple}$

Feline immunodeficiency virus (FIV$_{ple}$) is endemisch in de leeuwenpopulatie van het KNP. De relatief recente introductie van *M. bovis* zou een extra risico voor de leeuwenpopulatie kunnen betekenen als vergelijkbare versterkende effecten van een co-infectie worden gevonden voor de leeuwen als bij mensen met een co-infectie met *M. tuberculosis* en HIV het geval is. Van 1993-2008 zijn gegevens verzameld van 669 leeuwen in het KNP. In hoofdstuk 6 wordt de analyse van deze gegevens beschreven. Er was een hogere prevalentie van *M. bovis* in de leeuwen in het zuiden van het KNP ten opzichte van die in het noorden (72% versus 19% over de totale studieperiode), en in het noorden was in de loop van de tijd een sterke stijging van de prevalentie (0% tot 41%) te zien. Bij het beoordelen van de lichaamsconditie en relevante bloedwaarden werden geen aanwijzingen gevonden voor een versterkend effect van een co-infectie van *M. bovis* en FIV$_{ple}$ infectie, zoals dat bij mensen wel wordt gezien. De reden hiervoor kan liggen in de verschillen in immuunreactie tussen leeuwen en mensen of bijvoorbeeld in de verschillen in evolutie van de twee ziekteverwekkers in leeuwen- en mensenpopulaties.

De dataset van hoofdstuk 6 is ook gebruikt in hoofdstuk 7 om referentie-intervallen voor zeven hematologische en 11 serum biochemische waarden te bepalen van klinisch gezonde wilde leeuwen. Deze kunnen in de toekomst door dierenartsen en onderzoekers worden gebruikt als referentiewaarden. De referentie-intervallen omvatten, behalve voor alkalische fosfatase bij subadulte leeuwen, de gemiddeldes van de bloedparameters zoals gemeten bij leeuwen in gevangenschap en kunnen dus waarschijnlijk ook dienen als referentiewaarden voor dierentuinleeuwen.

Conclusies

Op dit moment zijn de lange-termijn effecten van *M. bovis* op de leeuwenpopulatie en het ecosysteem nog onduidelijk. Een afwachtende houding is echter onverstandig omdat leeuwen een kwetsbare diersoort zijn. Daarom dienen maatregelen te worden genomen om potentieel negatieve effecten van *M. bovis* op de leeuwenpopulatie te beperken. Controle- en eradicatiemaatregelen worden echter bemoeilijkt door een gebrek aan kennis over de immuunpathogenese en het ontbreken van een praktische, accurate diagnostische test. Dit proefschrift draagt bij aan de kennis over *M. bovis* infectie bij leeuwen en beschrijft ontwikkelingen op het gebied van diagnostiek, immuunpathogenese en effecten van co-infectie met FIV$_{ple}$ en draagt zo bij aan het ontwikkelen van deze maatregelen. Daarnaast kunnen de onderzoeksresultaten en methodieken als basis dienen voor nieuw, bij voorkeur longitudinaal, onderzoek ten dienste van diagnostiek en vaccinontwikkeling.

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Dankwoord

“Je gaat je dromen waarmaken.”

Dat was een veelgeschreven zin in mijn afscheidsboekje toen ik als ET-student in 2007 naar Afrika vertrok om aan dit onderzoek te beginnen. En hoe moeilijk dit onderzoek soms ook is geweest, vooral wat betreft alle zaken er omheen, ik heb fantastische momenten meegemaakt, geweldige mensen leren kennen, veel geleerd en ja, dromen waargemaakt.

Dat zou niet hebben gekund zonder een aantal mensen. Allereerst Victor Rutten en Hans Heesterbeek, ET-begeleiders en later promotoren. Bedankt voor de mogelijkheid dit onderzoek te hebben kunnen uitvoeren en voor jullie begeleiding, ook op afstand. Victor, bedankt voor je oneindige inzet en de vele e-mails, vaak geschreven midden in de nacht, die daar getuigen van waren. Hans, bedankt voor het zelfvertrouwen dat je me gaf door me dingen op mijn eigen manier te laten doen, en voor het altijd stellen van kritische vragen tijdens overleggen: dacht ik dat ik het goed had voorbereid, wist je toch weer een vraag te stellen waar ik je het antwoord op schuldig moest blijven.
Thank you, Anita Michel, for the supervision in South Africa. Our collaboration was not extensive, but I hope to have one day the same analytical and sharp way of reasoning as you have.
Dewald Keet, thank you for giving me the opportunity to include field work in my research and for the collaboration on the dataset. Though you are likely the person who is the most difficult to get hold of that I know, I enjoyed the moments that we discussed the work and your wealth of knowledge about lions. I wish you all the best with your PhD!
Jacques Godfroid, thank you for your contribution as an ET supervisor and for guiding me in my first months in South Africa.

Met een gevarieerd onderzoek als dit heb je experts nodig op verschillende gebieden. Mirjam Nielen, bedankt voor jouw bijdrage van epidemiologische kennis en voor alle momenten dat ik even binnen kon komen om iets te bespreken. Ik liep altijd weer geïnspireerd en gemotiveerd uit je kantoor.
“*Dat weet Peter vast wel.*”, was een veelgehoorde zin op het lab. Bofte ik even met de hulp van Peter van Kooten op het lab. Bedankt voor de introductie in de wereld van hybridoma’s en alle gezellige momenten.
Statistici Jan van den Broek en Hans Vernooij, bedankt voor jullie uitleg en hulp bij het maken van de modellen.
Ildiko, bedankt voor je hulp in Zuid-Afrika, altijd leuk dan weer even iemand uit Nederland tegen te komen! Zo ook Janneke Schreuder: bedankt voor jouw werk als HP-student op dit onderwerp, wat ook in dit proefschrift is beschreven.
The veterinarians and technicians of 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: *baie dankie* for the great experience! At, thank you for all the times you drove me back in the middle of the night so I could work on my tests. I enjoyed all our talks and the insight they gave me in the complex country South Africa is, but most of all I enjoyed your sense of humor (though as a “kaaskop” I was often the subject of these jokes).
Many thanks as well for the veterinarians and staff of the Veterinary Wildlife Services for the use of their laboratory and the chance to help with the buffalo captures.
Thank you, colleagues at the Department of Veterinary Tropical Disease at Onderstepoort: it was hard to keep track of when I was there and when I was not, but I always felt part of the group. Specials thanks to Darshana Morar, Melvyn Quan, Paula Allsopp and Marinda Oosthuizen for

helping me with specific parts of the research.
(Dierentuin)dierenartsen en anderen, zowel in Nederland als in Afrika: sorry voor mijn gestalk, maar bedankt voor jullie bijdrages in leeuwenbloed of bloed van andere katachtigen. Zonder jullie hulp zou de leeuwspecifieke IFN-γ assay er niet zijn geweest.
Er zijn nog vele anderen betrokken geweest bij de verschillende onderdelen van dit proefschrift: voor een dagje een labtechniek leren, voor een discussie over een bepaald onderwerp, voor het lenen van een reagens, enz. Om te voorkomen dat ik mensen ga vergeten als ik namen ga neerschrijven, wil ik jullie hierbij allemaal bedanken!

Zeker zo belangrijk tijdens een promotietraject zijn de mensen die niet direct met je onderzoek te maken hebben. Wat had ik leuke kamergenoten! Marjolein en Gerrit, we hebben wat afgekletst: frustraties over datasets (of onderzoek in het algemeen), vreugde over geaccepteerde artikelen, codes van R, statistiek, maar ook vooral over "gewone" of juist bijzondere dingen uit het leven. Totaal verschillend alle drie, maar ik ben heel blij dat jullie mijn paranimfen zijn. Milou, gezellig dat je je ET tijd bij ons zat!
Allemaal leuke kleine weetjes kom je tegen tijdens je onderzoek, maar daar is in een proefschrift helaas geen plaats voor. Die kon ik dan gelukkig wel kwijt tijdens de gezellige koffiepauzes met mijn mede AiOs en LH collega's, die ik zo een aantal dagen lang bezig kon houden over bijvoorbeeld *Cervus elaphus* en de problematiek als hetzelfde beest op verschillende continenten andere namen krijgt. Dat werd moeiteloos ingepast in de gesprekken die toch al uiteenliepen van Oh Oh Cherso, de dagelijkse horoscoop en het nieuws in de Metro, tot de beste manier van scripts schrijven in R. Bedankt allemaal voor deze gezellige momenten!
Bij I&I kwam ik altijd met vlagen: soms dagelijks als er experimenten liepen, soms weer maanden niet. Toch werd ik altijd hartelijk ontvangen door iedereen en had ik ook het genoegen om mee te mogen met het jaarlijkse labuitje, waar je tenslotte je collega's het best leert kennen! Bedankt hiervoor!
Many thanks to the post grads at the DVTD, for the warm welcome every time, for the many braais and parties and for being my friends in a country far away from home. Thank you for giving me a second home!

Mijn nieuwe collega's op het RIVM, vooral de wildlife groep (Joke, Frits, Cecile), bedankt voor jullie interesse in mijn promotie en voor bemoedigende woorden tijdens de laatste loodjes!

Lieve vrienden en vriendinnen, bedankt dat jullie me juist soms even niet aan mijn promotie lieten denken. Het laatste jaar is in een redelijk sociaal isolement doorgebracht, maar straks hoop ik weer veel tijd te hebben voor thee, etentjes, klimmen en wat nog meer!

Tijdens dit promotieonderzoek heb ik veel verschillende dingen geleerd, maar de belangrijkste daarvan hebben niks met werk te maken. Een ervan is hoeveel geluk ik heb met mijn familie. Lieve pap en mam, bedankt voor jullie steun tijdens de moeilijke momenten en voor jullie blijdschap en trots op andere momenten. En nou ja, voor eigenlijk alles wat me tot dit punt heeft gebracht. Suus, ik heb nog vaak aan je Sinterklaasgedicht gedacht op momenten dat mijn hoofd (en agenda) over leek te lopen. Yf, bedankt voor al jouw wijze raad en interesse in mijn werk, telkens weer.
Lieve Chris, bedankt voor de rust en steun die ik bij jou kan vinden en voor het aanhoren van eindeloze verhalen over artikelen, promotieperikelen en kaften. Je hebt het afgelopen jaar minder aandacht gekregen dan je verdiende, maar nu is er weer tijd om nieuwe avonturen te beleven en dromen waar te gaan maken!

Curriculum vitae

Miriam Maas was born on March 9, 1985 in Oosterhout (The Netherlands). In 2003, she finished high school (VWO) *cum laude* at the Mgr. Frencken College in Oosterhout. This gave her direct admission to the study that she had dreamed of since primary school: veterinary medicine. Almost from the start of the study, she was actively involved in the "wildlife work group" of Archaeopteryx, the veterinary society for birds and exotic animals, of which she was later a board member as well. In her second year, she participated in the first Student Workshop of the European Wildlife Disease Association (EWDA). Though determined at first to work in practice, this experience opened her eyes for wildlife research. She became president of the EWDA Student Chapter for two years, co-organizer of the second EWDA Student Workshop in Greece and has been attending the EWDA conferences ever since. In those first four years of Veterinary Medicine, she also spent one year as a secretary of the veterinary committee for pet animals, as well as one year as vice-president of the Student Alpine Club (USAC) and also obtained the "artikel 9" certificate to perform animal experiments.

In 2007, Miriam finished her doctorandus degree, equivalent to a Master of Veterinary Science. She then was asked to undertake a one-year excellent track research (equivalent to a Master of Veterinary Research), for which she wanted a subject that involved infectious diseases and wildlife. She found a project in South Africa, where she worked on developing diagnostic methods for tuberculosis in lions, at the Department of Veterinary Tropical Diseases of the Faculty of Veterinary Science of Onderstepoort. Prof. Victor Rutten and Prof. Hans Heesterbeek were the Dutch supervisors of this project and local supervision was from Dewald Keet and, until his immigration to Norway, Prof. Jacques Godfroid. Miriam spent one year in South Africa, travelling up and down to the Kruger National Park, alternating various laboratory activities with lion captures. Part of the work performed in that year is described in this thesis.

After successfully completing her excellent track research in 2008, Miriam continued with her clinical rotations in Utrecht. Though enjoying the practical work, she gladly took on the chance to continue her tuberculosis work after she graduated as a veterinarian in July 2010. In October 2010, she started her PhD research, which would build on the work that was done previously. She conducted her research at both the Department of Farm Animal Health (Division Epidemiology), as well as the Department of Infectious Diseases and Immunology (Division Immunology). Besides working in Utrecht, she spent again twice 3 months in South Africa at the Department of Veterinary Tropical Diseases. Unfortunately, due to both financial constraints and lack of field samples, the project finished in March 2012. However, she continued working on the research to finish remaining work for her PhD.

In May 2012, she started working at the National Institute for Public Health and Environment (RIVM) at the Laboratory for Zoonoses and Environmental Microbiology (LZO). She works in the "Animal and Vector group" in the wildlife cluster and now works with diverse pathogens, mainly parasites, like *Echinococcus multilocularis* and *Toxoplasma gondii*. The wildlife she works with now (e.g. foxes and rodents) has become a bit smaller compared to lions and buffaloes, but the challenges that are associated with wildlife research are still the same.