Natural animal model systems to study tuberculosis

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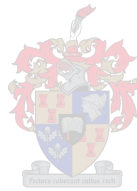

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Declaration

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“There is always a Cape Horn in one’s life that either one weathers or wrecks one’s self on. Thank God I think I may say I have weathered mine – not without a good deal of damage to spars and rigging though, for it blew deuced hard on the other side.”

Thomas Henry Huxley

Abstract

The growing global epidemic of human tuberculosis (TB) results in 8 million new cases of this disease and 2 million deaths annually. Control thereof will require greater insight into the biology of the causative organism, *Mycobacterium tuberculosis*, and into the pathogenesis of the disease. This will benefit the design of new vaccines and diagnostic assays which may reduce the degree of both disease transmission and progression.

Animal models have played a vital role in the understanding of the aetiology, pathogenesis, and treatment of TB. Much of such insight has been obtained from experimental infection models, and the development of new vaccines, for example, is dependant on these. Nonetheless, studies utilising naturally occurring TB in animals, such as those which have investigated the use of interferon-gamma release assays (IGRA) for its diagnosis, have contributed substantially to the body of knowledge in this field. However, there are few such examples, and this study sought to identify and investigate naturally occuring animal TB in South Africa as an opportunity to gain further insight into this disease.

During the course of this study, the dassie bacillus, a distinctly less virulent variant of *M. tuberculosis*, was isolated from a rock hyrax from the Western Cape Province of South Africa. This has provided new insight into the widespread occurrence of this organism in rock hyrax populations, and has given impetus to further exploring the nature of the difference in virulence between these pathogens.

Also investigated was *M. tuberculosis* infection in dogs in contact with human TB patients. In so doing, the first reported case of canine TB in South Africa was described,

a novel canine IGRA was developed, and a high level of *M. tuberculosis* infection in these animals was identified. This supports human data reflecting high levels of transmission of this pathogen during the course of human disease. Additionally, the fact that infected companion animals may progress to disease and potentially act as a source of human infection was highlighted. However, an attempt to adapt a flow cytometric assay to study cell-mediated immune responses during canine TB revealed the limitations of such studies in species in which the immune system remains poorly characterised.

The use of IGRAs to diagnose TB was further explored by adapting a human assay, the QuantiFERON-TB Gold (In-Tube Method), for use in non-human primates. These studies have shown that such an adaption allows for the sensitive detection of TB in baboons (*Papio ursinus*) and rhesus macaques (*Macaca mulatta*) and may be suitable for adaption for use in other species. However, they have also evidenced the limitation of this assay to specifically detect infection by *M. tuberculosis*.

Finally, to contextualise the occurrence of the mycobacterial infections described above, and other similar examples, these have been reviewed as an opinion piece.

Together, these investigations confirm that animal models will continue to make important contributions to the study of TB. More specifically, they highlight the opportunities that naturally occuring animal TB provides for the discovery of novel insights into this disease.

Opsomming

Die wêreldwye tuberkulose (TB) epidemie veroorsaak agt miljoen nuwe gevalle en twee miljoen sterftes jaarliks. Ingryping by die beheer hiervan vereis begrip van die biologie van die mikroörganisme *Mycobacterium tuberculosis*, die oorsaak van TB, asook van die patogenese van die siekte self. Hierdie kennis kan lei tot ontwerp van nuwe entstowwe en diagnostiese toetse wat gevolglik beide die oordrag- en vordering van die siekte mag bekamp.

Dieremodelle speel lankal 'n rol in ons begrip van die etiologie-, patogenese- en behandeling van TB. Insig is grotendeels verkry vanaf eksperimentele infeksiemodelle, en ontwikkeling van entstowwe, onder andere, is afhanklik van soortgelyke modelle. Desnieteenstaande, studies wat natuurlike TB voorkoms in diere ondersoek, byvoorbeeld dié wat op die ontwikkeling van interferon-gamma vrystellingstoetse (IGVT) fokus, het merkwaardige bydrae gemaak tot kennis en begrip in hierdie studieveld. Daar is slegs enkele soortgelyke voorbeelde. Om hierdie rede is die huidige studie uitgevoer waarbinne natuulike diere-TB geïdentifiseer en ondersoek is in Suid-Afrika om verdere kennis en insig te win aangaande TB.

Die "dassie bacillus", bekend om beduidend minder virulent te wees as *M. tuberculosis*, is tydens hierdie studie geïsoleer vanuit 'n klipdassie (*Procavia capensis*) in die Wes-Kaapse provinsie, Suid-Afrika. Insig in die wydverspreide voorkoms van hierdie organisme in klipdassie bevolkings is gevolglik verkry en verskaf momentum om die aard van verskil in virulensie tussen dié patogene te bestudeer.

Voorts is *M. tuberculosis* infeksie bestudeer in honde wat in kontak is met menslike TB pasiënte en word die eerste geval van honde TB dus in Suid-Afrika beskryf. In hierdie groep diere, is 'n hoë vlak van *M. tuberculosis* infeksie geïdentifiseer deur gebruik te maak van 'n nuut ontwikkelde IGVT vir die diagnose van honde TB. Gevolglik ondersteun dié studie bevindinge van menslike studies wat toon dat besondere hoë vlakke van *M. tuberculosis* oordrag voorkom gedurende die verloop van die siekte. Verder toon die studie dat geïnfekteerde troeteldiere 'n bron van menslike infeksie kan wees. 'n Poging om 'n vloeisitometriese toets te ontwikkel om die aard van selgefundeerde immuunreaksies te bestudeer in honde met TB toon die beperkings van dergelike studies in spesies waarin die immuunsisteem gebrekkig gekarakteriseer is.

Die gebruik van IGVT'e in die diagnose van TB is verder ondersoek deur 'n menslike toets (QuantiFERON-TB Gold, In-Tube Method) aan te pas vir die gebruik van nie-menslike primaat gevalle. Hierdie studies toon gevolglik dat so 'n aanpassing toepaslik is vir hoogs sensitiewe deteksie van TB in chacma bobbejane (*Papio ursinus)* en rhesus ape (*Macaca mulatta)*, en mag ook aangepas word vir gebruik in ander spesies. Tog word die beperkings van hierdie toets om infeksie wat spesifiek deur *M. tuberculosis* veroorsaak uitgelig.

Ter afsluiting word hierdie studie in konteks geplaas deur 'n oorsig te gee van bogenoemde- en soortgelyke gevalle van dierlike infeksie deur mikobakterieë in Suid-Afrika.

Hierdie studies bevestig dat dieremodelle steeds belangrike toevoegings maak tydens die bestudering van TB en lig veral die moontlikhede uit dat bestudering van natuulike TB in diere kan lei tot die ontdekking van nuwe insigte ten opsigte van die siekte self.

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CHAPTER 1

Introduction

1.1 Early animal models of tuberculosis

The disease tuberculosis (TB), classically typified by the "tubercle", or inflammatory granuloma, occurs in a broad range of animal hosts. It is caused by a group of closely related mycobacterial strains, such as *Mycobacterium tuberculosis* and *Mycobacterium bovis*, known collectively as the *Mycobacterium tuberculosis* complex (MTC). The infectious nature of TB, and the fact that it could be transmitted between species, was first proven in 1865, when Villemin transmitted the disease from human autopsy material to a rabbit[12]. However, depending on the species of host, and causative organism, a wide variety of disease outcomes are observed following infection by members of the MTC. As a result, interest in the comparative susceptibility of host species to these pathogens dates back to the first description of *M. tuberculosis* by Robert Koch in 1882, who reported on TB in a number of naturally infected host species and a wide range of experimental animals[26,31]. Subsequently, mice, rats, guinea-pigs and rabbits were established as common models for the study of the pathogenesis of this disease[26]. However, these species were regarded as generally susceptible to TB

and unusual examples of animal models of experimental infection, such as dogs, which were believed to be relatively resistant to TB, were periodically used[19,42].

A limited number of early studies examined comparative antibody responses during TB. In 1941 it was shown that following infection, rabbit strains which were relatively resistant to TB produced greater amounts of antibody, more rapidly, than more susceptible strains[36]. However, most early comparative studies focussed on differences in gross pathology, histopathology, and the rate of bacterial multiplication in the tissues of infected host species (or strains). In 1952, Lurie *et al.* compared the growth of *M. tuberculosis*, and the resulting pathology, in resistant and susceptible inbred rabbit strains, interpreting differences between the two as “all-or-nothing”[35]. In 1956, Francis compared the pathology induced in dogs, guinea pigs, rabbits and mice experimentally infected with *M. tuberculosis* or *M. bovis* and concluded that dogs exhibited a “native” resistance to the disease[19]. However, at these times, the limited insight into the specific mechanisms of the disease and almost non-existent tools for examining the immune response precluded a clearer understanding of the nature of these differences. Despite the development of more sophisticated experimental models, basic infection models are still utilised to examine the comparative susceptibility of various host species to disease. In 2002, infection experiments showed that racoons (*Procyon lotor*) were highly resistant to TB[50], and that they and North American opossums (*Didelphus virginiana*)[16] were unlikely to act as maintenance hosts of the disease in wildlife populations.

1.2 Elucidation of cell-mediated immunity to tuberculosis

In the 1970s, it was shown that the adoptive transfer of thymus-dependant lymphocytes (T cells) from *M. bovis* Bacille Calmette-Guérin (BCG)-vaccinated rats and mice, to naïve conspecifics, conferred immunity to the host animals, while the transfer of serum between these, did not[33,34]. Subsequently, this cell-mediated immunity (CMI) received greater attention in attempts to understand the nature of TB immunity. The identification of antigenic markers on phenotypically distinct immune cells, the so-called clusters of differentiation (CD) molecules, and the production of labelled antibodies against these have allowed for the study of the specific contribution of various cell types to TB-associated CMI.

The principle component of CMI, the T cell, recognises antigen via three classes of T cell receptor (TCR), namely the γδ receptor, CD4 receptor and CD8 receptor[5,30,51]. Unlike γδ cells, the majority of T cells only recognise foreign antigens presented by the major histocompatibility complex (MHC) molecules, or by the CD1 family of molecules, which are present on the surfaces of so-called antigen-presenting cells (APC)[6,30]. The MHC molecules present peptide antigens while the CD1 molecules present lipids and glycolipids. More specifically, $CD4^+$ or T helper (Th) cells recognise antigen presented in the context of MHC class II molecules, and upon activation, produce cytokines which regulate (i.e. help) the activity of other immune cells[30]. The classic example of such help is the production of interferon-gamma (IFN-γ) which enhances the killing of phagocytosed mycobacteria by macrophages[31]. Cytotoxic $CD8^+$ T cells, which recognise antigen presented in the context of MHC class I molecules, also produce cytokines, but additionally secrete granzyme and perforin which cause cell

lysis (i.e. cytotoxicity) of the specific APC[25]. This mechanism, too, contributes to the elimination of mycobacterium-infected cells[9]. To date, despite ever improving insight into the cellular and molecular processes involved in the immune response to mycobacteria, there is still a poor understanding of what factors contribute to the development of active mycobacterial disease, and what markers of immunity might be regarded as protective.

1.3 Lessons from comparative studies of cell-mediated immunity to tuberculosis

The basic model of the cells involved in CMI and the biology of their interactions has been derived from studies of humans and mice, however there can be great variation in these components between species. Unlike in humans and rodents, MHC class II molecules are expressed on resting T cells in a number of species, e.g. dogs and horses, and on resting T cells and granulocytes in rabbits, suggesting that these cells might be involved in antigen presentation to $CD4^+$ lymphocytes in these species[14]. In dogs, unlike other species, CD4 is strongly expressed on neutrophils[44], however the functional relevance of this is unknown[14]. In ruminants and pigs, two subpopulations of γδ cells exist, one of which is equivalent to those present in humans and mice, and one which is unique to each species[14]. Such species differences can have profound effects on the comparative immune response to specific diseases. For example, the group 1 CD1 molecules which present mycobacterial mycolic acids and lipoarabinomanan occur in humans and guinea pigs but not in mice[31]. With this in mind, conclusions drawn from animal models should be carefully considered before extrapolating these to other species. Nonetheless, such species differences may allow for the identification of specific factors which render host species either susceptible or resistant to TB disease.

In 1982, Thorns *et al* investigated TB immunity in experimentally infected guinea-pigs, rabbits, rats, ferrets and hedgehogs by comparing antibody responses, CMI (i.e. lymphoproliferative and tuberculin skin test responses), and the induced pathology in these species[59]. They found that in guinea-pigs and rabbits, intense CMI correlated with strong tissue reactions at the site of disease. Rats and hedgehogs displayed poorly detectable CMI and subclinical disease with no mortality. The authors concluded that the nature of the cell-mediated response was, in part, related to the comparative susceptibility to disease. In agreement, Dannenberg and Collins concluded that progressive disease in rabbits, mice and guinea-pigs was not due to increasing numbers of bacteria, but to the ongoing CMI response of the host[13].

Two studies, utilising immunohistochemistry techniques, investigated the lymphocyte phenotypes which were associated with granuloma formation in *M. tuberculosis*-infected mice and guinea-pigs. While granulomas in mice consisted largely of $CD4^+$ lymphocyte aggregations with $CD8^+$ lymphocytes scattered primarily around the periphery of the granuloma[23], those in guinea-pigs consisted of an apparently random mixture of $CD4^+$ and $CD8^+$ cells[60]. Such studies may shed light on the fact that mouse granulomas do not undergo caseous necrosis[53] while those of guinea-pigs do, with their subsequent development of pulmonary cavitations[10]. While $CD8^+$ T cells may contribute to necrosis of granulomas, other studies have pointed to their vital contribution to host protection[34,36]. In a study comparing the architecture of granulomas in rabbit strains, it was shown that the granulomas of inbred susceptible rabbits contained a lower frequency of $CD8^+$ T cells than did those of outbred controls[40]. In

agreement, Lyadova et al showed that significantly more CD8$^+$ T cells accumulated in lungs of resistant strains of mice than of susceptible strains during TB[37].

The growing availability of antibodies against cell markers for a wider variety of host species has meant that comparative studies of CMI have now become possible in hosts other than traditional laboratory animals. In a series of experiments, Waters *et al* examined *in vitro* lymphocyte proliferation following mycobacterial antigen stimulation of peripheral blood mononuclear cells (PBMCs) from *M. bovis*-infected cattle, reindeer (*Rangifer tarandus*) and white-tailed deer (*Odocoileus virginianus*)[63,62,64]. In white-tailed deer, CD4$^+$ lymphocytes proliferated in response to *M. bovis* purified protein derivative (PPD)[63], while this stimulus resulted in both CD4$^+$ and γδ TCR$^+$ lymphocytes proliferating in cattle[62]. A similar study in cattle also detected proliferation of predominantly CD4$^+$ lymphocytes, but also that of CD8$^+$ cells in response to both PPD and the mycobacterial proteins early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10)[39]. In reindeer, CD8$^+$ and γδ TCR$^+$ lymphocytes proliferated in response to ESAT-6/CFP-10[64]. Of interest is that disease progression in these species was markedly different. Reindeer presented as a distinctly resistant phenotype and the authors speculated that the *in vitro* findings of the study, i.e. the absence of a CD4$^+$ lymphocyte response and predominant CD8$^+$ T cell response, might represent a marker of a protective host response.

1.4 Lessons from comparative immunity to various members of the *Mycobacterium tuberculosis* complex

The MTC comprises genotypically closely related organisms which are phenotypically distinct, viz. *M. tuberculosis*, *Mycobacterium canettii*, *Mycobacterium africanum*, the dassie bacillus, the oryx bacillus, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, and *M. bovis*[29]. Members of the MTC reproduce clonally with limited evidence in strains other than *M. canettii* of the transfer of DNA or of DNA recombination[27,57,58]. It is therefore believed that sequential deletions of genetic material resulting in large sequence polymorphisms (LSP) have been the primary evolutionary events leading to the existence of the various MTC strains[46] which vary in geographical distribution, host preference and virulence[58].

Of these strains, *M. bovis* displays the greatest virulence, causing progressive disease in a wide variety of species following natural infection[48]. On the contrary, natural infection by *M. tuberculosis* apparently leads to disease much more rarely in most host species. Such differences in virulence between organisms have been exploited in order to more carefully understand immune responses to members of the MTC. Low-dose *M. tuberculosis* infection in rabbits, a species regarded as resistant to TB, results in negligible pulmonary disease and often in self-cure[28], and in rabbits infected with high doses of *M. tuberculosis*, disease is limited to the lungs[47]. However, *M. bovis* infection in rabbits results in extrapulmonary dissemination of disease[47]. This picture is mirrored in guinea pigs, a susceptible species, in which, in addition to severe lung pathology, lymphadenitis is a major element of *M. tuberculosis* infection[3]. Taken together, these studies suggest that protective immunological responses to pathogenic mycobacteria are

particularly poor in pulmonary tissues, while dissemination of disease acts as a marker of advanced immunological failure.

Variations in host susceptibility to other members of the MTC may contribute similar insights into the protective immunity to TB. *Mycobacterium microti* can cause severe disease in the field vole (*Microtus agrestis*)[7] yet experimental infection of this host with the dassie bacillus results in minimal pathology[56]. Interestingly, while these organisms display varying virulence characteristics in a single host species, both are believed to be primarily attenuated as a result of LSPs in the region of difference 1 (RD1) of the MTC genome[20,45]. As such, infection studies comparing host responses to these organisms may highlight aspects of immunological success or failure in mycobacterial disease.

1.5 Lessons from natural *Mycobacterium tuberculosis* complex infections in animals

Other than *M. tuberculosis*, the best known of the MTC stains is *M. bovis*, the primary causative organism of TB in cattle. The wide host range of *M. bovis* is evidenced by the fact that it has become endemic in numerous wildlife species[41,15]. Nonetheless, within natural multi-host systems, there is still great variation between hosts with respect to their susceptibility to this pathogen. For example, among ruminants in South Africa's Kruger National Park, members of the bovinae subfamily such as African buffalos (*Syncerus caffer*) and greater kudus (*Tragelaphus strepsiceros*) have proven highly susceptible hosts to *M. bovis*, maintaining, the disease indefinitely within infected herds[4,32]. In contrast, in this ecosystem, antelopes such as impalas (*Aepyceros capensis*),

while susceptible to TB, have not been reported to act as maintenance hosts of the disease[41,52]. Similarly, in England, free-living foxes which show evidence of exposure to *M. bovis* rarely progress to clinical disease, while badgers have been shown to be important maintenance hosts of this organism[15]. Natural *M. bovis* infection in cattle has also pointed to distinct variation in susceptibility to TB between breeds, with Holsteins showing a higher prevalence of infection, and increased severity of pathology compared to Zebu breeds[2].

Such variation between species provides an opportunity to more closely investigate immunological responses that might be associated with immunity to TB. As such, a limited number of studies have begun to explore these factors using material obtained from naturally infected animals. Utilising various cross-reactive anti-human and anti-dog antibodies, it was shown that in naturally infected badgers, as might be expected, $CD3^+$ lymphocytes were predominant in the peripheral cuff of tissue granulomas[8]. Of interest was the observation that IgA^+ and IgG^+ plasma cells were more common in larger than in smaller granulomas, suggesting that local (rather than generalised) disease conditions contributed substantially to the local recruitment, or proliferation, of specific cells. Also, these findings were consistent with those of an earlier study of naturally infected badgers which detected TB-specific antibody responses only in animals with advanced disease[38].

However, the limited availability of reagents suitable for use in comparative immunology has motivated the use of gene expression studies to detect cellular responses to TB. Fernández de Mera et al. (2008), investigating the expression of selected inflammatory and immune response genes in mesenteric lymph nodes of

naturally infected Iberian red deer (*Cervus elaphus hispanicus*), found 17 genes differentially expressed by either *M. bovis*-infected or non-infected animals[18]. Similarly, Galindo et al. (2009) investigated the expression of 20,400 genes in splenocytes of naturally *M. bovis*-infected and non-infected European wild boars[21]. They found that 14 of 17 differentially expressed immune genes were down-regulated in infected animals, suggesting TB-associated immune suppression in these individuals[21]. The population genetics of these animals was also shown to be associated with disease occurrence, with individuals with lower levels of heterozygosity appearing less capable of mounting protective responses to natural infection with *M. bovis*[1].

1.6 The application of cell-mediated immunity in the diagnosis of tuberculosis

Despite the relatively recent understanding of CMI, this component of the immune response to TB, in the form of the tuberculin skin test (TST), has long been the primary diagnostic immunological marker of mycobacterial infection and disease. First performed by Mantoux in 1908, the TST determines the inflammatory response to the intradermal injection of mycobacterial antigens in the form of purified protein derivative (PPD). The response is modulated by cytokines including interferon-gamma (IFN-γ), tumour necrosis factor (TNF) and lymphotoxin, which initially cause rapid infiltration of neutrophils and macrophages to the injection site[61]. However, by 48 hrs after antigen exposure, the cellular component consists primarily of T cells, of which the majority are PPD-specific $CD4^+$ T cells which have undergone local proliferation[17,61]. While the TST has formed the mainstay of immunological diagnosis

of TB in humans and in many animals, there is great variation in the sensitivity of the test between host species[11]. A range of TST-reactivity is observed among experimental animals with mice displaying a weak response to the TST while guinea pigs are strongly reactive[13]. The test has been rigorously evaluated in cattle[43], and its use in other animals is largely based upon knowledge of its use in this species. However, its clinical utility in many species is questionable, e.g. the European badger (*Meles meles*) and domestic dogs and cats, and it is inappropriate for TB screening of infected wildlife such as badgers and possums (*Trichosurus vulpecula*), both maintenance hosts of *M. bovis*[11].

More recently, the CMI response to TB has been exploited in the development of *in vitro* assays, the so-called interferon-γ release assays (IGRA). First described in 1990 for cattle[66], these tests quantify the secretion of IFN-γ by antigen-specific lymphocytes following the recognition of mycobacterial peptides by these cells. Field studies in cattle have variously determined the IGRA to be either more or less sensitive than the TST, however, studies have shown the use of both tests simultaneously to be useful for increasing the sensitivity of TB screening[67,65]. As a result, the test has been adapted for use in a wide range of hosts[11] including humans[49]. Animal adaptations of this assay have traditionally compared the IFN-γ response to *M. bovis* PPD and *Mycobacterium avium* PPD in order to specifically identify responses to MTC infection, while newer human assays have incorporated peptides simulating ESAT-6 and CFP-10, in place of PPD, as MTC-specific stimuli. Homologous genes encoding these proteins have been deleted from the BCG genome and are not present in the vast majority of

nontuberculous mycobacteria. As such, IFN-γ responses to these peptides are regarded as highly specific markers of infection by *M. tuberculosis* or *M. bovis*.

Although limited data is available on the use of IGRAs in species other than cattle, the test has shown promise for the diagnosis of TB in free-ranging wildlife such as African buffalo[24] and badgers[55], and in captive species in which the TST is unreliable such as monkeys[22] and cats[54].

1.7 Conclusion

Tuberculosis is a disease which affects a wide range of species to a greater or lesser degree and experimental infection studies, in several host species, have proven valuable for examining specific mechanisms of immunity to TB. The development of diagnostic tests in particular hosts have also shown enormous promise when adapted for use in other species. Thus, it can be expected that species-specific studies will continue to shed light on TB pathogenesis and diagnosis in general. As such, the identification and careful investigation of naturally occurring TB in species not conventionally used in experimental studies may contribute greatly to a more complete understanding of this disease. This thesis therefore seeks to explore the opportunities presented by such infections to gain greater insight into the aetiology, transmission, pathogenesis, and diagnosis of TB.

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CHAPTER 2

Pulmonary infection due to the dassie bacillus (*Mycobacterium tuberculosis* complex sp.) in a free-living dassie (rock hyrax – *Procavia capensis*) from South Africa

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2.1 Summary

We report a case of extensive necrogranulomatous pneumonia due to infection with the dassie bacillus (*mycobacterium tuberculosis* complex sp.) in a free-living pregnant adult female dassie (rock hyrax-Procavia capensis). A juvenile female dassie from the same colony also showed a focal lesion in the lungs suggestive of mycobacterial pneumonia. Our findings indicate the widespread occurrence of the dassie bacillus in free-living dassies and suggest very high infection rates in some populations. The introduction of South African dassies into novel environments should be considered in this light.

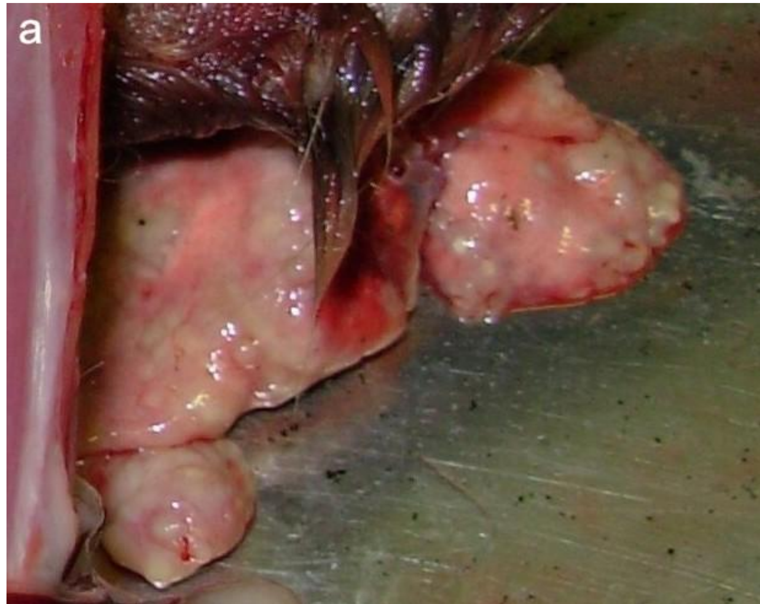
2.2 Keywords

Mycobacterium tuberculosis complex; dassie; hyrax; dassie bacillus

2.3 Case Report

In June 2006, two female dassies (*Procavia capensis*) were randomly chosen and euthanized by an accredited government conservation official on Dasklip Pass, Grootwinterhoek Mountains, Western Cape, South Africa (S32"53' 18.45; E19"01' 49.36), as part of an infectious disease survey of dassies. The dassies were sampled from a colony approximately 10 km from Porterville, the nearest human settlement. The animals were apparently healthy with no external injuries noted. Due to size difference and wearing of the teeth, it was determined that one dassie was an adult female while the other was a juvenile (under 3 years). Body condition was good in each female, weight was within the reported range for female *P. capensis* and all other signs were apparently normal. The adult female was identified as being pregnant with three foetuses.

Both were subjected to *post mortem* examination. Macroscopic lesions in the lungs of the pregnant adult female dassie consisted of numerous multifocal to confluent, round to irregular, 1–20 mm, dull whitish nodules distributed throughout the lung parenchyma with little normal lung tissue remaining (Figure 2.1a). The pleura was covered with similar slightly raised nodules and plaques. Many lesions showed central caseous necrosis with mild calcification. A few nodules showed mild central liquefactive necrosis with pus formation. The spleen showed similar lesions (Figure 2.1b).



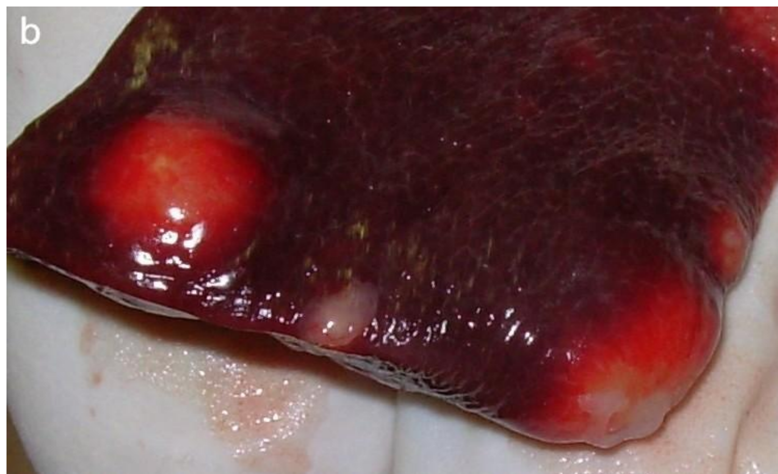


Fig 2.1 Extensive necrogranulomatous lesions observed in (a) the lungs and (b) the spleen of the adult female dassie.

The liver and placenta had a few small, multifocal, round, raised, dull whitish nodules, ≤ 1 mm. The lungs of the juvenile dassie contained a focal nodular lesion of 2mm in the cranial lobe of the left lung similar in appearance to the adult dassie. No other macroscopic lesions were detected in both animals. Samples from the lungs, spleen, liver, kidney and heart were collected from the adult dassie in 10% buffered formalin for microscopical examination. Fresh samples from the lung were collected aseptically from this animal for mycobacterial culture. No samples for histopathological examination or mycobacterial culture were collected from the juvenile dassie.

Microscopically, the lungs of the adult dassie showed numerous multifocal to confluent necrogranulomas throughout the parenchyma and the pleura. They consisted of a central area of caseous necrosis that was occasionally calcified. This was surrounded by a rim of moderate numbers of macrophages and epithelioid cells, and low numbers of multinucleated giant cells. Many macrophages and epithelioid cells contained single to multiple, small to large, round to oval, clear intracytoplasmic lipid vacuoles. There was an outer layer of lymphocytes and plasma cells within a mildly developed fibrous capsule. Numerous smaller, multifocal to confluent, often indistinctly outlined granulomas, without necrosis and calcification, consisting of vacuolated and normal macrophages and epithelioid cells, surrounded by lymphocytes and plasma cells were also visible. Some granulomas and necrogranulomas showed central infiltration of low to moderate numbers of neutrophils. Similar microscopic lesions were present in the spleen. The liver showed a few multifocal granulomas that consisted of moderate to large numbers of vacuolated and normal macrophages and epithelioid cells, surrounded by low to moderate numbers of lymphocytes and plasma cells. The centre of the liver

granulomas showed a small area of coagulation necrosis that was infiltrated by low numbers of neutrophils. Ziehl–Neelsen staining of the lung and spleen tissue revealed scanty medium-sized, slender, acid-fast bacilli in the cytoplasm of macrophages and epithelioid cells comprising the smaller granulomas, and those along the edge of the necrotic centre of necrogranulomas.

Affected lung tissue was prepared and cultured in triplicate with the BACTEC MGIT culture system (Becton Dickinson, USA) as previously described[16]. A multiplex polymerase chain reaction (PCR) test was performed on heat-killed culture lysates as previously described[16], and identified the organism as a member of the *Mycobacterium tuberculosis* complex with genomic characteristics consistent with the dassie bacillus. A subsequent PCR to detect a novel deletion in the dassie bacillus genome, $RD1^{das}$, using previously published primers[10], confirmed the isolation of this organism. Spoligotyping showed a pattern similar to that of a previous isolate of the dassie bacillus[14] and dissimilar to *M. tuberculosis* H37Rv, *Mycobacterium bovis* BCG, *Mycobacterium microti*[14] and *Mycobacterium africanum* subtype I[7] (Figure 2.2).

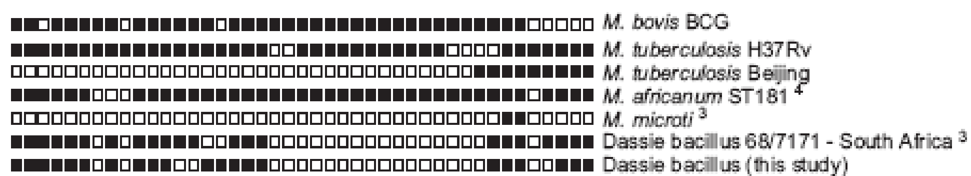


Fig 2.2 Spoligotype pattern for the dassie bacillus isolate is similar to a previously reported dassie bacillus spoligotype pattern and distinct from *M. tuberculosis*, *M. bovis* BCG, *M. microti* and *M. africanum* subtype I.

The *Mycobacterium* sp. defined as the dassie bacillus was first isolated from the lungs of a free-living dassie from Nieu Bethesda, in the Great Karoo, Eastern Cape, South Africa in the 1950s[15]. It is a member of the *M. tuberculosis* complex closely related to *M. microti* and *M. africanum* subtype Ia[2,6,12]. It has been more recently isolated from a few captive dassies and a single captive suricate (*Suricata suricatta*) all of which originated from unknown locations in South Africa[2,10,15] (Göran Bölske, personal communication), but it has not been found elsewhere. There are also no further reports of it being found in the wild since the original isolation, although no surveys have been conducted since then. It appears to have little virulence in rabbits or guinea pigs, species which are normally highly susceptible to pathogenic *Mycobacteria*[13,15].

The genome of the dassie bacillus has nine major regions of difference (RD) to that of *M. tuberculosis*, of which five are shared with *M. microti* and *M. africanum* subtype I[6,10]. A probably crucial difference is the small $RD1^{das}$ deletion in the ESX-1 region. This region, which contains the immunopathologically important T-cell antigens of the ESAT-6 gene family[4,9], has been extensively studied and all evidence suggests that it is implicated in virulence[3,5,8,11]. Other mycobacterial species which show virulence in limited hosts, e.g. *M. microti* in voles, also have RD1 deletions in the ESX-1 region[1,11].

Here we report that the dassie bacillus can be a pulmonary pathogen in free-living dassies. Whether this organism is sufficiently pathogenic to contribute in a meaningful way to the ecology of the dassie is currently unknown, but a study in this regard is probably justified as a general decline in dassie numbers has been observed over the last few years (unpublished observations). These animals live in colonies of up to 80 individuals, which are divided into smaller groups headed by one male and consisting

of up to 20 females with their young[17]. The large numbers living in close proximity in natural crevices of rocks or boulders, together with the fact that they spend a significant proportion of their time huddling together for warmth (they have a poorly developed internal temperature regulation), probably provides ample opportunity for the spread of the bacillus. The dassie has a relatively slow reproduction rate, giving birth to only two or three young after a 6–7 month gestation period[17]. Young are only sexually mature after 16 months, reach adult size at 3 years, and typically live about 10 years. The pregnant dassie in this case was severely infected despite previous observations that this mycobacterial species did not appear significantly virulent. This could perhaps be attributed to immune suppression during pregnancy, although more work would need to be done in this area. It may be significant, as it would specifically cause a decrease in the breeding population of dassies, with a subsequent decline in numbers, as has been observed over the last few years (unpublished observations). Future studies aimed at elucidating the association between the decline in the population and the prevalence of the dassie bacillus is currently planned.

It is clear from the captive suricate example that interspecies transmission of the dassie bacillus is possible. Whether the dassie bacillus can infect and affect predator species (of the dassie or suricate), such as eagles, caracal (*Felis caracal*) and leopard (*Panthera pardus*), has never been reported, although it seems possible as dassies and suricates are important prey species for these animals. The identification of the bacillus in an additional geographically distinct population of dassies (situated approximately 600 km from the original isolation in Nieu Bethesda) suggests the widespread occurrence of this organism in South Africa. The fact that random sampling of two free-living dassies

revealed infection in one and suspected lesions in the other, also suggests high rates of infection in at least the Dasklip population. These findings contrast markedly with the results of the Nieu Bethesda survey in which only 4 of 86 animals were found to have granulomatous lung lesions. Of these, the dassie bacillus was isolated from only a single animal[15]. Importantly, these findings, combined with the isolation of the organism from captive animals originally from South Africa, highlight the need for vigilance in preventing the translocation of mycobacterial pathogens during the movement of host species. No skin test assays have been done on dassies before, so we do not know whether this can be used as a diagnostic or survey instrument. However, given the limited genetic differences between the dassie bacillus and the rest of the members of the *M. tuberculosis* complex, the use of bovine or tuberculosis PPD would probably be justified. Further studies are underway to determine the relevance of this organism in the dassie population.

2.4 Acknowledgements

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CHAPTER 3

Pulmonary Mycobacterium tuberculosis (Beijing strain) infection in a stray dog

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3.1 Abstract

Mycobacterium tuberculosis infection in dogs is rarely reported and has not previously been documented in South Africa. A case of a stray Maltese crossbreed dog with extensive multifocal pulmonary tuberculosis due to *M. tuberculosis* is described. Pulmonary granulomas in this case were poorly encapsulated and contained large numbers of acid-fast bacteria, highlighting the potential for infected companion animals to excrete the pathogen. Treatment of canine tuberculosis is generally not advised, and for this reason, euthanasia of diseased animals must be advocated in most instances. Physicians and veterinarians must be aware that companion animals with active disease caused by *M. tuberculosis* could act as a potential source of infection.

3.2 Keywords

Canine, dog, *Mycobacterium tuberculosis,* tuberculosis, zoonosis.

3.3 Introduction

Mycobacterium tuberculosis is one of a number of closely related intracellular bacterial pathogens, grouped together as the *M. tuberculosis* complex (MTC) which cause granulomatous disease in a broad range of host species. It is the principal cause of human tuberculosis and the extraordinary success of this pathogen is reflected by its distribution. It is believed to infect a third of the world's population, causing 8 million new cases of human tuberculosis each year[7]. Despite the widespread occurrence of *M. tuberculosis,* infection by this organism is rarely diagnosed or maintained in free-living non-human hosts[1]. The precise nature of this apparent host-adaptation is unresolved, presumably involving aspects of both host physiology and ecology[19].

'Spillover' of *M. tuberculosis* infection to animals requires prolonged and close contact between humans and susceptible animal species and this scenario is classically illustrated by the prevalence of *M. tuberculosis* infection in zoo animals[17]. Companion animals living in close contact with tuberculosis patients represent a group at particular risk of high levels of exposure to this bacterium; however, cases of canine and feline tuberculosis are rarely described[9,22]. South Africa currently experiences an extremely high incidence rate for all forms of TB (600/100 000), suggesting that the risk of spillover of disease to contact animals will be significant in this country[24].As far as is known this is the 1st documented case of tuberculosis caused by *M. tuberculosis* in a dog in South Africa and the implications of this disease in this high-incidence setting are discussed.

3.4 Case History

An adult Maltese crossbreed dog was presented as a stray with an unknown history. The dog exhibited generalised alopecia and a multifocal superficial dermatitis. A skin scraping failed to identify a cause for the dermatitis. Clinical signs were judged as suggestive of *Sarcoptes scabei* infestation and the dog was treated with doramectin (Dectomax, Pfizer AH) at a dose of 200 μg/kg body weight. The following day the dog exhibited severe dyspnoea, of apparently acute onset, and died before further treatment could be implemented.

Gross abnormalities detected at *post mortem* examination were restricted to the respiratory tract. The lungs were generally congested and all lobes contained multifocal, pale grey areas of consolidation, 2–4mmin diameter. A single tracheobronchial lymph node that measured 12 mm in diameter was firm and pale yellow in appearance. Specimens from the lungs and bronchial lymph nodes were collected for histopathological examination. Lung sections showed multifocal to confluent necrogranulomas consisting of central coagulative to occasionally liquefactive necrosis that were infiltrated by low numbers of neutrophils. Some necrotic foci showed mild central calcification. The necrotic areas were surrounded by a moderately developed granulomatous layer consisting of large numbers of macrophages and epithelioid cells, moderate numbers of lymphocytes and plasma cells, and low numbers of fibroblasts, with the formation of an indistinct and poorly developed outer fibrous capsule (Fig. 3.1). Scanty Langhans' multinucleated giant cells were present. The remainder of the lungs showed moderate numbers of small multifocal to confluent granulomas consisting of macrophages, as well as widespread alveolar and interstitial infiltration of numerous

macrophages, lymphocytes and plasma cells, with moderate fibrinous oedema. There was mild to moderate epithelialisation of pneumocytes. The pleura was moderately thickened as a result of fibrosis and the infiltration of low numbers of macrophages, lymphocytes and plasma cells. Ziehl-Neelsen (ZN) staining revealed numerous acid-fast bacilli in the cytoplasm of macrophages and epithelioid cells of the necrogranulomas and granulomas, and in individual macrophages throughout the parenchyma (Fig. 3.2). The tracheobronchial lymph node sampled showed effacement of the normal architecture, which was replaced by extensive caseous necrosis with prominent central calcification. The area of necrosis was surrounded by large numbers of macrophages and epithelioid cells, moderate numbers of lymphocytes and plasma cells, low numbers of fibroblasts, and scanty Langhans' multinucleated giant cells. Numerous acid-fast bacilli were demonstrated, using ZN staining, in the cytoplasm of macrophages and epithelioid cells, as well as in the caseous necrotic centre.

Samples of the tracheobronchial lymph node, mesenteric lymph node, and lung tissue were homogenised separately, decontaminated and subjected to culture in BACTEC mycobacterial growth indicator tube (MGIT) medium containing polymixin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) (Becton Dickinson, USA) as previously described[23]. Acid-fast bacilli were identified in each culture. A multiplex polymerase chain reaction (PCR) test performed on heat-killed culture lysates, as previously described[23], identified the bacteria as *M. tuberculosis.* This isolate was shown to belong to the Beijing strain of *M. tuberculosis* by the IS*6110* restriction fragment length polymorphism (RFLP) genotyping technique, as previously described[18] (Fig. 3.3).

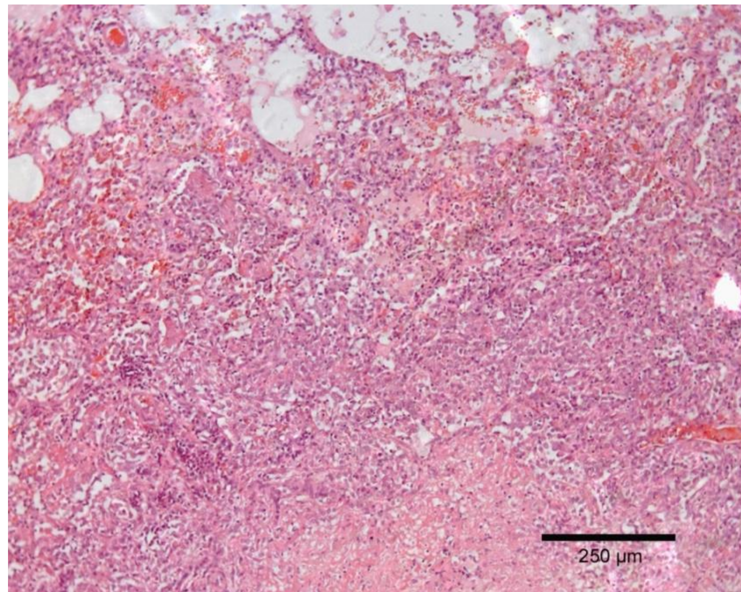

Fig. 3.1: Periphery of a typical pulmonary granuloma showing an indistinct division between the lymphocyte cuff and surrounding lung tissue (haematoxylin and eosin)

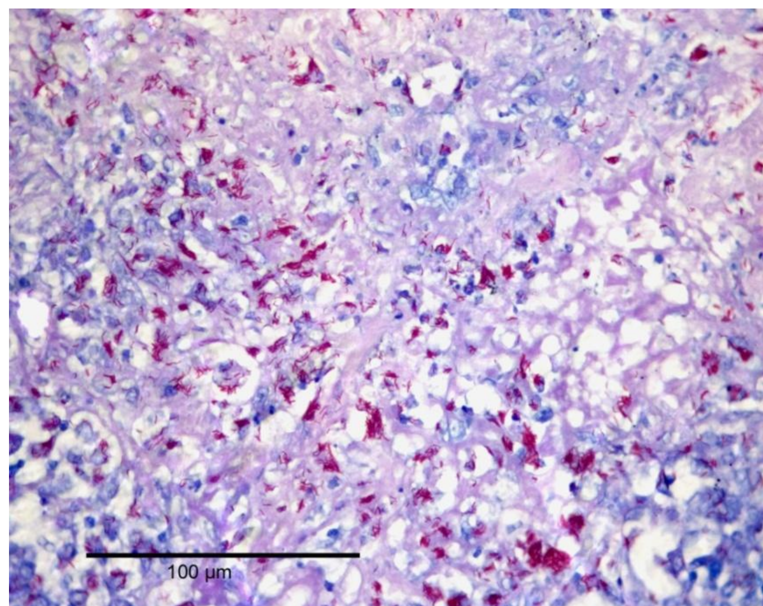

Fig. 3.2: Pulmonary granuloma showing numerous acid-fast bacilli (stained bright red), demonstrated by using Ziehl Neelsen staining.

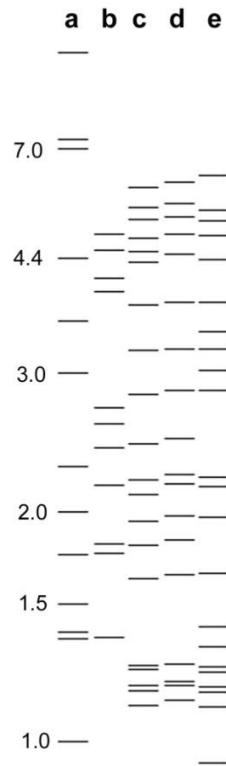

Fig. 3.3: IS*6110* restriction fragment length polymorphism patterns of (a) a reference strain of *M. tuberculosis* (Mt14323) with selected fragment lengths indicated in kilobases, (b) a non-Beijing strain of *M. tuberculosis*, (c, d) *M. tuberculosis* Beijing strains isolated from human subjects, and (e) the *M. tuberculosis* strain isolated in this case.

3.5 Discussion

Canine tuberculosis is rarely diagnosed worldwide and as far as could be established has not previously been reported in South Africa. In the present case, as is most commonly found in reported cases of canine tuberculosis, the causative organism was *M. tuberculosis,* and such infections have been associated with close contact between companion animals and human tuberculosis patients[9,14]. Notably in this case, the infection was caused by a Beijing strain of the pathogen, a genotype associated with high transmission rates and pathogenicity[15].

An estimation of the prevalence of canine TB in South Africa must be speculative. Human tuberculosis is most prevalent in resource-poor environments in which veterinary services are also rarely readily available. Given that the diagnosis of canine TB is time-consuming and relies on sophisticated and costly procedures, it will remain under-diagnosed. Reports from the 1st half of the 20th century estimated prevalence rates of canine TB, based on necropsy studies, at between 0.1 and 6.7 % (median 1.9 %) in various European cities[20]. These figures may approximate the scenario in South Africa, where the national incidence of human pulmonary TB[24] is more than double that of the European settings described above[2]. In the modern setting, however, antibiotic treatment of human TB, by reducing the severity and chronicity of disease, will probably affect the likelihood of disease transmission to contact animals. Also, differences in pet population age structures and differences in pet-owner interactions will affect the probability of transmission and disease in different settings. Additionally, control of *Mycobacterium bovis* has reduced the contribution of this pathogen to the incidence of canine TB[8].

Ante mortem prevalence rates in dogs in the European settings described above are recorded as varying between 0.04 and 1 %16 and 0.15 and 2 %[20]. These figures are spurious, however, as dogs often present with sub-clinical mycobacterial disease or with vague, non-specific symptoms which vary according to the organ systems affected. Clinical signs of canine tuberculosis are most commonly associated with respiratory disease and may include pyrexia, listlessness, inappetance, weight loss, a non-productive cough, retching, vomition and dyspnoea[16]. Non-respiratory signs can include diarrhoea, hepatomegaly, polyuria and polydypsia[16]. Radiographic evidence of canine TB can often be non-specific but may include signs of pleural and/or pericardial effusion, tracheobronchial lymph node enlargement, multifocal interstitial pneumonia (with a nodular to mixed nodular/alveolar pattern), hepatomegaly, splenomegaly and ascites[14].

The *ante mortem* diagnosis of canine TB is also complicated by the fact that dogs are poor responders to the intradermal tuberculin test commonly used in cattle and humans. In a recent study, 14 dogs experimentally infected with *M. tuberculosis* failed to respond to intradermal injections of *M. tuberculosis* and *M. bovis* purified protein derivative (PPD) tuberculin[3]. The use of higher concentrations of *M. tuberculosis* PPD in the form of Old Tuberculin, as used in the diagnosis of primate TB, may be more appropriate for eliciting a skin response[21]. Alternative *in vitro* diagnostic assays are available for a wide variety of host species, but their use in dogs has not been reported[4].

Definitive diagnosis of tuberculosis relies on histopathology of affected tissues and culture of the causative bacterium. The lesions of tuberculosis in carnivores differ from those in other species. Nonspecific granulomatous inflammation comprising

macrophages is the usual manifestation and the lesions often have a sarcomatous macroscopic appearance. Typical tubercles are not commonly found and caseation necrosis, calcification and giant cells are rare findings. Fibrous encapsulation and calcification are usually not present, while acid-fast bacilli may be numerous[6]. The present case generally exhibited similar pathology but microscopic caseation necrosis with calcification was quite prominent in the 1 tracheobronchial lymph node sampled for histopathology.

The treatment of canine tuberculosis must be considered in light of the fact that diseased animals present a potential source of human infection. The transmission of *M. tuberculosis* from companion animals to humans has not been described; however, the potential risk for zoonotic disease is apparent. This report has shown that infected dogs may carry high mycobacterial loads and granulomas may be poorly contained in these animals, allowing for the excretion of large numbers of bacteria (Figs 3.1 and 3.2). This is evidenced by the isolation of *M. tuberculosis* from laryngeal and rectal samples from 7 of 48 dogs and cats living in close contact with tuberculosis patients[13]. Also, Bonovska *et al.*(2005) have shown transmission of *M. tuberculosis* from infected to healthy dogs under experimental conditions[3].

Multiple-drug therapy must always be used in the treatment of tuberculosis in order to reduce the potential for the development of bacterial drug resistance. Current recommendations are a 6 to 9 month regimen combining a fluoroquinolone (*e.g.* enrofloxacin or ciprofloxacin) (5–15 mg/kg *per os,* daily), clarithromycin (5–10 mg/kg *per os,* daily) and rifampicin (10–20 mg/kg *per os,* daily)[10]. Alternatively, a combination of rifampicin (10–20 mg/kg *per os,* daily) isoniazid (10–20 mg/kg *per os,*

daily) and ethambutol (15 mg/kg *per os,* daily) given for 2 months followed by a combination of rifampicin and isoniazid for at least a further 4 months can be used[10]. Such lengthy treatments require intense commitment by the pet owner and this scenario presents a serious risk of noncompliance with treatment protocols. The dangers of this include the ongoing potential for zoonotic transmission of infection and importantly, the opportunity for the development of drug resistant mycobacterial strains. This is of particular importance for drugs such as the fluoroquinolones that are used in the treatment of recurrent and multi-drug resistant (MDR) TB in humans[5].Additionally, treatment may require careful monitoring for the development of severe side-effects to drugs such as isoniazid[11] and rifampicin[12]. For these reasons, it is widely believed that the treatment of canine TB is ill advised and euthanasia of diseased animals must be advocated in the majority of cases[8,21] (D Gunn-Moore, University of Edinburgh, pers. comm., 2007).

The implications of canine tuberculosis within the context of the South African human tuberculosis epidemic remain undefined. However, veterinarians and physicians must be aware of the potential for companion animals to act as reservoirs of *M. tuberculosis.* This is particularly true for veterinarians and veterinary staff working with animals from communities experiencing high levels of human tuberculosis.

3.6 Acknowledgements

The authors thank the management and staff of the Animal Anti-Cruelty League, Cape Town, and in particular Dr P. Naylor, for their contribution to the detection of this case.

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CHAPTER 4

The detection and epidemiology of *Mycobacterium tuberculosis* transmission to dogs in a high-risk setting

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4.1 Abstract

Mycobacterium tuberculosis infection in dogs can result in clinical tuberculosis (TB), and diseased companion animals pose a risk as a reservoir of human infection. Assessment of this risk is hampered by the fact that there are currently no validated immunological assays for diagnosing TB in this species. A limited postmortem canine TB survey was performed, and a canine TB-specific interferon-gamma release assay (IGRA) was developed to assess the prevalence of canine *M. tuberculosis* infection and disease in high-risk dogs. The prevalence of canine TB in a random sample of stray dogs was 1% (95% CI: 0-5%), while the prevalence of immunological sensitivity to *M. tuberculosis* in dogs in contact with smear-positive TB patients was 50%. Our findings suggest that the risk of progression to TB in *M. tuberculosis* infected dogs is low. Nonetheless, the risk of transmission of this pathogen from smear-positive TB patients to contact dogs is high and this should be considered when assessing the zoonotic risks associated with such companion animals.

4.2 Keywords

Canine, dog, interferon-gamma release assay, *Mycobacterium tuberculosis*, tuberculosis, zoonosis

4.3 Introduction

Mycobacterium tuberculosis, the primary agent of human tuberculosis (TB), is uncommonly isolated from cases of animal TB. However, natural infection by this organism can occur in a wide variety of animal hosts following close, prolonged contact with infectious humans[18]. Indeed, *post mortem* surveys performed in European cities in the first half of the 20th century determined the prevalence of canine TB as varying between 0.1 and 6.7 % (median 1.9%) with the majority of these being caused by *M. tuberculosis*[20]. South Africa currently experiences one of the world's highest national incidence rates for all forms of TB (600/100 000)[26] with extremely high levels of culture-positive pulmonary TB (1000/100 000) diagnosed in some settings[9].

In such environments, epidemiological investigations of both infection and disease have evidenced high levels of *M. tuberculosis* transmission between people[16,24], and it can be expected that companion animals living in such environments will be at high risk of infection by this pathogen. Infected animals can act as reservoirs of TB and transmission of this disease between dogs has been shown under experimental conditions[1]. However, TB is prevalent in resource-poor settings in which sophisticated veterinary services are generally unavailable and where cases of canine TB will remain largely undetected.

In addition to the lack of clinical data about canine TB, a comprehensive understanding of this disease is further limited by the absence of practical immunological tests for its diagnosis. These typically rely on the detection of antigen-specific T lymphocyte-mediated responses as surrogate markers of exposure to the causative organism[7,15]. This principle is employed in the *in vivo* tuberculin skin test (TST) which characterises the

inflammatory response to mycobacterial purified protein derivative (PPD). Similarly, the interferon-gamma (IFN-γ) release assay (IGRA) quantifies the *in vitro* release of IFN-γ by antigen-specific lymphocytes stimulated by mycobacterial peptides. In cattle, the TST has been adapted to determine either the response to a single injection of *Mycobacterium bovis* PPD (PPD^{bov}) or the comparative response to PPD^{bov} and *Mycobacterium avium* PPD (PPD^{av}) in order to distinguish between infection by *M. bovis* and environmental mycobacteria[7]. Historically, IGRAs have measured comparative responses to PPD^{bov} and PPD^{av} [7], while more recently, proteins largely specific to *M. tuberculosis* and *M. bovis* (early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10)) have been used in IGRAs to detect infection by these organisms[5,7]. No standard protocols exist for the TST in canines as it has long been believed that this test is unreliable in dogs[11] and that the use of regular *M. tuberculosis* and *M. bovis* PPD as TST stimuli are uninformative[1,21]. Additionally, there are no reports of the use of IGRAs in this species[6].

The aim of this study was to investigate and develop a suitable protocol (using the TST and IGRA) for identifying *M. tuberculosis* exposure in dogs, and to determine the prevalence of canine TB and the risk of *M. tuberculosis* transmission to dogs in a high-risk population in South Africa.

4.4 Materials and Methods

4.4.1 Post mortem survey

One hundred stray dogs euthanised at an animal welfare organisation servicing communities experiencing high transmission rates of TB in Cape Town, South Africa[24,16], were included in this study. A complete post mortem examination was performed on each animal and samples of retropharyngeal and ileo-caecal lymph nodes, lung tissue, and all abnormal tissues, were processed routinely for histopathology and stained with Haematoxylin and Eosin, and Ziehl-Neelsen (ZN) stains. As previously described, additional samples of these tissues were processed for mycobacterial culture[19]. Cultures were speciated by multiplex polymerase chain reaction (PCR)[25] and 16S ribosomal RNA gene sequencing[12], and genotyping of *M. tuberculosis* isolates was done by IS*6110* restriction fragment length polymorphism (RFLP) methods[23]. Culture of *M. tuberculosis* was regarded as definitive of *M. tuberculosis* infection.

4.4.2 Animals

Dogs at high risk of exposure to *M. tuberculosis* were identified as animals living on the same property as a person being treated for sputum smear-positive pulmonary TB. Tuberculosis patients were approached through an existing human TB research program of Stellenbosch University (SU) and written consent was given by owners to examine their dogs. Testing of animals was performed at the owner's home. Data relevant to the degree of exposure of pets to human patients was recorded: i.e. whether dogs had access to the patient's home, whether they slept in- or outside the home, and whether they had regular access to left-over human meals. Control animals, with a negligible risk of *M. tuberculosis* exposure (i.e. putatively unexposed), were identified through a private

veterinary practice situated in a high-income, low TB-incidence area, and owners of these gave consent for their inclusion in the study. Ethical approval to approach TB patients in contact with dogs was obtained from the SU Committee for Human Research, and to perform animal work, from the SU Committee for Experimental Animal Research.

4.4.3 Single and Comparative TST

Prior to the TST, dogs were sedated with medetomidine hydrochloride (30 µg/kg, i/m) (Domitor, Novartis SA Animal Health, Isando, South Africa). The TST was performed by intradermal injections of 0.1 ml of a 50% solution (in phosphate buffered saline (PBS)) of *M. tuberculosis* Old Tuberculin (OT) (1250 IU) (Statens Serum Institut, Copenhagen, Denmark) in the right medial thigh and 0.1 ml of a 50% solution of *M. avium* PPD (WDT, Hoyerhagen, Germany) (1250 IU) in the left medial thigh. A 50% solution was used as optimisation of the testing protocol showed that intradermal injection of full-strength OT resulted in a moderate non-specific dermatitis, with focal necrosis at the injection site. Sedation was reversed with Atipamezole hydrochloride (150 µg/kg, i/m) (Antisedan, Novartis AH). Sites of the TST were examined after 72 hours and the diameter of any ensuing tissue reaction was recorded. A cut-off of 5 mm between positive and negative responses, for either the comparative or single TST, was predicted based on previous studies of intradermal skin testing in dogs[4,21].

4.4.4 Interferon gamma release assay

At the time of the TST, blood was collected into Vacutainer® tubes containing sodium heparin (Becton Dickinson, Plymouth, England). Within two hours of collection, blood was diluted 1:5 in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml

penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, Kempton Park, South Africa). Aliquots of the whole blood suspension were incubated in triplicate in 96-well plates (200 µl/well) with 10 µg/ml PPD^{Av} (Statens Serum Institut (SSI), Copenhagen, Denmark), 10 µg/ml *M. tuberculosis* PPD (PPD^{Tb}) (SSI), 10 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich), and in medium alone, respectively. Additionally, whole blood suspensions from 13/24 TB-exposed dogs and all control dogs were incubated with 10 µg/ml recombinant ESAT-6/CFP-10 fusion protein from *M. tuberculosis* (a kind gift from T. Ottenhoff, Leiden University). Following 5 days of incubation, supernatants from each treatment group were harvested, pooled, and stored at -20°C. Interferon-gamma concentrations ([IFN-γ]) of pooled samples were determined using the Quantikine® canine IFN-γ enzyme-linked immunoassay (ELISA) kit (R&D Systems, Minneapolis, USA). Antigen-stimulated samples were assayed in duplicate. For each animal, the [IFN-γ] of the unstimulated sample ($[\text{IFN-}\gamma]^{nil}$) was subtracted from those of each stimulated sample to obtain final IFN-γ response results, i.e. $[\text{IFN}]^{esat6/cfp10}$, $[\text{IFN}]^{tb}$, $[\text{IFN}]^{av}$, and $[\text{IFN}]^{pha}$. Comparative IGRA results were calculated as $[\text{IFN}]^{tb}$ - $[\text{IFN}]^{av}$.

4.4.5 Statistical analysis and determination of IGRA cut-offs

Differences in $[\text{IFN}]^{esat6/cfp10}$ between the TB-exposed and control groups were analysed using the unpaired t-test. Differences in comparative IGRA responses between these groups were similarly analysed by comparing their relative PPD^{tb} responses (calculated as: $[\text{IFN-}\gamma]^{tb}$ / $[\text{IFN-}\gamma]^{tb}$ + $[\text{IFN-}\gamma]^{av}$).

The cut-off between positive and negative [IFN]$^{esat6/cfp10}$ was defined as the upper limit of the 95% CI of the [IFN-γ]$^{esat6/cfp10}$ values of all control animals. Due to the specificity of the ESAT-6/CFP-10 assay[3,5] this test was used as a gold standard to determine cut-off values for the comparative IGRA using an ROC curve analysis.

4.5 Results

4.5.1 Post mortem survey

Acid-fast (ZN positively-stained) bacteria were cultured from tissue samples from six animals. Four cultures were confirmed as *M. tuberculosis* (4% prevalence of infection, 95% CI: 1-10%) (Fig. 4.1), one as *Mycobacterium intracellulare*, and one as a *M. avium* species. Macroscopic and microscopic lesions consistent with TB were detected in a single culture positive animal (described by Parsons *et al.*[19]) (1% canine TB disease prevalence, 95% CI: 0-5%).

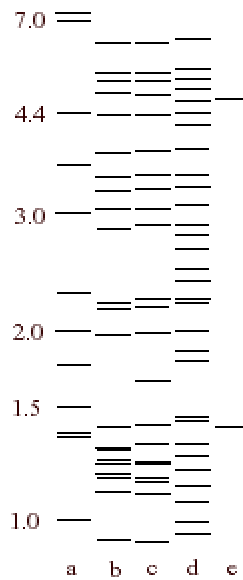

Fig. 4.1 IS*6110 Mycobacterium tuberculosis* restriction fragment length polymorphism patterns of (a) a reference strain, with selected fragment lengths indicated in kilobases, (b) the strain isolated from a dog with clinical tuberculosis, and (c, d, e) strains isolated from dogs with no gross or histopathological lesions of TB.

4.5.2 Animals

Of 23 TB-exposed animals, 18/23 (78%) slept outside the home. However, 12/23 (52%) had some access to the home, and 19/23 (83%) received remains of human meals.

4.5.3 Tuberculin skin testing

Of 17 TB-exposed dogs tested, 14 had OT TST responses of less than 5 mm, and none had responses greater than 10mm. In 12 of these animals, the PPD^{av} TST gave no response at all, while 2 had responses greater than 5 mm. The limited TST responses to both OT and PPD^{av} precluded further analysis of these data.

4.5.4 Interferon-gamma release assay

In vitro IFN-γ responses to mycobacterial PPD and ESAT-6/CFP-10, and the IGRA results for selected animals are presented in Tables 4.1 and 4.2. For this study, mean $[\text{IFN-}\gamma]^{nil}$ was 8 pg/ml (S.D. +/-22 pg/ml) and $[\text{IFN-}\gamma]^{pha}$ was greater than 500 pg/ml for all samples. A positive ESAT-6/CFP-10 IGRA result was calculated as $[\text{IFN}]^{esat6/cfp10} >$ 200 pg/ml. Using an ROC analysis, with the ESAT-6/CFP-10 IGRA as a gold standard, a cut-off for the comparative IGRA result was calculated as 161 pg/ml (sensitivity 0.75, specificity 0.9, AUC = 0.93). Mean $[\text{IFN}]^{esat6/cfp10}$ and mean relative PPD^{tb} responses were significantly higher in TB-exposed dogs than un-exposed dogs ($p < 0.05$) (Figs 4.2 and 4.3). These statistics gave us confidence to accept a positive result from either IGRA (according to the criteria described) as a true marker of immunological responses to *M. tuberculosis* in dogs. As such, the prevalence of *M. tuberculosis* infection in dogs in South Africa was determined as 12/24 (50%) within a high risk population and 1/16 (6%) within a population at low risk of exposure to TB.

Table 4.1 IFN-γ concentrations in antigen-stimulated blood samples, and IGRA results, for selected putatively TB-unexposed dogs.

animal ID	IFN-γ (pg/ml)				IGRA result	
	$[IFN]^{tb}$	$[IFN]^{av}$	$[IFN]^{tb-av}$	$[IFN]^{esat6/cfp10}$	Comparative assay[1]	ESAT-6/CFP-10 assay[2]
C1	89	159	-70	89	-	-
C2	262	905	-643	125	-	-
C3	464	1414	-950	192	-	-
C4	0	262	-272	132	-	-
C5	120	119	1	55	-	-
C6	1561	813	748	191	+	-
C7	248	185	63	71	-	-
C8	100	187	-87	126	-	-
C9	6	109	-103	27	-	-
C10	356	195	161	25	-	-
C11	71	145	-74	15	-	-
C12	537	1189	-652	104	-	-
C13	110	614	-504	33	-	-
C14	160	7	153	60	-	-
C15	1967	1893	74	45	-	-
C16	100	2	98	68	-	-

-, negative = [1]$[IFN]^{tb-av} < 161$ pg/ml; [2]$[IFN]^{esat6/cfp10} < 200$ pg/ml

+, positive = [1]$[IFN]^{tb-av} \geq 161$ pg/ml; [2]$[IFN]^{esat6/cfp10} \geq 200$ pg/ml

Table 4.2 IFN-γ concentrations in antigen-stimulated blood samples, and IGRA results, for selected TB-exposed dogs.

	IFN-γ (pg/ml)				IGRA result	
animal ID	$[IFN]^{tb}$	$[IFN]^{av}$	$[IFN]^{tb-av}$	$[IFN]^{esat6/cfp10}$	Comparative assay[1]	ESAT-6/CFP-10 assay[2]
E1	130	212	-82	ND	-	ND
E2	390	382	8	ND	-	ND
E3	0	143	-143	ND	-	ND
E4	89	120	-31	ND	-	ND
E5	2874	1528	1346	ND	+	ND
E6	141	239	-98	ND	-	ND
E7	1159	224	935	ND	+	ND
E8	161	134	27	ND	-	ND
E9	0	0	0	ND	-	ND
E10	605	416	189	ND	+	ND
E11	131	82	49	ND	-	ND
E12	470	72	398	64	+	-
E13	58	169	-111	98	-	-
E14	35	0	35	0	-	-
E15	1676	1545	131	2242	-	+
E16	249	266	-17	197	-	-
E17	4540	108	4432	4104	+	+
E18	216	0	216	265	+	+
E19	1593	992	601	574	+	+
E20	1453	136	1317	503	+	+
E21	1133	438	695	1156	+	+
E22	3758	68	3690	4642	+	+
E23	0	0	0	0	-	-
E24	294	189	105	361	-	+

-, negative = [1]$[IFN]^{tb-av} < 161$ pg/ml; [2]$[IFN]^{esat6/cfp10} < 200$ pg/ml

+, positive = [1]$[IFN]^{tb-av} \geq 161$ pg/ml; [2]$[IFN]^{esat6/cfp10} \geq 200$ pg/ml

ND, not done

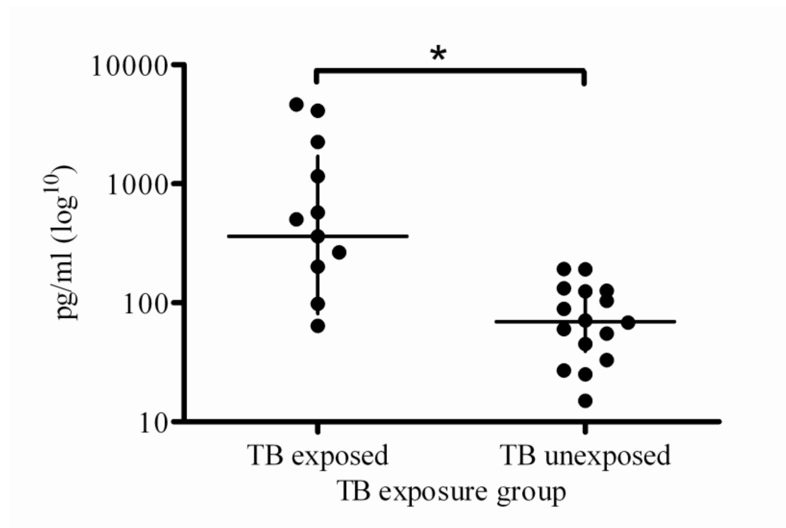

Fig 4.2 ESAT-6/CFP-10 stimulated whole blood interferon gamma responses in TB-exposed and unexposed dogs showing significant differences in these ($p < 0.05$).

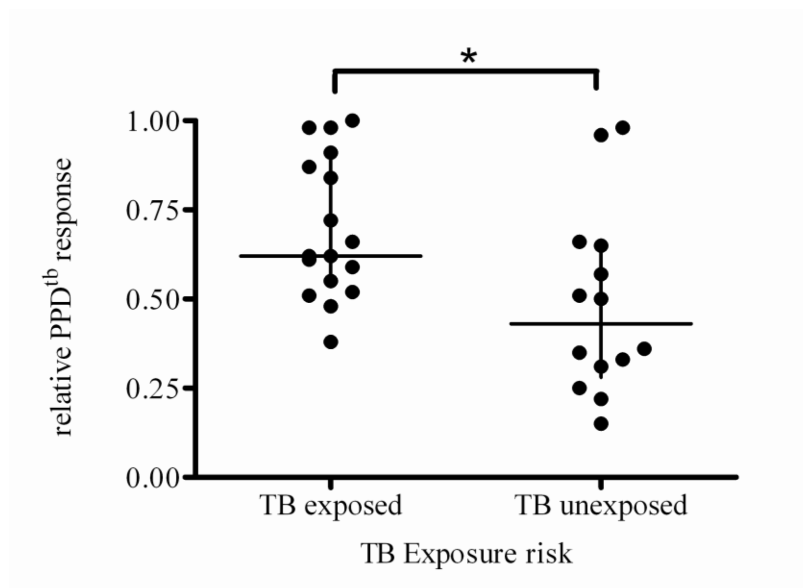

Fig 4.3 Relative PPD^{tb} interferon gamma responses ($[IFN\text{-}\gamma]^{tb}$ / $[IFN\text{-}\gamma]^{tb}$ + $[IFN\text{-}\gamma]^{av}$) from whole blood assays performed on TB-exposed and unexposed dogs showing significant differences in these ($p < 0.05$).

4.6 Discussion

In order to investigate the prevalence of canine TB in an urban South African setting, and to mitigate possible ethical and emotional concerns surrounding such a study, we performed a *post mortem* survey of stray dogs presented to an animal welfare organisation. Such sampling is not subject to the bias inherent in examining owner-presented animals only, and its value is supported by the fact that we have identified the first documented case of canine TB in South Africa[19]. The prevalence of this disease in the South African context (1%, 95% CI: 0-5%) is similar to those in European cities, as determined at necropsy, in the early part of the 20th century (median 1.9%, range 0.1 to 6.7%)[20]. Indeed, the higher historical European values are attributable, to some degree, to the prevalence of *M. bovis* infections in these settings[20].[7] These data are from randomly sampled animals and it should be noted that the prevalence of TB in dogs with known TB exposure can be expected to be higher.

In addition, we cultured *M. tuberculosis* from lymph nodes of 3/100 dogs with no gross or histopathological lesions of TB. Similarly, *M. bovis* was cultured from the carcases of 23/756 foxes (*Vulpes vulpes*) in a study which identified only a single case of gross TB[8] and from 3/62 coyote (*Canis latrans*) carcasses in a study in which no animals presented with macroscopic TB lesions[2]. These data indicate that in canids, true infection levels by pathogenic mycobacteria can be substantially higher than those determined by the detection of gross pathology at necropsy. *Mycobacterium tuberculosis* strains identified in this study (Fig 1) were representative of strains commonly associated with human infection in Cape Town[22].

To assess *M. tuberculosis* infection rates in dogs, we made use of TB tests widely used and well characterised in other species[6,7]. In the majority of tested animals, intradermal injection of 0.1 ml of a 50% solution of OT in PBS (1250 IU) resulted in inflammatory responses of 4 mm in diameter or less, and did not allow for a clear distinction between responses. Also, TST results were inconsistent with those of the IGRA. Together, these findings support those of other studies which have found the TST ineffective in dogs[1,11].

Additionally, using a novel IGRA to detect immunological sensitivity to *M. tuberculosis* antigens in dogs, we identified a 50% infection rate in dogs in contact with smear-positive TB patients. While overnight whole blood IGRAs have been successfully validated in a number of species, we chose to stimulate blood cultures for 5 days, since longer culture periods have been shown to be more sensitive for the detection of antigen-specific IFN-γ responses[5]. The IGRA has a number of advantages over the TST. In our experience, sedation of dogs was required to perform the TST, while this is not the case for the IGRA. Furthermore, animals need only to be examined once, test outcomes are objective, the test can be designed to be highly specific by using well-defined antigens, and the test allows for the inclusion of positive and negative controls. The significant distinction in IFN-γ responses between TB-exposed and control animals is indicative that the assay detects true infection in dogs ($p < 0.05$) and a significant agreement between the ESAT-6/CFP-10 and comparative IGRA results implies that this test is robust. As such, this assay appears to be highly suited for use in canines, and may prove useful in future investigations.

Many reports of canine TB highlight intimacy of contact between owners and their pets as an important risk factor for TB transmission. Hawthorne *et al.*, in a study

investigating animals living with TB patients in Edinburgh, found that 37/37 (100%) of dogs lived in the owner's house and 18/37 (51%) slept in the same room as the owner[13]. However, we have shown that in a setting where contacts between pets and humans are less intimate than previously described[10,13,17], levels of *M. tuberculosis* transmission may remain high.

This study has identified high levels of *M. tuberculosis* transmission to dogs living in contact with smear-positive TB patients and a canine TB prevalence rate of 1% in an urban South African setting. Our findings indicate that in many cases, the risk of transmission of *M. tuberculosis* infection, from humans with pulmonary disease to contact animals, should be regarded as high. In humans, fewer than 10% of infections result in clinical disease[15] and our data supports previous experimental and clinical findings that indicate that the risk of progression to active TB in infected dogs is similarly low[11,14,21]. Nonetheless, this risk must be evaluated on a case-by-case basis, as diseased animals could act as reservoirs of human infection. Such consideration is of particular importance where animals are living in contact with high-risk individuals such as children or immunocompromised patients, or have been exposed to multidrug-resistant strains of *M. tuberculosis*.

4.7 Acknowledgements

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CHAPTER 5

Development of an *in vitro* lymphoproliferative assay for the characterisation of the canine immune response to tuberculosis

5.1 Abstract

Specific lymphocyte response patterns during tuberculosis (TB) may reflect markers of host susceptibility or resistance to this disease. In order to characterise the canine cell-mediated immune response during TB, the possibility of adapting a flow cytometric lymphoproliferative assay used in various ruminant species was explored. A combination of direct and indirect cell labelling techniques was developed to allow multi-colour flow cytometric analysis of canine PBMCs. Significant neutrophil contamination of these cells was identified as a limitation for the development of the proliferative assay.

5.2 Introduction

Cell-mediated immunity (CMI) has been recognised as the major component of the protective immune response to tuberculosis (TB)[8]. As such, a clearer understanding of the mechanisms of this response may allow for a more rational approach to the development of new vaccines and diagnostic assays, or the identification of markers of host susceptibility or resistance, to this disease. One such field of inquiry has been the elucidation of the contribution of the various lymphocyte subsets to the CMI of TB. Classically, $CD4^+$ T-helper (Th) 1 lymphocytes have been regarded as central to the protective immunity to TB[8], however, more recently, focus has been placed on $CD8^+$ cytotoxic lymphoctes as being important in this response[7].

In a series of studies, Waters et al. investigated lymphoproliferative responses to various *M. bovis* antigens in experimentally-infected white-tailed deer (*Odocoileus virginianus*), cattle, and reindeer (*Rangifer tarandus grantii*)[15,16,17]. They reported that in reindeer, a species that is regarded as being resistant to TB[11], $CD8^+$ and γδ T cell receptor $(TCR)^+$ lymphocytes were the predominant cells which proliferated on exposure to *M. bovis* antigens[17]. In contrast, $CD4^+$ and γδ TCR^+ lymphocytes in cattle[15], and $CD4^+$ lymphocytes in white-tailed deer[16], both regarded as species which are susceptible to TB[17,12], were the predominant lymphocyte subsets responding to these antigens. They have speculated that the predominant $CD8^+$ lymphoproliferative response, in the absense of a significant $CD4^+$ response, might represent a marker of resistance to TB[17].

Tracking of proliferation of specific lymphocyte subsets is possible by flow cytometric analysis of cell division. One such protocol requires the isolation of peripheral blood

mononuclear cells (PBMCs) followed by the marking of these with an intercalating fluorescent dye, such as PKH26. Cell proliferation can then be detected as a reduction, by half, in the fluorescence of daughter cells. The isolation of human PBMCs is most commonly done by the centrifugation of diluted whole blood over a solution of polysucrose and sodium diatroziate Ficoll-Hypaque with a density of 1.077 g/ml[3,13]. This achieves the separation of red cells (1.093 – 1.096 g/ml) and granulocytes (1.087 – 1.092 g/ml) from lymphocytes (1.070 g/ml)[13]. The use of this technique has been reported in canine studies. However, using this technique, high levels of granulocyte contamination of PBMCs were noted by Bonnefont-Rebeix et al. (2006)[2], and the inconsistency in canine PBMC separation protocols in the published literature suggests poor reproducibility of this procedure[9,14].

Buurman et al. (1982) measured the mean densities of canine lymphocytes (1.071 g/ml) and neutrophils (1.077 g/ml) and concluded that the limited difference between these precludes the simple adoption of human PBMC separation protocols for use with canine samples[4]. Further complicating the development of such a protocol were their findings of substantial variation in cell densities between individual animals, and the fact that some individuals showed transient changes in granulocyte densities over time. Nonetheless, it has been reported that separation of canine PBMCs over a diluted solution of FH (1.070 g/ml) was effective in reducing the degree of neutrophil contamination[2].

A further complication of flow cytometric analysis of canine PBMCs is the limited availability of suitably labelled antibodies directed against canine leukocyte epitopes. As such, studies describing normal ranges of canine lymphocyte subsets have only used

single-parameter staining protocols[1,5,6].Such limitations preclude the detailed analysis of canine immune responses.

This study aimed to optimise the isolation of canine PBMCs and develop a methodology for the flow cytometric characterisation of the canine adaptive immune response to *M. tuberculosis* by investigating modifications to the standard immunological techniques discussed above.

5.3 Materials and Methods

5.3.1 Animals

Twenty dogs were used in this study. They were both male and female, of various ages and breeds, privately owned and randomly selected from households with or without a primary human TB case. Ten ml of blood was obtained by venipuncture of the cephalic or jugular vein and transferred to Vacutainer tubes containing sodium heparin (Becton-Dickinson, Franklin Lakes, NJ, USA). Ethical permission for the study was obtained from the Committee for Experimental Animal Research of the Faculty of Health Sciences, Stellenbosch University (Ref: P05/08/011).

5.3.2 Multi-parameter flow cytometric phenotyping of canine lymphocyte subsets

Canine PBMCs were incubated with unlabelled mouse IgG anti-dog CD4, mouse IgG anti-dog CD8, or mouse IgG anti-dog γδ T cell receptor (Leukocyte Antigen Biology Laboratory, University of California, Davis, CA, USA)) at 4°C for 30 min. Subsequently, these cells were sequentially incubated with Cyanine 5 (Cy5) conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL, USA) and fluorescein

isothionate (FITC) conjugated rat anti-dog CD5 (Serotec, Kidlington, UK), a canine lymphocyte marker, under the same conditions. Following each incubation step, cells were washed twice with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Quantification of Cy5- and FITC-labelled cells was done on a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ,USA).

5.3.3 Characterisation of neutrophil contamination of canine PBMCs separated over Ficoll-Hypaque

Canine PBMCs were resuspended in RPMI 1640 (Sigma-Aldrich, Saint Louis, MO, USA) and 10 µl of this suspension was diluted in 10 µl of Trypan Blue (Sigma-Aldrich). Ten µl of this suspension was loaded onto a haemocytometer and after 2 min. (in order to allow for cell adherence to the cover slip), cells were visualised at 40 x magnification. Differential counts of adherent and non-adherent cells were made according to cell morphology (Table 5.1).

Additionally, PBMC subsets were analysed by flow cytometry by direct and indirect two-colour fluorescent labling of cells using anti-CD5 and anti-CD4 antibodies as described above. Neutrophils were identified as $CD5^{-}/CD4^{+}$cells and relative numbers of these cells were recorded.

Table 5.1 Morphological characterisation of canine leukocytes isolated over Histopaque-1077

Cell type	Morphological description
Unadherent	Small (approx. 10 μm in diameter), round to oval, bright cell
Adherent	Large (> 40 μm in diameter), dark, spreading cell with "fried-egg appearance"

5.3.4 Optimisation of canine PBMC isolation from whole blood

For each animal, ten ml of blood was diluted 1:1 in phosphate-buffered saline (PBS). Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA) was diluted 95:5 and 9:1 with sterile water to obtain Ficoll-Hypaque solutions with densities of 1.073 and 1.069 g/ml, respectively. Subsequently, conditions for the isolation of PBMCs from randomly selected blood samples were varied according to Ficoll-Hypaque density (1.077, 1.071, and 1.069 g/ml), centrifugal force applied (350, 500, 1200 x g), and duration of centrifugation (20, 30, and 40 min.). In all cases, diluted blood was layered over Histopaque-1077 solutions and centrifuged at room temperature. Using a Pasteur pipette, PBMCs were harvested from the interface of the PBS/plasma and Histopaque-1077 fractions and washed three times in PBS. For selected samples, relative PBMC counts and relative neutrophil counts were visually made as described above.

5.3.5 PKH26 staining of PBMCs for lymphoproliferation studies

Briefly, PBMCs (2 x 10^7) were suspended in 1ml of Diluent C (provided in the PKH26 kit) and added to 1ml of a 4 x 10^{-6} molar PKH26 solution (Sigma-Aldrich, Saint Louis, MO, USA), a fluorescent dye which is incorporated into the lipid regions of the cell membrane. Following incubation at room temperature for 4 min., PKH26 staining was stopped by addition of 2 ml 1% BSA-PBS. Cells were then washed 3 times in RPMI 1640 before resuspension at 1 x 10^6/ml in RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). Aliquots of the cell suspension were incubated in 48-well plates with either 10 µg/ml phytohaemagglutinin (PHA) or in medium alone for 5 days. Following incubation, cells were harvested, washed, and prepared for flow cytometric analysis.

5.4 Results

5.4.1 Multi-parameter flow cytometric phenotyping of canine lymphocyte subsets

Two-colour labelling of canine PBMCs was achieved by a combination of indirect and direct methods. Such labelling allowed for highly reproducible two-parameter phenotypic analysis of these cells with insignificant cross-reactivity of the secondary antibody (Fig 5.1). In a randomly selected animal, differential $CD4^+$ lymphocyte counts in PBMC samples obtained under 3 different separation conditions were 32%, 33%, and 33%, respectively (Fig 5.1).

5.4.2. Characterisation of neutrophil contamination of canine PBMCs separated over Ficoll-Hypaque

In all cases, flow cytometric analysis of canine PBMCs revealed a significant number of $CD5^-/CD4^+$ neutrophils in this population (e.g. Fig 5.2). For all samples, relative adherent cell counts determined using a haemocytometer were approximately half those of the relative neutrophil counts obtained by flow cytometric analysis, and were uncorrelated with monocyte counts (Table 5.2). While these data do not specifically identify the adherent cells as neutrophils, it confirms that the adherent cell count can be used as a useful proxy for neutrophil contamination of PBMCs. This technique was used subsequently in evaluating and optimising separation conditions for canine PBMCs.

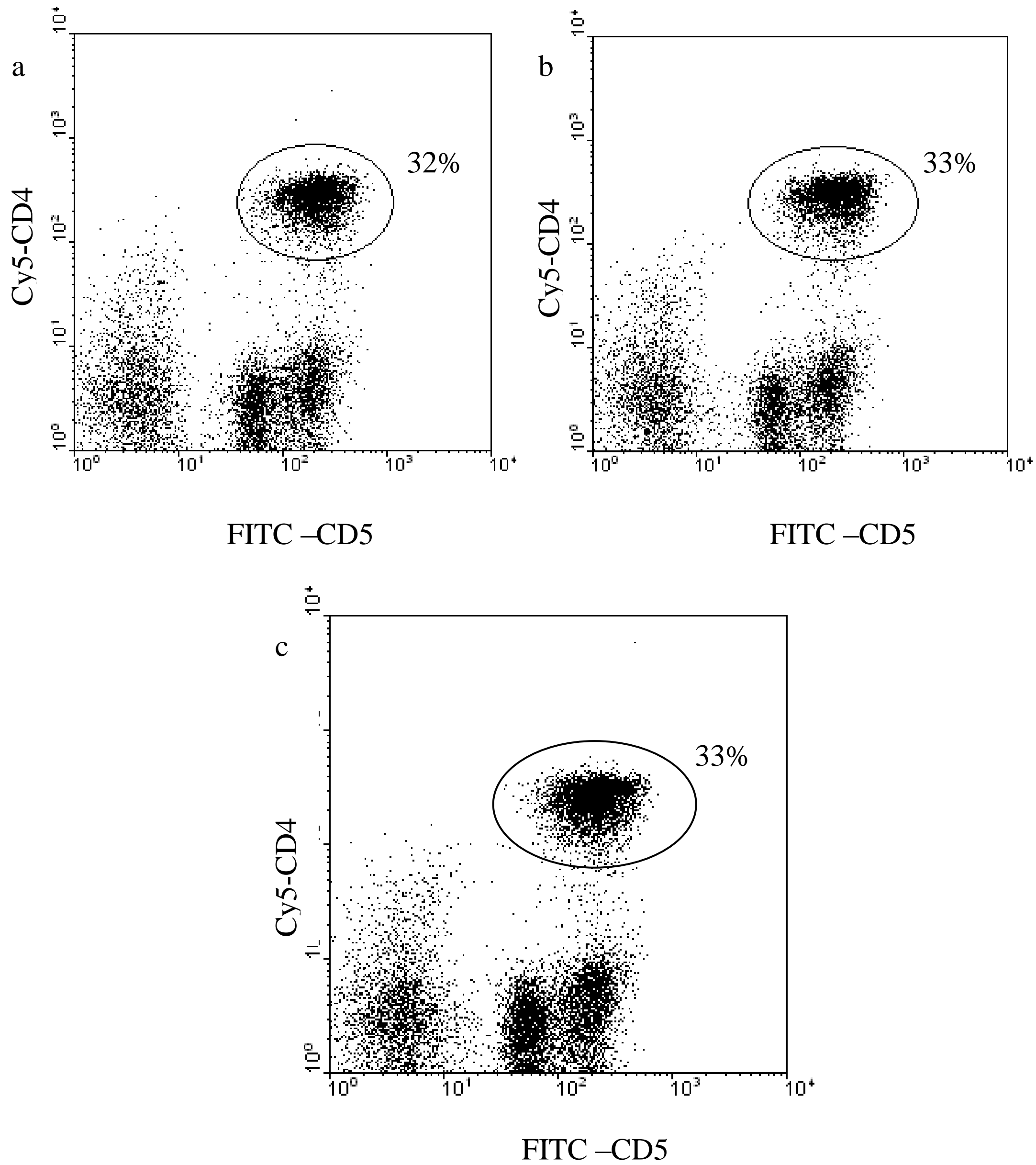

Fig 5.1 Flow cytometric analysis of $CD5^+/CD4^+$ cells showing reproducibility of results using a combination of direct and indirect staining technique. PBMCs, from a single animal, were separated under various conditions: (a) 600 x g, 90% Histopaque, (b) 600 x g, 95% Histopaque, (c) 1000 x g, 95% Histopaque.

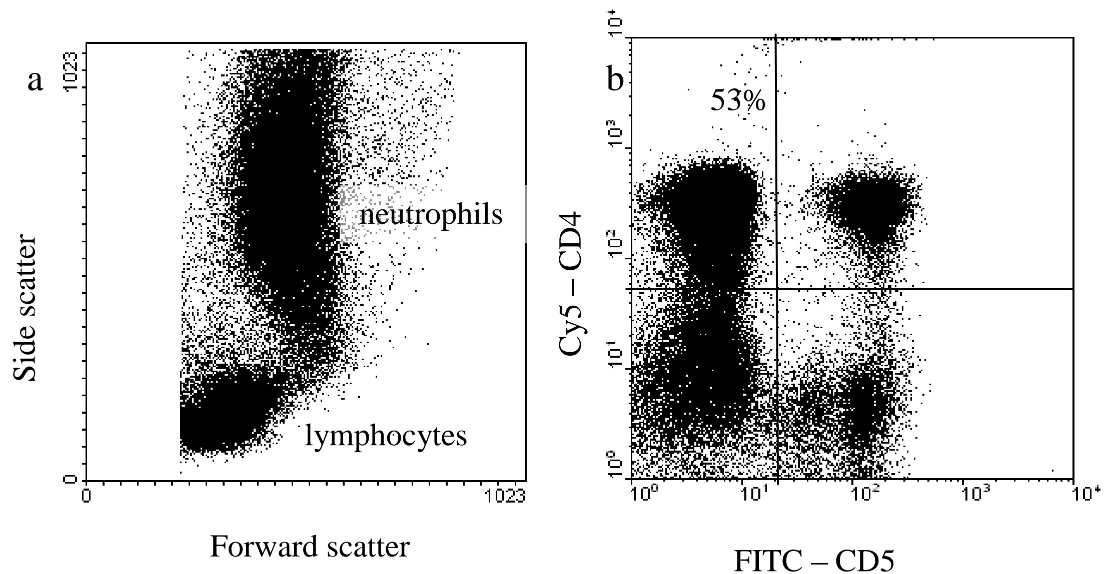

Fig 5.2 Flow cytometric scatter plots of PBMCs from a randomly selected individual indicating (a) typical light scatter characteristics of canine lymphocytes and neutrophils in PBMCs and (b) relative $CD5^-/CD4^+$ neutrophils in this sample.

Table 5.2 Relative cell counts in canine PBMCs as determined by visual cell counting and flow cytometry.

	Separation conditions		PBMC subsets (flow cytometry)			
Animal	Centrifugal force (x g)	Ficoll (%)	lymphocytes	monocytes	neutrophils	Adherent cell count
1	400	100	32	14	54	28
2	400	100	25	9	66	31
3	600	95	40	33	25	17
3	1500	95	44	32	22	11

5.4.3 Optimisation of canine PBMC isolation from whole blood

Neutrophil contamination of PBMCs isolated under similar conditions from blood samples from various dogs ranged between 6 and 78% (Table 5.3). In all cases, neutrophil contamination of PBMCs was less in samples separated over a Ficoll-Hypaque density of 1.069 g/ml than 1.077 g/ml (Table 5.4); however, separation over 1.069 g/ml resulted in a profound decrease in total PBMC isolation (Table 5.5). For a single animal, whole blood separated over 1.077 g/ml for either 20 or 30 min., showed a large reduction in contaminating neutrophils, but with a negligible reduction in the number of PBMCs isolated (Table 5.5). However, increasing the duration of centrifugation from 30 to 40 minutes resulted in both an increase in neutrophil contamination in PBMCs and a marked reduction in total PBMCs recovered (Table 5.6).

Table 5.3 Neutrophil counts, inferred by enumeration of glass-adherent cells, in canine PBMCs separated over a density of 1.077 g/ml.

Animal	Duration (min)	Centrifugal force (x g)	Adherent cells (%)	Neutrophils (%)
1	30	350	5	10
2	30	500	8	16
3	30	500	3	6
4	30	500	25	50
5	25	500	21	42
6	25	500	39	78
7	25	400	29	58
8	25	400	31	62

Table 5.4 Relative PBMC yields from 5 dogs (A-E) separated over various Ficoll-Histopaque densities.

Ficoll-Hypaque density (g/ml)	Relative PBMC yield (%)				
	A	B	C	D	E
1.077	100	100	100	100	100
1.073	26	41	93	100	90
1.069	20	65	30	82	—

Table 5.5 Relative neutrophil counts, from 5 dogs (A-E), in PBMCs separated over various Ficoll-Histopaque densities.

Ficoll-Hypaque density (g/ml)	Relative neutrophil count (%)				
	A	B	C	D	E
1.077	10	16	28	42	60
1.073	10	62	14	46	43
1.069	14	12	10	26	—

Table 5.6 Relative neutrophil counts and PBMC yields from 4 animals (A-D) separated over Ficoll-Histopaque (1.077 g/ml) for various lengths of time.

	Relative neutrophil count (%)				Relative PBMC yield (%)			
Duration (min.)	A	B	C	D	A	B	C	D
20	—	—	—	28	—	—	—	100
30	10	16	50	6	100	100	100	97
40	36	26	61	—	37	61	68	—

5.4.4 PKH26 staining of PBMCs for lymphoproliferation studies

PKH26 staining of PBMCs resulted in bright, homogeneous fluorescence of all cells, and allowed for the determination of PKH26 fluorescence in various lymphocyte subsets (Fig 5.3). Following 4 days of incubation with PHA, all lymphocytes, and all lymphocyte subsets showed no loss in PKH26 fluorescent intensity (e.g. Fig 5.4).

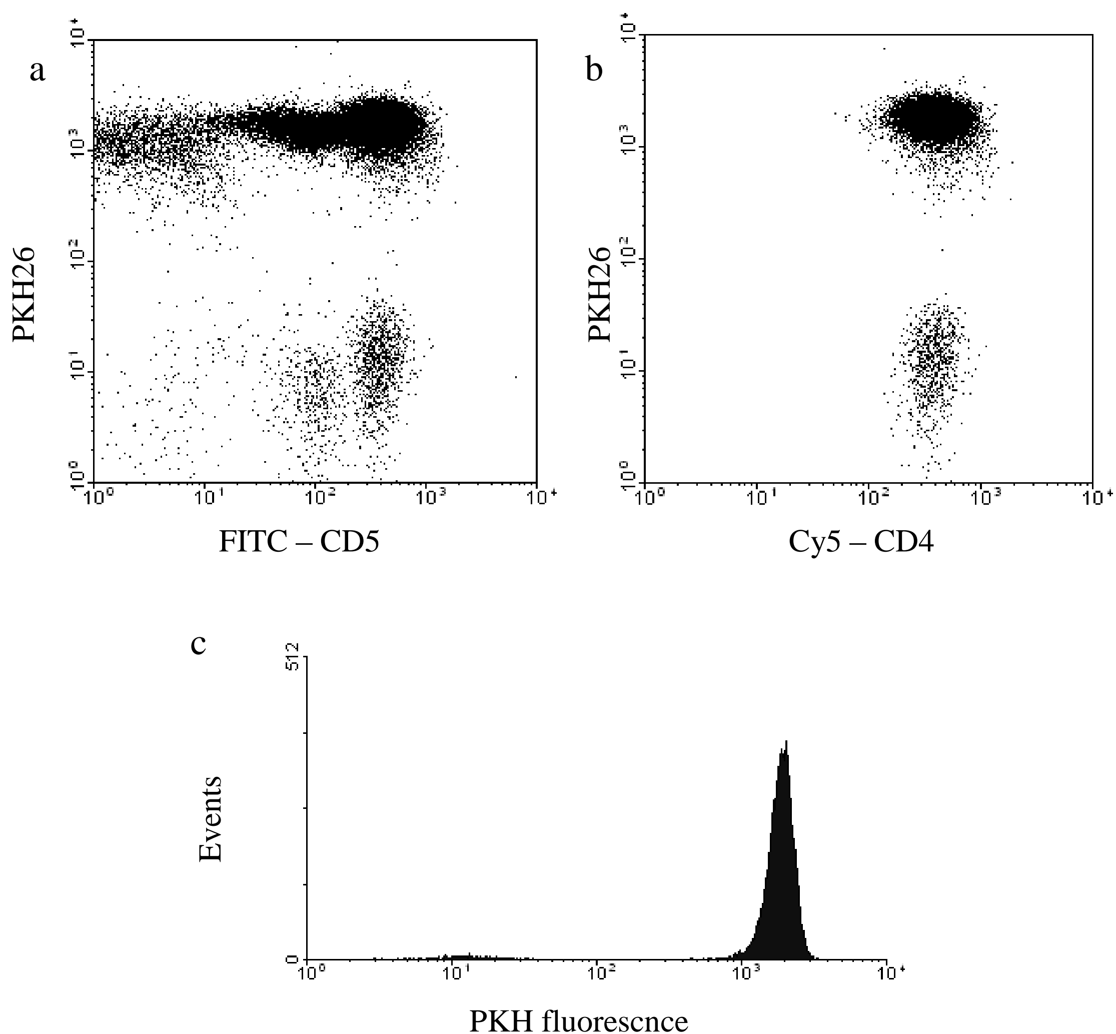

Fig 5.3 Flow cytometric analysis of PKH26 fluorescence of unstimulated canine PBMCs: (a) scatter plot showing intense PKH26 fluorescence of both $CD5^+$ and $CD5^-$ cells, (b) scatter plot showing PKH26 fluorescence of $CD4^+$ (and $CD5^+$) cells, and (c) histogram of PKH26 fluorescence of $CD5^+/CD4^+$ cells indicating homogenous PKH26 staining.

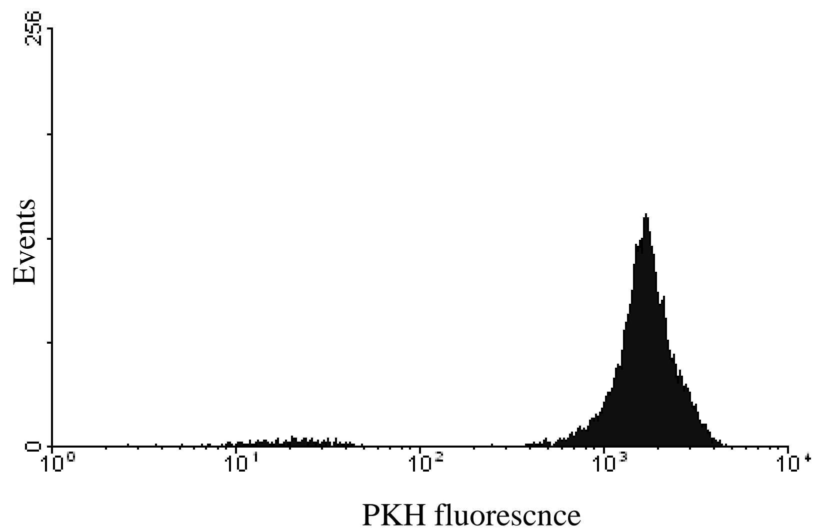

Fig 5.4 Histogram of PHA-stimulated canine CD5$^+$/CD4$^+$ lymphocytes showing negligible loss of PKH26 fluorescence.

4.5 Discussion

The difficulty in obtaining a pure canine PBMC population has been noted by numerous investigators[2,2] and is reflected by the wide variation in conditions under which these cells are obtained[9,14]. In this study, using dogs of various breeds, no optimal Ficoll-Hypaque density was identified for the separation of PBMCs. Separation of canine PBMCs over 1.069 g/ml Ficoll-Hypaque resulted in a dramatic reduction in the number of PBMCs isolated, while separation over 1.073 g/ml Ficoll-Hypaque resulted in inconsistent changes in the relative proportion of contaminating neutrophils (Tables 5.4 and 5.5). However, centrifugation of whole blood over Ficoll-Hypaque for 30 min. was identified as optimal for this procedure (Table 5.6).

Using a combination of direct and indirect labelling techniques, two-parameter flow cytometric leukocyte analysis was shown to be possible for dogs despite the limited spectrum of canine reagents (Fig 5.1). Canine neutrophils express the cell marker CD4[10] making differentiation between these and $CD4^+$ lymphocytes impossible using single-parameter labelling. Two-parameter labelling of cells, i.e combining the CD4 marker with a lymphocyte marker (CD5), allows for highly specific of immunophenotyping of these cells as evidenced by this study (Fig. 5.1)

However, it was not possible to adapt the PKH26 assay to track canine PBMC proliferation. This may, in part, have been due to the neutrophil contamination of PBMCs. PKH26, released by neutrophils undergoing apoptosis during the course of the assay, would be available for uptake by proliferating lymphocytes, thereby masking the loss of fluorescence by these cells, and negating the assay signal. Under these circumstances, the use of a flow cytometric proliferation assay which labels only those

cells undergoing proliferation, rather than all cells, would be more useful. As such, bromodeoxyuridine BrDU proliferation assays, which quantify the utake of BrDU by proliferating cells, may prove to be a more suitable for use in canines.

In conclusion, while two-colour labelling of canine leukocytes was achieved by a combination of direct and indirect labelling, this study was complicated by unforseen species-specific factors. The potential for substantial biological differences in leukocyte subsets between species should therefore be considered when adapting immunological assays for use in new animal models.

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CHAPTER 6

Detection of Mycobacterium tuberculosis infection in chacma baboons (*Papio ursinus*) using the QuantiFERON-TB Gold (In-Tube) assay

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6.1 Abstract

Background Early diagnosis of simian tuberculosis (TB) is vital to prevent transmission of this disease. We evaluated the ability of the QuantiFERON-TB Gold (In-Tube Method) assay (QFG-IT) to detect TB in chacma baboons (Papio ursinus). *Methods* Fifty-one baboons were tested using the Tuberculin Skin Test (TST) and the QFG-IT. Baboons testing positive, and animals exposed to infected individuals, were euthanised and subjected to necropsy. Selected tissues were processed for histopathology, mycobacterial culture and genetic speciation. *Results* Tuberculosis was confirmed in one TST positive/QFG-IT positive animal and one TST negative/QFG-IT positive animal. One TST positive/QFG-IT negative animal and five TST negative/QFG-IT negative animals were confirmed uninfected following necropsy. *Conclusion* The QFG-IT correctly detected TB in two baboons, including one TST negative individual and correctly identified six baboons as uninfected, including one TST positive individual. The QFG-IT shows promise as a sensitive, specific test for TB in chacma baboons.

6.2 Keywords

ELISA; ESAT-6/CFP-10; interferon-gamma release assay; non-human primate; tuberculin skin test; tuberculosis

6.3 Introduction

Old World monkeys are highly susceptible to tuberculosis (TB) caused by the acid-fast mycobacteria *Mycobacterium tuberculosis* and *Mycobacterium bovis* (members of the *M. tuberculosis* complex (MTC))[15,20]. Both pathogens have been associated with severe disease in chacma baboons (*Papio ursinus*)[8,10]. Typically, exposure of baboons to *M. tuberculosis* occurs in captive environments where close contact between infected humans and animals allows for aerosol transmission of the bacterium[8,14]. We have, however, detected cases of TB caused by *M. tuberculosis* in freeliving baboons in a peri-urban environment in Cape Town, South Africa (T. A. Gous, unpublished data), presumably as a result of these animals scavenging on human waste. This behaviour has previously been associated with outbreaks of TB caused by M. bovis in free-living baboons elsewhere[10,19].

Tuberculosis in Old World monkeys commonly results in debilitating pulmonary disease[9,15]. Given suitable conditions, infected monkeys can therefore act as a source of transmission of TB to other individuals and pose a health risk to humans[9,10]. As such, early detection of TB in captive non-human primates (NHP) is vital.

The tuberculin skin test (TST) has been the mainstay of TB detection in many mammals and is widely used in NHP[2,7]. The test measures cell-mediated delayed-type hypersensitivity in response to the intradermal injection of mammalian old tuberculin (MOT) derived from M. tuberculosis or purified protein derivative (PPD) derived from either *M. tuberculosis* or *M. bovis*. However, MOT and PPD are poorly defined and immunological cross-reactivity to various other mycobacterial species can cause false

positive results to this test. Also, false negative results (skin test anergy) have been described in a number of monkey species including baboons[3,8,13,20].

More recently, in vitro whole blood assays have been developed to diagnose TB in a wide range of species, including NHP[7,9]. These tests quantify the production of the cytokine interferon-gamma (IFN-γ) by antigen-specific lymphocytes in response to stimulation by mycobacterial proteins. Such tests have classically compared the differential IFN-γ responses to *M. bovis* PPD and *M. avium* PPD in order to differentiate infection by members of the MTC from infections by nontuberculous mycobacteria respectively. Alternatively, proteins which are highly specific to M. tuberculosis and M. bovis can be used as antigenic stimuli in order to identify infection by one of these pathogens. This strategy is employed in the human diagnostic test, the Quantiferon-TB Gold (In-Tube Method) assay (QFGIT) (Cellestis Limited, Carnegie, Australia), which utilises peptide antigens simulating proteins which are highly specific to most members of the MTC[16]. Genes encoding these proteins have been deleted from the genome of the attenuated strain of *M. bovis* Bacille Calmette-Guérin (BCG), which is used as a live vaccine against TB[12]. Accordingly, this test is useful in humans as it distinguishes *M. tuberculosis* infection from vaccination with BCG.

This study aimed to determine if the QFG-IT might be used for the diagnosis of TB in captive chacma baboons, some of which had previously been vaccinated with recombinant BCG (r-BCG).

6.4 Materials and methods

6.4.1 Animals

Fifty-one chacma baboons were included in this study. The baboons were housed singly, outdoors, with full visual, olfactory and auditory contact with conspecifics at the Delft Animal Facility of the South African Medical Research Council in accordance with the South African National Guidelines for the Care and Use of Animals for Scientific Purposes. Tactile contact, including grooming with adjacent individuals, was possible through grooming panels of wire mesh. The diet consisted of 200 g of a pelleted feed (Aquafeeds, Cape Town, South Africa) each morning and a 200 g portion of fresh fruit or vegetables each afternoon. Once per week the diet was supplemented with a 100 g slice of bread covered with 5 ml Vitamin C syrup. Fresh water was supplied twice daily. This colony had intermittently experienced cases of TB and the current study was initiated following the identification of two TST positive individuals during routine screening of the colony. A number of individuals in the colony had previously been vaccinated with BCG during a clinical trial[6]. Prior to TB testing and euthanasia, animals were tranquilised by intramuscular injection of 10 mg/kg ketamine hydrochloride.

6.4.2 Tuberculosis tests

Tuberculin skin tests were performed by intradermal injection of 0.1 ml (5000 IU) of *Mycobacterium bovis* PPD (WDT, Hoyerhagen, Germany) into the left upper palpebrum. The site of the TST was monitored daily for 72 hours and the inflammatory response characterised as previously described[2]. Concurrently, 1 ml of blood was

collected into QFG-IT TB Antigen, Mitogen Control (positive control), and Nil Control (negative control) tubes, respectively, and processed according to the manufacturer's instructions. The TB Antigen tubes contain peptide antigens simulating the MTC-specific proteins 6 kD Early Secretory Antigenic Target (ESAT-6), Culture Filtrate Protein 10 (CFP-10), and TB 7.7(p4)[1]. The Mitogen tubes contain phytohaemagglutinin (PHA), a potent stimulus for IFN-c production by lymphocytes in a wide variety of species[18]. Briefly, following vigorous shaking, tubes were incubated at 37°C for 16 to 24 hours, after which supernatants from these tubes were harvested and stored at -20°C. Interferon-gamma concentrations in supernatants were determined by enzyme-linked immunosorbant assay (ELISA) using the QFG-IT ELISA kit and test results were interpreted according to the manufacturer's criteria for human patients. Additionally, IFN-γ concentrations in supernatants from selected individuals were determined using the ELISA^{PRO} kit (Mabtech AB, Nacka Strand, Sweden) utilising antibodies previously shown to detect IFN-γ of chacma baboons[5]. Samples were assayed in duplicate and the minimum detectable dose (MDD) was calculated as twice the average IFN-γ concentration of the Nil Control samples of all animals. A positive result was determined by the formula: $[\text{IFN-}\gamma]^{\text{Antigen}} - [\text{IFN-}\gamma]^{\text{Nil}} > \text{MDD}$. Finally, blood from a randomly selected chacma baboon was diluted 1:5 in RPMI 1640 supplemented with 2 mm L-glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Aliquots of the whole blood suspension were stimulated in 96-well plates (200 µl/well) with either 10 µg/ml phytohaemagglutinin (PHA) for 5 days or 2 µg/ml Staphylococcal Enterotoxin B (SEB) for 2 days. All reagents were purchased from Sigma-Aldrich, South Africa. Interferon-gamma concentrations in supernatants from PHA- and SEB-stimulated wells were determined using both QFG-IT ELISA and Mabtech ELISA^{PRO} kits.

6.4.3 Postmortem examination

All animals which tested positive with either the TST or QFG-IT were euthanised by intravenous injection of 200 mg/kg sodium pentobarbitone. Additionally, five animals in close contact with confirmed TB cases were euthanised as a biosecurity precaution. Carcases were subjected to complete postmortem examinations. Samples of all major organs and macroscopically diseased tissues were routinely prepared for histopathological examination. Samples of tracheobronchial lymph nodes, pulmonary tissue and macroscopically diseased tissues were processed for mycobacterial culture and genetic speciation according to the presence of genomic regions of difference (RD) 1, 4, 9 and 12 as previously described[17,21].

6.5 Results

6.5.1 Detection of baboon IFN-γ by enzyme-linked immunosorbant assay

Positive signals for IFN-c were obtained from both the PHA- and SEB-stimulated blood samples using both the QFG-IT and Mabtech ELISA kits (Table 6.1). Notably, the IFN-γ concentration from samples stimulated with PHA for 5 days was surprisingly low. Similarly, IFN-γ concentrations from 25 samples incubated overnight in QFG-IT Mitogen Control tubes (containing PHA) were negligible or undetectable in all but a single case, i.e. B515 (Table 6.1).

6.5.2 Tuberculosis tests

Two animals tested positive by the TST: B750 (grade 4 response) and B675, an animal previously vaccinated with r-BCG (grade 3 response) (Table 6.2). All other animals were TST negative. Because IFN-γ responses were undetectable in the mitogen-stimulated samples, QFG-IT results were interpreted according to an algorithm provided by the manufacturer using only results from the Nil Control and TB Antigen tubes. Two animals tested positive by the QFG-IT (B750 and B749), while all other animals, including those previously vaccinated with r-BCG, tested negative with this test (Tables 6.2 and 6.3). The MDD for the ELISA^{PRO} assay was determined as 43 pg/ml (Nil Control result range: 0–83.7 pg/ml; mean: 21.5 pg/ml). Results obtained for those animals tested using the ELISAPRO assay concurred with the QFG-IT results (as shown in Table 3).

Table 6.1 Mitogen-stimulated whole blood interferon-γ responses in chacma baboons as determined by the QFG-IT and Mabtech ELISA^{PRO} kit (Mabtech) systems.

		Interferon-gamma (mean (+/- S.D.))			
Stimulation conditions	Duration	QFG-IT (IU/ml)	Mabtech (O.D.)	Mabtech (pg/ml)	Comment
10 µg/ml PHA in 96-well plate	5 days	1.08	0.74	n/d	randomly selected baboon
2 µg/ml SEB in 96-well plate	2 days	20.11	3.68	n/d	randomly selected baboon
Mitogen Control Tube (PHA)	overnight	6.03	n/d	n/d	1/25 baboons
Mitogen Control Tube (PHA)	overnight	0.17 (+/- 0.10)	n/d	n/d	9/25 baboons
Mitogen Control Tube (PHA)	overnight	n/d	0.19 (+/- 0.16)	40.6 (+/- 27.5)	15/25 baboons

PHA, phytohaemagglutinin; SEB, Staphylococcal Enterotoxin B (SEB); n/d, not done

Table 6.2 QFG-IT results, Tuberculin Skin Test (TST) results, and Post Mortem examination (PM)/mycobacterial culture results for chacma baboons euthanised during this study.

		QFG-IT (IU/ml)				
Category	Baboon	Nil Control Tube	TB Antigen Tube	Result	TST	PM/culture
TB Infected	B749	0.15	5.94	+	-	+
TB Infected	B750	0.68	21.58	+	+	+
BCG-vaccinated	B675	0.05	0.06	-	+	-
TB Contact	B515	0.41	0.29	-	-	-
TB Contact	B748	0.14	0.09	-	-	-
TB Contact	B755	0.08	0.08	-	-	-
TB Contact	B787	0.2	0.16	-	-	-
TB Contact	B788	0.07	0.13	-	-	-

-, negative; +, positive

Table 6.3 Tuberculosis test results for chacma baboons previously vaccinated with recombinant BCG.

	Mabtech ELISA^{PRO} (pg/ml)			QFG-IT (IU/ml)		
Baboon ID	Nil Control Tube	TB Antigen Tube	Result	Nil Control Tube	TB Antigen Tube	Result
B675	n/d	n/d	n/d	0.05	0.06	-
B687	18.6	14.0	-	0.12	0.13	-
B692	10.4	8.8	-	0.16	0.09	-
B710	11.2	7.0	-	0.09	0.09	-
B732	0	0	-	0.07	0.07	-
B733	37.8	44.1	-	0.16	0.15	-
B734	15.3	12.0	-	0.11	0.12	-
B738	6.4	4.4	-	0.12	0.08	-

-, negative

6.5.3 Pathology, histopathology and mycobacterial culture

Animals B675, B749 and B750 (positive responders) and B515, B748, B755, B787 and B788 (contact animals) were euthanised. Postmortem examination of B750 showed marked, multifocal to confluent, subacute to chronic, necrogranulomatous to pyogranulomatous inflammation affecting the lungs, pleura, lymph nodes (tracheobronchial, mediastinal and intrathoracic along the aorta), spleen and focally in the epicardium of the left ventricle of the heart. A prominent, round to oval, 150 mm · 50 mm, raised granuloma filled with 8 ml of pus, and attached to the lungs by a few fibrous adhesions, was visible in the diaphragmatic pleura.

Histopathology confirmed the macroscopic findings and also revealed multifocal microgranulomas in the liver and kidneys. The large granulomas consisted of occasional central caseous necrosis surrounded by a moderately developed layer of macrophages and epithelioid cells, and moderate to large numbers of multinucleated giant cells. This layer was surrounded by low to moderate numbers of lymphocytes and plasma cells and a poorly to well developed fibrous capsule. Some granulomas showed central liquefaction with infiltration of moderate to large numbers of neutrophils. The microgranulomas in the liver and kidney consisted of nodular aggregates of macrophages, epithelioid cells and multinucleated giant cells and low numbers lymphocytes and plasma cells. Ziehl-Neelsen staining was negative for acid-fast bacilli in all sections of all organs.

Postmortem examination of B749 showed a single, mildly encapsulated, round, 7 mm, raised necrogranuloma in the dorsal surface of the right cranial lung lobe (Fig. 1). The tracheobronchial lymph node showed a focal, indistinctly outlined, 3 mm, poorly

encapsulated, slightly raised oval granulomatous area in the cortex. Microscopically, the lung granuloma revealed a caseous necrotic centre that was infiltrated by low numbers of neutrophils, and surrounded by a layer consisting of low to moderate numbers of macrophages and epithelioid cells. This was surrounded by an indistinct outer layer consisting of low numbers of lymphocytes and plasma cells. There was an incomplete but fairly well developed fibrous capsule that extended into the necrotic centre. Some sections from macroscopically normal lung samples showed multifocal granulomatous bronchopneumonia.

These microgranulomas consisted of nodular aggregates of macrophages, epithelioid cells and low numbers of multinucleated giant cells, with some microgranulomas that were surrounded by a thin layer of lymphocytes and plasma cells, and a poorly developed fibrous capsule. Some microgranulomas showed central coagulative necrosis with infiltration of low numbers of neutrophils and mild to moderate calcification of the necrotic centre was occasionally noted. The microgranulomas were occasionally situated in the mucosa of bronchioles where they often extended through the respiratory epithelium to communicate with the airway lumen. There were numerous microgranulomas that consisted of macrophages, epithelioid cells and low numbers multinucleated giant cells in the cortex and paracortex of the tracheobronchial lymph node. Ziehl-Neelsen staining was negative for acid-fast bacilli in all sections of all organs.

Postmortem examinations of B515, B675 B748, B755, B787 and B788 did not show any macroscopic and microscopic lesions suggestive of tuberculosis, and all sections of all organs examined were negative for acid-fast bacilli with ZN-staining.

Acid-fast bacteria were cultured from lymph node and lung lesions from B749 and B750 and confirmed as *M. tuberculosis*.

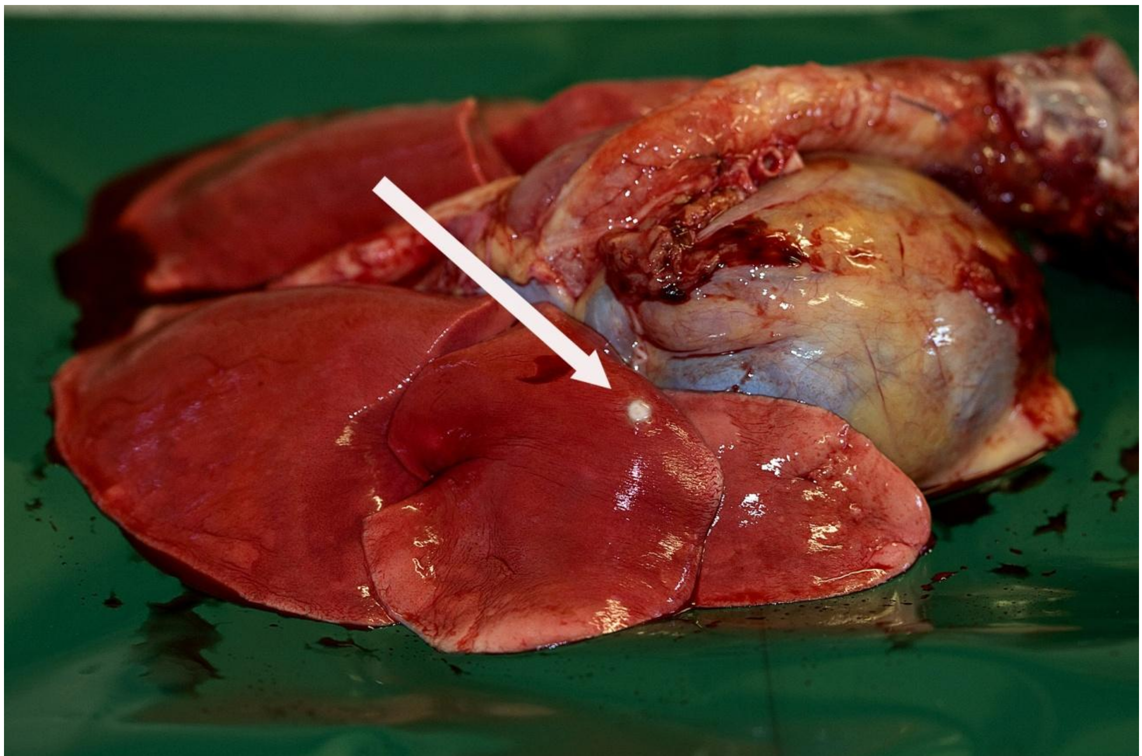

Fig 6.1 A single, mildly encapsulated, raised necrogranuloma (indicated by arrow) in the dorsal surface of the right cranial lung lobe (B749).

6.6 Discussion

The early diagnosis of TB is essential to prevent outbreaks of this disease in NHP colonies. In settings where a colony is presumed to be TB-free, and where the risk of infection is low, the TST provides for a simple, cost-effective assay for monitoring the infection status of the colony. However, the sensitivity of the TST has been reported in various species to vary between 60–85%[7]. Thus, this test may fare poorly at controlling an epizootic or for monitoring a population at high risk of infection. In such scenarios the use of a second test in conjunction with the TST can improve the sensitivity of infection screening[9,20].

Our study is the first to demonstrate that a human in vitro TB assay, the QFG-IT, can be used to detect TB infection in chacma baboons. The study investigated naturally occurring TB in a captive colony of baboons and as such the number of cases contributing to our findings is limited. Nonetheless, the use of the QFG-IT in conjunction with the TST improved the sensitivity of infection screening of the colony, and our results support a presumption that the QFG-IT may have greater specificity than the TST. The specificity of the QFG-IT is attributable to the use, as antigenic stimulants, of peptide antigens simulating the proteins ESAT-6, CFP-10, and TB 7.7(p4)[1]. Few non-tuberculous mycobacteria contain genes which encode homologous proteins, and these genes are deleted from the TB vaccine strain BCG[1,12]. As such, an immune response to these proteins may, with a great degree of confidence, be attributed to prior exposure to *M. tuberculosis* or *M. bovis*. In our study, this specificity was demonstrated in the case of B675, a baboon previously vaccinated with r-BCG. While TST positive, this animal was QFG-IT and tissue culture negative. This suggests that

the TST result was probably caused by immunological priming to BCG proteins contained in the M. bovis PPD. These findings suggest that the QFG-IT could be useful in differentiating BCG vaccination from TB infection in baboons.

The sensitivity of the QFG-IT is indicated by the ability of the test to correctly identify TB in a TST negative individual (B749). In this case, gross tuberculous lesions were limited to a single granuloma in a lung lobe and indistinct granulomatous inflammation in the tracheobronchial lymph node (Fig. 1). This picture suggests early infection or recent progression of disease, as in our experience, at the time of TB diagnosis by TST, baboons often present with advanced disease, as in the case of B750. It has been reported that for some monkey species, most infected individuals will convert to TST positivity within 8 weeks of infection[11,20]. However, this is not true for all individuals, and some previously TST positive animals can revert to being TST negative[3,13,20]. While the cause of the TST anergy is speculative, our results suggest that in this case the QFG-IT may have detected infection prior to the development of a positive TST.

A proviso for the use of the QFG-IT system for the diagnosis of TB in baboons (and possibly in other monkeys) is that the positive control tube (Mitogen Control) cannot be used for the assay in accordance with the manufacturer's instructions. Of 25 animals tested, a positive control result was obtained for only a single individual, B515 (Table 1). Additionally, 5-day whole blood culture with PHA resulted in a low IFN-c response in a single baboon, and weak whole blood responses to PHA stimulation in other monkeys has been reported[20]. This means that a positive control can not be conveniently included in the assay and negative test results must be interpreted in this light. In itself, the lack of a positive control does not significantly affect the validity of

the QFG-IT test in healthy humans, and this may be the case for NHP[4]. Indeed, the more common NHP TB test, the TST, is performed without a positive control. However, the lack of positive control responses in this study detracts from the significance of our results as negative TB Antigen responses can not be critically evaluated. This limitation of the QFG-IT must be considered if further attempts are made to validate the use of this assay in NHP, possibly by the addition of an appropriate mitogen, such as SEB, to the Mitogen Control tube.

The QFG-IT offers numerous advantages as an ancillary TB test. Blood samples are taken directly into tubes containing the antigens and incubated in these closed tubes. Incubation conditions need not be sterile and do not require CO_2 or humidification, and a standard blood tube centrifuge is used to harvest supernatants. Initial processing of samples, therefore, may be possible in moderately equipped field laboratories, zoos, and primate handling facilities. Of particular utility is the fact that the QFG ELISA kit can be used to determine test results. Commercial human laboratories offering the test can therefore process baboon samples without modification to the human protocol. If the QFG-IT were to be appropriately validated for baboons, these attributes would make this assay highly suitable for TB testing of individuals or small numbers of animals on an ad hoc basis.

In conclusion, while the sensitivity of the QFG-IT to diagnose TB in chacma baboons is undetermined, this highly specific test shows promise as a simple, practical addition to the TST, and would be particularly useful in settings in which commercial in vitro NHP TB tests are unavailable. As such, our experience strongly suggests that further

investigation of the use of the QFG-IT for the diagnosis of TB in NHP may prove highly rewarding.

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6.8 References

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CHAPTER 7

Detection of *Mycobacterium kansasii* infection in a rhesus macaque (*Macaca mulatta*) using a modified QuantiFERON-TB Gold assay

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7.1 Abstract

A modification of a highly practical human interferon-gamma release assay for the diagnosis of tuberculosis (TB), the QuantiFERON-TB Gold (In-Tube Method) (QFG-IT), was evaluated for diagnosing natural mycobacterial infection in rhesus macaques (*Macaca mulatta*). All animals in a captive colony were tested using the QFG-IT and tuberculin skin test (TST). Animals testing positive to these tests were euthanised and necropsied. Selected tissues were processed for histopathology and mycobacterial culture, and positive cultures were speciated by *Mycobacterium tuberculosis* complex polymerase chain reaction (PCR) and 16S rRNA gene PCR sequencing techniques. *Mycobacterium tuberculosis* was cultured from a TST-positive/QFG-IT-positive animal which showed gross pulmonary pathology typical of TB. Additionally, *Mycobacterium kansasii* was cultured from a TST-negative/QFG-IT-positive animal which had no pathological or histopathological signs of mycobacterial infection. The detection of *M. kansasii* infection in a QFG-IT-positive animal which showed no evidence of disease indicates that this test might be a highly sensitive tool for the diagnosis of mycobacterial infection in rhesus macaques. However, these findings highlight the limitations of the QFG-IT to specifically detect infection by the pathogens *M. tuberculosis* and *Mycobacterium bovis*.

7.2 Keywords

Diagnosis; interferon-gamma; *Mycobacterium kansasii*; *Mycobacterium tuberculosis*; rhesus macaque; tuberculosis

7.3 Introduction

Rhesus macaques (*Macaca mulatta*) are highly susceptible to tuberculosis (TB) caused by the bacteria *Mycobacterium tuberculosis* and *Mycobacterium bovis*[4,15]. In these monkeys the disease almost invariably results in severe pulmonary pathology and coughing is commonly an early clinical sign of illness[4]. This symptom, resulting in aerosolisation of the pathogen, provides a mechanism for the transmission of infection between hosts. These factors contribute to the high risk of epizootic disease in captive monkeys as confirmed by reported outbreaks of TB in rhesus macaque colonies[4,15,17].

In order to prevent or control TB outbreaks, detection of infected individuals prior to their progression to clinical disease is vital. While the tuberculin skin test (TST) has long been used to detect mycobacterial infection in non-human primates (NHP), the sensitivity of this test has been questioned[3,5,9] and these authors have suggested combining the TST with alternative immunological assays.

Recently, it has been reported that a human diagnostic TB test, the QuantiFERON-TB Gold assay (In-Tube Method) (QFG-IT), appears valuable for the diagnosis of TB in chacma baboons (*Papio ursinus*)[11]. This test detects immunological sensitisation to the mycobacterial proteins early secretory antigenic target 6 kDa (ESAT-6), culture filtrate protein 10 kDa (CFP-10), and TB 7.7(p.4) by quantifying the secretion of IFN-γ by lymphocytes recognising these antigens[10]. The relative specificity of this test for *M. tuberculosis* and *M. bovis* is determined by the fact that the genes encoding these proteins have been deleted in the vaccine strain *M. bovis* Bacille Calmete-Guérin (BCG) and highly homologous genes encoding these proteins have been detected in only a limited number of nontuberculous mycobacteria including *Mycobacterium*

kansasii, *Mycobacterium szulgai*, and *Mycobacterium marinum*[1]. Immunological sensitivity to these latter organisms can therefore cause false positive results to the QFG-IT[1,19] and it has been argued that other mycobacterial species may cause the same phenomenon[6]

Following the detection of a TST-positive animal from a captive colony of rhesus macaques, we investigated the usefulness of a modified QFG-IT for the diagnosis of mycobacterial infection in this species.

7.4 Materials and Methods

7.4.1 Animals and housing

Animals were housed outdoors in a coral and five group cages, and indoors, singly and in pairs, in galvanised wire mesh cages at the Delft Animal Facility of the Medical Research Council of South Africa in accordance with the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386). Permission to perform this study was obtained from the Committee for Experimental Animal Research of Stellenbosch University. Prior to TB testing and euthanasia, animals were tranquilised with 5 mg/kg ketamine hydrochloride (Kyron Laboratories, Benrose, South Africa).

7.4.2 Tuberculin Skin Test

Tuberculin Skin Tests were performed by intradermal injection of 0.1ml of bovine PPD (PPD^{bov}) (WDT, Hoyerhagen, Germany) diluted in 0.9% saline to a final concentration

of 3000 IU/0.1 ml. The site of the TST was monitored daily for 72 hrs and any inflammatory response was scored as previously described[3].

7.4.3 QuantiFERON-TB Gold (In-Tube) Assay

Blood sampling for the QFG-IT took place prior to the TST in 90/104 animals, three days after the TST in 13/104 animals, and both prior to and three days after the TST in a single animal (macaque No. 28). Three ml of blood was aspirated from the femoral vein of each animal via a 21G needle into a 5 ml syringe. Immediately, 1 ml aliquots of blood were transferred to a TB Antigen tube (containing ESAT-6, CFP-10 and TB7.7 (p.4)), a Mitogen (positive control) tube (containing phytohaemagglutinin (PHA)), and a Nil (negative control) tube, respectively, of the QFG-IT system. Following vigorous shaking, blood collection tubes were maintained at room temperature until further processing. In agreement with others [15], we found PHA to be a poor stimulus for IFN-γ production in rhesus macaque blood (data not shown). Accordingly, using an insulin syringe, 0.1 ml of staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, South Africa) diluted in RPMI 1640 (Sigma) was aseptically injected into the Mitogen tubes at a final concentration of 1 μg/ml as a positive control for IFN-γ production. All tubes were then incubated for 16-24 hrs at 37°C after which plasma fractions were aspirated following centrifugation (at 2500 x g) and stored at -20°C.

Interferon-gamma concentrations in plasma samples, i.e. $[\text{IFN-}\gamma]^{nil}$, $[\text{IFN-}\gamma]^{tb}$ and $[\text{IFN-}\gamma]^{mitogen}$, were determined in duplicate by enzyme-linked immunosorbant assay (ELISA) using a commercial kit according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden, code: 3420M-1HP-10). The 95th percentile of all $[\text{IFN-}\gamma]^{nil}$ values ($[\text{IFN-}\gamma]^{nil95}$) was selected as a cut-off between non-specific background

responses and a positive assay result. Additionally, QFG-IT results were regarded as positive if $[\text{IFN-}\gamma]^{tb\text{-}nil}/[\text{IFN-}\gamma]^{nil} > 0.25$ (as recommended by the QFG-IT manufacturers). In one instance, a second QFG-IT was performed in a TST-negative/QFG-IT-positive animal in order to confirm this result (i.e. macaque No. 28).

7.4.4 Postmortem examination

All animals which tested positive to either the TST or the QFG-IT assay were euthanised by intravenous injection of 200 mg/kg sodium pentobarbitone (Eutha-naze, Bayer Animal Health, Isando, South Africa). In addition, four animals in close contact with a confirmed TB case were euthanised as a biosecurity precaution. Carcases were subjected to complete postmortem examinations and tissue samples from all major organs, the tracheo-bronchial and ileo-caecal lymph nodes, and macroscopically diseased tissues were routinely prepared for histological examination and mycobacterial culture as previously described[12]. Mycobacterial speciation was performed by multiplex polymerase chain reaction (PCR)[18] and 16S rRNA gene PCR sequencing [7].

7.5 Results and Discussion

7.5.1 Use of the QFG-IT in rhesus macaques

For all animals, $[\text{IFN-}\gamma]^{nil}$ ranged from 0 – 873 pg/ml. Using box plot analysis, outliers in this range were excluded from the study as an unusually high $[\text{IFN-}\gamma]^{nil}$ value is recommended as an exclusion criterion by the the QFG-IT manufacturers. The 95th percentile of the modified $[\text{IFN-}\gamma]^{nil}$ range (0 – 179 pg/ml) was calculated as 155 pg/ml. Positive QFG-IT results were therefore defined as: $[\text{IFN-}\gamma]^{tb} > 155$ pg/ml where

$[\text{IFN-}\gamma]^{\text{tb-nil}}$ / $[\text{IFN-}\gamma]^{\text{nil}} > 0.25$. No specific cause for the high $[\text{IFN-}\gamma]^{\text{nil}}$ values was identified, however activation of leukocytes during syringe sampling and transfer of blood to the QuantiFERON tubes was suspected. Collection of blood directly into the QFG-IT collection tubes may mitigate this phenomenon. For all animals, $[\text{IFN-}\gamma]^{\text{mitogen}}$ was > 681 pg/ml.

7.5.2 Detection of TB using the QFG-IT

The identification of a TST-positive monkey (No. 12) during routine TB screening of the colony initiated this study. The QFG-IT performed on this individual three days after the TST resulted in a highly positive test result (Table 7.1). The animal was euthanised and a postmortem examination revealed multifocal to coalescing necrogranulomatous to pyogranulomatous inflammation of the left ventral lung lobe. The tracheo-bronchial lymph nodes were enlarged, firm, irregular, and on incision contained inspissated pus. The histopathology of affected tissues was typical of TB, consisting of multifocal lesions of caseous necrosis surrounded by macrophages, giant cells and lymphocytes with acid-fast organisms occasionally present. *Mycobacterium tuberculosis* was cultured from samples of these tissues.

Four QFG-IT-negative and TST-negative animals in contact with this case (No. 11, P48, P60, and P92), euthanised as a biosecurity precaution, showed no gross pathology or histopathology, and all sampled tissues from these animals were negative for mycobacterial culture (Table 7.1). These findings confirm those of Parsons et al. (2009) that the QFT-IT shows promise as a useful tool for the diagnosis of TB in NHP.

Table 7.1 Results of TST, QFG-IT (post-TST), postmortem examinations, and mycobacterial tissue cultures from rhesus macaques euthanised during this study.

		QFG-IT (pg/ml)						
macaque	TST	$[IFN]^{tb}$	$[IFN]^{nil}$	$[IFN]^{tb-nil}$	$[IFN]^{tb-nil}/[IFN]^{nil}$	Test Result	Postmortem	Speciation
11	-	19	19	0	0	-	nad	-
12	+	3120	84	3036	36	+	pulmonary granulomas	*M. tuberculosis*
28	-	557 (246[a])	40 (75[a])	517 (171[a])	12.9 (2.3[a])	+ (+[a])	nad	*M. kansasii*
P48	-	32	32	0	0	-	nad	-
P60	-	29	34	-5	-0.1	-	nad	-
P92	-	22	35	-13	-0.4	-	nad	-

-: negative; +: positive; nad: no abnormality detected
[a] pre-TST results

7.5.3 Detection of M. kansasii infection using the QFG-IT

A low positive QFG-IT result was obtained for a single TST-negative animal (macaque No. 28) (Table 7.1). A second QFG-IT performed on this animal 3 days following the TST gave a highly positive result (Table 7.1). This increased response was presumably due to boosting of existing immunological sensitivity to the test antigens following re-exposure to these proteins in the PPD^{bov} as it has been shown that two-weekly TST and IGRA testing in rhesus macaques over a 12 week period did not sensitise healthy animals to PPD^{bov} [13], and in the present study, no significant difference was detected in the QFG-IT responses between pre-TST and post-TST QFG-IT-negative monkeys ($p = 0.257$) (e.g. Table 7.2). Such boosting of IFN-γ responses has been shown to occur, although less rapidly, in humans[14] and the rapid onset of this effect observed in this monkey may be due to the composition of PPD^{bov} or the fact that a much greater PPD concentration is used in animal testing.

Postmortally, no evidence of gross pathology or histopathology was detected in this animal. However, *M. kansasii* was cultured from both a tracheo-bronchial lymph node and pooled samples of pulmonary and renal tissues. Together, the QFG-IT and tissue culture findings suggest true infection by this organism. Additionally, contamination of the samples during processing is unlikely as other tissues from this animal were culture negative as were samples from two other animals processed at the same time. Notably, *M. kansasii* is periodically cultured from TST-positive cattle[16] and experimental *M. kansasii* infection in calves results in immunological sensitivity to recombinant ESAT-6/CFP-10 protein in these animals[19]. More specifically, the QuantiFERON-TB Gold assay has been shown to be an effective tool for the diagnosis of *M. kansasii* disease in

humans[8]. Such findings are expected given the high degree of homology between ESAT-6 and CFP-10 proteins of *M. kansasii*, *M. bovis*[16] and *M. tuberculosis*[2].

Table 7.2 Results of the QFG-IT for selected rhesus macaques tested either pre- or post-TST

	QFG-IT		
macaque	$[IFN]^{tb}$ (pg/ml)	$[IFN]^{nil}$ (pg/ml)	Test Result
Pre-TST tested			
P50	28	34	-
P51	26	42	-
P52	111	141	-
P53	64	102	-
P54	7	9	-
Post-TST tested			
9	115	130	-
21	91	85	-
25	17	25	-
P47	6	11	-
P82	20	20	-

-: negative

7.5.4 Summary

This is the first report of the use of a modified QFG-IT for the diagnosis of mycobacterial infection in rhesus macaques and our findings provide further evidence that the QFG-IT is suitable for adaption for use in animals. This assay appears highly sensitive when used in rhesus macaques, detecting non-clinical infection by a mildly

virulent mycobacterium, *M. kansasii*. Hoever, this finding highlights the limitation of the QFG-IT assay to specifically detect *M. tuberculosis* or *M. bovis* infection and this must be considered when interpreting test results. A limitation of our study was the necessary use of an unvalidated cut-off point for defining test results. This limitation will remain a reality of NHP studies, complicated by the fact that validation of such parameters may require species-specific optimisation [5]. However, in the absence of suitable cut-off data, our findings suggest that an observation of post-TST boosting of QFG-IT responses might be used to confirm test results. Additionally, performing the QFG-IT assay 3 days following a TST may increase its sensitivity and this protocol could be suitable for testing high-risk or quarantined animals. Despite the limitations identified, the QFG-IT has numerous benefits over other IFN-γ release assay formats [11], allowing initial processing of samples in moderately equipped laboratories and possibly even under field conditions. As such, our findings suggest that that this assay, modified as described here, could prove highly valuable in diagnosing and controlling mycobacterial infections in rhesus macaques and other species.

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CHAPTER 8

"Emerging" Mycobacteria in South Africa

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8.1 Introduction

A number of incidents (both published and unpublished) regarding the unusual occurrence of tuberculosis (TB) and TB-like disease in animals in South Africa have emerged recently, highlighting infections caused by mycobacterial species.

These incidents include reports on *Mycobacterium goodii* in a spotted hyaena[22], *M. xenopi* in a ruffed lemur (unpublished), *M. intracellulare* in healthy wild-caught chacma baboons[1], the so-called "dassie bacillus" in a free-ranging dassie[16], as well as another dassie from the Western Cape (unpublished). In addition, reports on the "oryx bacillus" from an African buffalo in KZN (Gey van Pittius *et al.*, manuscript submitted), *M. bovis* from a black rhinoceros belonging to the National Zoo[3], *M. tuberculosis* in meerkats in the northern Cape (unpublished), *M. tuberculosis* in a domestic dog from Cape Town[15], 2008), and *M. tuberculosis* in baboons[14]. None of these are necessarily surprising or alarming for a variety of reasons. This communication is an attempt to put these in context.

8.2 The Mycobacteria

The genus *Mycobacterium* currently consists of 128 validly-published species and 5 subspecies, of which some are fast growers and mostly non-pathogenic, while others are slow growers and usually pathogenic (J.P. Euzéby: List of Prokaryotic names with Standing in Nomenclature (LPSN) - http://www.bacterio.cict.fr/). This latter branch includes those in the *M. tuberculosis* complex (MTC), such as *M. canettii*, *M. tuberculosis*, *M. africanum* (subtype Ia and Ib), dassie bacillus, oryx bacillus, *M. microti* (the vole

bacillus), *M. pinnipedii* (the seal bacillus), *M. caprae* (the goat bacillus), *M. bovis* (the bovine bacillus) and *M. bovis* BCG, (see Fig 8.1)[7]. Those mycobacterial species that are not usually pathogenic may become pathogens under unusual circumstances. In humans, these circumstances may include HIV infection, diabetes and steroid treatment, which suppresses the immune system and can allow usually non-pathogenic organisms to become pathogens[17].

The mycobacteria are regarded as very successful organisms and occupy a wide range of niches in the environment (soil and water) as well as being obligate pathogens in some cases[13]. Some mycobacterial species have even been found to colonize extremely harsh environments, such as extreme acidity (pH 3.0) and temperature (56ºC) in acidic hot springs in Yellowstone National Park[19]. In fact, 37% of gene clones analysed from a highly acidic (pH 1.0) volcanic environment were found to originate from previously unidentified mycobacterial species[23], signifying their ability to survive under extreme conditions. New species of mycobacteria are constantly being identified (9 new species were described in 2006 alone) and 52% of known species have been identified in the last 20 years (data not shown). We have recently found at least 5 new species from human sampling in the Western Cape in South Africa, which we are in the process of characterizing (NC Gey van Pittius, personal communication).

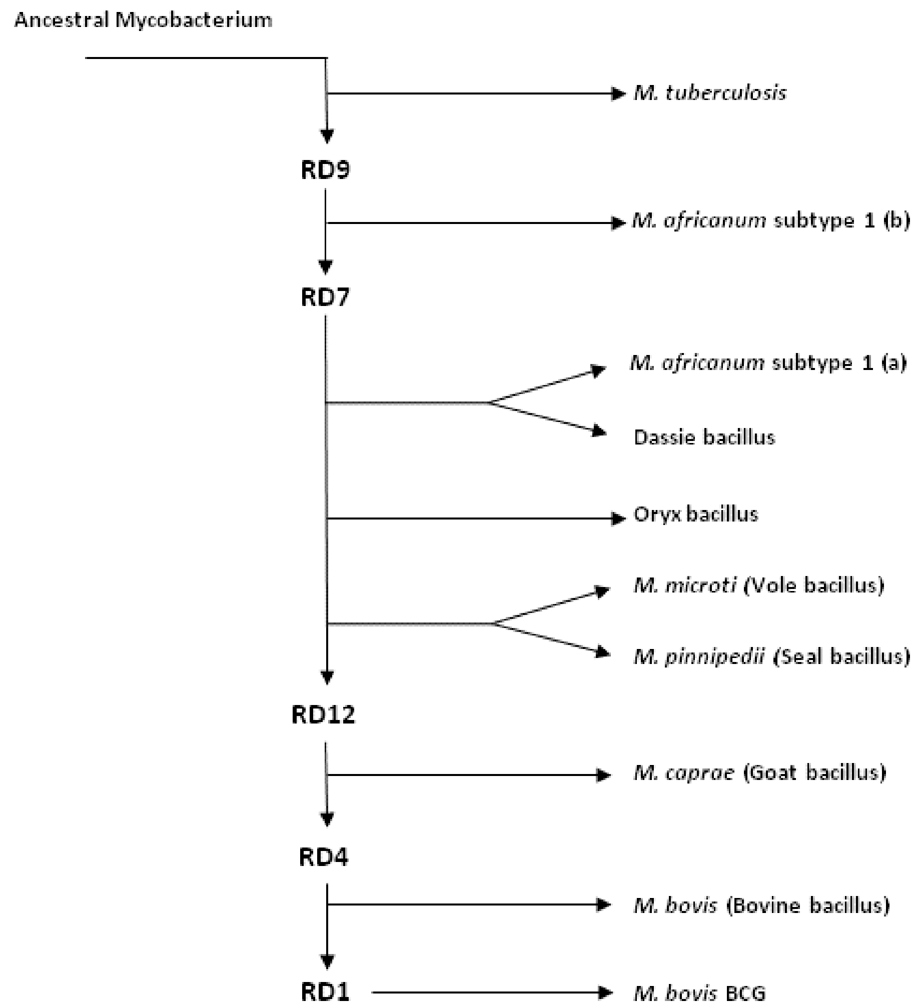

Fig. 8.1 Phylogenetic tree of the members of the *Mycobacterium tuberculosis* complex. "RD" denotes specific "regions of difference" detected between the genomes of different species.

The mycobacteria that are able to colonize animals have evolved such that many specialise, forming host-adapted ecotypes with preferred hosts[20]. These include *M. tuberculosis* and *M. africanum*, which tend to be found almost exclusively in humans. *M bovis,* on the other hand, has a very broad range of hosts, although it is found primarily in bovids. It is interesting to note that although many of these species (that are pathogenic to mammals) have evolved from a common ancestor (see fig 1), they are rarely, under natural circumstances, found outside of a defined host or host family (e.g. *M. microti* in voles, *M. pinnipedii* in seals, and dassie bacillus in dassies). In addition, there is strong evidence for an original home range or home population for some species or subspecies[20]. It should be noted that even *M. tuberculosis* has evolved extensively, leading to various strains and substrains that have individual differentiating characteristics. Some strain types are seen in abundance in certain populations of people or geographical regions and are absent or almost absent in other regions. Accordingly, we have the well-known "Beijing" strains of *M. tuberculosis* that are highly abundant in Asia and have now spread worldwide. This strain family is highly successful[6,21], prone to becoming drug resistant[8] (Johnson et al., manuscript in preparation), and is increasing in numbers in the Western Cape relative to other strains[21]. On the other hand, the so-called KZN (XDR) TB strain, which caused much publicity recently with high numbers of deaths in Tugela ferry[4], is not a new strain at all, but is part of a family of *M. tuberculosis* strains found all over South Africa[21]. This XDR strain variant of *M. tuberculosis* has found a vulnerable population in an extremely high HIV incidence area and has thus been able to cause increased mortality in this population[4].

8.3 New technologies highlight emerging disease

While many basic microbiological techniques to define mycobacterial species were developed decades ago, these are extremely labour intensive and cannot discriminate all species or subspecies and certainly not strain types. However, these techniques were able to define important pathogens such as *M. tuberculosis* and *M. bovis* in the late 1800's and even less important ones such as the dassie bacillus in the late 1950s. The dassie bacillus (which has never received a "proper" name), was found first in dassies from the Nieu Bethesda area, was tested for virulence by deliberate infection in a number of TB susceptible animal species and found to be non-pathogenic in these.

However, newly developed molecular-based (genome) techniques allow one to develop entirely new diagnostic and typing techniques and to identify species and subspecies more easily and more accurately. Such technologies, which include PCR-sequencing of target genes, as well as line probe hybridization assays, have been successfully applied to the mycobacterial field. These are usually based on detection of known specific DNA sequences, and include the newly-developed Hain Genotype product series which is used to speciate some Mycobacteria and to detect certain drug resistance markers[18]. These easy to use, fast and accurate techniques have allowed an explosion of new investigations, aimed at re-examining many of the old established dogmas in mycobacterial disease. A vast body of this type of work is done in research-based laboratories, where traditionally, research results often do not reach a wider audience. This paradigm is shifting, with researchers now putting more effort into translational research and dissemination of information. Some effort has also been directed to check that past species identification is in fact correct.

This was not possible in the past because of old and crude techniques which were not adequately discriminatory or simply too labour intensive and expensive to utilise.

It should be noted that with the advent of antibiotics (mostly from the late 1950s up to the mid-1960s), TB incidences largely stabilised or declined in the human population in many countries[10]. Similarly, in the veterinary field, prevalence of *M. bovis* declined, since skin testing and slaughter policies were quite effective[11]. The consequence was that TB was thought to be conquered and it became a neglected area of research for quite some time. This is nowhere clearer illustrated than in the words of the Nobel prize winner Selman A. Waksman (discoverer of over twenty antibiotics) who in 1964 said in his "The Conquest of Tuberculosis" that "*…the ancient foe of man, known as consumption, the great white plague, tuberculosis, or by whatever other name, is on the way to being reduced to a minor ailment of man. The future appears bright indeed, and the complete eradication of the disease is in sight…*"

8.4 The "new" TB epidemic

For reasons that are not clear, the incidence of *M. tuberculosis* in the South African population increased dramatically from the mid-1970s even prior to the introduction of HIV (South African National Department of Health). Similar increases were noted worldwide, even in developed countries such as the USA where socio-economic conditions were good and healthcare was improving steadily. This prompted new investment and a resurgence of research in TB, with the additional development of new technology.

Using these new sensitive tools, we are now able to find new mycobacterial species in previously unidentified niches. It is likely that many of these were present in the past, but simply not identified or misidentified using the crude techniques of the past. Essentially, we find them because we are now actively looking for them and because recent research has allowed more detailed speciation. We have identified a number of these rare species in our laboratory at Stellenbosch University, but this is part of our advanced research programme and one should not expect such sophistication from a routine diagnostic lab. We recently embarked on a large TB prevalence survey in humans in the Western Cape and while we found many individuals with *M. tuberculosis* infection who were asymptomatic, as expected, we found essentially as many who were infected by non-tuberculous mycobacteria (unpublished). Some of these mycobacteria are known pathogens, but no apparent signs of clinical disease were evident in the individuals infected by them. We do not know the impact of these infections and this will require extensive research to try to understand. Some of these individuals were followed up to ascertain if disease developed, but no symptoms are yet evident in those checked (unpublished results).

Additionally, because of "failure to cure", we have diagnosed *M. intracellulare* in two human cases recently (unpublished data). If these samples had not been sent to our research laboratory, they would have been classified as drug resistant TB and treated accordingly, or the patient may have died, as the case may be. Both patients were of advanced age, with the indication therefore that *M. intracellulare* may primarily be a pathogen of immunocompromised individuals.

8.5 Relevance to animals

The “new or emergent” TB in animals should be seen in a similar light. There have been many cases of cross-species infection from zoos. The most common perhaps is *M. tuberculosis* moving into animals from handlers or the viewing public[12], or *M. bovis* moving between animals or to human handlers from the animal. We have found *M. tuberculosis* in captive baboons[14]) and vervet monkeys in South Africa and even in free living baboons in close contact with farm workers or farmyards. This is not at all surprising, given the cavalier attitude to TB evinced by many South Africans, with spitting and unsanitary behaviour being common, and a TB incidence rate which is arguably the highest in the world.

We were aware of the dassie bacillus which has more recently been isolated in Australia from captive dassies originating from the RSA[2]. We were therefore keen to obtain a dassie carcass that showed visible lesions to see if we could “rediscover” this species in South Africa, since we were not able to obtain a culture from outside the RSA for our basic research. This opportunity occurred recently when 2 apparently healthy dassies in good condition were shot as part of a leopard research project in the Winterhoek Mountains of the Western Cape. The dassie had extensive necrogranulomatous pneumonia due to infection with the dassie bacillus[16]. Since previous reports of the dassie bacillus do not point to the Western Cape, this most likely shows that the dassie bacillus is wide-spread in the RSA. However, this should be confirmed, since we do not have final proof of its distribution.

We recently diagnosed a case of *M. bovis* infection in a black rhinoceros held in one of the facilities of the National Zoological Gardens[3]. In the case of this rhino, it should be noted that the rhinoceros was elderly and in poor condition. It cannot be said unequivocally that *M. bovis* caused the poor condition of the rhinoceros at all, since only two relatively small lesions were found at necropsy.

It is perhaps pertinent to note at this point that tuberculosis in humans is a complex disease. We know that after infection three possible routes can be taken: 1) the person does not establish an infection at all and the bacteria are killed, 2) a primary infection is established, but contained and no symptoms occur, 3) there is immediate progression to disease. In the absence of immunosuppression, only 5% of infected individuals fall into the latter class. Of those who initially contain infection, only 5% develop disease in their lifetime. In developed countries such as Europe and the USA, one can see this phenomenon in the elderly, once the immune system functionality declines. Thus, fewer than 10% of infected humans will develop active TB after infection (in the absence of immunosuppression).

We do not know what the situation is in different animal species, or whether different mycobacteria can do the same as *M. tuberculosis* in humans. However, it is highly likely that a similar situation prevails in many animals with perhaps quantitative differences in their relative proportions that progress to active disease soon after infection compared to those which can be latently infected and reactivate later.

Thus we speculatively explain the recent “oryx bacillus” event in which an oryx bacillus was isolated from a healthy, skin test positive African buffalo on a farm known to have

previously imported buffalo from a zoo in Portugal (Gey van Pittius et al., manuscript in preparation). The oryx bacillus was originally isolated from Arabian oryx and camelids, and thus far appeared to be restricted to the northern hemisphere. Buffalo captive in a Portuguese zoo were probably in contact with Arabian oryx, which allowed infection. The oryx bacillus infection was presumably kept in check by the innate or adaptive immune system of the buffalo. Some buffalos from the Zoo in Portugal were then transferred to South Africa approximately 20 years ago, in all likelihood bringing the dormant oryx bacillus in at least one animal. It is unlikely that we have sampled such an animal, but transmission between animals without observed symptoms might have occurred and we detect it in a second or third generation animal. Given the rare reactions seen in this herd, as well as the lack or apparent lack of disease noted over nearly 20 years, as far as our information extends, it is likely that this is not a serious pathogen in buffalo. However, we do not know that for certain, and we also do not know what could happen if it were to be excreted by the buffalo and infect other wildlife. The pathogenicity of the organism in the Southern African oryx species called the Gemsbok (*Oryx gazella*) has never been established, but given their relatedness to the Arabian oryx, the preferred host species of this organism, it can be hypothesized that it would also be pathogenic for gemsbok. Perhaps the oryx bacillus (like the dassie bacillus) is less virulent than *M. tuberculosis* or *M. bovis*. (Virulence is not understood at all well in mycobacteria, particularly when the species is not in its preferred host). Buffalo may even have reasonable innate resistance to oryx bacillus, although clearly not to *M. bovis*. Note that *M. tuberculosis* infection is extremely rare in bovids and that transfer of *M. tuberculosis* from one bovid to another is

not a problem in infected bovids, as far as we know. We should not lose sight of the fact that *M. bovis* is probably a far more serious mycobacterial pathogen compared to the oryx bacillus, however. We do not even know whether buffalo infected by the oryx bacillus will mount a positive skin test response in all cases. The only way to know is to test this experimentally.

From a management point of view, this is a difficult problem. One cannot know whether the oryx bacillus has crossed into another host with potential to infect buffalo again. Therefore to slaughter all buffalo on the affected property may not work. On the other hand, a typical response to a BTB herd would be recommended, i.e. initial test and slaughter, with regular follow up if possible.

Finally, it should be noted that in many instances, cases of TB in animals do not involve the lungs and therefore unless the animal is eaten uncooked (e.g. predated), it presents only a remote chance for transmission, particularly to humans.

8.6 Dog TB

In a recent case[15], a stray Maltese crossbreed domestic dog with extensive multifocal pulmonary tuberculosis due to *M. tuberculosis* was detected as part of an intensive research project being done in our centre to investigate innate resistance to TB. The rationale is that if we can understand innate or acquired resistance, we may be able to develop entirely new therapies. Since TB has been reported very rarely in dogs, we hypothesize that dogs have innate resistance to TB. In order to do this study, we selected dogs which would come from

communities in Cape Town that have amongst the highest reported TB incidence in the world. We know that at least 80% of adults in these communities show infection by *M. tuberculosis* (exposure)[9] and therefore the dogs are almost certainly also exposed to TB infection. Owing to poverty and many other social factors, hundreds of dogs from such communities are euthanized by animal welfare organisations every year and these animals provide an important resource for biosurveillance of zoonotic diseases such as TB. A limited post mortem survey of 100 stray dogs, using the "gold standard" of diagnosis, viz., culture, detected 1 animal with pulmonary tuberculosis, and infection in 3 others which showed no gross or histopathological signs of disease. Apart from bacterial culture, there is no validated test for TB infection in dogs; however, we have detected a high prevalence of immunological sensitivity to *M. tuberculosis* antigens among dogs living with TB patients suggesting a high level of transmission of this pathogen from owners to their pets. Our interpretation of these early findings is that despite frequent transmission of *M. tuberculosis* to dogs, a relatively small number of infected animals progress to active TB and that these animals contribute negligibly to the national TB epidemic. As such, our current data suggests no need for a concerted intervention in this regard.

However, there may be particular circumstances where the potential for pets to act as reservoirs of infection should be carefully considered. HIV positive individuals are highly susceptible to TB and, where possible, companion animals living in close contact with immunocompromised individuals should be screened for all zoonotic diseases. Animals that have potentially been infected by multi drug-resistant (MDR) strains of *M. tuberculosis* warrant concern. The management of MDR-TB requires enormous resources

and in extreme cases, this disease may be virtually untreatable. Companion animals infected with these strains therefore potentially pose a significant danger to human health. However, since we have found only one case of TB with fulminating lesions in a dog after 3 years of working in a high risk environment, transmission of TB from dogs can be regarded to be highly unlikely in most cases. Given the infection pressure of *M. tuberculosis* from human to human, particularly in South Africa where the disease is so prevalent, dog-to-human transmission risk is almost certainly therefore vanishingly low. However, it may be of interest to practising vets, who may suspect TB occasionally in a dog or related wild animal. In our opinion, it does not justify any intervention strategies at this stage.

8.7 Why did we not find these before?

It should be noted that new technology, particularly in genomics, has made identification easier and more accurate, and more mycobacterial species are differentiated and described every year. Mycobacteria are common in the environment[11] and under the right circumstances can become pathogens. We may therefore expect more, but probably rare, interesting and unusual cases to emerge over time.

It is estimated that there are approximately four to six nonillion (4-6 x 10^{30}) bacteria on Earth[25], and that we have only described around 4000 prokaryotic species to date, with recent estimates for the number of species in this domain of life ranging from 10^5 to 10^{75} Clearly, identification and description of these is beyond the remit and capabilities of

routine laboratories. It is also costly to establish advanced technology, particularly for rare events, and therefore not within the boundaries of most routine investigations. Most practising clinicians and veterinarians cannot be expected to be aware of the complexities of the *Mycobacteria* as a genus, nor of what might constitute an appropriate request for an advanced level test nor appropriate response to an unusual result. This is still a highly specialised research area. Our laboratory has developed a very deep and thorough speciation technology[24,22] involving genomics and bioinformatics. As such, we do not offer a routine diagnostic service, but a research based collaborative service. We deliberately search for interesting cases and mycobacterial species as part of our interest and mandate. Even this has only very recently been made possible by increased funding and new technologies that we have developed.

8.8 Conclusion

Much research still needs to be done to understand TB in humans and animals. Vigilance needs to be exercised, but not at the cost of trying to keep in check the major epidemics of *M. tuberculosis* in humans in SA, and *M. bovis* in domestic stock and wildlife. We are increasingly finding cases of TB-like disease due to species of mycobacteria not within the *M. tuberculosis* complex, but this may simply represent our success in searching deliberately for these. Many of these were almost certainly here before recent reports, but remained undetected because of rare occurrence, or the absence of clinical disease and of tools or funding to identify them. At this stage, in our opinion, it is doubtful that they

represent a serious threat to animal or human health other than at an occasional individual level. Nevertheless, many of the Mycobacteria can be regarded as “alien” to the RSA and as such should be kept under surveillance. Any veterinarian or biologist who finds an “unusual” case that is a suspect mycobacterium sp. infection is most welcome to contact us in this regard.

8.9 References

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CHAPTER 9

Conclusion

Comparative studies have long contributed to the understanding of the aetiology, pathogenesis, and diagnosis of tuberculosis (TB). With this in mind, opportunities to investigate naturally occurring mycobacterial infections in animals were identified in order to gain further insight into this disease. During the course of this study, the dassie bacillus, an unusual member of the *Mycobacterium tuberculosis* complex (MTC), was isolated from a free-living rock hyrax; the occurrence, epidemiology and immunology of canine *M. tuberculosis* infection was studied; and a novel method for the diagnosis of TB in non-human primates was investigated. Additionally, the documentation of unusual mycobacterial infections in various animals in South Africa was reviewed.

The identification and documentation of the occurrence of the dassie bacillus in a population of rock hyraxes in the Western Cape provides impetus and opportunity to further investigate this pathogen. Prior to this finding, the dassie bacillus had been isolated in the late 1950s from rock hyraxes from Nieu-Bethesda in the Eastern Cape Province of South Africa and from zoo specimens captured at unknown sites in this country. Our finding therefore suggests that this bacillus is widespread in the South African rock hyrax population. Subsequently, Gudan et al. (2008) have reported the

isolation of *M. africanum* from a rock hyrax originating from the United Arab Emirates (UAE)[4]. The authors used a commercial speciation assay, the GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany) which uses DNA sequence polymorphisms of the gene DNA gyrase subunit B (*gyrB*) to differentiate between members of the MTC[6]. However, criteria for the speciation of the dassie bacillus are not included in this test. Given that the dassie bacillus and *M. africanum* share common single nucleotide polymorphisms (SNPs) in *gyrB*, and that *M. africanum* has been almost exclusively isolated in West Africa and only occasionally elsewhere[5], it seems highly probable that the organism isolated from the UAE rock hyrax was in fact the dassie bacillus, suggesting that this bacillus is more widespread than previously documented.

Unfortunately, following initial growth of this bacillus in our laboratory, stocks thereof stored both at - 80°C and at 37°C could not be re-cultured for further analysis. However, our identification of this organism in local rock hyrax populations presents the possibility of actively attempting to re-establish such cultures. The dassie bacillus displays limited virulence in many laboratory animals which are susceptible to *M. tuberculosis* and *M. bovis*[7] and comparative genetic studies of these organisms could reveal genetic traits which confer such virulence. While known virulence factors (i.e. ESAT-6 and CFP-10) are encoded by the genetic region of difference 1 (RD1) of the MTC genome[2], *M. microti*, *M. bovis* BCG and the dassie bacillus all contain large sequence polymorphisms (LSP) in this region. The variation in virulence of these mycobacteria[7] therefore suggests that other important virulence factors may be identified through comparative studies of these organisms.

Vaccination of guinea pigs with the dassie bacillus has been shown to confer partial immunity to *M. bovis* infection, almost doubling their time to death following infection with *M. bovis*[7]. This is similar to the protection conferred by *M. bovis* BCG vaccination which reduces time to death in guinea-pigs following *M. tuberculosis* infection[3]. It has been suggested that only an effective vaccine program will control or eliminate TB[1]. It is therefore tempting to consider whether the dassie bacillus would not be a suitable candidate for development as an alternative TB vaccine, especially in ecosystems in which the bacillus naturally occurs, for example in African wildlife.

An investigation of the virulence of the dassie bacillus in rock hyraxes may also prove valuable. It has been reported that in recent years there has been a sharp decline in rock hyrax populations in some regions of South Africa (pers. com. P.D. van Helden). The cause for this is unknown, but the finding of severe TB in a free-living rock hyrax suggests that a study of the impact of this disease on the population dynamics of this species may be warranted.

The investigation of the spillover of *M. tuberculosis* from human TB patients to contact dogs provided an opportunity to explore the occurrence, epidemiology, and immunology of TB in this species. As a result of this study, the first known example of this disease in South Africa was recorded. Additionally, in the particular social setting in which this study was performed, high levels of *M. tuberculosis* transmission were shown to occur between smear positive TB patients and their pet dogs, even when interaction between humans and pets was not particularly close. A potential danger of canine tuberculosis is the fact that diseased animals could act as a source of human infection and early detection of these animals may be important. However, no

immunological tests have been validated for the diagnosis of canine TB and development of such tests would prove highly valuable. In this regard, ElephantTB STAT-PAK, a serological test for TB has shown some promise (pers. com. M. L. Boschiroli), however, validation of this or other tests will require further studies.

Despite the documentation of high levels of *M. tuberculosis* transmission to companion dogs, the rarity with which canine TB is diagnosed during human TB epidemics supports experimental data that this species is naturally resistant to this disease. As such, an attempt was made to further characterise the cell-mediated responses to TB in infected dogs, thereby potentially identifying markers of such resistance. Such an undertaking had three major limitations. Firstly, no validated assay was available for the identification of truly infected animals. Nonetheless, immunological sensitivity to the proteins ESAT-6 and CFP-10, both reasonably specific to the MTC, might have provided a suitable proxy for a definitive diagnosis. A second limitation was the limited availability of suitable canine reagents, meaning that convoluted and time-consuming techniques had to be employed to suitably label peripheral blood mononuclear cells (PBMC) for analysis by flow cytometry. Thirdly, the differences in the biology of canine immune cells compared to other species precluded the planned use of lymphoproliferative assays used to investigate the CMI of TB in humans and a number of ruminant species. While the availability of species-specific and cross-reactive reagents will improve, the infection status of animals naturally exposed to TB will often remain unverified, and unpredictable biological differences between species will continue to hamper the investigation of TB immunology in non-experimental and poorly characterised species.

The diagnosis of TB in such species may, however, benefit from the modification of a commercial human TB interferon-gamma release assay (IGRA). We have shown that the use of the QuantiFERON-TB Gold (In Tube Method) assay (QFG-IT) shows great promise as a sensitive test for TB in baboons, and that a modification of this assay was able to detect both active TB and non-clinical mycobacterial infection in a rhesus macaque. Because the QFG-IT is performed in closed test tubes containing the test antigens, processing and incubation of samples does not require sterile conditions, making the test suitable for veterinary applications. As such, further investigation of the use of this modified test in other species may prove valuable.

Together, these studies have systematically investigated natural mycobacterial infection in various species in South Africa, identifying the possibilities and limitations of such studies to provide insights into TB pathogenesis, epidemiology, and diagnosis.

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