TUBERCULOSIS IN WILD AND DOMESTIC ANIMALS IN SOUTH AFRICA

Tuberculose in wilde en gedomesticeerde dieren in Zuid Afrika
(met een samenvatting in het Nederlands)

Tuberkulose in Wild- und Haustieren in Sűdafrika
(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 23 juni 2008 des middags te 2.30 uur

door

Anita Luise Michel

geboren op 20 juli 1963
te Zőschingen, Duitsland

Promotoren: Prof.dr. W. van Eden
Prof.dr. J.A.W. Coetzer
Prof.dr. V.P.M.G. Rutten

This thesis was partly accomplished with financial support from the South African National Department of Agriculture, Directorate Animal Health.

In memory of

Mrs Ruth Gerhold,

my mentor, who planted the seed of self-confidence

which enabled me to pursue my dream to study veterinary science.

We will never know where my path would have led me without you teaching me positive thinking and courage, but I do know that your approach to life has guided me in much of my personal and professional career.

ISBN/EAN: 978-90-393-4803-1

The majority of studies presented in this thesis were performed at the Bacteriology Section of the ARC-Onderstepoort Veterinary Institute. Buffalo studies were conducted in collaboration with South African National Parks in the Kruger National Park.

Contents

Chapter 1

Overview

Chapter 1.1

General introduction

Aetiology

Bovine tuberculosis is caused by *Mycobacterium bovis*, which forms part of the *Mycobacterium tuberculosis* complex along with other closely related mycobacteria. *M. tuberculosis, M. africanum* and *M. canettii* are human pathogens, while *M. microti* affects rodents and *M. pinnipedii* have been isolated from seals (Smith et al. 2006). *M. caprae* has been initially classified as subspecies of *M. bovis* but was recently recognised as a species on its own. *M. bovis* has the widest host range of all mycobacteria, although susceptibility and pathological changes vary vastly between animal species. Modern genome analysis of *M. tuberculosis* complex mycobacteria has revealed, against the previously held dogma, that human tuberculosis has not evolved from *M. bovis* (Brosch et al. 2002) but that a separate lineage, represented by *M. africanum*, *M. microti* and *M. bovis,* branched from the progenitor of *M. tuberculosis* strains. This process was accompanied by a successive loss of DNA, which may have contributed to the appearance of more successful pathogens in new host species. The last common ancestor representing a much broader progenitor species of tubercle bacilli could already have affected early hominids in East Africa at least 2.6 million years ago (Gutierrez et al. 2005).

Pathogenesis

Mycobacterium bovis may infect the host via different routes, depending on the host species and infection pressure in the affected population. A common mode of transmission in most species is through aerosols, which usually leads to the formation of a primary tubercle in the lymph nodes of the upper respiratory tract, mostly the tonsils and/or retropharyngeal lymph nodes. Very small aerosol droplets may also directly penetrate into and colonise the bronchi of the lungs. Involvement of the gastrointestinal tract can occur as a primary event if infected material is ingested or as a consequence of swallowing infectious mucus secreted from the lungs. Lesions are typically manifested in bovine species as granulomas or tubercles, which comprise a caseous necrotic core surrounded by a zone of

inflammation. Their size can vary from microscopic to several centimetres or they may even partially or fully replace the functional tissue of the affected organ. The distribution, appearance and severity of lesions differ widely between host species and were described for African buffalo and other wildlife species by many different investigators (Keet et al. 1996, Keet et al. 2001, de Vos et al. 2001, de Lisle et al. 2002). It is virtually impossible to provide information on the length of time necessary from infection to the formation of lesions as the onset and severity of disease appears to be inversely related to the inoculation dose, both in cattle (Buddle et al. 1994) and African buffalo (de Klerk et al. 2006). The age of the animal, route of infection and strain of the organism are additional factors which may critically influence the time period required for the disease to develop (Thoen & Himes 1986). Experimental infections in cattle with $9x10^3$ bacilli resulted in lesions in lungs and lymph nodes similar to cases of field tuberculosis after a period of one to two months (Neill et al. 1988).

Chapter 1.2

The burden of tuberculosis in animals in South Africa

Historical background of bovine tuberculosis

Bovine tuberculosis was a well-known livestock disease during colonisation in South Africa, most frequently observed in imported cattle from Europe as well as from South America and Australia (Cousins *et al.* 2004). Especially dairy farmers in the Western Cape, but also in other parts of the country, suffered significant economic losses due to condemned carcasses at the abattoirs (Viljoen 1927). As a result bovine tuberculosis was among the first notifiable diseases in the Diseases of Stock Act, promulgated in 1911 in the then Union of South Africa and the tuberculin skin test was introduced using Old Tuberculin imported from Europe. The widespread application of the skin test prompted local production of first Heat Concentrated Tuberculin and in 1947 of Purified Protein Derivative (PPD) using *M. tuberculosis* strains. Field trials conducted in the country a short while later showed that tuberculin produced from *Mycobacterium bovis* was more specific and in 1960 South Africa was one of the first countries worldwide, after The Netherlands, to issue bovine tuberculin (Kleeberg 1960). However, the herd prevalence of bovine tuberculosis could not be effectively combated until the National Eradication Scheme was introduced in 1969 and driven efficiently (Michel *et al.* 2008).

Bovine tuberculosis in domestic cattle

When the occurrence of bovine tuberculosis in commercial herds had been successfully decreased and resources were diverted to controlling other diseases, the test and slaughter programme was no longer sufficiently enforced and the disease continued to persist in the country. The endemic character is evident in the occurrence of sporadic outbreaks discovered most often during routine slaughter and sometimes *post* movement testing. In one case the incidental diagnosis of *M. bovis* in fallen stock triggered the diagnosis and culling of close to

6000 reactor cattle on a single farm in the Eastern Cape between 2004 and 2006 in an attempt to eradicate a major outbreak of bovine tuberculosis (PRO/AH/EDR>Tuberculosis, bovine – South Africa (Eastern Cape) 24 May 2004). The farm had been stocked with cattle sourced from several auctions in the area during the years preceding the outbreak. Despite bovine tuberculosis being a controlled disease under the Animal Diseases Act, law enforcement is increasingly difficult if animal owners are not willing to accept and carry the economic burden of disease control measures. According to the compensation policy in South Africa the monetary value of reactor cattle is, by nature of their infection status, reduced from market to mere slaughter value. The resulting income deficit can be significant and is often seen as reason for farmers to illegally move and trade infected cattle, which contributes to the increased spread of bovine tuberculosis in the country.

Mycobacterium bovis in wildlife

Tuberculosis in wildlife had first been reported (Paine & Martinaglia 1929) in greater kudus in the Eastern Cape region but was not considered a significant problem until a high prevalence of *M. bovis* infection was discovered among herds of African buffaloes (*Syncerus caffer*) in both the Hluhluwe-iMfolozi Park (HiP) (Jolles 2004) and the Kruger National Park (KNP) (Bengis et al. 1996). Nowadays the infection has been diagnosed in 14 different wildlife species, some of which develop serious clinical signs. While transmission occurs in most cases through spillover from buffalo, either by predation, scavenging or contaminated habitat, intra-species transmission is a possibility especially in lions and kudus, as described in more detail in chapter 2. Also addressed in the subsequent chapters are recent advances in our knowledge on the epidemiology, diagnosis and first attempts to control tuberculosis in wildlife.

Mycobacterium bovis infection in humans

Mycobacterium bovis can be transmitted to people by one of two major routes, either via the alimentary tract or through aerosol transmission during close contact with infected cattle. In recent decades human tuberculosis caused by *M. bovis* has become uncommon in developed countries as a consequence of the compulsory pasteurisation of milk and the large scale eradication of infected cattle herds during the second half of the 20th century. The rapid success in combating cattle tuberculosis was, however, not immediately paralleled by the disappearance of *M. bovis* cases in humans, especially in adults. Possible explanations include long latency periods in adult *M. bovis* infection and reactivation of previous foci of infection acquired before compulsory pasteurisation (Meissner & Schroeder 1974, Cotter et al. 1996). Although the source of *M. bovis* is almost exclusively of environmental or animal origin, a recent report from the UK of an outbreak in young, epidemiologically linked

patients suggests that person-to-person transmission is a possibility (Evans et al. 2007).

In developing countries the bovine tuberculosis status of cattle herds is monitored less frequently, if at all, and control measures are often minimal (Cosivi et al. 1998). In South and Southern Africa only limited testing of especially communal cattle herds is carried out. As a result, of the 1.7 million inhabitants of the magisterial districts adjacent to the KNP and HiP, an estimated 165000 people live in close contact with livestock and on a daily basis consume unpasteurised milk and other products (Michel et al. 2003a). At the same time the HIV prevalence in this region is among the highest in Africa and the world and known to drive the tuberculosis epidemic in this part of the globe (UNAIDS 2006). Once transmitted, *M. bovis* might be more likely to establish disease in immunocompromised people than in healthy individuals (Grange 2001). Tuberculosis in humans caused by *M. bovis* or *M. tuberculosis* is indistinguishable clinically, radiologically and pathologically and location of lesions depends on the route of infection (Moda et al. 1996, Wedlock et al. 2002). Although rare, M. *bovis* can cause disseminated infection in immunocompromised as well as immunocompetent patients (Albrecht et al. 1995, Schübel et al. 2006). The role of *M. bovis* in human tuberculosis is still unknown in the majority of countries in Africa, including South Africa, but it is more likely to occur where there is a high prevalence of *M. bovis* in the cattle population (Sjörgren & Sutherland 1975). Vice versa, human-to-cow transmission of *M. bovis* has been known for a long time in Europe and recently the possibility of two-way transmission between cattle and their owners has been suggested in Ethiopia (Schmiedel 1968, Regassa et al. 2007). Molecular studies by Isabel et al. (2007) indicated that IS*6110* transpositions within the *M. bovis* genome might act as a driving force for adaptation of the organism from the animal to the human host. *M. bovis* infections in humans have also been confirmed in Nigeria and Tanzania (Cadmus et al. 2006, Cleaveland et al. 2007).

Tuberculosis in animals caused by *M. tuberculosis*

Animals with tuberculosis most often live in close contact with domestic animals or humans, with the highest prevalence rates occurring in zoological collections. It is therefore one of the most frequently recorded infectious diseases of captive wildlife (Griffith 1928). Although in some of the early reports no speciation of tubercle bacilli was mentioned, it is reasonable to conclude that outbreaks were equally often caused by *M. tuberculosis* and *M. bovis* (Griffith 1928, Kovalec 1980) With improved test and control measures in the livestock as well as the captive wildlife populations the incidence of *M. bovis* decreased in zoological collections, while *M. tuberculosis* appeared to remain a reason for high concern in all countries where tuberculosis in humans continued to be of great public health concern . In South Africa, tuberculosis presently accounts for 80% of all notifiable

diseases in humans and the incidence rate ranges between 500 and 1500/100000 inhabitants. The risk of spillover of *M. tuberculosis* from humans to animals is hence considered high wherever the conditions for transmission exist. A molecular study of the *M. tuberculosis* cases in the National Zoological Gardens of Pretoria over a period of eleven years indicated that the disease was more frequently transmitted from visitors than between animals of the same or different species (Michel et al. 2003b).

Free-ranging wildlife is believed to be less prone to *M. tuberculosis* as compared to those in captivity (Griffith 1928). An increasing number of isolations has been made in South Africa in recent years, including meerkat and banded mongoose (Alexander et al. 2002), sable antelope, springbuck and bontebok (Michel, 2007). These observations may be an early indicator for an emerging negative impact of human activities and interactions at the interface with wildlife and require more in-depth investigation of particular settings. Although probably less likely, it is possible under certain circumstances that wildlife infected with *M. tuberculosis* can transmit the infection back to humans.

M. tuberculosis is only occasionally found in domestic pigs where it causes caseous lesions in the parotid lymph nodes, detected during routine meat inspection (Fourie et al. 1950). Kleeberg & Nel (1969) reported a marked increase in lymphadenitis cases in slaughter pigs of which 6% were caused by the human tubercle bacillus. In contrast, during the years 1970 to 1985, close to 20% of lymph node samples submitted to the Tuberculosis Laboratory of the then Veterinary Research Institute at Onderstepoort for mycobacterial culture from slaughter pigs in South Africa yielded *M. tuberculosis*, indicating the extent of the health problem in humans (Huchzermeyer, pers. comm.).

Other examples of animals serving as sentinels for human tuberculosis were witnessed through the laboratory isolations at OVI and included fatal cases of *M. tuberculosis* infection in each of the following species: a privately owned parrot, a free-ranging baboon living in a farming area, a wild vervet monkey from an urban area and a pet marmoset monkey (Michel & Huchzermeyer 1998) as well as several antelope species kept under semi-free ranging conditions.

References

Albrecht H, Stellbrink HJ, Eggers C, Rüsch-Gerdes S, Greten H. 1995. A case of disseminated Mycobacteruim bovis infection in an AIDS patient. European Journal of Clinical Microbiological Infectious Diseases.

Alexander KA, Pleydell E, Williams MC, Lane EP, Nyange JFC, Michel AL. 2002. *Mycobacterium tuberculosis*: An emerging disease of free-ranging wildlife. Emerging Infectious Diseases 8:592-595.

Bengis RG, Kriek NPJ, Keet DF, Taath JP, de Vos V, Huchzermeyer HFAK. 1996. An outbreak of bovine tuberculosis in a free-living buffalo (*Syncerus caffer*-Sparrman) population in the Kruger National Park. Onderstepoort Journal of Veterinary Research 63:15-18.

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser CK, Garnier T, Gutierrez C, Hewnison G, Kremer K, Parson LM, Pym AS, Samper S, van Soolingen D, Cole ST. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proceedings of the National Academy of Science 99:3684-3689.

Buddle BM, Aldwell FE, Pfeffer A, de Lisle GW, Corner LA. 1994. Experimental *Mycobacterium bovis* infection of cattle – effect of dose of *Mycobacterium bovis* and pregnancy on immune responses and distribution of lesions. New Zealand Veterinary Journal 42:167-172.

Cadmus S, Palmer S, Okker M, Dale J, Gover K, Smith N, Jahans K, Hewinson G, Gordon SV. 2006. Molecular analysis of human and bovine tubercle bacilli from a local setting in Nigeria. Journal of Clinical Microbiology 44:29-34.

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

Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D, Robinson RA, Huchzermeyer HF, de Kantor I, Meslin FX. 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerging Infectious Diseases 4:59-70.

Cotter TP, Seehan S, Cryan B, O'Shaughnessy E, Cummins H, Bredin CP. 1996. Tuberculosis due to *Mycobacterium bovis* in humans in the south-west of Ireland: Is there a relationship with infection prevalence in cattle? Tuberculosis and Lung Disease 77:545-548.

Cousins, D.V., Skuce, R.A., Kazwala, R.R., van Embden, J.D.A., 1998a. Towards a standardized approach to DNA fingerprinting of *Mycobacterium bovis*. International Journal of Tuberculosis and Lung Disease 2:471-478.

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

De Lisle GW, Bengis RG, Schmitt SM, O'Brien DJ. 2002. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. Revue Scientifique et Technique des Office International des Epizooties 21:317-334.

De Vos V, Raath JP, Bengis RG, Kriek NJP, Huchzermeyer H, Keet DF, Michel A 2001. The epidemiology of tuberculosis in free ranging African buffalo

(*Syncerus caffer*) in the Kruger National Park, South Africa. Onderstepoort Journal of Veterinary Research. 68:119-130.

Evans JT, Smith EG, Banerjee A, Smith RMM, Dale J, Innes JA, Hunt D, Tweddle A, Wood A, Anderson C, Hewinson RG, Smith NH, Hawkey PM, Sonnenberg P. 2007. Cluster of human tuberculosis caused by *Mycobacterium bovis*: evidence for person-to-person transmission in the UK. The Lancet 369:1270-1276.

Fourie PJ, de Wet GJ, van Drimmelen GC. 1950. Tuberculosis in pigs caused by *M. tuberculosis* var, hominis. Journal of the South African Veterinary Medical Association, 21:70-73.

Gutierrez MC, Brisse S, Brosch R, Fabre M, Omais B, Marmiesse M, Supply P, Vincent V. 2005. Ancient Origin and Gene Mosaicism of the Progenitor of *Mycobacterium tuberculosis.* PloS pathogens. 1:e5 doi:10.1371/journal. ppat. 0010005

Grange JM. 2001. *Mycobacterium bovis* infection in human beings. Tuberculosis 81:71-77.

Griffith AS. 1928. Tuberculosis in captive wild animals. Journal of Hygiene (Camb) 28:198-218.

Isabel O, Gomez AB, Kremer K, de Haas P, Garcia MJ, Martin C, van Soolingen D. 2007. Mapping of IS*6110* insertion sites in *Mycobacterium bovis* isolates in relation to adaptation from the animal to human host. Veterinary Microbiology (ahead of print).

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

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

Keet DF, Kriek NPJ, Bengis RG, Michel A. 2001. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park. Onderstepoort Journal of Veterinary Research 68:225-230.

Kleeberg HH. 1960. The tuberculin test in cattle. Journal of the South African Veterinary and Medical Association 31:213-226.

Kleeberg HH & Nel EE. 1969. Porcine mycobacterial lymphadenitis. Journal of the South African Veterinary and Medical Association 40:233-250.

Kovalev GK. 1980. Tuberculosis in wildlife. Journal of Hygiene, Epidemiology and Microbiological and Immunology 24:495-503.

Meissner G, Schroeder KH. 1974. Bovine tuberculosis in man and its correlation with bovine cattle tuberculosis. Bulletin of the International Union against Tuberculosis 49:145-148.

Michel AL, Huchzermeyer HFAK. 1998. The zoonotic importance of *Mycobacterium tuberculosis*: transmission from human to monkey. Journal of the South African Veterinary Association 69:64-65.

Michel A. Meyer S, McCrindle CM, Veary T. 2003a. FAO Expert Consultation on Community Based Veterinary Public Health Systems. http://www.fao.org/ag/ againfo/programmes/en/vph/events/expert_consult_report.pdf. Rome, Italy, 27 – 28 October 2003.

Michel AL, Venter L, Espie IW, Coetzee ML. 2003b. *Mycobacterium tuberculosis* infections in eight species in the National Zoological Gardens of South Africa, 1991-2001. Journal of Zoo and Wildlife Medicine, 34:364-370.

Michel AL. 2007. The human/wildlife interface in a high tuberculosis incidence country. Proceedings of the 38th Conference of the International Union against Tuberculosis and Lung Disease, 8–12 November 2007, Cape Town, South Africa. No pages.

Moda G, Daborn CJ, Grange JM, Cosivi O. 1996. The zoonotic importance of *Mycobacterium bovis*. Tuberculosis and Lung Disease 77:103–108.

Neill SD, Hanna J, O'Brien JJ, McCracken RM. 1988. Excretion of *Mycobacterium bovis* by experiementally infected cattle. The Veterinary Record 123:340-343.

Paine R. & Martinaglia G. 1929. Tuberculosis in wild buck living under natural conditions. Journal of Comparative Pathology and Therapeutics. 42:1-8.

Regassa A, Medhin G, Ameni G. 2007. Bovine tuberculosis is more prevalent in cattle owned by farmers with active tuberculosis in central Ethiopia. Veterinary Journal (ahead of print)

Schmiedel A. 1968. Rapid decline in human tuberculosis and persistence of widespread tuberculosis of cattle: an unusual epidemiologic situation and its consequences. Bulletin of the International Union against Tuberculosis 41:297-300.

Schübel N, Rupp J, Gottschalk S, Zabel P, Dalhoff K. 2006. Disseminated *Mycobacterium bovis* infection in an immunocompetent host. European Journal of Medical Research. 11:163-166.

Sjörgren I & Sutherland I. 1975. Studies of tuberculosis in man in relation to infection in cattle. Tubercle 56:113-127.

Smith NH, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, van Soolingen D, Hewinson RG, Smith JM. 2006. Ecotypes of *Mycobacterium tuberculosis* complex. Journal of Theoretical Biology. 239:220-225.

Thoen CO & Himes EM. 1986. Pathogenesis of *Mycobacterium bovis* infection. Progress in Veterinary Microbiological Immunity 2:198-214.

UNAIDS Fact sheet 06, sub-saharan Africa, December 2006.

Viljoen PR. 1927. Tuberculosis in South Africa. Journal of the South African Veterinary and Medical Association 1:20-26.

Wedlock DN, Skinner MA, de Lisle GW, Buddle BM. 2002. Control of *Mycobacterium bovis* infections and the risk to human populations. Microbes and Infection 4:471–480.

Wilson GS, Miles AA. 1946. Topley and Wilson's Bacteriology, 3rd ed., reprint 1947.

Chapter 1.3

Aims and contents of the thesis

There is a need to shift the focus of bovine tuberculosis control in South Africa from an exclusively livestock based and economically driven scheme to an integrated strategy, which takes into account the role of wildlife as a reservoir for the disease transmission to cattle but does at the same time not neglect the social and economic value of the wildlife itself. This is a new and complex concept for South Africa and in fact the world. Irrespective of the type of control strategy aspired by boards of zoological collections, game parks and national governments, it is imperative to have suitable and reliable tools available for the detection and diagnosis of bovine tuberculosis in both the domestic and wild maintenance hosts. A basic understanding of the risks to conservation of indigenous wildlife, human health in the wildlife/ livestock/human interface and the livestock industry - as well as the principles guiding the transmission of *Mycobacterium bovis* between domestic and free-ranging wild animals, and amongst free ranging wild animals - is key to implementing effective preventive measures especially in the livestock/wildlife interface around conservation areas. The studies described in the following chapters will contribute to the knowledge in the specific areas of epidemiology, diagnosis and vaccinology of bovine tuberculosis in wildlife with an ultimate benefit to the livestock economy and human health.

Chapter 2 presents an overview on tuberculosis in both domestic and wild animal species. This is followed in Chapter 3 by results of epidemiological investigations in domestic and wild animal populations using molecular tools. In Chapter 4 diagnostic methods, in the standard or modified format, are validated and compared regarding their value for use in wildlife and domestic cattle and Chapter 5 deals with the first steps in the direction of developing a vaccination approach as the ultimate control measure of bovine tuberculosis in wildlife. It describes the establishment of an infection model and an initial vaccination trial with BCG in buffaloes in the context of a mycobacteria rich environment.

The main findings and their implications on the future control of bovine tuberculosis in South Africa are discussed in Chapter 6 with special reference to the challenges associated with this disease in a developing country.

Chapter 2

Wildlife tuberculosis in South African conservation areas: implications and challenges

A.L. Michel[1], R.G. Bengis[2], D.F. Keet[2], M. Hofmeyr[3], L.M. de Klerk[3], P.C. Cross[4], A.E. Jolles[5], D. Cooper[6], I.J. Whyte[3] and P. Buss[3] and J. Godfroid[7]

[1] Department of Bacteriology, ARC-Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa
[2] Directorate Veterinary Services, Skukuza, P.O. Box 138, South Africa
[3] South African National Parks, Skukuza, P.O. Box 402, South Africa,
[4] U.S. Geological Survey, Northern Rocky Mountain Science Center, 229 AJM, Johnson Hall, Bozeman MT 59717
[5] Department of Ecology & Evolutionary Biology, Princeton University, USA,
[6] Chief Veterinarian, Ezemvelo KwaZulu/Natal Wildlife - KZN Wildlife. Private Bag x01, St Lucia, 3936
[7] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Private Bag X04, Onderstepoort 0110, South Africa

Veterinary Microbiology, 2006, 112:91-100

Abstract

Tuberculosis, caused by *Mycobacterium bovis*, was first diagnosed in African buffalo in South Africa's Kruger National Park in 1990. Over the past 15 years the disease has spread northwards leaving only the most northern buffalo herds unaffected. Evidence suggests that ten other small and large mammalian species, including large predators are spillover hosts. Wildlife tuberculosis has also been diagnosed in several adjacent private game reserves and in the Hluhluwe-iMmfolozi Park, the third largest game reserve in South Africa.

The tuberculosis epidemic has a number of implications, for which the full effect of some might only be seen in the long term. Potential negative long term effects on the population dynamics of certain social animal species and the direct threat for the survival of endangered species pose particular problems for wildlife conservationists. On the other hand, the risk of spillover infection to neighboring communal cattle raises concerns about human health at the wildlife-livestock-human interface not only along the western boundary of Kruger National Park, but also with regards to the joint development of the Greater Limpopo Transfrontier Conservation Area (GLTFCA) with Zimbabwe and Mozambique. From an economic point of view, wildlife tuberculosis has resulted in national and international trade restrictions for affected species. The lack of diagnostic tools for most species and the absence of an effective vaccine make it currently impossible to contain and control this disease within an infected free-ranging ecosystem. Veterinary researchers and policy-makers have recognized the need to intensify research on this disease and the need to develop tools for control, initially targeting buffalo and lion.

1. Introduction

A number of reports of tuberculosis, caused by *Mycobacterium bovis*, in free-ranging African wildlife during the 20th century illustrate the susceptibility of a wide range of free-ranging mammals to this disease which has been primarily recognized as a disease of livestock (Thorburn and Thomas, 1940; Francis, 1957; Guilbride et al., 1963; Gallagher et al., 1972). Some affected species including African buffalo in the Queen Elizabeth National Park in Uganda and Lechwe in Zambia's Kafue National Park proved to act as maintenance host for *M. bovis* (Woodford, 1972; Krauss et al., 1984).

In 1880, Hutcheon made the first reference of bovine tuberculosis, which is caused by infection with *Mycobacterium bovis*, in cattle in South Africa. It is most likely that the disease was introduced by imported European cattle breeds mainly during the 18th and 19th centuries. A potential link between tuberculosis in livestock and game was first suggested by Paine and Martinaglia in 1929 when they reported bovine tuberculosis in kudu and small ungulates in the Eastern Cape Province of South Africa. Subsequently, the increasing economic importance

of tuberculosis as a disease of cattle led to the implementation of a national bovine tuberculosis control and eradication scheme in South Africa in 1969 (Huchzermeyer et al., 1994). Retrospective outbreak investigations suggested that the disease was transmitted to buffalo in Kruger National Park (KNP) from domestic cattle in the southeast corner of KNP between 1950 and 1960 (Kloeck, 1998). The Crocodile River formed a natural barrier between KNP and the farmland to the south, but sightings of buffalo and cattle grazing in close proximity of one another were not uncommon. The presence of the disease was, however, only discovered in 1990. In 1992, the prevalence of bovine tuberculosis was estimated to be 0, 4.4 and 27.1% in the north, central and south zones, respectively. Spread of infection to lion, cheetah, kudu, leopard and chacma baboon became evident by 1995 (Keet et al., 1996, 2000). By 1998 the prevalence of bovine tuberculosis had increased significantly to 16 and 38.2% in the central and south zones, due to increases in both the average herd prevalence and the total number of herds infected with bovine tuberculosis (Rodwell et al., 2000). In the Hluhluwe-iMfolozi-Park (HiP), bovine tuberculosis was first diagnosed in buffalo in 1986 and spillover to lion, chacma baboon, bushpig and greater kudu was later documented. Bovine tuberculosis herd prevalence in HiP varies from <10 to > 40% (Jolles, 2004). In Table 1 all free-ranging species diagnosed with BTB in HiP, KNP as well as adjacent reserves and farms are listed.

Table 1. Wildlife species in which *M. bovis* infection has been confirmed to date in South Africa

African buffalo *(Syncerus caffer*)
Greater kudu *(Tragelaphus strepsiceros*)
Lion *(Panthera leo)*
Eland *(Taurotragus oryx*)
Warthog (*Phacochoerus aethiopicus*)
Bushpig (*Potamochoerus porcus*)
Large spotted genet (*Genetta tigrina*)
Leopard (*Panthera pardus*)
Spotted hyena (*Crocuta crocuta*)
Cheetah (*Acinonyx jubatus*)
Chacma baboon (*Papio ursinus*)
Impala (*Aepyceros melampus*)
Honey badger (*Mellivora capensis*)

2. Area descriptions

2.1. Kruger National Park

Kruger National Park with an area of 19,485 km^2 is South Africa's largest wildlife refuge and a critical biodiversity resource. The Park's game population supports

147 mammal species, incl. approximately 27 000 African buffalo and 1700 lions. Bordering on Zimbabwe to the north and Mozambique to the east the KNP stretches 320 km from north to south and 65 km from east to west. More recently several private game reserves, situated on the western border, have been incorporated to form the Greater Kruger National Park Complex (GKNPC).

2.2. Hluhluwe-iMmfolozi Park

The Hluhluwe-Imfolozi Park (HiP) is situated in the province of Kwazulu/Natal and is South Africa's third largest game reserve. It covers an area of almost 100 000 ha. HiP has a buffalo population of approximately 3000 and is entirely surrounded by communal farm land.

3. Implications of bovine tuberculosis

3.1. Effect on wildlife populations

African buffalo can act as maintenance host of *M. bovis* and propagate bovine tuberculosis in large ecosystems in the absence of cattle (de Vos et al., 2001). Their social behaviour provides favourable conditions for aerosol transmission of *M. bovis* to members of the same herd. Buffalo herds in the Kruger National Park range in size from 50 to 1000 individuals with an average of roughly 250. In addition, males frequently disperse between herds via bachelor groups, while females and juveniles move to different herds via splinter groups (Halley et al., 2002; Cross et al., 2005a). Recent studies showed that these events may occur more frequently than previously thought, promoting the spatial spread of *M. bovis* (Cross et al., 2004, 2005a). Cross et al. (2005b) described how drought conditions may favour spatial spread of the disease by prompting herds to explore new areas and mix with previously unassociated herds. In HiP, buffalo bulls spent only a limited period, generally not exceeding 3–4 months, with breeding herds, but their *M. bovis* infection rates were higher than those of cows (Jolles, 2004).

On examination of mortality rates and calf:cow ratios in both infected and non-infected buffalo in HiP, Jolles et al. (2005) found that mortalities due to clinically advanced bovine tuberculosis occurred at an annual rate of 11%. Over time this is expected to shift the age distribution towards younger animals. On the other hand, bovine tuberculosis was found to reduce pregnancy rates in infected females which has an opposite effect on age distribution. As a result, bovine tuberculosis may have no overall affect upon the age structure of the buffalo population. Due to the chronic nature of bovine tuberculosis and the long lifespan of African buffalo, it is not surprising that results from studies conducted earlier in the epidemic may differ from those conducted later, and some effects may only be detectable later in the epidemic. Results from a cross-sectional

survey in 1998 by Rodwell et al. (2001) suggested that bovine tuberculosis may have no effect on buffalo fecundity, while data from HiP (Jolles, 2004) and a later study of known individuals from 2001-2005 suggest otherwise (Cross, unpublished data).

Caron et al. (2003) found a compelling correlation between increasing bovine tuberculosis herd prevalence in buffalo and a decrease in overall body condition. The association was even stronger during the dry season when herds of higher prevalence lost condition faster than herds of low bovine tuberculosis prevalence. Weak, old and debilitated prey animals are more vulnerable to predation by lions and other large predators (Mills et al., 1995; Funston 1998). Hence buffalo worst affected by the disease are the most likely targeted during lion predation because they are easiest to kill (Caron et al., 2003).

Since buffalo are considered to be one of four preferential prey species of lions (Mills, 1995), the frequent exposure of lions to large amounts of infectious buffalo tissue lead to a spatial spread of bovine tuberculosis within lion prides in areas where the BTB prevalence is high in buffalo (Keet, unpublished data). It is thus difficult to determine at present whether lions are a maintenance or spillover host. Although infection occurs predominantly via the oral route, sociality and intra-species aggression between lions are specific behaviour patterns that may facilitate and predispose to aerosol and percutaneous transmission. The role of these horizontal and possible of vertical transmission in perpetuating the infection cannot be excluded sufficiently. In a study comparing identified lion prides in the high buffalo TB prevalence zone, with a similar cohort in the low TB buffalo prevalence zone, disease effect parameters determined for buffalo were found to be true for infected lions. These include disease mortality, correlations between age and infection with bovine tuberculosis as well as between infection and body condition. Further and probably even more importantly, bovine tuberculosis was found to be driving social changes within prides which contributed to lower lion survival and breeding success (Keet, unpublished data). A faster territorial male coalition turnover was seen with consequent infanticide. The eviction of entire male and female prides from territories was also documented. This is in total contradiction with lion behaviour patterns described from elsewhere in Kruger and the rest of Africa. An abnormal sex ratio was seen – 2 males for every female (adults). It should be 1 male for every 2 females. The infected sub-population was significantly younger that the non-infected sub-population. The non-infected subpopulation lived significantly longer than the infected subpopulation, especially males. Cub survival was higher in the non-infected sub-population but birth rate was higher in the infected sub-population (Keet, unpublished data).
Research conducted in South Africa and elsewhere shows that infected buffalo serve as source of direct infection to large predators and scavenging omnivores. A less obvious link in the transmission between the maintenance and spillover host not living in the same habitat, has been demonstrated in greater kudu

(Michel, 2002). The *M. bovis* genotype commonly found in KNP buffalo has been isolated from kudu, suggesting either faecal-oral transmission as discussed by previous authors (Thorburn and Thomas, 1940), or alternatively, infection could have been carried over by ingestion of contaminated browse or water. More often a *M. bovis* strain genetically unrelated to the one characterized in buffalo, was associated exclusively with tuberculosis in KNP kudu, strongly indicating the maintenance host potential of this species. Cooper (unpublished data) concluded that a resident population of greater kudu were the most likely source of bovine tuberculosis infection in previously disease-free buffalo one year after they had been introduced into a Kwazulu/Natal game reserve.

The source of re-current infections in solitary predators such as cheetah and leopard is only partially understood. We have numerous observations where cheetahs and leopards were scavenging and it has been confirmed that they were infected with the same *M. bovis* genotype as buffalo (Michel, unpublished data). A possibility remains that they contract bovine tuberculosis from a currently undiagnosed infection in a smaller antelope species. Other carnivores such as hyenas, as well as certain omnivores (baboons, warthogs and honey badgers) are considered to contract *M. bovis* through scavenging on bovine tuberculosis infected carcasses (Bengis, unpublished data). Greater kudu appear to be the only species which show distinct clinical signs of bovine tuberculosis characterised by bilateral abscessation of parotid lymph nodes, frequently accompanied by formation of draining fistulae (Keet et al., 2001).

With the exception of greater kudu none of the infected species known to date has shown maintenance host potential. However, as bovine tuberculosis prevalence continues to increase there is also a greater risk of spillover to new vulnerable and rare species.

3.2. The wildlife-livestock-human interface

The farmland on the 390 km long western border of GKNPC is largely under communal land use. The livelihood of rural communities relies to a large extend on livestock farming. A game deterrent fence separates the two landscapes but despite great efforts and costs for its maintenance this man made barrier cannot guarantee the absolute separation of livestock from infected wildlife populations. Elephant activities or natural disasters such as the water floods experienced early in the year 2000, can cause damage to the fence, allowing buffalo to mingle with domestic cattle. On the other hand, fences cannot prevent the movement of wild animals in all cases, e.g. greater kudu and warthogs. Once contact between infected wild animals with livestock is established, the potential of *M. bovis* transmission to cattle exists, as demonstrated in New Zealand and Great Britain and North America (Cheeseman et al., 1989; Morris and Pfeiffer 1994).

To date no evidence of BTB outbreaks in communal cattle herds was demonstrated, despite intensified monitoring of cattle health at the interface (du Plessis, pers. comm.). However, unlike in commercial productions, communal livestock and their products areas are largely excluded from veterinary and veterinary public health control measures (Michel et al., 2004). Infection of communal cattle with bovine tuberculosis could be detrimental to the livelihood of small scale farmers. The objectives of livestock keeping in rural areas of sub-Saharan Africa, over and above that of food production, also include the generation of traditional wealth, social status and marriage dowries. As a result of this value system life expectancy of livestock is generally higher than on commercial farms, livestock are moved in exchange of goods or services and owners often live in close proximity with their animals. Bovine tuberculosis as a chronic and progressive disease manifests itself more often in older animals, under nutritional or productive stress. Taking this into account, people who are frequently exposed to either livestock infected with bovine tuberculosis or infected products such as unpasteurised milk, should be considered at risk of contracting zoonotic tuberculosis. This risk increases considerably in individuals with an immuno-suppression induced by HIV infection, as documented previously (Raviglione et al., 1995). A report published in South Africa in 2001 stated the overall HIV prevalence in this country at between 15% (total prevalence) and 30% (age group 30–34 years) (Dorrington et al., 2001). At the end of 2003 an estimated 5.3 million South Africans were living with HIV. As a result of the HIV epidemic the crude incidence rate of human tuberculosis has not only increased drastically (Cosivi et al., 1998) but 50% or more of new cases of tuberculosis in South Africa can be ascribed to prior infection with HIV (Maartens, 2001). In Hlabisa Hospital, situated in rural Kwazulu/Natal close to the HiP, the number of African HIV-positive patients with tuberculosis increased from six in 1989 to 451 as early as in 1993 (Walker et al., 2003). Although the role of zoonotic tuberculosis in humans has not been investigated in South Africa, the wildlife-livestock-human interface as a risk factor should not be underestimated.

3.3. Implications on conservation and trade

The diagnosis of bovine tuberculosis in a game species has severe implications on the national and international trade in wildlife due to movement restrictions and results in revenue losses for both KNP and HiP. It may be argued that bovine tuberculosis has partially turned the KNP into a conservation island thereby not only jeopardizing conservation efforts in endangered species but also prohibiting the free exchange of genetic resources between conservation areas.

3.4. Greater Limpopo Transfrontier Conservation Area (GLTFCA)

In December 2002 an international treaty to establish the Greater Limpopo Transfrontier National Park (GLTNP) was signed, bringing the parks of Gaza in

Mozambique, Kruger National Park in South Africa and Gonarezhou in Zimbabwe together under a joint management. The three countries also reached agreement on creating a transfrontier conservation area (TFCA) that encompasses the GLTNP and the intervening matrix of conservancies and wildlife ranches on freehold land, together with the communal farming areas. Covering an area of approximately 100 000 km^2 the Greater Limpopo Transfrontier Concervation Area (GLTFCA) will be the second transfrontier park in southern Africa and one of the biggest conservation areas in the world. The longer term plans for this vast area currently focus on the development of wildlife based tourism with freedom of movement for wildlife and tourists across international borders. Interactions between wildlife, livestock and humans living in the conservation area can be expected to increase drastically. The management of wildlife and livestock diseases such as bovine tuberculosis within the individual parks and the envisaged larger landscape has remained unresolved and presents a new challenge on approaches to disease control with an impact on existing disease control policies. Currently efforts are undertaken to gain information on geographical distribution and prevalence rates of bovine tuberculosis in domestic and wild species in the countries concerned. The need for an integrated, inter-disciplinary approach on animal health issues including bovine tuberculosis has been identified in a framework document by the *AHEAD* Working Group (Osofsky et al., 2005).

4. Progress

4.1. Surveillance and monitoring

In the absence of a management strategy policy for bovine tuberculosis in KNP buffalo, resources have been focussed on surveillance projects to determine the distribution and rate of spread of the disease. A progressive northward spread of bovine tuberculosis as well as an increase in disease prevalence have been documented. A monitoring project in buffalo in a dedicated study area in the medium prevalence zone revealed that the prevalence of bovine tuberculosis increased in this sub-population from 13% in 2001 to 25% in 2003. For minimal invasiveness as well as ethical and ecological considerations both surveys in the low prevalence north zone were based on live sampling making use of the modified gamma interferon assay as described by Grobler et al. (2002). In 2000 the infection had spread to an additional three herds. By 2003 a total of ten out of 29 buffalo herds in the northern region of KNP had a culture confirmed positive bovine tuberculosis status. Up to date the status of two further herds has remained suspect after positive interferon-gamma test results for one buffalo in either herd could not be confirmed (Hofmeyr, unpublished data). In 2004 the most northern case of bovine tuberculosis in buffalo was diagnosed approximately 40 km south of the Limpopo River, which forms the border between South Africa and Zimbabwe (Hofmeyr, unpublished data).

In Kwazulu/Natal a control programme for managing bovine tuberculosis in HiP was initiated in 1999 which is currently still ongoing. The programme is aimed at reducing buffalo herd prevalence below 10%, as well as reducing the risk of spillover into key species and to domestic livestock in areas surrounding the park. It is based on limited intervention in the form of mass capture of buffalo followed by tuberculin testing and removal of positive animals which appears to help reduce the prevalence of infection in individual herds (Cooper, unpublished data). To date a total of 4431 tests have been performed on buffalo identifying 850 reactors. The programme was successful in reducing the prevalence in some buffalo herds from previously 10 – 20% to below 10%, and in high prevalence herds from approximately 55% in 2000/2001 to an estimated 20 – 30%. (Jolles & Cooper, unpublished data).

Laboratory diagnosis of suspect cases of bovine tuberculosis in wildlife is essential for confirmation of infection and, in combination with molecular characterization of *M. bovis,* provides a powerful tool to assist in studying spatial, temporal and inter-species transmission of *M. bovis*. Restriction fragment length polymorphism has been used to track transmission from cattle to KNP buffalo, from buffalo to lion and other spillover species (Michel 2002). At present, results from the genetic analysis of *M. bovis* isolates from most of the infected species support the hypothesis that the bovine tuberculosis epidemic originated from a point source and subsequently spread through the park. In contrast, at least two epidemiologically unrelated *M. bovis* strains were found to circulate in HiP buffalo. The bovine tuberculosis epidemics in KNP and HiP were shown to be epidemiologically unrelated. Genotyping of *M. bovis* will become instrumental in the bovine tuberculosis control of the future transfrontier conservation area with Zimbabwe and Mozambique.

4.2. Control

Once bovine tuberculosis has established itself in a native, free-ranging maintenance host, eradication of the disease becomes highly unlikely. The choice of suitable control measures depends on the primary objectives for the particular ecosystem. KNP has an obligation to protect the species that host the pathogen. Although there is presently no evidence of a population level decline in the buffalo due to bovine tuberculosis (Whyte, 1998) various implications have to be considered which include the preservation of protected species, the minimization of risk of transmission to domestic cattle and a potentially devastating impact on population dynamics in other maintenance and spillover species. Vaccination is undisputedly the control measure of choice in achieving these objectives, but in the absence of an effective vaccine alternative strategies have to be decided upon. Currently bovine tuberculosis is managed in KNP with minimal interference, meaning that no active control efforts have been implementd, but surveillance,

monitoring and research activities are conducted to investigate the major epidemiological determinants (de Lisle et al., 2001). This strategy is likely to change following the recent classification of bovine tuberculosis as an alien species in the KNP ecosystem (SANParks, unpublished information). The broad objective of the policy on alien species is to minimize the impact on, and maintain the integrity of indigenous biodiversity. Thresholds for potential concerns (TPCs) e.g. influence of the disease on biodiversity, the spatial and temporal impact of bovine tuberculosis on population dynamics, the animal and public health implications at the interface, etc. have been determined for bovine tuberculosis in buffalo and TPCs for other species, especially lions, are expected to be included over time. A monitoring programme has been proposed to determine whether and to what extend the thresholds have been reached or exceeded. This monitoring programme is linked to objectives of the Southern Africa Working Group of *AHEAD* (Animal Health for the Environment and Development) (http://www.wcs-ahead.org) and the veterinary research objectives of the Peace Parks Foundation, both of which are concerned with the socio-political and socio-economic aspects of this and other livestock diseases and the impact they may have at the wildlife-livestock-human interface in the (GTFCA).

Vaccination remains the ultimate control measure for bovine tuberculosis in wildlife reservoirs. Despite the close relatedness between domestic cattle and African buffalo it is mandatory that the effectiveness of potential vaccine candidates can be demonstrated in buffalo. To determine adequate infectious challenge doses an infection model was developed in which a local *M. bovis* strain was used for intra-tonsillar infection of buffalo. Lesions induced were comparable in size, number and distribution to those found in naturally infected buffalo (de Klerk, unpublished data). The evaluation of BCG as a vaccine in African buffalo has recently commenced. Despite the fact that initial experiments did not yield statistically significant differences in the number of lesioned buffaloes between the groups of vaccinated and control animals, they have provided us with crucial insight instrumental to the design of subsequent trials.

For monitoring and control purposes availability of reliable diagnostic tests for affected species is essential. Despite its many limitations in wildlife, the intradermal tuberculin test is currently used to diagnose bovine tuberculosis in buffalo and lions (Jolles et al., 2005; Keet, unpublished data). Following a slight modification the bovine gamma interferon assay has proved to be a valuable alternative to the tuberculin test (Grobler et al., 2002). A project has recently been initiated to explore the potential of this technique for bovine tuberculosis testing in rhinoceros and elephants (Morar, 2003). For many other animal species, however, there are no ante mortem tests available to date and the diagnosis of *M. bovis* infection relies on culture and histopathology.

5. Discussion

Bovine tuberculosis is most well known as a disease of livestock and the role of wildlife reservoirs in its persistence has been recognized (Bengis et al., 1996; Schmitt et al., 2002). Countries' approaches to address and resolve this problem are largely determined by economic and socio-political driving forces. In the case of New Zealand, where the wildlife reservoir is considered an alien species, culling as a management option for bovine tuberculosis does not warrant ecological or ethical concerns. In contrast, wildlife tuberculosis in South and Southern Africa may, in the medium to long term, threaten the viability of indigenous, protected and even endangered species in ecosystems such as the KNP and HiP. Although direct effects of bovine tuberculosis are difficult to detect and appear to be developing slowly at the species population level, research conducted in buffalo and lion has revealed distinct adverse effects of bovine tuberculosis on individual and sub-population level which cannot be ignored. Organisations with the responsibility to maintain biodiversity in these ecosystems have the obligation to protect species, regardless of or despite the fact that they may be hosts of bovine tuberculosis. Wildlife-based tourism and trade are important economic lifelines for South Africa and can be adversely affected by bovine tuberculosis. At the same time governments have an obligation to protect human health at the interface of humans, domestic livestock and wildlife. The significance of zoonotic tuberculosis in humans in southern Africa is currently unknown. In the light of the current HIV/AIDS burden, however, zoonotic tuberculosis should be considered a health risk factor in immuno-compromised people, since human tuberculosis is not only the commonest cause of HIV-related deaths but HIV infection is driving the tuberculosis epidemic in sub-Saharan Africa.

Whatever the current limitations are in terms of resources, effective bovine tuberculosis control measures, scientific information, research tools, etc., the development of the GLTFCA requires an understanding of the complex systems influencing both human livelihoods and wildlife health across international borders.

6. Further challenges

The pioneer work of Anderson and May paved the way for the study of wildlife disease ecology. They pointed out that the parasite-host relationship was not simply the impact a parasite had on an individual, but formed an integral of those interactions at the population level and at the same time a dynamic process where parasites were flowing from one host to the next. The rate at which this took place was determined by host behaviour and abundance (Anderson and May, 1978). Nowadays, numerous workers apply these ideas to explore the

impact of diseases in naturally fluctuating wildlife populations, particularly in the context of conservation biology. Therefore, a major challenge is to link our understanding of individual level of infections to how disease flows through susceptible host populations and may possibly influence host dynamics. Due to its importance and its sustainability in the population, bovine tuberculosis in buffalo highlights some of the challenges posed by bovine tuberculosis in wildlife to ecologists and veterinarians.

In order to understand the epidemiology of bovine tuberculosis in the buffalo population, a fundamental parameter is the R_0 ('R nought'), the basic reproductive number that defines a threshold ($R_0 > 1$) for a pathogen to invade a population or the number of new infections arising from an infected individual. Obviously this parameter is linked to the density of the population allowing contact between susceptible and infected members (Hudson et al., 2002). In the buffalo population, the natural unit would be either a herd, or the number of individuals living in a defined geographical area.

Contradicting the often-presented hypothesis that *M. tuberculosis* evolved from *M. bovis*, recent work suggests that the common ancestor of the tubercle bacilli resembled *M. tuberculosis* and could well already have been a human pathogen (Brosch et al., 2002). Domestication of bovidae, in turn, allowed the adaptation of *M. bovis* to cattle. This study re-enforces the hypothesis of a recent introduction of *M. bovis* in buffalo related to the introduction of bovine tuberculosis infected cattle in Africa some 200 years ago and subsequent contact with naïve buffalo, 40 years ago (Bengis et al., 1996). As a consequence there has been no co-evolution between *M. bovis* and its new host and thus there are numerous unknowns in the short natural history of bovine tuberculosis in buffalo. Therefore, the pathobiology of the infection in buffalo has to be studied in detail, particularly immune responses, in order to ascertain that assumptions we make, based on our knowledge of the infection in cattle, are valid for buffalo, too. Critical questions like transmission of the infection, induced pathology and conditions prevailing for overt disease (and hence shedding and infectivity) in buffalo have to be addressed. This can only be achieved by identifying the host immune responses that are likely to protect the host or conversely that are likely to promote invasion of the buffalo population by the newly introduced pathogen. Ecological immunology opens new avenues of research for invasion biology (Lee and Klasing, 2004): how do buffalo cope with the shift from native, co-adapted pathogens to a preponderance of a novel challenge and how does this affect the potential of *M. bovis* to become invasive? Based on the temporal distribution pattern following the entry of bovine tuberculosis into KNP it was suggested in 2000 that it could take another 30 years for the infection to reach the northern most point of KNP, but that due to a higher buffalo density the spread might occur faster (de Vos et al., 2001). In 2004, bovine tuberculosis was diagnosed in buffalo just 40 km form the northern boundary of KNP. Such a phenomenon cannot be explained by transposing our knowledge of the epidemiology of bovine tuberculosis in cattle to buffalo. Indeed, most (if not all) our recent knowledge of

the epidemiology of bovine tuberculosis in cattle has been acquired in the context of control or eradication programs (Phillips et al., 2003). The epidemiology of bovine tuberculosis in buffalo is in essence different: there has been no co-evolution between the host and the newly introduced pathogen and no such control programs exist. Hence infection and disease are allowed to progress. Recent data suggest that bovine tuberculosis has invaded a vast proportion of the KNP buffalo population.

It is generally accepted that tuberculosis in humans results from a single infection with a single *M. tuberculosis* strain. Such infections are thought to confer protective immunity against exogenous re-infection. These assumptions were recently challenged. Indeed, a South African study published in 2004, showed that patients with active tuberculosis often have different strains in the same sputum specimen. These results suggest that multiple infections are frequent, implying high re-infection rates and the absence of efficient protective immunity conferred by the initial infection (Warren et al., 2004).

What is the potential role of multiple infections in bovine tuberculosis, particularly in buffalo in KNP where no control program exists and where the infection pressure is high? Is overt disease (and as a consequence, the shedding of *M. bovis*) a result of progressive disease following infection acquired early in life, possibly after reactivation, or could it be due to multiple infections? Is a shift of dominance of a Th1 towards a Th2 immune response associated with the progression of the disease as recently suggested for cattle (Welsh et al., 2005)? Answers to these questions are critical in order to better understand the epidemiology of bovine tuberculosis in buffalo.

Acknowledgements

The authors are grateful to the South African Veterinary Foundation for funding of fundamental studies on bovine tuberculosis in lions, and to the US NSF-NIH Ecology of Infectious Disease Grant for studies on the ecology of bovine tuberculosis in buffalo. We are grateful for financial assistance provided by the Smithsonian Institution for vaccine trials in buffalo and from the National Geographic Society, the Theresa Heinz Foundation, the Mellon Foundation and the Wildlife Conservation Society in support of research related to bovine tuberculosis buffalo in HiP.

References

Anderson, R.M., May, R.M. 1978. Regulation and stability of host-parasite population interactions. I. Regulatory processes. J. Anim. Ecol., 47, 219-247.

Bengis, R.G., Kriek, N.P.J., Keet, D. F., Raath, J.P., de Vos, V., Huchzermeyer, H., 1996. An outbreak of tuberculosis in a free-living African buffalo (*Syncerus caffer* SPARRMAN) population in the kruger National Park: a preliminary report. Onderstepoort J. Vet. Res. 63, 15-18.

Brosch, R, Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D., Cole, S.T. 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. USA., 99, 3684-3689.

Caron, A., Cross, P.C., du Toit, J.T. 2003. Ecological implications of bovine tuberculosis in African buffalo herds. Ecological Applications, 13, 1338-1345.

Cheeseman C.L., J.W. Wilesmith, F.A. Stuart. 1989. Tuberculosis: the disease and its epidemiology in the badger – a review. Epidemiol. Infect. 103, 113-125.

Cosivi, O, Grange J.M., Daborn, C.J., Raviglione, M.C., Fujikura, T., Cousins, D., Robinson, R.A, Huchzermeyer, H.FA.K., de Kantor, I. & Meslin, F.X. 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg. Infect. Dis. 4, 59-70.

Cross, P.C., Lloyed-Smith, J.O., Bowers, J.A., Hay, C.T. Hofmeyr, M. Getz, W.M. 2004. Integrating association data and disease dynamics in a social ungulate: bovine tuberculosis in African buffalo in the Kruger National Park. Ann. Zool. Fennici, 41, 879-892.

Cross, P.C., Lloyed-Smith, J.O., Getz, W.M. 2005a. Disentangling association patterns in fission-fusion societies using African buffalo as an example. Anim. Behav. 69, 499-506.

Cross, P.C., Lloyed-Smith, J.O., Johnson, P.L.F., Getz, W.M. 2005b. Duelling timescales of host movement and disease recovery determine invasion of disease in structured populations. Ecology Letters, 8, 587-595.

de Lisle, G.W. , Mackintosh, C.G., Bengis, R.G. 2001. *Mycobacterium bovis* in free living and captive wildlife, including farmed deer. In: Mycobacterial infections in domestic and wild animals. Rev. Sci. Tech. Off. Int. Epiz. 20,86-111.

De Vos, V., Bengis, R.G., Kriek, N.P.J., Michel, A., Keet, D.F., Raath, J.P., Huchzermeyer, H.F.K.A. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. Onderstepoort J. Vet. Res. 68, 119 – 130.

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

Francis, J. 1957. Tuberculosis in animal sand man. Cassell and Company Limited, London,pp. 3-10, 70-82.

Funston, P. 1998. Predator-prey relationships between lions and large ungulates in the Kruger National Park. PhD tesis, University of Pretoria, South Africa.

Gallagher, J., MacAdam, I., Sawyer, J. & van Lavieren, L.P. 1972. Pulmonary tuberculosis in free living leche antelope in Zambia. Tropical Animal Health and Production, 4, 204-213.

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

buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J. Vet. Res., 69, 221-227.

Guilbride, P.D.L., Rollison, D.H.L., Mcanulty, E.G., Alley, J.G. & Wells, E.A. 1963. Tuberculosis in free living African (Cape) buffalo (*Syncerus caffer* Sparrman). J. Comp. Path. Ther., 73, 337-348.

Halley, D.J., Vandewalle, M.E.J. & Taolo, C. 2002. Herd-switching and long-distance dispersal in female African buffalo (*Syncerus caffer*). African Journal of Ecology, 40, (1) p.97-99.

Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterbeek, H., Dobson, A.P. 2002. The Ecology of Wildlife Diseases. Oxford University Press. ISBN 0 19 850620 1.

Huchzermeyer, H.F.A.K., Brueckner, G.K., van Heerden, A., Kleeberg, H.H., van Rensburg, I.B.J., Koen, P. and Loveday R.K. 1994. Tuberculosis In: Infectious Diseases of Livestock. Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (Eds) pp. 1425 – 14445.

Hutcheon, D. 1880. Tering: consumption, tables mesenterica. Annual Report, Colonial Veterinary Surgeon, Cape of Good Hope.

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

Jolles, A.E., Cooper, D., Levin, S.A. 2005. Hidden effects of chronic tuberculosis in African buffalo. Ecology 86 (9), 2358-2364.

Keet, D.F., Kriek, N.P.J., Penrith, M.-L., Michel, A. & Huchzermeyer, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. Onderstepoort J. Vet. Res. 63, 239-244.

Keet, D.F., Kriek, N.P.J., Bengis, R.G., Grobler, D.G., Michel A.L. 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. Onderstepoort. J. Vet. Res., 67, 115-122.

Keet, D.F., Kriek, N.P.J.,. Bengis R.G & Michel, A. 2001. Tuberculosis in kudus (Tragelaphus strepsiceros) in the Kruger National Park Onderstepoort J. Vet. Res. 68, (3) 225-230.

Kloeck, P.E. 1998. Tuberculosis of domestic animals in areas surrounding the Kruger National Park. In Proceedings of "The challenges of managing tuberculosis in wildlife in Southern Africa, Zunkel, (Ed.), 30-31 July 1998, Nelspruit, South Africa

Krauss, H., Roetcher, D., Weis, R., Danner, K & Hubschle, O.J.B. 1984. Wildtiere als Infektionsquelle fuer Nutztiere: Untersuchungen in Zambia. Beitraege der Klinischen Veterinaermedizin zur Verbesserung der tierischen Erzeugung in den Tropen, Band 10, Justus-Liebig-Universitaet, Giessen.

Lee, K.A., Klasing, K.C. 2004. A role for immunology in invasion biology. Trends Ecol. Evol., 19: Doi: 10.1016/j.tree.2004.07.012.

Maartens, G. 2001. HIV and tuberculosis. Joint Congress: HIV Clinicians, Infectious Diseases, Infection Control, Travel Medicine, Sexually Transmitted Diseases Societies and Veterinary and Human Public Health, 2-6 December 2001, Stellenbosch, South Africa

Michel, A.L. 2002. The epidemiology of *M. bovis* infection in South African wildlife. Abstracts of the Veterinary European Network on Mycobacterium (VENOM) Symposium: "DNA Fingerprinting of Bovine TB strains" , Belfast, Northern Ireland, October 24-26, 2002.

Michel, A.L., Meyer, S., McCrindle, C.M., Veary, C.M. 2004. Community based veterinary public health systems, current situation, future trends and recommendations. In: FAO Expert Consultation on Community Based Veterinary Public Health Systems. http://www.fao.org/ag/againfo/programmes/en/vph/ events/expert_consult_report.pdf.

Mills, M.G. L., 1995. Notes on wild dog (Lycaon pictus) and lion (Panthera leo) population. Trends during a drought in the Kruger National Park. Koedoe, 38, 95-99.

Mills, M.G. L., Biggs, H. C. & Whyte, I. J., 1995. The relationship between rainfall, lion predation and population trends in African herbivores. Sixth Internation Theriological Congress, University of New South Wales in Sydney, Australia, 1995. Wildlife Research, 22, 75-88.

Morar, D., 2003. The development of an interferon-gamma (IFNg) assay for the diagnosis of tuberculosis in African elephants (Loxodonta Africana) and black rhinoceros (Diceros bicornis). MSc thesis. Dept. of Veterinary Tropical Diseases, Faculiy of Veterinary Sciences,University of Pretoria

Morris, R.S., Pfeifer, D.U., 1994. The epidemiology of Mycobacterium bovis infections. Vet. Microbiol. 40, 153-177.

Osofsky, S. A., Cleaveland, S., Karesh, W. B., Kock, M. D., Nyhus, P. J., Starr, L., and Yang A., (eds.) 2005. Proc. Southern and East African Experts Panel on Designing Successful Conservation and Development Interventions at the Wildlife/Livestock Interface: Implications for Wildlife, Livestock, and Human Health. AHEAD (Animal Health for the Environment And Development) Forum.

Paine, R., Martinaglia, G., 1929. Tuberculosis in wild buck living under natural conditions. J. Comp. Path. Ther. XLII, 1,1-8.

Phillips, C.J., Foster, C.R., Morris, P.A., Teverson, R., 2003. The transmission of *Mycobacterium bovis* infection to cattle. Res. Vet. Sci. 74, 1-15.

Raviglione, M.C., Snider, D.E., Kochi, A., 1995. Global epidemiology of tuberculosis. J. Am. Med. Assoc. 273, 220-226.

Rodwell, T.C., Kriek, N.P., Bengis, R.G., Whyte, I.J., Viljoen, P.C., de Vos, V., Boyce, W.M., 2000. Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. J. Wildlife Dis. 37, 258-264.

Rodwell, T.C., Whyte, I.J., Boyce, W.M., 2001. Evaluation of population effects of bovine tuberculosis in free-ranging African buffalo (*Syncerus caffer*). J. Mammal., 82, 231-238.

Schmitt, S.M., O'Brien, D.J., Bruning-Fann, C.S., Fitzgerald, S.D., 2002. Bovine tuberculosis in Michigan wildlife and livestock. Ann. N.Y. Acad. Sci., 969, 262-268.

Thorburn, J.A. and Thomas, A.D., 1940. Tuberculosis in Cape Kudu. J. S. Afr. Vet. Med. Ass., 11, 3-10.

Walker, A.R.P., Walker, B.F., Wadee, A.A., 2003. The HIV/AIDS infection – the public health burden particularly in Southern Africa. South. Afr. J Epidemiol. Infect., 18, 26-28.

Warren, R.M., Victor, T.C., Streicher, E.M., Richardson, M., Beyers, N., van Pittius, N.C., van Helden, P.D., 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. Am. J. Respir. Crit. Care Med., 169, 610-614.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G., Pollock, J.M., 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology, 114, 101-111.

Whyte, I.J., 1998. Effects of bovine tuberculosis on the dynamics of the buffalo population in Kruger National Park. Preliminary report. Scientific Report 07/1998. Kruger National Park, South Africa.

Woodford, M.H., 1972. Tuberculosis in the African buffalo (*Syncerus caffer*) in the Queen Elizabeth National Park, Uganda. Thesis presented to the Faculty of Veterinary Medicine of the University of Zurich for the Degree of Doctor of Veterinary Medicine. Zurich: Juris Druck + Verlag.

Chapter 3

Molecular epidemiology of tuberculosis in wildlife and domestic cattle in South Africa

Chapter 3.1

High Mycobacterium bovis genetic diversity in a low prevalence setting

Michel, A. L.[1] Hlokwe, T.M.[1], Coetzee, M. L.[1*], Maré, L.[1#], Connoway, L.[2] V.P.M.G. Rutten[3] and Kremer, K.[4]

1 Tuberculosis Laboratory of the ARC- Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa,

2 Directorate Veterinary Services, Mpumalanga Province, South Africa,

3 Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands,

4 National Mycobacteria Reference Laboratory, National Institute of Public Health and the Environment, Bilthoven, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands.

Veterinary Microbiology, 2008, 126:151-159

Abstract

The genetic diversity among South African *Mycobacterium bovis* isolates from cattle was determined by genetic fingerprinting. The restriction fragment length polymorphism (RFLP) markers IS*6110* and polymorphic GC-rich sequence (PGRS) as well as spoligotyping and determination of variable number of tandem repeats (VNTR) were used to characterize sub samples of 91 *M. bovis* field isolates. PGRS RFLP was the single most discriminatory method and combinations of typing methods, which included IS*6110* and/or PGRS had the highest discriminatory power, able to reveal 29 distinct genotypes among 35 farms with no epidemiological link. Three of the farms were co-infected with two genetically unrelated strains.

In contrast to reports from European and also other colonised countries on the African continent our findings are suggestive of a high genetic diversity of *M. bovis* in South Africa's cattle population, implying a variety of unrelated ancestor strains. Despite effective intervention through test-and slaughter campaigns no indication of a 'founder effect' was apparent in the panel of isolates derived from all infected provinces.

Keywords: *Mycobacterium bovis*, Cattle, RFLP, IS*6110*, PGRS, Spoligotyping, VNTR typing, Genetic diversity

1. Introduction

Bovine tuberculosis is believed to have been introduced into South Africa and possibly the sub region by European settlers (Hutcheon, 1880). In addition, cattle imports from Australia, Argentina and Madagascar in the early 20th century were reported to often include infected animals (Cousins et al., 2004). The introduction of a National Tuberculosis Scheme in 1969 resulted in the reduction of infected commercial cattle herds from 11.85% in 1971 to 0.39% in 1995, but ever since sporadic outbreaks continued to occur. Between 1993 and 2005 a total of 209 outbreaks were reported, with a peak of 20 outbreaks in 2003 (Animal disease statistics, National Dept of Agriculture). Presently, the control of bovine tuberculosis is based on intradermal tuberculin testing and slaughter as well as on abattoir surveillance. Genetic typing of *Mycobacterium bovis* has contributed to a greatly improved knowledge of inter-bovine and interspecies transmission of bovine tuberculosis *(*Durr et al., 2000*)*. This understanding is crucial to the effective management of bovine tuberculosis control schemes and the wildlife-livestock interface in countries where wildlife reservoirs for *M. bovis* have been identified, including South Africa (Haddad et al., 2004, Skuce and Neill, 2001). In the absence of a 'best' technique, the most widely used DNA typing techniques

for *M. bovis* include IS*6110* and PGRS restriction fragment length polymorphism (RFLP) typing, spoligotyping and variable number of tandem repeat (VNTR) typing (van Soolingen, 2001). In this study these techniques were used to conduct the first comparative genetic analysis of *M. bovis* isolates from South African cattle. We aimed at determining the genetic diversity of *M. bovis* among domestic cattle in South Africa using established typing methods with proven reproducibility in our setting.

2. Material and methods

2.1. Sample collection

A total of 90 *M. bovis* isolates from 42 South African cattle herds in six provinces and one additional strain from Swaziland were used in this study. Thirty-nine isolates were derived from a field investigation in the year 2003 into a multiple farm outbreak assumed to involve 12 farms (Mpekwane et al., 2004). Fifty-two *M. bovis* isolates from 35 infected farms in all six provinces with known occurrence of bovine tuberculosis had been collected as a result of routine sample submissions by state veterinary officials between 1993 and 2000. This represents 23% of the 148 outbreaks reported in South Africa during this period. No selection took place but all viable *M. bovis* isolates available in the laboratory were used for a retrospective characterization study involving combinations of RFLP typing, spoligotyping and VNTR typing. It was postulated that the typing method or combination of methods distinguishing the highest number of known unrelated outbreak strains would be considered the most discriminative approach. To facilitate this interpretation within the epidemiological context, isolates were classified into the following panels and evaluated against their specific background:

2.1.1. Isolates from epidemiologically related herds

2.1.1.1. Isolates from the same herd

Between two and four isolates were analysed from each of 15 farms (farms 3, 4, 7-11, 19, 22, 25, 26, 36-39). In addition, 28 isolates from farm 40 were examined.

2.1.1.2. Isolates from different but epidemiologically linked herds

Several months after a dispersal sale of an infected cattle herd in the Mpumalanga Province a back tracing investigation by the veterinary field officials indicated the possible spread of *M. bovis* to 12 different farms in various districts, and another province. Following slaughter of tuberculin reactors *M. bovis* was isolated from 39 cattle from eight of the suspected farms (farms 36-43) (Fig. 1).

An epidemiological link had also been suspected between two other farms. Bovine tuberculosis had been diagnosed on farm 26 which had introduced cattle

from farm 27 several months before. Subsequent skin testing confirmed the presence of bovine tuberculosis infection on farm 27.

2.1.2. Isolates from epidemiologically unrelated herds

Thirty-three of the 35 farms sampled between 1993 and 2000 were, to the best of our knowledge, epidemiologically unrelated. Farms 26 and 27 were linked to each other but not any other farm. The eight herds described under 2.1.1.2. were considered as one epidemiological unit and unrelated to the other 34 unlinked herds. The total number of outbreaks considered unrelated was 35.

2.2. Bacterial isolation

All samples were processed in the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute for culture according to standard procedures (Bengis et al., 1996; Alexander et al., 2001). Pure subcultures from all *M. bovis* isolates on Löwenstein-Jensen medium containing pyruvate were routinely stored at -20°C.

2.3. DNA extraction

DNA extraction from *M. bovis* isolates was accomplished from colonies of either fresh or revived subcultures on Löwenstein-Jensen medium with pyruvate. Following heat-inactivation at 80°C for 60 minutes, the colonies were scraped off and suspended in 5 ml of extraction buffer (50 g/l Mono Sodium Glutamic Acid; 6.06 g/l Tris.HCl (pH 7.4); 9.3g/l EDTA) (R. Warren, personal communication), to which lysozyme (50 mg/ml) and RNAseA (10 mg/ml) were added. The suspensions were incubated for two hours at 37°C after which proteinase K at a final concentration of 0.5 mg/ml was added. After incubation at 45°C overnight phenol/chloroform extraction was performed. The final pellet was resuspended in 40µl TE (1mM Tris.HCl (pH 7.6); 0.1 mM EDTA).

2.4. Genetic typing

2.4.1. IS*6110* RFLP typing

For IS*6110* RFLP typing approximately 1.5 µg of mycobacterial DNA was digested overnight with 1.5 units of *Pvu*II. Subsequently, the resulting fragments were separated by electrophoresis on a 0.8% agarose gel. DIG-labelled molecular weight size marker VII (Roche) was loaded in the first, middle and last lane of the gel. Southern blot transfer was performed as described by Skuce et al. (1994). IS*6110*-containing DNA fragments were detected through hybridization with the entire IS*6110* sequence as a probe, according to the manufacturer's instructions (Roche Molecular Biochemicals-The DIG System User's Guide,1995).

2.4.1.1. Analysis of IS*6110* RFLP patterns

The GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to determine the level of similarity between the IS*6110* RFLP patterns by

using the similarity coefficient of Dice and the unweighted pair group method with arithmetic averages (UPGMA) for clustering maximum tolerance 1.2%). DIG-labelled Molecular Weight Marker VII (Roche Diagnostics) was used as external marker for normalisation. Strains with a similarity coefficient of greater than 90% were considered identical provided they contained the same number of bands.

2.4.2. PGRS RFLP typing

For PGRS RFLP typing 1.5 µg of *M. bovis* DNA was digested with AluI and electrophoresis of DNA fragments was performed on a 1.2% agarose gel (Cousins et al., 1998 Southern blotting and detection after hybridization at 55°C with a DIG-labelled PGRS oligonucleotide probe (5'GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC 3') were performed according to the manufacturer's instructions (Roche Diagnostics). Banding patterns were analyzed manually.

2.4.3. Spoligotyping

Spoligotyping was performed according to the protocol of Kamerbeek et al. (1997). With this method, the direct repeat region of the isolates was amplified by PCR, and the PCR products were hybridized to 43 oligonucleotides of known spacer sequences by reversed-line blot hybridization. Because one of the primers for PCR was biotin labelled, and hence the PCR product, the presence of spacers was detected after incubation with streptavidin-peroxidase and enhanced chemiluminescence detection (ECL Detection kit, Amersham Biosciences). The spoligopatterns were compared and assigned a *M. bovis* spoligotype number (SP number). The spoligopatterns were also compared to the international database on http://www.mbovis.org and new patterns were reported and assigned a unique SB code.

2.4.4. VNTR typing

VNTR typing was performed according to the method of Frothingham and Meeker-O'Connell (1998), at the Veterinary Laboratory Agencies Weybridge, United Kingdom. Primer pairs for ETR loci A to F were similar to those published previously (Frothingham Meeker-O'Connell (1998), except for the following minor changes: the ETR-B forward primer () had a GGTT extension and the ETR-B reverse primer () had a CTATA extension to improve annealing. The ETR-F forward and reverse primers were shortened by 3 and 4 bp respectively at the 5' side of the probes (ETR–B forward: 5' GCGAACACCAGGACAGCATCATGGGTT3', ETR-B reverse: 5' GGCATGCCGGTGATCGAGTGGCTATA 3', ETR-F forward: 5' GGTGATGGT CCGGCCGGTCAC 3' and ETR-F reverse 5' GTGCTCGACAACGCCATGCC 3'). Each PCR reaction consisted of 10 µl of Qiagen Hotstar Mastermix (Qiagen), 0.5 µl of each of the primers for each locus (at 10 pmol/µl for all primers except for ETR-A primers which were used at 20 pmol/µl) and 2 µl of heat-killed cell supernatant in a final volume of 20 µl. Following an initial denaturation at 94°C for 15 min each sample was subjected to 30 cycles of 94°C for 30 s, 68°C for 60 s, and 72°C for 2 min. Followed by an extended

annealing temperature of 72°C for 10 min. Heat-killed cell supernatants of *M. tuberculosis* H37RV were used in each set of reactions as a positive control. PCR products were separated on an ABI 377 Sequencer, and analysed using ABI Prism Genescan software. The size of VNTR loci alleles were estimated by comparison to a ROX size standard (Applied Biosystems). The PCR products were compared to size standards and converted to repeat numbers at each loci using standard allele naming tables (ABI Genotyper software).
The VNTR genotype of a strain, representing the number of repeat elements at each locus, is presented as a series of integers representing the A to F VNTR loci, respectively. The ETR-D locus contains a 24 bp deletion in one of the repeats and the naming convention indicates the presence of this deletion by a * i.e. 4* (= 3x77 bp repeats and one 53 bp repeat). The ETR-F locus contains 79 bp tandem repeats and 55 bp tandem repeats. The naming convention indicates the number of 79 bp repeats followed by 55 bp repeats separated by a period.

3. Results

3.1. Identification of bacterial isolates

All isolates from cattle tissues were identified as *M. bovis* by either confirming the biochemical characteristics of microaerophilic growth, lack of niacin production and nitratase reductase, and pyrazinamide resistance or alternatively by PCR amplification of targets specific for the *M. tuberculosis* complex and *M. bovis* in particular.

3.2. Genetic typing

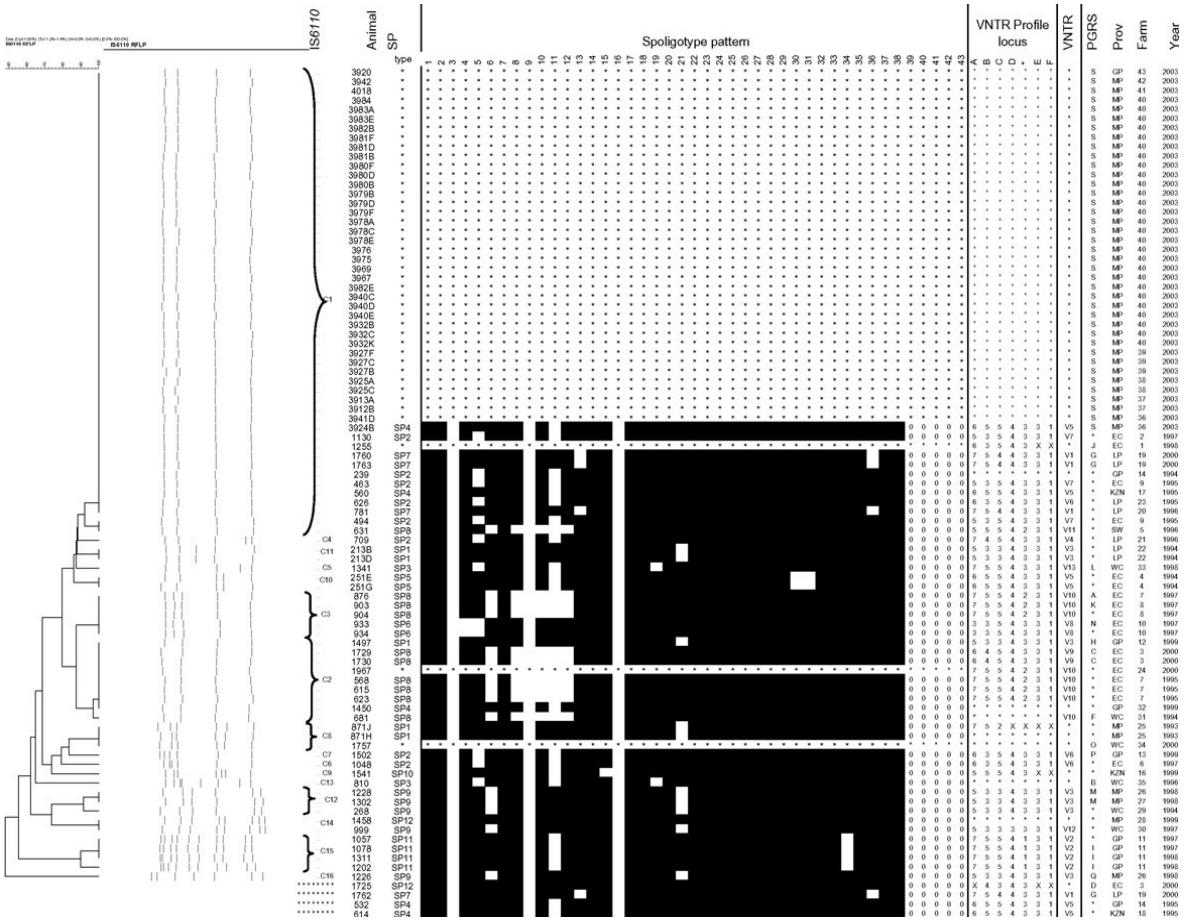
IS*6110* and PGRS RFLP analysis, spoligotyping and VNTR typing were applied to different subsamples of 92 *M. bovis* isolates. For most isolates typing procedures could not be synchronized but had to be performed partially on frozen and revived cultures. This is considered the most likely cause of failures to obtain sufficient DNA of good quality for typing. Complete analysis involving all four typing methods was carried out on 17 isolates from 12 farms, while only three or less typing methods could be applied to the remaining isolates. Results are summarized in Fig. 1.

3.2.1. Isolates from epidemiologically related herds
3.2.1.1. Isolates from the same herd

Genetic typing rendered identical typing patterns for multiple isolates from the same farm in 15 of the 16 herds in this panel. With the exception of two farms this was true for all "same herd" isolates. Both in herd 3 and 7 one strain was

isolated which had a genotype that did not match with the genotype of the isolates of the herd mates, suggesting the co-existence of two *M. bovis* strains those herds. In the case of farm 3, the PGRS RFLP pattern as well as spoligotyping data of isolate 1725 differed from those of the other isolates from this farm. In case of farm 7, isolate 876 could be distinguished by an additional band in the IS*6110* RFLP pattern, but not by spoligotyping nor VNTR typing (PGRS RFLP typing data lacked). In contrast, the only two isolates from farm 26 (1228 and 1226) were found to be unrelated regarding both the PGRS and IS*6110* RFLP patterns (similarity coefficient of 0.60). Unique DNA fingerprints remained unchanged for the period between samplings from the same herd, which was on average two years (see Fig. 1).

Fig. 1. DNA fingerprinting results of 91 *M. bovis* isolates from cattle ordered by IS*6110* RFLP similarity, followed by spoligopatterns. VNTR profile and PGRS RFLP types as well as farm identifications, provinces of origin, year of collection.



* Data not available; MP: Mpumalanga Province, EC: Eastern Cape Province, GP: Gauteng Province, LP: Limpopo Province, KZN: Kwazulu/Natal Province and WC: Western Cape Province.

3.2.1.2. Isolates from different but epidemiologically linked herds

The genetic relatedness of 39 isolates from eight infected herds, assumed to be linked through a dispersal sale of an infected cattle herd, was investigated. Initial IS*6110* RFLP analysis grouped all samples in the genotype C1, which contains only two IS copies (Fig. 1, farms 36 to 43). In subsequent PGRS RFLP typing all isolates again displayed an identical PGRS banding pattern, hence supporting the outcome of the epidemiological field investigation of a common source of infection for all eight farms.

Because of cattle movement from farm 27 to farm 26, an epidemiological link was also suspected between these two farms. Indeed, the PGRS and IS*6110* RFLP patterns of one of the isolates from farm 26 (isolate 1228) were identical to those of the isolate of farm 27 (isolate 1302), confirming the epidemiological link (Fig. 1).

3.2.2. Genetic profiles among epidemiologically unrelated *M. bovis* isolates

M. bovis strains isolated from herds with no known link are assumed to be genetically different. This section therefore served to evaluate the results for the various typing methods against this hypothesis. The study identified 29 genetically distinct *M. bovis* strains among 35 herds with no known epidemiological link (Table 1). The highest level of discrimination was achieved when RFLP typing with IS*6110* and/or PGRS was used.

3.3. IS*6110* RFLP

Among the 49 *M. bovis* isolates from 34 unrelated farms subjected to IS*6110* RFLP analysis, 16 distinct banding patterns were identified. These patterns comprised four to ten bands, which, due to the use of the entire sequence of IS*6110* probe, as described previously (Skuce et al., 1994), relates to two to five copies of IS*6110*. Unique IS*6110* RFLP types, not shared with any other herd, were found for ten infected farms (C4-C7, C9-C11, C13, C15, and C16), four IS*6110* RFLP types (C3, C8, C12, C14) were shared between two or three farms, and two patterns, both resembling *M. bovis* strains with two copies of IS*6110*, were found in ten and six unrelated outbreaks, respectively (C1 and C2) (Fig. 1).

3.4. PGRS RFLP

PGRS RFLP typing was only applied to 25 isolates from 16 unrelated farms which yielded a total of 18 unique patterns (Fig. 1 and Table 1). All PGRS types were unique and were not found in more than one epidemiologically unlinked herd.

Among the isolates of two farms (farms 3 and 26), two distinct PGRS RFLP types were observed.

Table 1. Comparison of the discriminatory power of different genetic markers used individually and in combination to characterize *M. bovis* isolates from epidemiologically unrelated farms

Typing method(s) used in parallel	Number of unrelated outbreaks (isolates) analysed	No. of genotypes identified
IS*6110* + PGRS + Spoligotyping + VNTR	11 (17)	12
IS*6110* + PGRS + Spoligotyping	14 (22)	15
PGRS + Spoligotyping + VNTR	11 (18)	12
IS*6110* + Spoligotyping + VNTR	23 (39)	21
IS*6110* + PGRS + VNTR	11 (17)	12
Spoligotyping + PGRS	14 (23)	16
Spoligotyping + VNTR	25(41)	15
IS*6110* + Spoligotyping	30 (45)	24
IS*6110* +PGRS	16 (24)	17
IS*6110* + VNTR	24 (39)	21
IS*6110*	34 (49)	16
VNTR	26 (42)	13
PGRS	16 (5)	18
Spoligotyping	33 (50)	12
Total (IS*6110* and/or PGRS and/or Spoligotyping and/or VNTR)	35 (53)[a]	29

[a]One isolate from the multiple farm outbreak (2.1.1.2.) was included in the analysis

3.5. Spoligotyping

Spoligotyping was performed on 50 isolates from 33 farms resulting in the identification of 12 spoligotypes, all of which lacked spacers 3, 9, 16, and 40–43 (Fig. 1). Seven types were each associated with several unrelated herds (SP1, SP3, SP4, SP7-SP9, SP12), while four spoligotypes were each associated with one outbreak only (SP5, SP6, SP10, SP11). Spoligotype SP8 was found along with SP12 on the same farm (farm 3) (see also Section 2.1).

Two of the 12 spoligopatterns identified (SP7 and SP11) had not been reported in the international *M. bovis* database before and were assigned the SB codes 1163 and 1164 (Table 2).

3.6. VNTR typing

VNTR typing was performed on 43 isolates resulting in 13 distinct patterns relating to 26 epidemiologically unrelated outbreaks (Table 1). As a result, seven farms revealed unique VNTR types (V2, V4, V8, V9, and V11–V13) while other types were shared by between two and five farms. Further discrimination of the more common patterns was possible if either PGRS RFLP patterns were available (V3, V10), or if IS*6110* banding patterns comprised at least six bands (V3, V5, and V6). For VNTR types V1 and V7, both detected in two herds, no further discrimination was possible due to a lack of PGRS data and corresponding low copy number IS*6110* patterns. With the exception of VNTR types V3 and V5, VNTR typing revealed superior or equal differentiation between strains compared to spoligotyping.

Table 2: *M. bovis* spoligotype and VNTR patterns frequencies among isolates in 35 unrelated cattle herds

Spoligotype	Frequency	SB code[a]	VNTR	Frequency	SP/VNTR	Frequency
SP1	3	SB0121	V1	2	SP4/V5	4
SP2	7	SB0131	V2	1	SP2/V6	3
SP3	2	SB0267	V3	4	SP7/V1	2
SP4	5	SB0130	V4	1	SP9/V3	3
SP5	1	SB0163	V5	5	SP8/V10	2
SP6	1	SB0134	V6	3	SP1/V3	2
SP7	2	SB1163	V7	2	SP2/V4	1
SP8	5	SB0140	V8	1	SP2/V7	2
SP9	3	SB0265	V9	1	SP8/V11	1
SP10	1	SB0678	V10	2	SP9/V12	1
SP11	1	SB1164	V11	1	SP3/V13	1
SP12	2	BCG	V12	1	SP11/V2	1
*	*	*	V13	1	SP5/V5	1
*	*	*	*	*	SP6/V8	1

[a] http://www.mbovis.org

* End of table

4. Discussion

The present study is the first to investigate DNA polymorphism among *M. bovis* isolates from cattle in South Africa. Four of the most commonly used genetic markers (IS*6110* RFLP, PGRS RFLP, spoligotyping and VNTR typing) provided high levels of both, reproducibility and genetic diversity in our setting. Although the study did not permit a true comparative evaluation of the methods due to incomplete typing data for several of the isolates, we are of the opinion that the study allows first conclusions regarding the genetic diversity among South African *M. bovis* isolates.

We found IS*6110* RFLP to be highly discriminatory for all *M. bovis* strains which contained more than three copies of the IS sequence. However, 44% of the outbreaks examined in this study were caused by a strain comprising only two copies of IS*6110* (C1 or C2), hence limiting the value of this probe. PGRS RFLP was the single most discriminatory method as it was able to distinguish between all 16 epidemiologically unrelated outbreaks subjected to this method. Furthermore two of the outbreaks (farms 3 and 26) were found to be associated with two genetically different *M. bovis* strains, bringing the total of PGRS types identified to 18. Previous investigators reported a similar superior performance of PGRS (Cousins et al., 1998; van Soolingen et al., 1994). The technically much less demanding spoligotyping provided the lowest level of differentiation between strains in our study. It was possible to increase the level of discrimination of spoligotyping by second stage IS*6110* RFLP, PGRS RFLP or VNTR typing, as suggested previously for *M. tuberculosis* isolates with low copy numbers of IS*6110* (Rasolofo-Razanamparany et al., 2001). Both the IS*6110* and PGRS RFLP typing methods confirmed transmission of infection between farms 26 and 27 as well as the co-infection of farm 26 with two genetically distinct *M. bovis* strains.
VNTR typing appeared to be less discriminatory than PGRS and IS*6110* RFLP typing in our study but comparable to spoligotyping. However, certain spoligotypes and IS*6110* RFLP types with three or less insertion elements could be subdivided by VNTR typing. In conclusion, the current VNTR typing protocol can provide a valuable first stage screening tool as recently suggested for *M. tuberculosis* strains (Kremer et al., 2005b). Strains with different VNTR patterns will most likely represent genetically distinct strains, but strains with the same VNTR type should be sub-typed with IS*6110* RFLP typing or even PGRS RFLP typing to determine whether they represent the same clone or not. Alternatively, the resolution of VNTR typing can be increased by the number and configuration of loci most appropriate for the locally prevalent strains.

Three of the herds examined were found to be co-infected with two distinct genotypes (3, 7 and 26) as demonstrated by various of the typing methods employed. Multi-genotype infections may not be a rare event, especially in countries where bovine tuberculosis occurs at a prevalence of >1% (Serraino et

al., 1999). Costello et al. found that 10% of cattle herds examined in Ireland harboured more than one strain (Costello et al., 1999). We were unable to reliably estimate the percentage of herds with multiple strain involvement due to the small number of outbreaks analysed with all four markers. However, the fact that such events were detected in the small sample analyzed in this study may either suggest a relatively high frequency of outbreaks with multiple sources of infection probably due to purchase of infected animals (Skuce et al., 1994, Neill et al., 1994) or, alternatively, persistence and evolution of "old" *M. bovis* strains within the country's cattle population (Milian-Suazo et al., 2002). Both scenarios appear plausible in the South African context. Despite an initial sharp decline in the bovine tuberculosis herd prevalence to below 0.4%, the disease was never eradicated from the country but continued to occur and more recently the spread of the disease to all nine provinces of South Africa has been confirmed (Michel, unpublished data).

The genetic diversity detected among the *M. bovis* isolates in this study appears to be high compared to studies conducted in European countries where test-and-slaughter is enforced more strictly. Genotyping of 233 *M. bovis* isolates from cattle in Ireland yielded 17 spoligotypes (Costello et al., 1999), Skuce et al. (2005) found 14 spoligotypes among 461 isolates of *M. bovis* in Northern Ireland and spoligotyping of 1349 *M. bovis* isolates in France identified 161 spoligotypes (Haddad et al., 2001). It has recently been reported that clonal expansion following a bovine population bottleneck is a major determinant of the reduced strain diversity of *M. bovis* in Great Britain (Smith et al., 2006). In the central African region the degree of heterogeneity appears to be low despite the absence of eradication programmes. Possible explanations are limited cattle imports from Europe and fairly recent introduction of the disease, (Njanpop-Lafourcade et al., 2001; Cadmus et al., 2006; Diguimbaye-Djaibé et al., 2006). The findings of our study do not fit any of these epidemiological scenarios for South Africa. During colonial times and into the 20th century cattle imports from different European countries and other continents were responsible for multiple introductions of *M. bovis* and most probably a high strain heterogeneity. On the other hand, the rinderpest pandemic, killing 66% of South African cattle (Rossiter, 2004), as well as an effective 'test-and-slaughter' scheme should be considered powerful population and diversity reducing factors, commonly facilitating. a founder effect characterised by the establishment of a successful genotype in a geographical region (Smith et al., 2006). Our study did not present any indicators of such an effect and it may be speculated that many genotypes were only introduced in the 20th century and that incomplete eradication of outbreaks during test-and slaughter' campaigns may have allowed survival of 'old' strains. In addition, it cannot be ruled out that undetected spillover of strains into wildlife occurred at the wildlife/livestock interface, possibly re-infecting cattle at a later stage.

In conclusion, the data obtained in this retrospective study show that IS*6110* and PGRS RFLP represented powerful markers in revealing a high genetic diversity

among cattle strains in South Africa, where the overall bovine tuberculosis prevalence is low compared to countries with a more limited strain diversity.

Acknowledgements

The authors want to thank Dr N. Smith and staff at the Veterinary Laboratories Agency Weybridge, England, for kindly performing the VNTR typing.

References

Alexander, K.A., Pleydell, E., Williams, M.C., Lane, E.P., Nyange, J.F.C. Michel A.L., 2002. *Mycobacterium tuberculosis*: An emerging disease of free-ranging wildlife. Emerg. Infect. Dis. 8, 592-595.

Bengis, R.G., Kriek, N.P.J., Keet, D.F., Raath, J.P., De Vos, V., Huchzermeyer, H.F.A.K., 1996. An outbreak of bovine tuberculosis in a free-living buffalo population in the Kruger National Park. Onderstepoort J. Vet. Res. 63, 15 - 18.

Cadmus, S., Palmer, S., Okker, M., Dale, J., Gover, K., Smith, N., Jahans, K., Hewinson, G., Gordon, S.V., 2006. J. Clin. Microbiol. 44, 29-34.

Costello, E., O'Grady, D., Flynn, O., O'Brien, R., Rogers, M., Quigley, F., Egan, J., Griffin, J., 1999. Study of restriction fragment length polymorphism analysis and spoligotyping for epidemiological investigation of *Mycobacterium bovis* infection. J. Clin. Microbiol. 37, 3217-3222.

Cousins, D., Williams, S., Liebana, E., Aranaz, A., Bunschoten, A., van Embden, J., Ellis, T., 1998. Evaluation of four DNA typing techniques in epidemiological investigations of bovine tuberculosis. J. Clin. Microbiol., 36, 168-178.

Cousins, D.V., Huchzermeyer, H.F.K.A, Griffin, J.F.T., Brueckner, G.K., van Rensburg, I.B.J., Kriek, N.P.J., 2004. Tuberculosis. In Infectious Diseases of Livestock. Edited by Coetzer J.A.W. & Tustin R.C. Oxford University Press Southern Africa, Cape Town.

Diguimbaye-Djaibé, C., Hilty, M., Ngandolo, R., Mahamat, H.H., Pfyffer, G.E., Baggi, F., Hewinson, G., Tanner, M., Zinstag, J., Schelling, E., 2006. *Mycobacterium bovis* isolates from tuberculous lesions in Chadian zebu cattle. Emerg. Infect. Dis. 12, 769-771.

Durr, P.A., Hewinson, R.G., Clifton-Hadley, R.S., 2000. Molecular epidemiology of bovine tuberculosis I. *Mycobacterium bovis* genotyping. Rev. Sci. Tech. Off. Int. Epiz. 19, 675-688.

Frothingham, R., Meeker-O'Connell, W.A., 1998. Genetic diversity in the M. TB complex based on variable numbers of tandem DNA repeats. Microbiol. 144, 1189-1196.

Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M.F., Hughes, S.L., Inwald, J., Hewinson, R.G., Durand, B., 2001. Spoligotype diversity of

Mycobacterium bovis strains isolated in France from 1979 to 2000. J. Clin. Microbiol. 39, 3623-3632.

Haddad, N., Masselot, M., Durand, B., 2004. Molecular differentiation of *Mycobacterium bovis* isolates. Review of main techniques and applications. Res. Vet. Sci., 76, 1-18.

Hutcheon, D., 1880. Tering, consumption, tables mesenterica. Annual Report, Colonial Veterinary Surgeon, Cape of Good Hope.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. J. Clin. Microbiol. 35, 907-914.

Kremer, K., Au, B.K., Yip, P.C., Skuce, R., Supply, P., Kam, K.M., 2005b. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and spoligotyping. J. Clin. Microbiol. 43, 314-320.

Milian-Suazo, F., Banda-Ruiz, V., Ramirez-Casillas, C., Arriaga-Diaz, C., 2002. Genotyping of *Mycobacterium bovis* by geographic location within Mexico. Prev. Vet. Med. 55, 255-264.

Mpekwane, T., Michel, A.L., Connoway, L., 2004. Preliminary results of a bovine tuberculosis outbreak investigation in Mpumalanga and Gauteng Provinces using *M. bovis* genetic strain typing. Proceedings of the Annual conference of the Southern African Society of Veterinary Epidemiology and Preventive Medicine. 25 – 27 August 2004. Intundla Lodge, Pretoria, South Africa.

National Department of Agriculture, Veterinary Services. Monthly and Annual disease reports www.nda.agric.za/vetweb/Animal%20Disease/reports/. Last accessed February 21, 2006.

Neill, S.D., Pollock, J.M., Bryson, D.B., Hanna, J., 1994. Pathogenesis of *Mycobacterium bovis* infection in cattle. Vet. Microbiol. 40, 41-52.

Njanpop-Lafourcade, B.M., Inwald, J., Ostyn, A., Durand, B., Hughes, S., Thorel, M.F., Hewinson, G., Haddad, N., 2001. Molecular typing of *Mycobacterium bovis* isolates from Cameroon. J. Clin. Microbiol. 39, 222-227.

Rasolofo-Razanamparany, V, Ramarokoto, H., Aurégan, G., Gicquel, B., Chanteau, S., 2001. A combination of two genetic markers is sufficient for restriction fragment length polymorphism typing of *Mycobacterium tuberculosis* complex in areas with a high incidence of tuberculosis. J. Clin. Microbiol. 39, 1530-1535.

Rossiter, P.B., 2004. Rinderpest. In: Infectious Diseases of Livestock. Edited by Coetzer J.A.W. & Tustin R.C. Oxford University Press Southern Africa, Cape Town.

Serraino, A., Marchetti, G., Sanguinetti, V., Rossi, M.C., Zanoni, R.G., Catozzi, L., Bandera, A., Dini, W., Mignone, W., Franzetti, F., Gori, A., 1999. Monitoring transmission of tuberculosis between wild boars and cattle: genotyipcal analysis of strains by molecular epidemiology techniques. J. Clin. Microbiol. 37, 2766-2771.

Skuce, R.A., Brittain, D., Hughes, M.S., Beck, L.A., Neill, S.D., 1994. Genomic fingerprinting of *Mycobacterium bovis* from cattle by restriction fragment length polymorphism analysis. J. Clin. Microbiol. 32, 2387-2392.

Skuce, R.A., Neill, S.D., 2001. Molecular epidemiology of *Mycobacterium bovis*: exploiting molecular data. Tuberculosis 81, 169-175.

Skuce, R.A., McDowell, S.W., Mallon, T.R., Luke, B., Breadon, E.L., Lagan, P.L., McCormick, C.M., McBride, S.H., Pollock, J.M., 2005. Discrimination of isolates of *Mycobacterium bovis* in Northern Ireland on the basis of variable tandem repeats (VNTRs). Vet. Rec. 157, 501-504.

Smith, N.H., Gordon, S.V., de la Rua-Romenech, R., Clifton-Hadley, R.S., Hewinson, R.G., 2006. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat. Rev. Microbiol. 4, 670-681.

Van Soolingen D., 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections; main methodologies and achievements. Review. J. Int. Med. 249, 1-26.

Van Soolingen, D., de Haas, P.E.W., Haagsma, J., Eger, T., Hermans, P.W.M., Ritacco, V., Alito, A., van Embden, J.D.A., 1994. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. J. Clin. Microbiol. 32, 2425-2433.

Chapter 3.2

Molecular typing reveals important clues on the transmission of Mycobacterium bovis to and among free-ranging African wildlife species

Michel, A. L.[1*] Coetzee, M. L.[1$] , Keet, D.F[2]., Maré, L.[1#], Warren, R.[3] Cooper, D.[4], Bengis, R.G.[2] , Kremer, K.[5], van Helden P.[3]

[1]Tuberculosis Laboratory; Food, Feed & Veterinary Public Health Programme, ARC- Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa

[2]Directorate Veterinary Services, Skukuza, P.O. Box 138, South Africa

[3]DST/NRF Centre of Excellence for Biomedical TB Research, US/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg 7505, South Africa.

[4]Chief Veterinarian, Ezemvelo Kwazulu-Natal Wildlife - KZN Wildlife. Private Bag x01, St Lucia, 3936, South Africa

[5]National Mycobacteria Reference Laboratory, National Institute of Public Health and the Environment, Bilthoven, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands.

Submitted

Abstract

Bovine tuberculosis is endemic in African buffalo and a number of other wildlife species in the Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HiP) in South Africa. It was thought that the infection had been introduced into the KNP ecosystem through direct contact between cattle and buffalo, a hypothesis which was confirmed in this study by IS*6110* and PGRS restriction fragment length polymorphism (RFLP) typing. The molecular characterisation of 189 *M. bovis* isolates from nine wildlife species in the HiP, including three smaller associated parks, and the Kruger National Park with adjacent areas showed that the respective epidemics were each caused by an infiltration of a single *M. bovis* genotype. The two *M. bovis* strains had different genetic profiles, demonstrated by hybridisation with the IS*6110* and PGRS RFLP probes, as well as with regard to evidence of evolutionary changes to the IS profile. While the *M. bovis* type in HiP was transmitted between buffaloes and to at least baboon, bushpig and lion without obvious genetic changes to the RFLP patterns, in the KNP a dominant strain was represented in 73% of the *M. bovis* isolates, whilst the remaining 27% were variants of this strain. No species-specific variants were observed, except for one IS*6110* type which was found only in a group of five epidemiologically related greater kudu. This finding was attributed to species-specific behaviour patterns rather than an advanced host-pathogen interaction.

Keywords: *M. bovis*, wildlife, molecular epidemiology, IS*6110* RFLP

Introduction

Although tuberculosis caused by *Mycobacterium bovis* is known mainly as an economically important disease of cattle, it can affect a wide range of domestic and wild animal species. In South Africa, *M. bovis* infection in antelope species was first diagnosed in the Eastern Cape in 1928 as it occurred frequently on large commercial farms under semi-free ranging conditions where bovine tuberculosis was rife in cattle (Paine & Martinaglia 1929, Thorburn & Thomas 1940).

In 1986 and 1990 bovine tuberculosis (BTB) was first diagnosed in African buffalo (*Syncerus caffer*) in two of South Africa's largest free-ranging conservation areas, the Hluhluwe-iMfolozi Park (HiP) and Kruger National Park (KNP) (Bengis et al., 1996, Michel et al., 2006). Although the disease was detected in both reserves within a few years of each other, there was no known epidemiological link between the two epidemics. HiP and other game reserves in Kwazulu-Natal are entirely surrounded by communal land where livestock farming provides a sustainable income to local farming communities. The bovine tuberculosis status of communal cattle is largely unknown due to the practical constraints associated

with the mustering of cattle for the skin test. Therefore, their involvement in the disease epidemic in HiP and other smaller reserves is considered highly likely.

In the case of the bovine tuberculosis outbreak in KNP, local veterinary authorities expressed the opinion that the disease most probably entered the KNP ecosystem during the 1950's – 1960's through infected cattle mingling with buffalo. High infection rates had been found previously in several commercial cattle herds in the vicinity of the southern part of KNP. At the time the southern boundary of KNP was not fully fenced and intermingling of cattle and buffalo was frequently observed along the Crocodile River. On one particular farm, bovine tuberculosis was repeatedly diagnosed between 1958 and 1993, even after depopulation (Kloeck 1998).

Following the discovery of bovine tuberculosis in the KNP, extensive surveys including 1974 buffaloes were conducted between 1991 and 1993 (de Vos et al., 2001) which revealed that some animals in virtually all buffalo herds south of the Sabie River (southern region) were infected with bovine tuberculosis (BTB). Additional surveys were carried out in 1996 as well as in 1998 (Rodwell et al., 2001). During the latter, a stratified, two-stage cluster sampling was used (Thrusfield, 1995). It was shown that the disease had spread in a northern direction, such that the incidence of BTB in the southern region had possibly attained a prevalence of over 90% in some individual buffalo herds (de Vos et al., 2001). Between the Sabie and the Olifants rivers (central region) the incidence increased from 4% to 16% while the initially BTB free area north of the Olifants River showed infection in only one herd, resulting in an overall prevalence of 1.5% in 1998.

With the increasing *M. bovis* infection rate in the buffalo population, the infection spilled over into other wildlife species and was first reported in lion (*Panthera leo*), baboon (*Papio ursinus*) and cheetah (*Acinonyx jubatus*) in the KNP in 1995 (Keet et al., 1996). In subsequent years, transmission of *M. bovis* to an additional seven wildlife species including predators, herbivores and omnivores was demonstrated (Keet et al., 2000, Keet et al., 2001, Michel et al., 2006).

The spatial spread of bovine tuberculosis within and between species in the KNP was mainly northwards but occurred also outwards into neighbouring private game reserves as wildlife can move freely between the parks belonging to the greater KNP complex (GKNPC) (de Vos et al., 2001). Wildlife also occurs abundantly in the farming areas south of the KNP and cases of BTB had been encountered in these areas in warthog (*Phacochoerus aethiopicus*) and greater kudu (*Tragelaphus strepsiceros*) as well as in domestic cattle (Michel. unpublished data, du Plessis, pers. comm.).

It was reported by de Vos *et al*. (2001) and Michel (2002) that most of the *M. bovis* genotypes seen in isolates from the buffalo were identical, and that variants were >70% homologous to this genotype. In this initial study, only one genotyping probe (IS*6110*) was used for analysis, and since *M. bovis* has few IS*6110* copies, the analysis is indicative of a single introduction event of *M. bovis,* but it was not possible to show unequivocally whether the BTB epidemic was the result of a single introduction event into the KNP, or whether a number of events had taken place.

In this paper we report on an integrated approach of genotyping and spatial analysis to study the BTB epidemic in the KNP by characterising *M. bovis* isolates from wild animals and comparing them with those recently characterised from domestic cattle (Michel et al., 2008) in South Africa. We show that we can use molecular epidemiological techniques to establish the extent of *M. bovis* strain diversity in isolates collected in the KNP and HiP, including associated game parks. From this data, we recreate a putative evolutionary history of these *M. bovis* isolates, thereby providing evidence that the present BTB epidemics in KNP and HiP have been caused by two different progenitor strains.

Materials and Methods

Animals and tissue samples

Kwazulu-Natal Parks

Twenty-eight tissue samples had been collected during bovine tuberculosis surveys from tuberculin skin test and/or interferon gamma test positive buffaloes (Table 1) in the Spioenkop Nature Reserve (SP; n=2), in western Kwazulu-Natal and the Hluhluwe-iMfolozi Park (HiP; n=14), Munyawana Game Reserve (MGR; n=6) and Eastern Shores of Lake St Lucia (at present part of the iSimangaliso Wetland Park) (ESL; n=6) in northern Kwazulu-Natal. All samples were collected between 1992 and 2000. Buffalo populations in the latter two parks originated from HiP and were translocated in 1977 and 1997 – 2000, respectively. Four additional samples were collected during *ad hoc* post mortem examinations of one bushpig (*Potamochoerus larvatus)* and one baboon from HiP , one lion from MGR (TB 1199) and one lion (TB 613) which was translocated from HiP to the National Zoological Gardens in Pretoria (NZG) where it succumbed to tuberculosis and was euthanased. The last sample originated from a buffalo that was moved from HiP to a reserve in the Northern Cape province, where it was destroyed after both the tuberculin test and interferon gamma tests showed positive test results.

Greater Kruger National Park Complex

Standard sets of tissue samples from the head, thoracic and mediastinal lymph nodes and, where applicable, lesions from any other affected tissues were collected as described by Bengis et al. (1996). A total of 122 animals in KNP, 17 in two private reserves within the GKNPC and adjacent to the western boundary of the central region of KNP and 17 animals on four properties neighbouring the southern region were sampled as listed in Table 1. The majority of buffalo were sampled during BTB surveys while other species were subject to passive surveillance only and tissue samples were collected at *ad hoc post mortem* examinations. All samples examined were collected between 1994 and 2003.

Bacteriology and genetic characterisation of *M. bovis* isolates

Tissue samples were processed and cultured, followed by species identification, as reported by Bengis et al. (1996) and Alexander et al. (2002). *M. bovis* isolates were genetically characterised using standard IS*6110* and PGRS RFLP methods as well as described previously (Michel et al., 2008). Spoligotyping was performed according to the method of Kamerbeek et al. (1997). Forty-two of the *M. bovis* isolates were additionally genotyped in the research laboratory at the Stellenbosch University for quality control purposes, using alternative methods (Warren et al., 1996).

Table 1. Summary of wildlife species and numbers of samples analysed from KwaZulu-Natal parks (KZN) and the Greater Kruger National Park Complex (GKNPC)

Species	KZN				GKNPC			Total
	HiP	MGR	ESL	SP	KNP	properties west	properties south	
Buffalo	15	6	6	2	82	5	14	**130**
Lion	1	1			22	5		29
Kudu					7	2	1	10
Baboon	1				8		1	10
Leopard					2	1		3
Cheetah					1	1		2
Hyena						3		3
Warthog							1	1
Bushpig	1							1
Total	18	7	6	2	122	17	17	189

HiP = Hluhluwe iMfolozi Park, KNP = Kruger National Park, MGR = Munyawane Game Reserve, ESL = Eastern Shores of Lake St. Lucia, SP = Spioenkop Nature Reserve

Data analysis

IS*6110* profiles were analysed using computer assisted (GelCompar II) comparative analysis using the UPGMA (unweighted pair group method with arithmetic mean), Dice coefficient (Hermans et al., 1995) and 1.2% optimisation. The blots probed by MTB484 (Felsenstein 1985) and PGRS were visually analyzed by two independent persons (Warren et al., 2001). Spoligotype patterns were assigned SB numbers after comparison with the international database of spoligotype patterns of *M. bovis* strains (www.mbovis.org).

Genetic relationship analysis

The evolutionary state(s) for the IS*6110* and MTB484 (Felsenstein 1985) RFLP data of 42 *M. bovis* isolates (Fig. 4) were assigned according to the presence (indicated by "1") or the absence (indicated by "0") of a hybridizing band. The complete set of evolutionary states were subjected to phylogenetic analysis using the neighbour joining algorithm (PAUP 4.0*; Phylogenetic Analysis Using Parsimony (*Other Methods) Version 4b10. Sinauer Associates, Sunderland, Massachusetts). Bootstrapping was performed to establish a degree of statistical support for nodes within each phylogenetic reconstruction (Felsenstein 1985). A consensus tree was generated using the program contree (PAUP 4.0*) in combination with the majority rule formula. The resulting trees were rooted to isolate TB 1067 because this genotype represents the most ancestral IS*6110* fingerprint. It has been suggested by Dale et al. (2003) that the original IS*6110* inserted into the DR region giving rise to a single copy strain and thereafter evolved to have either more than one copy of IS*6110* or lost the original IS*6110*. In the absence of genotypic data on an unrelated mycobacterial strain we have accepted this strain as an outgroup. Only branches which occurred in > 50 % of the bootstrap trees were included in the final tree and all branches with a zero branch length were collapsed.

Results

A combination of three genetic markers, IS*6110* RFLP, PGRS RFLP and spoligotyping was used to characterise and compare 189 *M. bovis* isolates from wildlife in Kwazulu-Natal game reserves and the GKNPC. Figures 1, 2 and 3 illustrate the typing patterns which were representative of the strains found in each of the game reserves for these markers. Figure 4 shows an evolutionary tree of selected *M. bovis* isolates from the two major ecosystems. Overall the phylogenetic analysis based on IS-3' RFLP showed that most of the isolates (including *M. bovis* BCG) shared a common IS*6110* insertion identified by a DNA fragment at approximately 1900 base pairs (bp), which is indicative of the ancestral insertion in the direct repeat (DR) region (data not shown). It was also shown that a number of the isolates shared a second common IS*6110* insertion,

suggestive of a subsequent replicative transposition event. In addition, some of these isolates have acquired additional IS*6110* insertions (up to 5 in total), most likely through replicative transposition. The IS-5' RFLP data (data not shown) confirmed the conclusions made from the IS-3' RFLP data and provided additional support for the sequential acquisition of IS*6110* elements by transposition.

For maximum sensitivity of the culture method multiple tissues were cultured per animal wherever possible and isolation of *M. bovis* from more than one lymph node or lesion per animal was not uncommon (data not shown). A comparison of IS*6110* fingerprints between 'same animal isolates' was carried out for ten animals: viz. six buffaloes, three lions and one hyena (*Crocuta crocuta*). In nine cases the comparison yielded identical IS*6110* RFLP patterns, while one buffalo generated two different IS*6110* types, a strain of the C8 type (the most common type found in this study and also in a previous study, where it was designated C8 (Michel et al., 2008) was isolated from the pool of lymph nodes (TB 1865B). From the pool of head lymph nodes of the thoracis cavity a strain (TB 1865A) was isolated that was similar to TB 1865B, but differing by the addition of one band. These genotype variants that were different from, but similar to the previously observed C8 type are referred to C8v in this study (Fig. 1 & Fig. 5).

The banding patterns observed in PGRS RFLP analysis also clearly identified two major groups (eight isolates shown in Fig 3). One group comprised the isolates from KZN with one IS*6110* insertion, while the second group comprised isolates which originated from the GKNPC and which had more than one IS*6110* insertion. Spoligotyping divided the isolates into the same two groups, yielding one spoligotype (SP4/SB0130) among isolates analysed from HiP and one spoligotype (SP1/SB0121) among KNP isolates (Fig. 2). In summary, the genotypic variation seen between these two groups (KZN and KNP) demonstrated that they are distinct. However, the genotypic relatedness observed within the second (KNP) group confirmed that these isolates share a common progenitor and have evolved by acquiring additional IS*6110* insertions.

Kwazulu-Natal Parks

RFLP typing using IS*6110* as a probe generated three distinct IS*6110* profiles among the 33 *M. bovis* isolates from the four parks. All isolates from HiP produced the same banding pattern, which comprised two copies of the insertion sequence. This fingerprint was described previously in cattle and designated C2 (Michel et al., 2008). All isolates from ESL and MGR (total n=13) showed the same banding pattern. The two isolates from buffaloes in the SP reserve were identical to each other but different from C2. They represented a single evolutionary variant with a number of genotype changes (TB1531 & TB 1532) whereby the 3' insertion of IS*6110* was identical to the other KZN parks isolates,

but the 5' insertion was shifted. When hybridised with the PGRS oligonucleotide probe these two isolates yielded a unique fingerprint (TB 1532 shown in Fig. 3), while 18 *M. bovis* isolates from the other three KZN parks (represented by TB 954J shown in Fig. 3) were found to share a common fingerprint. Spoligotyping of eleven HiP isolates all yielded spoligotype 4 (Fig. 2), which is identical to SB0130 in the international *M. bovis* database (Michel et al., 2008).

Greater Kruger National Park Complex

Strain typing of *M. bovis* isolates from 156 animals using the IS*6110* RFLP probe generated a total of 22 fingerprinting patterns (Fig. 1 & Fig. 5). Seventy-three percent of animals, representing all eight species examined in GKNPC (Table 1), shared a single banding pattern. This genotype had been isolated from cattle from an epidemiologically related farm, situated on the southern boundary of KNP, and was previously described as IS*6110* type C8 (Michel et al. 2008). In addition, 21 variants of C8 (referred to as C8v types) with variable genome changes were identified among the remaining 42 isolates. Five of these variants accounted for between three and seven isolates each with identical IS*6110* profiles, while two C8v fingerprints were shared by two isolates (animals) each and 14 were found in single cases (Fig 1 & Fig 5). One of the multi-isolate groups comprised exclusively isolates from five kudus (TB 647, TB 747, TB 905, TB 1081, TB 1088) clustered within a 35 km radius (Fig. 5). Another C8v type formed a geographical cluster of six isolates (representing buffalo, leopard (*Panthera pardus*) and lion) in the central region with one remote isolate located in the northern region (Fig. 5).

PGRS RFLP typing profiles were generated for 78 of the 156 animals studied, 47 isolates from the southern and 31 isolates from the central and northern regions. These included 19 isolates with C8v profiles (Fig 1). For all isolates a common banding pattern was observed (Fig. 3). As for the IS*6110* type C8, this common PGRS pattern had been previously described in the cattle herd south of KNP (Michel et al., 2008).

Spoligotyping was performed for 44 isolates, 32 from the southern and 12 from the central and northern regions, respectively , and yielded one spoligotype (SP1, Fig 2) only, resembling the international SB code SB0121 (Michel et al. 2008).

Comparison of the observed frequencies of C8 and C8v profiles in the southern versus the central/northern regions of GKNPC (Table 2), showed that significantly more C8v types were found in the central/northern regions than south of the Sabie river. This difference was statistically significant ($p<0.05$) and based on the assumption that C8 and C8v types can occur equally often throughout the GKNPC ecosystem.

Table 2. Breakdown of the numbers of C8 and C8v IS*6110* typing patterns in the Greater Kruger National Park Complex and adjoining private properties**.**

Location	C8 IS*6110* profile	C8v IS*6110 p*rofiles	Total
Southern regions	70	18	88
Central & northern regions	44	24	68
Total	114	42	156

Discussion

Disease detection and prevalence surveys conducted in KNP during the 1990's showed that the prevalence distribution followed a descending south to north gradient with herds in the south having an infection rate of up to 92% while it had not been detected in buffaloes north of the Olifants River until late 1996 (de Vos et al., 2001). Together with historical epidemiological information on the BTB status of cattle herds across KNP's southern boundary some fifty years ago as well as their interactions with KNP buffalo, these findings provided sufficient circumstantial evidence to suggest the source, mode and time of the introduction of *M. bovis* into the KNP ecosystem. The question whether the epidemic in KNP was caused by a single or multiple introduction event(s) at different times and ports of entry remained, however, open. The data provided in our study clearly suggest an epidemiological link between the BTB outbreaks in KNP buffalo and a neighbouring cattle herd, based on identical IS*6110* and PGRS fingerprints found in both species. The fingerprints were readily distinguishable from typing patterns in cattle in other geographical regions of South Africa (Michel et al., 2008). It can

Figure 1. Dendogram comprising IS*6110* fingerprints of 189 *M. bovis* isolates from Kwazulu-Natal Parks and the Greater Kruger National Park Complex.

The KZN group comprised 33 isolates from four parks (SP; n=2, ESL; n=6, MGR; n=7, HiP; n=18), and 156 isolates were analysed for the GKNPC of which 122 originated from wildlife in KNP, 17 from wildlife on private properties south and the same number from properties west of the KNP.
Identical IS typing patterns with more than 25 representative isolates are depicted as collapsed branches. The collapsed branch for type C8 was reduced in size by graphically removing parts of the branch for improved visibility of genetically diverse fingerprints.

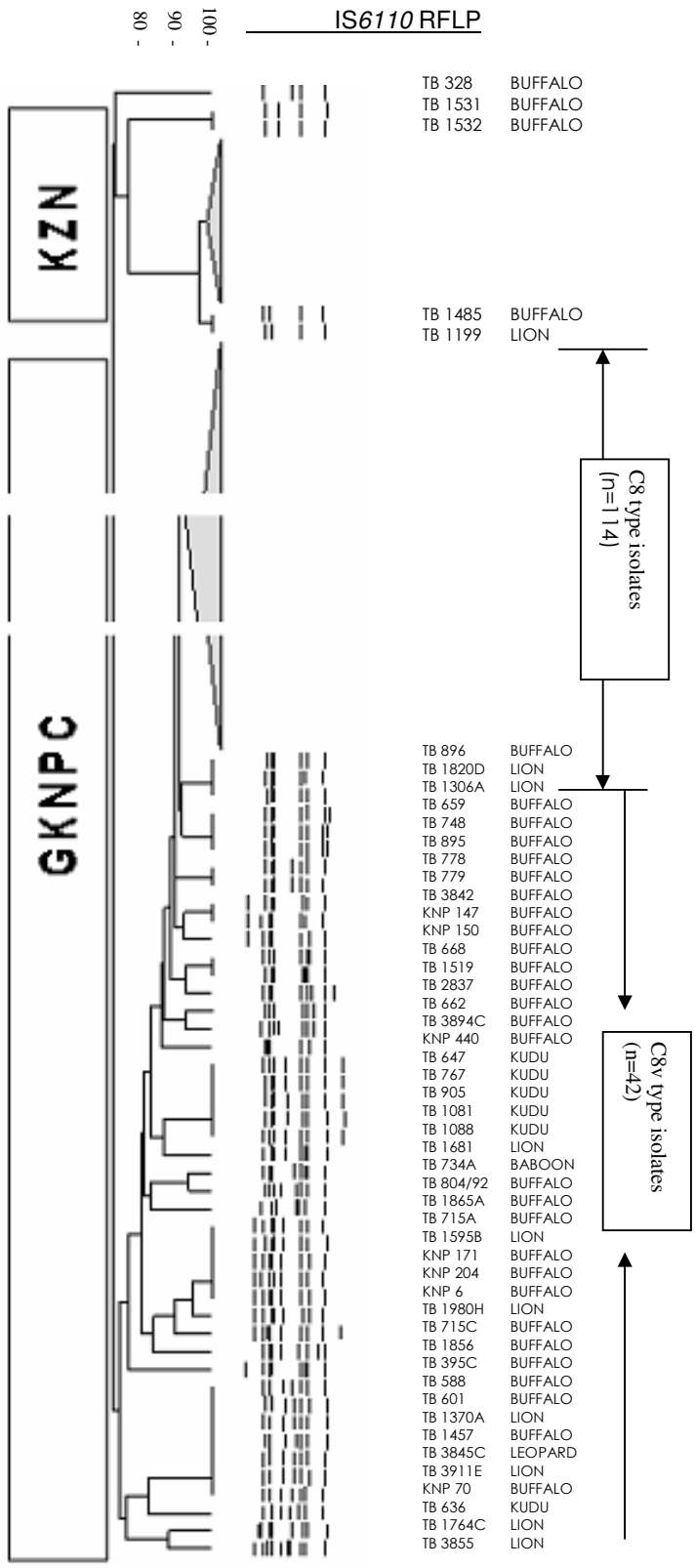
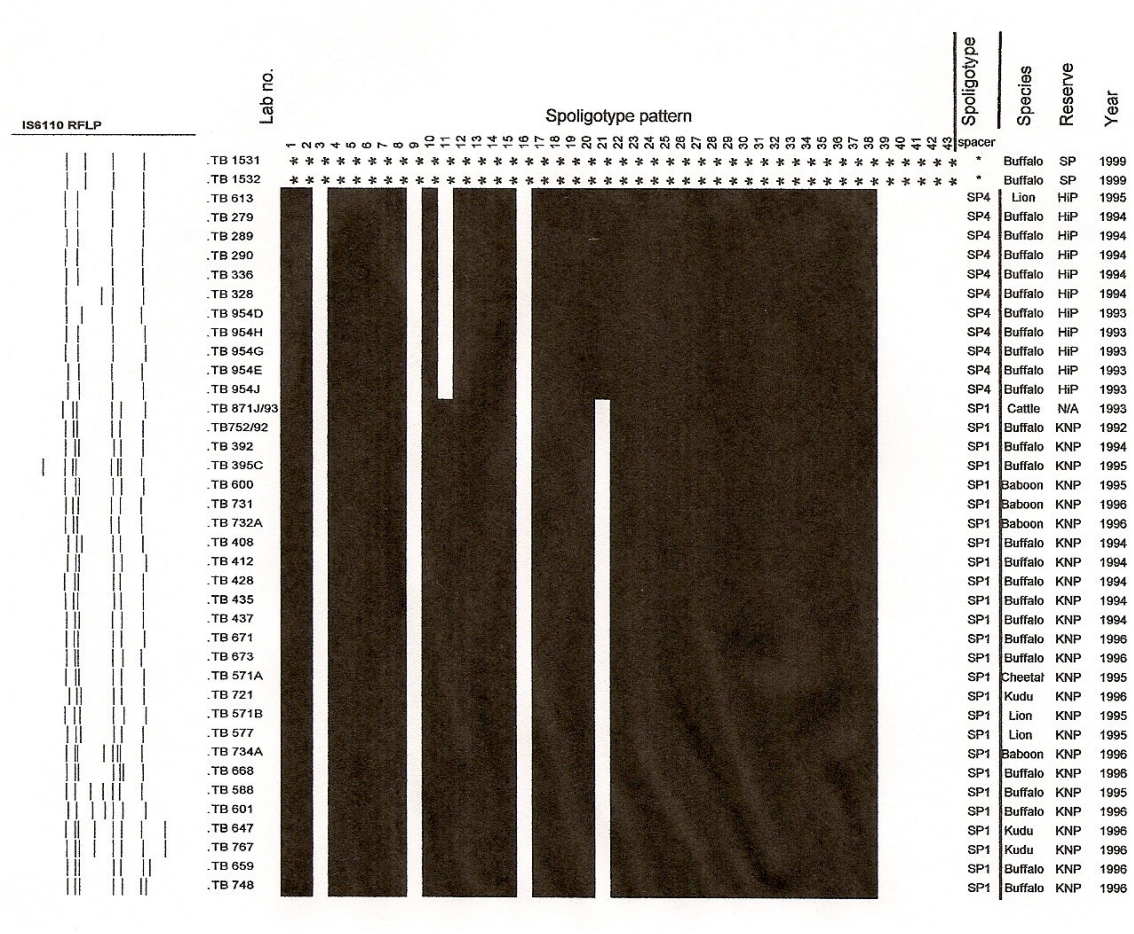

IS6110 RFLP
100 -
90 -
80 -
KZN
GKNPC
TB 328 BUFFALO
TB 1531 BUFFALO
TB 1532 BUFFALO
TB 1485 BUFFALO
TB 1199 LION
C8 type isolates (n=114)
TB 896 BUFFALO
TB 1820D LION
TB 1306A LION
TB 659 BUFFALO
TB 748 BUFFALO
TB 895 BUFFALO
TB 778 BUFFALO
TB 779 BUFFALO
TB 3842 BUFFALO
KNP 147 BUFFALO
KNP 150 BUFFALO
TB 668 BUFFALO
TB 1519 BUFFALO
TB 2837 BUFFALO
TB 662 BUFFALO
TB 3894C BUFFALO
KNP 440 BUFFALO
TB 647 KUDU
TB 767 KUDU
TB 905 KUDU
TB 1081 KUDU
TB 1088 KUDU
TB 1681 LION
TB 734A BABOON
TB 804/92 BUFFALO
TB 1865A BUFFALO
TB 715A BUFFALO
TB 1595B LION
KNP 171 BUFFALO
KNP 204 BUFFALO
KNP 6 BUFFALO
TB 1980H LION
TB 715C BUFFALO
TB 1856 BUFFALO
TB 395C BUFFALO
TB 588 BUFFALO
TB 601 BUFFALO
TB 1370A LION
TB 1457 BUFFALO
TB 3845C LEOPARD
TB 3911E LION
KNP 70 BUFFALO
TB 636 KUDU
TB 1764C LION
TB 3855 LION
C8v type isolates (n=42)

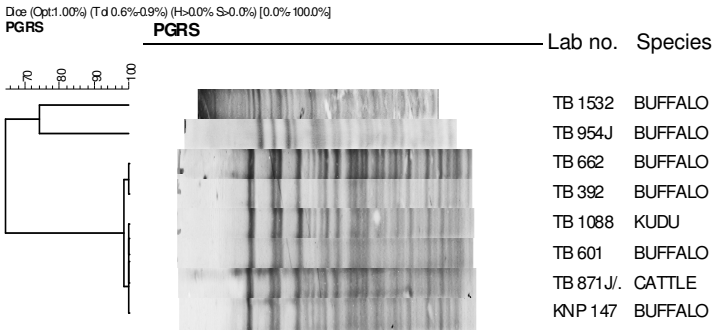
Figure 2. IS*6110* RFLP typing and spoligotyping patterns of *M. bovis* isolates from HiP (n=11), SP (n=2), KNP (n=25) including one isolate from an epidemiologically related cattle farm



SP: Spioenkop Nature Reserve, HiP:Hluhluwe iMfolozi Park; KNP: Kruger National Park

be argued that this finding does not necessarily confirm the genotype C8 as the original outbreak strain in this particular cattle herd, since the herd may have contracted C8 through spillover from buffalo, as a secondary event to the already established epidemic in the KNP buffalo. In that case the primary infection would, mostly probably , still have originated from an infected cattle herd adjacent to KNP's southern boundary, in order to allow for *M. bovis* to cross into buffalo, amplify in the new host population and re-infect the cattle herd examined in this study. The time line places these events well ahead of its first detection in the south of KNP in 1990.

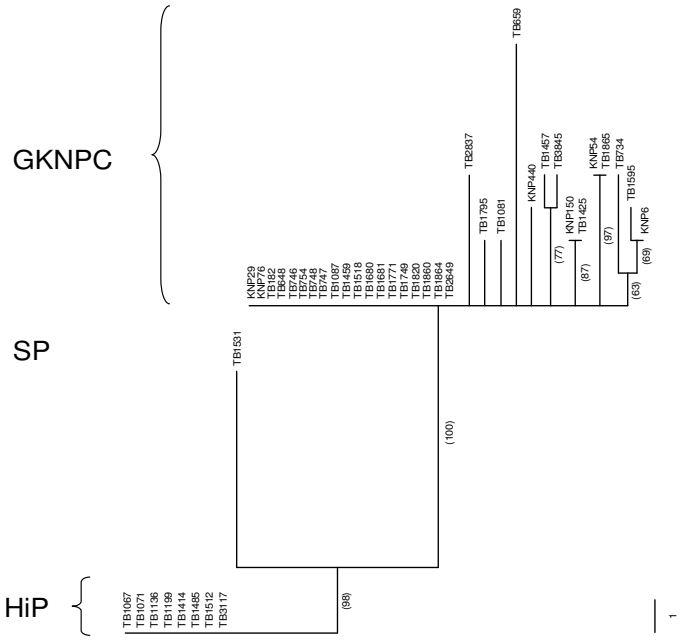
Figure 3. Dendogram showing the relationship between eight PGRS RFLP profiles of representative samples from Spioenkop Nature Reserve (TB 1532), Hluhluwe iMfolozi Park (TB 954J), Kruger National Park (TB 662, TB 392, TB 601, KNP 147) and an epidemiologically related cattle farm (TB 871J).



Phylogenetic analysis of *M. bovis* isolates from KNP and HiP revealed that the BTB epidemics that had occurred were caused by non-related strains which strongly suggests an independent introduction of a single *M bovis* strain in each of the parks. From the RFLP data it is evident that these two groups show significant genetic differences (Fig. 1 and 4) but at the same time must have had a common ancestor, given that they share a common IS*6110* insertion.

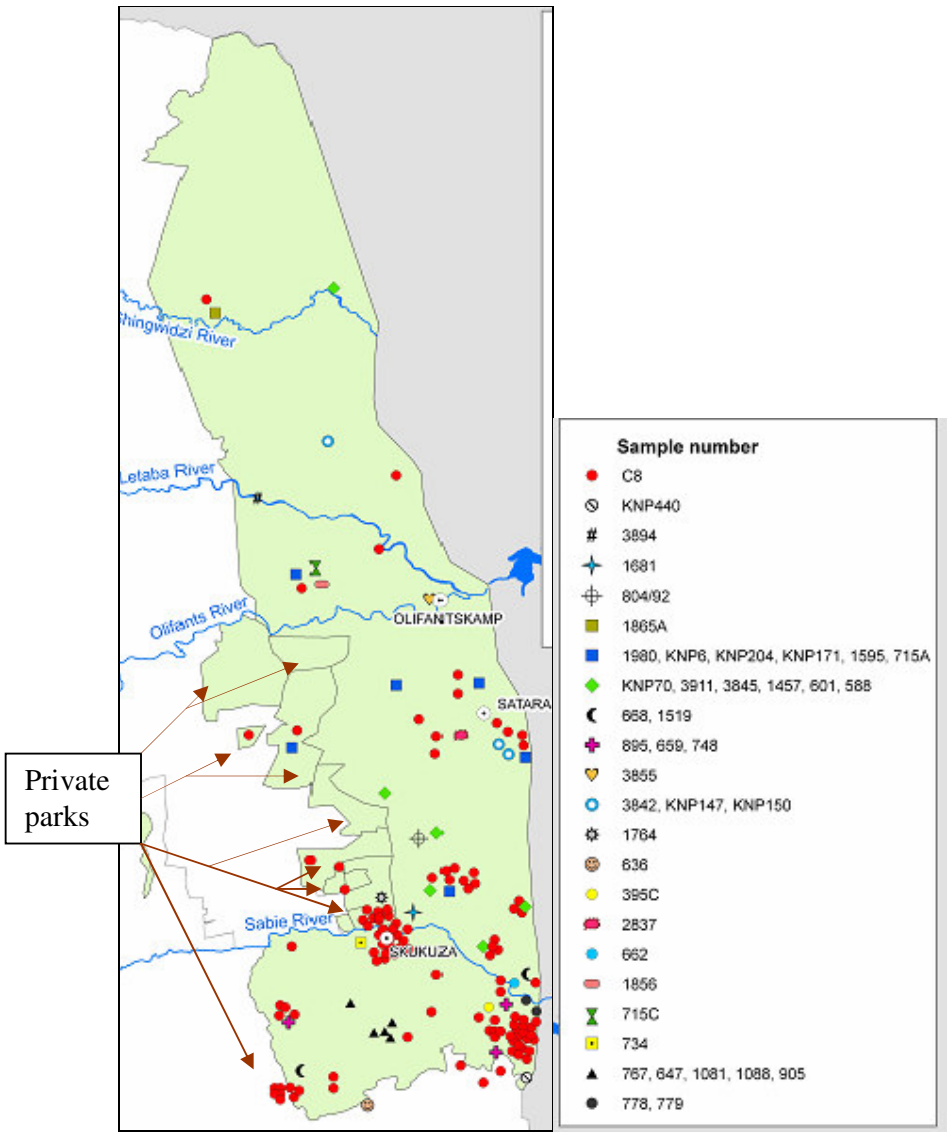
The results suggest a confidence for this of 98-100%. Unfortunately, little is known about the rate of genome diversification in *M. bovis.* In *M. tuberculosis*, the fingerprint rate change for IS*6110* is estimated at 0.0139 changes per copy per year (Warren et al., 2002). Assuming the same rate for *M. bovis*, we may expect variants with one or two extra copies to arise over 30 years, which is more than the time that *M bovis* has been present in South Africa and approximately the time it is thought to have occurred in KNP. From these results it is not possible to say whether evolution from the common ancestor occurred in South Africa, or whether these variants were introduced to the country and became endemic to different areas, to be introduced to wildlife in due course. However, the variants detected in KNP almost certainly arose within the KNP, which is supported on the one hand by the appearance of some of the C8v types in geographical clusters (Fig. 5). On the other hand, the spatial distribution of IS*6110* types in the GKNPC pictured as an ascending south-north gradient in terms of the relative frequency of C8v types. At the same time the dominant C8 type occurred throughout the KNP (Fig. 5, Table 2). It supports our suggestion that the C8v RFLP patterns have evolved from genotype C8 through mutation events in the form of a clonal expansion. This was made possible by the high BTB prevalence in buffalo (reservoir host) which led to a high infection pressure on the ecosystem with a resulting high number of intra- and interspecies transmission events (Michel et al., 2006, Warren et al., 2000).

Figure 4. Phylogenetic tree of *M. bovis* isolates from KZN parks and the greater Kruger National Park Complex. Genetic data from two different genotyping methods were subjected to phylogenetic analysis using the bootstrapping and neighbour joining algorithm in methods. The tree was rooted isolate TB1067. Bootstrap values are given in brackets at internal nodes. All branches with a zero length were collapsed. The scale indicates the number of steps per unit length.



In addition, the KNP strain C8 has more than one copy of IS*6110*, with concomitant higher likelihood of mobility and generation of additional copies (McEvoy et al.., 2007). The rate of change is not identical in different strains of mycobacteria and even for different locations of IS*6110*. Therefore, it is not necessarily surprising that variants are observed in KNP but not HiP. Transposition events can lead to decreased virulence, or gain of fitness, even in *M. bovis* (Soto et al., 2004), transposition rate change or evolutionary change, particularly if integration affects promoter activity (Tanaka & Rosenberg 2001). In *M. tuberculosis*, some strain families appear to be almost fixed with low copy numbers of IS*6110* whereas others readily gain extra copies, to a limit. Thus, in KNP we witness active evolution, but not in the KZN parks, with the tools applied and the limited sample size examined here.

Figure 5. Geographical distribution of the dominant *M. bovis* IS*6110* type (C8) and its 21 variants (C8 variants) in the greater Kruger National Park Complex GKNPC).



The demonstrated association between IS*6110* profiles and spatial distribution also highlighted the usefulness of IS*6110* RFLP typing for isolates from this particular epidemiological setting. In contrast, spoligotyping could not detect any genetic polymorphism between the related KNP variants identified as C8v types, but was useful in pointing out the genetic differences between the BTB epidemics in KNP and KZN parks.

Inter-species spread of bovine tuberculosis is most likely where different species, either wild or domesticated, share the same habitat. In both KNP and KZN parks transmission of *M. bovis* was demonstrated in a number of species including baboon and bushpig in HiP, lion in MGR (TB 1199) and greater kudu, warthog, baboon, hyena, lion, cheetah and leopard in GKNPC. The mode of transmission in most of these species is in all likelihood predominantly via the oral route during scavenging or predation. This raises concerns regarding the risk of bovine tuberculosis to other obligate or opportunistic predators and scavengers, especially those living in social structures. Recent studies conducted on colonies of meerkats (*Suricatta suricatta*) in the Northern Cape Province of South Africa, and of banded mongooses (*Mungos mungo*) in the Chobe National Park of Botswana revealed the maintenance host potential of both species for *M. tuberculosis* complex organisms (Alexander et al., 2002, Drewe et al., 2007).
Two different epidemiological cycles of BTB appeared to exist in greater kudu in the KNP. Apart from transmission of the dominant C8 type to kudu and other affected wildlife species, a unique variant of this genotype was found in a cluster of five kudus sampled within a range of 35 km. This "kudu strain" was neither found in buffalo nor any other species in this study nor thereafter. Given the divergent evolution of *M. bovis* found in the KNP ecosystem, the "kudu strain" is likely to be the result of a series of genotype changes to C8 after introduction into the kudu subpopulation. As a result of the fundamental differences between browsers and grazers in terms of their behavioural and feeding patterns there is an assumed low risk for *M. bovis* transmission from kudu to buffalo in KNP. This may, however, not be necessarily true for smaller reserves and mixed game and livestock farms, where more limited feed and water sources are confounding factors to closer contact between the species (Thorburn & Thomas 1940). To this effect it is believed that kudus resident in the Spioenkop Nature Reserve may have contracted bovine tuberculosis from cattle while roaming in adjacent farming areas and transmitted the disease to a herd of newly introduced, BTB negative buffaloes (represented by TB 1531 & 1532) during or prior to 1997 (Cooper, unpublished data). Kudus are considered a BTB maintenance host and powerful transmitters of the disease because they move over significant distances, cross game fences with ease and infected individuals excrete large amounts of infectious material (Bengis et al., 2001).
Since various genotypes were found in a variety of animal species, there is currently no evidence to suggest that evolution may be accelerated during cross-over into species, nor to suggest a species specific variant as suggested by Costello et al. (1999).

The molecular typing results of the buffalo isolates 1865A & B suggest that free-ranging wild animals, like humans, can be simultaneously infected with strains of different IS*6110* types (du Plessis et al., 2001).

In conclusion, molecular typing provided valuable epidemiological information regarding the transmission of *M. bovis* from livestock to buffalo, buffalo to buffalo and to other wildlife species.

Acknowledgements

The authors want to thank the personnel of the ARC-OVI TB laboratory and Ms A Venter and Mrs M De Kock from the Stellenbosch University for culturing isolates of *M bovis*. We appreciate the technical support from Dr R Williams in illustrating the geographical distribution of cases in GKNPC.

References

Alexander, K.A., Pleydell, E., Williams, M.C., Lane, E.P., Nyange, J.F.C., Michel, A.L., 2002. *Mycobacterium tuberculosis*: An emerging disease of free-ranging wildlife. Emerg. Infect. Dis. 8, 592-595.

Bengis, R.G., Kriek, N.P.J., Keet, D.F., Raath, J.P., De Vos, V., Huchzermeyer, H.F.A.K., 1996. An outbreak of bovine tuberculosis in a free-living buffalo population in the Kruger National Park. Onderstepoort J. Vet. Res. 63, 15-18.

Bengis, R.G., Keet, D.F., Michel, A.L., Kriek, N.P.J., 2001. Tuberculosis caused by *Mycobacterium bovis* in a kudu (*Tragelaphus strepsiceros*) from a commercial farm in the Malelane area of the Mpumalanga Province, South Africa. Onderstepoort J. Vet. Res. 68, 239-241.

Costello, E., O'Grady, D., Flynn, O., O'Brien, R., Rogers, M., Quigley, F., Egan, J., Griffin, J., 1999. Study of restriction fragment length polymorphism analysis and spoligotyping for epidemiological investigation of *Mycobacterium bovis* infection. J Clin Microbiol. 37, 3217-3222.

Dale, J.W., Al-Ghusein, H., Al-Hashmi, S., Butcher, P., Dickens, A.L., Drobniewski, F., Forbes, K.J., Gillespie, S.H., Lamprecht, D., McHugh, T.D., Pitman, R., Rastogi, N., Smith, A.T., Sola, C., Yesilkaya, H. 2003. Evolutionary relationships among strains of *Mycobacterium tuberculosis* with few copies of IS6110. J. Bacteriol. 185, 2555-2562.

De Lisle, G.W., Mackintosh, C.G., Bengis, R.G. 2001. Mycobacterium bovis in free-living and captive wildlife including farmed deer. Rev. Sci. Tech. Off. Int. Epiz. 20, 86-111.

De Vos, V., Bengis, R.G., Kriek, N.P.J., Michel, A., Keet, D.F., Raath, J.P., Huchzermeyer, H.F.A.K., 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. Onderstepoort J. Vet. Res. 68, 119 – 130.

Drewe, J., Pearce, G., Clutton-Brock, T., Dean, G., Michel, A., 2007. Establishing the disease ecology of tuberculosis in meerkats at the wildlife-domestic animal-human interface. Proceedings of the 38th World Coference on Lung Health of the International Union against Tuberculosis and Lung Disease. 8 – 12 November 2007. Cape Town, South Africa. p S 26.

Du Plessis, D.G., Warren, R., Richardson, M., Joubert, J.J., van Helden, P.D., 2001. Demonstration of reinfection and reactivation in HIV-negative autopsied cases of secondary tuberculosis: multilesional genotyping of *Mycobacterium tuberculosis* utilizing IS*6110* and other repetitive element-based DNA fingerprinting. Tuberculosis (Edinb). 81, 211-20.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783-793.

Hermans, P. W., Messadi, F., Guebrexabher, H., van Soolingen, D., de Haas, P. E., Heersma, H., de Neeling, H., Ayoub, A., Portaels, F., Frommel. D., 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA typing for global tuberculosis epidemiology [see comments]. J. Infect.Dis. 171, 1504-1513.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. J. Clin. Microbiol. 35, 907-914.

Keet, D.F., Kriek, N.P.J., Penrith, M.-L., Michel, A., Huchzermeyer, H., 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. Onderstepoort J. Vet. Res. 63, 239-244.

Keet, D.F., Kriek, N.P.J., Bengis, R.G., Grobler, D.G., Michel A.L., 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. Onderstepoort. J. Vet. Res. 67, 115-122.

Keet, D.F., Kriek, N.P.J., Bengis, R.G., Michel, A., 2001. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park Onderstepoort J. Vet. Res. 68, 225-230.

Kloeck, P.E., 1998. Tuberculosis of domestic animals in areas surrounding the Kruger National Park. In: Proceedings 'The challenges of managing tuberculosis in wildlifein Southern Africa, Ed Zunkel, July 30-31, Nelspruit, South Africa.

McEvoy, C.R.E., Falmer, A.A., Gey van Pittius, N.C., Victor, T.C., van Helden, P.D., Warren, R.M., 2007. The role of IS*6110* in the evolution of *Mycobacterium tuberculosis*. Tuberculosis 87, 393-404.

Michel, A.L., 2002. The epidemiology of M. bovis infection in South African wildlife. Abstracts of the Veterinary European Network on Mycobacterium (VENOM) Symposium: "DNA Fingerprinting of Bovine TB strains" , Belfast, Northern Ireland, October 24-26, 2002.

Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., de Klerk L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P., Godfroid, J., 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. Vet. Microbiol. 112, 91-100.

Michel, A. L., Hlokwe, T.M., Coetzee, M. L. , Maré, L., Connoway, L., Rutten, V.P.M.G., Kremer, K., 2008. High *Mycobacterium bovis* genetic diversity in a low prevalence setting. Vet. Microbiol. 126, 151-159.

Paine, R., Martinaglia, G., 1929. Tuberculosis in wild buck living under natural conditions. J. Comp. Path. Ther. XLII, 1,1-8.

Rodwell, T.C., Whyte, I.J., Boyce, W.M., 2001. Evaluation of population effects of bovine tuberculosis in free-ranging African buffalo (*Syncerus caffer*). J. Mammal., 82, 231-238.

Smith N.H., Dale J., Inwald J., Palmer S., Gordon S.V., Hewinson R.G., Smith J.M., 2003. The population structure of Mycobacterium bovis in Great Britain: clonal expansion. Proc. Natl. Acad. Sci. USA. 100, 15271-15275.

Soto, C.Y., Menendez, M.C., Perez, E., Samper, S., Gomez, A.B., Garcia, M.J., 2004. IS6110 mediates increased transcription of the phoP virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. J. Clin. Microbiol. 42, 212-219.

Tanaka, M.M., Rosenberg, N.A., 2001. Optimal estimation of transposition rates of insertion sequences for molecular epidemiology. Statist. Med. 20, 2409-2420.

Thorburn, J.A., Thomas, A. D., 1940. Tuberculosis in Cape Kudu. J. S. Afr. Vet. Med. Ass. 11, 3-10.

Thrusfield, M., 1995. Veterinary Epidemiology. Second Edition. Blackwell Science Ltd. Oxford, pp 181-191.

Warren, R., Richardson, M., Sampson, S., Hauman, J.H., Beyers, N., Donald, P.R., van Helden, P.D., 1996. Genotyping of *Mycobacterium tuberculosis* with additional markers enhances accuracy in epidemiological studies. J.Clin.Microbiol. 34, 2219-2224.

Warren RM, Sampson SL, Richardson M, van der Spuy, GD, Lombard CJ, Victor, TC, van Helden PD., 2000. Mapping of IS*6110* flanking regions in clinical isolates of *Mycobacterium tuberculosis* demonstrates genome plasticity. Mol. Microbiol. 37, 1405-1416.

Warren, R. M., M. Richardson, S. L. Sampson, G. D. van der Spuy, W. Bourn, J. H. Hauman, H. Heersma, W. Hide, N. Beyers, and P. D. van Helden. 2001. Molecular evolution of *Mycobacterium tuberculosis*: phylogenetic reconstruction of clonal expansion. Tuberculosis. (Edinb.) 81, 291-302.

Warren, R.M., van der Spuy, G.D., Richardson, M., Beyers, N., Booysen, C., Behr, M.A., van Helden, P.D. 2002. Evolution of the IS*6110* - based restriction fragment length polymorphism pattern during the transmission of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 40, 1277-1282.

Chapter 4

Improved diagnosis of bovine tuberculosis in wildlife and domestic cattle

Chapter 4.1

Approaches towards optimising the gamma interferon assay for diagnosing Mycobacterium bovis infection in African buffalo (*Syncerus caffer*)

A.L. Michel[1], D. Cooper[2], J. Jooste[3], L-M. de Klerk[4], A. Jolles[5]

[1]Tuberculosis Laboratory; Food, Feed & Veterinary Public Health Programme, ARC- Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa

[2]Chief Veterinarian, Ezemvelo Kwazulu-Natal Wildlife - KZN Wildlife. Private Bag x01, St Lucia, 3936, South Africa

[3]State Veterinarian, Directorate Veterinary Services Limpopo Province, Hoedspruit 1380, South Africa

[4]South African National Parks, Private Bag X402, Skukuza, 1350 South Africa

[5]Department of Ecology and Evolutionary Biology, Princeton University, USA

Manuscript in preparation

Abstract

The application of diagnostic tests for bovine tuberculosis in wildlife poses formidable technical difficulties and the use of the gamma interferon assay offers a technically much more practical approach to testing wild animal species. We compared the performance of the gamma interferon assay in African buffalo under the recommended guidelines for interpretation of test results and found a high sensitivity (92.1%) at the cost of a greatly reduced specificity (68.3%). An optimised cut-off value for positive test results was identified at 0.38 as the preferred compromise between sensitivity and specificity. Additional optimisation approaches to improve test performance were examined and showed that the application of 'a priori exclusions' of test results on the basis of reactivity to avian and fortuitum PPD (sensitin produced from *Mycobacterium fortuitum*) increased specificity without losing sensitivity. The implications of this and other interpretation schemes are discussed.

Keywords: Bovine tuberculosis, cattle, *Mycobacterium fortuitum*, Fortuitum, interferon gamma assay, African buffalo

Introduction

The intradermal tuberculin test (IDT), or skin test, is still the most widely used method to diagnose bovine tuberculosis in cattle in countries worldwide. Limitations of the IDT in cattle have been mainly described in developed countries and include aspects relating to test performance (Wood, 1991, Neill et al., 1992, Monaghan et al., 1994), source of tuberculin PPD (Cagiola et al., 2000) as well as to logistical drawbacks in terms of repeated handling of animals and the minimum testing interval (Radunz et al., 1985).

Developing countries face a number of constraints in implementing and maintaining a bovine tuberculosis (BTB) control scheme. In remote areas difficult accessibility, long travelling distances and large, scattered herds are aggravating logistical constraints such as the lack of veterinary capacity and handling facilities for cattle. In the communal farming systems of sub-Saharan Africa, BTB testing is typically performed at communal diptank stations where local cattle owners muster their herds in weekly or two-weekly intervals to receive general veterinary extension services. Failure of owners to present the injected cattle for test interpretation three days after injection of tuberculin is among the common causes of the limited efficacy of BTB control in those areas. These factors constitute a high financial burden and render BTB testing in developing countries less efficient and affordable.

The development of the gamma interferon (IFNg) assay as an ancillary test for bovine tuberculosis has improved the sensitivity of BTB testing (Wood et al., 1991). Cattle with early *M. bovis* infections are more readily detected by the gamma interferon assay than the IDT (Neill et al., 1994) and parallel interpretation of both tests exceeded their individual diagnostic sensitivities (Whipple et al., 1995). The achieved specificity of approximately 96% was generally considered sufficient for BTB control purposes in cattle and could not be increased further without compromising on the sensitivity (Wood et al., 1991, Buddle et al., 2001).

Once bovine tuberculosis has established itself in a wildlife population it is difficult to control and probably impossible to eradicate. Despite its status as maintenance host for *M. bovis* the African buffalo (*Syncerus caffer*) is of high commercial and ecological value and diagnostic tools used towards its control are required to possess maximum sensitivity and specificity (Michel et al., 2006). We have previously observed false positive test results in free-ranging buffaloes when using the standard protocol for the IFNg assay. We have further established that false positive test results were caused by sensitisation of the animals with environmental mycobacteria (Michel, in press). Subsequently the commercial assay was modified into a triple comparative test setup. In addition to the standard test format based on stimulation of whole blood with bovine and avian tuberculin PPD, IFNg produced by white blood cells in response to sensitin derived from *M. fortuitum*, (Fortuitum), was evaluated. The results suggested that Fortuitum could be of potential value in detecting non-specific sensitisation in cattle and buffalo, hence possibly allowing improved test specificity in uninfected herds and populations. It was therefore the aim of this study to use data sets generated from the field application of the IFNg assay in buffalo to determine measures to predict the BTB status and subsequently to improve test validity in this species by determining the most appropriate cut-off value(s) for the IFNg test under local conditions.

Materials and methods

Animals

Known uninfected buffalo were sourced from registered operations on game farms and parks aimed at breeding buffaloes which are free from specified controlled diseases, including bovine tuberculosis. The infection status of the breeding stock is monitored by means of annual IFNg tests. Offspring are tested for BTB according to a five-phase protocol applied over a two year period.

Infected buffaloes were sampled during bovine tuberculosis surveys in the endemically infected KNP and HiP between 1996 and 2007. Additional samples were sourced from three different research trials involving experimentally or

naturally infected buffaloes (de Klerk et al., 2006, Michel et al., 2007, de Klerk, in prep.).

Necropsy and bacteriological confirmation

All culled buffaloes from infected herds were subjected to a detailed post mortem examination and tissue samples were collected for bacterial culture. Isolation and identification of mycobacteria was performed as described previously (Bengis et al., 1996, Michel et al., 2007).

Production of sensitin from *M. fortuitum* (Fortuitum)

Mycobacterium fortuitum cultures (ATCC strain 6841) were grown in 7H9 Middelbrook medium supplemented with OADC (Biolab Diagnostics, Wadeville, South Africa) at a final concentration of 0.1%. The cultures were incubated at 37°C for three to four weeks with loosened caps and occasional shaking of the flasks until the turbid cultures started to form a sediment. Before harvesting the cultures were autoclaved at 121°C for 15 minutes and filtered through Whatman 40 filter paper. The culture filtrates were precipitated overnight with trichloracetic acid (TCA) at a final concentration of 4%. On the following day the protein precipitate was concentrated by centrifugation (4000 rpm, Bechman-Coulter, Allegra X22R) and washed in succession twice with 1% TCA and once with PBS. The concentrated, wet Fortuitum pellet was weighed and dissolved in PBS containing 0.01% Tween 20, ph 7.2, to give a final concentration of 20 mg/ml (wet weight/volume).

Assay for bovine gamma interferon

The Bovigam IFNg assay was performed as described by the manufacturer with the following modification. During the processing step an additional aliquot of 1.5 ml whole blood was stimulated with 500 µg of Fortuitum and incubated as recommended for the standard blood cultures. All plasma samples were assayed in parallel according to the manufacturer's instructions.

Data analysis

The sensitivity of the IFNg assay was determined from data collected from infected buffalo defined as those from which *M. bovis* was isolated. The sensitivity was calculated as the proportion of test positive infected animals from the total number of known infected animals examined (Toma et al., 1999).

The specificity of the IFNg assay was determined using test data from cattle and buffalo herds with a bovine tuberculosis free status (negative). To obtain further certainty on their negative status for the second stage evaluation of the specificity only herds for which a minimum data set of two but mostly of 3 – 4 consecutive IFNg tests as well as their negative IDT status were available. The specificity was defined as the proportion of test negative animals from the total number of known negative animals examined.

Logistic regression analysis was used with BTB status as the dependent variable and each of the optical density (OD) values as independent variables, alone and in combination with each other. USE Akaike Information Criterion and/or negative loglikelihood (nll) was used to select the best model (lower AIC is better, lower nll is better), and examine p-values to document statistical significance of each factor.

The optimal cut-off value for a positive test result was defined as the absorbance value that minimizes erroneous test results, e.g. the percentage of animals generating test results equal or greater than the cut-off value is higher in infected than in uninfected groups of animals. For optimizing the cut-off value initially only one test variable, starting with the OD value for bovine PPD was taken into account, followed by addition of absorbance information on avian and Fortuitum PPD as well as the nil control.

Stage two optimisation of the test validity was based on "a priori exclusions": if OD-x>=OD-bov, the test was interpreted as negative, based on the assumption that any OD-bov response is primarily a cross-reaction due to sensitisation by x (*M. avium*, *M. fortuitum*, etc.).

Table 1. 2 x 2 table showing IFNg test results among 344 uninfected and 149 known infected buffalo using standard test interpretation*

Test	**BTB status**	
	Infected	Uninfected
Test positive	138	109
Test negative	12	235
Total	149	344

* Whipple et al. (2001)

Results

Animals

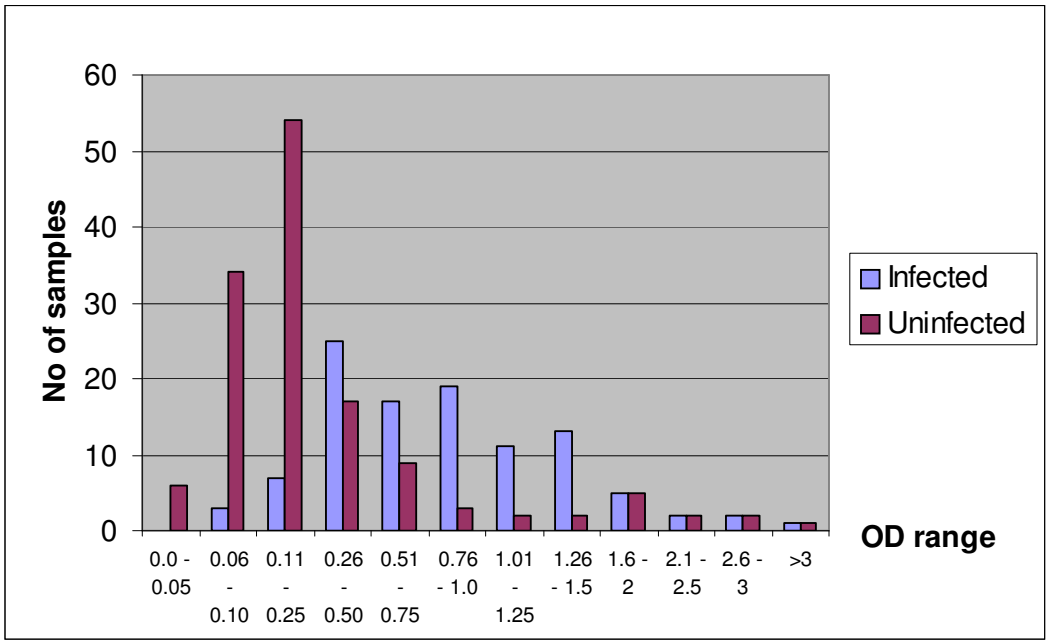
Data from 149 infected buffaloes from known infected herds were examined, which included 69 animals with culture confirmed *M. bovis* infection. In 80 animals bovine tuberculosis was diagnosed macroscopically at necropsy and 77 of those had been tested with the comparative intradermal tuberculin test and found positive.
A total of 344 known negative buffalo (all IDT negative) had been tested with the standard IFNg assay. Test data from an additional 1531 buffaloes were analysed retrospectively for optimisation of the cut-off value for OD-bov.

Assay for gamma interferon

The evaluation of the test performance was done in two stages. Initially 149 infected and 344 uninfected buffaloes were tested with the IFNg assay using the criteria for test interpretation reported by Wood et al. (1992) and Whipple et al. (2001). In brief, animals were classified as positive if OD-bov minus OD-control was greater than 0.099 and if OD-bov was greater than OD-av. The sensitivity and specificity of this standard IFNg assay were found to be 92.1% and 68.3%, respectively (Table 1).

A total of 32 uninfected buffaloes showed a pronounced false positive reactivity to bovine tuberculin of OD-bov > 0.40, comparable to infected animals. They also mounted a significant IFNg response to stimulation with Fortuitum which was largely absent in test negative animals (Fig. 3).

Figure 1. Frequency distribution of OD-bov absorbances in infected and uninfected buffalo



Prediction of BTB status

The analyses were based on and evaluated the OD values of all the available data in both groups of buffalo. It is evident that although OD-bov, ODav, and ODnil help in predicting the BTB status in buffalo, the absorbance for bovine PPD was identified as the most predictive measurement. Examination of composite single factors e.g. OD-bov – OD-nil did not improve the predictability. Two-factor models (OD-bov plus each of the others in turn) showed that adding OD-av improved the model significantly while the effect of OD-nil was low and that of

OD-fort was not statistically significant. Other modifications of the factor-model had an adverse effect (e.g. OD-bov x OD-av). Among the three-factor models the combination OD-bov + OD-av + OD-nil led to a slight improvement of predictability.

Table 2. Example calculations of 'a priori' exclusions in the determination of the cut-off for a positive IFNg test result

	A priori exclusions: TB- if ODbov*0.9<=	Cut-off: TB+ if ODbov>	specificity (%)	sensitivity (%)	overall validity (%)
optimum	***none***	***0.385***	***91.86***	***86.17***	***89.17***
	ODav	0.375	93.6	83.11	88.36
	ODav or ODnil	0.375	93.53	82.43	87.98
	ODav or ODfort	0.375	95.09	80.41	87.75
	ODav or ODnil or ODfort	0.375	95.09	79.73	87.41
To achieve specificity > 95%	none	0.525	95.35	73.65	84.5
	ODav	0.425	95.35	77.7	86.53
	ODav or ODnil	0.425	95.29	77.03	86.16
	ODav or ODfort	***0.375***	***95.09***	***80.41***	***87.75***
	ODav or ODnil or ODfort	0.375	95.09	79.73	87.41
To achieve sensitivity > 95%	***none***	***0.235***	***79.07***	***95.27***	***87.05***
	ODav	n/a	n/a	max < 95	n/a
	ODav or ODnil	n/a	n/a	max < 95	n/a
	ODav or ODfort	n/a	n/a	max < 95	n/a
	ODav or ODnil or ODfort	n/a	n/a	max < 95	n/a

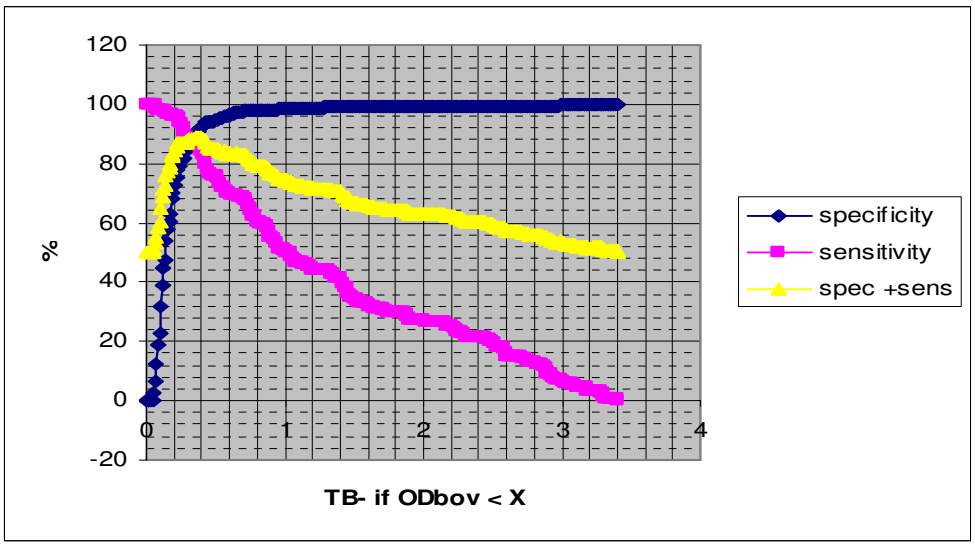
** best test for each situation in bold italics*

Optimising the cut-off value for a positive test result

An arbitrary approach was followed by determining the most suitable cut-off value for test positive animals by adding twice the standard deviation to the mean optical density of 1875 bovine PPD stimulated plasma samples from uninfected buffaloes (Richardson et al., 1983). As a result the cut-off value for test positive OD values was defined as 0.385 (Figure 1). At this cut-off the test sensitivity was 86.5% and the specificity 91.9%. Setting the cut-off lower led to an increase in both the sensitivity and the total error rate, e.g. a cut-off at OD-bov >=0.235 resulted in a sensitivity of 95.3% and a specificity 79.1%. Likewise, selection of a higher cut-off value yielded a higher specificity at the cost of sensitivity (Figure 2).

To improve overall test validity information on OD-av, OD-fort and OD-nil were taken into account, based on "a priori exclusions". The data showed that adding information on OD-av or OD-fort can reduce the loss of sensitivity associated with maximising specificity (Table 2 and Fig 3).

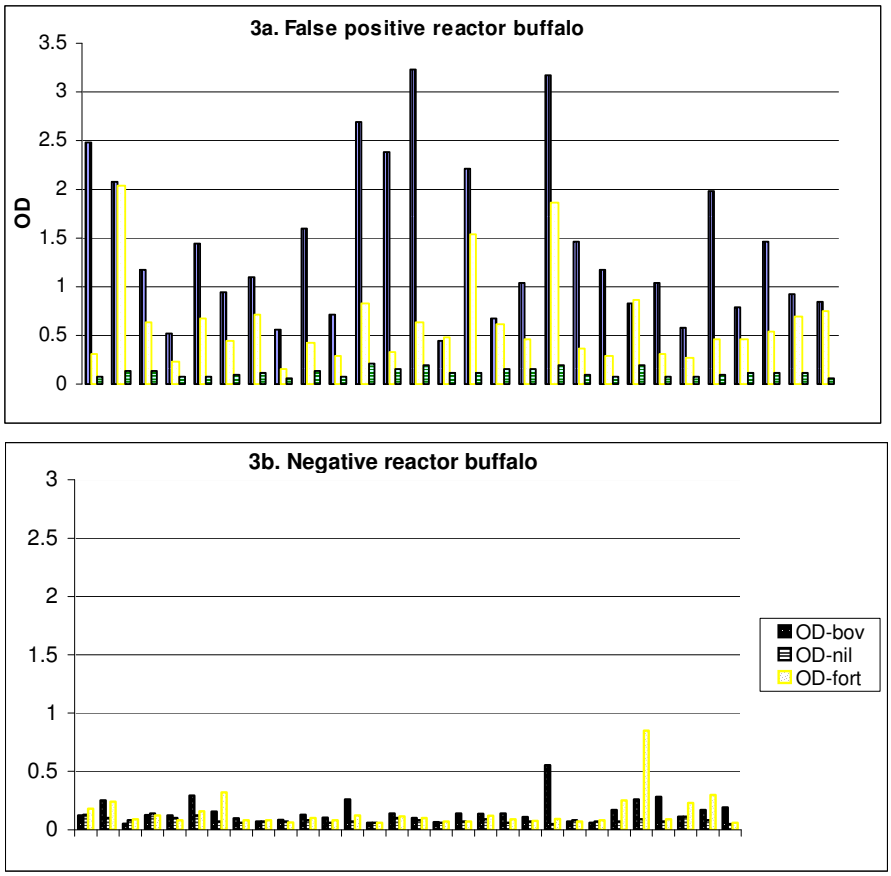
Figure 2. Test validity by cut-off values for OD-bov



Discussion

Bovine tuberculosis control in protected wildlife reservoir species such as the African buffalo in South Africa introduces a new challenge for Government, conservation organisations and the wildlife industry. An overkill of buffaloes in order to reduce the herd and regional BTB prevalence is only acceptable in known infected populations with a high prevalence, such as the Hluhluwe-iMfolozi Park (Michel et al., 2006). In all currently uninfected populations the culling of false positive buffaloes as a result of a lack of test specificity is an ethically and financially unacceptable sacrifice. The IFNg assay has many practical advantages over the skin test, especially in wildlife, and was found valuable in a preliminary evaluation in buffalo in the KNP (Michel, unpublished data). In contrast to the livestock sector, there is a high demand for both maximum sensitivity and specificity.

Figure 3. IFNg reactivity in uninfected buffaloes classified as false positive (a) and negative (b).



There is no perfect discrimination between infected and uninfected populations. The desired compromise in our situation should offer optimum specificity but at the same time the flexibility to opt for high sensitivity when required. When applying the standard interpretation criteria recommended by the supplier it was found that the standard protocol of the IFNg assay could not meet these requirements. The test validity was improved in this study by firstly identifying the absorbance of bovine PPD as the dominant variable and by optimising the cut-off value for a positive test result. By applying this OD value (0.385) which is significantly higher than in the standard protocol, the specificity was increased from 68.3% to 91.9%. As expected this was paralleled by a decrease in sensitivity from 92.1% to 86.5%. To be able to achieve a further increase in specificity without losing sensitivity it is important to understand the mechanisms which modulate the immune responses in cattle and buffalo.

Exposure of cattle to environmental mycobacteria has been previously implied as underlying cause of non-specific reactivity during skin testing as well as IFNg testing (Kleeberg 1960, Cagiola et al., 2004, Kormendy 1995, Donoghue et al.,

1997, Michel in press). We have recently reported the isolation of environmental mycobacteria from infected and uninfected buffalo as well as from surface water (Michel et al., 2007) and we may therefore speculate that the false positive reactivity in the buffaloes examined in the present study was caused by antigenic cross-reactivity with mycobacteria other than tuberculosis. Consecutive sampling and testing of non-specific reactors furthermore substantiated the transient character of this sensitisation in buffalo which rarely persisted for periods longer than three months (data not shown). The results of this study further showed that non-specific sensitisations occur more often in the IFNg assay than in the IDT (results not shown).

The misclassification of 32 out of 344 uninfected buffaloes (9.3%) as positive reactors with OD-bov values of greater than 0.40 was associated with high reactivity to OD-Fort (Figure 3a & b). This finding indicated that a differential interpretation scheme based on the discrimination of buffaloes reacting to avian or Fortuitum PPD may be a useful measure to increase test specificity of the IFNg assay in buffalo. Further optimisation of the IFNg test validity was therefore pursued in this study by examining 'a priori' exclusions which allow for certain bovine reactors to be classified as test negative, based on the level of reactivity to avian or fortuitum PPD (Table 2). A slight modification of this approach has already been applied very successfully in BTB surveys in buffalo in the KNP (Grobler et al., 2002, Hofmeyr et al., 2003, de Klerk unpublished data) as well as in interpreting immune status of experimentally infected and vaccinated buffaloes as described recently (de Klerk et al., 2006, Michel et al., 2005, de Klerk in prep.) The interpretation scheme suggested here therefore promises to provide further improvement in the IFNg test performance by maintaining the required level of specificity and ensuring satisfactory sensitivity. The decision what error rate (in either direction) is acceptable, depends on the epidemiological setting and management strategy. While maintenance of BTB free populations will require maximum specificity, the control of BTB in medium and high prevalence herds in infected ecosystems will call for highest sensitivity. The use of two different interpretation schemes for infected versus uninfected populations is not new and generally accepted for the IDT (Kleeberg 1960). It is therefore our next objective to determine appropriate cut-off values for the IFNg assay in these contrasting situations, which we believe will add value to the use the IFNg assay in supporting the control of BTB in buffalo and cattle in South Africa.

References

Bengis RG, Kriek NPJ, Keet DF, Raath JP, de Vos V, Huchzermeyer HFAK. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer* – Sparrman) population in the Kruger National Park: a preliminary report. Onderstepoort J Vet Res 63:5–18.

Buddle BM, Ryan TJ, Pollock JM, Andersen P, de Lisle GW. 2001. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol 80:37-46.

Cagiola M, Feliziani F, Severi G, Pasquali P, Rutili D. 2000. Analysis of possible factors affecting the specificity of the gamma interferon test in tuberculosis-free cattle herds. Clin Vaccine Immunol 11:952–956.

Donoghue HD, Overend E, Stanford JL. 1997. A longitudinal study of environmental mycobacteria on a farm in south-west England. J Appl Microbiol 82:56-67.

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

Greiner M. 1996. Two-graph receiver operating characteristic (TG-ROC)_ update version supports optiisation of cut-off values that minimise overall misclassification costs. J Immunol Methods 191:93-94.

Grobler DG Michel AL, de Klerk, L-M, Bengis, RG 2002. The gamma interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J. Vet. Res. 69: 221-227.

Hofmeyr M, Bengis R, de Klerk LM, Buss P. 2003. BTB survey in buffalo herds in the northern half of the Kruger National Park from 4 – 27 July 2003. South African National Parks Management report. 20 September 2003.

Kleeberg HH, 1960. The tuberculin test in cattle. J S Afr Vet Med Assoc, 31:213-226.

Kormendy B. 1995. Achievements and difficulties in maintaining the tuberculosis free status of Hungarian cattle herds. Acta Vet Hungarica 43:377-384.

Michel AL, de Klerk LM, Gey van Pittius NC, Warren RM, van Helden PD. 2007. Bovine tuberculosis in African buffaloes: Observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. BMC Vet Res 3:23.

Michel AL, Bengis RG, Keet DF, Hofmeyr M, de Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte IJ, Buss P, Godfroid J. Wildlife tuberculosis in South African conservation areas: implications and challenges. Keynote address presented

at the IVth International *Mycobacterium bovis* conference. Dublin. 22–26 August 2005.

Monaghan ML, Doherty ML, Collin JD, Kazda JF, Quinn PJ. 1994. The tuberculin test Vet Microbiol 40:111-124.

Neill SD, Hanna J, Mackie DP, Bryson TGD. 1992. Isolation of *Mycobacterium bovis* from the respiratory trat s of skin test negative cattle. The Veterinary Record, July 18:45-47.

Neill SD, Cassidy J, Hanna J, Mackie DP, Pollock JmcA, Clements A, Walton E, Bryson DG. 1994. Detection of *Mycobacterium bovis* infection in skin test negative cattle with an assay for bovine interferon-gamma. Vet Rec 135:134-135.

Radunz BL, Lepper AWD. 1985. Suppression of skin reactivity to bovine tuberculin in repeat tests. Aust Vet J, 62:191-194.

Richardson MD, Turner A, Warnock DW, Liewellyn PA. 1983. Computer assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. J Immunol Methods 56:201-207.

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

Whipple, DL, Palmer MV, Slaughter RE, Jones SL, 2001. Comparison of purified protein derivates and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. J Vet Diagn Invest 13:117-122.

Wood PR, Corner LA, Rothel JS, Baldock C, Jones SL, Cousins DV, McGormick BS, Francis BR, Creeper J, Tweddle NE. 1991. Field comparison of the interferon gamma assayand the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Aust Vet J 68:286-290.

Wood PR, Corner LA, Rothel JS, Ripper JL, Fifis T, McGormick BS, Francis B, Melville L, Small K, de Witte K, Tolson J, Ryan TJ, de Lisle GW, Cox JC, Jones SL. 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Vet Microbiol 31:71-79.

Chapter 4.2

Mycobacterium fortuitum infection interference with *Mycobacterium bovis* diagnostics: natural infection cases and a pilot experimental infection

Anita L. Michel

Tuberculosis Laboratory, Bacteriology Section, ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa

J Veterinary Diagnostic Investigation, 2008; in press

Abstract

Mycobacterium fortuitum and at least one unidentified species of soil mycobacteria were isolated from lymph nodes from four out of five African buffalo (*Syncerus caffer*), which had been culled due to positive test results using the Bovigam assay. The buffalo were part of a group of 16 free-ranging buffalo captured in the far north of the Kruger National Park (South Africa) assumed to be free of bovine tuberculosis. No *Mycobacterium bovis* was isolated. To investigate the possible cause of the apparent false-positive diagnosis, the *Mycobacterium* isolates were inoculated into four experimental cattle and their immune responses monitored over a 13-week period, using the gamma interferon assay. The immune reactivity was predominantly directed toward avian tuberculin purified protein derivative (PPD) and lasted for approximately eight weeks. During that period three of the four cattle yielded positive test results on one or two occasions. The immune responsiveness was boosted when the inoculations were repeated after 15 weeks, which led to two subsequent positive reactions in the experimental animal that did not react before. Including an additional stimulatory antigen, sensitin prepared from *M. fortuitum* in the gamma interferon assay, showed that it was able to elicit a detectable gamma interferon response in all four experimentally inoculated cattle when applied in parallel with bovine and avian tuberculin PPD for the stimulation of blood samples. The implications of occasional cross-reactive responses in natural cases of infection with environmental mycobacteria in the diagnosis of bovine tuberculosis in African buffalo and cattle in South Africa are discussed.

Key words: Bovine tuberculosis; buffalo; cattle; gamma interferon assay; *Mycobacterium fortuitum*.

Mycobacterium fortuitum is an environmental, nontuberculous mycobacterium, which has been repeatedly isolated from cattle. Previously, infections with other atypical mycobacteria were shown to result in positive skin tests in cattle [1,2,5,13]. Since it must be assumed that buffalo are abundantly confronted with environmental mycobacteria, such exposure may bias immunoreactivity towards infection with *M. bovis*. It may also influence diagnostic assays for *M. bovis*, including both the skin test and the gamma interferon (IFNg) assay. The associated risk of encountering false-positive diagnoses in buffalo is unacceptably high when measured against the economical and ethical consequences. It is therefore important to identify a test strategy able to distinguish between specific, truly infected, and nonspecific reactors. The commercial Bovigam kit is an in vitro IFNg assay for the diagnosis of *Mycobacterium bovis* infection in bovines that has proven extremely useful as an ancillary assay to the skin test in different countries.[4, 10,11] Its use in free-ranging buffalo populations is preferred

over the intradermal tuberculin test because it requires only a single manipulation of the animals.[9] In infected buffalo herds in the KNP the IFNg assay proved suitable to correctly identify infected individuals based on their immune response towards bovine PPD[7].

The aim of the present study was to describe various cases of natural *M. fortuitum* infection in buffalo and to perform a pilot experimental infection with *M. bovis* diagnostic follow up.

Five buffalo from a group of 16 animals captured in the far northern tuberculosis-free region of the Kruger National Park (South Africa) reacted strongly to bovine tuberculin purified protein derivative (PPD)a, in the gamma interferon assay. The bovine absorbance values were more than 0.1 greater than the absorbance values of avian PPD. The remaining eleven buffalo tested negative. Results for the positive and negative control samples included in the kitb as well as the nil controls of the test samples were within acceptable ranges and according to the manufacturer's recommendations for interpretation of test results, these animals were classified as test positive. The buffaloes were euthanized, and pooled lymph node samples from the head, thorax, and mesenterium were cultured according to standard procedures.3 No visible lesions were observed at necropsy. Bacterial culture yielded fast growing Mycobacterium spp. from four of the five reactor buffalo and a presumptive diagnosis of M. fortuitum complex was made for two isolates based on biochemical characteristics (growth at 25°C, production of arylsulphatase and nitrate reductase). The diagnosis was confirmed in a line probe assay8 performed at the Mycobacteriology Unit of the Prince Leopold Institute of Tropical Medicine (Antwerp, Belgium). The two unidentified *Mycobacterium* isolates comprised unclassified soil mycobacteria. No isolation of *M. bovis* was made from any samples collected from the test positive buffalo.

The potential role of environmental mycobacteria, including *M. fortuitum* as the cause of the false-positive reactions in buffalo, was investigated by way of experimental infection. Four IFNg- and skin test–negative cattle (10-month-old oxen of mixed breed) were inoculated intravenously with 2 ml of phosphate buffered saline (PBS) containing a mixture of the *Mycobacterium* isolates grown from the lymph nodes of the test positive buffalo at a final concentration of 10^7 bacteria/ml. Two skin test– and IFNg-negative control animals were inoculated with PBS and kept on the farm, separate from the experimental group. Blood in heparin was collected in intervals of 1–2 weeks for a period of 13 weeks. Inoculation was repeated for the experimental group after 15 weeks, while monitoring of the control animals was stopped for operational reasons. Blood

---

Sources and manufacturers

a. Institute for Animal Science and Health, Lelystad, The Netherlands,

b. Commonwealth Serum Laboratories, Victoria, Australia.

samples were processed and tested according to the standard IFNg protocol provided by the manufacturer[b]. Starting in week 5 *post inoculation*, an additional aliquot of 1.5 ml whole blood was stimulated with 50 µg Fortuitum[b], a sensitin derived from *M. fortuitum*.

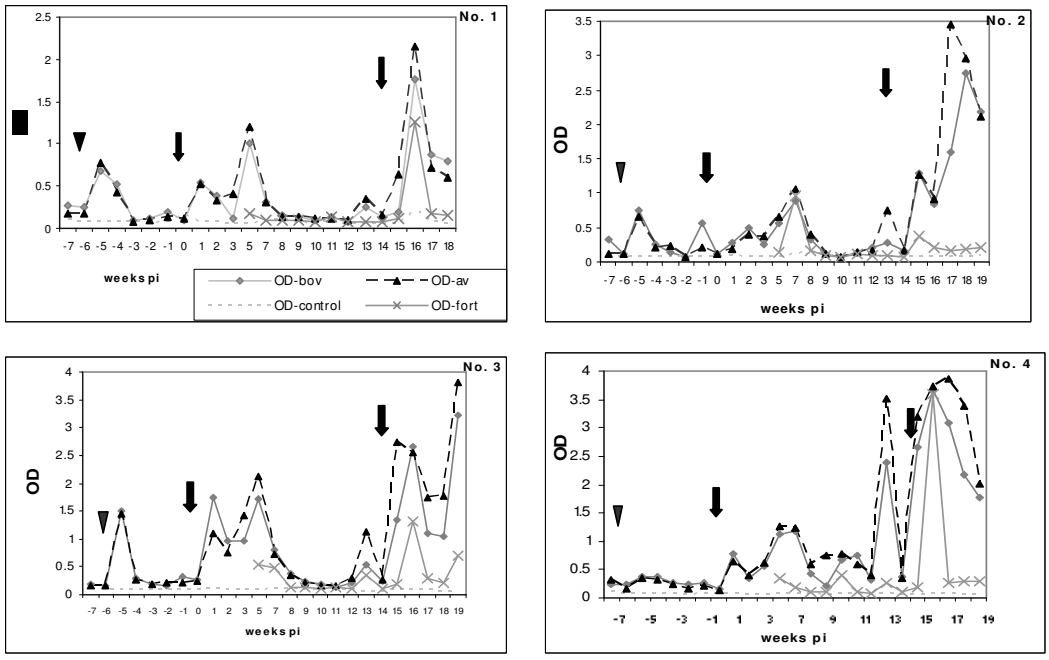
The kinetics of the IFNg responses of the experimental cattle are illustrated in Figure 1 (charts 1–4) and show that inoculation with environmental mycobacteria resulted in an at least 2-fold increase of the bovine and avian absorbance values within 1 week in animals 1, 3, and 4, and within 2 weeks in animal 2. For most of the experiment the IFNg response was primarily directed against avian tuberculin. However, all experimental animals showed episodes when bovine and avian absorbance values were equally strong and 1–2 test occasions when the IFNg response to bovine tuberculin exceeded that of avian tuberculin by at least 0.1, resulting in a positive test outcome (Fig. 1). Animal no. 1 tested positive on the last two test occasions in weeks 17 and 18, while animals no. 2 and 3 did so in week 1 and week 2. Animal no. 4 showed one peak of bovine IFNg reactivity in week 1 and a second peak in week 11. Eight weeks after inoculation the IFNg responses of the experimental animals had normalized to preinoculation levels in 3 of the 4 experimental animals. Following the second inoculation with the *Mycobacterium* cocktail the IFNg reactivity increased markedly (3- to 10-fold) in all experimental oxen and remained high for the remaining period of the experiment. In the IFNg assay the control cattle kept separately did not show any significant immune response to stimulation with any of the antigens, except for two occasions when one of the animals mounted a sporadic, short-lived response to avian tuberculin PPD, not exceeding an optical density (OD) value of 0.53 (data not shown).

In summary, following natural or experimental exposure to environmental mycobacteria including at least *M. fortuitum* buffalo and cattle may become sensitized to stimulation with bovine and avian tuberculin PPD when tested in the IFNg assay. The resulting cross-reactive immune responses can lead to a reduction in IFNg test specificity and occasional misclassification of animals as test positive. Since both infected and uninfected buffalo populations are exposed to the same environmental mycobacteria, it is not surprising that similar patterns of IFNg reactivity have also been observed occasionally in a small number of buffalo in naturally infected populations.[7] No indication for the induction of IFNg reactivity to Fortuitum by *M. bovis* has, however, been found in subsequent studies in infected buffaloes (Michel, unpublished data).

The findings of the current study have shown that stimulation of whole blood with Fortuitum has led to the production of detectable amounts of IFNg in sensitised cattle, indicating that Fortuitum may be a suitable antigen for the detection of exposure to related environmental mycobacteria when using this

diagnostic tool. A previous study on sensitisation of cattle by nontuberculous mycobacteria in South Africa found that skin test reactivity to Fortuitum was the third most frequently encountered sensitisation in "problem herds" (history of nonspecific reactors) after that of *M. avium* and *M. kansasii*.[12] While the most probable source of natural infection of buffalo and cattle with *M. fortuitum* would be soil and water, it has actually been shown that oral administration of *M. fortuitum* in cattle led to skin reactivity to mammalian and avian tuberculin as well as Fortuitum.[6] Worthington (1967) experimentally sensitized cattle with five different non-tuberculous mycobacteria and measured their skin reaction to intradermal injection of sensitins prepared from the same strains. He found that the homologous sensitin caused distinctly larger reactions than heterologous sensitins[12]. It is therefore relevant in this context to investigate the diagnostic value of including Fortuitum as an additional stimulatory antigen in the IFNg assay protocol in order to improve the specificity of the IFNg assay in buffalo and cattle under South African conditions.

Figure 1. Kinetics of the early gamma interferon responses of four cattle inoculated with a mixture of environmental mycobacteria.



Panels 1–4 illustrate the gamma interferon responses of 4 experimental cattle to inoculation with environmental mycobacteria. OD: optical density; p.i.: post infection; arrowhead: skin test; arrow: inoculation with environmental mycobacteria.

Acknowledgements
The authors want to thank Prof. F. Portaels for kindly identifying the *Mycobacterium* strains isolated from the buffalo, as well as Central Commonwealth Serum Laboratories, Australia, for providing the Fortuitum. The study would not have been possible without the excellent technical support from the staff at the Onderstepoort Veterinary Institute Tuberculosis Laboratory and the Animal Provision Unit led by Mr. C. van Vuuren.

References

1. Alhaji I: 1976, Bovine tuberculosis: a general review with special reference to Nigeria. Vet Bull 46:829–841.
2. Amadori M, Tagliabue S, Lauzi S, et al.: 2002, Diagnosis of *Mycobacterium bovis* infection in calves sensitized by mycobacteria of the avium/intracellulare group. J Vet Med B Infect Dis Vet Public Health 49:89–96.
3. Bengis RG, Kriek NPJ, Keet DF, et al.: 1996, An outbreak of bovine tuberculosis in a free-living buffalo population in the Kruger National Park. Onderstepoort J Vet Res 63:15–18.
4. Cagiola M, Feliziani F, Severi G, et al.: 2000, Analysis of possible factors affecting the specificity of the gamma interferon test in tuberculosis-free cattle herds. Clin Vaccine Immunol 11:952–956.
5. Cooney R, Kazda J, Quinn J, et al.: 1997, Environmental mycobacteria in Ireland as a source of non-specific sensitization to tuberculins. Irish Vet J 50:370–373.
6. Freerksen E, Lauterback D, Rosenfeld M, Wolter H: 1961, Testung mit homologen und heterologen Tuberkulinen nach Sensibilisierung mit "atypischen" Mykobakterienstämmen (Versuche am Meerschweinchen und am Rind) [Testing of guinea pigs and cattle with homologues and heterologous tuberculins following sensitization with atypical Mycobacterium strains]. Article in German. Jahresberichte Borstel 5:185–208.
7. Grobler DG, Michel AL, de Klerk LM, Bengis RG, 2002. The gamma interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J. Vet. Res. 69:221-227.
8. Portaels F, de Rijk P, Jannes G, Lemans R, Mijs W, Rigouts L, Rossau R. The 16S-23S rRNA spacer, a useful tool for taxonomical and epidemiological studies of the M. chelonae complex. Proceedings of the Conference on Global Lung Health and the 1996 Annual Meeting of the International Union against Tuberculosis and Lung Disease. Tubercle and Lung Dis 1996. 77: 17-18.
9. Raath JP, Bengis RG, Bush M, et al.: 1995, Diagnosis of tuberculosis due to *Mycobacterium bovis* in the African Buffalo (*Syncerus caffer*) in the Kruger National Park. In: Tuberculosis in wildlife and domestic animals, ed. Griffin

F, de Lisle G, pp. 313–315. University of Otago Press, Dunedin, New Zealand.
10. Whipple DL, Palmer MV, Slaughter RE, Jones SL: 2001, Comparison of purified protein derivates and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. J Vet Diagn Invest 13:117–122.
11. Wood PR, Corner LA, Plackett P: 1990, Development of a simple, rapid in vitro cellular assay for bovine tuberculosis based on the production of gamma interferon. Res Vet Sci 49:46–49.
12. Worthington RW: 1967, Mycobacterial PPD sensitins and the non-specific reactor problem. Onderstepoort J Vet Res 34:345–437.
13. Worthington RW, Kleeberg HH: 1965, Practical problems in tuberculin testing in cattle. J S Afr Vet Med Assoc 36:191–196.

Chapter 4.3

The gamma interferon assay: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park

D.G. Grobler[1], Anita L. Michel[2], Lin-Mari De Klerk[3] And R.G. Bengis[4]

[1] *P.O. Box 1148, Highlands North, 2037 South Africa*

[2] *Tuberculosis Laboratory, ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110 South Africa*

[3] *Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa*

[4] *National Directorate of Veterinary Services, P.O. Box 12, Skukuza, 1350 South Africa*

Onderstepoort Journal of Veterinary Research, 69:221–227

ABSTRACT

A survey to determine the bovine tuberculosis status of buffalo herds north of the Olifants River in the Kruger National Park was conducted, using a new diagnostic approach. Diagnosis of Mycobacterium bovis infection was accomplished using the gamma-interferon assay technique in 608 adult buffaloes out of a total of 29 discreet herds. The animals were immobilized in groups of 10–15, bled, individually marked and then revived and released on site. As soon as test results were available (after 26–36 h), the same buffalo herd was relocated by tracking the frequency of a radio-collar previously fitted to one adult cow per group during the initial operation. Bovine reactors were identified, darted and euthanased from the helicopter. Necropsy and culture findings of all culled buffaloes showed excellent correlation with the results of the ante-mortem gamma-interferon test. The survey revealed that over and above the two positive herds that had been identified during a previous survey carried out in 1996, there were three additional, but previously unidentified, infected herds in the region north of the Olifants River.

Keywords: African buffalo, bovine tuberculosis, gamma-interferon test, Kruger National Park, *Mycobacterium bovis*

INTRODUCTION

In the absence of suitable control measures, bovine tuberculosis (BTB) can progressively infect increasing numbers of cattle in a given population, resulting in significant economic losses as well as a zoonotic risk. Infection by the causative agent, Mycobacterium bovis, is by no means restricted to cattle, and spillover into a wide range of domestic and wild species, as well as humans, has been reported (Collins 1995; O'Reilly & Daborn 1995). There is strong circumstantial evidence, confirmed by molecular typing of Kruger National Park (KNP) *M. bovis* isolates, that BTB was introduced into the Park by cattle-to-buffalo (*Syncerus caffer*) transmission across the southern Crocodile River boundary near Hectorspruit during the late 1950s (Kloeck 1998; De Vos, Bengis, Kriek, Michel, Keet, Raath & Huchzermeyer 2001). Once BTB had established itself in the buffaloes, spatio-temporal spread occurred within and between buffalo herds resulting in a gradient of infection, with prevalence rates ranging from 1.5% (northern herds) to 55% (southern herds).

"Spillover" of infection by direct and indirect transmission occurred in a number of other wildlife species (Keet, Kriek, Penrith, Michel & Huchzermeyer 1996; Keet, Kriek, Penrith & Michel 1998; Bengis, Keet, Michel & Kriek 2001) in this national park. Buffaloes have proved to be true maintenance hosts of the disease and today, more than half of the buffalo herds in the KNP are infected.

In order to contain BTB in the KNP and reduce further spatial spread, it is of crucial importance to possess a sensitive, specific and practical ante mortem test to diagnose *M. bovis* infection under field conditions and with minimal

manipulation of the buffalo herds.

During the 20th century many countries worldwide successfully eradicated BTB from their cattle populations using control (test-and-slaughter) measures based mainly on the intradermal tuberculin (IDT) skin test (Collins 1995). Although the IDT has also proved both sensitive and specific in free-ranging buffaloes (J.P. Raath, unpublished data 1996), this technique is costly and impractical, and has inherently more risk owing to the necessity of repeated chemical immobilization and animal holding facilities required for it. During a comparative field evaluation of the commercial gamma-interferon test (Bovigam™, CSL, Australia) and the skin test in cattle and buffaloes in South Africa, a non-specific reactor problem caused by cross-reactions with environmental mycobacteria was identified. It was found that the specificity of the test could be increased considerably when the test was modified in such a way that animals whose immune response was stimulated by environmental mycobacteria could be differentiated from true bovine reactors (A.L. Michel, unpublished data 2000). In the same evaluation the sensitivity of the IFNg assay in buffaloes was found to be 84.6 %. Based on this study it was decided to use this technique to determine the BTB status of all buffalo herds in the northern part of the KNP. Pending the outcome of this project the test could form an integral part of the future BTB management strategy in the KNP.

MATERIALS AND METHODS

Identifying buffalo herds

The 1999 aerial census results (Whyte 1999) were used as a basis to identify all the buffalo herds in the area north of the Olifants River. A Eurocopter Colibri EC120 was used for all the aeronautical requirements, including aerial darting. Some of the herds in the far north had been fitted with radio-collars in 1999 before the present study commenced and the remaining herds were marked with radio-collars (MOD-600 transmitter, Telonics, 932 E. Impala Av., Mesa, Arizona, 85204-6699, USA) transmitting a specific unique frequency, during the study.

Capture

Once a buffalo herd was located, a group of about 25–40 animals was selected and cut out of the herd. Target animals in the group to be sampled were then darted. Only adult animals were selected for the study and, depending on various factors, such as the terrain and the workability of the group, 10–15 animals were immobilized together. The KNP-developed aluminium dart system (4 mldarts each fitted with a 45 mm collared needle), fired from a modified 20 gauge shotgun was used to deliver the anaesthetic "cocktails" at the following dosage rates and composition:

Adult bull: 8 mg etorphine hydrochloride (M99; Logos Agvet) + 100 mg azaperone (Stresnil; Janssen Pharmaceutica)

Adult cow: 7 mg etorphine hydrochloride + 80 mg azaperone

Down times (the time for the animal to become immobilized after being darted) ranged from 5–8 min. After the samples had been collected and the animals suitably marked for future identification (see below), anaesthesia was reversed by administrating 20 mg (bulls) or 18 mg (cows) diprenorphine hydrochloride (M5050; Logos Agvet) intravenously into an ear vein. All the animals were observed until they were mobile, a process that generally took about 2–5 min.

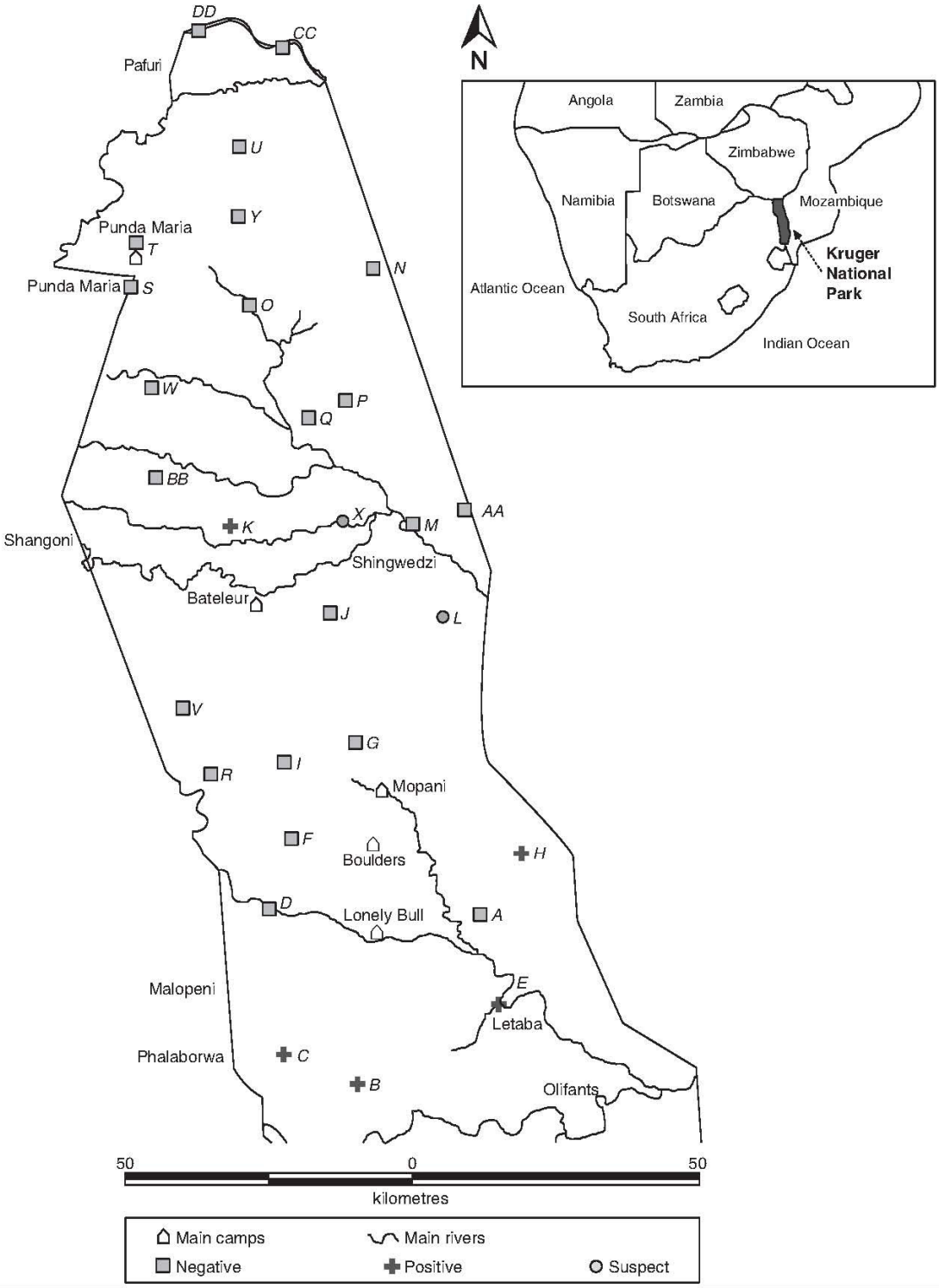
Collection of samples

Blood samples were collected by venopuncture of the jugular vein using a 38 mm 18 G Vacutainer needle. Ten millilitres of blood was collected from each animal into clearly marked tubes containing heparin for the purposes of the gamma-interferon test.

Marking of individual buffaloes

The allocated identification number of each immobilized buffalo was painted on its back using aluminium paint. These numbers were large enough (25 cm) to be visible from the air, making it possible to identify positive animals after the test results had become available. In some cases the numbers were still legible after 1 week. Each herd was allocated an alphabetical letter that was boldly painted on the right shoulder of each buffalo sampled from that herd. In addition, a hot "Z" brand was used to brand all buffaloes permanently on the right rump as a retrospective means of linking to the BTB survey.

FIGURE. 1 Locations and BTB results of buffalo herds in the northern Kruger National Park



Gamma-interferon test

The commercial Bovigam kit was used in a modified form (Michel, Nel, Cooper & Morobane 2000) to detect buffaloes infected with *M. bovis*. Briefly, between 1 h and 4 h after collection the heparinized blood samples from buffaloes were stimulated with bovine and avian tuberculin PPD (ID-Lelystad, The Netherlands) as well as a crude protein extract of M. fortuitum to assist with the differentiation of specific and non-specific reactions. Incubation and detection of the gamma-interferon assay were carried out according to the manufacturer's instructions. All handling and testing of blood samples were performed in a mobile laboratory near the capture sites.

Necropsies and culture

Buffaloes that tested positive on gamma-interferon assay were then traced by helicopter using the herd radio-collar frequency and identified from the air by their herd designation and specific individual numbers. These positive reactors were then removed from the herds by darting with an overdose of succinyl dicholine. Necropsies were performed on these animals in the field and samples from all the lymph nodes of the head and thorax, as well as from suspect lesions were taken for histopathology and bacteriological culture. The samples for culture were stored at –20 °C until transferred and processed at the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute according to standard procedures (Bengis, Kriek, Keet, Raath, De Vos & Huchzermeyer 1996).

RESULTS

All results are summarized in Table 1 and the locations of herds in Fig. 1.

Interpretation of the gamma-interferon assay

During extensive studies in buffaloes it was previously shown that cross-reactivity with environmental mycobacteria could be detected by the additional stimulation of blood cultures with a crude protein extract from M. fortuitum which modified the commercial Bovigam into a triple comparative gamma-interferon assay (Michel, Nel, Cooper & Morobane 2000)

In brief, a test result was considered positive for infection with *M. bovis* infection if the following criteria were met:

$OD_{bovine} - OD_{avian} > 0.20$ and if $OD_{fortuitum} - OD_{nil} < 0.15$, provided that $OD_{nil} < 0.25$.

In cases where $OD_{fortuitum} - OD_{nil} > 0.15$ the buffalo was classified as multiple reactor (MR). In our previous studies we found this pattern of multiple reactions in infected as well as in uninfected buffaloes. For the purpose of this survey it was decided to exclude this group of reactors from culling.

Gamma-interferon tests (IFNg)

Out of a total of 29 buffalo herds comprising approximately 8 390 animals, 608 were tested using the IFNg assay. A total of nine test-positive buffaloes were identified (Tables 1 and 2). Seven out of eight bovine reactors were shown to have macroscopic lesions of tuberculosis in the lymph nodes associated with the head and the respiratory tract or the lungs and *M. bovis* was isolated on culture. One test-positive animal could not be retrieved for culling, as it had not joined up with its parent herd. Necropsy of another test positive buffalo failed to reveal macroscopic lesions of tuberculosis and culture of the lymph nodes collected was negative. A total of three multiple reactors and 26 avian reactors were detected but not identified for culling.

DISCUSSION

In previous BTB surveys in the KNP, the TB status of selected buffaloes was determined either by necropsy or by the intradermal tuberculin test that required the test animals to be contained in a holding facility (boma) for 72 h. BTB-monitoring practices based on culling might be acceptable in high prevalence herds such as in the southern part of the KNP. It has, however, met with growing opposition in low prevalence herds because of the large sample sizes needed to detect infection, the possible adverse effects on the genetic diversity of the herds, and other ecological and ethical considerations.

The intradermal tuberculin test is associated with high costs and a high level of handling stress to animals due to the double immobilization and containment in the boma.Animals often refuse to drink after capture leading to dehydration and occasionally death. The interpretation of the skin test is inevitably compromised in dehydrated animals (Raath, Bengis, Bush, Huchzermeyer, Keet, Kernes, Kriek & Michel 1993).

TABLE 1. IFNg test results obtained in the survey on bovine tuberculosis in buffaloes in KNP

Herd name	Herd symbol	Herd size n	No. of buffaloes tested	Herd % tested	No. of test positives	No. of culture-positive animals
Letaba	A	145	14	9.65	3	2
Macetse	B	320	23	7.18	3	2*
Masorini	C	550	27	4.90	1	1
Blach Heron dam	D	180	23	12.77	0	N/A
Maloponyane	E	290	26	8.96	0	N/A
Stapelkop dam	F	310	21	6.77	0	N/A
Grysbok	G	650	20	3.07	0	N/A
Shawu	H	430	22	5.11	1	1
Stamp en stoot	I	240	20	8.33	0	N/A
Nkokodzi	J	250	19	8.63	0	N/A
Tussen-in	K	230	20	8.69	1	1
Mahlati	L	800	28	3.50	0	N/A
Shingwedzi	M	240	21	8.75	0	N/A
Shirombi pan	N	300	20	6.66	0	N/A
Magamba	O	190	21	11.05	0	N/A
Nkulumbeni	P	400	18	4.50	0	N/A
Boyela	Q	200	17	8.50	0	N/A
Klein Letaba	R	190	19	10.00	0	N/A
Punda Maria	S	210	19	9.04	0	N/A
Mahonie loop	T	260	18	6.92	0	N/A
Nkovakulu	U	120	21	17.50	0	N/A
Shangoni koppies	V	140	19	13.57	0	N/A
Malahlapanga	W	345	32	9.27	0	N/A
Shipande	X	280	25	8.92	0	N/A
Klopperfontein	Y	210	24	11.42	0	N/A
Gadzingwe	AA	280	26	9.28	0	N/A
Mooigesig dam	BB	400	19	4.75	0	N/A
Gwalali	CC	110	13	11.81	0	N/A
Makwadzi	DD	120	13	10.83	0	N/A
Total			608		9	

N/A Not applicable

* One test-positive buffalo could not be retrieved for necropsy

TABLE 2. OD values detected in the IFNg assay in 15 buffaloes with various diagnostic results

Animal no.	ODbov	ODav	ODfort	ODnil	Result
A5	0.54	0.18	0.23	0.12	Positive
A7	1.43	0.33	0.13	0.09	Positive
A8	0.83	0.23	0.11	0.08	Positive
B3	0.46	0.22	0.13	0.08	Positive
B6	2.86	0.38	0.22	0.09	Positive
B22	0.43	0.14	0.07	0.07	Positive
C22	0.93	0.20	0.06	0.05	Positive
H12	0.79	0.11	0.10	0.09	Positive
K6	0.89	0.13	0.09	0.09	Positive
B16	0.19	0.16	0.07	0.05	Negative
D15	0.13	0.18	0.10	0.09	Negative
B7	1.36	0.37	0.55	0.20	MR
Q1	0.80	0.39	0.31	0.09	MR
H14	0.88	1.40	0.51	0.08	AV

The gamma-interferon technique has been used and evaluated in buffaloes in South Africa mostly to overcome the problems associated with the skin test and to avoid unnecessary culling of healthy buffaloes. It was previously found that in buffaloes, the IFNg assay had similar sensitivity and specificity ranges to the comparative intradermal skin test (Raath et al. 1993; Michel, personal communication 2000). In the present survey the diagnosis of bovine tuberculosis in buffalo herds was, for the first time, based exclusively on the IFNg assay. The strong correlation between test-positive and culture-positive buffaloes confirms the high specificity of the IFNg test (99.3 %) found in the comparative field evaluation (Michel, Nel, Cooper & Morobane 2000), although cross-reactivity with environmental mycobacteria did not seem to be a major complicating factor in this study, since only three buffaloes (0.5 %) showed a "multiple reaction". In comparison, the previous field evaluation revealed a multiple reactor rate of 4 %, meaning that under standard test conditions, which lack the use of fortuitum protein, those buffaloes would have been falsely classified as bovine reactors (Table 2). These buffaloes' blood samples were collected in different geographical and climatic areas in South Africa throughout the year. The animals either roamed free or semi-free or were kept in a boma for varying periods of time. It is possible that any of these factors may have influenced the non-specific reactor rate in the present survey which was carried out in KNP during the dry winter season. In conclusion, the sensitivity could not be determined for this study as no gold standard method was included in the study design. However, *M. bovis* infection was confirmed in the Macetse and Letaba herds, previously known to be

affected. In addition, three new infected herds, namely Masorini (Phalaborwa), Shawu (Mopanie) and Tussen-in (Woodlands) were identified. This indicates an increase in the overall BTB prevalence in the northern region of the KNP compared to the findings of a survey conducted in 1998 in which 1.45% of buffaloes were found to be infected (Rodwell 2000). In retrospect, in January 1999 during routine buffalo capture operations, a young cow from the Nkokodzi herd was found to be positive on both the gamma-interferon and the skin tests. On necropsy a tubercular lesion (20 mm) was found in the lung and *M. bovis* isolated from it. This was the first recorded and confirmed case north of the Letaba River. However, during the 2000 survey, no positive reactors were found in the sample from this herd.
Based on these observations as well as on the data provided by the previous validation (Michel et al. 2000; A.L Michel, unpublished data 2000) the results of this study are believed to demonstrate a satisfactory sensitivity of the IFNg test under field conditions. Our data further show that the use of fortuitum protein in a triple comparative IFNg test is of distinct advantage in our situation where pressing ethical and economic considerations do not allow an "overkill" of buffaloes due to reduced specificity of the bovine tuberculosis control measure.

In the KNP, the average buffalo herd comprises 200–300 individuals (Whyte 1999). The sampling technique employed in the survey described here allowed for the capture and testing of 5–10 % of each herd in the study area. This correlates with the required sample size for detecting infection in herds with an infection prevalence of between 10% and 15 %, at a confidence level of 95 % and using total random sampling (Thrusfield 1995). Although the expected prevalence of BTB in the northern part of KNP is below 5% the selection of adult buffaloes could help to increase the probability of disease detection at this sample size as previous investigations have revealed a positive correlation between age and likelihood of infection (De Vos et al. 2001).

The BTB lesions that were found were suggestive of "early" infections as the lesions were small and found mainly in the lymph nodes of the head and lungs. Discrete focal tubercular lesions were also found in the lungs of four animals. These results indicate active infection, probably with temporospatial spreading of the disease. In addition, a single cow in both the Shipande and Mahlati herds tested positive on gamma-interferon, but could not be recovered for necropsy purposes.

CONCLUSION

In conclusion, we believe that the results of this survey are encouraging for detecting BTB-infected herds in a geographical area, bearing in mind the limitations of sample size. The use of the gamma-interferon test as described in this paper may be an important tool for future "test and remove" actions to control bovine tuberculosis in free-ranging buffalo populations. The modified gamma-interferon test has significant advantages over the skin test and culling methods with regard to financial, conservation and ethical considerations.

ACKNOWLEDGEMENTS

This survey would not have been possible without the dedicated efforts of a large group of key individuals: Dr Markus Hofmeyr (veterinarian and radio-collars); Li-Ann Small (technician); Fanie Jordaan and Piet Otto (pilots); Schalk van Dyk (blood samples and overall help); Johan Malan and Marius Kruger (ground team) and Johan Oosthuizen, At Dekker and Kenneth Muchocho (State Veterinary Services). Their collective contribution to ensure the efficient success of this major operation is highly appreciated. Members of the capture team worked long hours under difficult circumstances, as did the drivers Petrus Mashile, Johannes Khoza and Joao Muhlanga. The assistance provided by the Section Rangers (especially Louis Olivier and Arrie Schreiber) and their staff is recognized and appreciated. Sandra McFayden is thanked for her help in creating the KNP map.

REFERENCES

BENGIS, R.G., KRIEK, N.P.J., KEET, D.F., RAATH, J.P., DE VOS, V. & HUCHZEMEYER, H.F.A.K. 1996. An outbreak of bovine tuberculosis in a free-living buffalo population in the Kruger National Park. Onderstepoort Journal of Veterinary Research, 63:15–18.

BENGIS, R.G., KEET, D.F., MICHEL, A.L. & KRIEK, N.P.J. 2001. Tuberculosis, caused by Mycobacterium bovis, in a kudu (Tragelaphus strepsiceros) from a commercial game farm in the Malelane area of the Mpumalanga Province, South Africa. Onderstepoort Journal of Veterinary Research, 68:239–241.

COLLINS, J.D. 1995. Ireland, in *Mycobacterium bovis* infections in animals and humans, edited by C.O. Thoen & J.H. Steele, 1st ed. Ames, Iowa: Iowa State University Press: 224–238.

DE VOS, V., BENGIS, R.G., KRIEK, N.P.J., MICHEL, A., KEET, D.F., RAATH, J.P. & HUCHZERMEYER, H.F.K.A. 2001.
The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. Onderstepoort Journal of Veterinary Research, 68: 119–130.

GRIFFIN, J.F.T., CROSS, J.P., CHINN, D.N., RODGERS, C.R. & BUCHAN, G.S.

1994. Diagnosis of tuberculosis due to *Mycobacterium bovis* in New Zealand red deer (*Cervus elaphus*) using a composite blood test and antibody assays. Offprint from the New Zealand Veterinary Journal, 42:173– 179.

KEET, D.F., KRIEK, N.P.J., PENRITH, M.-L., MICHEL, A. & HUCHZERMEYER, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: spread of the disease to other species. Onderstepoort Journal of Veterinary Research, 63:239–244.

KEET, D.F., KRIEK, N.P.J., PENRITH, M.-L. & MICHEL, A. 1998. Tuberculosis in free-ranging lions in the Kruger National Park. Proceedings of the ARC-Onderstepoort OIE International Congress with WHO-Cosponsorship on Anthrax, Brucellosis, CBPP, Clostridial and Mycobacterial Diseases, 9–15 August 1998, Berg-en-Dal, Kruger National Park: 444– 453.

KLOECK, P.E. 1998. Tuberculosis of domestic animals in areas surrounding the Kruger National Park. Proceedings of a Colloquium on the Challenges of Managing Tuberculosis in Wildlife in Southern Africa, 30–31 July 1998, Mpumalanga Parks Board, Nelspruit, South Africa.

MICHEL, A.L., NEL, M., COOPER, D. & MOROBANE, R.N. 2000. Field evaluation of a modified "gamma interferon" assay in African buffalo (*Syncerus caffer*) and cattle in South Africa. Proceedings of the 3rd International Conference on *Mycobacterium bovis*, 14–16 August 2000, Cambridge, United Kingdom.

O'REILLY, L.M. & DABORN, C.J. 1995. The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. Tubercle Lung Disease (Suppl. 1), 76:1–46.

RAATH, J.P., BENGIS, R.G., BUSH, M., HUCHZERMEYER, H., KEET, D.F., KERNES, D.J., KRIEK, N.P.J. & MICHEL, A. 1993. Diagnosis of tuberculosis due to *Mycobacterium bovis* in the African buffalo (*Syncerus caffer*) in the Kruger National Park, in Tuberculosis in wildlife and animals, edited by F. Griffin & G. de Lisle. Otago Conference Series, University of Otago Press, Dunedin: 313–315.

RODWELL, T.C. 2000. The epidemiology of bovine tuberculosis in African buffalo (*Syncerus caffer*). Ph.D. dissertation, University of California, Davis, USA.

THRUSFIELD, M. 1995. Veterinary epidemiology, 2nd ed. Oxford, Halden, Massachusetts: Blackwell Science.

WHYTE, I.J. 1999. Elephant and buffalo census report for 1999. Scientific Services, Kruger National Park, Skukuza.

Chapter 4.4

Comparative field evaluation of two rapid immunochromatographic tests for the diagnosis of bovine tuberculosis in African buffaloes (*Syncerus caffer*)

A.L. Michel[1] & M. Simões[2]

1……Bacteriology Department, ARC-Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110

2…… UTAD (Universidade de Trás-os-Montes e Alto Douro) & ACD (Associação Ciência para o Desenvolvimento), Portugal

Submitted

Abstract

Panels of sera from African buffalo with confirmed bovine tuberculosis and from known uninfected controls were used to evaluate the performance of two commercial rapid chromatographic immunoassays (A & B) for the detection of antibodies to *Mycobacterium bovis.* The sensitivity was 33% and 23%, respectively, while the specificity was determined at 90% and 94%, respectively. Overall the performance of both diagnostic tests under field conditions was not found sufficiently high to support their use in bovine tuberculosis management and control strategies in South African game reserves.

Keywords: *African buffalo, bovine tuberculosis, rapid test, immunodiagnosis*

Introduction

Wildlife tuberculosis caused by *Mycobacterium bovis* is endemic in some of South Africa's large conservation areas where it is maintained in African buffaloes (*Syncerus caffer*). As a consequence of transmission to a wide range of spillover hosts and an emerging risk of spreading outside these parks, where it may be a potential threat to cattle and human populations, bovine tuberculosis (BTB) is considered an increasing wildlife health and management problem (Michel et al. 2006). Currently movement control and disease monitoring strategies rely on the intradermal tuberculin test and interferon gamma assay, which both have practical limitations (Grobler et al. 2002). Moreover, due to the nature of the immunological responses to infection these test systems detect only the cell mediated immune response developed relatively early after infection and therefore lack the ability to identify animals that no longer show cell mediated immunity but may produce antibodies (Wood et al. 1991).
In the absence of systematic test-and-remove interventions in free-living wildlife to curb the progression of bovine tuberculosis, a certain percentage of infected individuals are likely to develop advanced lesions and become effective shedders of *M. bovis* (Bengis 1999, de Vos et al. 2001). In light of the spectrum of disease stages in bovine tuberculosis (BTB) and their different immunological profiles represented in especially chronically infected populations it is the ultimate goal to develop a comprehensive diagnostic approach, in which cellular as well as humoral immune responses are detected.

The value of antibody detection as a fast, cost-effective and user-friendly alternative to the skin test has been explored in cattle populations in developed countries, but did not find widespread application in the past, mostly due to low sensitivity and/or specificity (Placket et al.1989), potentially as a result of the fact that antibody production occurs relatively late.

The aim of this study is to present the comparative evaluation of two commercially available rapid test systems, based on immunochromatographic detection of antibodies against *M. bovis* for their diagnostic value in African buffaloes.

Materials and Methods

Animals and serum samples

Panels of sera from BTB infected and uninfected buffaloes were selected from a serum collection maintained at –20°C. The positive panel consisted of serum from 100 naturally infected, free-ranging, culled buffaloes, aged between six months and 15 years. The buffaloes were sampled between 1996 and 2005 during BTB management surveys in the southern and central regions of the Kruger National Park, where the BTB prevalence is highest and ranges from 16% to 38% (Rodwell et al. 2001) with individual herds reaching up to 67% (de Vos et al. 2001) Eighty of the culled buffaloes had macroscopic lesions in at least one lymph node. BTB infection had been confirmed in all 100 buffaloes of the positive panel either by culture (65 animals) or gross- and histopathology (35 animals). For 44 of the culture positive buffaloes additional histopathological examination was performed with consistent positive results (Table 1).

The negative panel (N=100) originated from known uninfected buffaloes, 31 of which were culture negative buffaloes which were necropsied during surveys in reserves or regions with no history of BTB and 69 serum samples were collected from live buffaloes from BTB free game farms or reserves (Table 1), 34 of which had at least two rounds of skin tests and four rounds of IFNg assays, all with negative results, 31 buffaloes formed part of a larger herd with repeated negative skin tests and one negative IFNg assay and four IFNg negative animals were selected from a larger group of 65 IFNg negative buffaloes sampled and tested in a BTB free region.

Diagnostic assays including gold standard tests

Bacterial culture and histopathology (in combination with gross pathological examination) served as gold standard tests for confirming *M. bovis* infection in the buffaloes of the positive panel. Both methods have been described previously (Bengis et al. 1996).

The Bovigam™ commercial interferon gamma kit was used with modifications as described by Grobler et al. (2002) and applied on 20 infected and 69 uninfected buffaloes.

Immunochromatographic (Rapid) tests

Two commercial immunochromatographic test systems, based on the capture of antibodies against recombinant *M. bovis* antigens were included in the comparative assessment. In both these tests the antigens are immobilised on the test line of a sample pad and trap serum antibodies migrating through the test device by capillary forces. The tests were performed according to the manufacturer's instructions.

Rapid test A: BovidTB STAT-PAK, Chembio Diagnostic Systems, Inc., (Medford, NY,USA)

Briefly, 30µl serum were spotted in the sample window of the kit, followed by four drops of diluent dispensed from a dropper bottle supplied by the manufacturer.

Rapid test B: Anigen, Animal Genetics, Inc., South Korea

Four drops of serum were added to the sample window of the kit by means of a specimen dropper supplied by the manufacturer. No diluent was applied.

All reactions were recorded after 20 min and scored by comparing colour intensity of the test line with that of the positive control line. If the test line was continuous but of weaker intensity than the control line, it scored 1+, lines of equal intensities were classified 2+ and stronger lines than the control was classified 3+ (Tables 2 and 3). Suspect reactions, which constituted fuzzy or discontinuous lines were designated doubtful reactions and grouped separately.

Table1. Test results used to classify buffalo sera into positive (infected) and negative (uninfected) panels

BTB status	**No. test positive**					**Total**
	Culture (a)	Histopath (b)	(a) + (b)	IFNg	IFNg + IDT	
Infected	65	35	44	17 [20]	NA	100
Uninfected	0 [10]	NA	0 [21]	0 [4]	0 [65]	100

IFNg Interferon gamma assay
IDT Intradermal tuberculin test
Figures indicate number of test positive buffaloes in this category; [ ] = total no. of buffaloes tested in this group

Data analysis

For comparative evaluation of the diagnostic performance of the two rapid tests their sensitivity and specificity values were determined according to Toma et al. (1999).

The positive and negative predictive values for each of the rapid tests and under different prevalence situations were calculated using the following formula (Altman 1994):

$$PPV = \frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$$

$$NPV = \frac{\text{specificity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence}}$$

Cohen's kappa index for agreement between the rapid tests and with the gold standard methods was computed and interpreted according to Landis and Koch (1977).

Results

Rapid tests A and B detected antibodies to *M. bovis* in 33 and 23 of 100 infected and in ten and six of 100 uninfected buffaloes, respectively (Tables 2 and 3). Resulting sensitivity values for Rapid test A and B were 33% and 23%, specificities were 90 and 94 %, respectively. If suspect reactions were taken into consideration, their respective sensitivities could be increased to 38% (Rapid test A) and 32% (Rapid test B). At the same time the specificity for those testsdecreased from 90% and 94% to 77% and 86%, on the same account. When series interpretation (samples positive on both tests) was applied, 17/100 known positive sera and 86/100 known negative sera were correctly classified by both rapid tests. The tests also agreed in results for 2/14 false positive and 61/83 false negative results. Parallel interpretation (either test yielding a positive result) increased the number of test positive sera from 17 to 39 and decreased the number of test negative buffalo from 86 to 74. Inclusion of suspect reactions led to an increase in the number of true positive sera from 33 to 38 for Rapid test A and from 23 to 32 in the case of Rapid test B. However, the detection of 13 and eight suspect reactors in the negative serum panel for rapid tests A and B, respectively increased the false positive reactor rate for those tests (Table 3). The agreement between the two rapid tests was moderate (kappa index = 0.42) and slight to fair between the gold standard and Rapid test A (kappa index = 0.23) and Rapid test B (kappa index = 0.17), respectively.

The intensity of colour reactions was rated between 1+ and 3+. For both tests most true positive reactors yielded a colour intensity of 2+. Although the majority of false positive reactors fell into the category 1+, stronger reactions were observed (Tables 2 and 3).

Analysis of the age distribution of seropositive buffaloes (in either one or both tests) showed that antibody responses were detectable in animals of all age groups with the youngest buffalo tested being six months old. The proportion of seropositive buffaloes was highest in the age group above eight years (Fig. 1).

Table 2. Sensitivity values including rating of reaction intensities of Rapid test A and Rapid test B among panels of sera from infected buffaloes

	TOTAL	SE	FN	TP	Reactor classification			
					S	1+	2+	3+
Rapid test A	100	0.33	67 [62]	33 [38]	5	11	18	4
Rapid test B	100	0.23	77 [68]	23 [32]	9	8	12	3

SE Sensitivity IFN False negative reactors TP True positive reactors S Suspect reactors Figures represent number of reactors for this category. Figures in [] include suspect reactors.

On an individual test basis Rapid test A detected all seropositive buffalo in the categories <3 years and >8 years but failed to identify several of the Rapid Test B positive buffalo between 4 and 8 years (results not shown).

The IFNg assay was in agreement with at least one or both rapid tests in detecting six out of twenty infected buffaloes, while one buffalo from the infected group tested negative in all cellular and antibody tests and two buffaloes were antibody positive but IFNg and skin test negative (results not shown).

Table 3. Specificity values including rating of reaction intensities of Rapid test A and Rapid test B among panels of sera from uninfected buffaloes

	TOTAL	SP	TN	FP	Reactor classification			
					S	1+	2+	3+
Rapid test A	100	0.90	90 [77]	10 [23]	13	7	2	1
Rapid test B	100	0.94	94 [86]	6 [14]	8	4	2	0

SP Specificity TN True negative reactors FP False positive reactors S Suspect reactors
Figures represent number of reactors for this category. Figures in [] include suspect reactors.

Table 4. Serological responses among panels of infected and uninfected buffalo sera

BTB status	A+/B+	A-/B-	A+/B-	A-/B+	Total
Infected	17	61	16	6	100
Uninfected	2	86	8	4	100
Total	19	147	24	10	200

+ test positive; - test negative; A Rapid test A; B Rapid test B

The positive predictive values of both rapid tests were calculated for different prevalence rates of bovine tuberculosis and results are listed in Table 5. For true prevalences assumed at 5%, 20% and 50%, the positive predictive values were increasing from a minimum of 14.8% for Rapid test A to a maximum of 79.3% for Rapid test B. At the same time the negative predictive values were decreasing from 96.3% for Rapid test A to 54.9% for Rapid test B.

Table 5. Positive and negative predictive values for two rapid tests in relationship to prevalence of disease

P	PPV		NPV	
	A	B	A	B
0.05	14.80%	16.80%	96.28%	95.91%
0.20	46.5%	48.9%	84.40%	82.80%
0.50	76.70%	79.30%	57.30%	54.90%

P prevalence of disease PPV positive predictive value NPV negative predictive value
A Rapid test A B Rapid test B

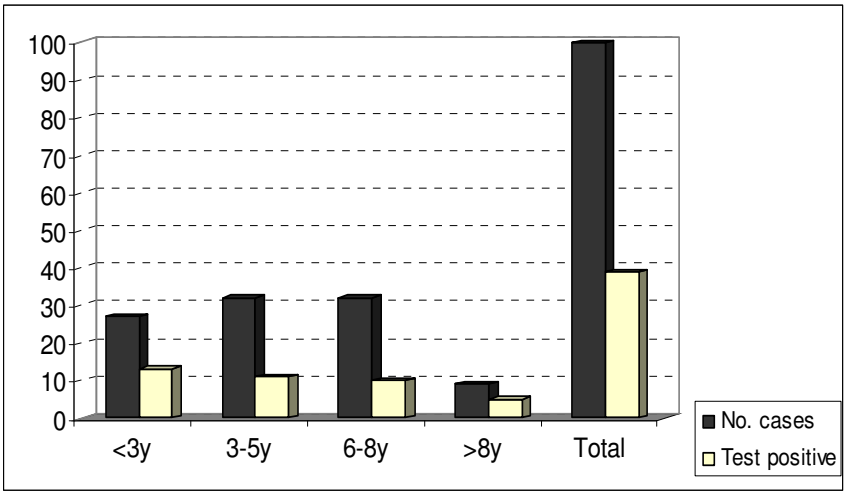
Discussion

The management of bovine tuberculosis in free ranging wildlife populations poses huge challenges in terms of disease management within wildlife conservation objectives on the one hand, and veterinary public health responsibilities on the other hand (Bengis et al. 2002). Against this setting reliable diagnostic tests are of paramount importance to identify infected buffaloes but also other infected species with high sensitivity. At the same time a high specificity is required to minimise unnecessary and unethical culling of valuable genetic resources. Immunochromatographic detection of anti-*M. tuberculosis* complex antibodies in the sera of infected animals provides a technically simple diagnostic approach without the need for species-specific secondary antibodies. Very recently

successful applications have been reported in deer, elephants and camels (Koo et al., 2005, Wernery et al., 2007).

In this study the comparative evaluation of two commercial immuno-chromatographic tests showed a very limited sensitivity of 33% and 23%, respectively, which could only be slightly enhanced by parallel interpretation (either test yielding a positive result) to 38%, since five buffaloes were identified by Rapid test B but not by the more sensitive Rapid test A. This finding may suggest that antibodies directed against MPB70 and various other *M. tuberculosis* complex antigens antibodies develop later in buffaloes in favour of a prolonged persistence of the cell mediated immune response indicated by only six seropositive out of 17 IFNg positive buffaloes (35%), all aged five years or older. Furthermore overall good body condition scores in necropsied, infected buffaloes and the rare observation of clinical signs had previously led to the conclusion that BTB is a slow, progressive disease in the individual buffalo as well as on herd level. This was supported by the fact that advanced lesions or generalised disease were observed primarily in older animals (de Vos et al. 2001). We found the highest prevalence of seropositive animals (55.6%) in the oldest age group (Figure 1), which concurs with the characteristics of a chronic, progressive disease and late onset of antibody production.

Fig. 1. Age distribution of infected and antibody positive buffaloes



y- axis: No. of animals; x-axis: age groups in years

On the other hand, it has been demonstrated in experimentally infected cattle that antibody production may also be a function of the infectious dose (Buddle et al. 1994). It was previously reported that the number of advanced respiratory tract lesions, and hence the likelihood of *M. bovis* shedding, was positively correlated with the BTB prevalence rates (de Vos et al. 2001). Thus, if the same

positive correlation between *M. bovis* dose and humoral immune response applies in buffalo, it may explain the high percentage of seropositive buffaloes (48%) in the age group below 3 years (Fig. 1) captured in the high BTB prevalence zone of KNP.

In conclusion, the findings of this study showed that neither of the two rapid tests possessed a sufficiently high diagnostic accuracy to be of diagnostic value in buffaloes. This is also demonstrated by the low predictive values (PVs) of the rapid tests under the actual prevalence scenarios in the KNP. The probability of BTB in a buffalo given a positive rapid test result is below 0.17 in herds in the northern region of the KNP, where the BTB prevalence is estimated at less than 5%, while the probability that an antibody negative buffalo in the high prevalence southern region is actually not infected could be less than 58%. The rapid tests may, however, have a limited ability to detect anergic buffaloes, as indicated by the detection of antibodies in two out of three IFNg and skin test negative buffaloes which is in agreement with the observations of Harboe et al. (1990) of an inverse relationship between antibody reactivity and cell-mediated immune response when employing MPB70 in the serodiagnosis of *M. bovis* infected cattle. Further investigations of the antibody reactivity particularly of buffaloes lacking cellular immune response but exposed to high levels of BTB would be useful to assess the value of rapid tests as ancillary tests in this wildlife species.

Acknowledgements

The authors want to thank the companies Chembio, USA, and Anigen, South Korea, including its distributor in South Africa, Molecular Diagnostic Services, for making available the rapid tests for this study. The study was partially financially funded by the Association Science for Development, Portugal.

References

Altman, D.G., Bland, J.M., 1994. Diagnostic tests 2: Predictive values. *BMJ* 309, 102

Bengis, R. G., 1999. Tuberculosis in free-ranging mammals. Pages 101-114 *in* M. E. Fowler and R. E. Miller, editors. Zoo and Wild Animal Medicine. W. B. Saunders Company, Philadelphia.

Bengis, R.G., Kock, R.A., Fischer, J., 2002. Infectious animal diseases: the wildlife/livestock interface. Rev. Sci. Tech. Off. Int. Epiz. 21, 53-65.

Buddle, B.M., Aldwell, F.E., Pfeffer, A., de Lisle, G.W., Corner, L.A., 1994. Experimental *Mycobacterium bovis* infection of cattle: effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. NZ Vet. J. 42,167-172.

Caron, A., Cross, P.C., du Toit J.T., 2003. Ecological implicaitons of bovine tuberculosis in African buffalo herds. Ecol. Appl. 13,1338-1345.

De Lisle, G.W., Cox, J.C., Jones, S.L., 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Vet. Microbiol. 31, 71-79.

De Vos, V., Raath, J. P., Bengis, R. G., Kriek, N.J. P., Huchzermeyer, H., Keet, D.F., Michel, A., 2001. The epidemiology of tuberculosis in free ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. Onderstepoort J. Vet. Res. 68, 119-130.

Grobler, D.G., Michel, A.L., de Klerk, L-M., Bengis, R.G., 2002. The gamma interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J. Vet. Res. 69, 221-227.

Harboe, M., Wiker, H.G., Duncan, J.R., Garcia, M.M., Dukes, T.W., Brooks, B.W., Turcotte, C., Nagai, S., 1990. Protein G-based enzyme-linked immuno-sorbent assay for the anti-MP70 antibodies in bovine tuberculosis. J. Clin. Microbiol. 28, 913-921.

Koo, H.C., Park, Y.H., Ahn, J., Waters, W.R., Palmer, M.V., Hamilton, M.J., Barrington, G., Mosaad, A.A., Park, K.T., Jung, W.K., Hwang, I.Y., Cho, S.N., Shin, S.J., Davis, W.C., 2005. Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme linked immunosorbent, immunochromatorgraphic, and latex bead aggluntination assays for serodiagnosis of bovine tuberculosis. J. Clin. Microbiol. 43, 4498-4506.

Landis JR, Koch GG., 1977. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics, 33, 363-374.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Olsen, J.H., Ball, R., Dumonceaux, G., Dunker, F., Buckley, C.,, Richard M., Murray, S., Payeur, J.B., Andersen, P., Pollock, J.M., Mikota, S., Miller, M., Sofranko, D., Waters, W.R., 2006. Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment. Clin. Vaccine Immunol, 13, 722–732.

Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., de Klerk L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P., Godfroid, J., 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. Vet. Microbiol. 112, 91-100.

Plackett, P., Ripper, J.L., Corner, L.A., Small, K., De Witte, K., Melville, L., Hides, S., Wood, P.R., 1989. An ELISA for the detection of anergic tuberculous cattle. Aust. Vet. J. 66, 15-19.

Rodwell,T.C., Kriek, N.P., Bengis, R.G., Whyte, I.J., Viljoen, P.C., de Vos, V., Boyce, W.M., 2001. Prevalence of bovine tuberculosis,in African buffalo at Kruger National Park. J. Wildl. Dis. 37, 258-264.

Toma, B., Dufour, B., Sanaa, M., Benet, J.J., Moutou, F., Louza, A., Ellis, P., 1999, In: Applied Veterinary Epidemiology and the control of disease in populations. AEEMA, Maisons-Alfort.

Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M.,Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M.

P. Andersen, and Lyashchenko, K. P., 2006. Early Antibody Responses to Experimental *Mycobacterium bovis* Infection of Cattle. Clin. Vaccine Immunol. 13, 648-654.

Wernery, U., Kinne, J, Jahans, K.L., Vordermeier, H.M., Esfandiari, J., Greenwald, R., Johnson, B., Ul-Haq, A., Lyashchenko, K.P., 2007. Tuberculosis outbreak in a dromedary racing herd and rapid serological detection of infected camels. Vet. Microbiol. 122, 108-115.

Wood, P.R., Corner, L.A., Rothel, J.S., Baldock, C., Jones, S.L., Cousins, D., McCormick, B.S., Francis, B.R., Creeper ,J., Tweddle, N.E., 1991. Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Aust. Vet. J. 68, 286-290.

Wood, P.R., Corner, L.A., Rothel, J.S., Ripper, J.L., Fifis, T., McGormick, B.S., Francis, B, Melville, L., Small, K., de Witte, K., Tolson, J., Ryan, T.J., de Lisle, G.W., Cox, J.C., Jones, S.L., 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Vet. Microbiol. 31, 71-79.

Chapter 5

Control of bovine tuberculosis in free-ranging buffalo

Chapter 5.1

An experimental intratonsilar infection model for bovine tuberculosis in African buffaloes, *Syncerus caffer*

L. DE KLERK, [1] A.L. MICHEL, [2] D.G. GROBLER,[1] R.G. BENGIS,[3] M. BUSH,[4] N.P.J. KRIEK,[5] M.S. HOFMEYR,[1] J.F.T. GRIFFIN,[6] C.G. MACKINTOSH[7]

[1] South African National Parks, Private Bag X402, Skukuza, 1350 South Africa

[2] ARC-OVI, Private Bag X05, Onderstepoort, 0110 South Africa

[3] Office of the State Veterinarian, Kruger National Park, P.O. Box 12, Skukuza, 1350 South Africa

[4] Chief of Veterinary Services, Conservation and Research Center, Smithsonian Institution, Front Royal, Virginia, USA

[5] Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[6] Disease Research Laboratory, Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand

[7] Deer Research Unit, AgResearch, Invermay Agriculture Research Centre, Mosgiel, New Zealand

Onderstepoort Journal of Veterinary Research, 2006, 73:293–303

ABSTRACT

An infection model for *Mycobacterium bovis* in African buffalo, *Syncerus caffer*, was developed, using the intratonsilar route of inoculation. Two groups of 11 buffaloes each, aged approximately 18 months, were infected with either 3.2 x 10^2 cfu (low dose) or 3 x 10^4 cfu (high dose) of a *M. bovis* strain isolated from a buffalo. A control group of six buffaloes received saline via the same route. The infection status was monitored *in vivo* using the comparative intradermal tuberculin test, and *in vitro* by the modified interferon-gamma assay. All buffaloes were euthanazed 22 weeks post infection and lesion development was assessed by macroscopic examination, culture and histopathology. It was found that the high dose caused macroscopic lesions in 9 out of 11 buffaloes. *Mycobacterium bovis* was isolated from all buffaloes in the high dose group and from 6 out of 11 in the low dose group.

Key words: African buffalo, Bacille Calmette-Guérin, bovine tuberculosis, intratonsilar infection model, Kruger National Park, *Mycobacterium bovis*, *Syncerus caffer*

INTRODUCTION

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, was first diagnosed in free-ranging buffalo, *Syncerus caffer*, in the Kruger National Park in 1991 (KNP) (Bengis, Kriek, Keet, Raath, De Vos & Huchzermeyer 1996). In this ecosystem, buffaloes are now considered to be the main reservoir and maintenance host (De Vos, Bengis, Kriek, Michel, Keet, Raath & Huchzermeyer 2001). Since the initial diagnosis, BTB infection has spilled over into a variety of other species (Keet, Kriek, Penrith, Michel & Huchzermeyer 1996) and has been confirmed in kudus, *Tragelaphus strepsiceros*, baboons, *Papio ursinus*, lions *Panthera leo*, leopards, *Panthera pardus*, hyenas, *Crocuta crocuta*, cheetahs, *Acinonyx jubatus*, warthogs, *Phacochoerus ethiopicus*, genets, *Genetta genetta* and honey badgers, *Mellivora capensis* (Keet 1996; Keet, Kriek, Bengis, Grobler & Michel 2000; Keet, Kriek, Bengis, & Michel 2001; Michel 2002).

Because of the number of species infected with BTB, the control and eventual eradication of BTB from the KNP will become increasingly difficult. Complicating factors include the widespread geographical ranges occupied by infected buffalo herds and the potential of other species to become maintenance hosts. The spread of infection both within and between herds is thought to be further enhanced by the gregarious lifestyle of buffaloes, as well as the dynamic fragmentation and coalescing of buffalo herds (De Vos *et al.* 2001). Various strategies to control and eventually eradicate the disease from the KNP have been considered. Of the strategies that may be effective, vaccination could be the

most feasible option provided a vaccine candidate can be validated in an experimental buffalo model.

The Bacille Calmette-Guérin (BCG) vaccine is the only vaccine currently available with proven safety and efficacy for the control of the infection in humans. The BCG vaccine has also been tested in a number of domesticated and wild species (Buddle, Keen, Thomson, Jowett, McCarthy, Heslop & De Lisle 1995; Buddle, Skinner, & Chambers 2000; Corner, Buddle, Pfeiffer & Morris 2001, 2002). Variability in the efficacy seen in these experimental studies has precluded the use of the BCG as a vaccine for domestic livestock or wildlife. Factors that affected the efficacy of BCG vaccine in experimental protocols included the age of the animals that were vaccinated, prior sensitization to environmental mycobacteria (Buddle, Wards, Aldwell, Collins & De Lisle 2002), delivery and dosage, and whether single or multiple booster doses were used (Griffin, Mackintosh, Slobbe, Thomson & Buchan 1999; Cross, Labes, Griffin & Mackintosh 2000). When a pathology scoring system was recently applied to cattle which had been experimentally challenged with *M. bovis*, BCG vaccination reduced disease severity by 75 % (Vordermeier, Chambers, Cockle, Whelan, Simmons & Hewinson 2002). The vaccine also gave excellent protection to red deer, *Cervus elaphus*, at the Disease Research Laboratory (DRL) in Dunedin, New Zealand, if a booster was administered eight weeks after the initial BCG vaccination (Griffin, Chinn, Rodgers & Mackintosh 2001). An added advantage of vaccination, even if the BCG vaccine does not provide full protection against the disease, is that it appears to reduce the severity of the disease and subsequent mycobacterial excretion. Reduced contamination of the environment could also limit the subsequent spread of infection (Cross *et al.* 2000). Indeed, under certain environmental conditions, *M. bovis* can survive outside its host for long enough to significantly increase the likelihood that other animals may become infected and develop disease (Tanner & Michel 1999).

To evaluate the efficacy of such a vaccine in buffaloes, a reliable infection model had to be developed which mimics natural infection in this species. The model should reproduce the typical range of lesions, progression of the disease and the immunological response seen in naturally infected animals (Mackintosh, Waldrup, Labes, Buchan & Griffin 1995). The model should be also repeatable, practical to execute, safe and economical.

Various methods of establishing experimental infection with *M. bovis* have been studied in different host species. The intra-tracheal route of infection has been used in cattle (Buddle *et al.* 1995), possums (Corner *et al.* 2002), the oral route in ferrets (Cross *et al.* 2000), and the intratonsilar inoculation in red deer (Mackintosh *et al.* 1995; Griffin, Mackintosh & Buchan 1995) and cattle (Palmer, Whipple, Rhyan, Bolin & Saari 1999). The intratonsilar method was selected for this study because it is easy to execute, safe and economical. It involves instilling

0.2 ml of an inoculum into the left tonsilar crypt while the experimental animal is under anaesthesia. The disease in infected cattle and deer mimicked the natural disease in terms of its pathogenesis and rate of progression and severity of lesions in deer (Griffin *et al.* 1995) and cattle (Palmer *et al.* 1999). The aim of this study is to establish an *M. bovis* experimental model of infection in buffaloes in order to evaluate further BTB vaccine candidates.

MATERIAL AND METHODS

ANIMALS

Twenty-eight buffalo calves, varying in age from 12-24 months, were captured in a tuberculosis-free area of the KNP. The calves were randomly divided into three groups, with comparable numbers of bull and heifer calves in each group. The two experimental groups comprised eleven animals each while the control group comprised six animals. The three groups were housed separately. The control group was placed in a facility furthest away from the group that received the higher infectious dose of virulent *M. bovis*.

Husbandry and monitoring

The calves were housed in bomas of 600 m^2 with an inner and an outer fence designed to keep out predators. The animals were observed three times a day, and interactions, general health, and condition scores were recorded. Injuries and acute illnesses were treated appropriately. They were fed twice a day with teff hay and lucerne mixed at a ratio of 2:1, and water was available *ad libidum.* At capture all the calves were tested with the comparative intradermal tuberculin skin test as well as the gamma-interferon assay. All calves tested negative for bovine tuberculosis although sensitization to environmental mycobacteria was seen in some animals. After four weeks the gamma-interferon assays was repeated and all the results were comparable to those obtained at capture.

Mycobacterium bovis strain

During a survey in 1998 to determine the prevalence of BTB in buffaloes in the KNP, suspect tissue samples were submitted for culture (Rodwell, Kriek, Bengis, Whyte, Viljoen, De Vos & Boyce 2001). Mycobacterial isolates were identified using biochemical and PCR tests followed by restriction fragment length polymorphism (RFLP) characterisation of the *M. bovis* isolates (De Vos *et al.* 2000). One *M. bovis* isolate (Case no. KNP 182) classified as representative of the dominant KNP genotype ZA-01 (De Vos *et al.* 2000), was selected for use as the challenge strain for the trial. Subcultures of this isolate had been stored at –20 °C on Lőwenstein-Jensen slopes containing pyruvate. For preparation of the different inocula used for challenge, growth from fresh subcultures was carefully suspended in saline containing 0.5 % Tween 80 on the day of the experimental

infection. The concentration of bacteria was adjusted by microscopically counting of serial dilutions in a Neubauer counting chamber to 3 x 10^2 (low dose inoculum) and 3 x 10^4 (high dose inoculum) per 200 μℓ, respectively. Tween 80 was used to avoid clumping of bacteria, allowing for accurate counting. Aliquots of each serial dilution were plated out in triplicate onto Petri dishes containing Lőwenstein-Jensen medium with pyruvate. To avoid desiccation during the prolonged incubation at 37°C, Petri dishes were sealed and placed in a humid chamber for 10 weeks.

ANAESTHESIA
A combination of etorphine hydrochloride (M99, Novartis SA Animal Health) and xylazine (Chanazine 2%, Centaur, Bayer Animal Health) at standard dosages used for routine buffalo immobilization was used to anaesthetize the buffalo calves. Xylazine was used because it is a good muscle relaxant, and it enhanced the relaxation of the jaw muscles, facilitating the opening of the mouth during the infection procedure. During the rest of the study period, a combination of M99 and azaperone (Stresnil, Janssen Animal Health) was used as described previously (Bengis & Raath 1993).

Experimental infection procedure

The control animals were handled first. Blood samples were collected from the jugular vein into vacuum tubes containing appropriate solutions for preservation and/or preventing coagulation, as required. The calves were then rolled onto strong tarpaulin stretchers, and moved to a separate pen to reduce the likelihood of contact with the animals infected with live *M. bovis*.

The two groups to be infected, each comprising 11 calves, were anaesthetized and inoculated with live *M. bovis* culture material as follows. The anaesthetized calves were placed in sternal recumbency with their heads lowered to allow any oral fluid resulting from the administration of xylazine, to drain before the instillation of the bacterial suspension into the left tonsilar crypt. Each animal's mouth was opened and its tongue was reflected to the left side of the operator. The base of the tongue was depressed using a 400 mm laryngoscope, so that the left tonsilar crypt could be seen. The *M. bovis* suspension was instilled into the crypt with a 1 mℓ syringe fitted with a 300 mm long 18 G needle with a ball tip. Any spillage or haemorrhage from the crypt following instillation was recorded. The eleven calves in the low dose group received 0.2 mℓ of a suspension containing about 3 x 10^2 cfu of *M. bovis* and the 11 calves in the high dose group received 0.2 mℓ of a suspension containing 3 x 10^4 cfu of *M. bovis*. The six calves in the control group received 0.2 mℓ of saline into the left tonsilar crypt following the same overall procedure.

After the procedure was completed, the animals were revived by administering the antidote diprenorphine hydrochloride (M50/50, Novartis SA Animal Health) at twice the dosage of the M99. Whenever xylazine was used to immobilize the calves, 3 – 5 mℓ of yohimbine were also administered. The different experimental groups of calves were kept in separate bomas.

Laboratory tests

Interferon-γ assay (Bovigam)

The interferon-□ assay (IFN-γ) is a rapid, blood-based assay of cell-mediated immunity (CMI) used for the diagnosis of BTB in cattle (Woods *et al.* 1991). However, when used for the diagnosis in buffaloes there was a lack of specificity (Michel *et al.* 2000). Through subsequent modification of the commercial kit protocol into a triple comparative test (i.e. use of *Mycobacterium fortuitum* besides *Mycobacterium avium* and *M. bovis* purified protein derivatives (PPD), as an ancillary antigen), discrimination between specific and non-specific immune reactions was significantly improved (Grobler *et al.* 2002). In this experiment the modified protocol was used as described previously by Grobler *et al.* (2002).

Whole blood count

Whole blood was collected in an EDTA tube from every animal and assayed within 6 hours of blood collection to perform whole blood counts on the T-890 coulter counter (Beckman Coulter). Blood smears were prepared on glass slides, stained with Diff-Quick (Kyro-quick, Kyron Laboratories) and examined for *Babesia* and *Theileria* parasites.

Bacteriology

Specimens for culture were collected from the following lymph nodes irrespective of whether lesions were present. The left and right tonsils and both medial retropharyngeal lymph nodes were processed separately and equally divided for mycobacterial culture and histopathology. All lesions detected in any other of the lymph nodes or organs, were collected and processed for mycobacterial culture and histopathology. Specimens from other lymph nodes (as indicated below) were pooled for culture:

- Pooled head lymph nodes: Mandibular and parotid lymph nodes
- Pooled thoracic lymph nodes: Mediastinal and bronchial lymph nodes
- Pooled abdominal lymph nodes: Mesenteric, hepatic, renal, omasal, and abomasal lymph nodes

- Pooled carcass lymph nodes: Cervical, prescapular, axillary, popliteal, inguinal and mammary or scrotal lymph nodes

Samples were transferred to and processed at the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute, where they were cultured and identified as described by Bengis *et al.* (1996). Cultures were considered negative for *M. bovis* when no growth was detected after 12 weeks of incubation. In this study the culture result was used as the gold standard to indicate if an animal was infected with *M. bovis.* The same isolation and identification protocols were applied to swabs that were taken during the course of infection.

Histopathology

Specimens were collected and preserved in 10 % buffered formalin, and were later prepared routinely for light microscopy by embedding them in paraffin wax. Sections cut to a thickness of 4 – 6 μm were routinely stained with haematoxylin and eosin, and selected sections with the Ziehl-Neelsen acid-fast stain. The Ziehl-Neelsen stained histopathology sections were examined microscopically for the presence of acid-fast bacilli (AFB).

Intradermal tuberculin test

A comparative intradermal tuberculin test using 0.1 mℓ of bovine PPD (0.1 mg/mℓ) and 0.1 mℓ of avian PPD (0.05 mg/mℓ, Lelystad, The Netherlands) was done on all the calves six weeks prior to infection and again three months after infection. Injection sites on both sides of the lower neck were shaved with a battery-operated hair clipper. As a rule bovine tuberculin PPD was injected intradermally on the left and avian PPD on the right side of the neck with a McClintok syringe. The skin thickness was measured with a calliper before and 72 hours after injection. Both manipulations necessitated the calves to be anaesthetised, firstly to give the injections and secondly to palpate and assess the nature of the skin reaction.

The intradermal tuberculin test results were interpreted as recommended by the OIE:

- Increase of skin thickness in response to injection of bovine tuberculin PPD < 2 mm = negative

Provided the reaction to bovine tuberculin PPD was greater than that elicited by the avian tuberculin PPD the following interpretation was applied:

- If the increase in skin thickness at the bovine tuberculin injection site minus the increase at the avian injection site is 2-4 mm, the result is suspect.
- If the increase in skin thickness at the bovine tuberculin injection site minus the increase at the avian injection site is > 4 mm, the result is positive.

Routine monitoring

After infection, the calves were monitored three times daily for evidence of clinical disease or abnormal behaviour. No acute onset of disease was observed.

Blood specimens, nasal swab collection and weight gain during the course of infection

Apart from the day of experimental *M. bovis* infection and euthanasia, the buffalo calves were anaesthetized at three further occasions between the 5th and the 15th week post infection (p.i.), at which time they were weighed, and blood specimens and nasal swabs collected. Whole blood was collected from each experimental animal and preserved in heparin as well as in EDTA for the IFN-γ assay and routine haematology. Vacuum tubes without preservative were used to collect serum for serum banking. Nasal swabs were collected for bacteriology. On the day of euthanasia the same sampling procedures were applied as mentioned previously. In total, five sets of samples were collected for each individual, the last being taken on the day of euthanasia.

Each anaesthetized calf was weighed regularly by being lifted onto a tarpaulin stretcher that was connected to a scale attached to a hydraulic crane. The scale was zeroed before each calf was weighed. The weight of each calf was recorded to reflect its change in mass over the duration of the experiment.

Necropsy procedure

All the experimental animals were euthanazed with succinyl dicholine chloride (scoline) and then immediately subjected to a detailed necropsy in the abattoir at Skukuza. Five animals (two each from the low and high dose groups and one from the control group) were necropsied 18 weeks after infection, to establish whether the infection technique developed for deer was valid in buffalo. Previous studies showed that characteristic pathological changes are evident in deer 18 weeks after experimental infection (Griffin, 2002 unpublished data). At the end of the study, 22 weeks post infection, all the remaining calves were euthanazed.

Selected lymph nodes were removed from the carcass and after being thinly sliced with a scalpel blade, were visually examined for the presence of macroscopic tuberculous lesions. The tonsils and the mandibular, parotid, and medial retropharyngeal lymph nodes together with the mediastinal and bronchial lymph nodes and the pre-scapular, axillary, popliteal, inguinal, mammary or scrotal, hepatic, ruminal, omasal, abomasal, mesenteric, ileo-caecal and rectal lymph nodes were collected. The medial retropharyngeal lymph nodes of each animal were labelled and photographed.

The lungs were initially carefully palpated to detect any nodules or lesions, after which they were systematically sliced with a knife to detect any lesions. All lesions that were detected in the lungs, lymph nodes and tonsils were collected and submitted for histopathology and bacterial culture. Irrespective of whether macroscopic lesions were detected in specimens of the tonsils and lymph nodes listed above, these tissues were collected for histopathology and culture. Specimens for histopathology were preserved in 10% buffered formalin and submitted to the Department of Pathology of the Faculty of Veterinary Science, University of Pretoria at Onderstepoort for processing and examination.

Grading of macroscopic lesions

The following criteria were used to grade the macroscopic lesions of each individual animal:

Grade 0: No visible lesions
Grade 1: Less than 50% of a single lymph node or tonsil affected
Grade 2: More than 50% of a single lymph node or tonsil affected
Grade 3: Two or three lymph nodes and/or tonsils involved
Grade 4: More than three lymph nodes involved
Grade 5: Multiple lymph nodes as well as organs involved (Miliary disease)

Statistical methods

An ANOVA was used because the response variables were all continuous variables and the aim was to determine whether there were any significant differences between the positive and negative individuals over the specified time period. This method of analysis was used for both the haematology parameters and the body mass data sets. There were no significant differences in any of the parameters between the positive and negative individuals.

RESULTS

Mycobacterium bovis inoculum

Plate counts of serial dilutions of the *M. bovis* inoculum 6 weeks after experimental infection revealed that the infectious doses administered were 3.2 x 10^2 and 3 x 10^4 cfu, respectively.

Interferon-γ assay

The results are shown in Table 1. All blood samples collected on the day of the experimental infection (Day 0) tested negative for bovine PPD. Five weeks post infection (p.i.) seven out of 11 animals inoculated with the high dose tested positive with IFN-γ. All control calves as well as those receiving the low dose tested negative. The intradermal tuberculin test was applied at 11 weeks p.i. During weeks 13 – 17 p.i., the number of infected calves testing IFN-γ positive increased until at 22 weeks post infection all calves in the high dose group and four out of the 11 in the low dose group tested positive on IFN-γ. Some non-specific sensitization was observed in all the groups during the trial, but was disregarded, and a false positive result occurred in one animal from the control group after the intradermal tuberculin test.

Whole blood count

Throughout the study whole blood and total lymphocyte counts remained stable for all animals in the study. At 95 % confidence levels no difference could be detected between the haematological parameters of diseased and non-diseased animals.

Tuberculin skin test

Two calves tested positive for avian PPD prior to infection (Table 2). The second skin test was carried out 11 weeks post-infection which identified all infected calves in the high dose group and six of the 11 calves in the low dose group. None of the control calves tested positive. The increase in skin thickness at the test sites in seven of the nine animals from the high dose group was no longer measurable because of the pronounced swelling that produced an increase of skin thickness in excess of 33 mm. Many of these skin reactions also demonstrated superficial necrosis covered by a sero-fibrinous exudate. Two of the 11 animals in the low dose group also had reactions that could not be measured.

Body mass and condition

The average daily body mass gain was between 0.34 and 0.32 kg/animal/day. No statistically significant difference in body mass gain or condition could be detected between infected and control animals.

Table 1. Results of the gamma-interferon assay from the control, low dose and high dose group animals

Number	Day 0	Week 5	Week 11*	Week 15	Week 21/22
Control					
LM4	-	-	-	-	AV
LM5	-	-	Invalid	-	-
LM8	-	-	-	+	-
LM9	-	-	-	AV	AV
LM18	-	-	-	-	-
LM26	-	-	-	-	-
Low dose					
LM1	-	-	-	+	+
LM2	-	-	-	-	-
LM7	-	-	-	Invalid	-
LM12	-	-	+	-	-
LM14	-	-	-	-	-
LM17	-	-	+	+	+
LM21	-	-	-	-	+
LM22	-	-	+	+	+
LM23	-	-	-	-	-
LM24	-	-	-	-	-
LM27	-	-	-	+	-
High dose					
LM3	-	-	+	+	+
LM6	-	+	+	+	+
LM10	-	-	+	+	+
LM11	-	+	+	+	+
LM13	-	+	+	+	+
LM15**	-	+	+		
LM16	-	-	-	+	+
LM19**	-	+			
LM20	-	-	+	+	+
LM25	-	+	-	+	+
LM28	-	+	Suspect	+	+

+ (positive) : Bovine – avian (OD) > 0.2
– (negative) : Bovine – avian (OD) < 0.17 provided that bovine (OD) < 0.3
Suspect : Bovine – avian (OD) > 0.17 but < 0.2 provided that bovine (OD) > 0.3
AV (avian reactor) : Avian – bovine (OD) > bovine (OD) + bovine/10
Invalid : Control (OD) > 0.25
Week 11* : Animals were skin tested during Week 11
LM15** & LM19** : Two animals died due to causes unrelated to bovine tuberculosis

Macroscopic lesions and histopathology

Control group

No macroscopic or microscopic lesions were detected in the control animals.

Low dose group

Macroscopic lesions typical for *M. bovis* infection were detected in four out of 11 calves infected with the low dose. Histopathology confirmed BTB infection in these animals. Generally, lymph node lesions varied in size and distribution with small granulomas that had central caseation and calcification.

High dose group

Two animals in this group died prematurely and were necropsied. LM19 died 5 weeks p.i., but no macroscopic tuberculous lesions were noted. On histological sections of the left retropharyngeal lymph node a few giant cell granulomas couldbe seen and numerous small acid-fast bacteria were present in the cytoplasm of multinucleate giant cells. Mycobacterium bovis was cultured from lymph node samples. LM15 died at 11 weeks p.i., and mild to moderate lymphadenitis with multiple caseo-necrotic foci was present. Histopathology showed a few acid-fast bacilli in the cytoplasm of the giant cells and epithelioid macrophages. Only one calf (LM16) in the high dose group did not show macroscopic lesions, while all the remaining calves in this group had varying degrees of caseo-necrotic lymphadenitis. Two calves in the high dose group (LM16 & LM25) were histopathologically negative for BTB.

The grading of the macropathology was higher in the animals from the high dose group than in animals from the low dose group. Generally, a larger percentage of lymph node mass was affected in animals in the high dose group and histopathology revealed larger numbers of acid-fast bacilli organisms (AFBs) in tissue sections. However, there was no difference in the degree of dissemination of lesions between the two infected groups. Indeed, lesions beyond the left retro-pharyngeal lymph nodes were only seen in mediastinal lymph nodes in one animal of each of the challenge groups.

DISCUSSION

The experimental procedure described here was successful in inducing an infection in which the tuberculous lesions were comparable to those seen in natural infections of buffaloes in low BTB prevalence herds. The model showed that *M. bovis* could be recovered from at least 5 weeks p.i. from the regional lymph node associated with portal of entry. The overall macroscopic pathology of

Table 2. A comparison of the skin tests that were done 2 weeks prior to and 11 weeks post intratonsilar infection, of all three groups of animals from the Infection model (results in mm)

	Skin test results: 2 weeks prior to infection					Skin test results: 11 weeks post infection					
Nr.	**Bov 0hr**	**Bov 72hr**	**Av 0hr**	**Av 72hr**	**Result**	**Bov 0hr**	**Bov 72hr**	**Av 0hr**	**Av 72hr**	**Result**	**Test site appearance**
Control											
LM 4	18	16	17.7	19.7	Neg.	21.7	23	21.7	23.4	Neg.	Normal
LM 5	15.3	15.5	15.1	16.2	Neg.	17.2	19.5	16	19.4	Neg.	Normal
LM 8	14.4	13.5	14.4	14.3	Neg.	14.6	14.5	14	15	Neg.	Normal
LM 9	13.5	13.8	13.6	19.4	Avian	13.4	15.4	11.6	15.5	Neg.	Normal
LM 18	14.3	14.5	13.9	14.6	Neg.	13.5	15.1	13.8	18.3	Avian	Normal
LM 26	11.3	12.1	11.3	12.3	Neg.	10.1	9.6	10.7	12.8	Neg.	Normal
Low dose											
LM 1	12.3	12.5	13.3	18.8	Avian	13.3	31.4	13	15.5	Pos.	Oedema
LM 2	12.5	12.9	13.9	14.9	Neg.	15.2	15.4	15.5	15.3	Neg.	Normal
LM 7	16.3	17.1	15.9	18	Neg.	15.5	16.2	15.9	16.6	Neg.	Normal
LM 12	15.5	15.4	15.2	17	Neg.	15	14.9	15.1	16.5	Neg.	Normal
LM 14	12.4	11.8	13.4	14.2	Neg.	12.6	16.6	12.2	13.4	Susp.	Oedema
LM 17	11.9	12.9	11.8	14	Neg.	11	>33	10.8	13.1	Pos.	Oedema, necrosis
LM 21	14.5	15.6	15.1	15.8	Neg.	12.8	13.5	15	18.1	Neg.	Small avain nodule
LM 22	13	12.8	14	13.3	Neg.	12.1	>33	13.3	18.5	Pos.	Oedema, necrosis
LM 23	14.1	14.2	14.4	16.5	Neg.	10.6	12.6	14.2	17	Neg.	Small avian nodule
LM 24	13	13	12.9	13.3	Neg.	12.2	17.5	11.7	12.8	Pos.	Oedema
LM 27	12.4	12	12.5	12.6	Neg.	11.4	16.7	10.7	12.7	Susp.	Oedema
High dose											
LM 3	18	17.3	17.3	19.8	Neg.	17.2	>33	16.4	20.5	Pos.	Oedema, necrosis
LM 6	15	15.7	15.9	15.5	Neg.	16.1	>33	16.7	17.4	Pos.	Oedema, necrosis
LM 10	13.3	14.8	12.5	14.7	Neg.	13.3	>33	12.8	17.3	Pos.	Oedema, necrosis
LM 11	11.5	11.4	11.7	11.7	Neg.	10	>33	11	12.5	Pos.	Oedema, necrosis
LM 13	17.4	18.5	17.1	18.3	Neg.	18.1	>33	17.3	18.4	Pos.	Oedema, necrosis
LM 16	14.5	14.3	14.4	13.9	Neg.	16.1	>33	14.8	22.2	Pos.	Oedema, necrosis
LM 20	11.4	10.8	11	10.8	Neg.	10.4	30.4	11.8	14.5	Pos.	Oedema
LM 25	14.5	14.3	14	14.2	Neg.	13	19.7	12.6	14.4	Pos.	Oedema
LM 28	12.4	12	12.5	12.6	Neg.	17.7	>33	11.5	14.8	Pos.	Oedema, necrosis

Positive : Increase in bovine measurement – increase in avian measurement > 4 mm
Suspect : Increase in bovine measurement – increase in avian measurement > 2 mm; < 4 mm
Avian reactor : Increase in avian measurement – increase in bovine measurement > 4 mm
Negative : Increase in bovine measurement – increase in avian measurement < 2 mm

the experimentally infected animals compared well to the necropsy findings of an adult buffalo with BTB (Keet, Kriek, Huchzermeyer & Bengis 1994). Contrary to findings reported from intra-nasal infections of domestic cattle with 10^7 cfu of live*M. bovis,* none of the buffalo calves in our study developed pulmonary disease (Cassidy, Bryson, Pollock, Evans, Forster & Neill 1998). A direct correlation between severity of disease and infectious dose, described by Cassidy *et al*. (1998) was also demonstrated in our experiment, although much lower infectious doses were used. The grading of the gross pathology of affected tissues revealed significant differences between animals in the high and low dose groups (Table 3).

In agreement with previous similar *M. bovis* infection experiments by Palmer *et al.* (1999), the retropharyngeal lymph node that drains the palatine tonsil, was the most likely node to develop tuberculous lesions. Lesions were found in the left retropharyngeal lymph nodes of 13 of the 22 infected animals, four and nine from the low dose and high dose groups, respectively. *Mycobacterium bovis* was cultured from all the macroscopic lesions submitted for culture. In addition, the macroscopically negative retropharyngeal lymph nodes from two animals in the low dose group were positive for *M. bovis* on culture. This indicates that viable *M. bovis* can be harboured within the lymphoid tissue without the development of macroscopically detectable lesions for at least 22 weeks following infection.

At an early stage the IFN-γ test was able to differentiate at an early stage between infected and non-infected animals. As early as 5 weeks p.i. the IFN-γ test classified seven animals as positive in the high dose group. Only four animals did not show any sensitization. In the low dose group, none of the animals was positive at 5 weeks p.i., whereas at 11 weeks p.i., two animals were positive. Since the intradermal tuberculin test was performed at 11 weeks p.i., it is likely that this has had an influence on the IFN-γ results. This has also been demonstrated in cattle in experimental conditions (Walravens, Wellemans, Weynants, Boelaert, Debergeyck, Letesson, Huygen & Godfroid 2002). The intradermal tuberculin test could also have been the cause of the false positive test result in one of the uninfected controls at 15 weeks p.i.. Ryan, Buddle & De Lisle (2000) found the IFN-γ assay to be a valuable ancillary test in cattle and that it was able to accurately predict the BTB status of an animal that was skin tested 8 – 28 days previously. The IFN-γ assay can be repeated at regular intervals with the advantage of only a single anaesthesia per animal. The intradermal tuberculin test on the other hand is associated with high costs because of the double anaesthesia as well as a time limit due to the required interval of 3 months between tests.

At the end of the study, *M. bovis* could only be isolated from six animals from the low dose group, while five animals were negative. Four of the six animals that

were positive on culture were also classified positive by the IFN-γ test at time of euthanasia. In the high dose group all nine animals were positive. Alltogether these results show that the IFN-γ test detects BTB as early as 5 weeks p.i. in the

Table 3. Macroscopic pathology, histopathology and culture results of the three different animal groups from the Infection model

Number	Gross pathology			Histopathology	Culture
	Left retro.	Other	Grading	Result (AFB**)	
Control					
LM 4	Neg.	Neg.	0	Neg. (0)	Neg.
LM 5	Neg.	Neg.	0	Neg. (0)	Neg.
LM 8	Neg.	Neg.	0	Neg. (0)	Neg.
LM 9	Neg.	Neg.	0	Neg. (0)	Neg.
LM 18	Neg.	Neg.	0	Neg. (0)	Neg.
LM 26	Neg.	Neg.	0	Neg. (0)	Neg.
Low dose					
LM 1	Pos.	Mediast. ln.	3	Pos. (<5)	Pos.
LM 2	Neg.	Neg.	0	Neg. (0)	Pos.
LM 7	Neg.	Neg.	0	Neg. (0)	Neg.
LM 12	Neg.	Neg.	0	Neg. (0)	Neg.
LM 14	Neg.	Neg.	0	Neg. (0)	Pos.
LM 17	Pos.	Neg.	2	Pos. (<5)	Pos.
LM 21	Pos.	Neg.	1	Pos. (<5)	Pos.
LM 22	Pos.	Neg.	2	Pos. (<5)	Pos.
LM 23	Neg.	Neg.	0	Neg. (0)	Neg.
LM 24	Neg.	Neg.	0	Neg. (0)	Neg.
LM 27	Neg.	Neg.	0	Neg. (0)	Neg.
High dose					
LM 3	Pos.	Neg.	2	Pos. (0)	Pos.
LM 6	Pos.	Neg.	2	Pos. (<5)	Pos.
LM 10	Pos.	Mediast. ln.	3	Pos. (0)	Pos.
LM 11	Pos.	Neg.	2	Pos. (0)	Pos.
LM 13	Pos.	Neg.	2	Pos. (>20)	Pos.
LM 15	Pos.	Neg.	2	Pos. (0)	Pos.
LM 16	Neg.	Neg.	0	Neg. (0)	Pos.
LM 19	Neg.	Neg.	0	Pos. (0)	Pos.
LM 20	Pos.	Neg.	1	Pos. (>20)	Pos.
LM 25	Pos.	Neg.	1	Neg. (0)	Pos.
LM 28	Pos.	Neg.	2	Pos. (10 - 20)	Pos.

** The numbers in the histopathology result column indicates the number of acid fast organisms present per slide

high dose group, as was the case in cattle. When performed at 21-22 weeks p.i., all the animals with macroscopic lesions and positive culture results also tested positive with the IFN-γ test. In this model positive IFN-γ results correlate with the

establishment of the infection in the host. All the animals in the low dose group that were able to clear the infection, were classified negatively by the IFN-γ test at slaughter.

Throughout the study the infected buffalo calves had growth rates comparable to those of the controls. The lesions that developed after infection did have any detectable effect on their general health or the average daily body mass gain. Similar findings have been reported in deer, where equivalent doses of virulent *M. bovis* were administered by the intratonsilar route (Griffin, Mackintosh & Buchan 1995). Weight loss has been recorded in advanced stages of BTB. (Bengis *et al.* 1996; De Vos *et al.* 2001). In buffalo BTB usually has a sub-clinical development, and manifestations such as poor body condition, coughing and weight loss is only found in animals with advanced disease. Our results suggest that experimental *M. bovis* infection at doses up to 3×10^4 CFU will not affect the body condition or weight gain in young animals for at least 6 months p.i.

Because of the time frame of the experiment and the challenge route of choice, it was expected that very few animals would develop disseminated lesions. This infection model appears to be safe to work with, and effective in producing tuberculous lesions comparable with early natural infections in wild buffaloes. This model may be considered for use in further research studies where a longer time frame might allow for secondary spread of lesions. The higher infectious dose is considered the "dose of choice" for future trials since 3×10^4 cfu produced consistent and reliable infections with associated lesion development. The successful completion of the infection model will facilitate further studies of this disease problem, including evaluation of the efficacy of BCG vaccination or other vaccine candidates.

ACKNOWLEDGEMENTS

The authors are grateful for the input and co-operation of the following groups of people, for without them this project would not have been possible. Field staff of the Office of the State Veterinarian for the care and feeding of the animals and daily cleaning of the boma facilities; J. Malan, M. Kruger, J. van Altena, P. Mashile and J. Muhlanga from the Game Capture team for capture, transport and weighing of the calves; veterinary technicians (Schalk van Dyk, At Decker, Johan Oosthuizen and Kenneth Muchocho); laboratory assistant (Li-Ann Small) and students (Nerina Belcher, Justin Bowers, Matthew Brownie, Jana Meyer) for their help during the sampling sessions. A special thank you to Albert Linsky (Senior Meat Inspector) and his team for the slaughtering and processing of the buffalo carcasses, Judith Kruger for statistical analysis of the data and Prof. Jacques Godfroid for extensive overview and recommendations. This project would not have been possible without the major financial contributions of the Smithsonian Institute and Shirley Sichel, in the USA.

REFERENCES

BENGIS, R.G. & RAATH, J.P. 1993. Chemical capture of the African buffalo (*Syncerus caffer*), in *The capture and care manual*, edited by Andrew A. McKenzie. Section B4: 583–597.

BENGIS, R.G., KRIEK, N.P.J., KEET, D.F., RAATH, J.P. DE VOS, V. & HUCHZERMEYER, H.F.A.K. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer* – Sparrman) population in the Kruger National Park:a preliminary report. *Onderstepoort Journal of Veterinary Research*, 63:15–18.

BUCHAN, G.S. & GRIFFIN, J.F.T 1990. Tuberculosis in domesticated deer (*Cervus elaphus*): A large animal model for human tuberculosis. *Journal of Comparative Pathology,* 103:11-23.

BUDDLE, B.M., KEEN, D., THOMSON, A., JOWETT, G., MCCARTHY, A.R., HESLOP, J. & DE LISLE, G.W. 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed M*ycobacterium vaccae. Research in Veterinary Science,* 59:10–16.

BUDDLE, B.M., SKINNER, M.A. & CHAMBERS, M.A. 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. *Veterinary Immunology and Immunopathology,* 74:1–16.

BUDDLE, B.M., WARDS, B.J., ALDWELL, F.E., COLLINS, D.M. & DE LISLE, G.W., 2002. Influence of sensitization to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine,* 20:1126 – 1133.

CASSIDY, J.P, BRYSON, D.G., POLLOCK, J.M., EVANS, R.T., FORSTER, F. & NEILL, S.D. 1998. Early lesion formation in cattle experimentally infected with *Mycobacterium bovis*. *Journal of Comparative Pathology,* 119:27–44.

CORNER, L.A.L., BUDDLE, B.M., PFEIFFER, D.U. & MORRIS, R.S. 2001. Aerosol vaccination of the brushtail possum (*Trichosurus vulpecula*) with bacille Calmette-Guérin: the duration of protection. *Veterinary Microbiology,* 81:181–191.

CORNER, L.A.L., BUDDLE, B.M., PFEIFFER, D.U. & MORRIS, R.S. 2002. Vaccination of the brushtail possum (*Trichosurus vulpecula*) against *Mycobacterium bovis* infection with bacille Calmette-Guérin: the response to multiple doses. *Veterinary Microbiology,* 84:327–336.

CROSS, M.L., LABES, R.E. & MACKINTOSH, C.G. 2000. Oral infection of ferrets with virulent *Mycobacterium bovis* or *Mycobacterium avium*: susceptibility, pathogenesis and immune responses. *Journal of Comparative Pathology,* 123:15–21.

CROSS, M.L., LABES, R.E., GRIFFIN, J.F.T. & MACKINTOSH, C.G. 2000. Systemic but not intra-testinal vaccination with BCG reduces the severity of tuberculosis infection in ferrets (*Mustela furo*). *International Journal of Tuberculosis and Lung Disease,* 4:473–480.

DE VOS, V., BENGIS, R.G., KRIEK, N.P.J., MICHEL, A., KEET, D.F., RAATH, J.P. & HUCHZERMEYER, H.F.K.A. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research,* 68:119–130.

GRIFFIN, J.F.T., MACKINTOSH, C.G. & BUCHAN, G.S. 1995. Animal models of protective immunity in tuberculosis to evaluate candidate vaccines. *Trends in Microbiology,* 3:418–422.

GRIFFIN, J.F.T, MACKINTOSH, C.G., SLOBBE, L., THOMSON, A.J. & BUCHAN, G.S. 1999. Vaccine protocols to optimise the protective efficacy of BCG. *Tubercle and Lung Disease,* 79:135–143.

GRIFFIN, J.F.T., CHINN, D.N., RODGERS, C.R. & MACKINTOSH, C.G. 2001. Optimal models to evaluate the protective efficacy of tuberculosis vaccines. *Tuberculosis*, 81:133–139.

GROBLER, D.G., MICHEL, A.L., DE KLERK, L. & BENGIS, R.G. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 69:221–227.

KEET, D.F., KRIEK, N.P.J., HUCHZERMEYER, H. & BENGIS, R.G. 1994. Advanced tuberculosis in an African buffalo (*Syncerus caffer* Sparrman). *Journal of the South African Veterinary Association,* 65:79–83.

KEET, D.F., KRIEK, N.P.J., PENRITH, M.L., MICHEL, A.L. & HUCHZERMEYER, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. *Onderstepoort Journal of Veterinary Research,* 63:239–244.

KEET, D.F., KRIEK, N.P.J., BENGIS, R.G., GROBLER D.G. & MICHEL, A.L. 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 67:115–122.

KEET, D.F., KRIEK, N.P.J., BENGIS, R.G. & MICHEL, A.L. 2001. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 68:225–230.

MACKINTOSH, C.G., WALDRUP, K., LABES, R., BUCHAN, G. & GRIFFIN, J.F.T. 1995. Intra-tonsil inoculation: an experimental model for tuberculosis in deer. *Tuberculosis in wildlife and domestic animals.* Dunedin: Otago University press: 121–123.

MICHEL, A.L., NEL, M., COOPER, D. & MOROBANE , R.N. 2000. Field evaluation of a modified "gamma interferon" assay in African buffalo (*Syncerus caffer*) and cattle in South Africa. *Proceedings of the 3rd International Conference on Mycobacterium bovis,* 14 – 16 August 2000, Cambridge, United Kingdom.

MICHEL, A.L. 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences,* 969:251–255.

PALMER, M.V., WHIPPLE, D.L., RHYAN, J.C., BOLIN, C.A. & SAARI, D.A. 1999. Granuloma development in cattle after intratonsilar inoculation with

Mycobacterium bovis. *American Journal of Veterinary Research*, 60:310–1315.

RODWELL, T.C., KRIEK, N.P., BENGIS, R.G., WHYTE, I.J., VILJOEN, P.C., DE VOS, V. & BOYCE, W.M. 2001. Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. *Journal of Wildlife Diseases,* 37:258–264.

RYAN, T.J., BUDDLE, B.M. & DE LISLE, G.W. 2000. An evaluation of the gamma interferon test for detecting bovine tuberculosis in cattle 8 to 28 days after tuberculin skin testing. *Research in Veterinary Science,* 69:57–61.

TANNER, M. & MICHEL, A.L. 1999. Investigation of the viability of *M.bovis* under different environmental conditions in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 66:185–190.

VORDERMEIER, H.M., CHAMBERS, M.A., COCKLE, P.J., WHELAN, A.O., SIMMONS, J. & HEWINSON, R.G. 2002. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection and Immunity,* 70:3026–3032.

WALRAVENS, K., WELLEMANS, V., WEYNANTS, V., BOELAERT, F., DEBERGEYCK, V., LETESSON, J-J., HUYGEN, K. & GODFROID, J. 2002. Analysis of the antigen-specific IFN-γ producing T-cell subsets in cattle experimentally infected with *Mycobacterium bovis*. *Veterinary Immunology and Immunopathology,* 84:29–41.

Chapter 5.2

BCG vaccination failed to protect yearling African buffaloes (*Syncerus caffer*) against experimental challenge with *Mycobacterium bovis*

Lin-Mari de Klerk [a], Anita L. Michel[b*], Roy G. Bengis[c], Nick P.J. Kriek[d], & Jacques Godfroid[d]

[a] South African National Parks, Private Bag X402, Skukuza, 1350 South Africa

[b] Tuberculosis Laboratory, Food, Feed & Veterinary Public Health programme, ARC-Onderstepoort Veterinary Institute , Private Bag X05, Onderstepoort, 0110 South Africa

[c] Office of the State Veterinarian, Kruger National Park, P.O. Box 12, Skukuza, 1350, South Africa

[d] Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

Submitted

ABSTRACT

Vaccination has been discussed as a practical option to control bovine tuberculosis in countries where a wildlife reservoir of the disease is present. African buffaloes (*Syncerus caffer*) are the main wildlife reservoir of *Mycobacterium bovis* in South African game parks and vaccination is not only the most promising but the only ethically acceptable control measure currently available. The use of bacillus Calmette Guerin vaccine (Pasteur strain) to vaccinate fourteen African buffalo yearlings and their reactions to subsequent challenge with a field strain of *M. bovis* are described. The BCG vaccine was administered twice intramuscularly, six weeks apart. All vaccinates and thirteen control buffaloes were euthanazed and necropsies performed nine months after the challenge. Standard sets of lymph nodes from the head, the thoracic cavity and abdomen were cultured and examined histopathologically. No significant reduction in number of lesions or severity of disease was noted, concluding that the BCG vaccine did not induce sufficient protection able to limit the shedding of organisms. The age of the buffaloes, route of vaccination and prior exposure to environmental mycobacteria are among the possible reasons for vaccination failure.

Key words: Kruger National Park (KNP), African buffalo (*Syncerus caffer*), bovine tuberculosis, *Mycobacterium bovis*, BCG vaccine

1. Introduction

The African buffaloes are the main reservoir and maintenance hosts of bovine tuberculosis in the Kruger National Park (KNP) ecosystem [1,2]. The disease has also spilled over into several other sympatric mammalian species since its discovery in 1990 [3-6]. Bovine tuberculosis (BTB) is a multi-host disease in a multi-species system and because of the large number of species in which BTB has already been detected, it is reasoned that the control of BTB in the KNP will become increasingly difficult and eradication unlikely. Various strategies to control and possibly eradicate this disease from KNP have been considered. Of the limited options available in the complex KNP situation, vaccination currently appears to be the most practical, promising and ethically acceptable option.

Vaccination might be a good option for the control of *Mycobacterium bovis* infection in certain wildlife hosts worldwide [7]. When attempting to control this disease by vaccination, the immediate aim is to reduce the transmission of infection among the reservoir hosts and as well as spread to other species. The spread of infection from diseased to uninfected animals may be interrupted if the shedding of bacilli is reduced or prevented. To achieve this, the vaccine does not

necessarily have to prevent primary infection, but more importantly, it should rather reduce the number of mycobacteria shed to below that required to maintain the disease in these populations [7].

The need for an effective tuberculosis vaccine for animals is immense, but the target species for such a vaccination programme will probably vary from one country to another [8]. An added advantage of vaccination, even if the BCG vaccine does not provide full protection against the disease, is that it appears to reduce the severity of the disease and subsequent mycobacterial shedding and contamination of the environment [9]. In addition, it is important to realise that under cool, moist and shady environmental conditions, *M. bovis* can survive outside its host for long enough to significantly increase the likelihood that other animals may become infected and develop disease [10,11]. Reduced contamination of the environment may therefore also limit the subsequent spread of disease.

BCG (Bacille Calmette-Guérin) is the only vaccine currently available for the control of tuberculosis in humans and animals that has proven safety and efficacy [12]. In the past, this vaccine has been tested in a number of domesticated and wildlife species, with a marked variability in efficacy [7,13-15], which may limit its suitability for use as a standard vaccine in domestic livestock and wildlife. Factors that have been shown to affect the efficacy of BCG vaccine in experimental protocols include the age of the animals when vaccinated, the method of vaccine formulation, delivery system and dosage, and whether single or multiple booster doses were used [16,17]. Experimental vaccination induced excellent protection in red deer (*Cervus elaphus*), when a booster was administered eight weeks after the initial BCG vaccination [18].

At present, many of the practical issues that are central to the successful vaccination of wildlife are currently being researched [7,19]. Ideally, a vaccine should provide long-term protective immunity, after a single immunization. It should also be cost-effective and an appropriate delivery system should be available. The vaccination of rare and endangered species in the Kruger National Park against anthrax using remote injection and the successful vaccination of foxes against rabies using oral bait vaccines in Europe, suggest that the concept is feasible. The efficacy of a vaccine for wildlife should preferably be evaluated in the target species itself [7]. When evaluating vaccine efficacy the experimental challenge should mimic natural transmission, in both route and dose under controlled conditions. Likewise, lesions produced should resemble those found in natural infections. This approach has the additional advantage of excluding complications associated with prior *M. bovis* infection, an intangible factor that may confound field studies of vaccine efficacy [19]. The intratonsilar method has been shown to be effective in creating BTB lesions that mimic natural infection in cattle [20], red deer [21] and African buffaloes [22]. With a suitable challenge

model in place for buffalo, it was possible to aim for a study to evaluate the efficacy of the BCG (Pasteur 1173P2) vaccine in this species.

2. Materials and methods

Two different studies were carried out for the evaluation of the efficacy of BCG in buffalo. In the first study, groups of 15 vaccinated and 15 unvaccinated buffaloes were kept in enclosures of 600 m^2 each, which were located within the research facility in the KNP. Following two vaccinations, during the period nine to 18 weeks after *M. bovis* challenge, a clinical outbreak of foot-and-mouth disease occurred in both treatment groups, accompanied by pronounced lesion development and associated pain and discomfort in the affected buffaloes [23]. On analysis of the results of the pathological examination and bacterial cultures it was found that, although the total number of lymph nodes affected in animals from the control group was higher than that of the vaccine group, the difference was not statistically significant ($p > 0.05$) (de Klerk, unpublished data). We are of the opinion that the outbreak of clinical disease could have influenced the outcome of the vaccination trial and the data obtained will therefore not be included in this report.

The design of the second study aimed at documenting transmission patterns of *M. bovis* from adult infected buffalo cows to the treatment groups of buffalo yearlings under semi-free ranging conditions. Given that no indication of transmission of *M .bovis* to the yearlings could be demonstrated after one year of contact, it was decided to conduct an experimental infection. This report describes the design and findings of the second vaccination trial, carried out under semi-free ranging conditions and providing opportunities for natural as well as experimental *M. bovis* challenge.

2.1. Animals, husbandry and monitoring

Thirty buffalo yearlings, varying in age from 10–20 months were captured during one week in October 2002 from four different BTB-negative buffalo herds in the northern districts of the Kruger National Park: Mahonie Loop (22° 40′ 10.3″ south; 31° 01′ 40.2″ east); Elandskuil (22° 42′ 55.7″ south; 31° 10′ 09.5″ east); Masakhosa Pan (22° 37′ 47.3″ south; 31° 16′ 53.5″ east) and Shingomeni (22° 54′ 12.9″ south; 31° 26′ 35.8″ east). At the capture site each animal was ear-tagged and skin-branded. Blood in heparin was collected from each buffalo by venipuncture for the interferon-gamma (IFNg) assay as well as bovine and avian tuberculin PPD injected intradermally for the comparative skin test as described previously [22]. Immediately following capture the yearlings were transferred to an enclosure near the Shingwedzi camp (northern region of KNP) where they were kept for seven days until test results for the IFNγ assay and

skin test were available. All animals tested negative and were transported to the State Veterinary Unit enclosure in Skukuza, where they were randomly divided into two groups of 15 animals each. One group received a defined dose of BCG vaccine while animals in the control group were sham vaccinated (PBS). After a period of three months in these enclosures the yearlings were released into a larger camp (100 hectares) under semi-free ranging conditions, where they joined 27 adult buffalo cows that originated from known BTB infected buffalo herds in the south of the KNP [1,24] in the Crocodile Bridge region of the Park, namely: Mpanamanadam (25° 17′ 40.0″ south; 31° 58′ 34.2″ east); Mhlanganzwanedam (25° 13′ 28.7″ south; 31° 58′ 55.1″ east) & Makambene spruit (25° 19′ 20.3″ south; 31° 54′ 08.2″ east). Animals were ear-tagged and branded at the capture site and all cows were skin tested while inside a smaller enclosure within the semi-free range camp. The test positive buffaloes were intended to serve as natural source of infection to the yearling buffalo.

Although the buffaloes could graze freely, supplementary feed (lucerne and teff hay) was also provided due to the large number of animals present within the camp. The camp was inspected twice daily for fence breakages and to ensure sufficient feed and fresh water supplies to the buffaloes. Several cows were pregnant at the time of capture and all the calves born to the cows in the semi-free range camp were retained in the study. Altogether 16 calves were born during the two-year study, of which ten survived.

2.2. Anaesthesia

A combination of etorphine hydrochloride (M99; Novartis SA Animal Health) and xylazine (Chanazine 2%; Centaur, Bayer Animal Health) at standard dosages used for routine buffalo immobilization was used to anaesthetize the yearlings for intratonsilar infection. Xylazine was used because of its muscle relaxant properties, which enhanced the relaxation of the jaw muscles, facilitating the opening of the mouth during the intratonsilar infection procedure. Thereafter a combination of M99 and azaperone (Stresnil; Janssen Animals Health) was applied to immobilize the buffalo. In all instances the animals were revived by administering the antidote diprenorphine hydrochloride (M50/50; Novartis SA Animal Health) at twice the dosage of the M99. When xylazine was used to immobilize calves, 3 – 5ml of yohimbine (Kyron Laboratories) was administered additionally to reverse the effects of this component at the same time.

2.3. Blood specimens and weight gain

During 2003 (the first year of the semi-free range study), blood samples were collected at capture (two months prior to primary vaccination) (-2 mths), at primary vaccination (0 mths), at booster vaccination (1.5 mths) and six months after primary vaccination (6 mnths) from all experimental buffalo yearlings, the calves as well as from cows that tested negative in the IFNg assay.

After the experimental groups were challenged in 2004 (14 months after primary vaccination), blood samples were collected every eight weeks (16 mths, 18 mths, 20 mths, 22 mths) until one month prior to slaughter. Body conditions were scored throughout the study and all the carcasses were weighed at the abattoir after euthanasia at conclusion of the study.

2.4. BCG vaccine

The BCG seed stock (Pasteur 1173P2) was grown in 7H9 Middlebrook broth (Difco Laboratories, Detroit, USA) supplemented with 10% OADC (3.83g NaCl, 25g BSA, 15ml sodium oleate and 20ml of 50% glucose in 465ml of distilled water) and 0.05% Tween 80 (VWR International, Merck House, UK) at 37°C, without shaking, to the mid-logarithmic growth phase. Bacteria were harvested by centrifugation, washed three times and the re-suspended bacteria were enumerated using a phase contrast microscope. The actual number of colony-forming units was determined retrospectively by plating out serial dilutions of the inoculum dose. Plate counts for the semi-free range study reflected a dosage of 3.2×10^7cfu for the primary and of 4.4×10^7cfu for the booster vaccination.

2.5. Experimental infection procedure

After one year of the study no evidence of natural *M. bovis* transmission was evident by IFNg assay and it was hence decided to challenge the buffaloes via the intratonsilar route. All the buffalo yearlings were experimentally infected on two consecutive days with the first group receiving 1×10^3 cfu and the second group 6×10^2 cfu of *M. bovis*, respectively. using the method as previously described [22].

2.6. Mycobacterium bovis strain

To be able to distinguish between experimentally and natural challenged animals, a genetically different *M. bovis* field strain was used for the experimental challenge of the two treatment groups. This challenge strain (TB 1088), a field isolate from KNP, possessed a unique IS*6110* RFLP pattern, different from the genotypes commonly found prevalent in the buffalo herds of the southern region of KNP (Michel, in prep.).

2.7. Laboratory tests

Interferon-gamma (IFNg) assay A modified protocol for the IFNg assay applicable in buffaloes was used [25,26]. The responses to mycobacterial antigens were used as markers of response to BCG vaccination [27,28].

2.8. Comparative intra-dermal tuberculin test

The method for the intra-dermal tuberculin test and its interpretation was applied as described previously [22] on all cows and both treatment groups of buffalo yearlings on the day of capture. This test was carried out once only, with the aim of determining the BTB status of each animal at that specific time and to avoid any influence of this test on IFNg results during the course of the study.

2.9. Euthanasia

At the end of the study, all experimental and in-contact buffaloes were euthanased using 2 - 3ml of a saturated solution (850mg/ml) of succinyl dicholine chloride (Scoline) intramuscularly by darting. The jugular veins were cut for exsanguination.

2.10. Necropsy procedure

The buffalo carcases were taken to the abattoir and immediately subjected to a detailed meat inspection and post mortem examination. Selected lymph nodes were removed from the carcases and after being thinly sliced with a trimming blade (Accu-Edge, Bayer Healthcare) the cut surfaces were carefully examined with the naked eye for the presence of macroscopic granulomatous lesions. The left and right palatine tonsils and both medial retropharyngeal lymph nodes were processed separately and equally divided for mycobacterial culture and histo-pathology. Specimens of all lesions detected in any of the other lymph nodes or organs, were collected and processed for mycobacterial culture and histopathology. Specimens from other lymph nodes (as indicated below) were pooled for culture:

- Pooled head lymph nodes: Mandibular and parotid lymph nodes
- Pooled thoracic lymph nodes: Mediastinal and bronchial lymph nodes
- Pooled abdominal lymph nodes: Mesenteric, hepatic, renal, omasal, and abomasal lymph nodes
- Pooled carcass lymph nodes: Superficial cervical, axillary, popliteal, inguinal, and mammary or scrotal lymph nodes.

The lungs were systematically palpated to detect granulomas, and then systematically sliced with a sharp knife to aid visualisation of any undetected lesions. All visible lesions were collected for histopathology and culture. Specimens for histopathology were preserved in 10% buffered formalin and transferred to the Department of Pathology at the Faculty of Veterinary Science, University of Pretoria, for histopathological examination.

2.11. Grading of macroscopic lesions

The following system was used to grade macroscopic tuberculous lesions:

Grade 0: No visible lesions
Grade 1: Less than 50% of one lymph node or tonsil affected
Grade 2: More than 50% of one lymph node or tonsil affected, but not more than 2 lymph nodes involved
Grade 3: More than two lymph nodes affected, but no organs involved
Grade 4: Pulmonary lesions and/or multiple lymph nodes affected
Grade 5: Lesions in multiple lymph nodes, lungs and other organs (milliary disease)

2.12. Bacterial isolation

Pooled specimens were collected as indicated and stored in sterile containers at minus 20°C. At the end of the study the frozen specimens were transferred to the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute. The specimens were processed and mycobacterial culture and identification of isolates were performed as described by Bengis et al. [29]. Cultures were considered negative for *M. bovis* if no growth was detected after 10 weeks incubation.

2.13. Statistical methods

Analysis of variance (ANOVA) was the method of choice for statistical analyses performed on the IFNg data [30].

3. Results

3.1. Clinical signs and weight loss

Due to the nature of the semi free-range study body mass could not be determined but condition scores were recorded whenever animals were captured to collect blood samples. Three animals were euthanased following severe loss of body weight. One, an adult cow (LM 47), had extensive lesions typical of BTB at necropsy. The other two were yearlings (LM30~ and LM35~) and both showed dental malocclusion involving the mandibular molars, interfering with normal mastication. No lesions consistent with BTB were observed in these two animals.

3.2. Laboratory tests

Interferon-gamma assay

All the experimental yearlings tested negative for BTB on the IFNg assays at capture. Two months later, at the time of administering the primary vaccination with BCG, the buffaloes were tested again and all were found negative after stimulation with bovine PPD. Differences between the optical density values (bovine PPD stimulated plasmas only) of the two treatment groups (vaccine and control), are indicated in Fig. 1.

Table 1. Gross pathology and histopathology results for control and vaccinated buffaloes

Number	Gender	Gross pathology					Histopathology	Culture
		Head lnn.	Thoracic lnn.	Lung	Other	Grading		
Control group								
LM1~	Male	+	-	-	-	2	+	-
LM2~	Male	-	-	-	-	0	-	+
LM3~	Female	+++	-	+	-	4	+	+
LM5~	Female	+++	+	-	-	3	+	+
LM7~	Male	+++	-	-	-	2	-	+
LM24~	Male	-	-	-	-	0	-	-
LM25~	Male	-	-	-	-	0	-	-
LM26~	Male	++	-	-	-	2	+	+
LM27~	Male	+++	-	-	-	2	+	+
LM28~	Female	+	-	-	-	1	+	+
LM29~	Female	-	-	-	-	0	-	-
LM32~	Male	-	-	-	-	0	+	+
LM35~	Female	++	-	-	-	2	+	+
Vaccine group								
LM4~	Female	+++	++	-	-	3	+	+
LM9~	Male	-	-	-	-	0	-	-
LM10~	Male	-	+	-	-	1	+	+
LM11~	Male	-	-	-	-	0	-	-
LM12~	Male	-	-	-	-	0	+	-
LM14~	Male	+++	+	-	-	2	+	+
LM15~	Female	+	-	-	-	2	+	+
LM17~	Male	-	-	-	-	0	-	-
LM18~	Male	-	-	-	-	0	-	-
LM19~	Female	+	-	-	-	1	+	+
LM20~	Female	+	-	-	-	1	+	+
LM21~	Male	-	-	-	-	0	+	+
LM22~	Female	-	-	-	-	0	-	-
LM23~	Female	+	-	-	-	3	+	+

Histopathology + : Granuloma associated with BTB infection in buffaloes;
Histopathology - : No granuloma typical for BTB could be detected

Five months after the booster vaccination a significant difference could be detected between the IFNg responses to bovine PPD ($P < 0.01$) between the vaccinated and control animals and was still detectable one year post vaccination when the animals were challenged (Fig. 1). Following challenge with live *M. bovis*, an increase in IFNg responses was observed in vaccinated and control

Table 2. Gross pathology and histopathology results of in-contact cows and calves

Number	Gross pathology					Histopathology	Culture
	Head lnn.	Thoracic lnn.	Lung	Other	Grading		
Adult cows							
LM 40~	-	-	+	-	4	+	+
LM 41~	-	+	-	-	1	+	-
LM 42~	-	-	-	-	0	+	+
LM 43~	-	-	-	-	0	+	-
LM 44~	-	-	-	-	0	+	+
LM 45~	-	-	-	-	0	-	-
LM 46~	+++	++	+++	-	5	+	+
LM 47~	+++	+++	+++	-	5	+	+
LM 48~	+	-	++	-	4	+	+
LM 49~	+	-	-	-	1	+	MOTT
LM 50~	-	-	++	-	4	+	+
LM 51~	-	-	-	-	0	-	-
LM 52~	+	-	++	-	4	+	+
LM 53~	-	-	-	-	0	-	-
LM 54~	+++	+	++	++	4	+	+
LM 55~	-	-	-	-	0	-	-
LM 56~	+	++	++	-	4	+	+
LM 59~	-	-	-	-	0	+	-
LM 60~	-	+++	+++	-	4	+	+
LM 61~	-	++	+	-	4	+	+
LM 62~	-	-	-	-	0	-	+
LM 63~	-	-	++	-	4	+	+
LM 65~	-	-	-	-	0	-	-
LM 66~	-	-	-	-	0	-	-
In contact calves							
C 1	-	-	-	-	0	-	-
C 4	-	-	-	-	0	-	-
C 8	-	-	-	-	0	-	-
C 9	-	-	-	-	0	-	-
C 10	-	+	+	-	4	+	-
C 11	-	-	pneumonia	-	0	+	+
C 12	-	-	-	-	0	-	+
C 14	-	-	-	-	0	-	-
C 15	-	-	-	-	0	-	-
C 16	-	-	-	-	0	-	-

animals. The responses in control animals were higher than in vaccinated ones. Simultaneous IFNg reactivity to avian PPD was observed sporadically in some animals but at a significant level 12 months post vaccination in six vaccinated and two control buffaloes (results not shown).

3.3. Comparative intra-dermal tuberculin test

All buffalo yearlings were found to be negative in the comparative tuberculin skin test at capture. Fifteen of the 24 in-contact cows had positive results on the intra-dermal tuberculin test (results not shown).

3.4. Pathology and culture

Control animals

Eight of the thirteen animals had macroscopic lesions while nine were culture positive for *M. bovis.* For one buffalo (LM1~) with macroscopic lesions, confirmed by histopathology to be typical for bovine tuberculosis, the culture result was negative.

Vaccinated animals

Seven of the fourteen animals had macroscopic lesions while eight were positive for *M. bovis* on bacterial culture. One male buffalo (LM12~) which showed no macroscopic lesions and was culture negative for *M. bovis*, histopathological examination revealed a typical granulomatous mycobacterial reaction. The strain used for experimental infection, which showed a unique IS*6110* RFLP profile, was isolated from both control and vaccinated animals. Results for gross pathology and histopathology are summarised in Table 1.

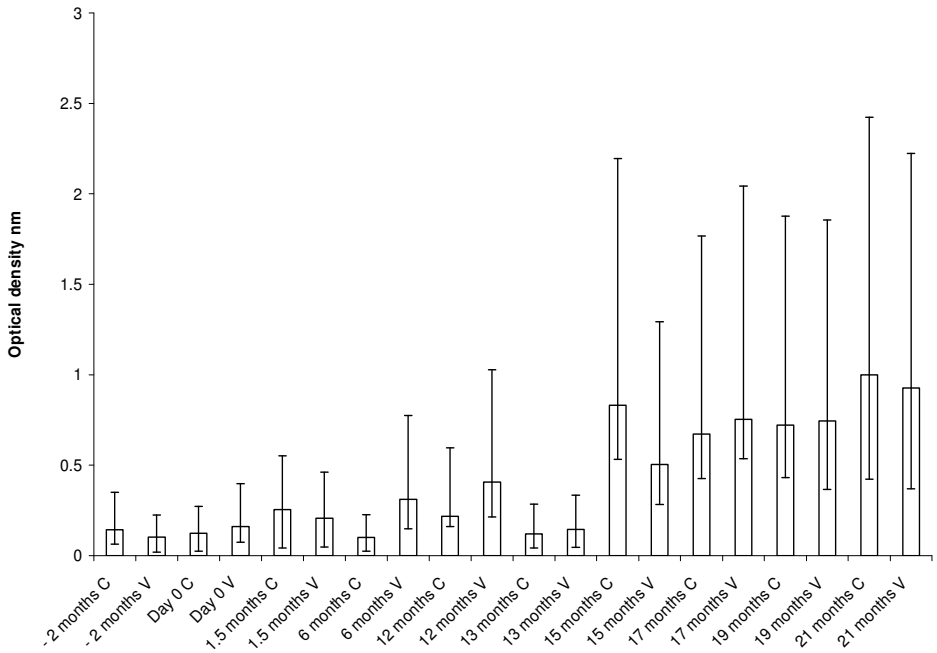
In-contact cows

Fourteen of the 24 cows were found to be positive for *M. bovis* on culture (Table 2). *Mycobacterium bovis* was cultured from eleven of the thirteen cows with macroscopic lesions consistent with bovine tuberculosis. In addition, *M. bovis* was isolated form three cows with non-visible lesions. The strains were typed and showed the same IS*6110* RFLP profile typical for strains isolated from buffaloes in the southern region of KNP (strain C8, Michel in prep.). This profile was indeed different from the profile of the strain (TB 1088) used for experimental infection. Histopathological examination of two cows with macroscopic lesions revealed granulomatous lesions consistent with those of bovine tuberculosis.

4. Discussion

Horizontal disease transmission between the in-contact cows and the experimental buffaloes failed, despite the fact that the majority of in-contact cows could be considered as potent source of infection. Eleven of the 17 culture positive cows presented with macroscopic lung lesions, of which nine represented cases of disseminated tuberculous pneumonia (Table 2). One of these cows even had to be euthanased due to severe emaciation because of generalised tuberculosis (LM 47) half-way through the study. The distribution of lesions confirms that aerosol is the most important mode of natural disease transmission between buffaloes and this is strongly supported by the presence of macroscopic lesions and isolation of *M. bovis* from pulmonary tissues of very young calves born to the in-contact cows during the study (Table 2). But it also demonstrated that the opportunity for close contact with infected animals was, on its own, not sufficient to effect disease transmission.

Figure 1: Comparison between the optical densities of bovine PPD stimulated plasmas in control (c) and vaccinated (v) animals from capture until slaughter



-2 months: Day of capture; Day 0: Primary vaccination; 13 months: Day of experimental challenge with live *M. bovis*

It should be noted here that the yearlings did not interact closely with the infected in-contact cows, because they were introduced from different social groupings and this was aggravated by the fact that the majority of cows had their

own offspring they were associated with. Essentially the same observation was reported for natural transmission of *M. bovis* between captive brushtail possums [31]. We therefore conclude that regular and close physical contact i.e. by licking, grooming, suckling between animals over a longer period of time is necessary for successful disease transmission to occur. Our results suggest that after one year of contact, no transmission from infected cows to in-contact calves occurred, whereas transmission from infected cow to offspring took place. Typing of the strains isolated after experimental and natural infection in the different groups of animals strongly supports this.

If vaccination of animals can lead to a lesser degree of disease resulting in a reduced risk of animals becoming shedders of *M. bovis,* there may be merit in pursuing vaccination as the most or only feasible option for controlling BTB in free-ranging buffalo populations. The establishment of an infection model for BTB in African buffaloes [22] enabled us to conduct a vaccination trial to assess the efficacy of BCG in this reservoir species. The outcome of this study indicates that the BCG vaccine does not protect buffaloes under semi-free-ranging conditions from BTB infection and disease.

In trying to identify the potential causes and contributing factors for the failure, we reviewed those with a reported impact on vaccine efficacy including route of administration, booster applications, infection dose, immunogenicity, exposure to non-tuberculous mycobacteria, age and type of target species [32]. The intramuscular vaccine delivery has been preferred over the more commonly used subcutaneous route for reasons of practicality in vaccinating large buffalo herds from the air. Boostering was previously found necessary to protect against establishment of infection in deer [19] and since no data were available for buffalo the same concept was adopted. The infectious dose of *M. bovis* that was used, induced typical lesions of tuberculosis in our previous study, establishing the *M. bovis* experimental infectious model in buffalo. The use of an *M. bovis* strain showing a different RFLP profile than the anticipated natural infection strain was critical in assessing transmission patterns.

Recently, it has been shown that different BCG strains (lyophilised or in solution formulations) induced the same level of protection although the immune responses measured in the IFNg test was different. This particular study suggested that there is no correlation between the amount of IFNg release after in vitro stimulation and the degree of protection conferred [27].

We compared the increase in IFNg response between the vaccinated and control animals at pre- and post-vaccination intervals. There was a significant difference between IFNg response in the bovine PPD stimulated plasmas of the vaccinated and control groups at six months post vaccination, demonstrating a biological activity of BCG in the vaccinated buffalo. This was still persistent one year after

vaccination. It must, however, be mentioned that IFNg reactivity in the vaccinated animals was not only detected to bovine PPD but also to avian PPD, Twelve months after BCG vaccination responses to both bovine and avian PPD occurred in six animals in the vaccination group as compared to two animals in the control group (results not shown), Buddle et al. [33] reported similar observations for the skin test in BCG vaccinated cattle.

A strong IFNg response does not necessarily mean protection to subsequent challenge [27], but may be the result of non-specific stimulation following infection with environmental mycobacteria [34]. We have shown in a parallel investigation to the vaccination study that the water in the buffalo camp contained various environmental non-tuberculous mycobacteria (NTM) [24], which could have negatively influenced the immune response of the buffaloes to *M. bovis*. Buddle et al [35] in fact reported that sensitisation to *Mycobacterium avium* adversely affected the protective efficacy of the BCG vaccine. Similar findings have been published on the effects of other environmental mycobacteria [36, 37]. All together these findings suggest that sensitisation to NTM took place and may have adversely affected the protective efficacy of the BCG vaccine.

Literature reports provide indications that the age of the vaccinates has a direct influence on the vaccine efficacy. Neonates and very young animals are usually better vaccine responders [12, 38], which may be due to younger animals having less exposure to environmental mycobacteria and because maternal immunity may have protected them from infection. The ages of the experimental animals were generally underestimated during selection of the groups for capture and the majority were older than 12 months at the start of the study, which could have compromised vaccine efficacy.

Gender was previously not found to be a risk factor in buffalo for developing bovine tuberculosis [1]. In the present study it was striking that 9 out of 11 challenged female buffaloes had tuberculous lesions, while only six out of 16 males showed lesions (Table 1). All buffaloes with the highest lesion scores of (3) and (4) were female, in contrast to males for which the maximum lesion score was (2) (Table 1). The reason for the observed difference in lesion development between the gender groups is not clear at this stage, but continued social stress in the young heifers due to competition with the older cows may have played a significant role.

The virulence of the KNP *M. bovis* strain used for challenge might have had an influence on vaccine efficacy but unfortunately very little is known about the virulence of different *M. bovis* strains throughout the world [39].

Finally, the process of identifying and applying an effective vaccine for BTB in buffaloes poses considerable challenges, both logistical and developmental. According to Cross & Getz [40] the success of a vaccination programme will depend largely on the half-life and ability of protection of the vaccine as well as the number of new calves that will need to be vaccinated every year. Because no vaccine is currently available which meets these requirements, vaccination alone cannot be expected to successfully limit the spread of the disease and should ultimately be used in combination with other control measures. A combined strategy of vaccinating the young cohorts and culling the more severely affected older individuals could be considered as a possible future solution to limit the spread of BTB and to reduce the prevalence of the infection in the KNP buffalo populations.

Acknowledgements

The authors are grateful for the input and co-operation of the following groups of people, for without them these projects would not have been possible. Field staff of the Office of the State Veterinarian for the care and feeding of the animals and daily cleaning of the boma facilities as well as the veterinary technicians for skin test and necropsy procedures; the veterinarians and operational staff from the Veterinary Wildlife Services team for capture, transport and weighing of the calves; the laboratory assistants for blood processing and banking, and all the conservation students for their help during the sampling sessions. A special thanks to Albert Linsky (Senior Meat Inspector) and his team for the slaughtering and processing of the buffalo carcasses. This project would not have been possible without the major financial contributions of the Smithsonian Institution and Shirley Sichel, in the USA. We would also like to thank the Peace Parks Foundation for their contribution towards funding of laboratory procedures and diagnostics.

References

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

[2] Rodwell TC, Kriek NP, Bengis RG, Whyte IJ, Viljoen PC, De Vos V. et al. Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. J Wildl Dis 2001; 37:258–264.

[3] Keet DF, Kriek NPJ, Penrith ML, Michel AL, Huchzermeyer H. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. Onderstepoort J Vet Res 1996;63:239–244.

[4] Keet DF, Kriek NPJ, Bengis RG, Grobler DG, Michel AL. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. Onderstepoort J Vet Res 2000; 67: 115–122.
[5] Keet DF, Kriek NPJ, Bengis RG, Michel AL. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park. Onderstepoort J Vet Res 2001; 68: 225–230.
[6] Michel AL. Implications of tuberculosis in African wildlife and livestock. Ann NY Acad Sci 2002; 969:251–255.
[7] Buddle BM, Skinner MA, Chambers MA. Immunological approaches to the control of tuberculosis in wildlife reservoirs. Vet Immun Immunopath 2000; 74:1–16.
[8] McMurray DN. A coordinated strategy for evaluating new vaccines for human and animal tuberculosis. Tuberculosis 2001; 81:141–146.
[9] Cross ML, Labes RE, Griffin JFT, Mackintosh CG. Systemic but not intra-testinal vaccination with BCG reduces the severity of tuberculosis infection in ferrets (*Mustela furo*). Int J Tuberc Lung Dis 2000;4: 473–480.
[10] Wray C. Survival and spread of pathogenic bacteria of veterinary importance within the environment. Vet Bull 1975; 45: 543–550.
[11] Tanner M., Michel AL. Investigation of the viability of *M.bovis* under different environmental conditions in the Kruger National Park. Onderstepoort J Vet Res 1999; 66:185–190.
[12] Hope JC, Vordermeier HM. Vaccines for bovine tuberculosis: current views and future prospects. Expert Rev Vaccines 2005; 4:891–903.
[13] Buddle BM, Keen D, Thomson A, Jowett G, Mccarthy AR, Heslop J., De Lisle GW. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae.* Res Vet Sci 1995; 59:10–16.
[14] Corner LAL, Buddle BM, Pfeiffer DU, Morris RS. Aerosol vaccination of the brushtail possum (*Trichosurus vulpecula*) with bacille Calmette-Guérin: the duration of protection. Vet Microbiol 2001; 81:181–191.
[15] Corner LAL, Buddle BM, Pfeiffer DU, Morris RS. Vaccination of the brushtail possum (*Trichosurus vulpecula*) against *Mycobacterium bovis* infection with bacille Calmette-Guérin: the response to multiple doses. Vet Microbiol 2002;84:327–336.
[16] Griffin JFT, Mackintosh CG, Slobbe L, Thomson AJ, Buchan GS. Vaccine protocols to optimise the protective efficacy of BCG. Tuber Lung Dis 1999; 79 :135–143.
[17] Cross ML, Labes RE, Griffin JFT, Mackintosh CG. Oral infection of ferrets with virulent *Mycobacterium bovis* or *Mycobacterium avium*: susceptibility, pathogenesis and immune responses. J Comp Pathol 2000; 123;15–21.
[18] Griffin JFT, Chinn DN, Rodgers CR, Mackintosh CG. Optimal models to evaluate the protective efficacy of tuberculosis vaccines. Tuberculosis 2001; 81:133–139.

[19] Griffin JFT. Veterinary tuberculosis vaccine development. *Clin Infect Dis* 2000; 30:223–228.

[20] Palmer MV, Whipple DL, Rhyan JC, Bolin CA, Saari DA. Granuloma development in cattle after intratonsilar inoculation with *Mycobacterium bovis*. Am J Vet Res 1999; 60:310–1315.

[21] Mackintosh CG, Waldrup K, Labes R, Buchan G, Griffin JFT. Intra-tonsil inoculation: an experimental model for tuberculosis in deer. Tuberculosis in wildlife and domestic animals. Dunedin, New Zealand: Otago University press, 1995:121-123.

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

[23] Vosloo W, de Klerk L-M, Boshoff CI, Botha B, Dwarka RM, Keet D, Haydon DT. Characterization of a SAT-1 outbreak of foot-and-mouth disease in captive African buffalo (*Syncerus caffer*): Clinical symptoms, genetic characterisation and phylogenetic comparison of outbreak isolates. Vet Microbiol 2006; 120:226-240.

[24] Michel AL, de Klerk LM, Gey van Pittius NC, Warren RM, van Helden PD. Bovine tuberculosis in African buffaloes: Observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. BMC Vet Res 2007;3:23.

[25] Michel AL, Nel M, Cooper D, Morobane RN. Field evaluation of a modified "gamma interferon" assay in African buffalo (*Syncerus caffer*) and cattle in South Africa. *Proceedings of the 3rd International Conference on Mycobacterium bovis,* 14 – 16 August 2000, Cambridge, United Kingdom.

[26] Grobler DG, Michel AL, de Klerk L, Bengis RG. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J Vet Res 2002; 69:221–227.

[27] Wedlock DN, Denis M, Vordermeier HM, Hewinson RG, Buddle BM. Vaccination of cattle with Danish and Pasteur strains of *Mycobacterium bovis* BCG induce different levels of IFN gamma post-vaccination, but induce similar levels of protection against bovine tuberculosis. Vet immunol Immunopathol 2007; 118:50-58.

[28] Weir RE, Fine PEM, Floyd S, Stenson S, Stanley C, Branson K, Britton WJ, Huygen K, Singh M, Black GF, Dockrell HM. Comparison of IFN-γ responses to mycobacterial antigens as markers of response to BCG vaccination. Tuberculosis (in press)

[29] Bengis RG, Kriek NPJ, Keet DF, Raath JP, de Vos V, Huchzermeyer HFAK. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer* – Sparrman) population in the Kruger National Park: a preliminary report. Onderstepoort J Vet Res 1996; 63:5–18.

[30] Zar JH, 1974. Multisample hypotheses in *Biostatistical Analysis,* Pretence-hall Inc., Englewood Cliffs.

[31] Corner LA, Pfeiffer DU, de Lisle GW, Morris RS, Buddle BM. Natural transmission of *Mycobacterium bovis* infection in captive brushtail possums (*Trichosurus vulpecula*). N Z Vet J 2002; 50:154-162.

[32] Suazo FM, Escalera AMA, Gallegos Torres RM. A review of *M. bovis* BCG protection against TB in cattle and other animals species. Prevent Vet Med 2003; 58:1-13.

[33] Buddle BM, Wedlock DN, Denis M, Skinner MA. Identification of immune response correlates for protection against bovine tuberculosis. Vet Immunol Immunopathol 2005; 108:45-51.

[34] Demangel C, Garnier T, Rosenkrands I, Cole ST. Differential effects of prior exposure to environmental mycobacteria on vaccination with *Mycobacterium bovis* BCG or a recombinant BCG strain expressing RD1 antigens. Infect Immun 2005; 73:2190-2196.

[35] Buddle BM, Wards BJ, Aldwell FE, Collins DM, de Lisle GW. Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. Vaccine 2002; 20:1126–1133.

[36] Kamala T, Paramasivan CN, Herbert D, Venkatesan P, Prabhakar R. Immune response & modulation of immune response induced in the guinea-pigs by *Mycobacterium avium* complex (MAC) & *M. fortuitum* complex isolates from different sources in the south Indian BCG trial area. Appl Environ Microbiol 1996; 103:201-211.

[37] Brandt L, Cunha JF, Weinreich Olsen A, Chilima B, Hirsch P, Appelberg R. et al. Failure of the *Mycobacterium bovis* BCG Vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect Immun 2002; 70:672-678.

[38] Buddle BM, Wedlock DN, Parlane NA, Corner LA, De Lisle GW, Skinner MA. Revaccination of neonatal calves with *Mycobacterium bovis* BCG reduces the level of protection against bovine tuberculosis induced by a single vaccination. Infect Immun 2003; 71:6411-6419.

[39] Hewinson RG, Vordermeier HM, Smith NH, Gordon SV. Recent advances in our knowledge of *Mycobacterium bovis*: a feeling for the organism. Vet Microbiol 2006; 112:127-139.

[40] Cross PC, Getz WM. Assessing vaccination as a control strategy in an ongoing epidemic: Bovine tuberculosis in African buffalo. Ecol Modell 2006; 196:494–504.

Chapter 5.3

Bovine tuberculosis in African buffaloes: observations regarding Mycobacterium bovis shedding into water and exposure to environmental mycobacteria

Anita L Michel[1], Lin-Mari de Klerk[2], Nico C Gey van Pittius[3], Rob M Warren[3], Paul D van Helden[3]

[1] *Tuberculosis Laboratory, ARC-Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa*

[2] *Game Capture Unit, South African National Parks, Private Bag x402, Skukuza 1350, South Africa*

[3] *DST/NRF Centre of Excellence in Biomedical Tuberculosis Research, US/MRC Centre for Molecular and Cellular Biology, Department of Biomedical Science, Faculty of Health Sciences - Stellenbosch University, P.O. Box 19063, Tygerberg 7505, South Africa*

BioMedCentral: Veterinary Research, 2007, 3:23

Abstract

Background

African buffaloes are the maintenance host for *Mycobacterium bovis* in the endemically infected Kruger National Park (KNP). The infection is primarily spread between buffaloes via the respiratory route, but it is not known whether shedding of *M. bovis* in nasal and oral excretions may lead to contamination of ground and surface water and facilitate the transmission to other animal species. A study to investigate the possibility of water contamination with *M. bovis* was conducted in association with a BCG vaccination trial in African buffalo. Groups of vaccinated and nonvaccinated buffaloes were kept together with known infected in-contact buffalo cows to allow natural *M. bovis* transmission under semi-free ranging conditions. In the absence of horizontal transmission vaccinated and control buffaloes were experimentally challenged with *M. bovis.* Hence, all study buffaloes in the vaccination trial could be considered potential shedders and provided a suitable setting for investigating questions relating to the tenacity of *M. bovis* shed in water.

Results

Serial water samples were collected from the drinking troughs of the buffaloes once per season over an eleven-month period and cultured for presence of mycobacteria. All water samples were found to be negative for *M. bovis*, but 16 non-tuberculous *Mycobacterium spp.* isolates were cultured. The non-tuberculous *Mycobacterium* species were further characterised using 5'-16S rDNA PCR-sequencing, resulting in the identification of *M. terrae, M. vaccae* (or *vanbaalenii*), *M. engbaekii, M. thermoresistibile* as well as at least two species which have not yet been classified.

Conclusion

The absence of detectable levels of *Mycobacterium bovis* in the trough water suggests that diseased buffalo do not commonly shed the organism in high quantities in nasal and oral discharges. Surface water may therefore not be likely to play an important role in the transmission of bovine tuberculosis from buffalo living in free-ranging ecosystems. The study buffalo were, however, frequently exposed to different species of non-tuberculous, environmental mycobacteria, with an unknown effect on the buffaloes' immune response to mycobacteria.

Background

Tuberculosis in wildlife, caused by *Mycobacterium bovis,* has emerged as an increasingly important disease of free-ranging wildlife populations [1-3]. The

African buffalo (*Syncerus caffer)* has established itself as a maintenance host for *M. bovis* in two of South Africa's largest conservation areas, namely the Kruger National Park (KNP) and the Hluhluwe iMfolozi Park (HiP) [4,5]. Transmission of *M. bovis* between herd members occurs most frequently by aerosol, whereas spillover to other species requires different modes of transmission [6]. Predators and scavengers alike contract the infection commonly by ingestion of infected tissues [3]. Other pathways may apply only to particular animal species. The secretion of infectious pus from draining fistulae of parotid lymph glands, for example, has been suggested as a mode of transmission between greater kudu (*Tragelaphus strepsiceros*) [7], as well as between cattle and kudu [8]. Contaminated faeces have been implicated in the spread of bovine tuberculosis (BTB) within a troop of baboons (*Papio ursinus*) [9]. Hence, environmental *M. bovis* contamination may be a side effect of events leading to spillover or it may be the cause of spillover itself. If pathogenic microorganisms can retain their viability for some time outside the animal host, environmental sources could play a significant role in their spread to a wide range of animal species from different habitats and ecological niches. To this effect, it has been shown that *M. bovis* can survive for 42 days in tissues with lesions and up to four weeks in faecal material from buffalo [10]. The tenacity of tubercle bacilli in effluents from sanatoria and dairies and its significance in the spread of infection to cattle were major public health concerns prior to eradication of BTB in Europe [11,12].

No information is, however, available on the role of surface water in the epidemiology of bovine tuberculosis in an endemically infected ecosystem, especially where limited water sources cause a variety of animal species to gather in high densities for most of the year.

The present study was conducted during a BCG vaccination trial in buffalo involving *M. bovis* challenge. We used this opportunity to determine whether naturally and/or experimentally infected buffalo were shedding detectable levels of *M. bovis* into the drinking water, and if so, to provide an estimate of the organism's tenacity.

Table 1. Bovine tuberculosis culture and lesion status of the different groups of study buffalo

No. of buffalo	Group	No. of buffalo surviving	Culture positive	No. buffalo with lesions
27	Experimental	27	17	15
27	In-contact	24	14	13
11	Calves*	10	2	2
Total	*	61	33	30

*One calf which died before the end of the study presented with lesions consistent with pneumonia and yielded *M. bovis* on culture of lung tissue

Results

Animals

The results of macroscopic and culture examination of all surviving study animals are summarised in Table 1. *M. bovis* infection was confirmed by culture and subsequent PCR identification of acid-fast isolates in 14 of the 24 surviving in-contact buffalo cows. Three culture positive buffalo did not yield macroscopic lesions and two buffalo with lesions in a single lymph node were culture negative. A total of 13 buffaloes presented with macroscopic lesions, two of which had lesions restricted to lymph nodes. Eleven cows presented with macroscopic lung lesions varying from pinpoint foci in two cases, to disseminated tuberculous pneumonia in nine animals (de Klerk, unpublished data).

From the experimental group, fourteen buffaloes showed visible lesions in mainly the lymph nodes of the head, with only three having secondary spread to thoracic lymph nodes. One additional buffalo had a single lung lesion. However, 17 buffaloes yielded *M. bovis* on culture. *M. bovis* infection was confirmed in two out of the ten surviving calves, both showed involvement of the thoracic lymph nodes. Another calf, which died a few weeks after birth, yielded *M. bovis* from lung tissue.

Isolation and identification of Mycobacterium spp. by polymerase chain reaction (PCR)

In contrast to the tissue samples, *M. bovis* was not cultured from any of the water samples except the spiked water used for quality control. Four inoculated trough water samples containing *M. bovis* concentrations ranging from $2 \cdot 10^5$ to $2 \cdot 10^2$/ml yielded abundant growth of *M. bovis* after three weeks for the highest and after eight weeks for the lowest concentration.

However, 16 other mycobacterial isolates were recovered from water samples (Table 2) and two others were cultured from lymph nodes of vaccinated buffalo. These 18 *Mycobacterium spp.* isolates were acid-fast on microscopic smear examination but failed to amplify the expected 372 bp product in the PCR protocol used to identify *M. tuberculosis* complex bacteria (data not shown). Subsequent analysis using 5'-16S rDNA PCR-sequencing revealed that these *Mycobacterium spp.* belonged to the species *M. terrae*, *M. engbaekii*, *M. vaccae* (or *vanbaalenii*) and two previously unidentified species closely related to *M. moriokaense* and *M. kansasii* (and *M. szulgai*), respectively (Table 2 and Figure 1). The mycobacterial species isolated from the buffalo tissues were identified as *M. thermoresistibile* and an unidentified species closely related to *M. moriokaense* (Fig. 1). Three NTM isolates could not be further identified to species level.

Table 2. Culture of water samples collected from drinking troughs of buffalo

Sampling occasion		Culture result	Water temp in cyath trough
1	October 2003	N	25
	Day 1	*M. vaccae or M. vanbaalenii*	35
	Day 2	N	37
	Day 3	N	33
	Day 4	NTM*	36.5
	Day 5	N	33
	Day 6	N	25
	Day 7	N	31
	Day 14	N	46
	Day 21	N	42.5
2	January 2004	Unknown *Mycobacterium* species closely related to *M. moriokaense*	28
	Day 1	P/C	37
	Day 2	C	36.5
	Day 3	*M. terrae*	30
	Day 4	N	29
	Day 5	P/C	34
	Day 6	*M. engbaekii*	36
	Day 7	N	34
	Day 14	P/C	30
	Day 21	P/C	32
3	26 April 2004	C	23.6
	Day 1	*M. terrae*	20.0
	Day 2	N	22.0
	Day 3	*M. engbaekii*	26.5
	Day 4	N	26.5
	Day 5	*M. terrae*	26.0
	Day 6	NTM*	26.5
	Day 7	*M. terrae*	24.5
	Day 14	*M. engbaekii*	23.0
	Day 21	N	23.6
4	02 August 04	N	18.5
	Day 1	Unknown *Mycobacterium* species closely related to *M. kansasii* and *M.*	24
	Day 2	N	25
	Day 3		N/A
	Day 4		24
	Day 5	N	25
	Day 6	Mixed culture of *M. engbaekii* and *M. terrae*	26
	Day 7	NTM*	25
	Day 14	N	26.5
	Day 21	*M. vaccae or M. vanbaalenii*	19.5

Figure 1. Multiple sequence alignment of a part of the 16S rRNA gene of Mycobacterium species isolated from water samples.

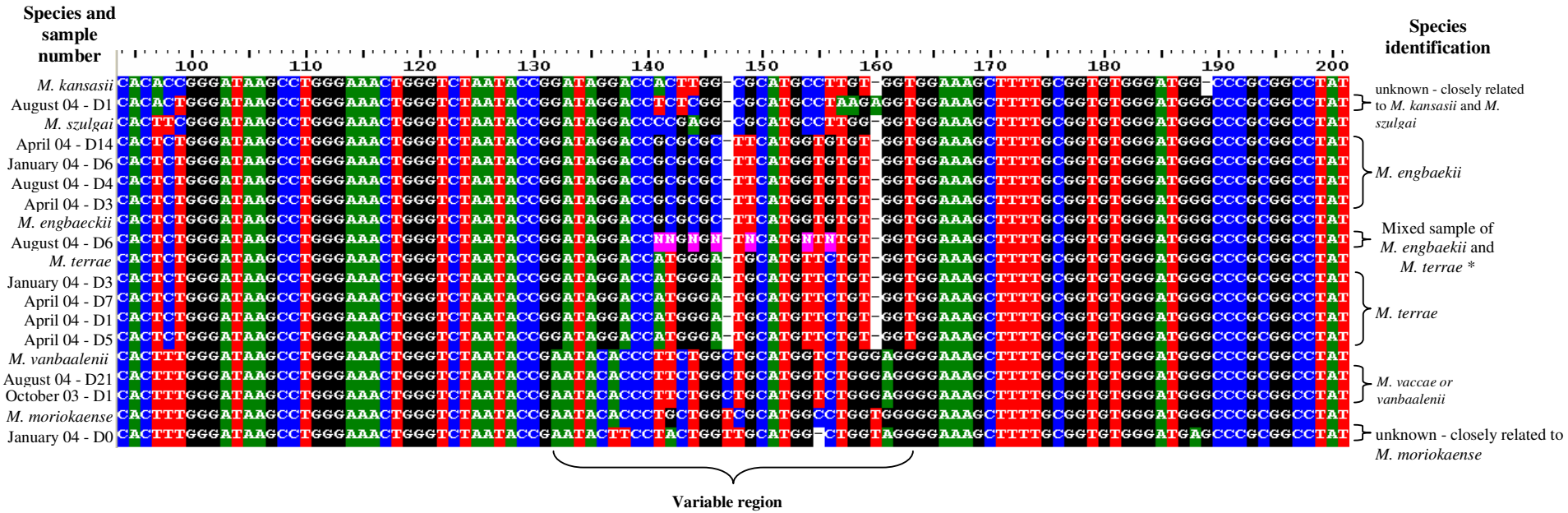


Fig. 1. Sample sequences obtained through PCR sequencing of the 16S rRNA gene were aligned with those of type strains of species resembling the isolated mycobacteria. Species-specific variable region is indicated. * - mixed sample in April F identified by two different overlapping chromatograms at the positions indicated by N and highlighted in pink. Chromatogram 1 - ATGGGATGCATGTTC = *M. terrae* Chromatogram 2 - GCGCGCTTCATGGTG = *M. engbaekii*

Discussion

To assess the risk of *M. bovis* spillover from buffalo to other species it is critical to determine the mode, frequency and level of shedding by infected buffalo herds. Neill et al. [14,15], concluded from experimental infections in calves that excretion of *M. bovis* in nasal mucus is a consistent feature in all infected cattle and can continue for weeks and even months. Furthermore, shedding rates ranging from 6% to 20% were found among naturally infected, tuberculous cattle in different countries, whereby the occurrence of lung lesions may be very low [14]. If the same was true for African buffalo, for which lesion types and distribution are generally comparable to those in cattle, they could possibly spread bovine tuberculosis by contaminating surface water such as water holes, dams and pools formed in stagnant rivers, as they commonly spend extended periods in and along the various water points. Especially large buffalo herds with high *M. bovis* infection rates as documented for the southern region of the Kruger National Park [3], could pose a significant risk to all susceptible species in the area.

Based on the abovementioned shedding rate for cattle, our study could have contained at least two to six shedders among the 31 tuberculous buffalo at any time during the study (Table 1). The actual conditions for shedding in buffalo were, however, more favorable than reported for cattle [14], since our study population included nine in-contact buffalo cows with advanced ('open') lung lesions, which is considered a sign of infectiousness in cattle [16,17]. If *M. bovis* shedding was an intermittent but common feature in infected buffalo, we had expected the *M. bovis* load in the trough water to be well above the confirmed detection limit of bacterial culture.

Our study did, however, not provide any indication of detectable amounts of *M. bovis* being present in the water troughs. Despite a degree of sampling uncertainty e.g. mycobacteria trapped in sediments or biofilms may have escaped sampling, we believe that the culture method used was suitable since it supported isolation of 16 *Mycobacterium spp*. isolates and of *M. bovis* from all spiked water samples. We are therefore of the opinion that shedding of *M. bovis* in nasal and oral discharges is an infrequent event in African buffalo, possibly limited to animals with clinical signs or to very low bacterial loads below the detection limit. This finding suggests a low to negligible risk for buffalo to serve as transmitters of *M. bovis* via water under free-ranging conditions.

Our conclusions are furthermore supported by the fact that no evidence of horizontal transmission of *M. bovis* between in-contact cows and the experimental buffalo could be demonstrated (results not shown). This includes potential spread by water or aerosolised droplets. Spread by aerosolised droplets may be dependent on frequent, close physical contact and social interactions, which, although well described characteristics in buffalo behaviour, were not observed between the two study groups. The in-contact and experimental buffalo

had been sourced from different herds. Apart from grazing and resting in relative proximity to each other, the two groups remained separate social entities throughout the vaccination trial. This observation is important as it may indicate that social and behavioural patterns are key determinants in driving transmission within and between buffalo herds and warrants more in-depth investigations.

Unlike members of the *M. tuberculosis* complex, NTMs are rarely associated with invasive disease, but they may temporarily colonise the host and cause transient infection accompanied by non-specific stimulation of the host's immune system [18]. In our study we isolated an unidentified *Mycobacterium* species closely related to *M. moriokaense* from both the trough water and lymph node tissue from one of the buffalos, suggesting the environment as the source of infection.
The specific effect of this particular, or other, NTM species on the immune response of buffalo is unknown at this stage. In both, cattle and buffalo, environmental mycobacteria have been suspected to cause non-specific sensitisation to the tuberculin skin test [19,20]. Corner et al. [21,22] showed that cattle inoculated with atypical mycobacteria isolated from either soil or bovine origin developed a significant level of sensitivity to both bovine and avian PPD in the tuberculin skin test. The immune response lasted for between four and ten weeks and was generally higher for avian PPD than bovine PPD. Demangel et al. [23] have implied a potential adverse effect of environmental mycobacteria on vaccination efficacy of BCG, depending on the extent to which these mycobacteria share cross-reacting antigens with the vaccine. In calves, sensitisation with environmental mycobacteria prior to vaccination had an antagonistic effect on BCG efficacy [24]. Initial vaccination trials using BCG in buffalo did not yield a significant reduction of infection, questioning the efficacy of BCG in these animals (de Klerk, unpublished data) and raising the question whether this effect might be due to the presence of NTMs. Our microbiological examination of water, pumped from a tributary of the Sabie River into the water trough for the study buffaloes, yielded five species of NTM, including two previously unidentified species (Table 2). *M. vaccae* and *M. terrae* are reportedly among the most frequently isolated organisms from fresh water [25], along with *M. engbaekii* and a number of unclassified mycobacteria [26]. Their natural habitat, however, is more likely to be wet soil [27], which may suggest that the NTMs, especially *M. terrae*, did not all originate from the river water but could have been introduced via the soiled muzzles or feet of the buffalo while drinking. Favourable water temperatures throughout the experiments (Table 2) and the presence of sufficient nutrients in river water are known to facilitate replication of mycobacteria [27].

Conclusion

The findings of this study suggest that contamination of surface water by infected buffalo may not be likely to play a significant role in the spread of *M. bovis* in a free-ranging ecosystem. The study also demonstrated that buffalo were exposed to different environmental NTMs in river-water without producing any signs of infection or disease.
Further studies will be required to investigate the potential effects of these and other NTM species on the immune response of buffalo especially in the context of BTB control strategies involving vaccination and diagnostics.

Methods

Study site

The present study was dovetailed with a BCG vaccination trial in buffalo, and was carried out in a 100 hectare fenced camp with natural habitat near Skukuza in the KNP. Two concrete drinking troughs (inner troughs) with a capacity of 500 liters each, were located in an enclosure situated within one corner of the camp and were the only permanent water source for the buffalo during the trial. Fresh water was pumped daily from a tributary of the nearby Sabie River to replenish the water in both drinking troughs. A separate concrete trough (experimental trough) with a capacity of approximately 250 liters was located next to the inner troughs but on the outside of the enclosure and camp. This trough was used for collection of serial water samples as described below. Access to the inner water troughs was restricted for several hours before each new sampling experiment to ensure that all buffaloes would consume water before sampling took place later on that same day.

Animals

A trial to evaluate the efficacy of BCG vaccination in African buffalo was conducted between January 2003 and November 2004 with prior approval by the Animal Care and Use Committee of the South African National Parks. Before the start of the project twenty-seven PPD skin test and interferon gamma negative buffalo (experimental buffalo), aged about two years, were translocated from the northern part of KNP into a holding facility (boma) at Skukuza. Prior to introduction into a 100 hectare camp, 14 animals were randomly selected and vaccinated twice, six weeks apart, with BCG (Pasteur strain P1172) via the

intramuscular route (de Klerk, unpublished data). The initial vaccination protocol anticipated natural *M. bovis* challenge from close contact with infected herd members. For this purpose a group of 27 adult buffalo cows (in-contact buffalo) was captured from a high prevalence herd in the south of the KNP and introduced into the same camp to join up with the 27 experimental buffalo. After a period of eleven months without any evidence (skin test, interferon gamma test) of horizontal transmission of *M. bovis* to the nonvaccinated buffalo, both the vaccinated and nonvaccinated groups were challenged with a field strain of *M. bovis* via the intra-tonsilar route in January 2004 [13]. For the purpose of the present study the vaccination status of the experimental buffalo was considered insignificant and hence no distinction is made hereafter between the two treatment groups. Twenty-four of the 27 in-contact buffalo cows as well as all 27 experimental buffalo survived. Three cows died of undetermined causes. During the trial period 16 calves were born to the in-contact cows, of which ten survived. Three months after the last water sampling was conducted, all buffalo (n=61) were slaughtered in November 2004.

Water sample collection plan

One sampling experiment was conducted each in October 2003, as well as in the months of January, April, and August 2004 (Table 2). The experimental troughs were emptied and dried between sampling experiments. At the start of each sampling experiment, the buffalo were allowed to drink from both inner troughs and subsequently moved out of the enclosure. Following mixing of the water and collection of the first water sample (Table 2), approximately 100 liters of water was transferred manually from each inner trough into the experimental trough outside the fence, using a bucket. The water temperature in both troughs was recorded daily. Each experiment was designed to determine the survival time of potentially excreted *M. bovis*, by collecting ten serial water samples into 400 ml sterile containers. On day 1 the water sample was taken from the inner trough within two hours after buffalo contact. Thereafter water samples were collected from the experimental trough on a daily basis up to day 7 as well as on day 14 and day 21. The water samples were stored at –minus 20°C until transferred to the Tuberculosis laboratory at the ARC-Onderstepoort Veterinary Institute (OVI) for culture. For quality control purposes, four aliquots of 50 ml trough water each, were spiked with serial dilutions from 10^7 to 10^4 organisms from a *M. bovis* field strain and frozen until processing.

Tissue sample collection plan

At slaughter, a standard set consisting of nine lymph node samples was collected for histopathology and culture from each buffalo. The samples included lymph nodes of the head (incl. tonsils), thorax, abdomen and carcass. Samples were

also collected from any other tissues with visible lesions. All tissue samples for culture were individually packed in sterile containers and frozen at minus 20°C until processed in the Tuberculosis laboratory at the OVI.

Bacterial isolation

Tissue samples were processed and cultured according to the protocol described by Bengis et al. [6]. A modification of the standard protocol was used to isolate *M. bovis* from the water samples and the quality control samples. Briefly, the water was centrifuged at 3500 rpm for 10 min and the pellet resuspended in 25 ml of sterile, double distilled water. Decontamination was effected by adding 25 ml of sodium hydroxide (4% w/v). The mixture was left for 10 minutes before centrifugation for 15 min at 3500 rpm. The pellet was neutralised by adding 5% oxalic acid for 15 minutes, followed by centrifugation as before. The pellet was mixed and inoculated onto four slants of Lőwenstein-Jensen medium, two of which contained pyruvate to facilitate growth of *M. bovis*. All cultures were evaluated for colony growth on a weekly basis up to 10 weeks. Culture slants, which showed contamination on less than 50% of the medium surface, were classified as partially contaminated. These cultures were maintained unless the contamination covered more than 50% of the medium slant in which case it was discarded for contamination.

Identification of Mycobacterium spp. by polymerase chain reaction (PCR)

All acid-fast isolates were subjected to a PCR assay specific for *M. tuberculosis* complex bacteria [28]. All isolates, which failed to yield the expected 372 bp amplification product, were subjected to a 5'-16S rDNA PCR-sequencing assay, which is able to identify non-tuberculous *Mycobacterium spp*. [29].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ALM designed and supervised the experiment as well as the bacteriological analyses and drafted the manuscript. LMDK was the project leader of the BCG trial and performed the field work. NGVP performed the sequence analysis. NGVP and RW added value through introduction of critical technical considerations. PVH

was instrumental in the inter-institutional collaboration and contributed overall to the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors want to thank the staff of the OVI-Tuberculosis laboratory for excellent technical support.

References

1. De Lisle GW, Bengis RG, Schmidt SM, O'Brien DJO: **Tuberculosis in free-ranging wildlife: detection, diagnosis and management.** *Rev Sci Tech Off int Epiz* 2002, 21:317-334.
2. Schmitt SM, O'Brien DJ, Bruning-Fann CS, Fitzgerald SD: **Bovine tuberculosis in Michigan wildlife and livestock.** In: *The domestic animal/wildlife interface. Issues for disease control, conservation, sustainable food production and emerging diseases.* Edited by Gibbs, E.P.J., Bokma, B.H., *Ann NY Acad Sci* 2002, 969:262-268.
3. Michel AL, Bengis RG, Keet DF, Hofmeyr M, de Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte IJ, Buss P, Godfroid J: **Wildlife tuberculosis in South African conservation areas: implications and challenges.** *Vet Microbiol* 2006, 112:91-100.
4. De Vos V, Raath JP, Bengis RG, Kriek NJP, Huchzermeyer H, Keet DF, Michel A: **The epidemiology of tuberculosis in free ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa.** *Onderstepoort J Vet Res* 2001, 68:119-130.
5. Michel AL: **Implications of tuberculosis in African wildlife and livestock.** *In: The domestic animal/wildlife interface. Issues for disease control, conservation, sustainable food production and emerging diseases. Edited by* Gibbs, E.P.J., Bokma, B.H., (Eds.) Ann NY Acad Sci 2002, 969:251-255.
6. Bengis RG, Kriek NPJ, Keet DF, Raath JP, de Vos V, Huchzermeyer HFAK: **An outbreak of bovine tuberculosis in a free-living buffalo population in the Kruger National Park.** *Onderstepoort J Vet Res* 1996, 63:15-18.
7. Keet DF, Kriek NPJ, Bengis RG, Michel AL: **Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park**. *Onderstepoort J Vet Res* 2001, 68:225 – 230.
8. Thorburn JA, Thomas AD: **Tuberculosis in Cape Kudu.** *J S Afr Vet Med Ass* 1940, 11:3-10.

9. Keet DF, Kriek NPJ, Bengis RG, Grobler DG, Michel AL: **The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park.** *Onderstepoort J Vet Res* 2000, 67:115-122.
10. Tanner M, Michel AL: **Investigation of the viability of *Mycobacterium bovis* under different environmental conditions in the Kruger National Park.** *Onderstepoort J Vet Res* 1999, 66:185-190.
11. Kraus E: **Untersuchungen über die Verbreitung der Tuberkelbazillen aus den Abwässern einer Lungenheilstätte.** *Arch Hyg Bakt* 1942, 128:112-122.
12. Jensen KE: **Presence and destruction of tubercle bacilli in sewage.** *Bull Wld Hlth Org* 1954, 10:171-179.
13. De Klerk LM, Michel AL, Grobler DG, Bengis RG, Bush M, Kriek NPJ, Hofmeyr MS, Griffin JFT, Mackintosh CG: **An experimental intratonsilar infection model for bovine tuberculosis in African buffalo (*Syncerus caffer*)**. Onderstepoort J Vet Res 2006, 73: 293-303.
14. Neill SD, O'Brien JJ, McCracken RM: ***Mycobacterium bovis* in the anterior respiratory tracts in the heads of tuberculin-reacting cattle.** *Vet Rec* 1988, 122:184-186.
15. Neill SD, Hanna J, O'Brien JJ, McCracken RM: **Transmission of tuberculosis from experimentally infected cattle to in-contact calves.** *Vet Rec* 1989, 124:269-271.
16. Kleeberg HH: Tuberculosis and other mycobacterioses. In Hubert, W.T.McCulloch, W.F. and Schnurrenberger, D.R. (eds)6th edition. Diseases transmitted from animal to man. Springfield, Illinois: Charles C. Thomas. 1975.
17. Cousins DV, Huchzermeyer HFKA, Griffin JFT, Brueckner GK, van Rensburg IBJ, Kriek NPJ: Tuberculosis. In *Infectious Diseases of Livestock.* Edited by Coetzer J.A.W. & Tustin R.C. Oxford University Press Southern Africa, Cape Town, 2004:1973-1993.
18. Woods GL, Washington JA: **Mycobacteria other than *Mycobacterium tuberculosis*: Review of microbiologic and clinical aspects.** *Rev Infect Dis* 1987, 9:275-294.
19. Cooney R, Kazda J, Quinn J, Cook B, Müller K, Monaghan M: **Environmental mycobacteria in Ireland as a source of non-specific sensitization to tuberculins.** *Ir Vet J* 1997, 50:370-373.
20. Michel AL, Jones S: **False positive reactions to the gamma-Interferon assay in buffalo in the Kruger National Park**. In: Proceedings of the *ARC-Onderstepoort OIE International Congress with WHO-Cosponsorship on Anthrax, Brucellosis, CBPP, Clostridial and Mycobacterial Diseases:* 9-15 August 1998: Berg-en-Dal, Kruger National Park, South Africa. 388-392.
21. Corner LA, Pearson CW : **Response of cattle to inoculation with atypical mycobacteria from soil.** *Aust Vet J* 1979, 55:6-9.

22. Corner LA, Pearson CW : **Response of cattle to inoculation with atypical mycobacteria from bovine origin**. *Aust Vet J* 1978, 54:379-382.
23. Demangel C, Garnier T, Rosenkrands I, Cole ST: **Differential effects of prior exposure to environmental mycobacteria on vaccination with Mycobacterium bovis BCG or a recombinant BCG strain expressing RD1 antigens.** *Infect Immun* 2005, 73:2190-2196.
24. Buddle BM, Wards BJ, Aldwell FE, Collins DM, de Lisle GW: **Influence of sensitization to environmental mycobacteria on subsequent vaccination against bovine tuberculosis.** *Vaccine* 2002, 20:1126-1133.
25. Viallier J, Viallier G: **Inventaires des mycobakteries de la nature.** *Ann Soc Belge Med Trop* 1973, 53:361-371.
26. Marranzano M: **Atypical mycobacteria isolated from the water of the Simeto River at Catania.** *Boll 1st Sieroter Milan* 1978, 57:128-133.
27. Collins CH, Grange JM, Yates MD: **Mycobacteria in water. A review.** *J Appl Bacteriol* 1984, 57:193-211.
28. Cousins DV, Wilton SD, Francis BR: **Use of DNA amplification for the rapid identification of *Mycobacterium bovis*.** *Vet Microbiol* 1991, 27:187-195.
29. Harmsen D, Dostal S, Roth A, Niemann S, Rothganger J, Sammeth M, Albert J, Frosch M, Richter E: **RIDOM: comprehensive and public sequence database for identification of *Mycobacterium* species.** *BMC Infect Dis* 2003, 3:26.

Chapter 6

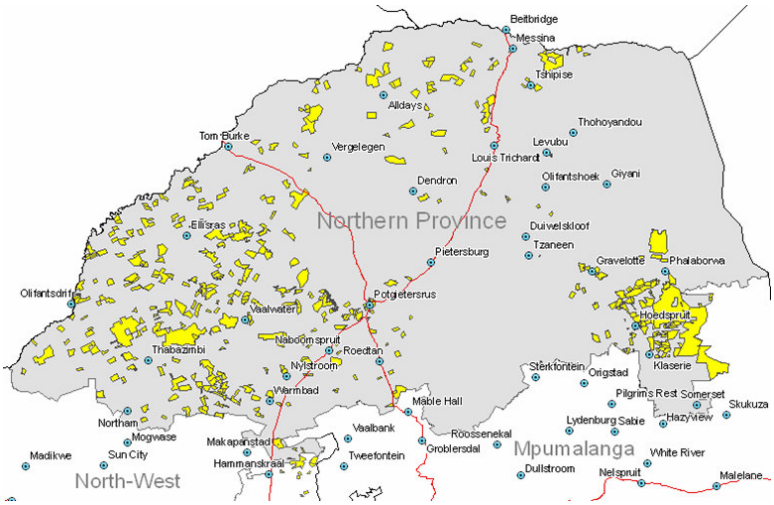
Discussion

Animal tuberculosis is a disease of high relevance within the South African context of wildlife conservation, commercial game farming, livestock farming and not least of all at the interface with human health. Although implications of animal tuberculosis on any of these commodities are not unique to South Africa and have been documented in a number of countries and continents (de Lisle et al. 2002), there is a fundamental difference in the complexity and level at which they impact on South Africa's economy at large. The accepted driving forces for the control and eradication of bovine tuberculosis from the national herd, especially in developed countries, are indisputably of economic and socio-political nature, based mainly on the negative impact of the disease on international trade, and, to a lesser extent, on its potential as zoonosis. In situations outside Africa where control efforts are hampered by the existence of wildlife reservoirs for bovine tuberculosis, these maintenance host species score a low priority on their national wildlife conservation listings and enjoy, at best, the status of valued, sought-after hunting trophies such as the white tailed deer in Michigan, USA, wild boar in Spain or deer in the Czech Republic. In some cases these reservoir species are even classified as alien or feral with well documented examples from the brushtail possums in New Zealand (Morris et al. 1994) and feral water buffalo in Australia (Cousins et al. 1998). As a direct result control programmes were designed to exclusively or primarily benefit the livestock sector, at the cost of the affected wildlife species, which is not recognised in its own right.

In sharp contrast with the spectrum of scenarios found in developed countries worldwide, bovine tuberculosis is not only an endemic disease in South African livestock but has established itself in the African buffalo (*Syncerus caffer*), a wildlife species of outstanding economic and ecological value, also reflected in its ranking among the 'Big Five' wildlife species. Bovine tuberculosis in buffalo poses a threat not only to species conservation efforts but to commercial game farming which has, through the historically embedded prestige associated with keeping indigenous game, created a unique and sustainable niche in South African agriculture (Reilly et al. 2003). The sudden, sharp increase in wildlife-based tourism after 1994 further catalysed the rapid growth of the wildlife industry which nowadays enjoys the status of a fully-fledged agricultural activity. Wildlife ranching is further integrated into other sectors of the economy such as tourism and conservation (www.nda.agric.za). Today the land surface utilised for game farming is equal to or has, in parts of several of the country's provinces, exceeded that of livestock farming (Fig. 1).

The WHO in conjunction with FAO & OIE recently classified bovine tuberculosis, along with a few other diseases, as a neglected zoonosis, with special reference to developing countries (WHO Report 2006). In the world's most vulnerable communities animal diseases, which are transmissible between livestock and

Figure 1. Distribution of game farms registered to keep buffalo in Limpopo Province, South Africa



humans not only have the potential to impact on human health directly, but to threaten human livelihoods by compromising sustainable food supply, income and social status. Although reported evidence on *Mycobacterium bovis* as cause of human disease does exist, it is accepted that its role is negligible in most of the developed world (Grange & Yates 1994). The significance in developing countries in Africa is largely unknown but the situation may be profoundly different due to the existence of complex contributing factors (Ayele et al. 2004). In South Africa, like other countries in the region, communities facing a higher disease risk from *M. bovis* include those living at the livestock/human interface, consuming mostly unpasteurised milk and dairy products derived from cattle herds with an uncontrolled bovine tuberculosis disease status. At the same time they also include those population groups who are suffering from the world's highest HIV/AIDS infection rates and the associated increased susceptibility to co-infection with *M. tuberculosis*, cause of tuberculosis in humans (Narain et al. 1992).

Molecular epidemiology

A thorough understanding of the epidemiology of bovine tuberculosis in wildlife is essential for the development of suitable control strategies. Genetic characterisation of *M. bovis* strains has revolutionised the control of bovine tuberculosis internationally as it enables studies on the spatial spread and genetic relationship of strains. While the concerted efforts of international research teams have greatly contributed to the development and evaluation of a variety of genetic markers, such as those based on restriction fragment length polymorphism (RFLP) (IS*6110*, IS*1081*, PGRS), spoligo- and VNTR typing, the diverse approaches have led to varying degrees of success in different countries and no "ideal", standardised typing method for universal use globally exists to date (Aranaz et al. 1998, Roring et al. 2002, Allix et al. 2006, Hewinson et al.

2006). For South Africa, information on the genetic profiles of *M. bovis* strains causing outbreaks in local cattle and wildlife was lacking entirely. The studies presented in this thesis provided the first insight in the usefulness of specific markers and the genetic diversity among South African *M. bovis* isolates from cattle. The findings showed that IS*6110* RFLP, potentially supported by PGRS RFLP where necessary, provided a high discriminatory power between known unrelated strains and at the same time proved to generate reproducible results within groups of related strains. These markers revealed the existence of a genetically more diverse *M. bovis* population in South Africa than reported from parts of Europe or central Africa. This may be attributed to a route–cause association between the high number of *M. bovis* strains imports during colonial times and the initial lack or subsequent low efficiency of bovine tuberculosis control during the previous century. In conclusion the outcomes of these genetic markers were found suitable for investigating geographical distribution of particular genotypes and their spread to other species including wildlife. A database of typing patterns in cattle and different wildlife species in KNP and HiP was established, which showed that the BTB epidemics in these two ecosystems were each caused by a unique *M. bovis* strain. These *M. bovis* strains were genetically and epidemiologically unrelated. In the case of the KNP an association could be established on a genetic level between isolates from buffalo in KNP and cattle in adjacent farmland south of KNP. Through characterisation and comparison of the *M. bovis* isolates from wildlife in HiP and KNP it became evident that the dominant KNP strain C8 underwent evolutionary events leading to the description of 21 variant strains of C8 in this thesis, while all epidemiologically related strains in three game reserves in Kwazulu-Natal shared the same genotype. The investigations included inter-species spread of the C8 and several of its variant strains, revealing a clustered distribution of certain variants in defined geographical regions of KNP and adjacent private reserves of the GKNPC.

Future directions:
Further optimisation of the typing approach in the medium term will include evaluation of next generation VNTR typing strategies. A particular need for a molecular epidemiological support system has been recently identified in the context of the planned implementation of the Greater Limpopo Transfrontier Conservation Area (GLTFCA) joining national parks in South Africa, Mozambique and Zimbabwe. It will be an interesting challenge to extend the application of the typing methodology to link with large-scale comparative epidemiological studies across political borders and encompassing ecological and cultural landscapes.

Improved diagnosis
The intradermal tuberculin test (IDT) has proved to be very valuable for the diagnosis of bovine tuberculosis in cattle, but has severe limitations when applied to free-ranging wildlife, including the need for repeated physical manipulation of

the stressed animals within 72 hours and for a holding facility in which the buffaloes were to be contained during this period. In comparison with the labour intensive IDT the IFNg assay holds distinct advantages in terms of the simplification of test procedures in the field. On evaluation of the IFNg assay natural infections with *M. fortuitum* were encountered in free-ranging buffalo which were the possible cause for false positive reactions of these buffaloes in the IFNg assay. Due to the extremely high commercial value of buffalo a lack in diagnostic specificity is far less acceptable in this species than in cattle and could have led to the rejection of the IFNg assay from the national testing protocol for buffalo. This national guideline prescribes minimum requirements for testing for bovine tuberculosis and other notifiable diseases to private and public buffalo owners.

Chapters 4.1 to 4.3 of this thesis undertook to investigate the immune reactivity possible of buffalo in the IFNg assay and examined different interpretation criteria of the test results generated.

Serological tests have been used to detect tuberculosis in a range of wildlife hosts, but in the past none could overall compete in terms of sensitivity and specificity with cell mediated tests such as skin test and IFNg assay (Placket et al. 1989). The technical simplicity and ability to detect antibodies in different species without the need for species-specific conjugates justified the evaluation of two new, commercially available rapid tests. These immunochromatographic assays have been advocated for offering improved sensitivity in detecting *M. bovis* infection in cattle as well as a number of wildlife species, including deer, elephant, non-human primates and camel (Lyashchenko et al. 2006, Wernery et al. 2007). The findings described in this thesis, however, demonstrated a low diagnostic accuracy for both rapid tests, precluding their use as stand-alone tests, especially if the IDT or IFNg assay status of the relevant herd is unknown. These results re-enforce previous observations of poor sensitivity of antibody-based detection systems in buffalo. Previously, a non-commercial EIA produced by Commonwealth Serum Laboratories, Australia, detected 10.4% of culture positive buffaloes (Cleaveland et al. 2005). In sharp contrast to the rapid tests, the EIA had a very high specificity of 99% which resulted in a high positive predictive value even in herds with a low infection prevalence.

Future directions

Recently two in-house ELISA systems were developed, one based on rMPB70 and another based on rMPB83. Preliminary validation of the assays in panels of sera from cattle, buffalo and lion once again indicated very low sensitivities, especially in buffalo. On the other hand, both systems proved to be highly specific and further work should focus on the validation of this diagnostic parameter, with regard to particularly the screening of herds and populations for animals which are in advanced disease stages and more likely to shed and transmit *M. bovis.*

Another technology currently pursued is the fluorescent polarisation assay for which preliminary work has been performed in South African cattle in conjunction with an international comparison and showed promising results (Jolley et al. 2006). The major advantage of this alternative technology is that it is independent from secondary antibodies but based on the binding of a fluorescent low molecular weight moiety (tracer) to its high molecular weight binding partner (anti-*M. bovis* antibody) by determining the tracer's fluorescence polarisation (FP). In the absence of the specific antibodies the tracer has a low FP, while the tracer's FP is high if it binds to antibodies against *M. bovis* present in the serum. For the medium to long-term, research objectives should aim at identifying "early" and "late" antigens, defined by their first appearance in serum of buffalo infected with bovine tuberculosis. Whether the aim is to detect all infected animals or selectively those shedding the organism, through a series application of these different antigens and interpretation of the responses they elicit, the most valuable single or combination of *M. bovis* antigens can be determined.

Control of bovine tuberculosis

Bovine tuberculosis in free-ranging buffalo populations will need to be controlled to protect the biodiversity and especially endangered wildlife species in South African conservation areas and also to prevent the spread of infection to domestic livestock and ultimately to humans (Michel et al. 2006). The approval of tools for controlling *M. bovis* in buffalo, and in indigenous wildlife in general, is governed by considerations for ethical and ecological acceptance. Vaccination has been recognised not only as a possible but as the most promising means of controlling bovine tuberculosis in free-ranging wildlife, with the only vaccine currently available being bacillus Calmette-Guérin (BCG). Vaccination studies in new species require prior knowledge on the most effective route and dose of infection during challenge to allow a scientifically sound evaluation of the outcome. We have successfully established an experimental infection model in buffalo, demonstrating that a dose of 3×10^4 cfu was suitable for infection experiments as it led to visible lesions in the majority and culture isolation of *M. bovis* from all infected buffaloes. Subsequently two vaccine efficacy trials using BCG in conjunction with intra-tonsilar *M. bovis* challenge were conducted. The first trial, during which the groups of experimental buffaloes were kept in bomas, did not yield any significant protection against *M. bovis* challenge (results not presented). The failure to induce protective immunity was partially attributed to the stress imposed on the buffalo while housed in the bomas and partially on the concurrent outbreak of clinical foot-and-mouth disease in the experimental animals (Vosloo et al. 2006). During the second vaccine efficacy trial buffalo groups were maintained under semi-free ranging conditions in a 100-hectare enclosure. This experiment was set up to facilitate natural *M. bovis* challenge from infected buffalo cows kept together with the vaccinates and control animals, but required modification to needle challenge because no natural *M. bovis* transmission could be demonstrated. Once again, BCG vaccination did not result

in a significant reduction in the number or severity of lesions compared to the control animals.

Future directions:
Future challenges in vaccinating buffalo and possibly other wildlife species are believed to lie, apart from better vaccine candidates, mainly in the recruitment of very young animals into the experiment as prior stimulation with environmental mycobacteria may have an adverse effect on protective immune responses. Practical requirements such as means of vaccine delivery and the need to administer more than a single dose of vaccine also need to be addressed.

References

Allix C., Walravens K., Saegerman C., Godfroid J., Supply P., Fauville-Dufaux M. 2006. J Clin Microbiol 44:1951-1962.

Aranaz A., Liebana E., Mateos A., Dominguez L., Cousins D., 1998. Restriction fragment length polymorphism an spacer oligonucleotide typing: a comparative analysis of fingerprinting strategies for *Mycobacterium bovis*. Vet Microbiol 61:311-324.

Ayele W.Y., Neil S.D., Zinstag J., Weiss M.G., Pavlik I. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. Int J Tuberc Lung Dis 8:924-937.

Cleaveland S., Mlengeya T., Kazwala R.R., Michel A., Kaare M.T., Jones S.L., Eblate E., Shirima G.M., Packer C. 2005. Tuberculosis in Tanzanian wildlife. J Wildl Dis 41:446-453.

Cousins D.V., Corner L.A., Tolson J.W., Jones S.L., Wood P.R. 1998. Eradication of bovine tuberculosis from Australia: key management and technical aspects. Page 4. National Library of Australia Cataloguing-in Publication Entry. CSL Limited.

De Lisle G.W., Bengis R.G., Schmitt S.M., O'Brien D.J. 2002. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. Rev Sci Tech Off Int Epiz 21:317-334.

Grange J.M., Daborn C., Cosivi O. 1994. HIV-related tuberculosis due to *Mycobacterium bovis*. Eur Respir J 7:1564-1566.

Hewinson R.G., Vordermeier H.M., Smith N.H., Gordon S.V. 2006. Recent advancesin our knowledge of *Mycobacterium bovis*: a feeling for the organism. Vet Microbiol 112:127-139.

Lyashchenko K.P., Greenwald R., Esfandiari J., Olsen J.H., Ball R., Dumonceaux G., Dunker F., Buckley C., Richard M., Murray S., Payeur J.B., Andersen P., Pollock J.M., Mikota S., Miller M., Sofranko D., Waters W.R., 2006. Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment. Clin Vaccine Immunol 13, 722–732.

Michel A.L., Bengis R.G., Keet D.F. Hofmeyr M., de Klerk L.M., Cross P.C. Jolles A.E., Cooper D., Whyte I.J., Buss P., Godfroid J. 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. Vet Microbiol 112:91-100.

Morris RS, Pfeiffer DU, Jackson R. 1994. The epidemiology of *Mycobacterium bovis* infections. Vet Microbiol 40:153-177.

Narain J.P., Ravigione M.C., Kochi A. 1992. HIV-associated tuberculosis in developing countries: epidemiology and strategy for prevention. Geneva, World Health Organisation, WHO/TB/92.164.

Plackett P., Ripper J.L., Corner L.A., Small K., De Witte K., Melville L., Hides S., Wood P.R., 1989. An ELISA for the detection of anergic tuberculous cattle. Aust Vet J 66, 15-19.

Reilly B.K., Sutherland E.A., Harley V. 2003. The nature and extent of wildlife ranching in Gauteng province, South Africa. S Afr J Wildl Res 33(2): 141-144.

Roring S., Scott A., Brittain D., Walker I., Hewinson G., Neilll S., SKuce R. 2002. Development of Variable-Number Tandem Repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J Clin Microbiol 40:2126-2133.

The Control of Neglected Zoonotic diseases: a route to poverty alleviation. Report of a Joint WHO/DFID-AHP Meeting with the participation of FAO and OIE. Geneva, 20 and 21 September 2005. http://www.who.int/zoonoses

Vosloo W., de Klerk L.-M., Boshoff C.I., Botha B., Dwarka R.M., Keet D., Haydon D.T. 2006. Characterization of a SAT-1 outbreak of foot-and-mouth disease in captive African buffalo (*Syncerus caffer*): Clinical symptoms, genetic characterisation and phylogenetic comparison of outbreak isolates. Vet Microbiol 3499: 1 – 15.

Wernery U., Kinne J, Jahans K.L., Vordermeier H.M., Esfandiari J., Greenwald R., Johnson B., Ul-Haq A., Lyashchenko K.P., 2007. Tuberculosis outbreak in a dromedary racing herd and rapid serological detection of infected camels. Vet Microbiol 122: 108-115.

Summary

Bovine tuberculosis is an endemic disease with a low prevalence in South African cattle. This is mostly the result of a national bovine tuberculosis control scheme which has been in place for nearly 40 years and has prevented outbreaks from spreading and causing large-scale losses, thereby also minimising the risk in view of human health. However, with the establishment of African buffalo as a successful wildlife maintenance host a new, yet undetermined risk factor has been introduced. It poses an entirely new challenge on national and provincial authorities to meet the 'fit for purpose' principle in terms of reducing economic losses but also the potentially negative impact on conservation efforts and, most of all, to protect human health and livelihoods.

There is a need to shift the focus of bovine tuberculosis control from an exclusively cattle based to an integrated strategy, which takes into consideration the wildlife reservoir and the added risk of *Mycobacterium bovis* transmission between cattle and buffalo populations. In order to justify such a change in strategy, data regarding the epidemiology of bovine tuberculosis in wildlife reservoirs need to be available to underpin the interdependence of bovine tuberculosis in livestock and wildlife. This thesis describes the establishment of a set of molecular tools for researching the epidemiology of bovine tuberculosis in both livestock and wildlife and their crucial contribution to insight in the genetic diversity of *M. bovis* in South Africa. Furthermore it was demonstrated beyond doubt that spillover of *M. bovis* from cattle to wildlife has occurred and revealed important clues on the course of the epidemic within the Kruger National Park by intra- and interspecies spread of one dominant *M. bovis* strain.

Evaluation of new and improved diagnostic methods based on both the cellular immunity and humoral immune response were undertaken and showed that especially the gamma interferon assay holds a high potential for more practical and reliable testing of buffalo and cattle for bovine tuberculosis than conventional approaches. In the long-term, all diagnostic assays applied and evaluated will play a crucial role in the implementation and monitoring of a suitable control strategy in wildlife as well as cattle. The principal aim for controlling bovine tuberculosis is to eliminate the risk of infecting humans, but also to protect biodiversity in indigenous wildlife. Ultimately vaccination will be the most feasible, economical and ethically acceptable control option in South Africa and work presented in this thesis has resulted in both an experimental intratonsilar infection model in buffalo and provided the first insight into the immune responsiveness of buffalo to vaccination with BCG Pasteur vaccine. Both developments present crucial milestones in gaining a better understanding of the factors influencing the efficacy of vaccination regimens in this wildlife reservoir.

Samenvatting

Bovine tuberculose is een endemische ziekte, met lage prevalentie, in runderen in Zuid Afrika. Dit is voornamelijk te danken aan het nationale Tuberculose bestrijdingsprogramma, dat gedurende bijna 40 jaar ziekte-uitbraken en verspreiding beperkte en daarmee grote economische verliezen heeft voorkomen. Tegelijkertijd werd het besmettingsgevaar voor de mens grotendeels gereduceerd. Echter door de zich wijd verbreidende aanwezigheid van de Afrikaanse buffel, als "wildlife" reservoir voor bovine tuberculose, is een tot dusverre onbekende risicofactor geïntroduceerd. Een totaal nieuwe uitdaging voor autoriteiten op nationaal en provinciaal niveau om economische verliezen te beperken, een negatieve invloed op natuurbeschermingsinitiatieven, gericht op het behoud van biodiversiteit, te voorkomen en tevens de menselijke gezondheid te beschermen.

Het is noodzakelijk om het accent van tuberculose bestrijding te verleggen van een exclusief rund georiënteerde benadering naar een geïntegreerde strategie die het "wildlife" reservoir en de overdracht van *Mycobacterium bovis* tussen runderen en buffels in aanmerking neemt. Ten einde een accentverlegging te rechtvaardigen zijn overtuigende gegevens nodig over de epidemiologie van bovine tuberculose in "wildlife" en over de samenhang tussen bovine tuberculose in runderen en die in wilde diersoorten.

Dit proefschrift beschrijft de invoering van een set moleculaire technieken die epidemiologisch onderzoek van bovine tuberculose in runderen en "wildlife" reservoirs mogelijk maken en hun cruciale bijdrage aan inzicht in de genetische diversiteit van *M.bovis* in Zuid Afrika. Onomstotelijk werd vastgesteld dat overdracht van *M.bovis* van runderen naar "wildlife" plaats vindt. Daarnaast levert het proefschrift belangrijke aanwijzingen tav de verspreiding van een dominante *M.bovis stam* binnen en tussen diersoorten in het Kruger Nationaal Park.

Evaluatie van nieuwe en verbeterde diagnostische methoden ter bepaling van zowel cellulaire als humorale immuniteit liet zien dat vooral de interferon gamma test veelbelovend is en meer praktisch bruikbaar en betrouwbaar dan conventionele methoden voor diagnose van bovine tuberculose in buffels en runderen. Op langere termijn zullen al de geëvalueerde testen een belangrijke bijdrage leveren aan implementatie en monitoring van een bruikbare controle strategie in zowel wilde diersoorten als in runderen. De belangrijkste doelstelling van controle van bovine tuberculose is het elimineren van het risico van infectie van mensen, maar ook het behouden van de biodiversiteit in inlandse diersoorten.

Uiteindelijk zal vaccinatie de meest geschikte, economisch en ethisch geaccepteerde controle optie zijn voor Zuid Afrika. Onderzoek gepresenteerd in dit proefschrift resulteerde in zowel een experimenteel intratonsilair infectie model in buffels, als in een eerste inzicht in immuunreactiviteit van buffels na vaccinatie met BCG Pasteur als vaccin. Beide ontwikkelingen zijn belangrijke

mijlpalen op weg naar beter begrip van de factoren die de effectiviteit van vaccinatie strategieën in het "wildlife" reservoir kunnen beïnvloeden.

Zusammenfassung

Die bovine Tuberkulose ist eine endemische Krankheit in Südafrika, mit niedriger Prävalenz in Rinderbeständen. Dieser Zustand ist hauptsächlich dem nationalen Tuberkulosebekämpfungsprogramm zu verdanken, das seit knapp 40 Jahren Krankheitsausbrűche in Grenzen gehalten und damit weitgreifende wirtschaftliche Verluste verhindert hat. Dies hat gleichzeitig auch das Risiko fűr die menschliche Gesundheit auf ein Minimum reduziert. Die Entwicklung des afrikanischen Bűffels zum Wildtierreservoir fűr bovine Tuberkulose rief jedoch ein neues, bislang unbekanntes Risiko hervor. Damit stellte sich den Behörden auf nationaler- und Provinzebene eine gänzlich neue Herausforderung, da es jetzt gilt wirtschaftliche Verluste und mögliche negative Auswirkungen auf den Artenschutz weitmőglichst zu begrenzen sowie vor allem den Schutz der menschlichen Gesundheit und Lebensgrundlage zu sichern.

Aufgrund dieses neuen Risikos müssen die zukűnftigen Tuberkulose-bekämpfungsstrategien ihren Schwerpunkt nicht mehr nur auf die Viehbestände legen sondern auch das Wildtierreservoir mit einbeziehen, unter Einschluss der potentiellen Übertragung von *Mykobakterium bovis* zwischen Hausrindern und Bűffeln. Die Rechtfertigung eines solchen Strategiewechsels bedarf überzeugender Daten, die die Epidemiologie der bovinen Tuberkulose in Wildtierreservoiren aufzeigen und das Wechselspiel der Übertragung zwischen Haus- und Wiltieren untermauern kőnnen. In dieser Promotionsarbeit wird die Entwicklung von Molekulartechniken und Nachweismethoden beschrieben, die der Erforschung der Epidemiologie der bovinen Tuberkulose in sowohl Hausrindern als auch Wildtieren dienen. Ausserdem gewährten diese Erkenntnisse einen wichtigen Einblick in die genetische Vielfalt der südafrikanischen *M. bovis* Stämme. Zweifelsohne konnte bewiesen werden, dass eine Übertragung von *M. bovis* vom Hausrind auf Wildtiere stattgefunden hat. Ausserdem liefert die vorliegende These wichtige Hinweise zum Verlauf der Tuberkuloseepidemie im Krűger Nationalpark, die durch eine Verbreitung des dominanten *M. bovis* Stammes sowohl innerhalb als auch zwischen verschiedenen Wildtierpopulationen vorangetrieben wurde.
Die Evaluierung neuer, verbesserter Diagnostikmethoden auf dem Gebiet der zellulären sowie der humoralen Immunität zeigte, dass besonders der Gamma Interferontest eine Reihe praktischer Vorteile fűr die Tuberkulosediagnose in Bűffeln und Rindern innehält und zuverlässigere Testergebnisse anbietet als konventionelle Testmethoden.
Langfristig werden alle evaluierten und fűr tauglichen befundenen diagnostischen Untersuchungsmethoden eine Schlűsselfunktion bei der Einfűhrung und Überwachung einer zukűnftigen Kontrollstrategie fűr die bovine Tuberkulose in Wildtier- und Hausrindern spielen. Das Hauptziel der bovinen Tuberkulosekontrolle besteht sowohl in der Ausmerzung eines jeglichen Risikos fűr menschliche Ansteckungsgefahren als auch im Schutz der einheimischen

Artenvielfalt. Letztendlich ist anzunehmen, dass eine auf Impfung beruhende Kontrollstrategie die wirtschaftlich und ethisch meist akzeptable Option in Sűdafrika darstellen wird. In dieser Promotionsarbeit wurde deshalb auch Wert auf die Erstellung eines Infektionsmodelles (intra-tonsilär) für *M. bovis* in Bűffeln gelegt. Eine darauffolgende Impfstudie lieferte die ersten Erkenntnisse zur Immunantwort von Bűffeln auf den BCG Impfstoff (Pasteur). Beide diese Entwicklungen repräsentieren bedeutende Errungenschaften, die zu unserem Verständnis der Faktoren beitragen, die die Wirksamkeit verschiedener Impfstrategien in Wildtierreservoiren beinflussen kőnnen.

Acknowledgements

As a foreign student I am very grateful to the Utrecht University for permission to register for doctoral studies and to my promoters Prof. W. van Eden, Prof. K. Coetzer and Prof. V. P.M.G. Rutten for granting me the opportunity to obtain a higher degree.

My sincere gratitude goes to my promoter and mentor, Prof. V.P.M.G. Rutten, who not only encouraged me to embark on this doctoral thesis but who coached and navigated me on this journey with untiring efforts and in his gentle yet persistent way.

Thank you to my supervisor and Research & Technology Manager at OVI, Prof. Tony Musoke, for his moral support and to the Agricultural Research Council for providing travel funds to defend this thesis in The Netherlands.

My children, Tamara and Sascha, I thank you for your understanding during times when I could not attend to you as much as I would have liked to.

Finally I am endlessly grateful to Robert, my husband, who is always my pillar of support but was even more so during the time I was writing up this thesis. He simply took over all household and family responsibilities and spoiled me with the best food and cappuccinos one could wish for.

Curriculum vitae

Anita Luise Michel was born on July 20, 1963 in Zoeschingen, Germany. She matriculated in 1982 at the Hellenstein Gymnasium in Heidenheim, Germany and commenced her studies of veterinary medicine at the Ludwig-Maximilians University in Munich in the same year. After completing her studies she registered for her postgraduate research project in molecular virology at the Max-Planck Institute for Biochemistry in Martinsried near Munich and graduated as a Doctor of Veterinary Medicine in February 1989 from the aforementioned University. In 1989, Anita Michel emigrated, together with her husband Robert, to South Africa to take up a position as researcher in the Virology department of the then Veterinary Research Institute at Onderstepoort, which later became part of the Agricultural Research Council and is now known as ARC-Onderstepoort Veterinary Institute (OVI). In 1994 she joined the Tuberculosis Laboratory and later took over the management of this unit in 1996 and of the Bacteriology department in 2002. During this time she was responsible for the department's comprehensive diagnostic service. Implementation of a laboratory quality system and accreditation of the department to ISO 25 and 17025 followed as milestones between 2002 and 2005. Her research during this time focussed mostly on improvement of diagnostic methods for bovine tuberculosis and paratuberculosis and as well as the epidemiology of bovine tuberculosis in African buffalo and other wildlife species. Following restructuring of the research programmes at OVI in 2007, Anita Michel became Acting Programme Manager for Food, Feed & Veterinary Public Health.