Screening of banded mongooses (*Mungos mungo*) for mycobacterial infection in the Kruger National Park, South Africa

by

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And

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To my Family

Far away but always in my Heart

Contents

Declaration

I, Angela Caren Brüns, declare that the thesis, which I hereby submit for the degree of Master of Veterinary Medicine (Wildlife) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

…….. ……
Signature Date

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Summary

Dr Angela Caren Brüns

Screening of banded mongooses (*Mungos mungo*) for mycobacterial infection in the Kruger National Park, South Africa

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Bovine tuberculosis (bTB) was first diagnosed in the Kruger National Park (KNP) in 1990 and research has since focused primarily on the buffalo (*Syncerus caffer*) as the maintenance host and lion (*Panthera leo*) as a clinically affected species. However, little is known about the role that small predators might play in the tuberculosis epidemiology. The aim of this pilot study was to screen banded mongoose populations in the bTB high prevalence zone of the KNP for mycobacteria in general and for *Mycobacterium bovis* and other *Mycobacterium tuberculosis* complex members in particular to detect presence of infection.

Faecal swabs, tracheal swabs and tracheal lavage of 76 banded mongooses caught in cage traps within a two kilometre radius of Skukuza Rest Camp in the KNP were submitted for culture, isolation and speciation of *Mycobacterium* as the gold standard of bTB diagnosis. Blood was collected and serologically analysed for *M. bovis* and *Mycobacterium tuberculosis* antibodies using the ElephantTB STAT-PAK® Assay (STAT-PAK) and the Enferplex™ TB Assay (Enferplex). DPP® VetTB Assay for elephants (DPP) was used on STAT-PAK positive samples. To complement the

sample set obtained from live banded mongooses 12 animals were necropsied. Lesions and pooled lymph node samples together with a standard set of organ samples were submitted for culture and histopathology analysis.

Two banded mongooses had developed well demarcated, irregularly margined, grey-yellow nodules of up to 5 mm diameter located in the caudal lung lobes and/ or tracheo-bronchial, retropharyngeal or superficial cervical lymph nodes. These lesions were characterised by central necrosis in the one and calcification in the other animal. Histopathologically the lesions were described as caseating necrosis associated with epithelioid macrophages and necrogranuloma with calcified centre respectively. No acid fast bacteria were identified with Ziehl-Neelsen stain.

M. bovis was isolated from lung, lymph node and liver samples as well as tracheal lavages and tracheal swab from the same two banded mongooses but not from any other study animal. No other *Mycobacterium* of the *M. tuberculosis* complex was isolated. However, a variety of environmental mycobacteria, the most frequent from the *Mycobacterium avium* complex, *M. fortuitum* group, *M. simiae* group and *M. terrae* group, were cultured. *M. fortuitum* group was only and *M. terrae* group predominantly isolated from tracheal and faecal samples whereas *M. simiae* group and *M. avium* complex were the most frequent species isolated from post mortem samples, including tissue lesions and lymph nodes.

Serological analysis revealed 12 banded mongooses with a positive STAT-PAK result, confirmed with DPP. Enferplex was positive for MPB83 in four and MPB70 peptide in one animal. Only two banded mongooses, the ones with the strongest positive reaction on both STAT-PAK and DPP, reacted positively on all three serological assays. These were the same two animals that had developed granulomatous lesions and that *M. bovis* was cultured from ante and post mortem samples.

In conclusion, this study has provided the first evidence of bTB infection in banded mongooses in the KNP and demonstrated their ability to shed *M. bovis*. This finding has opened the discussion around possible sources of infection and its significance at the human/ wildlife interface in and around Skukuza.

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List of Abbreviations

abdom	abdominal
ca.	circa
CFP10	culture filtrate protein 10 kDa
Corp.	Corporation
i.e.	id est
Inc.	Incorporation
edn.	edition
e.g.	exempli gratia
ESAT-6	early secretory antigenic target 6 kDa
histopath	histopathology
KNP	Kruger National Park
Ln	Lymph node
MAC	*Mycobacterium avium* complex
macropath	macropathology
MFG	*Mycobacterium fortuitum* group
MPB70	Mycobacterial protein bovis 70
MPB83	Mycobacterial protein bovis 83
MSG	*Mycobacterium simiae* group
MTG	*Mycobacterium terrae* group
n.d.	no date
neg	negative
no	Number
NTM	nontuberculous *Mycobacterium*
NTMs	nontuberculous mycobacteria
path.	pathogenic
periph	peripheral
pers. comm.	personal communication
pos	positive
RLU	relative light units
rpm	revolutions per minute
Rv3616c	ESX-1 secretion system protein
SANParks	South African National Parks

thorac	thoracal
trach	tracheal
VWS	Veterinary Wildlife Services

Chapter 1: Introduction and Literature Review

1.1. Introduction

Tuberculosis is a slowly developing, chronic disease, which progresses if untreated and may result in a fatal outcome. It is caused by members of the *Mycobacterium* genus with *Mycobacterium tuberculosis* primarily causing infections in humans. Human tuberculosis (hTB) is decreasing globally (Kendall & Winthropp 2013); however, it is still highly prevalent in Africa with the world's highest prevalence and incidence rates recorded in South Africa (WHO 2013b). Infections in animals are mostly caused by *Mycobacterium bovis*. Historically bovine tuberculosis (bTB) has been one of the globally most important infectious diseases in cattle (OIE World Health Organisation 2009). Introduced into South Africa in the wake of the British colonization (Smith 2012), bTB has been reported as early as the 1920's in South African wildlife (Paine & Martinaglia 1929 in Michel *et al.* 2006). With the African buffalo *(Syncerus caffer*) established as a maintenance host (Rodwell *et al.* 2001) since first discovered in the Hluhluwe National Park in 1986 (Jolles, Cooper & Levin 2005), bTB has persisted in the wildlife conservation areas (Michel *et al*. 2006, 2009a). BTB thereby evaded country wide eradication programmes which were predominantly successful in cattle. A similar situation occurs in some other countries with bTB persisting in wildlife reservoirs e.g. in the European Badger (*Meles meles*) in the British Isles (Garnetta, Ropera & Delahay 2003) or the brushtail possum (*Trichosurus vulpecula*) in New Zealand (O'Brien *et al.* 2011). In South Africa, spill over infections have been recorded in a broad spectrum of antelope, pig, primate and predator species (Michel *et al.* 2006, Clifford *et al.* 2013). The spill over pathway with the most prominent impact on wildlife is the infection of lion (*Panthera leo*) predominantly from infected African buffalo especially evident in the Kruger National Park (KNP).

The KNP is situated in the summer rainfall area of a savannah ecosystem in the Mpumalanga lowveld. First proclaimed in 1926 after combining several smaller game reserves, it was originally set up to protect the diminishing fauna from hunting. Nowadays it encompasses 19624 km^2, measuring 350 km from north to south and 65 km from east to west. It provides a home to 147 mammal, 114 reptile and 507 bird species (SANParks n.d.). Across the Limpopo river these animals have access to the

Gonarezhou National Park in Zimbabwe in the north and Limpopo National Park in Mozambique to the east which were joined with the KNP to form the Great Limpopo Transfrontier Park in 2002. To the west several private and provincial game reserves adjoin the park. From farmlands bordering to the south and south west cattle infected with bTB most likely infected the KNP wildlife with *M. bovis*. Retrospective analysis places the introduction event in the late 1950s (Michel *et al.* 2006). This was most likely the only introduction event, as indicated by the cross species prevalence of only one dominant strain with few dependent variants suggesting a common ancestor (Michel *et al.* 2009a), in contrast to the high genetic diversity of *M. bovis* in South Africa's cattle population.

Only detected in 1990 for the first time in the KNP in an African buffalo (Bengis *et al.* 1996) bTB spread rapidly throughout the park and within 16 years of monitoring had reached and crossed the Zimbabwe border in the north (Palmer 2013). This demonstrates the potential threat of persisting bTB infection in the wildlife population with regards to recurrent outbreaks in livestock, transboundary spread or spill over to valuable or threatened game species.

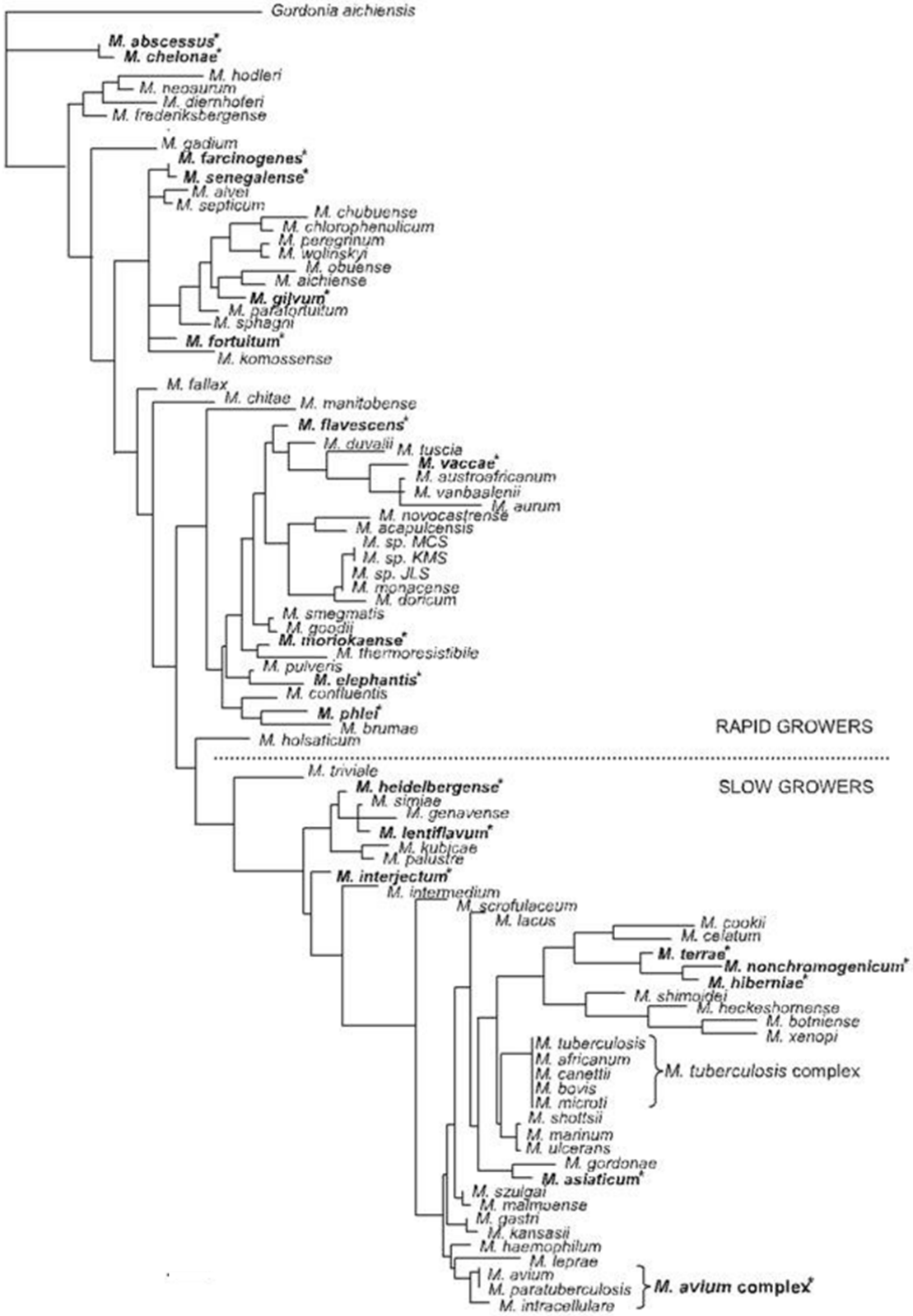
1.2. Aetiology

Up to date more than 120 species of *Mycobacterium* have been identified (Botha, Gey van Pittius & Van Helden 2013). They are aerobic, non-sporulating, immobile, 1.5 to 4 µm long and 0.3 to 0.5 µm wide pleomorph rods. Due to their thick outer lipid layer they are hydrophobic and stain acid fast (eds. Coetzer & Tustin 2004). They are ubiquitous and found as environmental saprophytes in soil, dust, aerosol and water (Falkinham 2009) or as opportunistic or obligate pathogens in animals and humans. Historically the genus was divided according to selected culturing properties, the most significant one being slow or fast growth. Those groups correlate with molecular relatedness and are still recognized today (Figure 1) (Botha e*t al.* 2013).

Nontuberculous mycobacteria:

Most of the obligate pathogenic mycobacteria are growing slowly with major pathogens and cause of tuberculosis in humans as well as animals found in the *Mycobacterium tuberculosis* complex (MTC). Mycobacteria not grouped in the MTC are called 'nontuberculous mycobacteria' (NTMs), 'mycobacteria other than tubercle

Figure 1: Phylogenetic tree of genus *Mycobacterium* depicting division between rapid and slow growing *Mycobacterium* species and indicating *Mycobacterium* species isolated in South Africa in animals in bold with asterisk (Botha *et al.* 2013).



bacilli' (MOTT) or 'potentially pathogenic environmental mycobacteria' (PPEM) (Wayne 1992). They encompass mostly environmental and saprophytic mycobacteria (Bercovier & Vincent 2001) but also opportunistic and some obligate pathogens. Concurrent immunosuppression or a local impairment such as chronic lung disease, trauma or surgical wounds of the host are a prerequisite for NTMs to cause clinical disease as an intact immune system will succeed in eliminating the less virulent bacteria (Falkinham 2009).

Clinical disease caused by NTM is called mycobacteriosis and does not differ from TB with lymphadenitis, pulmonary disease or skin granulomas, the predominant manifestation of infection (Katoch 2004). With the exception of the *Mycobacterium avium-intracellulare* complex (MAIC) members *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis* causing avian tuberculosis in birds and pigs and paratuberculosis in ruminants, respectively, little has been published regarding NTM infection in animals (Botha *et al.* 2013). Worldwide, *Mycobacterium kansasii* was found to cause lesions in wildlife such as deer, camels, birds and monkeys (Botha *et al.* 2013). *Mycobacterium marinum* (Falkinham 2009) and *Mycobacterium fortuitum* (eds. Coetzer & Tustin2004) cause mycobacteriosis in fish. In Africa, *Mycobacterium senegalense* and *Mycobacterium goodii* were described to cause pathology in cattle (Botha *et al.* 2013) and members of the *M. fortuitum* complex lesions in cattle and pigs (Bercovier & Vincent 2001). In a South Africa wide survey of buffalo, cattle soil and water *Mycobacterium terrae*, a group of *Mycobacterium moriokaense*-like mycobacteria, *Mycobacterium nonchromogenicum* and *Mycobacterium vaccae/ M. vanbaalenii* were most often isolated with a wide overlap occurring between animal and environment (Gcebe *et al.* 2013).

Mycobacterium tuberculosis complex:

Members of the *Mycobacterium tuberculosis* complex (MTC) are characterised by their slow growth as well as lack of pigment. They share an identical 16S rRNA sequence and with 99% DNA homology they are best differentiated by standard molecular tools (Botha *et al.* 2013). The most important members of the complex are *M. tuberculosis* and *M. bovis.* Species less common or affecting a narrower host range are the predominantly human pathogens *Mycobacterium africanum* and *Mycobacterium canettii* (eds. Coetzer & Tustin 2004), *Mycobacterium microti* causing

tuberculosis predominantly in voles (*Microtus agrestis*) and other selected small mammals (Skorik *et al.* 2007), *Mycobacterium caprae* isolated from goats and cattle in Central Europe (Prodinger *et al.* 2005), *Mycobacterium pinnipedii* from fur seals (*Arctocephalus* spp.) and sea lions (*Neophoca cinerea*) (Cousins *et al.* 2003), *Mycobacterium orygis* from antelope and camels in North Africa (Gey van Pittius *et al.* 2012), *Mycobacterium mungi* from the banded mongoose (Alexander *et al.* 2010), *Mycobacterium suricattae* from the suricate (*Suricata suricatta*) (Parsons *et al.* 2013) and the dassie *bacillus* (Parsons *et al.* 2008).

1.3. Epidemiology

Members of the MTC are found worldwide with primates and artiodactyla being more susceptible than e.g. horses or dogs. On a species level artiodactyla are more susceptible to *M. bovis* and primates and humans more susceptible to *M. tuberculosis* (eds. Coetzer & Tustin 2004) with the latter only sporadically recorded in a number of antelope, pig and primate species (Michel *et al.* 2013) as well as elephant and black rhinoceros (*Diceros bicornis*) (Mikota & Maslow 2011). With a mostly anthropozoonotic background hTB occurs predominantly in zoological collections or similar facilities with intensive management.

M. bovis induced bTB historically was one of the globally most important infectious diseases in cattle and humans during the 19th and 20th centuries and cattle are still recognized as the primary maintenance host of *M. bovis* (OIE 2009). Since the 1920s extensive eradication programmes in especially the developed world have succeeded in reducing the prevalence of bTB drastically. Nowadays bTB mainly persists in cattle in the developing world and some regional pockets in Canada, the United States, Europe and New Zealand (OIE n.d.). Where highly *M. bovis* susceptible wildlife occurs in sufficient numbers the obligate pathogen can circulate and persist. Those wildlife populations that develop into maintenance and reservoir hosts, make disease control and eradication difficult (Palmer 2013). Examples of these wildlife reservoir host species are the white tailed deer (*Odocoileus virginianus*), wood bison (*Bison bison athabascae*) and elk (*Cervus elaphus manitobensis*) in North America (Nishi, Shury & Elkin 2006, O'Brien *et al.* 2011), the European Badger in the British Isles (Garnetta, Ropera & Delahay 2003), the brushtail possum (*Trichosurus vulpecula*) in

New Zealand (O'Brien *et al.* 2011), the wild boar (*Sus scrofa*) in selected areas in Spain and selected deer populations throughout Europe (Gortazar *et al.* 2012).

M. bovis was introduced into South Africa via cattle in the wake of the British colonization as suggested by the exclusive isolation of European 1 clonal complex of *M. bovis* in South Africa (Smith 2012). Due to eradication programmes in the early 20ths century and subsequent diligent control efforts as implemented by the National Eradication Scheme introduced in 1969 (Michel *et al.* 2008) bTB in South Africa is nowadays mainly restricted to wildlife and wildlife areas (Michel *et al.* 2006, 2009a; Michel, Müller & van Helden 2010). It has been reported as early as the 1920 in the greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmia*) in the Eastern Cape Province (Paine & Martinaglia 1929 in Michel *et al.* 2006). However, eradication of bTB from wildlife was only considered after high prevalence was detected in the African buffalo population of the Hluhluwe National Park in 1986 (Jolles, Cooper & Levin 2005).

Only in 1990 was the first case of bTB detected in the Kruger National Park (KNP) buffalo (Bengis *et al.* 1996). Contact with cattle from adjoining farmlands in the south were most likely the source of *M. bovis* infection of KNP wildlife and the highest prevalence of bTB in the KNP is still found in the buffalo population in the south (Cross *et al.* 2009). Retrospective analysis places the introduction event in the late 1950s (Michel *et al.* 2006). This was most likely the only introduction event, as indicated by the cross species prevalence of only one dominant strain with few dependent variants suggesting a common ancestor (Michel *et al.* 2009a) in contrast to the high genetic diversity of *M. bovis* in South Africa's cattle population. Within 16 years of monitoring the disease reached and crossed the Zimbabwe border in the north (Palmer 2013) having spread rapidly throughout the park.

Over the decades buffalo (Rodwell *et al.* 2001) as well as greater kudu (Palmer 2013) have developed into maintenance hosts for bTB in South Africa with spill over documented in several antelope, pig, primate and predator species, namely eland (*Taurotragus oryx*), impala (*Aepyceros melampus*), Kirk's dik-dik (*Madoqua kirkii*), warthog (*Phacochoerus africanus*), bushpig (*Potamochoerus porcus*), chacma baboon (*Papio ursinus*), yellow baboon (*Papio cynocephalus*), vervet monkey

(*Chlorocebus pygerethrus*), lion, leopard (*Panthera pardus*), spotted hyaena (*Crocuta crocuta*) and cheetah (*Acinonyx jubatus*) (Michel *et al*. 2006, Clifford *et al.* 2013). Of the small predators *M. bovis* has been isolated from single cases in the large spotted genet (*Genetta tigrina*) and the honey badger (*Mellivora capensis*) (Michel *et al.* 2006). Novel members of the MTC were isolated from the suricate in the Kalahari and banded mongoose in northern Botswana named *M. suricattae* (Parsons *et al.* 2013) and *M. mungi* (Alexander *et al.* 2010), respectively.

Even though the route of infection for mongooses is not clearly established yet, similar to the badger (Nolan & Wilesmith 1994) and wild and domestic pigs (Palmer 2013) in Europe as well as the suricate (Drewe *et al.* 2009b), the chacma baboon (Keet *et al.* 2000) and large predators as lion and leopard (Keet *et al.* 1996) in Southern Africa spill-over infection is suspected to occur orally by scavenging or predation (Alexander *et al.* 2002). Alternatively urine, faeces or sputum contaminated food or water could be an infective source of *M. bovis* as postulated for cross species infection from the African buffalo to the greater kudu (Michel *et al.* 2006), with favourable moisture and temperature as well as availability of nutrients playing a key role for the survival of *M. bovis* in the environment (Tanner & Michel 1999).

Close contact as provided by herd, nursing or grooming behaviour within a herd or pack is the key driver that promotes respiratory transmission (Drewe *et al.* 2009b). The latter is the main mode of transmission for *M. bovis* in cattle but also in intra-species transmission in wildlife as demonstrated by bTB lesions found predominantly in the respiratory tract in African buffalo (Michel *et al.* 2007, 2009), water buffalo (*Bubalus bubalis*), bison, lechwe (*Kobus leche*), cervids (Bengis 1999), European badgers, brushtail possums (Palmer 2013) and suricate (Drewe *et al.* 2011).

A third means of infection is by the percutaneous route, shown to be an important mode of transmission in kudu, possums (Bengis 1999, 2001), mongooses and suricates (Alexander *et al.* 2002; Drewe e*t al.* 2009b). These species tend to develop draining fistulas releasing mucopurulent discharge from superficial lymph nodes that contaminate food or water. In the brushtail possum direct horizontal spread is promoted during grooming, nursing, mating or aggressive behaviour. Kudus are postulated to get infected through microlesions in the skin obtained when scratching

themselves behind the ears or injuring themselves on contaminated thorns while browsing. Other species where percutaneous transmission has been shown to be of importance for *M. bovis* infection are lion (Keet *et al.* 2010) and badgers (Kaneene *et al.* 2010) that get infected through bite wounds during aggressive feeding frenzies or territorial fights.

Ultimately the infective dose will be decisive in whether a pathogen can establish itself in the target species. Smaller doses are sufficient for aerosol transmission when compared to the oral or transdermal route of infection (Bengis 1999), explaining the predominance of intra-species over inter-species spread and the development of gregarious species into maintenance hosts.

1.4. Pathogenesis

Many factors will influence the course of the disease and whether disease as such will develop at all: these include mycobacterial species, species strain, route of infection, host species, host age and immune status and infective dose (Cousins et al. 2004). These factors together with the slow progressive character of the disease make it difficult to determine incubation times.

After respiratory, alimentary or percutaneous infection *M. bovis* for example is ingested by phagocytes at the site of entry, i.e. bronchioli or interstitium of the respective tissue (Ernst 1998). The more virulent the bacterial strain, the more resistant it is to cellular immune response (Cross *et al.* 1999). A compromised immune system or high infective dose will also promote persistence and propagation of the bacilli in the host organism. They will multiply in the phagocytes and destroy them, causing a local inflammatory reaction, the primary lesion (Bengis 1999). From there they are circulated mostly via the lymphatic route to the local lymph node, i.e. the tonsil, pharyngeal, tracheo-bronchial, mediastinal or superficial lymph nodes. Primary lesions and affected lymph node form the primary complex. The inflammatory process promotes itself further, enhanced by cytokine release and cellular hypersensitivity reaction, resulting in lesion formation with species specific characteristics (Dannenberg 1989). In bovids that is a typical granuloma known as a tubercle. This tubercle is characterised by the formation of a fibrous capsule around a caseous necrotic centre. The process is often accompanied by lesion calcification (Neill *et al.* 1994). In the enlarging granuloma the bacilli are initially contained locally

but with time, enzymatic action of the immune cells results in liquefaction and rupture of the lesion which leads to invasion of the airways, blood or lymphatic systems and spread to species specific predilection sites in the entire body (Neill *et al.* 1994). In this way secondary lesions are formed ranging from focal to multifocal to miliary or nodular character, and may progress to finally encompass the entire organ leading to organ failure and death (Bengis 1999; Cousins *et al.* 2004).

1.5. Clinical Signs

Tuberculosis in general is considered a chronic, slow, progressive and frequently fatal bacterial disease. Clinical signs are often inapparent, especially in wildlife, until cachexia becomes obvious in the terminal stage of the disease (Kaneene *et al*. 2010). As lesions progress, clinical signs of general disease such as loss of appetite, weight-loss and undulating fever might become apparent. Depending on lesion localization they may be accompanied by dyspnoea, coughing and respiratory distress or indigestion, colic, congestion and diarrhoea. This variety of clinical signs may be caused by enlarged internal lymph nodes that obstruct airways, blood vessels or the alimentary tract as well as by organ impairment itself as e.g. with granulomatous pneumonia. Granulomatous mastitis might develop in the mammary gland (OIE 2009). Only in species that frequently develop superficial lymph node swelling or draining sinuses might lesions be readily visible. An example is the greater kudu where swelling of the parotid lymph nodes (Bengis *et al.* 2001) might be visible from afar. In the possum swelling of the inguinal and axillary lymph node predominates (Bengis 1999). Similarly suricates develop superficial lymph node swelling, with abscessation occurring especially in the head and neck lymph nodes resulting in chronic, discharging skin wounds caused by *M. suricattae* (Alexander *et al.* 2002) or *M. bovis* (Drewe *et al.* 2009b), similar to what has been described in European badgers. Contrary to what is described in the suricate, only cachexia, ataxia and weakness were described to manifest clinically with *M. mungi* infection in the banded mongoose (Alexander e*t al*. 2002). Therefore, tuberculosis in wildlife is most frequently diagnosed on post mortem examination only, and often so in animals that are sampled as part of a routine surveillance programme or opportunistically, after having died of other causes.

1.6. Pathology

Typical gross lesions of bTB in bovids for example are the pale-white to light yellow nodular granulomas called tubercles that are surrounded by a thickening fibrous capsule. As the granulomas enlarge they develop a characteristic caseating central necrosis with occasional calcifying foci. The lesions can be focal, confluent or miliary and therefore vary in size from microscopically small to several centimetres in diameter. Ultimately they can encompass the entire organ in question, replacing all functional tissue. Lesions are found most frequently in lymphoid tissues of the head, lung and liver, but also commonly in the lung, liver, spleen, pleura and peritoneum (OIE 2009). Histopathologically the central necrosis with its white blood cell debris is surrounded by epithelioid cells that transform into giant Langhans' cells as the granuloma develops. Mycobacteria cannot always be illustrated in the histopathology sections in ruminants, but when present these acid fast bacteria appear as red rods with Ziehl-Neelsen stain.

Depending on the host species examined, macroscopic appearance will differ in regards to lesion localization and appearance, degree of encapsulation, caseation, liquefaction, mineralisation as well as progressive nature (Bengis 1999). Cervids, camelids (Kaneene *et al.* 2010) and kudu develop predominantly purulent lesions (Bengis *et al.* 2001). In buffalo bTB granulomas are poorly encapsulated which promotes the spread of infection and results in a more progressive character of disease in this species (De Vos *et al.* 2001). In large predators such as lion *M. bovis* lesions are described as proliferative and fibrous without significant necrosis but rather pulmonary cavitational lesions lined by mucoid exudate (Keet *el al.* 2010; Palmer 2013). However, in small predators such as ferrets necrosis has been described to be of rather coagulative than liquefactious character (Bengis 1999). In the suricate, lesions ranging from disseminated miliary white nodules to centrally caseous or liquefying granulomas were located in submandibular, retropharyngeal, axillary and mediastinal lymph nodes as well as in the lung, liver, spleen, parotis and pancreas (Alexander *et al.* 2002; Drewe *et al.* 2009b). Similarly in the banded mongoose lesions appeared as miliary grey-white nodules of 0.5 to 2 cm diameter with chalky central necrosis in enlarged mesenteric lymph nodes and in liver and spleen, causing hepato- and splenomegaly, as well as grey-white infiltrates in the lung, kidney and intestine (Alexander *et al.* 2002). Histopathology revealed lesions to

be granulomatous inflammation characterized by epithelioid macrophages, intracellular acid fast rods (Drewe *et al.* 2009b) and central necrosis in the larger lesions (Alexander *et al.* 2002). Those microscopic lesions were found in all liver, spleen, lymph node and lung samples examined as well as in some adrenal gland, kidney, myocardium, pancreas, epididymis, pleura, intestine, peritoneum and skin samples but not in brain, skeletal muscle, urinary bladder or testis (Alexander *et al.* 2002).

1.7. Diagnosis

A definitive diagnosis of tuberculosis is often only obtained from a post mortem examination. Indicative of mycobacterial disease are the presence of acid fast bacilli on Ziehl-Neelsen stained organ impression smears, exudate smears or tissue samples and the presence of a typical granulomatous inflammatory reaction with epithelioid and giant cells seen in histopathology. A definitive diagnosis is made by culture of a pathogenic *Mycobacterium* only, the gold standard for TB diagnostic. This can be followed by molecular biological techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP, i.e. DNA finger printing), spoligotyping and single nucleotide polymorphisms (SNP) genotyping for species or strain identification, which enables epidemiological tracing and detection of species spill-over (Bengis 1999).
Clinical diagnosis of TB is often difficult especially since clinical signs are mostly non-specific and clinically apparent only in the late stages of the disease. This has led to the investigation and development of a variety of immune meditated diagnostics based on either serology or cell mediated immunity, to aid in ante mortem diagnosis of TB.

Cell-mediated immune reactions are utilized in tests such as the tuberculin intradermal or skin test and interferon-gamma release assay (IGRA) (Chambers 2013). During early stages of infection macrophages attempt to eliminate the intracellularly persisting mycobacteria. However, the macrophages are only able to lyse the intracellular pathogens after having been activated by lymphokines released by activated T-helper cells (TH1); a process termed delayed type hypersensitivity reaction. In addition to activating macrophages lymphokines will attract additional mononuclear cells promoting the inflammatory reaction (Pollock *et al*. 2001).

The intradermal test evaluates the delayed tissue hypersensitivity reaction towards purified mycobacterial antigen, the so called purified protein derivative (PPD, avian and bovine), which results from previous exposure to MTC organisms. Signs of inflammation are measured quantitatively as increased skin thickness and qualitatively as necrosis, oedema, pain, attachment, lymph node enlargement and diffuse or circumscribed swelling. False positive reactions can occur if animals are exposed and sensitized to NTMs (Bengis 1999). Further drawbacks of this method are the time delay of 72 hours between test application and reading as well as species specific skin properties and differences in inflammatory reaction making it necessary to determine species specific cut off values.

To avoid animal handling and time delay necessary when implementing the intradermal test, cellular immunity can also be evaluated in vitro by measuring cytokine production, in particular gamma interferon. Using heparin whole blood, in vitro stimulation of *Mycobacterium* sensitized TH1 cells with PPD induce interferon gamma release which can be measured in the plasma. Drawbacks are that the detection of gamma interferon depends on the use of species specific antibodies in specifically developed detection assays. Such assays have a limited application across different species and up to date have only been developed for cattle/ buffalo, sheep/ goats, primates (Bengis 1999), elephant and rhinoceros (Morar 2009; Morar *et al.* 2013).

To enable rapid screening diagnosis of TB, serological approaches relying on antibody-antigen binding reactions have been developed. With mycobacteria being mostly tissue bound and even contained in granulomatous lesions, serological test approaches depend on antibody detection in the blood. Depending on the test assay used, different mycobacterial antigens as well as detection methods are employed to identify mycobacterial antibodies. Examples are lateral-flow devices as STAT-PAK® Assay (STAT-PAK) and DPP® VetTB Assay (DPP) (Chembio, Medford, NY, USA) that implement immunochromatography, blotting methods as the multi-antigen print immunoassay (MAPIA) as well as the traditional enzyme-linked immunosorbent assay (ELISA) and the less commonly used fluorescence polarization assay (FPA) (Chambers 2013). Limiting factors in the implementation of these serological tests are that species differences in antibody responses might result in limited cross reactivity of tests for species they were not developed for (Bengis 1999).

Furthermore, considering the predominantly cellular mediated immune reaction towards mycobacterial infection, humoral response, especially in early stages of infection, is often only weak. However, promising progress has been made with serological tests detecting antibodies with specific mycobacterial target proteins such as early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10 (Botha *et al.* 2013).

In summary, diagnosing mycobacterial disease in a wildlife species is met with several challenges. The varying degree of cross-reactivity between species renders serological tests not consistently reliable in a species they are not developed for (Michel & Simoes 2009). To address this issue the range of species, that serological tests have been evaluated for, has increased dramatically in the last five years and now includes South American camelids, black rhinoceros, lions and non-human primates (Chambers 2013). On the other hand cross reaction with NTMs and other MTC members as e.g. *M. mungi, M. caprae* and *M. orygis* (Chambers 2013) or bacteria closely related to *Mycobacterium* as e.g. *Nocardia* and *Corynebacterium* spp. (Cousins *et al.* 2004) can be confounding factors in immune mediated diagnostic. Therefore, especially in species that tests have not been validated for, the use of a combination of tests is recommended to enhance the chance for making the correct diagnosis (Drewe *et al.* 2009a), bearing in mind that the presence of antibodies does not necessarily indicate disease but mainly exposure.

Ultimately only the culturing of the causative agent, either from secretions, faeces or necropsy samples, is regarded to support a definitive diagnosis and even then the pathogen isolated still needs to be correlated to macroscopic and/ or histopathologic lesions to verify it as causative agent (OIE 2009).

1.8. Motivation for and aim of this study

With research on bTB in the KNP focused primarily on the buffalo as the maintenance host and lion as a clinically affected species, little is known about the role that small predators might play in the epidemiology of tuberculosis in the KNP.
That small predators are affected by tuberculosis is demonstrated by the fact that cases of bTB have been diagnosed in Southern Africa in the large spotted genet and the honey badger (Michel *et al.* 2006). Furthermore, members of the MTC were

isolated from the clinically diseased suricates in the Kalahari and banded mongooses in northern Botswana (Alexander *et al.* 2002; Drewe *et al.* 2009b), subsequently identified as novel *Mycobacterium* species *M. suricattae* (Parsons *et al.* 2013) and *M. mungi* (Alexander *et al.* 2010). If clinical bTB developed in small predators in the KNP, this might contribute to its spread comparable to the European badger in the United Kingdom spreading tuberculosis to cattle by contaminating feed with *M. bovis* (Garnetta, Ropera & Delahay 2003). In this way not only rare or endangered wildlife species in the KNP but also the livestock of communities adjoining the park might be threatened by infection. In the same context, the close contact between wildlife and humans in and around rest camps might pose a risk to wildlife from hTB, as described for animals in close proximity to tuberculosis infected humans (Michel *et al.* 2009c, 2013).

This study forms part of a long term research plan to study tuberculosis epidemiology in the KNP ecosystem, evaluating whether small predators possibly play a role in the bTB epidemiology in the KNP and whether there is any evidence of human tuberculosis transmission at the small predator/ human interface of rest camps. Restricted visibility of small predators as mostly nocturnal species as well as inconspicuousness of their small sized carcasses in case of disease related death called for the need of a representative study animal, the banded mongoose. However, conducting a lethal survey in a species whose role in the epidemiology had not yet been illustrated did not seem ethical or feasible. Therefore, a survey based on clinical samples and only selected necropsies was decided on.

The aim of this pilot study was to screen the banded mongoose population in the bTB high prevalence zone in certain high risk areas in the KNP, i.e. in and around rest camps, for mycobacteria in general and for *M. bovis* and other MTC members in particular to detect presence of infection or disease on the basis of the following hypothesis:

Banded mongooses in the KNP become infected with *M. bovis,* other members of the MTC or other *Mycobacterium* species potentially able to cause disease or serve as immune modulators of the host response to bovine tuberculosis.

The following key questions were identified:

- Are infection and/ or disease due to *Mycobacterium* species detectable in selected banded mongoose populations as determined by culture of in vivo and post mortem samples?
- If *Mycobacterium* species are isolated, what species are isolated (*M. bovis, M. tuberculosis, M. mungi* and/ or other *Mycobacterium* species)?
- Does infection with *Mycobacterium* species correlate to pathological organ changes as evaluated by histopathology?
- Can we detect antibodies to MTC organisms including cross-reacting antibodies in the mongooses captured from the selected populations using STAT-PAK® Assay and Enferplex^{TM} TB Assay?
- If antibodies are detected can we find evidence of infection with tuberculous or nontuberculous *Mycobacterium* species in these mongooses using culture and histopathological evaluation?

Chapter 2: Materials and Methods

2.1. Study animals

Mongooses including the suricates belong to the family of the herpestidae (Hoffmann 2008). Of the eleven South African mongoose species only seven occur in the Kruger National Park (KNP). Of these only the banded mongoose and the dwarf mongoose (*Helogale parvula*) are a relatively common mongoose species with a diurnal life style as well as living in social groups. The adult banded mongoose averages 1 to 1.6 kg body mass and 59 cm total body length and is therefore better suited for sampling purposes than the smaller dwarf mongoose (267 g average body weight) (ed. Apps 2000). Similar to other mongoose species the banded mongoose has a ferret like appearance and species specific grey to grey brown coat with characteristic narrow, regular, transverse grey stripes from shoulder to tail base. Pups younger than three months old are uniformly dark red-brown (Skinner & Smithers 1990). They leave the den to forage with the pack when four to five weeks old, are sexually mature at 10 months and fully grown at 13 months (ed. Apps 2000). Mean life expectancy for the banded mongoose has been recorded as seven years (Hinton & Dunn 1967). However, survival rates are only 50% up to three months of age and 90% in over six month old individuals (Skinner & Smithers 1990). Being a social species banded mongoose live in packs of about 30 animals with a maximum of 75 reported in the KNP (ed. Apps 2000). They occupy home ranges or territories at densities that mostly range around 2.4 to 3 individuals per km² (Hoffman 2008; Maddock 1988) in water associated savannah and woodland. Within their home range they will have several preferred den sites such as old termitaria, abandoned burrows of denning species, fallen trees, gullies or manmade shelters which they might use in rotation. During the day they forage in a scattered group keeping vocal contact while in search for their preferred food, mostly beetles and grubs but also other invertebrates as well as small reptiles, mammals and birds. Additionally they will eat fruit and opportunistically raid rubbish bins where close to human settlements (ed. Apps 2000; Hoffmann 2008). When on the lookout for potential danger, e.g. raptors, they will stand on their hind feet or on a raised vantage point. The IUCN categorizes the banded mongoose as of least concern and with a stable population (Hoffmann 2008).

Target n number for captures were determined by tabulating test sensitivity against different prevalence scenarios with a confidence level of 0.9 and assumed test specificity of 0.9. A target sampling size of 70 animals was selected for the study because statistically that would yield at least five positive test reactants should test sensitivity lie at 0.6 and prevalence at 0.05.

2.2. Capture Sites

All banded mongooses were caught within a two kilometre radius of the Skukuza rest camp (see figure 2 in 4.1) situated on the southern bank of the Sabie River at the confluence with the N'waswitshaka in the south west of the KNP. Encompassing the Skukuza rest camp and day visitors area as well as the KNP administrative head office and supporting staff village including a church and elementary school, the area is the largest settlement within the park. Situated within the Sabie River Thicket ecozone (SANParks 2008) the targeted banded mongoose population had access to natural bush veld as well as the human habitation. The latter comprises a mosaic of artificial landscapes within the natural bush veld and riverine vegetation of e.g. the water reservoir Lake Panic, golf course, nursery, church and school grounds, residential houses with surrounding gardens, clustered living compounds, administrative buildings, workshops, storage areas, animal holding facilities and the rest camp itself as well as day visitors area. Fencing ranging from none to 1 and 2.1 m depending on locality but did not prohibit access for the banded mongooses.

2.3. Procedures

2.3.1. Animal capture

Similar to the capture method described by Cant (2000) and De Luca & Ginsberg (2001) cage traps measuring 72x28x32 cm3 (Standard Humane Cage Trap AHATSD provided by SANParks, Scientific Services, available from, Animal Handling Support Systems, Greenside, South Africa) were baited with a mixture of peanut butter and oats to capture the banded mongooses. Locations within fenced areas were preferred for trapping as they would exclude large opportunistic foragers as e.g. warthogs from triggering the traps. The traps were set early in the morning in preferred foraging places of the troops, near sleeping hide outs or opportunistically at troop sightings. For example at trapping sites in the staff village mongooses would

forage in gardens and on heaps of garden litter or compost. At the SANParks Veterinary Wildlife Services (VWS) office bomas they would hunt for grubs and beetles on the dung heap of boma enclosed white rhinos (*Ceratotherium simum*) which were concurrently being studied at that site. In the living quarters, the day visitors area and the student accommodation at the VWS office the mongooses would raid rubbish bins. At the vehicle compound and the VWS office bomas they would sleep underneath storage containers and in the staff village in garages or gullies. Traps were checked every three to four hours for successful captures, malfunction or disturbance by non-target species as e.g. non human primates - and reset or rebaited if indicated. Trapped animals were recovered together with the now covered cage and brought to the VWS office laboratory for anaesthesia and processing.

Trapping site GPS coordinates were recorded together with location, date and cage number on the 'Mongoose Capture Sheet' (Appendix 1). Traps were closed in the late afternoon to avoid capture of non-target species during the night. Unsuccessful trapping as well as sightings and preferred localities were observed, noted and evaluated to improve trapping success. Animal welfare was evaluated according to primary injury defined as trauma due to the trapping event itself and secondary injury caused by attempts to escape as indicated by recent tooth damage, claw injury, nose and skin abrasions or other injuries rated as none (0), mild (1), moderate (2) to severe (3) with healed wounds rated as 1. Most likely capture related injury was recorded as fresh injury versus capture unrelated old or healed wounds. To identify recaptures all animals were marked with ear notches and a microchip transponder as described below (animal data and identification).

2.3.2. Anaesthesia

Modifying the trap into a squeeze cage, animals were restrained with a customized (VWS) wire-pane and handle insert. The anaesthetic agent was hand injected intramuscularly according to estimated weight as a mixture of commercially available drugs, diluted to the desired concentration at a volume feasible for once off injection. If more than one animal were caught in one cage or at the same time, they were anaesthetized and processed concurrently to minimize time in captivity.

The first 13 banded mongooses were anaesthetised with a mixture of ketamine (Kyron Laboratories, Benrose, South Africa) and medetomidine (Kyron; Domitor®, Pfizer, Sandton, South Africa) at mean dose of 3.14 mg per kg and 0.16 mg per kg respectively, resulting in recumbency within six to 12 minutes. Possibly due to adrenaline antagonism to medetomidine induced by capture stress, adult males did not respond well to this anaesthetic combination and butorphanol (Kyron) at 0.39 mg per kg mean dose was added to the anaesthetic mixture along with increasing ketamine to 3.28 mg per kg and medetomidine to 0.17 mg per kg mean doses for the following 23 anaesthesias. Due to varied results in heavier animals, the remaining 51 banded mongoose were anaesthetised with mean doses of Zoletil® (Virbac, Centurion, South Africa) of 2.86 mg per kg and medetomidine of 0.14 mg per kg, resulting in recumbency within two to 10 minutes.

Anaesthesia was monitored according to physiological responses including heart and respiratory rates, rectal body temperature, eye reflex, jaw tone and responsiveness to external stimuli or pain. Returning jaw tone or pain response indicating a light plane of anaesthesia required injection of an additional anaesthetic intramuscularly at 40 to 50% of the original dose. The banded mongooses were placed on a covered hot water bottle to help maintain body temperature. Procedures lasted for a mean of 80 minutes calculated from time of recumbency to the time that antidote was administered intramuscularly at five times the equivalent amount of medetomidine for atipamezole (Antisedan®, Pfizer) and 20 times the equivalent amount of butorphanol for naltrexone (Kyron). For recovery animals were replaced into the capture cages now padded and covered with sheets. Average time of recovery measured from time of antidote injection to time of first signs of head movement was four minutes and 53 seconds, with slightly longer recovery times of five minutes three seconds as well as prolonged times of ataxia when using the zoletil / medetomidine mixture. No food or water was offered during the wake-up period to avoid accidental aspiration or suffocation. Once fully recovered as indicated by absence of ataxia the banded mongoose were released at the capture site or in sight of their family group. If ataxia was still present at night fall the animal was kept overnight in a warm dark place and released in the early morning.

Data such as anaesthetic premix date and concentration, administered drug volume and mass, time and site of injection, dose, monitoring data, time and route of antidote administration, time of first signs of wake-up indicated by head lifting, time of recovery as indicated by locomotion was recorded on the 'Mongoose Capture Sheet' (Appendix 1).

2.3.3. Euthanasia

A maximum of 30 animals was planned for elective euthanasia and necropsy. Selection criteria were clinical signs of mycobacterial disease as listed below, advanced age or a reactive STAT-PAK® (STAT-PAK). To reduce bias towards the STAT-PAK the maximum proportion of STAT-PAK positive animals subjected to necropsy should not exceed 50%.

Criteria considered to indicate mycobacterial disease were lethargy or weakness as observed before anaesthesia or cachexia, enlarged lymph nodes especially in the head and neck region, chronic skin wounds, abnormal respiratory auscultation or abdominal palpation (Alexander *et al.* 2002; Drewe e*t al.* 2009b) evaluated during clinical examination.

Age was determined by adapting De Luca & Ginsberg's (2001) scale of relative tooth wear according to postulated mean life span of seven years in the banded mongoose (Hinton & Dunn 1967) as indicated in Table . Additional traits taken into account for ageing were coat colour change from red-brown to adult grey with species specific bands estimated to occur at three months, tooth eruption estimated at five to six months of age and maximum adult head measurement of 41 mm in females and 41 to 42 mm in males at 12 months (Cant 2000). Accordingly age groups were classified as baby (0 to 3 months), juvenile (3 to 5.5 months), subadult (5.5 to 12 months), young adults (12 to 18 months), prime adults I (18 months to 2 years), prime adults II (2 to 4 years), prime adults III (4 to 6 years) and old adults (>6 years) (Table).

Animals that expressed any one of the three qualifying criteria were euthanised while still under the influence of the anaesthetic via intracardial injection of pentobarbitone (Euthapent®, Kyron) at a dose of at least 200 mg per kg.

Table 1: Allocation of age and age category according to tooth wear, dentition, colour change and head width adapted from Cant (2000) and De Luca & Ginsberg (2001) taking a life span of seven years into account (Hinton & Dunn 1967).

Age category		Age	Tooth wear		Dentition	Colour	Head width [mm]
Baby	B	< 6 weeks	none	0	deciduous	brown	<41 (female) <42 (male)
Juvenile	J	< 5.5 months					
Subadult	S	> 5.5 months			eruption		
Young adult	Y	> 12 months	light	1	permanent	banded	41 (female) 42 (male)
Prime adult I	P1	> 18 months	light to moderate	2			
Prime adult II	P2	> 2 years	moderate	3			
Prime Adult III	P3	> 4 years	moderate to heavy	4			
Old adult	O	> 6 years	heavy	5			

2.3.4. Sample collection, processing and data recording

All captured animals older than three months of age as indicated by a change in coat colour as described above (Table) were subjected to sampling unless they had been sampled within the preceding six weeks. All samples were collected under anaesthesia. Each animal was allocated a unique study number ('Ax') at first capture, allocated continuously in order of capture (e.g. A1, A2). All sample containers were labelled with the study number, species, age, gender, date, type of sample and a VWS laboratory number. Animal data such as study number, measurements and identification as well as sampling, clinical evaluation and monitoring data were recorded on the 'Mongoose Capture Sheet' (Appendix 1). All field staff wore gloves and protective clothing while handling the animals or performing necropsies. Face masks were worn at necropsy. Work surfaces and work material such as instruments, cutting boards and hot water bottle were disinfected with F10SC Vet Disinfectant® (Health and Hygiene, Roodepoort, South Africa) at a dilution of 1:250.

Clinical evaluation and animal identification:

Gender, mass in gram and body condition score on a scale of 0 to 5 as described in Table 1 were recorded for each individual. Age was determined as described above (3.3.3). In addition to monitoring anaesthesia according to heart rate, respiratory rate, reflexes and body temperature as described above (3.3.2) and evaluating trapping injuries as listed in 3.3.1, dehydration in per cent was recorded. Other injuries were described qualitatively and captured on photograph (Olympus C765 ultra zoom digital camera, Olympus Deutschland GmbH, Hamburg, Germany). Peripheral lymph nodes were palpated and recorded as atrophied (-), not abnormal (0), mildly enlarged (1)

when asymmetry was palpated during thorough examination and moderately enlarged (2) when lymph nodes or asymmetry was easily palpated in at least one node, and severely enlarged (3) when readily visible. Lungs were auscultated either before or after tracheal lavage and intensity of respiratory noises recorded on a scale of no sounds audible (0), diminished sounds audible only after listening for a few seconds (1), diminished sounds readily audible (2), vesicular sounds readily audible (3), sounds including rales immediately audible (4) and sounds including stridor or rales audible without a stethoscope (5). External parasite loads were measured by presence or absence of fleas and estimated tick numbers categorised as absent (0), less than five (<5), ten (5-10), 20 (11-20), 50 (21-50) and 100 (51-100) ticks.

Table 1: Criteria used to allocate a body condition score.

Body condition score		Ribs/ spine/ hip bones
0	Emaciated	Very prominent
1	Poor	Prominent
2	Fair	Visible
3	Good	Palpable
4	Fat	Stout
5	Very fat	Rounded

To identify individual animals and recognize repeat captures a microchip transponder (Identipet® FDX-A 10 digit microchip small TX1440L10S, Identipet (Pty) Ltd, Muldersdrift, South Africa) with a unique number was inserted subcutaneously with the applicator syringe at the left tail base of each animal. The number was recorded on the capture sheet. For visual identification a triangular ear notch with two millimetre long sides was taken with small surgical scissors from the edge of the ear according to a positional notching system at the top, middle or bottom position of left or right ear or any combination thereof. Occasional bleeding from the incision was stopped with applying pressure and potassium permanganate crystals (Kyron).

Blood sampling and processing:

Anaesthetised animals were sampled for blood at a maximum of 1.5 ml from the brachycephalic vein following standard aseptic procedure by inserting a 23 to 21 gauge needle (Terumo®, Somerset, New Jersey, USA) into the raised vein and catching the free flowing blood into a serum and a lithium heparin MiniCollect® tube (Greiner bio-one, Kremsmünster, Austria), respectively. Blood samples were centrifuged for 10 minutes at 3000 rpm (DYNAC 10 000 rpm Centrifuge 420101, Clay

Adams, Becton Dickinson, USA) and serum as well as plasma harvested and stored as 60 to 100 µl aliquots in a steam-pressure sterilised (ALMOR P-09A, Albert Moore (Pty) Ltd, South Africa) 96 deep well minitube storage system (Axygen®, Corning Life Sciences, Union City, California, USA) at -80 ºC.

Laryngo-tracheal and faecal sampling and processing:

The banded mongoose was placed in sternal recumbency with moderately elevated abdomen to position thorax and trachea in a slightly downward angle. While a helper opened the jaws a headlamp served to illuminate and therewith visualise the caudal oral cavity. Steam pressure sterilised wooden skewers with their tip wrapped in cotton wool were used as laryngo-tracheal swabs. The cotton wool was wetted with two drops of sterile physiological sodium chloride solution and brushed over the laryngeal mucosa, avoiding the oral mucosa. After saturating the swab with saline it was placed in a sterile 1.8 ml cryotube (Cryo.s^{TM}, greiner bio-one, Kremsmünster, Austria) and the wooden skewer broken off. In the following a bronchio-tracheal lavage was performed adapted from Drewe *et al.* (2009b) by instilling two millilitre of sterile 0.9% saline with a three millilitre syringe (Terumo®) into the trachea with a sterile intravenous catheter (Jelco® I.V. Catheters, Smiths Medical UK, Ashford, United Kingdom) without stilette, measuring 18 gauge for adults and 22 gauge for sub-adults and juveniles. The fluid was immediately retrieved by gentle suction and transferred into a 1.8 ml cryotube. Tracheal lavage was considered successful if 0.5 to 1 ml of slightly opaque fluid was recovered. For samples of less than 0.5 ml the procedure was repeated once and if still insufficient 0.5 ml sterile saline added.

Preceding rectal manipulation for anaesthesia monitoring steam pressure sterilised cotton buds wetted with two drops of sterile saline were inserted into the rectum and turned twice. Colour change of the swab indicated successful sampling. The swab was then saturated with sterile saline, placed into a 1.8 ml cryotube and cut off. Faecal samples were collected opportunistically when animals defecated in the capture cage.

Tracheal swabs, tracheal washes, faecal swabs and faecal samples were stored at -80 ºC.

Necropsy and post mortem sampling:

Necropsies were performed in a closed room on a clean, steel necropsy table using F10 SC® disinfected plastic white board and 10% formalin sterilized surgical instruments. The animal was examined externally, then skinned and positioned in right lateral recumbency with the non-dependent limbs partially removed. Peripheral lymph nodes were evaluated and sampled. After opening the abdomen and thorax all inner organs and lymph nodes were evaluated macroscopically for presence of lesions. Aliquots of organ and lymph node lesions were collected into 50 ml sample bottles or 1.8 to 4 ml cryotubes and frozen for mycobacterial culture as well as placed in 10% buffered formalin, respectively. All remaining lymph nodes were pooled into head (mandibular, parotideal and retropharyngeal), peripheral (superficial cervical, axillary, inguinal and popliteal), thoracal (mediastinal, sternal and tracheo-bronchial) and abdominal lymph nodes (mesenterial, gastric, hepatic and renal) and stored at -80 °C. Samples of spleen, lung, liver, muscle, kidney, heart, adrenal gland, pancreas, small and large intestine and genital organs were collected in 250 ml wide necked formalin bottles. The head was split in half, the nasal septum evaluated for lesions and the brain sampled in formalin. Lesions were photographed (Olympus) and together with the samples taken recorded on the 'Necropsy Report' template (Appendix 2).

2.4. Analytical procedures

2.4.1. Histopathological evaluation of tuberculosis status

For histopathological evaluation the samples containing macroscopical lesions and enlarged lymph nodes that were stored in 10% buffered formalin were sectioned at 4-6 µm thickness across lesions, stained with haematoxylin/ eosin and Ziehl-Neelsen stain and evaluated microscopically for tuberculosis-like lesions and acid fast bacilli by the Pathology Section, Department of Paraclinical Sciences of the Faculty of Veterinary Science according to standard procedures (Prof. Mark Williams).

2.4.2. Cultural evaluation of tuberculosis status

Frozen tissue samples, laryngo-tracheal swabs, tracheal washes, faecal swabs and faecal samples were processed, cultured and speciated at the Division of Molecular

Biology and Human Genetics of Stellenbosch University (Louise Botha, Prof. Paul van Helden).

Sample processing and culture conditions:

The samples were thawed, homogenized and decontaminated by incubation in 4% sodium hydroxide and 2.9% sodium citrate for 15 minutes (Warren *et al.* 2006). Processed samples were transferred into Mycobacterial Growth Indicator Tubes (MGIT) (BD Biosciences, USA) and incubated at 37 °C until bacterial growth was detected using the automated Bactec 960 TB system (BD Biosciences, USA). Positive samples were screened for contamination using Ziehl-Neelsen staining as described by Kent and Kubica (1985).

Mycobacterial species identification:

For subsequent mycobacterial analysis boiled samples were prepared from uncontaminated culture samples by boiling one ml of culture for one hour at 95 °C. To identify mycobacterial species, polymerase chain reactions (PCRs) targeting 16S rRNA and *gyr*B gene were conducted. Each PCR reaction contained: 1 µl of DNA template, 2.5 µl of 10x PCR buffer, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs, 5 µl Q-buffer, 0.5 µl of each primer (50 pmol/ µl) as listed in Table 2 and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) in a 24.5 µl reaction with double distilled water. A negative control (no template) and a positive control (DNA template from *M. tuberculosis* H37Rv) were included to assess contamination of the reagents and successful PCR amplification, respectively. Amplification was done by activating the Taq polymerase at 95 ºC for 15 min, followed by 45 cycles of 94 ºC for 30 s, 60 ºC for 1 min and 72 ºC for 30 s, followed by a final elongation step at 72 ºC for 10 min. PCR was followed by sequencing of 16S rDNA (Harmsen *et al.* 2003) and *gyr*B gene (Huard *et al.* 2006) as previously described. Sequencing was done by the Central Analytical Facility (CAF) of Stellenbosch.

Table 2: Primer pairs used for PCR for *Mycobacterium* species identification.

Primer name	5'- 3'
16S rRNA forward	AGA GTT TGA TCC TGG CTC AG
16S rRNA reverse	GCG ACA AAC CAC CTA CGA G
*gyr*B forward	TCG GAC GCG TAT GCG ATA TC
*gyr*B reverse	ACA TAC AGT TCG GAC TTG CG

Mycobacterium species were identified by comparing the 16S rDNA derived sequence with the RIDOM (ribosomal differentiation of microorganisms) database (Harmsen *et al.* 2002) and GenBank's Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes). A species was recorded as definitively identified final culture result where RIDOM and BLAST search results matched or at least one search returned a 100% and the second 68% to 98% species identification. Searches that only identified *M. intracellulare* with 68% to 98% similarity on RIDOM and as *M. avium* complex on BLAST were finalised as *M. avium* complex.

Where one search identified a non-mycobacterial sequence and the other only 68% NTM similarity, the culture result was finalised as non-mycobacterial, i.e. negative on *Mycobacterium* culture, thereby excluding two *M. intermedium* 68% (R)/ Rhodococcus (B), two *M. phlei* (68%) (R)/ Rhodococcus (B), two *M. paraffinicum* 68% (R)/ Saccharopolyspora (B), one *M. septicum/ M. peregrinum* (R)/ Uncultured bacterium (B), eight *M. lentiflavum (68%)* (R)/ Cellulosimicrobium (B), one *M. sphagni (68%)* (R)/ Cellulosimicrobium (B), one *M. sphagni (68%)* (R)/ Prauserella (B), one *M. gilven* (68%) (R)/ Microbacterium spp. (B), one *M. septicum* (68%) (R)/ not *Mycobacterium* (B) and one *M. simiae* (68%) (R)/ not *Mycobacterium* (B) result. One sample identified as *M. avium* complex (R)/ *M. vulneris* (B) was incorporated as a *M. avium* complex result.

Taking phylogenetic relations into account searches identifying *M. fortuitum, M. farcinogenes, M. senegalense* or *M. conceptionense* in either RIDOM or BLAST were termed *M. fortuitum* group (Tortoli 2011). Those including any of *M. simiae, M. sherrisii, M. parascrofulaceum* or *M. interjectum* species were grouped as *M. simiae* group and for the phylogenetically more distant species *M. simiae* and *M. paraffinicum/ M. scrofulaceum* the result *M. simiae* group-like was recorded (adapted from Tortoli 2011). Where *M. terrae* and/ or *M. engbaeckii, M. hiberniae* and *M. heraklionense* were identified *M. terrae* group was assigned as result (Tortoli 2013). An isolate identified as *M. engbaeckii* 68% on RIDOM and *M. heckeshornense* on BLAST was recorded as M. *heckeshornense*-like, as was *M. senuense*-like for *M. intracellulare* 68% (R) versus *M. senuense* (B) and *M. intracellulare*-like for

M. szulgai 68% (R) versus *M. intracellulare*. An isolate identified on both RIDOM and BLAST as *M. asiaticum* 68% was termed *M. asiaticum*-like.
MTC members were identified by sequencing the entire genome.

NTM species were categorised as potentially pathogenic if pathology was reported also in non-immunodeficient animal or human patients as for those NTM from the *M. fortuitum* group, the *M. avium* complex, the *M. chelonae-abscessus* group, the *M. terrae* group, *M. szulgai, M. scrofulaceum* (Botha *et al.* 2013), *M. simiae, M. asiaticum* (Dostal, Richter & Harmsen 2003), *M. parascrofulaceum* (Liu e*t al.* 2014; Turenne *et al.* 2004) and *M. heckeshornense* (Roth *et al.* 2000). NTMs were considered not to be of clinical significance if mostly reported as incidental findings as those of the *M. elephantis group* (Michel pers. comm., 5 September 2014), *M. nonchromogenicum, M. phlei, M. smegmatis, M. hassiacum, M. moriokaense* (Dostal, Richter & Harmsen 2003), *M. senuense*-like (Mun *et al.*2008) and *M. paraffinicum* (Wang *et al.* 2008).

2.4.3. Serological evaluation of tuberculosis status

STAT-PAK® Assay:

As soon as lithium heparin blood was collected, the whole blood sample was tested for antibodies to recombinant proteins early secretory antigenic target 6 kDa (ESAT-6), culture filtrate protein 10 kDa (CFP10) and Mycobacterial protein bovis 83 (MPB83) *(*Lyashchenko *et al.* 2012) using the lateral flow immuno-chromatography ElephantTB STAT-PAK® Assay (STAT-PAK) (Chembio diagnostic systems, Medford, New York, USA) according to the test kit instructions (Appendix 6, Chembio Diagnostic Systems 2007). An aliquot of 30 µl undiluted heparin whole blood was pipetted onto the centre of the test unit sample well followed by three drops of the test kit diluent equivalent to approximately 100 µl. The result was read qualitatively after 20 minutes when adsorption of antibodies to the nitrocellulose membrane bound antigens was indicated by a blue latex bead-based detection system with test line area labelled ‘T’ and control line area labelled ‘C’ on the test cassette. A blue test line ‘T’ without apparent control line ‘C’ on the test cassette was interpreted as invalid result and the assay was repeated using a new test kit. One blue line in the control area without a coloured line in the test area was interpreted as non-reactive and recorded as ‘negative’. A blue control as well as test line were a considered reactive

result and termed positive. Faint test lines were recorded as '(+)' or 'very weak' when appearing only after 20 minutes and before 30 minutes, the cut of time for reading the test. Test lines appearing within 20 minutes and developing to a weaker intensity than the control line were recorded as '1+' or 'weak', those with same intensity as '2+' or 'strong' and those with stronger intensity than the control line as '3+' or 'very strong' (Table 3). All tests were labelled with the species, study number, date, type of blood sample used and result, recorded photographically (Olympus) no later than 30 minutes after test begin and stored at room temperature.

Table 3: Allocation of STAT-PAK result category to STAT-PAK test line appearance.

STAT-PAK result	Intensity of reaction		Comparison test line versus control line
negative			no test line, only control line visible
	(+)	very weak	test line appears weaker than control line and only after 20 minutes
positive	1+	weak	test line appears weaker than control line
	2+	strong	test line appears as strong as control line
	3+	very strong	test line appears stronger than control line

DPP® VetTB Assay:

Only plasma of STAT-PAK assay positive animals was tested with the dual path platform Chembio DPP® VetTB Assay for elephants (DPP) (Chembio Diagnostic Systems, Medford, New York, USA) for antibodies against antigen MPB83 coated to test area labelled 'T1' and antigen CFP10/ ESAT-6 fusion protein coated to test area labelled 'T2'. According to the test kit instructions (Appendix 6, Chembio Diagnostic Systems 2012) 5 µl of plasma were pipetted into the 'buffer + sample' 'well one' of the test cassette followed by two drops equivalent to approximately 65 µl buffer. After 5 minutes the sample-buffer-mix had migrated vertically on the nitrocellulose membrane and four drops of buffer were added to the 'buffer' only 'well two' to initiate lateral flow. The test result was evaluated qualitatively 15 minutes later when recombinant Protein A/G conjugated to colloidal gold particles would visualise bound antibodies as a pink to purple band. The test was considered invalid if no control line labelled 'C' on the cassette appeared and the test was repeated with a new test kit. A test with only the control line turning purple was denoted as negative, a test with control and any of the two test lines turning purple as positive. Result categories according to intensity of test line appearance were allocated as for STAT-PAK results (Table 3). The test cassettes were labelled with species, study number, date and test

result, recorded photographically (Olympus) no later than 30 min after test begin and stored at room temperature.

Enferplex™ TB Assay:

Serum samples were analysed using the Enferplex™ TB Assay (Enferplex), a multiplex ELISA for the detection of immune response to *M. bovis* infection in cattle using a panel of specific recombinant proteins and their peptides (Whelan *et al.* 2008), by Enfer Scientific (Newhall, Naas, Co. Kildare, Ireland) (Clare Whelan, John Clarke, Amanda O'Brien). The proteins included were ESAT-6, ESX-1 secretion system protein (Rv3616c), MPB83, CFP-10, Mycobacterial protein bovis 70 (MPB70) and MPB70 peptide. The method was modified for testing banded mongoose samples as follows (Whelan pers. comm., 30 January 2014): serum samples were diluted at a ratio of 1:500 with Enfer sample dilution buffer (Enfer Scientific) and mixed, and 50 µl of each diluted sample was added per microplate well, pre-coated with the panel of multiple antigens (Enfer Group n.d.). The plates were incubated for 60 minutes at 25 °C and agitated, then washed with Enfer wash buffer (Enfer Scientific) and the buffer aspirated. Protein G (Pierce® Protein Biological Products, Thermo Fisher Scientific Inc., Rockford, Illinois, USA) was used as detection antibody in a dilution of 1:5000 in the detection antibody dilution buffer. Of this dilution 50 µl were added to each test well and the plates were incubated for 30 minutes at 25 °C and agitated. After washing as above, 50 µl of chemiluminescent substrate, prepared at a ratio of 50:50 substrate and diluent, was added per well. Chemiluminescence signals were captured during 45 seconds of exposure to a Quansys Biosciences Imaging system (Quansys Biosciences, Logan, Utah, USA). Data was extracted as relative light units (RLU) with custom software (Quansys Q-View software, version 2.0) and analysed as previously described (Whelan *et al.* 2008) with an Enfer custom-made macro in Microsoft Excel (Excel Enfer multiplex macro, version 1.0.1.0) that defines a specific threshold for each antigen. In contrast to Whelan *et al.* (2008) defining a positive result as reaction above the threshold for at least two antigens and taking into account that the assay and reaction thresholds were not validated for mongoose sera, a positive result was defined as a reaction above the threshold towards at least one antigen to gain the highest sensitivity for the purpose of this study.

2.4.4. Data analysis

All data were entered into an excel (excel version 2007) spread sheet from which data were extracted to create tables or exported to statistical programmes.

Maps were generated using Q-GIS (Q-GIS 2014). Skukuza landscape feature metadata were provided by the SANParks GIS laboratory and an aerial photograph was extracted from google earth (Google Earth 2014).
Categorical data were described as frequency and percentages while quantitative data were described as medians and ranges. For statistical analysis animals were categorized as bTB positive if a positive result was obtained on any serological test or if a *Mycobacterium* culture was positive for *M. bovis*. Animals were classified as having a potentially clinically important nontuberculous mycobacterial infection if *M. fortuitum, M. fortuitum* group, *M. avium* complex, *M. avium, M. intracellulare, M. scrofulaceum, M. simiae, M. heckeshornense, M. chelonae-abscessus* group, *M. parascrofulaceum, M. asiaticum*-like, *M. asiaticum, M. szulgai, M. terrae or M. terrae* group were cultured from clinical specimens. Animals culture positive for *M. nonchromogenicum, M. phlei, M. senuense*-like, *M. smegmatis, M. hassiacum, M. moriokaense, M. paraffinicum* or *M. elephantis* group (clinically unimportant NTMs) were grouped with culture negative animals as the comparison group.

Categorical variables were compared between animal categories using chi-square and Fisher exact tests. Quantitative variables were compared between groups using Mann-Whitney U tests. Statistical analysis was performed in commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and freeware (Epi Info, version 6.04, CDC, Atlanta, Georgia, USA) at the Department of Production Animal Studies of the Faculty of Veterinary Science (Geoffrey Fosgate). Statistical significance was set at $P < 0.05$.

2.4.5. Study approval and ethics considerations

The study was approved by the SANParks Research Committee and registered with SANParks on 06 February 2012 with the reference number ‘BRUA986’ (Appendix 7) and title ‘Screening of small predators in the Kruger National Park for mycobacterial infection and disease: A pilot study in the banded mongoose (*Mungos mungo*)’. SANParks Animal Use and Care Committee approved the study on 23 February

2012 (Appendix 7). The research study was approved by the Research Committee and the Animal Use and Care Committee of the University of Pretoria on 23 April 2012 (Appendix 7) and registered as a MMedVet masters project with the University of Pretoria with the study number V085-11 and title as above. Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was granted by the Department: Agriculture, Forestry and Fisheries on 07 January 2013 (Appendix 7).

Chapter 3: Results

3.1. Study animals and capture sites

Between December 2011 and November 2012 77 individual banded mongooses (Table 4) were captured in cage traps in 91 capture events as described in 3.2.1. After release of 70 animals, 11 individuals were caught a second time and three a third time with one banded mongoose euthanised at the second capture event and one found dead in the cage at recapture. Also included in the study was a banded mongoose that was found dead in the Skukuza rest camp and samples of two banded mongooses that were retrieved from the South African National Parks (SANParks) Veterinary Wildlife Services (VWS) biobank. One of these had been found dead in the staff village and one had been brought in ill to the VWS and subsequently been euthanised.

Table 4: Total numbers of banded mongooses cage trapped, released, euthanised or found dead as well as retrieved from the VWS biobank.

		cage trapped			capture events total
		once	twice	thrice	
cage trapped	released	70	9	3	91
	euthanised	7	1		
	found dead		1		
total trapped		77	11	3	
found dead		1			
VWS biobank	euthanasia	1			
	found dead	1			
TOTAL ANIMALS		**80**			

The 80 banded mongooses originated from three main locations within a two kilometre radius of Skukuza rest camp (Table 5), namely the staff village (34 individuals), the VWS office (29 individuals) and from the extended area surrounding the living quarters (15 individuals) including the ware house, the transport depot and the day visitors area (Figure 2). Within these main areas two banded mongooses were recaptured within the staff village, five at the VWS offices, one at the day visitors area and two between the day visitors area and the transport depot (Figure 2). Two banded mongooses were not allocated to one of the main locations with one banded mongoose having been found dead in the Skukuza rest camp and one on the low water bridge between the staff village and the VWS office.

Table 5: Gender and age distribution of the study banded mongooses in correlation to the three main capture areas.

Location	subadult	adult	subadult	adult	Total	
	male		female		no	(%)
Staff Village	8	9	10	7	34	(42.5)
VWS Office	4	13	7	5	29	(36.3)
Living Quarters	7		5	3	15	(18.8)
Other				2	2	(2.5)
Total no (%)	19 (25)	22 (28.9)	22 (28.9)	17 (22.4)		
	41 (51.3)		39 (48.8)		80	100

subadult: younger than 12 months; adult: older than 12 months

Gender and age when grouped as subadults younger than 12 months (including age categories juvenile and subadult) and adults older than 12 months (including young, prime and old adults) were evenly distributed in the sample group. With 19 subadult males, 22 adult males, 22 subadult females and 17 adult females the study animals comprised a total of 41 males and 39 females (Table 5). When split into more detailed age groups the following age and gender classes were caught: 31 juveniles (18 females, 13 males), ten subadults (4 females, 6 males), 13 young adults (7 females, 6 males), ten prime adults I (4 females, 6 males), seven prime adults II (1 female, 6 males), six prime adults III (3 females, 3 males) and only three old adults (2 female, 1 male) (Appendix 5).

Banded mongooses submitted for necropsy showed a biased gender distribution with only three adult males (1 prime adult I, 1 prime adult II, 1 old adult) fitting the criteria for euthanasia in comparison to two subadult females (1 juvenile, 1 subadult) and four adult females (2 young adults, 1 prime adult II, 1 old adult). With an additional three adult females (1 young adult, 2 prime adults III) found dead, only one quarter of the animals submitted for necropsy were male and three quarters female (Table 6). Banded mongooses throughout the study area were submitted for euthanasia, namely two animals from the staff village, four from the VWS offices and three animals from around the living quarters. One additional banded mongoose was found dead at the VWS office and submitted to necropsy.

Figure 2: Capture sites and recaptured banded mongooses within the study area.

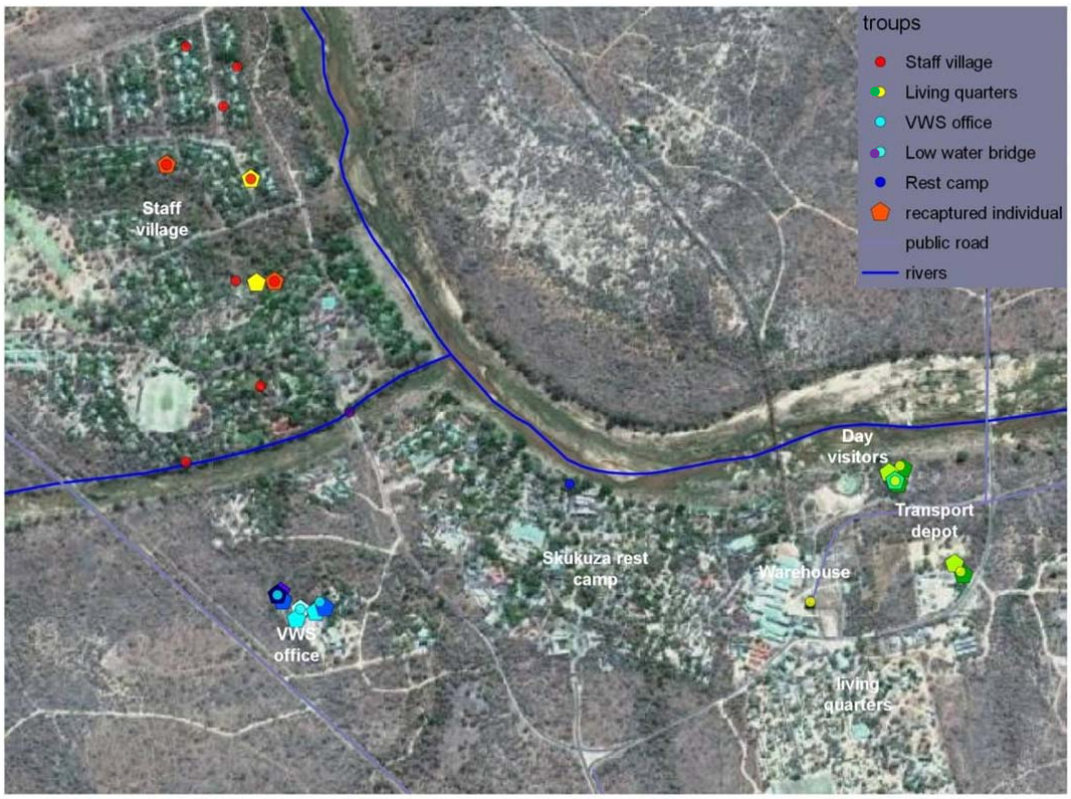


Table 6: Gender and age distribution of the study banded mongooses submitted to necropsy in correlation to the three main capture areas.

Location	subadult	adult	subadult	adult	Total	
	male		female		no	(%)
Staff Village			1	1	2	(16.7)
VWS Office		3	1	1*	5	(41.7)
Living Quarters				3	3	(25)
Other				2*	2	(16.7)
Total no (%)	0	3 (25)	2 (16.7)	7 (58.3)		
	3 (25)		9 (75)		12	(100)

subadult: younger than 12 months; adult: older than 12 months; *: found dead

3.2. Sample collection

All animals captured in cage traps were anaesthetised as described in 3.2.2. Animals recaptured within four weeks of previous sampling were not sampled again at recapture. The test and culture results for banded mongooses that had been sampled more than once were summarised and treated as one sample set per individual as e.g. for A67 where clinical samples from the first capture and post

mortem samples from the second capture when it was found dead in the cage trap, were combined.

Clinical samples were collected ante mortem from 73 of the 77 captured individuals as described in 3.2.4. The remaining four individuals were not sampled because they were the youngest of seven caught in one cage and prolonged anaesthesia time was to be avoided for animal welfare reasons. Animal A77 that was found dead in the Skukuza rest camp yielded a serum sample from intrathoracal haemorrhage and was further sampled, as if alive, for tracheal swab, tracheal lavage and faecal swab. Banded mongoose 11/844 had been sampled before onset of the project. It had been euthanised due to weakness indicating disease, was sampled for cardiac blood and the serum sample was retrieved from the VWS biobank. In summary, clinical samples collected originated from 75 individual banded mongooses (Table 7) with samples available to perform STAT-PAK® Assay (STAT-PAK) consisting of 71 lithium heparin whole blood samples, two lithium heparin plasma samples stored for banded mongoose A1 and A2 until test availability and two serum samples for animals A77 and 11/844. From the same animals excluding banded mongoose 11/844, 74 sera were submitted for Enferplex™ TB assay (Enferplex).

Table 7: Summary of individual banded mongoose numbers per test or sample type submitted for analysis according to origin of study animal.

		total animals	STAT-PAK	ENFER PLEX	TW	TS	FS	F	PM
cage trapped	released	64	64	64	55	62	63	8	0
	euthanised	8	8	8	8	8	8	1	8
	found dead	1	1	1	1	1	1	0	1
found dead		1	1	1	1	1	1	0	1
VWS biobank	euthanised	1	1	0	0	0	0	0	1
	found dead	1	0	0	0	0	0	0	1
TOTAL SAMPLES		76	75	74	65	72	73	9	12

TL: tracheal lavage; TS: tracheal swab; FS: faecal swab; F: faecal sample; PM: post mortem samples

Tracheal lavages were recovered from only 65 banded mongooses due to the need to refine the sampling technique, small size of the animal to be sampled or insufficient depth of anaesthesia. Including tracheal lavage samples from three recaptured banded mongooses 68 tracheal lavages were submitted for culture.

Small body size of two animals resulted in tracheal swabs collected from 73 banded mongooses in total adding up to a total of 75 tracheal swabs when including a tracheal swab from a recapture and a double sample taken from another animal.
One faecal swab was lost during storage leaving a total of 73 animals sampled. Together with faecal swabs collected at recapture from seven animals and on two occasions two and three results respectively reported for a swab, culture results were reported for a total of 83 faecal swabs.
Only nine animals defecated in the cage trap allowing for opportunistic faecal sampling.

Post mortem samples as described in 3.2.4 were collected from eight cage trapped animals that fit the criteria for euthanasia as described in 3.2.3 (Table 7). Of these banded mongooses one showed signs of clinical disease, namely weakness and ataxia. STAT-PAK tested positive for seven of these animals with one of these (A5) showing seroconversion at recapture only. Banded mongooses A1 and A2 had been tested for STAT-PAK only after release due to test unavailability at time of capture and therefore were not euthanised even though testing STAT-PAK positive. Additional post mortem samples were collected from banded mongoose A67 that was found dead in the capture cage at recapture and A77 that was found dead in the Skukuza rest camp. Post mortem samples collected from banded mongooses 11/844, that had been euthanised due to weakness, and 11/639 that had been found dead, were both sampled as described in 3.2.4 between August and October 2011 and samples were retrieved from the VWS biobank (Table 7). In summary, 12 animals were sampled post mortem as described in 3.2.4 making up a total sample set of nine spleen, eight liver, eight lung and seven other organ tissue samples, namely two from kidney, two from soft palate, two from the intestine and one amniotic fluid aliquot. From all 12 animals submitted for necropsy lymph nodes were collected for head, thorax, abdomen and periphery. Additional lymph nodes were collected separately for animal A5 from duodenal lymph nodes. Abdominal lymph nodes from animal A72 were the mesenteric and gastric lymph node, adding up to a total of 14 abdominal lymph node samples.

In addition to the pooled head lymph nodes separate samples were submitted to culture for banded mongoose A63 from mandibular lymph nodes and for A56 from

retropharyngeal lymph nodes. All head lymph nodes of banded mongoose A57 were submitted separately as left and right mandibular and left and right retropharyngeal lymph node. Therefore a total of 17 head lymph nodes were submitted for *Mycobacterium* culture.

Additional separate peripheral lymph nodes submitted for culture were superficial cervical and popliteal lymph nodes for animal A52, the left and right superficial cervical lymph nodes for animal A56, the left and right superficial lymph node of animal A57, the left superficial cervical and inguinal lymph nodes for animal A63 and the left and right popliteal as well as inguinal lymph nodes for animal A72, bringing up the total for peripheral lymph nodes sampled and cultured to 23.

Thirteen samples of thoracal lymph nodes were collected with lymph nodes of banded mongoose A57 sampled separately as sterno-mediastinal and tracheo-bronchial lymph node.

3.3. Clinical evaluation of banded mongooses

The body condition score (x/5) of banded mongooses included 1 (n=1), 2 (n=4), 3 (n=35), 3.5 (n=14), 4 (n=18) and 4.5 (n=2) (Appendix 5) with a median of 3 (Table 19). Two animals whose samples had been retrieved from the biobank had not been evaluated for body condition score or weight when sampled. The weight of the banded mongooses varied between 387 and 1591 g (Appendix 5) with a median of 953 g (Table 19).

Most banded mongooses were not dehydrated but three animals were 1% dehydrated, four animals 5% dehydrated and one animal 10% dehydrated (Appendix 5).

The majority of animals had no older external injuries (described in 3.3.1) but 14 animals had wounds including missing digits or claws and open wounds rated as mild, three animals had wounds categorised as moderate and one as severe. Thirty-seven banded mongooses sustained no injuries at capture, 32 animals had mild abrasions and cuts on the nose or feet and four animals sustained moderate external injury (Appendix 5).

Peripheral lymph nodes (rating as described in 3.3.4) were typically symmetrical and of physiological size. Mild asymmetry (1) was noted in 24 animals, moderate enlargement (2) in five animals and lymph node atrophy in one animal. No banded mongoose presented with visibly enlarged (3) lymph nodes (Appendix 5).

Lungs were auscultated before tracheal lavage in 50 animals with no sounds audible in 19 animals, diminished sounds only distinguishable after listening for a few seconds (1/5) in 13 animals, diminished sounds (2/5) and vesicular sounds (3/5) readily audible in seven animals each and sounds with rales immediately audible (4/5) in four animals. Twelve animals were evaluated after tracheal lavage with no lung sounds (0/5) and diminished sounds barely distinguishable (1/5) recorded for two banded mongooses each. Vesicular lung sounds (3/5) were readily audible in eight banded mongooses. No banded mongoose had lung sounds audible without use of a stethoscope (5/5) (Appendix 5).

External parasite evaluation (described in 3.3.4) documented fleas present on 38 study animals and fleas absent on 35 banded mongooses (Appendix 5). Four banded mongooses were tick-free, 33 had less than five ticks, 23 animals had 5-10 ticks, six animals 11-20, three animals 21-50 and four animals had 50-100 ticks (Appendix 5).

Only two banded mongooses that were captured had evidence of clinical disease. Weakness, hypothermia and oedema of the throat, neck and face were the only clinical signs in animal 11/844. Banded mongoose A72 was weak, slow and ataxic before capture. Clinical exam revealed a very poor body condition (1/5), thin and scruffy coat as well as multiple old but not yet healed bite wounds on the skull. Both animals were euthanised and submitted for necropsy.

Only three animals were identified as older adults based on dental examination. One of these animals was a lactating female and was therefore not euthanised.

3.4. Macroscopic and histopathological evaluation of tuberculosis status

The most significant findings in the 12 animals necropsied were lesions located in the lungs, lymph nodes and liver (Table 8). Lesions were most frequently located in the liver (in 75% of necropsied animals). Macroscopically they consisted of varying degrees (negative, 1+ mild, 2+ moderate and 3+ severe) of singly multifocal to miliary and disseminated, well demarcated necrosis, characterized by crème coloured, spherical lesions between 1 mm and 3 mm diameter that were distributed throughout the parenchyma of all lobes and showed central indentation when located at the liver surface. Histopathologically these lesions were described as having varying degrees of necrosis with round cell infiltration of most likely parasitic origin. However, no parasites were detected on histopathological sections. Additionally few giant cells were found in the liver lesions of banded mongoose 11/639. With the exception of an old male banded mongoose A29, that did not develop any liver lesions, the extent of the liver lesions correlated with age or concurrent lesions (A56 and A57).

Distinct nodular to plaque like lung lesions were present in four banded mongooses. In individuals A56 and A57 these nodules consisted of multifocal well demarcated, irregularly margined, round, grey-yellow granulomas of up to 5 mm diameter with central necrosis in the larger lesions. In mongoose A56 the singular lung lesion, located centrally in the right caudal lobe, was calcified (Figure 3). In banded mongoose A57 six lesions were located dorsal, caudal and caudo-lateral in the right caudal lung lobe (Figure 4), two lesions centrally in the left caudal lung lobe and one lesion dorso-medial in the left medial lung lobe.

Figure 3: Granulomatous lesions of banded mongoose A56 in right caudal lung lobe (left) and left superficial cervical lymph node (right).

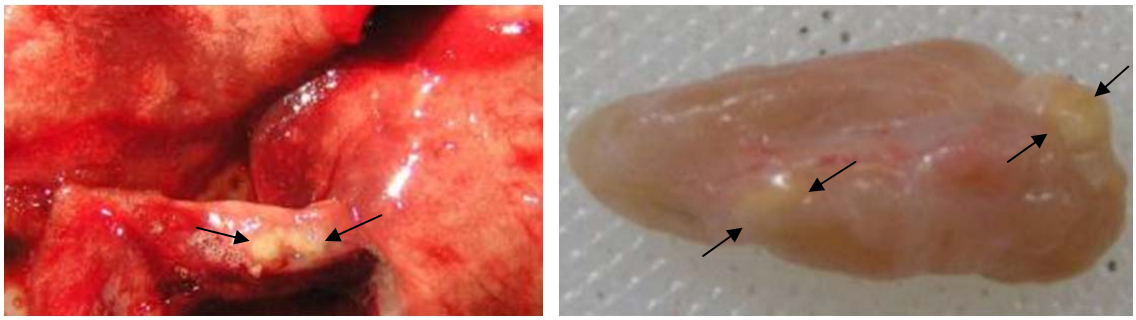


Table 8: Macropathology and histopathology findings of 12 banded mongooses.

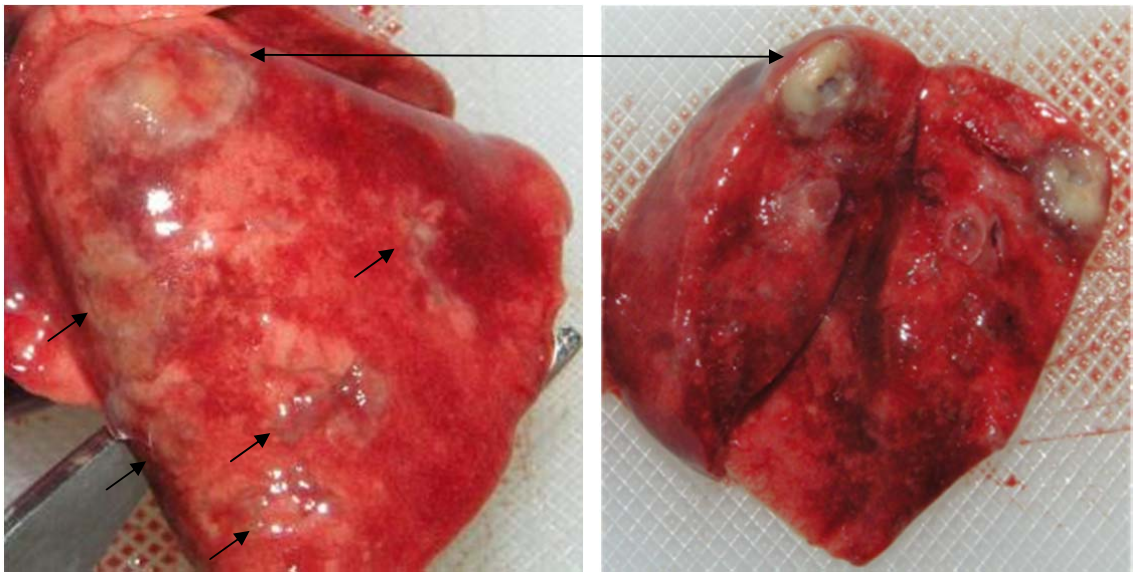
ID	Lung		Lymph nodes		Liver[1]		Other	Age class
	Macropathology	**Histopathology**	**Macropathology**	**Histopathology**	**Macro-pathology**	**Histo-pathology**	**Macro-pathology**	
A57	**Granuloma (9x)**	**caseating necrosis with epithelioid macrophages***	↑; hyperaemia, morph. change	-	3+	3+	gingivitis	P2
A56	**granuloma, calcification (1x)**	mucosal calcification in bronchioles	↑; **granuloma, calcification* (5x)**	**necrogranuloma, calcified**	2+	2+	-	Y
A72	soft nodular lesions (10x); A	bacterial granuloma	↑; A	A; hyperplasia	2+	-	toxo-plasmosis; trauma[a]	O
A52	opaque nodules (4x), haemorrhage	-	↑; morph. change	-	2+	2+	-	Y
11/639	Haemorrhage	-	haemorrhage	-	1+	2+	trauma[b]	Y
A5	-	-	↑	-	1+	-	-	J
A63	-	-	↑	-	1+	-	-	P2
A77	-	-	↑	-	1+	-	trauma[c]	P3
A24	-	-	-	-	1+	1+	-	P1
A29	plaque-like lesion (4x)	lipid granuloma; A	↑; A	A	-	-	arthropod skin granuloma	O
11/844	-	-	oedema	-	-	-	snake bite	S
A67	-	-	↑	-	-	-	heat stroke	P3

ID: identity; 1: severity of liver necrosis: - none/ negative, 1+ mild, 2+ moderate, 3+ severe, *: no acid-fast organism on Ziehl-Neelsen stain; □: some lymph nodes enlarged; A: anthracosis or anthrosilicosis; a: bite wounds to the head; b: road kill - skull fracture, haemorrhage; c: atrial rupture and haemorrhage; J: juvenile; Y: young adult; P1: prime adult I; P2 prime adult II; P3: prime adult III; O: old adult

Histopathologically these lesions were characterized as scattered caseating foci of necrosis associated with epithelioid macrophages in mongoose A57 and as scattered foci of mucosal calcification in the lumen of some bronchioles in mongoose A56. Ziehl-Neelsen staining could not depict acid fast organisms.

Lesions with the same macroscopic characteristics including calcification were identified in the left retropharyngeal lymph node (one nodule), the left superficial cervical lymph node (two nodules) (Figure 3) and in the tracheo-bronchial lymph nodes (two nodules) of banded mongoose A56. Histopathologically they were described as a necrogranuloma with calcified centre lacking acid fast bacteria on Ziehl-Neelsen stain.

Figure 4: Granulomatous lesions of banded mongoose A57 in the right caudal lung lobe, dorsal view (left) and cut surface (right).



Other lung lesions identified (Table 8) were four superficial, opaque off-white, oval to irregularly shaped, well demarcated, solid plaque-like lesions of up to 5 mm diameter and 1 mm depth identified as lipid granulomas with cholesterol clefts on histopathology on the dorsal and dorso-caudal aspect of left and right caudal lung lobe of banded mongoose A29. Animal A72 had ten, multifocal, fairly demarcated, light crème coloured, soft nodules of up to 2 mm diameter throughout the organ identified as luminal bacterial granulomas with filamentous organisms in the bronchioles. No significant lymph node lesions were associated with these lung lesions.

Lung lesions in animal A52 were more obscure and consisted of mild haemorrhage throughout the lung lobes as well as four white-opaque nodules, 1 mm in diameter embedded in an area of haemorrhage and slight consolidation, measuring 1 cm in diameter, in the right cranial lung lobe. No histopathological changes were noted and the tracheo-bronchial lymph nodes were inconspicuous macroscopically.

Other lesions identified in head, peripheral, thoracic or abdominal lymph nodes consisted predominantly of macroscopically enlarged nodes without significant histopathological changes.

The spleens of mongooses A56 and A57 showed mild white pulp hyperplasia macroscopically, but were unremarkable on histopathology. No significant pathology was noted in the spleen of any other banded mongoose examined.

Causes of death or underlying illnesses other than mycobacterial infection identified were cerebral toxoplasmosis together with bite wounds to the head (A72), snake bite (11/844), heat stroke (A67) and trauma including skull fracture (11/639) and atrial rupture (A77) (Table 8).

Histopathologically identified incidental lesions were nodular intestinal helminth lesions (11/844), foci of mucosal overgrowth into the intestinal submucosa (A72), a tail base arthropod granuloma, possibly caused by a demodex mite (A29) as well as anthracosis and anthrosilicosis in two old adult banded mongooses (A29, A72).

Descriptions exclude iatrogenic induced pathology as e.g. haemorrhage or barbiturate precipitation due to intracardial euthanasia.

3.5. Culture evaluation of tuberculosis status

3.5.1. ***Culture results per sample type***

Out of 334 samples from 76 individual banded mongooses cultured for mycobacteria nine samples (3%) from two animals (2.6%) (A56 and A57) were positive for *M. tuberculosis* complex (MTC). PCR amplification followed by sequencing of the *gyr*B gene confirmed all MTC members isolated to be *M. bovis* (Table 9).

Table 9: Isolation of *M. bovis* and NTM per sample type.

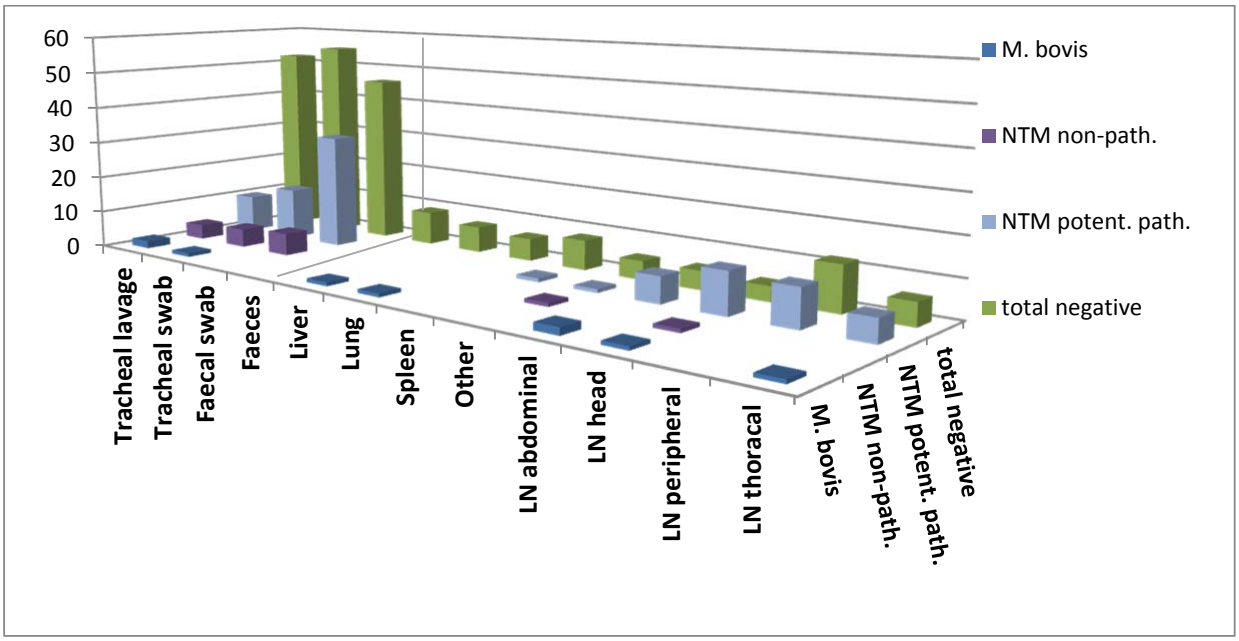
			Ante mortem samples				Post mortem samples									
			Animal no*	Trach lavage	Trach swab	Faecal swab	Fae-ces	Liver	Lung	Spleen	Other	Ln abdom	Ln head	Ln periph	Ln thorac	Total	
		M. bovis	2	2	1			1	1			2	1		1	9	*M. bovis*
slow growers	MTG	***M. terrae* group**	5	1	2	1					1 [1]					5	*M. terrae* group
		M. terrae	4	1	1	2								1		5	*M. terrae*
		M. senuense-like	1		1											1	*M. senuense*-like
		M. nonchromogenicum	4	1	2	2					1 [2]		1			7	*M. nonchromo.*
		M. heckeshornense	1										1			1	*M. heckeshorn.*
		***M. heckeshornense*-like**	1			1										1	*M. heckesh.*-like
		M. asiaticum	2	1	1	1										3	*M. asiaticum*
		***M. asiaticum*-like**	1			1										1	*M. asiaticum*-like
		M.paraffinicum/***M.scrofulaceum***	1	1												1	*M. paraf/M.scrof*
		M. szulgai	2									1		1		2	*M. szulgai*
	MAC	***M. avium complex***	9	4		2				1				1	1	9	*M. avium complex*
		M. avium	1									1				1	*M. avium*
		M. intracellulare	10	1		2						1	4	3	3	14	*M. intracellulare*
		***M. intracellulare*-like**	1		2											2	*M. intracell.*-like
	MSG	***M. simiae* group**	8		1	2			1			3	4	1	2	14	*M. simiae* group
		M. simiae	4		1							1	1	1		4	*M. simiae*
		M. parascrofulaceum	1										1	1		2	*M. parascrofulac.*
fast growers		*M. phlei*	3			3										3	*M. phlei*
		M. hassiacum	2	1	1											2	*M. hassiacum*
		M. elephantis group	2	1	1											2	*M. elephantis* gr.
		M. moriokaense	1			1										1	*M. moriokaense*
		M. smegmatis	1	1												1	*M. smegmatis*
	MFG	***M. fortuitum* group**	12		2	12										14	*M. fortuitum* gr.
		M. fortuitum	8	1	3	5										9	*M. fortuitum*
		M. septicum	1		1											1	*M. septicum*
		***M. chelonae* group**	3			2								2		4	*M. chelonae group*
total		*M. bovis*	2	2	1			1	1			2	1		1	9	(3%)
		NTM	48	14	19	37			1	1	2	7	12	11	6	110	(33%)
		NTM potentially pathogenic	41	10	14	31			1	1	1	7	11	11	6	93	(28%)
		NTM no clinical relevance	11	4	5	6					1		1			17	(5%)
		negative	28	52	55	46	9	7	6	8	5	5	4	12	6	215	(64%)
		samples	76	68	75	83	9	8	8	9	7	14	17	23	13	334	

MTG: *M. terrae* group; MAC: *M. avium* complex; MSG: *M. simiae* group; MFG: *M. fortuitum* group; Trach: tracheal; Ln: lymph node; 1: soft palate; 2: amniotic fluid; NTMs in bold are grouped as potentially pathogenic versus clinically not relevant NTMs; *: see Appendix 3 for detailed listing of *M. bovis* and NTM isolation per individual banded mongoose and sample type.

Nontuberculous mycobacteria (NTMs) were cultured from 110 (33%) samples in 48 (36.2%) individual banded mongooses. Of these isolates 84.5% (93 isolates) belonged to NTM species regarded as potentially pathogenic namely those from the *M. fortuitum* group, the *M. avium* complex, the *M. chelonae-abscessus* group, the *M. terrae* group, *M. szulgai, M. scrofulaceum, M. simiae, M. asiaticum*, *M. parascrofulaceum* and *M. heckeshornense* as indicated in Table 9 in bold and depicted in Figure 5. Only 15.5% of NTM culture positive samples (17 isolates) were positive for NTM considered not to be of clinical significance as those of the *M. elephantis group, M. nonchromogenicum, M. phlei, M. smegmatis, M. hassiacum, M. moriokaense, M. senuense*-like *and M. paraffinicum*.

Negative for *Mycobacterium* culture were 215 (64%) samples from 28 (36.8%) animals.

Figure 5: Frequency diagramme of isolates of *M. bovis,* non-pathogenic NTMs and potentially pathogenic NTMs versus samples that were *Mycobacterium* culture negative in the respective sample types of ante mortem samples to the left and post mortem samples to the right of the diagramme.



Out of 235 samples taken ante mortem including 68 tracheal lavages, 75 tracheal swabs, 83 faecal swabs and nine faecal samples *M. bovis* was isolated from three (1%) samples and NTMs from 70 (30%) samples whereas 162 (69%) ante mortem samples were negative. From a total of 99 samples taken post mortem, eight from liver and lung, nine from spleen, seven from other tissues as described in 4.2, 14 abdominal, 17 head, 23 peripheral and 13 thoracal lymph nodes, *M. bovis* was

isolated from six (6%) samples and NTMs from 40 (40%) samples whereas 53 (54%) samples were *Mycobacterium* culture negative.

NTMs in post mortem samples:

The only members of the fast growing NTMs isolated in post mortem samples, namely from two peripheral lymph node samples, were members of the *M. chelonae* group. The only NTM clinically not relevant isolated from post mortem samples was *M. nonchromogenicum*, isolated from one head lymph node sample and one amniotic fluid sample respectively.

All other post mortem samples that NTMs were isolated from, cultured positive for a potentially pathogenic NTM with the most frequent isolates from the *M. simiae* group (MSG) (16) including *M. simiae* group (11), *M. simiae* (3) and *M. parascrofulaceum* (2), closely followed by members of the *M. avium* complex (MAC) (15) including *M. intracellulare* (11), *M. avium complex* (3) and *M. avium* (1). Other isolates were *M. szulgai* (2), *M. terrae*-group (1), *M. terrae* (1) and *M. heckeshornense* (1). The majority of NTMs isolated from post mortem samples were found in the head lymph node pool (12), followed by peripheral (9), abdominal (7) and thoracal (6) lymph nodes as listed in Table 9. No NTMs were isolated from liver samples; only one lung sample was positive for *M. simiae* group and only one spleen sample positive for *M. avium* group.

NTMs in ante mortem samples:

All nine faecal samples were culture negative for *M. bovis.* Of the 70 NTMs isolated from tracheal lavage, tracheal swab and faecal swab 35 (50%) were fast growers and 35 (50%) were slow growers. The majority (79%) of NTMs isolated from ante mortem samples were potentially pathogenic with the most isolated from faecal swabs, namely 15 cultures positive for *M. fortuitum* group including *M. fortuitum,* also the species cultured the most from tracheal swabs (6). Isolates cultured the most in tracheal lavages originate from the *M. avium* complex, including *M. avium* complex (4) and *M. intracellulare* (1). Species isolated from both tracheal as well as faecal source include members of the *M. terrae* group, *M. avium* complex, *M. simiae* group, *M. fortuitum* group and *M. asiaticum.* The only NTMs isolated from faecal swabs exclusively were *M. chelonae* group and *M. asiaticum*-like whereas *M. paraffinicum/ M. scrofulaceum* was the only NTM cultured exclusively from a tracheal sample only.

A mere 15 (21%) cultures of ante mortem samples yielded NTMs of likely no clinical relevance. Notably *M. nonchromogenicum* was the only NTM found in ante mortem as well as post mortem samples as described above. Furthermore no other NTM of unlikely clinical relevance but *M. nonchromogenicum* was found in both a tracheal as well as faeces derived sample. *M. hassiacum, M. elephantis* group, *M. smegmatis* and *M. senuense*-like NTMs were the only ones isolated from tracheal samples, whereas *M. phlei* and *M. moriokaense* were exclusively found in faecal swabs.

3.5.2. Culture results per individual

Mycobacterium bovis was isolated from two banded mongooses from one tracheal swab and two tracheal lavages, two of the abdominal, one head and one thoracal lymph node sample as well as one liver and one lung sample as summarised in Table 10. NTMs were isolated from tracheal lavages from 13 banded mongooses, tracheal swabs from 19 animals and faecal swabs of 42 banded mongooses. Post mortem samples were positive for NTMs for six banded mongooses on abdominal lymph nodes, nine animals on head lymph nodes, nine animals on peripheral and four banded mongooses on thoracal lymph node samples. Two banded mongooses were positive for NTMs on the lung sample, two animals on the spleen sample, one on the soft palate sample and one on the amniotic fluid aliquot. The remaining animals were negative when cultured for *Mycobacterium* on tracheal swabs (52), tracheal lavages (50), faecal swabs (31) and faecal samples (9) as well as on post mortem samples of four abdominal, two head, three peripheral and four thoracal lymph node samples as well as seven liver, five lung, seven spleen, two kidney, two intestine and one soft palate sample.

Table 10: *Mycobacterium* culture results per sample type for 76 individual banded mongooses.

Sample type	Tracheal swab	Tracheal lavage	Faecal swab	Faeces	LNa	LNh	LNp	LNt	Liver	Lung	Spleen	Other*
positive												
M. bovis	**1**	**2**	0	0	**2**	**1**	**0**	**1**	**1**	**1**	0	0
NTM	19	13	42	0	6	9	9	7	0	2	2	2
negative	52	50	31	9	4	2	3	4	7	5	7	5
total no animals	72	65	73	9	12	12	12	12	8	8	9	7

NTM: nontuberculous *Mycobacterium*; LN: pooled lymph nodes of a: abdomen, h: head, p: periphery, t: thorax; * 2 kidney, 2 soft palate, 2 intestine, 1 amniotic fluid

M. bovis culture results and NTM coinfection:

All in vivo and post mortem samples that *M. bovis* was isolated from originated from two individual banded mongooses, A56 and A57. From no other banded mongoose was *M. bovis* isolated. Tracheal lavage for both animals yielded *M. bovis* on culture, whereas only tracheal swab of A56 *M. bovis* positive (Table 11). Liver and lung sample of A57 were also positive for *M. bovis.* The faecal swab of A56 was positive for *M. fortuitum* group. Tracheal and faecal swab of A57, liver and lung of A56 as well as spleen samples for both animals were negative on *Mycobacterium* culture (Table 11).

Table 11: *Mycobacterium* culture results for tracheal lavage, tracheal swab, faecal swab, liver, lung and spleen samples of banded mongooses A56 and A57.

ID	Tracheal lavage	Tracheal swab	Faecal swab	Liver	Lung	Spleen
A56	***M. bovis***	***M. bovis***	*M. fortuitum* group	neg	neg	neg
A57	***M. bovis***	neg	neg	***M. bovis***	***M. bovis***	neg

M. bovis was isolated from the retropharyngeal and abdominal lymph node of banded mongoose A56 as well as the abdominal and tracheo-bronchial lymph node of A57. NTMs were isolated from banded mongoose A56 from the pooled head lymph node sample (*M. simiae* group), the tracheo-bronchial lymph node (*M. avium* complex) and the pooled peripheral lymph node sample (*M.* szulgai); the left and right superficial cervical lymph nodes being *Mycobacterium* culture negative (Table 12).

Table 12: *Mycobacterium* culture results for lymph node samples of banded mongoose A56 and A57.

ID	LN head	LN mandibularis	LN retro-pharyngeal	LN tracheo-bronchial	LN sterno-mediastinal	LN abdominal	LN peripheral	LN superficial cervical
A56	*M. simiae* group	○	***M. bovis***	*M. avium* complex	○	***M. bovis***	*M. szulgai*	neg (le/ ri)
A57	○	*M. intra-cellulare* (le), *M. parascro-fulaceum* (ri)	*M. simiae* (le), *M. intra-cellulare* (ri)	***M. bovis***	neg	***M. bovis***	*M. para-scrofula-ceum*	neg (le), *M. intra-cellulare* (ri)

LN: lymph node; le: left sided lymph node; ri: right sided lymph node, ○: no sample

Banded mongoose A57 was positive for NTMs on the left (*M. intracellulare*) and right (*M. parascrofulaceum*) mandibular lymph node, the left (*M. simiae*) and right (*M. intracellulare*) retropharyngeal lymph nodes, the pooled peripheral lymph nodes (*M. parascrofulaceum*) and the right superficial cervical lymph node (*M. intracellulare*)

with sterno-mediastinal as well as left superficial cervical lymph nodes culturing negative for *Mycobacterium* (Table 12). In summary *M. bovis* was found together with *M. intracellulare, M. parascrofulaceum* and *M. simiae* or *M. fortuitum* group, *M. szulgai, M. simiae* and *M. avium* complex.

NTM culture results and co-infection:

Culture results for NTMs were highly variable throughout the examined population for in vivo as well as post mortem samples (Table 9, Appendix 3). The majority, i.e. 41 animals equivalent to 54%, was positive for potentially pathogenic NTMs as described above and only 11 banded mongooses (14%) were positive for clinically irrelevant NTMs with an overlap of four animals (5%) that were positive for both. A negative *Mycobacterium* culture result of all in vivo samples was noted in 28 (37%) banded mongooses. All 12 banded mongooses that post mortem samples were submitted for had at least one culture positive for NTMs.

In 27 animals only one of all the samples submitted for each animal was positive for NTMs. For most animals the positive testing sample was a faecal swab (n = 20) and NTMs isolated consisted predominantly of members of the *M. fortuitum* group (n = 10), followed by members of the *M. avium* complex (n = 5) and *M. terrae* group (n = 3). Other members isolated as the only positive result in one banded mongoose were *M. phlei, M. moriokaense, M. asiaticum, M. hassiacum, M. paraffinicum/ M. scrofulaceum, M. elephantis* group and *M. chelonae-abscessus* group. Only two animals of the 12 submitted for necropsy cultured positive for NTMs on only one sample with *M. intracellulare* isolated from the peripheral lymph nodes of banded mongoose 11/844 and *M. fortuitum* group from the faecal swab of animal A5.

In nine animals two samples cultured positive for NTMs, in four animals three and four samples each, in one animal each five and six samples and in two animals seven samples were positive for NTMs. Only in three animals was only one NTM species or NTM group isolated in more than one sample, namely in banded mongoose A33 *M. nonchromogenicum* and animals A19 and A47 members of the *M. fortuitum* group. In all other animals a mixture of NTMs was isolated. The combination of members of the *M. simiae* group and *M. avium* complex in six animals was the most frequent combination. In three animals the combination of *M. terrae*

group and *M. nonchromogenicum* was present, whereas *M. fortuitum* group and *M. avium* complex was a combination also found in another three animals.

Comparing NTM isolation with troop association *M. hassiacum, M. elephantis* and M. nonchromogenicum were exclusively and *M. phlei, M. terrae* group and *M. chelonae-abscessus* mostly isolated from individuals of the troop frequenting the VWS offices (Appendix 3), the latter two categorised as potentially pathogenic. The potentially pathogenic members of *M. fortuitum* group, *M. avium* complex and *M. simiae* group were isolated from all three troops.

3.6. Serological evaluation of the tuberculosis status

STAT-PAK® Assay:

The STAT-PAK Assay was performed for 75 banded mongooses (Appendix 4). As described in 3.4.3 and 4.2 heparin whole blood was used for 71 of these animals. For animals A1 and A2 the assay was run retrospectively on heparin plasma that had been stored at -80 ºC until test availability. Serum was used for the assay for animals 11/844 and A77, for the latter obtained from intrathoracal haemorrhage.

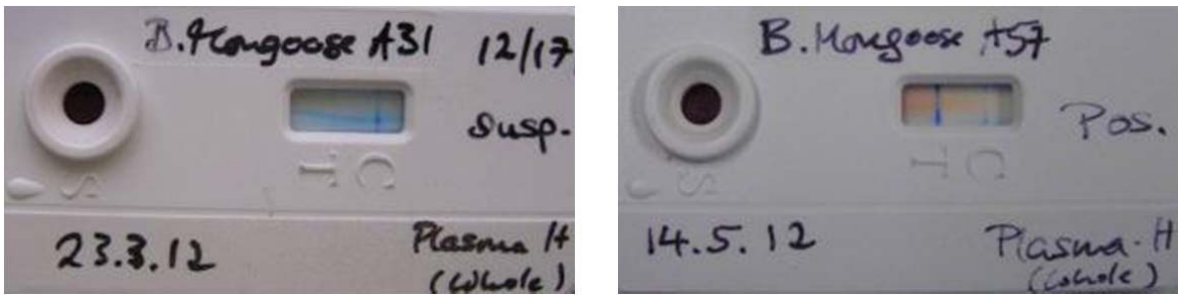
Twelve individuals (16% of the tested animals) showed a positive reaction (Table 13). Examples of test results for banded mongooses A31 and A57, depicted in
Figure 6, show the range of positive STAT-PAK reactions from very weak (+) to very strong 3+. A very weak (+) positive result was seen in five animals, a weak (1+) reaction in four animals, a strong (2+) one in one animal and a very strong positive result in the two animals A56 and A57 (Table 13).

Table 13: Serological responses of study animals measured by STAT-PAK assay.

STAT-PAK	n	intensity of reaction	n
Negative	63 (84%)		
Positive	12 (16%)	(+)	5
		1+	4
		2+	1
		3+	2
Total	75		

intensity of reaction:
(+): very weak; 1+: weak; 2+: strong; 3+ very strong

Figure 6: Examples for a very weak positive (left, A31) and very strong (right, A57) STAT-PAK test result.



DPP® VetTB Assay:

Stored heparin plasma of the twelve animals that had a positive STAT-PAK test result was also tested with the DPP® VetTB Assay (DPP). All twelve tests were positive with at least one positively reacting test line. Test line one (DPP 1) equivalent to an antigen-antibody reaction with protein MPB83 showed a positive result for all 12 animals (Table 15). For test line two (DPP 2) corresponding to a reaction with CFP10/ ESAT-6 fusion protein only ten out of the 12 sera tested were positive and animals A1 and A22 were negative. Figure 7 shows examples of weak (1+), strong (2+) and very strong (3+) DPP test results on the examples of animals A31 and A57.

Reaction intensities of STAT-PAK and DPP assays are compared further down together with the Enferplex assay. As the DPP test results did not rule out any of the weak or very weak positive STAT-PAK results all 12 STAT-PAK results were considered positive.

Figure 7: Examples for a weak 1+ (1) and strong 2+ (2) positive (left, A31) and very strong 3+ (1) and strong (2+) (right, A57) DPP test result.

Enferplex™ TB Assay:

Serum samples from 74 banded mongooses were analysed using the Enferplex assay. Reactions were captured with a Quansys Biosciences Imaging System and analysed with custom-made Excel Enfer multiplex macro version 1.0.1.0 that defines a specific threshold for each antigen as described before (3.4.3; Whelan *et al.* 2008). Sera of five individuals developed a positive reaction above the threshold for one antigen each, namely four sera to antigen MPB83 and one to the MPB70 peptide (Table 14). No sample reacted positive to more than one antigen and no antibodies were detected against the antigens ESAT-6, Rv3616c, CFP-10 or MPB70 in any of the samples leaving 69 negative results (Table 14; Appendix 4).

Table 14: Serological responses of 74 study animals to antigens MPB83, MPB70 peptide, ESAT-6, Rv3616c, CFP-10 and MPB70 measured by Enferplex assay.

Enferplex	Total	MPB83	MPB70 peptide	ESAT-6	Rv3616c	CFP-10	MPB70
Positive	**5**	**4**	**1**	0	0	0	0
Negative	69	70	73	74	74	74	74
Total	74						

Comparison of STAT-PAK®, DPP® VetTB and Enferplex™ TB Assay:

Only two banded mongooses, animals A56 and A57, reacted positively on all three serological assays (Table 15). In both of these animals the test results of STAT-PAK and DPP test line 1 (DPP1) equivalent to MPB83 had revealed the strongest positive reaction (3+) and correlated with a positive Enferplex assay result for MPB83. Positive test results for Enferplex assay for MPB83 in animals A49 and A78 as well as a positive result for MPB70 peptide in animal A70 correlated to a negative STAT-PAK assay and were not tested for with DPP assay. No other animal with a positive STAT-PAK or DPP test result of varying intensity had a positive Enferplex test result.
When comparing the reaction intensities of STAT-PAK and DPP tests (Table 15) only individuals A56 and A57 showed a very strong reaction (3+) on both STAT-PAK and DPP 1 and none reacted strongly on DPP 2. Individual A1 showed a strong reaction (2+) on both STAT-PAK and DPP 1, however, was negative on DPP 2. Banded mongoose A8 showed very strong reaction (3+) on DPP 1, however only weak (1+) reaction on STAT-PAK and DPP 2. The remaining eight, very weak to weak positive STAT-PAK reactants, showed a varying response on DPP 1 and DPP 2 without obvious correlation.

Table 15: Comparison of study animals with positive STAT-PAK, DPP and Enferplex test result.

ID	STAT-PAK	DPP		Enferplex					
	(ESAT-6 + CFP10 + MPB83)	**DPP1** (MPB83)	**DPP2** (CFP10/ ESAT-6)	MPB70 peptide	MPB83	ESAT-6	Rv3616c	CFP-10	MPB70
A56	**3+**	**3+**	1+	-	+	-	-	-	-
A57	**3+**	**3+**	2+	-	+	-	-	-	-
A1	2+	2+	-	-	-	-	-	-	-
A8	1+	**3+**	1+	-	-	-	-	-	-
A29	1+	2+	1+	-	-	-	-	-	-
A24	1+	2+	(+)	-	-	-	-	-	-
A5	1+	(+)	(+)	-	-	-	-	-	-
A52	(+)	2+	2+	-	-	-	-	-	-
A22	(+)	2+	-	-	-	-	-	-	-
A63	(+)	1+	2+	-	-	-	-	-	-
A31	(+)	1+	2+	-	-	-	-	-	-
A2	(+)	1+	1+	-	-	-	-	-	-
A49	-	○	○	-	+	-	-	-	-
A78	-	○	○	-	+	-	-	-	-
A70	-	○	○	+	-	-	-	-	-

ID: study animal number; DPP 1: test line one of DPP assay; DPP 2: test line two of DPP assay;
For Enferplex assay -: negative; +: positive;
For STAT-PAK and DPP assay (+) very weak positive; 1+: weak positive; 2+: strong positive; 3+: very strong positive; ○: not tested

Statistical evaluation of serological test performance was not possible due to the small number of *M. bovis* culture positive animals. The evaluation of test performance in the absence of a gold standard was also not possible without results from a third serological assay.

3.7. Comparative evaluation of *Mycobacterium bovis* culture results, macropathology and histopathology of banded mongooses subjected to necropsy

Banded mongooses A56 and A57 were the only animals that had developed granulomatous lesions resembling tuberculous lesions and at the same time had *Mycobacterium* culture results positive for *M. bovis* (Table 16). However, only lung lesions of A57 and retropharyngeal lymph node lesions of A56 were also *M. bovis* positive, whereas other granulomatous lesions identified in these two banded mongooses were either *Mycobacterium* culture negative (lung and left superficial cervical lymph node lesions of A56) or positive for NTMs (tracheo-bronchial lymph nodes of A56 – *M. avium* complex). Conversely organs with non-granulomatous lesions such as enlarged tracheo-bronchial lymph nodes of A56 as well as abdominal

lymph nodes of both A56 and A57 and the liver of A57 with multifocal necrosis were culture positive for *M. bovis.* The liver samples of banded mongooses A56, A72, A52, 11/639, A63, A77 with similar but decreasing degree of multifocal necrosis were *Mycobacterium* culture negative, as was the lesion-free liver sample of A29.

Lung lesions of non-granulomatous character were culture negative with the exception of banded mongoose A52 where opaque miliary nodules were observed on macropathology and culture was positive for *M. simiae* group but no lesion

Table 16: Comparison of macropathology, histopathology and *Mycobacterium* culture results for lung and lymph node lesions of banded mongooses subjected to necropsy.

ID	Lung			Lymph nodes		
	Macropath	**Histopath**	**Culture**	**Macropath**	**Histopath**	**Culture**
A56	**granuloma calcified**	mucosa calcified	neg	**granuloma calcified**	**necro-granuloma calcified***	***M. bovis/*** NTM
A57	**granuloma**	**caseating necrosis, epithelioid macrophages***	***M. bovis***	enlarged, morph. change	neg	***M. bovis/*** NTM
A52	Opaque nodule	neg	NTM	enlarged	neg	NTM
A29	plaque-like lesion	lipid granuloma	neg	enlarged	neg	NTM
A72	soft miliary nodules	bacterial granuloma	neg	enlarged	hyperplasia	NTM
11/639	haemorrhage	neg	neg	haemorrhage	neg	NTM
A63 A77	neg	neg	neg	enlarged	neg	NTM
A24 A67 11/844	neg	neg	not cultured	neg/ enlarged	neg	NTM
A5	neg	neg	not cultured	enlarged	neg	neg

* negative for acid fast bacteria

confirmed on histopathology. No lung sample of animals A5, A24, A67 and 11/844 had been submitted for culture.

All animals but A24 had at least one enlarged lymph node and also, with the exception of A5, had at least one lymph node sample cultured positive for a NTM, predominantly members of the *M. avium* complex and *M. simiae* group. No correlation between culture, macro- or histopathology was observed.

The spleen of banded mongoose 11/639 contained three nodular lesions and cultured positive for *M. avium* complex.

3.8. Comparative evaluation of *Mycobacterium bovis* culture and serology results

Only two animals, A56 and A57, showed an overlap for positive STAT-PAK, Enferplex assay and *M. bovis* positive culture from post mortem samples (Table 17).

Table 17: Comparison of serological and *Mycobacterium* culture results of tracheal lavage, tracheal swab, faecal swab and post mortem samples. NTM findings are described in detail in the text.

ID	STAT-PAK	Enferplex	Culture			
			Tracheal lavage	Tracheal swab	Faecal swab	Post mortem
A56	**pos 3+**	**pos**	***M. bovis***	***M. bovis***	NTM	***M. bovis/*** NTM
A57	**pos 3+**	**pos**	***M. bovis***	neg	NTM	***M. bovis/*** NTM
A1	**pos 2+**	neg	○	neg	*M. phlei*	○
A8	**pos 1+**	neg	○	neg	*M. fortuitum* group	○
A24	**pos 1+**	neg	neg	neg		NTM
A5	**pos 1+**	neg	neg	neg		neg
A29	**pos 1+**	neg	neg	NTM	NTM	NTM
A52,63	**pos (+)**	neg	NTM	NTM	neg	NTM
A2	**pos (+)**	neg	○	neg	*M. fort.* gr.	○
A22,31	**pos (+)**	neg	neg	neg	neg	○
A78	neg	**pos**	NTM	NTM	NTM	○
A49	neg	**pos**	neg	NTM	NTM	○
A70	neg	**pos**	neg	neg	neg	○
A67,72,77	neg	neg	neg	neg	neg	NTM
11/844	neg	neg	○	○	○	NTM
A30,32,35,54	neg	neg	NTM	NTM	NTM	○
A7,19,42,47	neg	neg	neg	NTM	NTM	○
15x	neg	neg	neg	neg	NTM	○
A33	neg	neg	*M. nonchromogenicum*		neg	○
A26,28,39,53,73	neg	neg	NTM	neg	neg	○
A16, A46	neg	neg	neg	NTM	neg	○
25x	neg	neg	neg	neg	neg	○
Total *M. bovis* positive	**12**	**5**	**2**	**1**	**0**	**2**

3+: very strong positive; 2+: strong positive; 1+: weak positive; (+): very weak positive;
○: no sample or result available;
15x: A6,9,10,11,14,23,38,44,48,50,51,55,59,61,68;
25x: A3,4,12,13,15,25,27,34,36,37,40,41,43,45,58,60.62,64,65,66,69,71,74,75,76

These were the only two banded mongooses where the STAT-PAK reacted very strongly positive. They were also the only ones from which *M. bovis* was isolated from an ante mortem sample, namely the tracheal lavage for both A56 and A57 and the tracheal swab for A56.

Other NTM isolated from the *M. bovis* positive animals were *M. fortuitum* group from ante mortem samples of A56 and *M. simiae* group, *M. avium* complex, *M. szulgai* and *M. parascrofulaceum* from post mortem samples of both A 56 and A57.

Of the remaining ten STAT-PAK positive banded mongooses all but animals A22 and A31 had cultured positive for NTMs from at least one sample. In the sampled banded mongooses (A2, A5, A8, A24) *M. fortuitum* group was isolated from the faecal swab with the exception of *M. phlei* isolated from A1 and *M. heckeshornense*-like from A29. *M. asiaticum* was isolated from both tracheal lavage and swab in banded mongoose A63 whereas *M. avium* complex and *M. simiae* group were isolated from post mortem samples. A52 was positive for *M. avium* complex in tracheal lavage and *M. simiae* group on tracheal swab, whereas post mortem samples cultured positive for *M. simiae* group and *M. chelonae-abscessus.*

Animals serologically positive on Enferplex assay only, were either *Mycobacterium* culture negative (A70) or cultured positive for NTMs from tracheal (A78: *M. avium* complex, *M. nonchromogenicum,* A49: *M. hassiacum*) as well as faecal samples (A78: *M. terrae*, A49: *M. phlei*).

Four (A67, A72, A77, 11/844) animals were positive for a variety of NTMs on post mortem samples only. No ante mortem sample was available from animal 11/844.
Lung or lymph node lesions were only present in animals that also had a STAT-PAK positive test result, whereas liver lesions were also found in animals with negative STAT-PAK result.

Of the sampled banded mongooses 35 were seronegative for both STAT-PAK and Enfer but positive for a variety of NTMs. In this group NTMs were isolated from both tracheal as well as faecal samples in eight animals. Only faecal swabs positive for a variety of NTMs were recorded in 15 banded mongooses (including A6 and A9

without a tracheal lavage sample), only tracheal samples positive in eight animals. The remainder in this group was only positive on post mortem samples.

Twenty-five animals (33.3%) were negative on all samples submitted for a serological test or *Mycobacterium* culture. These animals include A4 and A40 that were not sampled for tracheal lavage, A3 and A13 not sampled for tracheal lavage and swab and A15 without a faecal swab sample.

3.9. Comparative evaluation of demographics and clinical findings with *Mycobacterium* status

For statistical analysis animals with any one test positive result, either serology or *Mycobacterium tuberculosis* complex culture, were considered bTB positive because of the small number of animals testing positive on any one test.

Eighty-seven percent of the bTB positive group were older than 12 months versus only 42% of the bTB negative group (P = 0.002) (Table 18). The bTB positive group comprised more older animals than the bTB negative group when age was evaluated on an ordinal scale (P = 0.025) (Table 19).
Seventy-three percent of the bTB positive animals were heavier than 953g (sample median) compared to 42% of the bTB negative animals (P = 0.037) (Table 18). Weight was also significantly different between groups when compared on a quantitative scale (P = 0.008) (Table 19).

Banded mongoose that had *M. phlei* isolated from clinical specimens were more likely to be classified as bTB positive but the association was not significant (P = 0.103). A similar association was identified related to the *M. fortuitum* group (P = 0.095).

No significant difference was detected between bTB positive and bTB negative banded mongooses for the environmental mycobacteria of *M. avium* complex, *M. intracellulare, M. terrae* group, or any other NTMs or for the three different capture locations office, staff village and living quarters, gender, body condition, dehydration status, injuries or if found dead as well as flea or tick burden (Table 18).

Table 18: Descriptive statistics and comparison of categorical variables based on *Mycobacterium bovis* (bTB) status of sampled banded mongooses.

Variable	Level	Overall Percent (n)	bTB positive* Percent (n)	bTB negative Percent (n)	P value†
Location					
	VWS Office	39 (74)	47 (15)	37 (59)	0.506
	Staff village	41 (74)	27 (15)	44 (59)	0.220
	Living quarters	20 (74)	27 (15)	19 (59)	0.488
Signalment					
	Female	47 (76)	53 (15)	45 (60)	0.563
	Age (>12 months)	51 (76)	87 (15)	42 (60)	0.002
	Weight (>953 g)	49 (73)	73 (15)	43 (58)	0.037
	BCS (>3)	46 (74)	60 (15)	42 (59)	0.221
Health					
	Alive	84 (76)	53 (15)	93 (60)	<0.001
	Dead	4 (76)	0 (15)	3 (60)	1.0
	Euthanised	12 (73)	47 (15)	3 (58)	<0.001
	Dehydration	13 (72)	7 (15)	14 (57)	0.674
	Old injury	25 (72)	33 (15)	23 (57)	0.504
	Fresh injury	50 (72)	33 (15)	54 (57)	0.147
	Any injury	65 (72)	60 (15)	67 (57)	0.629
External parasites					
	Fleas	52 (73)	47 (15)	53 (58)	0.639
	Ticks (≥5)	49 (73)	67 (15)	43 (58)	0.132
Nontuberculous mycobacteria					
	M. avium complex	8 (74)	13 (15)	7 (59)	0.595
	M. intracellulare	5 (74)	0 (15)	7 (59)	0.576
	M. fortuitum group	23 (74)	40 (15)	19 (59)	0.095
	M. terrae group	8 (74)	7 (15)	8 (59)	1.0
	M. phlei	4 (74)	13 (15)	2 (59)	0.103
	Any	57 (74)	73 (15)	53 (59)	0.147

*Positive on any serological test or for *M. bovis* on bacterial culture; †Based on chi-square or Fisher exact tests; BCS: Body condition score

Table 19: Descriptive statistics and comparison of quantitative variables based on *Mycobacterium bovis* (bTB) status of sampled banded mongooses.

Variable	n	Overall Median (range)	bTB positive* Median (range)	bTB negative Median (range)	P value†
Age	76	Y (J, O)	Y (J, O)	S (J, O)	0.025
Weight (g)	73	953 (387, 1591)	1064 (537, 1444)	763 (387, 1591)	0.008
BCS	74	3 (1, 4.5)	3.5 (2.5, 4.5)	3 (1, 4.5)	0.115
Dehydration	72	0 (0, 0.10)	0 (0, 0.02)	0 (0, 0.10)	0.454
Old injuries	72	None (none, severe)	None (none, moderate)	None (none, severe)	0.340
Fresh injuries	72	None-mild (none, moderate)	None (none, mild)	Mild (none, moderate)	0.118
Ticks	73	<5 (0, 50-100)	5-10 (0, 50-100)	<5 (0, 50-100)	0.161

*Positive on any serological test or for *M. bovis* on bacterial culture; †Based on Mann-Whitney U tests; BCS: Body condition score

When comparing NTM positive versus NTM negative animals with locality, signalement and health status animals sampled at the VWS office were significantly more likely (P = 0.002) to harbour NTMs with 55% culturing positive and only 19% culturing NTM negative, whereas animals caught in the staff village were significantly

less likely (P = 0.006) to culture positive for NTMs with only 26% NTM culture positive versus 58% culture negative (Table 20).

Females were significantly (P = 0.043) more likely to culture negative for NTMs (59%). Furthermore, animals that had sustained fresh injuries were significantly less likely associated with NTM colonisation both when expressed as categorical data with 65% of banded mongooses with fresh injuries culturing negative for NTM versus 35% culturing positive (P = 0.032) (Table 20), as well as when comparing quantitative data with no fresh injuries associated with NTM positive and mild fresh injuries associated with NTM negative animals (P = 0.043) (Table 21).

Table 20: Descriptive statistics and comparison of categorical variables based on isolation of non-tuberculosis mycobacteria (NTM).

		Overall	NTM positive	NTM negative	
Variable	**Level**	**Percent (n)**	**Percent (n)**	**Percent (n)**	**P value***
Location					
	VWS Office	40 (73)	55 (42)	19 (31)	0.002
	Staff village	40 (73)	26 (42)	58 (31)	0.006
	Living quarters	21 (73)	19 (42)	23 (31)	0.712
Signalment					
	Female	46 (74)	36 (42)	59 (32)	0.043
	Age (>12 months)	51 (74)	50 (42)	53 (32)	0.790
	Weight (>953 g)	49 (73)	48 (42)	52 (31)	0.736
	BCS (>3)	46 (74)	45 (42)	47 (32)	0.889
Health					
	Alive	86 (74)	86 (42)	88 (32)	1.0
	Dead	3 (74)	6 (32)	0 (42)	0.184
	Euthanized	11 (72)	14 (42)	7 (30)	0.455
	Dehydration	13 (72)	14 (42)	10 (30)	0.726
	Old injury	25 (72)	24 (41)	26 (31)	0.891
	Fresh injury	50 (72)	39 (41)	65 (31)	0.032
	Any injury	65 (72)	56 (41)	77 (31)	0.060
External parasites					
	Fleas	52 (73)	45 (42)	61 (31)	0.175
	Ticks (≥5)	49 (73)	52 (42)	45 (31)	0.542

*Based on chi-square or Fisher exact tests

Table 21: Descriptive statistics and comparison of quantitative variables based on based on isolation of nontuberculous mycobacteria (NTMs).

		Overall	NTM positive	NTM negative	
Variable	**n**	**Median (range)**	**Median (range)**	**Median (range)**	**P value***
Age	76	Y (J, O)	Y (J, O)	Y (J, O)	0.238
Weight (g)	73	953 (387, 1591)	832 (422, 1394)	1019 (387, 1591)	0.349
BCS	74	3 (1, 4.5)	3 (2.5, 4.5)	3 (1, 4.5)	0.820
Dehydration	72	0 (0, 0.10)	0 (0, 0.05)	0 (0, 0.10)	0.564
Old injuries	72	None (none, severe)	None (none, moderate)	None (none, severe)	0.857
Fresh injuries	72	None-mild (none, moderate)	None (none, moderate)	Mild (none, moderate)	0.043
Ticks	73	<5 (0, 50-100)	5-10 (0, 50-100)	<5 (0, 50-100)	0.311

*Based on Mann-Whitney U tests

Animals that showed any injury, fresh or old, would show a tendency (P = 0.060) to culture negative for NTMs with no difference in regards to NTM isolation found in animals that showed old injuries (Table 20). No other signalement or health parameter showed association with NTM isolation.

Comparing animals harbouring NTMs of potential pathogenicity as categorised in chapter 3.5 versus animals with clinically irrelevant NTMs or NTM negative culture, summarised as NTM negative in the following, location again appeared to be an important factor, indicating a strong tendency (P = 0.062) that banded mongooses from the staff village were more likely (50%) to be NTM negative (Table 22).

Table 22: Descriptive statistics and comparison of categorical variables based on isolation of non-tuberculous mycobacteria (NTMs) with potential clinical importance.

		Overall	**NTM (path.) positive***	**NTM (path.) negative****	
Variable	**Level**	**Percent (n)**	**Percent (n)**	**Percent (n)**	**P value†**
Location					
	VWS Office	40 (73)	49 (35)	32 (38)	0.138
	Staff village	40 (73)	29 (35)	50 (38)	0.062
	Living quarters	21 (73)	23 (35)	18 (38)	0.639
Signalment					
	Female	47 (76)	37 (35)	54 (39)	0.150
	Age (>12 months)	51 (76)	49 (35)	54 (39)	0.650
	Weight (>953 g)	49 (73)	49 (35)	50 (38)	0.903
	BCS (>3)	46 (74)	46 (35)	46 (39)	0.970
Health					
	Alive	84 (76)	83 (35)	90 (39)	0.502
	Dead	4 (76)	0 (35)	5 (39)	0.495
	Euthanised	12 (73)	17 (35)	5 (37)	0.146
	Dehydration	13 (72)	9 (35)	16 (37)	0.480
	Old injury	25 (72)	18 (34)	32 (38)	0.217
	Fresh injury	50 (72)	38 (34)	61 (38)	0.059
	Any injury	65 (72)	50 (34)	79 (38)	0.010
External parasites					
	Fleas	52 (73)	49 (35)	55 (38)	0.567
	Ticks (≥5)	49 (73)	57 (35)	41 (39)	0.199

Path.: potential pathogenic; *positive culture for *M. fortuitum, M. fortuitum* group, *M. avium* complex, *M. avium, M. intracellulare*, *M. scrofulaceum, M. simiae, M. heckeshornense, M. chelonae-abscessus* group, *M. parascrofulaceum, M. asiaticum*-like, *M. asiaticum, M. szulgai, M. terrae* or *M. terrae* group; ** positive culture for *M. elephantis group, M. nonchromogenicum, M. phlei, M. smegmatis, M. hassiacum, M. moriokaense, M. senuense*-like *and M. paraffinicum* or *Mycobacterium* negative culture; †Based on chi-square or Fisher exact tests; BCS: Body condition score

Comparing the influence of health parameters animals that had sustained any injury, fresh or old, were significantly (P = 0.01) associated with NTM negative status (79%). Similarly animals that presented with fresh injuries showed a tendency to be NTM culture negative both for categorical (Table 22) as well as quantitative data (Table 23) (P = 0.059 and P = 0.088). On the other side tick counts between five and ten per

animal tended to be associated with animals culture positive for potentially pathogenic NTMs.

Table 23: Descriptive statistics and comparison of quantitative variables based on isolation of nontuberculous mycobacteria (NTMs) with potential clinical importance.

		Overall	**NTM (path.) positive***	**NTM (path.) negative****	
Variable	**n**	**Median (range)**	**Median (range)**	**Median (range)**	**P value†**
Age	76	Y (J, O)	S (J, O)	Y (J, O)	0.292
Weight (g)	73	953 (387, 1591)	901 (422, 1394)	986 (387, 1591)	0.398
BCS	74	3 (1, 4.5)	3 (2.5, 4)	3 (1, 4.5)	0.991
Dehydration	72	0 (0, 0.10)	0 (0, 0.05)	0 (0, 0.10)	0.372
Old injuries	72	None (none, severe)	None (none, mild)	None (none, severe)	0.128
Fresh injuries	72	None-mild (none, moderate)	None (none, mild)	Mild (none, mild)	0.088
Ticks	73	<5 (0, 50-100)	5-10 (0, 50-100)	<5 (0, 50-100)	0.087

Path.: potential pathogenic; *positive culture for *M. fortuitum, M. fortuitum* group, *M. avium* complex, *M. avium, M. intracellulare*, *M. scrofulaceum, M. simiae, M. heckeshornense, M. chelonae-abscessus* group, *M. parascrofulaceum, M. asiaticum*-like, *M. asiaticum, M. szulgai, M. terrae* or *M. terrae* group; ** positive culture for *M. elephantis group, M. nonchromogenicum, M. phlei, M. smegmatis, M. hassiacum, M. moriokaense, M. senuense*-like *and M. paraffinicum* or *Mycobacterium* negative culture; †Based on Mann-Whitney U tests; BCS: Body condition score

Chapter 4: Discussion and Conclusion

4.1. Discussion

The results of our study confirmed that banded mongooses in the vicinity of the Skukuza Rest Camp in the southern Kruger National Park (KNP) did indeed become infected with mycobacteria of the *Mycobacterium tuberculosis* complex (MTC), as results of mycobacterial culture of post mortem samples of two banded mongoose individuals from the troop frequenting the area around the Skukuza living quarters has shown. The culture isolates were identified as *M. bovis* and therefore confirmed the presence of a pathogenic *Mycobacterium* in the banded mongoose population investigated.

Lesions noted in the *M. bovis* infected banded mongooses resembled caseous-necrotic or calcified granulomas typical for bovines (Neill et al. 1994) rather than more fibroblastic lesions as described for large predators, e.g. lion (Keet *et al.* 2010). Macromorphological and histopathological similarities were noted in lesions in the banded mongoose caused by *M. bovis* as described by Drewe *et al.* (2009b) as well as lesions caused by *M. mungi* seen in the outbreak in northern Botswana (Alexander *et al.* 2002). Lesions described by Alexander *et al.* (2002) were generally larger, up to 2 cm in diameter in comparison to 0.5 cm in the KNP banded mongooses, most likely because disease had progressed further in the Botswana mongooses as indicated by the presence of clinical symptoms such as cachexia, ataxia and weakness. Also in contrast to this study, Alexander *et al.* (2002) found granulomas to be more prominent in the abdominal organs such as liver, spleen and mesenteric lymph nodes, whereas in the KNP study lesions typical for mycobacterial infection were only noted in the lung and head lymph nodes. Multifocal miliary necrosis of the liver in the KNP banded mongooses was ascribed to parasitic rather than *Mycobacterium* infection, according to macroscopic and histopathological features as well as the fact that similar lesions of varying degree were encountered in almost every animal examined on necropsy, irrespective of *Mycobacterium* isolation or serological status. To support this lung or lymph node lesions were only present in animals that also had a STAT-PAK® Assay (STAT-PAK) positive test result, whereas liver lesions were also found in animals with negative STAT-PAK results. Notably, the severest liver necrosis was observed in the two individuals that carried *M. bovis*,

possibly indicating a higher parasite load associated with decreased immunocompetency following co-infection with *Mycobacterium* (Cousins *et al.* 2004). Interestingly mongoose A56 and A57 were the only animals necropsied that showed signs of white pulp hyperplasia and splenomegaly, in accordance with findings of Alexander *et al.* (2002). However, histopathology did not confirm macroscopically identified lesions. In contrast to suricates where lymph node swelling seems to be a prominent clinical feature (Alexander *et al.* 2002) banded mongooses showed few clinical symptoms in accordance with findings in the mycobacterial outbreak in banded mongooses in Botswana. However, no weight loss or weakness were observed, possibly because the disease had not advanced to that stage yet.

Only the animals with macroscopic lesions were also positive for *M. bovis* culture. However, not all lesions yielded *M. bovis* on culture; e.g. in banded mongoose A56 lung lesion and cervical superficial lymph node lesion were *Mycobacterium* culture negative and tracheo-bronchial lymph node lesion yielded *M. avium* complex. Vice versa some organs such as abdominal lymph nodes of individuals A56 and A57 and liver and tracheo-bronchial lymph node of A57 were positive for *M. bovis* on culture, however, lacked macroscopic or microscopic typical mycobacterial lesions. Only from lesions in the lung of A57 and retropharyngeal lymph node of A56 was *M. bovis* isolated on culture. A similar finding was described by O'Brien *et al.* (2013) where the majority of routine lymph node samples of carcasses of Michigan fur bearers as e.g. black bear (*Ursus americanus*), bobcat (*Lynx rufus*) or red fox (*Vulpes vulpes*) (Schmitt *et al.* 2002) that were *M. bovis* culture positive did not show any lesions, indicating low screening sensitivity of macropathology, histopathology or presence of acid fast bacilli. As in the present study mycobacteria cannot always be illustrated in the histopathology section with Ziehl-Neelsen stain, but even if a pathogen is isolated, it still needs to be correlated to macroscopic and/ or histopathological lesions to verify it as causative agent (OIE 2009). Therefore, even though typical tuberculosis lesions were identified on macro- and histopathology, positive *M. bovis* culture and pathology correlated only inconsistently, resulting in failure to confirm *M. bovis* as the causative agent of the lesions (OIE 2009). This phenomenon is most likely related to early pauci-bacilliary disease due to *M. bovis* in the banded mongoose population in the vicinity of Skukuza in the KNP. Drewe *et al.* (2009b) on

the other hand could demonstrate intracellular acid fast rods and therefore link mycobacterial infection to disease.

Of particular interest was that *M. bovis* was not only isolated from post mortem samples, but also from in vivo samples, i.e. tracheal lavage for banded mongoose A56 and A57 and tracheal swab for A56. On the one hand this finding indicates the potential usefulness of non-lethal sampling for monitoring purposes. Unfortunately *M. bovis* positive culture results were too few to allow for statistical power analysis.

On the other hand, the cultural isolation of *M. bovis* from tracheal samples indicates that these individuals were able to shed the pathogen. This fact has enormous implications for elucidating the transmission modes of *M. bovis* in the banded mongoose and confirms, as proposed by Drewe *et al.* (2011), that aerosol transmission might play an important role in spreading the pathogen within the population e.g. via close contact as during intensive grooming behaviour or when sleeping. The fact that the only two animals that *M. bovis* was isolated from, originated from the same troop supports this route of transmission. Aerosol transmission as predominant mode of spread is also supported by the occurrence of lesions only in the lungs, lung lymph nodes or head lymph nodes, suggesting the formation of a typical primary complex (Cousins *et al.* 2004). Quantification of bacteria shed from the respiratory tract would be the first step into investigating the potential of the banded mongoose to develop into a maintenance host.

When investigating the intestinal tract no macroscopic lesions typical of *Mycobacterium* infection were noted. However, *M. bovis* was isolated from the abdominal lymph nodes in both *M. bovis* positive animals and in banded mongoose A57 from the liver, lesions of which were ascribed to parasite rather than *Mycobacterium* infection. The question arises if *M. bovis* isolation from the intestinal tract would indicate the occurrence of oral infection, or if perhaps more likely *M. bovis* shed from the respiratory tract was swallowed and invaded the gastrointestinal tract secondarily. Thirdly haematogenous spread, would have to be considered, especially as lung lesions are not encapsulated by a thick fibrous capsule in the banded mongoose. Notably no *M. bovis* was isolated from faeces or faecal swabs whereas NTMs were readily isolated from the latter. Interestingly, no *Mycobacterium* was

cultured from any of the nine opportunistically collected faecal samples, two of which correlate to NTM culture positive faecal swabs (*M. fortuitum* in A24 and *M. terrae* in A78). Limited survival in the environment due to opportunistic faecal sampling after animals defecated in the cage trap is unlikely the cause for failed isolation as survival times of *M. bovis* in faeces of up to one week have been indicated by Tanner & Michel (1999). Perhaps extraction technique or dilution factor are better explanations for a zero *Mycobacterium* yield from the collected faeces.

Contrary to reports from Drewe e*t al.* (2009b) who indicate the percutaneous route of infection as an important mode of transmission in banded mongooses, no indication of percutaneous infection was obvious in this study. One explanation might be that shedding loads in the KNP banded mongooses are too small for transdermal infection but have reached the threshold dose for aerosol transmission, the latter being the smallest when compared to oral or transdermal route of infection (Bengis 1999).

Determining the source of infection for these banded mongooses seems to be the most imperative question that is raised from the research results. The source of infection would give invaluable insight into the epidemiology of bTB in these small predators as well as the role that the banded mongoose may play in its epidemiology. Speculating, the buffalo population around Skukuza located in the high bTB prevalence zone could be a direct source to the banded mongoose population via environmental contamination, with banded mongoose e.g. scavenging in buffalo faeces for insects (Skinner & Smithers 1990).

Or banded mongooses could be infected by scavenging on abattoir scraps at the Skukuza Game Processing Plant (GPP) as postulated for a resident troop of baboon (Keet *et al.* 2000). However the abattoir in Skukuza had not been operational for 15 years prior to the conducting of the study, which raises the question of where the pathogen was maintained in the interim. New outbreaks of TB have been recorded in close temporal proximity to the conduction of the mongoose study in the area in the local baboon troop as well as resident warthog (De Klerk pers. comm., 9 September 2012). Interestingly the infected banded mongooses in this study roamed and slept in close proximity to the bTB infected baboon troop (Keet *et al.* 2000; De Klerk pers.

comm., 9 September 2012). The close proximity of infected baboons and banded mongooses raises the question of a possible common source of infection, perhaps human refuse or after its' reopening the GPP. Alternatively it might indicate a new maintenance host with possible spill over host relationship from baboon to banded mongoose, the latter scavenging in an environment contaminated by baboon dung.

A third possible source of infection for the banded mongoose with *M. bovis is* the local human population, considering that the banded mongooses regularly foraged in rubbish bins. The fact that *M. bovis* was present in only one troop, the one closest to the living quarters and picnic area, where rubbish bins are often not secured against baboon raids as in the staff village leaves room to speculation as to a possible source of infection, considering Alexander *et al.* (2002) also postulated that scavenging on human refuse might have led to infection with MTC. An alternative and perhaps more likely explanation, considering that Alexander *et al.* (2010) subsequently discovered the causative agent in the Botswana tuberculosis outbreak to be the novel MTC species *M. mungi* not demonstrated yet in human infection, might be that exposure of animals to increased human activity levels, their pathogens and exposure to atypical food sources might induce stress in wild animals (Keet *et al.* 2000). Stress as an immune-modulating factor (Cousins *et al.* 2004) might lead to disease manifestation as postulated for the baboon troop that was found to be positive for bTB in Skukuza in 1996 (Keet *et al.* 2000).

To investigate the epidemiology in regards to the human interface it would be interesting to investigate the tuberculosis epidemiology of the permanent population in the Skukuza staff village and living quarters. Elucidating if *M. bovis* or *M. tuberculosis* is the predominant causative agent of mycobacterial disease in humans in the KNP/ Skukuza might include or exclude the possibility for human-wildlife spill-over.

In contrast to the spread of bTB from buffalo to lion believed to occur predominantly directly via the oral route when lion prey on buffalo (Keet *et al.* 2010), infection of banded mongooses from either of the above speculated sources raises the question of survival time and persistence of *M. bovis* in the environment and indicates that environmental contamination might play an important and potentially underestimated

role in the bTB epidemiology at the wildlife interface of yet unidentified interspecies transmission, supported by recent evidence of predominantly indirect transmission of bTB via the environment between the European badger and cattle in the United Kingdom (UK) (Drewe *et al.* 2013). Whereas survival times of *M. bovis* in the environment for up to 15 months after host removal have been recorded in the UK (Sweeney *et al.* 2007), environmental conditions in South Africa might differ and further studies in addition to the ones conducted by Tanner & Michel (1999) using novel techniques as described from the UK would be necessary to elucidate the South African situation. Long persistence of *M. bovis* in the environment highlights the potential threat of *M. bovis* spill-over to threatened or endangered wildlife species along the wildlife-environment-wildlife route of transmission.

The alternative to infection from buffaloes, baboons or humans is that the banded mongoose itself is developing into a maintenance host. This possibility and its implications might have tremendous economic consequences, if the scenario is to be compared to the European situation where development of the European badger into a maintenance host has raised the costs of bTB control tremendously (Garnetta, Ropera & Delahay 2003). However, keeping in mind that only two animals infected with *M. bovis* were identified in this study, the banded mongoose as a maintenance host seems as yet highly unlikely.

Ultimately, genotyping of the *M. bovis* strain isolated in this study is necessary to investigate its epidemiology in the banded mongoose with a good chance for clarification of some of the above questions, considering that only one dominant strain with its few dependent variants is present in the KNP (Michel *et al.* 2009a).

No other member of the MTC was identified in this study. However, a variety of nontuberculous mycobacteria (NTMs) was isolated. When examining NTM and *M. bovis* coinfection, a tendency for *M. phlei* and *M. fortuitum* group to be associated with *M. bovis* was seen, with epidemiological implications yet obscure. Other coinfections observed for example with *M. avium* complex, *M. fortuitum* group or *M. simiae* group might influence various diagnostic tests by cross reactivity or contribute to immunosuppression and enhance disease manifestation of bTB.

Mostly a mixture of several NTM species was identified. The broad range of species encountered in the banded mongoose is in accordance with findings of Gcebe e*t al.* (2013), who investigated NTM species composition in buffalo and their environment, concluding that South Africa seems to have a more diverse NTM population when compared globally. Additionally, in this study we found that NTM species composition in the banded mongoose differed with organ system sampled as well as differing demographic drivers.

When comparing in vivo versus post mortem samples as indicators of differing invasive potential of the respective NTM, members of the *M. simiae* group (MSG), closely followed by members of the *M. avium* complex (MAC), often in combination, were the most frequently isolated NTMs from post mortem samples. In vivo samples most often yielded members of the *M. fortuitum* group from faecal and tracheal swabs, whereas tracheal lavages returned predominantly members of the MAC. Generally potentially pathogenic NTMs predominated and notably *M. nonchromogenicum* was the only NTM found in ante mortem as well as post mortem samples, giving cause for speculation as to whether it should not rather be classified as a potentially pathogenic NTM as well, considering its invasive potential. However, no pathology was associated with the *M. nonchromogenicum* culture positive samples. The only NTM species isolated from a mild lesion consisting of few opaque nodules and mild haemorrhage in the lung was a member of the *M. simiae* group. This is in accordance with *M. simiae* classified as a potentially pathogenic *Mycobacterium* (Dostal, Richter & Harmsen 2003). The low incidence of NTM induced pathology correlates to the overall good health encountered in the banded mongoose study population, indicating an intact immune system in the majority of the study animals with the ability to eliminate NTM infection.

Respiratory and alimentary tract also differed in their NTM composition with e.g. *M. chelonae* group, *M. asiaticum*-like NTMs, *M. phlei* and *M. moriokaense* exclusively isolated from faecal swabs, whereas *M. hassiacum, M. elephantis* group, *M. smegmatis* and *M. senuense*-like NTMs were the only ones isolated from tracheal samples.

The different distribution patterns might in future help elucidate certain transmission patterns. Already certain trends are evident. Comparing NTM isolation with troop association the potentially pathogenic members of *M. fortuitum* group, *M. avium* complex and *M. simiae* group were isolated from all three troops, whereas *M. hassiacum, M. elephantis* group and *M. nonchromogenicum* were exclusively and *M. phlei, M. terrae* group and *M. chelonae-abscessus* group mostly isolated from individuals of the troop frequenting the VWS offices. These localised NTM communities strongly indicate either habitat or social behaviour as e.g. grooming or nursing to be the driving factors behind the differing frequencies of NTM isolations in different troops/ localities. These demographic differences are highlighted by the statistical significance of isolating more NTMs from banded mongooses frequenting the VWS offices and less from those roaming in the staff village. Additionally, a trend towards less potentially pathogenic NTMs colonising the banded mongooses in the staff village was detected, possibly indicating fewer stressors compromising their immune system in that habitat. As Drewe *et al.* (2011) proposed for infection with MTC it also seems to be true for NTM infection, that aerosol transmission might play an important role in spreading the pathogen within the population e.g. via close contact as during intensive grooming periods or when sleeping. Interestingly though female banded mongooses were found to harbour fewer NTMs (Table 20), perhaps reflecting less aggression and therefore lower infection rate in that gender.

Contrary to expectations, animals that had sustained injuries, especially fresh injures were significantly less likely associated with NTM colonisation. A possible explanation is that after sustaining a fresh injury not enough time had elapsed for NTM colonisation to take hold. However, old injuries did not show higher association with NTMs. One might speculate that opportunistic NTM infection is eliminated during the healing process in an immunocompetent individual. Alternatively the need to search for an easy food source such as the bait in a cage trap is higher when injured. Therefore the driving force for an animal to be caught in a cage trap as it is in search for an easy food source is either compromised health due to injury or due to NTM colonisation without any causative relation between injury and NTM colonisation. Contrary to that tick counts between five and ten per animal tended to be associated with an animals' culture to yield potentially pathogenic NTMs, which is in accordance to a compromised immune system being more prone to NTM colonisation as well as

parasite infestation. The question remains if the potentially pathogenic NTM would be the cause for immunosuppression.

Comparing serological test results, antibodies to MTC were detected with STAT-PAK and Enferplex^{TM} TB Assay (Enferplex). All positive STAT-PAK test results were confirmed by DPP® VetTB Assay (DPP). MPB83 seemed to be the most important antigenic factor in the banded mongoose antibody response, with antibodies detected by all three tests. In accordance with *Mycobacterium* culture results in this study, MPB83 rather correlates with *M. bovis*, versus CFP10/ESAT-6 fusion protein a more general or *M. tuberculosis* marker antigen (Lyashchenko *et al.* 2012). In comparison to the results obtained from the banded mongoose, antibodies for *M. bovis* have been detected in the Eurasian badger, the Iberian lynx (*Lynx pardinus*) and the European red fox (*Vulpes vulpes*) using an indirect competitive enzyme-linked immunoassay targeting antibodies against the MPB70 protein of *M. bovis* (Martin-Atance *et al.* 2006) and although no lesions typical for tuberculosis were found in the red fox, *M. bovis* was cultured from the retropharyngeal lymph node (Martin-Atance *et al.* 2005). The same survey failed to identify antibodies in an Egyptian mongoose (*Herpestes ichneumon*), possibly indicating MPB83 to be a better target antigen. However, neither lesions typical for *Mycobacterium* infection were detected nor was *M. bovis* isolated via culture.

Serological tests correlated with a positive *M. bovis* culture result when serial interpretation of STAT-PAK and Enferplex was implemented, accepting a positive result only if both tests were positive. Additionally the intensity of DPP test reaction in comparison to the STAT-PAK test reaction was a further indicator for a positive *M. bovis* culture result. A56 and A57 were the only two animals where both tests developed a very strong positive reaction as well as expressed the same reaction intensity. Therefore, DPP was able to confirm the two individuals with highest antibody titres. However, as DPP was only performed on STAT-PAK positive sera, statistical analysis in the absence of a gold standard could not be performed, lacking a third analytical test. Nevertheless, the combination of either STAT-PAK or DPP with Enferplex seemed to be the most reliable serological diagnostic tool for identifying a *M. bovis* infected individual ante mortem.

No consistent cross reactions with NTMs would explain the different reaction intensities of either serological test, especially as all NTMs isolated from seropositive animals would also occur in seronegative individuals. Even though direct evidence of NTM cross reaction with the serological tests is not obvious, statistically the tendency of *M. phlei* and *M. fortuitum* to be linked to bTB positive serological status might indicate some degree of cross reaction or immune modulation towards *M. bovis*.

If combining in vivo sampling as a tracheal lavage and tracheal swab for *Mycobacterium* culture with at least two serological tests, non-lethal sampling of banded mongooses showed promising potential as a surveying and monitoring tool for detection of *M. bovis* infection in this species. This is in accordance with Drewe *et al.* (2009a) who recommends a combination of tests to enhance the likelihood of making the correct diagnosis.

Contrary to expectations for the demographic distribution of a chronic disease such as bTB to be more frequently encountered in older animals, the two *M. bovis* infected individuals identified in this study belonged to relatively young age groups (young adult being 12 to 18 months old and prime adult II relating to two to four years of age). However, the lesions identified were not far advanced (up to 0.5 cm in diameter) if compared to the larger lesions (0.5 to 2 cm in diameter) described for the clinically ill, i.e. further advanced in prevalence and progression, banded mongooses in the Botswana outbreak. Considering that the KNP study was a survey in contrast to the disease outbreak investigation in Botswana (Alexander *et al.* 2002) it seems logical that clinical disease had not progressed as far in the KNP animals. Animals were most likely caught in an early stage of infection and would have shown more severe lesions and clinical disease if progressed to an older age. Statistical analysis on the other side is in accordance with epidemiology of a chronic and slow progressive disease, linking animals positive for bTB as defined by any positive test result to age groups of older than 12 months (87%). Statistical correlation of bTB status to weight with 73% of the bTB positive animals weighing more than 953 g, is most likely an expression of age rather than health status, with mongooses reaching adulthood and therefore adult weight of 1 kg when older than 12 months of age (ed. Apps 2000).

Recaptured animals mostly retained their serological status. Interestingly animal A5 seroconverted from STAT-PAK negative to positive within 20 days. On first capture *M. phlei*-like or *Rhodococcus* and on recapture *M. fortuitum* was isolated from a faecal swab sample. Banded mongoose A5 was, however, negative on pathology. Seroconversion of this animal might reflect the statistically identified tendency for *M. fortuitum* status of an animal to be correlated with seropositive reaction. Furthermore, banded mongoose A5 belonged to the troop frequenting the VWS office. This troop was subjected to statistically significant higher NTM infection pressures, which could explain that this animal was infected with *M. fortuitum* and seroconverted during the study time. Interestingly banded mongoose A5 was a juvenile animal, at an age that it would encounter novel pathogens and develop its immune system. Other animals that were recaptured mostly harboured differing NTMs on the recapture event with capture interim times varying from 16 days to one month or even up to 6 months, indicating always changing environmental exposure and elimination by an intact immune system.

One of the reasons why necropsy numbers were so low in this study was that one of the euthanasia criteria was advanced age to increase the likelihood of lesion development and progression to a detectable stage. However, only three old adults (two females, one male) were caught. On the one hand this could indicate a relative short life span of the banded mongoose in the KNP, possibly induced by disease such as bTB. On the other hand catching only younger age groups could be the consequence of banded mongoose behaviour confounding the overall capture outcome. In that respect, older animals are most likely preoccupied with sentinel duties when entering a new foraging area (personal observation), resulting in the younger ones to be the first to explore traps and bait and therefore predominantly get caught rather than the older individuals.

4.2. Conclusion

In conclusion, two banded mongooses in the vicinity of Skukuza in the high bTB prevalence zone in the KNP were identified with typical mycobacterial lesions which could be associated with *M. bovis* infection. The isolate was speciated as *M. bovis,* confirming that the banded mongoose population in the KNP is indeed infected with *M. bovis.* Furthermore, the positive *M. bovis* culture correlated with serological

diagnostic, if STAT-PAK and Enferplex ELISA were interpreted in parallel, a positive serological result defined as both STAT-PAK and Enferplex reacting positive. Together with a positive *M. bovis* culture of an in vivo sample such as tracheal lavage or tracheal swab, given the consensus of in vivo and post mortem results of the study, the potential for non-lethal diagnostic is given, enabling monitoring or surveillance programmes.

Most interestingly, the isolation of *M. bovis* from a tracheal sample indicates that individual banded mongooses shed the pathogen and therefore potentially transmit it via aerosol formation.

The way forward would be to confirm the source of infection, e.g. by identifying the *M. bovis* genotype, to then elucidate the role of the banded mongoose in bTB epidemiology. Is the banded mongoose a spill over host and contracts the pathogen through scavenging or could it be a maintenance host as indicated by his potential to shed *M. bovis*?

References

1. 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', *Emerging Infectious Diseases* 8(6), 598–601.
2. Alexander, K.A., Laver, P.N., Michel, A.L., Williams, M., Van Helden, P.D., Warren, R.M. & Gey Van Pittius, N.C., 2010, 'Novel *Mycobacterium tuberculosis* Complex Pathogen, *M. mungi', Emerging Infectious Diseases* 16(8), 1296-1299.
3. Apps, P. (ed.), 2000, *Smithers' mammals of Southern Africa – A field guide*, Struik Publishers, Cape Town.
4. Bengis, R.B., 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.
5. Bengis, R.G., 1999, 'Tuberculosis in free-ranging mammals', in M. E. Fowler & R. E. Miller (eds.), *Zoo and wild animal medicine: Current Therapy 4*, pp 101–114, W. B. Saunders Company, Philadelphia, Pennsylvania.
6. 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, 225-230.
7. Bercovier, H. & Vincent, V., 2001, 'Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum, M. fortuitum, M. chelonae, M. porcinum, M. farcinogenes, M. smegmatis, M. scrofulaceum, M. xenopi, M. kansasii, M. simiae* and *M. genavense*', *Revue Scientifique et Technique de l Office international des Epizooties* 20(1), 265-290.
8. Botha, L., Gey van Pittius, N.C. & van Helden, P.D., 2013, 'Mycobacteria and Disease in Southern Africa', *Transboundary and Emerging Diseases* 60, 147-156.
9. Cant, A.M., 2000, 'Social control of reproduction in banded mongooses', *Animal Behaviour* 59, 147–158.

10. Chambers, M.A., 2013, 'Review of the Diagnosis of Tuberculosis in Non-Bovid Wildlife Species Using Immunological Methods - An Update of Published Work Since 2009', *Transboundary and Emerging Diseases* 60, 14-27.
11. Chembio Diagnostic Systems, 2007, *Mycobacterium bovis – Mycobacterium tuberculosis, Antibody Test Kit, ElephantTB STAT-PAK® Assay, A Rapid Immunochromatographic Test for the Detection of Antibodies to Mycobacterium tuberculosis and Mycobacterium bovis in Elephant Serum, Plasma or Whole Blood,* brochure, CDS, Medford, New York, USA, viewed 20 January 2012, from http://www.invitech.co.uk/downloads/instructions/ElephantTB50Kit.pdf.
12. Chembio Diagnostic Systems, 2012, *Mycobacterium bovis – Mycobacterium tuberculosis, Antibody Test Kit DPP® VetTB Assay for Elephants, A Rapid Immunochromatographic Test for the Detection of Antibodies to Mycobacterium tuberculosis and Mycobacterium bovis in Elephant Serum, Plasma or Whole Blood,* brochure, CDS, Medford, New York, USA, viewed 3 May 2014, from http://chembio.com/wp-content/uploads/2013/09/65-9111-0-DPP-VetTB-Elephant-Insert-5-Test-Kit-Rev-2.pdf.
13. Clifford, D.L., Kazwala, R.R., Sadiki, H., Roug, A., Muse, E.A., Coppolillo, P.C. & Mazet, J.A.K., 2013, 'Tuberculosis infection in wildlife from the Ruaha ecosystem Tanzania: implications for wildlife, domestic animals, and human health', *Epidemiology and infection* 141(7, SPECIAL ISSUE: TB in Wildlife), 1371-1381.
14. Coetzer, J.A.W. & Tustin R.C. (eds.), 2004, *Infectious diseases of livestock,* 2nd edn., Oxford University Press Southern Africa, Cape Town.
15. Cousins, D.V., Bastida, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., Duignan, P., Murray, A., Dupont, C., Ahmed, A., Collins, D.M., Butler, W.R., Dawson, D., Rodriguez, D., Loureiro, J., Romano, M.I., Alito, A., Zumarraga, M. & Bernadellia, A., 2003, 'Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov.', *International Journal of Systematic and Evolutionary Microbiology* 53, 1305–1314.
16. Cousins, D.V., Huchzermeyer, H.F.K.A., Griffin, J.F.T., Brückner, G.K., Van Rensburg, I.B.J. & Kriek, N.P.J., 2004, 'Tuberculosis', in J.A.W. Coetzer &

R.C. Tustin (eds.), *Infectious diseases of livestock,* 2nd edn., pp. 1973-1993, Oxford University Press Southern Africa, Cape Town.

17. Cross, M.L., Aldwell, F.E., Griffin, J.F.T. & Mackintosh, C.G., 1999, 'Intracellular survival of virulent *Mycobacterium bovis* and *M. bovis* BCG in ferret macrophages', *Veterinary Microbiology* 66(3), 235-243.
18. Cross, P.C., Heisey, D.M., Bowers, J.A., Hay, C.T., Wolhuter, J., Buss, P., Hofmeyr, M., Michel, A.L., Bengis, R.G., Bird, T.L.F., Du Toit, J.T. & Getz, W.M., 2009, 'Disease, predation and demography: assessing the impacts of bovine tuberculosis on African buffalo by monitoring at individual and population levels', *Journal of Applied Ecology* 46, 467–475.
19. Dannenberg, A.M., 1989, 'Immune Mechanisms in the Pathogenesis of Pulmonary Tuberculosis', *Reviews of Infectious Diseases* 11 (suppl. ser. 2), S369-S378.
20. De Luca, D.W. & Ginsberg, J.R., 2001, ,Dominance, reproduction and survival in banded mongooses: towards an egalitarian social system?' *Animal Behaviour,* 61, 17–30.De Vos, V., Bengis, R.G., Kriek, N.P.J., Michel, A.L., 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(2), 119–130.
21. Dostal, S., Richter, E. & Harmsen, D., 2003, *Concise Guide to Mycobacteria and Their Molecular Differentiation,* Ridom Press, Würzburg, Germany.
22. Drewe, J.A, Dean, G.S., Michel, A.L. & Pearce, G.P., 2009a, 'Accuracy of three diagnostic tests for determining *Mycobacterium bovis* infection status in live-sampled wild meerkats (*Suricata suricatta*)', *Journal of Veterinary Diagnostic Investigation* 21, 31-39.
23. Drewe, J.A., Foote, A.K., Sutcliffe, R.L. & Pearce, G.P., 2009b, 'Pathology of *Mycobacterium bovis* Infection in Wild Meerkats (*Suricata suricatta*)', *Journal of Comparative Pathology* 140, 12-24.
24. Drewe, J.A., Eames, K.T., Madden, J.R. & Pearce, G.P., 2011, 'Integrating contact network structure into tuberculosis epidemiology in meerkats in South Africa: implications for control', *Preventive Veterinary Medicine* 101, 113–120.
25. Drewe, J.A., O'Connor, H.M., Weber, N., McDonald, R.A. & Delahay, R.J., 2013, 'Patterns of direct and indirect contact between cattle and badgers

naturally infected with tuberculosis', *Epidemiology and Infection* 141, 1467–1475.

26. Enfer Group, n.d., *Enferplex™,* brochure, Newhall, Naas, Co. Kildare, Ireland, viewed 14 November 2011, from http://www.2013.ie/enfer/wp-content/uploads/2013/09/ Enferplex-brochure.pdf.
27. Ernst, J.D., 1998, 'Macrophage Receptors for *Mycobacterium tuberculosis*', *Infection and Immunity* 66(4), 1277-1281.
28. Falkinham, J.O., 2009, 'The biology of environmental mycobacteria', *Environmental Microbiology Reports* 1(6), 477–487.
29. Garnetta, B.T., Ropera, T.J. & Delahay, R.J., 2003, 'Use of cattle troughs by badgers (*Meles meles*) A potential route for the transmission of bovine tuberculosis (*Mycobacterium bovis) to cattle', Applied Animal Behaviour Science* 80, 1–8.
30. Gcebe, N., Rutten, V., Gey van Pittius, N.C. & Michel, A. 2013, "Prevalence and Distribution of Non-Tuberculous Mycobacteria (NTM) in Cattle, African Buffaloes (*Syncerus caffer*) and their Environments in South Africa", *Transboundary and Emerging Diseases* 60, 74-84.
31. Gey van Pittius, N.C., Perrett, K.D., Michel, A.L., Keet, D.F., Hlokwe, T., Streicher, E.M., Warren, R.M. & van Helden, P.D., 2012, 'Infection of African buffalo (*Syncerus caffer*) by oryx bacillus, a rare member of the antelope clade of the Mycobacterium tuberculosis complex' Journal of Wildlife Diseases 48(4), 849–857.
32. Gortazar, C., Delahay, R.J., Mcdonald, R.A., Boadella, M., Wilson, G.J., Gavier-Widen, D. & Acevedo, P., 2012, 'The status of tuberculosis in European wild mammals', *Mammal Review* 42(3), 193-206.
33. Google Earth 7.2.1, 2014, *Skukuza 21°58'15.15"S, 31°33'57.67"E elevation 297m,* Landsat, viewed 5 May, 2014 from . http://www.google.com/earth/index.html.
34. Harmsen, D., Rothgänger, J., Frosch, M. & Albert, J., 2002, 'RIDOM: Ribosomal Differentiation of Medical Microorganisms Database', *Nucleic Acids Research* 30, 416-417.
35. Harmsen, D., Dostal, S., Roth, A., Niemann, S., Rothgänger, J., Sammeth, M., Albert, J., Frosch, M. & Richter, E., 2003, 'RIDOM: comprehensive and public sequence database for identification of Mycobacterium species', *BMC*

Infectious Diseases 3(26), viewed 4 May 2014, http://www.biomedcentral.com/1471-2334/3/26.

36. Hinton, H.E. & Dunn, A.M.S., 1967, *Mongooses: Their natural history and behaviour*, Oliver and Boyd LTD, Edinburgh and London.
37. Hoffmann, M., 2008, '*Mungos mungo*', in IUCN 2013, *IUCN Red List of Threatened Species*, Version 2013.2, viewed 7 May 2014 from <www.iucnredlist.org>.
38. Huard R.C., Fabre, M., De Haas, P., Lazzarini, L.C.O., Van Soolingen, D., Cousins, D. & Ho, J.L., 2006, 'Novel Genetic Polymorphisms That Further Delineate the Phylogeny of the *Mycobacterium tuberculosis* Complex', *Journal of Bacteriology* 188(12), 4271–4287.
39. Jolles, A.E., Cooper, D.V. & Levin, S.A., 2005, 'Hidden Effects of Chronic Tuberculosis in African Buffalo', *Ecology* 86(9), 2358-2364.
40. Kaneene, J.B., Miller, R., de Kantor, I.N. & Thoen, C.O., 2010, 'Tuberculosis in wild animals [Serialised article. Tuberculosis: a re-emerging disease in animals and humans. Number 4 in the series]', *The International Journal of Tuberculosis and Lung Disease* 12(14), 1508-1512.
41. Katoch, V.M., 2004, 'Infections due to non-tuberculous mycobacteria (NTM)', *Indian Journal* of *Medical Research*, (abstract), 120, 290–304.
42. Keet, D.F., Kriek, N.P.J., Penrith, M., 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.
43. Keet, D.F., Kriek, N.P.J., Bengis, R.G., Grobler, D.G. & Michel, A., 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.
44. Keet, D.F., Michel, A.L., Bengis, R.G., Becker, P., Van Dyk, D.S., Van Vuuren, M., Rutten, V.P.M.G. & Penzhorn, B.L., 2010, 'Intradermal tuberculin testing of wild African lions (*Panthera leo*) naturally exposed to infection with *Mycobacterium bovis'*, *Veterinary Microbiology* 144, 384–391.
45. Kendall, B.A. & Winthrop, K.L., 2013, 'Update on the epidemiology of pulmonary nontuberculous mycobacterial infections', *Seminars in Respiratory and Critical Care Medicine* 34, 87-94.

46. Kent, P. & Kubica, G., 1985, *Public Health Microbiology: a Guide for the Level III Laboratory*, Centers for Disease Control, Atlanta.
47. Liu, G., Wang, G.-R., Yu, X., Liang, Q., Mu, J., Shang, Y.-Y., Ling, Y., Zhang, H.-Q., Zheng, S.-H. & Huang, H.-R., 2014, 'Bacteriological characterization of a *Mycobacterium parascrofulaceum* strain isolated from a Chinese pneumonia patient', *International Journal of Infectious Diseases*, (abstract), 25, 82–87.
48. Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Mikota, S., Miller, M., Moller, T., Vogelnest, L., Gairhe, K.P., Robbe-Austerman, S., Gai, J. & Waters, W.R., 2012, 'Field Application of Serodiagnostics To Identify Elephants with Tuberculosis prior to Case Confirmation by Culture', *Clinical and Vaccine Immunology* 19(8), 1269–1275.
49. Maddock, A.H., 1988, 'Resource partitioning in a viverrid assemblage', PhD thesis, University of Natal, quoted in *Wikipedia*, viewed 7 May 2014, from http://en.wikipedia.org/wiki/Banded_mongoose.
50. Martin-Atance, P., Palomares, F., Gonzalez-Candela, M., Revilla, E., Cubero, M. J., Calzada, J. & Leon-Vizcaino, L., 2005, 'Bovine Tuberculosis in a Free Ranging Red Fox (*Vulpes vulpes*) from Donana National Park (Spain)', *Journal of Wildlife Diseases* 41(2), 435–436.
51. Martin-Atance, P., Leon-Vizcaino, L., Palomares, F., Revilla, E., Gonzalez-Candela, M., Calzada, J., Cubero-Pablo, M.J. & Delibes, M., 2006, 'Antibodies to *Mycobacterium bovis* in Wild Carnivores from Donana National Park (Spain)', *Journal of Wildlife Diseases* 42(3), 704–708.
52. Michel, A.L., De Klerk, L.-M., Gey van Pittius, N.C., Warren, R.M. & Van Helden, P.D., 2007, 'Bovine tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria', *BMC Veterinary Research* 3:23, http://www.biomedcentral.com/ 1746-6148/3/23.
53. 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', *Veterinary Microbiology* 112, 91–100.
54. Michel, A.L., Hlokwe, T.M., Coetzee, M.L., Mare, L., Connoway, L., Rutten, V.P.M.G. & Kremer, K., 2008, 'High *Mycobacterium bovis* genetic diversity in a low prevalence setting', *Veterinary Microbiology* 126, 151–159.

55. Michel, A.L., Coetzee, M.L., Keet, D.F., Maré, L., Warren, R., Cooper, D., Bengis, R.G., Kremer, K. & van Helden, P., 2009a, 'Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves', *Veterinary Microbiology* 133(4), 335-343.
56. Michel, A.L. & Simoes, M., 2009b, 'Comparative field evaluation of two rapid immunochromatographic tests for the diagnosis of bovine tuberculosis in African buffaloes (*Syncerus caffer*)', *Veterinary Immunology and Immunopathology* 127, 186–189.
57. Michel, A.L., Hlokwe, T.M., Espie, I.W., Van Zijl Langhout, M., Koeppel, K. & Lane, E., 2009c, 'Human Tuberculosis in Captive Wild Animal Populations: Trends in South Africa and Implications for "One Health"', *Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics,* Durban, South Africa, 224-228.
58. Michel, A.L., Müller, B. & Van Helden, P.D., 2010, '*Mycobacterium bovis* at the animal–human interface: A problem, or not?', *Veterinary Microbiology* 140(3–4), 371-381.
59. Michel, A.L., Hlokwe, T.M., Espie, I.W., Van Zijll Langhout, M., Koeppel, K. & Lane, E., 2013, '*Mycobacterium tuberculosis* at the Human/Wildlife Interface in a High TB Burden Country', *Transboundary and Emerging Diseases* 60, 46–52.
60. Mikota, S.K. & Maslow, J.N., 2011, 'Tuberculosis at the human-animal interface: An emerging disease of elephants', *Tuberculosis* 91, 208-211.
61. Morar, D., 2009, 'Development of ELISAs for the detection of interferon-gamma in rhinoceroses and elephants as diagnostic tools for *Mycobacterium bovis* and *Mycobacterium tuberculosis* infections', PhD thesis, Dept. of Veterinary Tropical Diseases, University of Pretoria.
62. Morar, D., Schreuder, J., Meny, M., Van Kooten, P.J.S., Tijhaar, E., Michel, A.L. & Rutten, V.P.M.V., 2013, 'Towards establishing a rhinoceros-specific Interferon-Gamma (IFN-c) assay for diagnosis of tuberculosis', *Transboundary and Emerging Diseases,* 60 (suppl.1), 60-66.
63. Mun, H.S., Park, J.H., Kim, H., Yu, H.K., Park, Y.G., Cha, C.Y., Kook, Y.H. & Kim, B.J., 2008, 'Mycobacterium senuense sp. nov., a slowly growing, non-chromogenic species closely related to the Mycobacterium terrae complex',

International Journal of Systematic and Evolutionary Microbiology, (abstract), 58(Pt 3), 641-646.

64. Neill, S.D., Pollock, J.M., Bryson, D.B. & Hanna, J., 1994, 'Pathogenesis of *Mycobacterium bovis* infection in cattle', *Veterinary Microbiology* 40, 41-52.
65. Nishi, J.S., Shury, T. & Elkin, B.T., 2006, 'Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: Strategies for management and research', *Veterinary Microbiology* 112(2–4), 325-338.
66. Nolan, A. & Wilesmith, J.W., 1994, 'Tuberculosis in badgers (*Meles meles*)', *Veterinary Microbiology* 40, 179-191.
67. O'Brien, D.J., Schmitt, S.M., Rudolph, B.A. & Nugent, G., 2011, 'Recent advances in the management of bovine tuberculosis in free-ranging wildlife', *Veterinary Microbiology* 151(1–2), 23-33.
68. O'Brien, D.J., Fierke, J.S., Cooley, T.M., Fitzgerald, S.D., Cosgrove, M.K. & Schmitt, S.M., 2013, 'Performance of Diagnostic Tests for Bovine Tuberculosis in North American Furbearers and Implications for Surveillance', *Transboundary and Emerging Diseases* 60, 67-73.
69. OIE World Organisation for Animal Health, 2009, 'Bovine tuberculosis', in *OIE Terrestrial Manual 2009*, viewed 15 May 2014, from http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.07_BOVINE_TB.pdf.
70. OIE, n.d., *Bovine tuberculosis – General disease information sheets,* brochure, viewed 15 May 2014, from http://www.oie.int/fileadmin/home/eng/media_center/docs/pdf/disease_cards/bovine-tb-en.pdf.
71. Palmer, M.V., 2013, '*Mycobacterium bovis*: Characteristics of Wildlife Reservoir Hosts', *Transboundary and Emerging Diseases* 60, 1-13.
72. Parsons, S., Smith, S.G., Martins, Q., Horsnell, W.G., Gous, T.A., Streicher, E.M., Warren, R.M., Van Helden, P.D. & Gey van Pittius, N.C., 2008, 'Pulmonary infection due to the dassie bacillus (*Mycobacterium tuberculosis* complex sp.) in a free-living dassie (rock hyrax - *Procavia capensis*) from South Africa' *Tuberculosis* 88, 80–83.
73. Parsons, S.D.C., Drewe, J.A., Gey van Pittius, N.C., Warren, R.M. & Van Helden, P.D., 2013, 'Novel Cause of Tuberculosis in Meerkats, South Africa'. *Emerging Infectious Diseases* 19(12), 2004-2007.

74. Pollock, J.M., McNair, J., Welsh, M.D., Girvin, R.M., Kennedy, H.E., Mackie, D.P. & Neill, S.D., 2001, 'Immune responses in bovine tuberculosis', *Tuberculosis* 81, 103-107.
75. Prodinger, W.M., Brandstatter, A., Naumann, L., Pacciarini, M., Kubica, T., Boschiroli, M.L., Aranaz, A., Nagy, G., Cvetnic, Z., Ocepek, M., Skrypnyk, A., Erler, W., Niemann, S., Pavlik, I. & Moser, I., 2005, 'Characterization of *Mycobacterium caprae* isolates from Europe by mycobacterial interspersed repetitive unit genotyping' *Journal of Clinical Microbiology* 43, 4984–4992.
76. *Q-GIS* version 2.2.0-Valmiera, 2014, computer software, Open Source Geospatial Foundation, Beaverton, Oregon, United States.
77. 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(2), 258–264.
78. Roth, A., Reischl, U., Schönfeld, N., Naumann, L., Emler, S. Fischer, M., Mauch, H., Loddenkemper, R. & Kroppenstedt, R.M., 2000, '*Mycobacterium heckeshornense* sp. nov., a New Pathogenic Slowly Growing *Mycobacterium* sp. Causing Cavitary Lung Disease in an Immunocompetent Patient', *Journal of Clinical Microbiology* 38 (11), 4102–4107.
79. Skinner, J.D. & Smithers, R.H.N., 1990, *The mammals of the Southern African subregion,* 2nd edn., University of Pretoria, Pretoria.
80. Smith, N.H., 2012, 'The global distribution and phylogeography of *Mycobacterium bovis* clonal complexes,' *Infection, Genetics and Evolution* 12(4), 857-865.
81. South African National Parks (SANParks), 2008, *Kruger National Park visitors guide*', 2nd edn., Jacana Media, Melville South Africa.
82. SANParks, n.d., *Kruger National Park – Introduction*, viewed on 20 May 2014, from http://www.sanparks.org/parks/kruger/.
83. Sweeney, F.P., Courtenay, O., Hibberd, V., Hewinson, R.G., Reilly, L.A., Gaze, W.H. & Wellington, E.M.H., 2007, 'Environmental Monitoring of *Mycobacterium bovis* in Badger Feces and Badger Sett Soil by Real-Time PCR, as Confirmed by Immunofluorescence, Immunocapture, and Cultivation', *Applied and Environmental Microbiology* 73 (22), 7471–7473.

84. 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(3), 185-190.
85. Tortoli, E., 2011, 'Phylogeny of the genus *Mycobacterium*: Many doubts, few certainties', *Infection, Genetics and Evolution* 12(4), 827-831.
86. Tortoli, E., Gitti, Z., Klenk, H.-P., Lauria, S., Mannino, R., Mantegani, P., Mariottini, A. & Neonakis, I., 2013, 'Survey of 150 strains belonging to the *Mycobacterium terrae* complex and description of *Mycobacterium engbaekii* sp. nov., *Mycobacterium heraklionense* sp. nov. and *Mycobacterium longobardum* sp. nov.', *International Journal of Systematic and Evolutionary Microbiology* 63, 401–411.
87. Turenne, C.Y., Cook, V.J., Burdz, T.V., Pauls, R.J., Thibert, L., Wolfe, J.N. & Kabani, A., 2004, '*Mycobacterium parascrofulaceum* sp. nov., novel slowly growing, scotochromogenic clinical isolates related to *Mycobacterium simiae*', *International Journal of Systematic and Evolutionary Microbiology,* (abstract), 54(Pt 5), 1543-1551.
88. Wang, S.-H., Mangino, J.E., Stevenson, K., Yakrus, M.A., Cooksey, R., Butler, W.R., Healy, M., Wise, M.G., Schlesinger, L.S. & Panchol, P., 2008, 'Characterization of "*Mycobacterium paraffinicum*" Associated with a Pseudo-Outbreak', *Journal of Clinical Microbiology*, (abstract), 46(5), 1850–1853.
89. Wayne, L.G. & Sramek, H.A., 1992, 'Agents of infrequently encountered mycobacterial diseases', *Clinical Microbiology Reviews* 5(1), 1-25.
90. Whelan, C., Shuralev, E., O'Keeffe, G., Hyland, P., Kwok, H.F., Snoddy, P., O'Brien, A., Connolly, M., Quinn, P., Groll, M., Watterson, T., Call, S., Kenny, K., Duignan, A., Hamilton, M.J., Buddle, B.M., Johnston, J.A., Davis, W.C., Olwill, S.A. & Clarke, J., 2008, 'Multiplex Immunoassay for Serological Diagnosis of *Mycobacterium bovis* Infection in Cattle', *Clinical and Vaccine Immunology* 15(12), 1834–1838.
91. WHO, 2013a, *Global tuberculosis report 2013*, *Annex 2 Country profiles*, viewed 15 May 2014, from http://www.who.int/tb/publications/global_report/gtbr13_annex_2_country_profiles.pdf?ua=1.
92. WHO, 2013b, *Global tuberculosis report 2013*, *Annex 3 Regional profiles*, viewed 15 May 2014, from http://www.who.int/tb/publications/global_report/gtbr13_annex_3_regional_profiles.pdf?ua=1.

Appendices

Appendix 1: Capture Data Sheet

Mongoose Capture Sheet

Date ..... Time ..... VWS lab no/ study no/ field no ...../ A..... / .....

Camp ..... Weather ..... Cage no ..... GPS S.....E..... Location .....

Baby / Juvenile / Subadult / Young Adult /Adult / Old Adult age estimate ..... male/ female

Microchip ..... Ear notch .....

Body condition Score 1 / 2 / 3 / 4 / 5 head btw. Ears..... widest ..... Mass ..... g

Anaesthesia Premix: Date ..... Drugs ..... final Conc. .....

Anaesthesia Drugs	Dosages	Premix (ml)	Time	Injection site

Time: first signs ..... Recumbency ..... Surgical plane .....

Treatment - Drug	Dose	route			Reason
		im	sc	iv	

Antidote	Dose	Time	route			First wake up @	Recovery @	Release @
			im	sc	iv			

Euthapent		Dose (ml)	route				Time	Time of death (HR)	Reason		
no	yes		iv	ic	it	ip			STAT-PAK	age	clinic

SAMPLING (*tick or ml/ no*)

Serum			
Heparin			
EDTA			
STAT PAK	pos	susp	neg
Faecal	swab	faeces	
Trach. Swab			
Trach. Lav.			
Trach. Paras.	yes	no	
Hair			
Tissue			
Bl. Smear			
Pictures	yes	no	

CLINICAL EXAM

Injuries	
Skin	
Dehydration	
Parasites	
Nose	
Teeth	
Claws	
Lungs	
Abdomen	
Faeces	
Periph. Lnn.	
Other	

MONITORING

(T in °C; Reflex -/+/++/+++)

TIME		
Heart Rate		
Respiration		
Temperature		
Reflex eye		
Reflex jaw		
Reflex pain		

Comments

Name ..... Signed .....

Appendix 2: Necropsy Report Template

Necropsy Report	PM no/Ref no: Date:	SAMPLES			
***Person conducting PM*: Dr. AC Brüns**		FORMALIN		FROZEN	
Time of PM:		normal	lesion	normal	lesion
Animal information Date of death: Euthanasia: Species: Banded Mongoose Gender: Age: Condition score Weight: ID/other identification:					
History / Capture Location					
Interim ***Stage of decomposition/ pm signs:***					
***External* (parasites)** Natural body openings **PLANUM NASI** Abdomen **SKIN** Mucous membranes Subcutis Blood Bone marrow			0		0
***Abdominal cavity*/wall** Mesentery ***Thoracic cavity*/wall** Diaphragm		**0**			
LYMPHOID SYSTEM **Lymph nodes (specify)** **HEAD LN**					
Mandibular		0	0	**0**	0
Retropharyngeal		0	0	**0**	0
Parotideal		0	0	**0**	0
PERIPHERAL LN					
Axillary		0	0	**0**	0
Superf. Cerv.		0	0	**0**	0
Inguinal		0	0	**0**	0
Poplital		0	0	**0**	0
THORACAL LN					
Mediastinal		0	0	**0**	0
Sternal		0	0	**0**	0
Tracheal		0	0	**0**	0
Bronchial		0	0	**0**	0
ABDOMINAL LN					
Mesenterial		0	0	**0**	0
Gastric		0	0	**0**	0
Hepatic		0	0	**0**	0
Renal		0	0	**0**	0
Spleen		**0**	0	0	0
Thymus		0	0	0	0

NOTE – TAKE IMPRESSION SMEARS OF LESIONS/ enlarged Lnn take ½ Formalin ½ Frozen				
Salivary glands				
Endocrine glands				
Adrenal glands	**0**	0	0	0
Thyroid	0	0	0	0
Parathyroid	0	0	0	0
Hypophysis	0	0	0	0
Respiratory system				
Nasal cavity/ Sinuses/**Septum**				
Larynx				
Trachea				
Lungs	**0**	0	0	0
Cardiovascular system				
Heart	**0**	0	0	0
Blood vessels				
Liver	**0**	0	0	0
Gall bladder				
Pancreas	**0**	0	0	0
Gastro-intestinal system				
Oral cavity/ **Palate**	0	0	0	0
Teeth				
Tongue				
Pharynx				
Oesophagus (SPIROCERCA?)	0	0	0	0
Stomach	0	0	0	0
Fore-stomachs	0	0	0	0
Small intestine	**0**	0	0	0
Large intestine	**0**	0	0	0
Kidneys	**0**	0	0	0
Urinary bladder	0	0	0	0
Genital organs				
ovaries/testes	**0**	0	0	0
Uterus	**0**	0	0	0
Mammary Gland	**0**	0	0	0
Nervous system				
Eye	0	0	0	0
Ear	0	0	0	0
CNS	0	0	0	0
Spinal cord	0	0	0	0
Peripheral nervous system				
Autonomic system				
Musculo-skeletal system				
Muscle	**0**	0	0	0
Bones				
Joints				
Other				
Morphological diagnoses (main/incidental)				
Aetiological/provisional diagnosis				
Comments/Discussion				
Name of Veterinarian				
Signature				

Appendix 3: Culture results per individual banded mongoose and sample type, with colour coded troop association

ID	PM	NTM path.	NTM non-path.	Tracheal lavage	Tracheal swab	Faecal swab	Lymph nodes	other
A51	no		1	neg	neg	*M. moriokaense*		
A1	no		1		neg	*M. phlei*		
A50	no		1	neg	neg	*M. phlei*		
A73	no		1	*M. hassiacum*	neg	neg		
A46	no		1	neg	*M. elephantis* group	neg		
A49	no		2	neg	*M. hassiacum*	*M. phlei*		
A33	no		2	*M. nonchromo-genicum*	*M. nonchromo-genicum*	neg/ neg		
A7	no	1	1	neg	***M. terrae* group**	*M. nonchromo-genicum*		
A78	no	2	1	***M. avium* complex**	*M. nonchromo-genicum*	***M. terrae***		
A67	yes	1	2	neg	neg	neg	*M. nonchromogenicum* (h), ***M. terrae* (p),** neg (a,t)	*M. nonchromoge-nicum* (amniotic fluid)
A32	no	1	2	*M. elephantis* group/ *M. smegmatis*	*M. senuense*-like	***M. chelonae-abscessus*** group		
A53	no	1		*M. paraffinicum*-***M.scrofulaceum***/ neg	neg	neg/ neg		
A10	no	1		neg	neg	***M. asiaticum*/** neg		
A14	no	1		neg	neg	***M. asiaticum*-like**		
A55	no	1		neg	neg	***M. simiae* group**		

grey back ground for banded mongooses sampled post mortem; more than one result per cell equals multiple samples
lymph nodes: a - abdominal, h - head, p - peripheral, t - thoracal, rp – retropharyngeal; red: individuals from staff village, blue: individuals from VWS offices; green: individuals from living quarters

ID	PM	NTM path.	NTM non-path.	Tracheal lavage	Tracheal swab	Faecal swab	Lymph nodes	other
A9	no	1		neg	neg	***M. avium complex***		
A38	no	1		neg	neg	***M. avium complex***		
A59	no	1		neg	neg	***M. intracellulare***		
11/844	yes	1					***M. intracellulare* (p),** neg (a,h,t)	
A28	no	1		***M. intracellulare***	neg	neg		
A24	yes	1		neg	neg	***M. fortuitum***	***M. intracellulare* (t),** neg (a,h,p)	neg (soft palate, spleen)
A5	yes	1		neg	neg	***M. fortuitum* group/** neg	neg/ neg (a), neg (h,p,t)	neg (spleen)
A2	no	1			neg	***M. fortuitum* group**		
A6	no	1			neg	***M. fortuitum* group**		
A8	no	1			neg	***M. fortuitum* group**		
A11	no	1		neg	neg	***M. fortuitum* group/** neg		
A23	no	1		neg	neg	***M. fortuitum***		
A68	no	1		neg	neg	***M. fortuitum* group**		
A16	no	1		neg	***M. fortuitum***	neg		
A39	no	1		***M. fortuitum***	neg	neg		
A26	no	1		***M. terrae*** group	neg	neg		
A44	no	1		neg	neg	***M. terrae***		
A61	no	1		neg/ neg	neg/ neg	***M. terrae* group**		

grey back ground for banded mongooses sampled post mortem; more than one result per cell equals multiple samples
lymph nodes: a - abdominal, h - head, p - peripheral, t - thoracal, rp – retropharyngeal; red: individuals from staff village, blue: individuals from VWS offices; green: individuals from living quarters

ID	PM	NTM path.	NTM non-path.	Tracheal lavage	Tracheal swab	Faecal swab	Lymph nodes	other
A48	no	1		neg	neg	***M. chelonae-abscessus* group**		
A77	yes	2		neg	neg	neg	***M. simiae* (a), *M. intracellulare* (h),** neg (p,t)	neg (liver, lung, kidney)
A42	no	2		neg	***M. fortuitum***	***M. intracellulare***		
A19	no	2		neg	***M. fortuitum***	***M. fortuitum* group**		
A47	no	2		neg	***M. fortuitum* group**	***M. fortuitum***		
A54	no	3		***M. avium complex***	***M. simiae***	***M. simiae* group**		
A30	no	3		***M. avium* complex**	***M. intracellulare*-like**	***M. fortuitum* group**		
A35	no	3		***M. terrae***	***M. terrae* group/ *M. terrae***	***M. fortuitum* group**		
11/639	yes	4					***M. simiae* group (a,h), *M. intracellulare* (t),** neg (p)	***M. avium* complex** (spleen), neg (liver, lung, kidney)
A72	yes	5		neg	neg	neg	***M. szulgai* (a), *M. simiae* group (a), *M. intracellulare* (h), *M. simiae* (p), *M.simiae* group (t),** neg/ neg/ neg (p)	neg (liver, lung, spleen, brain, duodenum)

grey back ground for banded mongooses sampled post mortem; more than one result per cell equals multiple samples
lymph nodes: a - abdominal, h - head, p - peripheral, t - thoracal, rp – retropharyngeal; red: individuals from staff village, blue: individuals from VWS offices; green: individuals from living quarters

ID	PM	NTM path.	NTM non-path.	Tracheal lavage	Tracheal swab	Faecal swab	Lymph nodes	other
A52	yes	6		***M. avium* complex**	***M. simiae* group**	neg	***M. simiae* group (a,h),** ***M. chelonae-abscessus* group (p),** neg (p,t)	***M. simiae* group** (lung), neg (liver, spleen)
A63	yes	7		***M. asiaticum***	***M. asiaticum***	neg	***M. intracellulare* (a,p,t), *M. simiae group* (h), *M. avium* complex (p),** neg (h,p)	neg (liver, lung, spleen, jejunum)
A29	yes	7		neg	***M. fortuitum* group**	***M. heckeshorn-ense*-like/ *M. fortuitum*/ *M. fortuitum* group**	***M. avium*(a),** ***M. heckeshornense*(h)** ***M. simiae* group (p,t)**	***M. terrae* group** (soft palate), neg (liver, lung, spleen)
A57	yes	2		***M. bovis***	neg	neg	***M. bovis* (a,p),** ***M. intracellulare* (h,h,p)** ***M.parascrofulaceum* (h,p),** ***M. simiae* (h),** neg (p,t)	***M. bovis*** (liver, lung), neg (spleen)
A56	yes	4		***M. bovis***	***M. bovis***	***M. fortuitum* group**	***M. bovis* (a,rp),** ***M. simiae* group (h),** ***M. szulgai* (p), *M. avium* complex (t),** neg (p,p)	neg (liver, lung, spleen)

neg for tracheal lavage, tracheal swab and faecal sample:
A3, A4, A12, A13, A15, A22, A25, A27, A31, A34, A36, A37, A40, A41, A43, A45, A58, A60, A62, A64, A65, A66, A69, A70, A71, A74, A75 and A76
with no tracheal lavage cultured for A3, A4, A13 and A40, no tracheal swab submitted for A3 and A13 and no faecal swab submitted for A15

grey back ground for banded mongooses sampled post mortem; more than one result per cell equals multiple samples
lymph nodes: a - abdominal, h - head, p - peripheral, t - thoracal, rp – retropharyngeal; red: individuals from staff village, blue: individuals from VWS offices; green: individuals from living quarters

Appendix 4: STAT-PAK, DPP and ENFERPLEX results per study animal

ID	STAT-PAK	STAT-PAK	DPP	DPP 1	DPP 2	Enfer-plex	MPB70 peptide	ESAT-6	Rv3616c	MPB83	CFP-10	MPB70
11/844	neg											
11/639												
A1	**pos**	++	pos	++	neg	neg						
A2	**pos**	(+)	pos	+	+	neg						
A3	neg					neg						
A4	neg					neg						
A5	pos	+	pos	(+)	(+)	neg						
A6	neg					neg						
A7	neg					neg						
A8	pos	+	pos	+++	+	neg						
A9	neg					neg						
A10	neg					neg						
A11	neg					neg						
A12	neg					neg						
A13	neg					neg						
A14	neg					neg						
A15	neg					neg						
A16	neg					neg						
A19	neg					neg						
A22	**pos**	(+)	pos	++	neg	neg						
A23	neg					neg						
A24	**pos**	+	pos	++	(+)	neg						
A25	neg					neg						
A26	neg					neg						
A27	neg					neg						
A28	neg					neg						
A29	**pos**	+	pos	++	+	neg						
A30	neg					neg						
A31	**pos**	(+)	pos	+	++	neg						
A32	neg					neg						
A33	neg					neg						
A34	neg					neg						
A35	neg					neg						
A36	neg					neg						
A37	neg					neg						
A38	neg					neg						
A39	neg					neg						
A40	neg					neg						
A41	neg					neg						
A42	neg					neg						
A43	neg					neg						
A44	neg					neg						
A45	neg					neg						
A46	neg					neg						
A47	neg					neg						
A48	neg					neg						
A49	neg					**pos**				+		
A50	neg					neg						
A51	neg					neg						

ID: study animal number; DPP1: MPB83 antigen; DPP 2: CFP10/ESAT-6 fusion protein; (+) very weak positive; +: weak positive; ++: strong positive; +++: very strong positive

ID	STAT-PAK	STAT-PAK	DPP	DPP 1	DPP 2	Enfer-plex	MPB70 peptide	ESAT-6	Rv3616c	MPB83	CFP-10	MPB70
A52	**pos**	(+)	pos	++	++	neg						
A53	neg					neg						
A54	neg					neg						
A55	neg					neg						
A56	**pos**	+++	pos	+++	+	**pos**				+		
A57	**pos**	+++	pos	+++	++	**pos**				+		
A58	neg					neg						
A59	neg					neg						
A60	neg					neg						
A61	neg					neg						
A62	neg					neg						
A63	**pos**	(+)	pos	+	++	neg						
A64	neg					neg						
A65	neg					neg						
A66	neg					neg						
A67	neg					neg						
A68	neg					neg						
A69	neg					neg						
A70	neg					**pos**	+					
A71	neg					neg						
A72	neg					neg						
A73	neg					neg						
A74	neg					neg						
A75	neg					neg						
A76	neg					neg						
A77	neg					neg						
A78	neg					**pos**				+		

ID: study animal number; DPP1: MPB83 antigen; DPP 2: CFP10/ESAT-6 fusion protein; (+) very weak positive; +: weak positive; ++: strong positive; +++: very strong positive

Appendix 5: Age, gender and clinical findings of banded mongooses sampled for the study

ID	euthanasia	Age class	Sex	mass [g]	Body condition	dehydration [%]	injuries old	injuries fresh	fleas	Ticks	LN peripher	lungs prae lavage	lungs post lavage
11/844	yes	S	Female	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne
11/639	found dead	Y	Female	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne
A1	no	Y	Male	744	2.5	0	2	0	no	no	0	0	ne
A2	no	P1	Female	1217	4	0	0	0	no	no	0	0	ne
A3	no	J	Female		3	ne	ne	ne	ne	ne	ne	ne	ne
A4	no	P1	Male	1031	4	0	0	0	no	<10	1	0	ne
A5	yes	J	Female	537	3	0	0	0	yes	<10	1	0	ne
A6	no	J	Female	608	3	0	0	1	no	<10	0	1	ne
A7	no	J	Male	742	3.5	0	1	1	no	<5	1	ne	ne
A8	no	P2	Male	1064	3	0	1	0	yes	<50	1	1	ne
A9	no	Y	Male	1012	3.5	0	0	0	yes	<5	0	1	ne
A10	no	Y	Male	1155	4	0	0	1	no	<5	0	0	ne
A11	no	J	Male	456	3	0	0	0	no	<100	1	ne	ne
A12	no	P3	Male	1354	4	0	1	1	no	<5	0	ne	3
A13	no	P1	Female	1150	3	0	0	0	no	no	0	0	ne
A14	no	P3	Male	1058	3	0	1	0	no	<10	0	3	ne
A15	no	P2	Male	1098	3	0	0	0	no	<5	0	0	ne
A16	no	J	Male	718	3	0	0	0	no	<5	0	0	ne
A19	no	J	Male	692	3	0	0	0	no	<5	0	0	ne
A22	no	Y	Female	896	3	0	0	1	no	<5	1	1	ne
A23	no	J	Male	422	3	0	0	0	no	<5	0	0	ne
A24	yes	P1	Male	1024	4	0	0	0	yes	<10	0	ne	3
A25	no	J	Male	513	3	0	0	1	yes	<10	0	ne	ne
A26	no	J	Female	446	3	0	0	0	yes	<10	0	ne	ne
A27	no	J	Female	387	2.5	0	0	1	yes	<10	0	ne	ne
A28	no	O	Female	990	3	5%	1	0	yes	<10	0	4	ne
A29	yes	O	Male	1189	3	0	1	1	no	<20	0	4	ne
A30	no	Y	Female	966	3	0	0	0	no	<10	0	3	ne
A31	no	Y	Male	1444	4	0	1	0	no	<10	0	ne	ne
A32	no	J	Female	685	3.5	0	0	2	yes	<5	0	ne	3

J: juvenile; S:subadult; Y: young adult; P1: prime adult I; P2: prime adult II; P3: prime adult III; O: old adult; ne: not evaluated; LN: lymph node

ID	euthanasia	Age class	Sex	mass [g]	Body condition	dehydration [%]	injuries old	injuries fresh	fleas	Ticks	LN peripher	lungs prae lavage	lungs post lavage
A33	no	J	Female	558	3	1%	0	1	yes	<5	1	ne	3
A34	no	P2	Male	1363	4	0	0	1	no	<5	0	1	ne
A35	no	J	Male	550	2.5	0	0	1	no	<5	1	0	ne
A36	no	P1	Female	1026	3.5	0	0	1	yes	<10	0	2	ne
A37	no	Y	Female	1026	4	0	0	0	no	<5	0	ne	ne
A38	no	Y	Male	1158	3.5	0	0	0	no	<5	0	0	ne
A39	no	P2	Male	1166	4	0	0	1	no	<5	0	0	ne
A40	no	J	Female	463	2.5	0	0	0	no	no	2	0	ne
A41	no	P2	Male	1230	3	0	1	2	no	<10	2	0	ne
A42	no	S	Male	901	3	0	0	2	yes	<20	1	0	ne
A43	no	J	Female	523	3	0	0	1	yes	<10	2	0	ne
A44	no	J	Male	501	3	0	0	1	yes	<10	0	2	ne
A45	no	J	Female	395	3	0	0	1	yes	<5	0	1	ne
A46	no	S	Male	706	3.5	2%	1	0	no	<5	1	1	ne
A47	no	P1	Male	1183	4	0	1	0	yes	<5	0	ne	ne
A48	no	P1	Male	1394	4	0	0	0	no	<5	0	ne	ne
A49	no	P1	Male	1245	4.5	0	0	0	no	<5	0	ne	ne
A50	no	Y	Male	1192	3.5	0	1	0	no	<10	1	1	ne
A51	no	J	Female	557	3	2%	2	1	yes	<5	0	2	ne
A52	yes	Y	Female	1277	4	0	0	0	yes	<100	-	ne	3
A53	no	J	Female	665	3.5	0			yes	<5	0	1	ne
A54	no	J	Male	681	3.5	0	0	0	yes	<10	0	1	ne
A55	no	J	Female	523	3	2%	0	0	no	<20	0	3	ne
A56	yes	Y	Female	1052	4	2%	0	1	no	<20	0	3	ne
A57	yes	P2	Female	1356	4	0	2	0	yes	<5	1	ne	3
A58	no	J	Male	739	3	0	0	1	yes	<5	1	ne	1
A59	no	J	Male	763	3	0	0	1	yes	<5	0	ne	0
A60	no	J	Female	700	3	0	0	1	yes	<5	0	3	ne
A61	no	S	Female	762	3	0	0	1	yes	<50	0	4	ne
A62	no	P3	Female	1381	4	0	1	0	no	<10	1	ne	0
A63	yes	P2	Male	1296	3.5-4	0	0	1	no	<100	1	1	ne
A64	no	S	Female	807	3.5	0	0	1	yes	<100	1	ne	3

J: juvenile; S:subadult; Y: young adult; P1: prime adult I; P2: prime adult II; P3: prime adult III; O: old adult; ne: not evaluated; LN: lymph node

ID	euthanasia	Age class	Sex	mass [g]	Body condition	dehydration [%]	injuries old	injuries fresh	fleas	Ticks	LN peripher	lungs prae lavage	lungs post lavage
A65	no	S	Male	706	3.5	0	0	1	yes	<10	1	1	ne
A66	no	P3	Male	1435	4	0	1	1	yes	<5	1	2	ne
A67	found dead	P3	Female	1254	4	0	0	1	yes	<5	1	ne	1
A68	no	S	Male	642	3	0	0	0	yes	<10	2	2	ne
A69	no	S	Male	1019	3.5	0	0	0	yes	<5	0	2	ne
A70	no	S	Female	953	3	0	0	1	yes	<10	0	3	ne
A71	no	P1	Female	1212	3.5	0	0	1	no	<5	1	3	ne
A72	yes	O	Female	840	1	10%	3	0	yes	<5	1	ne	3
A73	no	P1	Male	1048	3	0	0	1	no	<20	1	1	ne
A74	no	J	Female	476	3	0	0	2	yes	<20	2	4	ne
A75	no	S	Male	717	3	1%	0	1	yes	<5	1	2	ne
A76	no	J	Male	482	3	1%	1	1	yes	<10	0	2	ne
A77	found dead	P3	Female	1591	4.5	ne	0	0	yes	<50	0	ne	ne
A78	no	Y	Female	1056	3.5	0	0	0	yes	<10	1	0	ne

J: juvenile; S:subadult; Y: young adult; P1: prime adult I; P2: prime adult II; P3: prime adult III; O: old adult; ne: not evaluated; LN: lymph node

Appendix 6: Test Kit Instructions

ElephantTB STAT-PAK® Assay

CHEMBIO
DIAGNOSTIC SYSTEMS, INC.

CATALOG # 60-9680-0
5 Test Kit

3661 Horseblock Road
Medford, New York 11763
U.S. Veterinary License No. 645

Mycobacterium bovis – Mycobacterium tuberculosis
Antibody Test Kit
ElephantTB STAT-PAK® Assay

A Rapid Immunochromatographic Test for the Detection of Antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in Elephant Serum, Plasma or Whole Blood

FOR *IN VITRO* VETERINARY DIAGNOSTIC USE
READ INSTRUCTIONS FOR USE CAREFULLY BEFORE PERFORMING TEST

INTENDED USE

The ElephantTB STAT-PAK Assay is a qualitative, single use, two-step, immunochromatographic screening test for the detection of antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in serum, plasma or whole blood from African elephants (*Loxodonta africana)* and Asian elephants (*Elephas maximus)*. The test is used as an aid in the diagnosis of active tuberculosis (TB) in conjunction with other diagnostic methods.

If specific antibodies are present in the sample, the expected test result is reactive. A reactive result is suggestive of active TB. In the absence of antibodies, the expected test result is nonreactive.

SUMMARY AND EXPLANATION

Tuberculosis (TB) in elephants is a re-emerging zoonotic disease caused primarily by *Mycobacterium tuberculosis* and, in some cases, by *Mycobacterium bovis*. The only USDA-recommended diagnostic test for TB in elephants is mycobacterial culture of trunk wash samples. However, there is a growing body of evidence indicating that this method has poor sensitivity, as it can only identify animals with extensive shedding of the organism that typically occurs late in the course of disease. Rapid detection of infected elephants is a crucial prerequisite for more effective control of TB, as early diagnosis allows timely initiation of chemotherapy [1-3].

Serological methods constitute an attractive alternative as they are simple, inexpensive, relatively non-invasive, and they do not depend on detection of mycobacteria [4-5]. None of the existing TB tests alone is sufficient to diagnose disease. Therefore, new TB diagnostic algorithms are being developed, in which serological assays may play an important role (see PERFORMANCE CHARACTERISTICS below).

The Chembio ElephantTB STAT-PAK Assay is a rapid immunochromatographic test for antibody detection that is safe, simple, and easy to perform.

PRINCIPLE OF TEST

The Chembio ElephantTB STAT-PAK Assay is based on immunochromatographic (lateral-flow) technology. The test employs a unique cocktail of recombinant *M. tuberculosis* proteins that are bound to the membrane solid phase. Blue latex particles conjugated with protein are used as the detection system. The ElephantTB STAT-PAK Assay can be used with serum, plasma or whole blood. Once a test sample is applied to the SAMPLE (S) well followed by the addition of a diluent, it flows laterally through the membrane strip. When it reaches the conjugate pad, antibodies, if present, bind to protein-latex conjugate and then the migrating immune complex binds to the antigens on the solid phase in the TEST (T) area producing a blue line. In the absence of antibodies there is no line in the TEST (T) area. The sample continues to migrate along the membrane and produces a blue line in the CONTROL (C) area demonstrating that the reagents are functioning properly.

MATERIALS PROVIDED

Each kit contains the following items:

- 5 ElephantTB STAT-PAK test devices
- 5 Disposable pipettes
- 1 Diluent vial (5mL)
- 1 Product insert

Additional Material Required But Not Provided

- Clock, watch or other timing device
- Disposable gloves
- Biohazard disposal container
- Collection devices for specimens

STORAGE AND STABILITY

The ElephantTB STAT-PAK Assay should be stored at 8 to 30°C in the original sealed pouch. The diluent should be stored in the original vial at 8 to 30°C. The kit is stable until the date printed on the box label and/or pouch.
NOTE: Do not use expired test kits.
CAUTION: Do not freeze test kits.

PRECAUTIONS

1. The test is designed FOR *IN VITRO* DIAGNOSTIC USE only. Use the test only in accordance with instructions supplied with the kit.
2. Handle all specimens as recommended for any potentially infectious serum or blood specimen in the CDC-NIH manual, *Biosafety in Microbiological and Biomedical Laboratories, 4th ed., 1999.*
3. Use suitable protective clothing (gloves, lab coat, safety glasses) when handling samples or test devices after samples have been applied. Avoid any contact between hands, eyes, nose or mouth during specimen collection and testing.
4. Do not pipette any material by mouth. Do not smoke, eat or drink in areas where specimens or kit material are kept.
5. All testing should be performed at a temperature of 18 to 30°C.
6. After the completion of the assay, carefully dispose of materials treating them as biohazardous waste.
7. Do not use expired test kits. Do not freeze test kits.
8. Do not mix reagents from different kit lots.

SPECIMEN COLLECTION

The ElephantTB STAT-PAK Assay can be performed on whole blood, serum or plasma.

Whole Blood: Collect whole blood into tubes containing heparin or EDTA. Be sure to thoroughly mix whole blood by inverting capped tube several times just prior to testing. Follow test procedure instructions.

Serum: Serum is used from whole blood collected aseptically by venipuncture into a clean tube without anticoagulant. Allow the blood to clot at room temperature, centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and separate the serum from the clot.

Plasma: Collect whole blood with anticoagulant (heparin, EDTA or sodium citrate), centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and isolate the plasma supernatant.

Samples perform best when tested immediately after collection. Specimens should be immediately refrigerated at 2 to 8°C following collection and can be used up to 3 days. If testing within 3 days is not possible, the specimens should be frozen at -20°C or colder until use. Avoid repeated freezing and thawing. DO NOT FREEZE WHOLE BLOOD.

NOTE: If specimens are to be shipped, they should be packed in compliance with regulations covering the transportation of etiologic agents. Venous whole blood, serum and plasma specimens should be shipped refrigerated with cold packs or wet ice.

TEST PROCEDURE

1. If test samples are refrigerated, remove them from the refrigerator and allow them to come to a temperature of 18 to 30°C before testing.
2. Remove the required number of ElephantTB STAT-PAK Assay devices from their pouches and place the devices on a flat surface area. It is not necessary to remove the desiccant from the package. **NOTE**: If desiccant packet is missing, DO NOT USE, discard the test device and a new test device should be used.
3. Label test units with sample names and/or identification numbers. (see Figure 1 below)

Figure 1

4. Using a disposable pipette, draw the specimen to be tested (whole blood, serum or plasma), into the pipette being careful not to draw up any air and add one full drop of specimen onto the center of the SAMPLE (S) well. (See Figure 2 below)

Figure 2

5. Once the specimen has been applied to the SAMPLE (S) well, remove the cap, invert the diluent bottle and hold it vertically (not at an angle) over the SAMPLE well. Add the diluent slowly dropwise; add **3 drops (~100 µl)** into SAMPLE (S) well. (See Figure 3)

Figure 3

6. Read results at **20 minutes** after the addition of diluent. **Do not read any results after 30 minutes.** Refer to INTERPRETATION OF RESULTS section below.
7. Discard the used disposable pipette, test device and any other test materials into a biohazard waste container.

QUALITY CONTROL

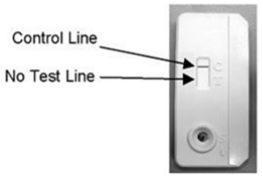
A blue colored line should always appear in CONTROL (C) area if the test has been performed correctly and the device is working properly. It serves as an internal test procedural control.

Good Laboratory Practice (GLP) recommends the use of control materials along with the test samples to ensure proper performance of the test kit. Positive and Negative serum or plasma based commercial controls should be used for this purpose. Use controls as per the TEST PROCEDURE instructions of this insert.

INTERPRETATION OF RESULTS

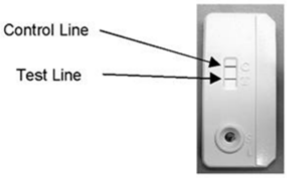
Nonreactive Result

One blue colored line in the CONTROL (C) area, with no visible colored line in the TEST (T) area indicates a nonreactive result. A nonreactive result at 20 minutes means that neither *Mycobacterium tuberculosis* nor *Mycobacterium bovis* antibodies were detected in the specimen. A nonreactive result does not preclude the possibility of TB infection.



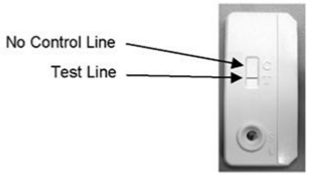
Reactive Result

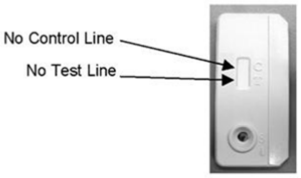
Two blue lines - one in the TEST (T) area and one in the CONTROL (C) area - indicate a reactive result. Intensities of the TEST and CONTROL lines may vary. Even a very faint line in the TEST (T) area of the device within 20 minutes is indicative of a reactive result. A reactive result means that *Mycobacterium tuberculosis* and/or *Mycobacterium bovis* antibodies were detected in the specimen



INVALID RESULTS

A blue line should always appear in the CONTROL (C) area, whether or not a line appears in the TEST (T) area. If there is no distinct blue line in the CONTROL (C) area, the test is invalid and should be repeated using a new device.





LIMITATIONS OF THE PROCEDURE

1. The assay is designed for detecting antibodies against *M. tuberculosis* and *M. bovis* only from elephant plasma, serum or whole blood. Any other body fluids or pooled samples or specimens from other than elephant species should not be used.
2. A reactive result suggests the presence of antibodies to *M. tuberculosis* and/or *M. bovis*.
3. For a reactive result, the intensity of the test line does not necessarily correlate with the titer of antibody in the specimen.
4. Reading nonreactive results earlier than 20 minutes or any results later than 30 minutes may yield erroneous results.
5. Do not use hemolyzed blood samples.
6. Blood specimens must be thoroughly mixed just prior to testing.
7. Be careful to add only 30 µL of specimen and 3 drops of diluent after applying the specimen to the SAMPLE (S) well.
8. Do not open the sealed test pouch until just prior to use.
9. Do not use kit contents beyond labeled expiration date.
10. Read results in a well-lit area.

PERFORMANCE CHARACTERISTICS

Highly specific and sensitive antibody binding proteins are used in the ElephantTB STAT-PAK Assay. The diagnostic performance was compared to the standard USDA-recommended method, trunk wash culture, and the ElephantTB STAT-PAK Assay was found to be superior.

Further, it was shown that both Asian and African elephants infected with either *M. tuberculosis* or *M. bovis* could be detected by ElephantTB STAT-PAK Assay up to several years prior to finding positive culture in trunk washes [3].

Sensitivity and Specificity

Sensitivity of the ElephantTB STAT-PAK Assay was determined by testing 23 culture positive elephants. Of these samples, 23/23 were reactive by the Chembio ElephantTB STAT-PAK antibody test kit (Table 1).
The specificity of the ElephantTB STAT-PAK Assay was determined by testing 131 serum, plasma, and/or whole blood samples. Of these samples 127/131 were non-reactive by the Chembio ElephantTB STAT-PAK antibody test kit (Table 2).

Table 1.
Diagnostic sensitivity of ElephantTB STAT-PAK Assay

Elephant	Mycobacterial species	ElephantTB STAT-PAK reactive
African	*M. tuberculosis*	3/3
African	*M. bovis*	1/1
Asian	*M. tuberculosis*	19/19

Table 2.
Specificity studies of ElephantTB STAT-PAK Assay

Elephant	ElephantTB STAT-PAK non-reactive	Trunk Lavage Culture negative
African	58/58	58/58
Asian	50/54	54/54
Unknown	19/19	19/19

REPRODUCIBILITY STUDIES

Reproducibility was tested at three independent laboratories using three serials of ElephantTB STAT-PAK Assay. A reference panel of 30 blinded samples representing negative, weakly reactive and reactive were tested 3 different times on 3 different days. The compiled results from 3 laboratories demonstrated 98.5% accuracy.

REFERENCES

1. Lewerin, S.S., Olsson, S-L., Eld, K., Röken, B., Ghebremichael, S., Koivula, T., Källenius, G., and Bölske, G. (2005) Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. *Vet. Rec.* 156: 171-175.
2. Montali, R.J., Mikota, S.K., and Chemg, L.I. (2001) *Mycobacterium tuberculosis* in in zoo and wildlife species. *Rev. sci. tech. Off. int.Epiz.* 20: 291-303.
3. Mikota, S.K., Peddie, L., Peddie, J., Isaza, R., Dunker, F., West, G., Lindsay, W., Larsen, R.S., Salman, M.D., Chatterjee, D., Payeur, J., Whipple, D., Thoen, C., Davis, D. S., Sedgwick, C., Montali, R.J., Ziccardi, M., and Maslow, J. (2001) Epidemiology and diagnosis *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). *J. Zoo Wildl. Med.* 32: 1-16.
4. Lyashchenko, K.P., et al., (2006) Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment. *Clinical And Vaccine Immunology* 13: 722-732.
5. Larsen, R.S., Salman, M.D., Mikota, S.K., Isaza, R., Montali, R.J., and Triantis, J. (2000) Evaluation of a Multiple-Antigen Enzyme-Linked Immunosorbent Assay for the Detection of *Mycobacterium Tuberculosis* Infection in Captive Elephants. *J. Zoo Wildl. Med.* 31: 291-302.
6. Lyashchenko K.P., Singh M., Colangeli R., and Gennaro M.L. (2000) A multi-antigen print immunoassay for the serological diagnosis of infectious diseases. *J. Immunol. Methods* 242: 91-100.

FOR MORE INFORMATION, CONTACT:

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ORDERING INFORMATION

Cat #	Product
60-9680-0	ElephantTB STAT-PAK® 5 Test Kit
60-9682-0	ElephantTB STAT-PAK® 20 Test Kit
60-9683-0	ElephantTB STAT-PAK® 50 Test Kit

DPP® VetTB Assay for Elephants

CHEMBIO
DIAGNOSTIC SYSTEMS, INC.

CATALOG # 65-9111-0
5 Test Kit

3661 Horseblock Road
Medford, New York 11763
U.S. Veterinary License No. 645

Mycobacterium bovis – Mycobacterium tuberculosis
Antibody Test Kit
DPP® VetTB Assay for Elephants

A Rapid Immunochromatographic Test for the Detection of Antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in Elephant Serum, Plasma or Whole Blood

FOR *IN VITRO* VETERINARY DIAGNOSTIC USE
READ INSTRUCTIONS FOR USE CAREFULLY BEFORE PERFORMING TEST

INTENDED USE

The DPP VetTB Assay is a single use immunochromatographic rapid test for the detection of antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in serum, plasma or whole blood from African elephants (*Loxodonta africana)* and Asian elephants (*Elephas maximus)*. The test is used as an aid in the diagnosis of active tuberculosis (TB) in conjunction with other diagnostic methods.

If specific antibodies are present in the sample, the expected test result is reactive. A reactive result is suggestive of active TB. In the absence of antibodies, the expected test result is nonreactive.

SUMMARY AND EXPLANATION

Tuberculosis (TB) in elephants is a re-emerging zoonotic disease caused primarily by *Mycobacterium tuberculosis* and, in some cases, by *Mycobacterium bovis* [1]. The only USDA-recommended definitive diagnostic test to detect TB in live elephants is mycobacterial culture of trunk wash samples. However, this method has poor diagnostic sensitivity, as it can only identify animals with extensive shedding of the organism that typically occurs late in the course of disease [2]. Rapid detection of infected elephants is a crucial prerequisite for more effective control of TB, as early diagnosis allows timely isolation and/or initiation of chemotherapy [1-3].

Serological methods constitute an attractive alternative as they are relatively simple, inexpensive, non-invasive, and they do not depend on detection of mycobacteria [3-4]. None of the existing TB tests alone is sufficient to diagnose disease. Therefore, new TB diagnostic algorithms are being developed, in which serological assays may play an important role [3-5] (see PERFORMANCE CHARACTERISTICS below).

The use of Chembio DPP VetTB Assay is an effective approach for rapid animal-side identification of elephants infected with *M. tuberculosis* or *M. bovis*, as this test is highly accurate, user-friendly, safe, and easy to perform.

PRINCIPLE OF TEST

The Chembio DPP VetTB Assay is based on immunochromatographic technology. The test employs two recombinant antigens, an *M. tuberculosis* and an *M. bovis* antigen, which are separately immobilized on the membrane solid phase. It also utilizes recombinant Protein A/G conjugated to colloidal gold particles for antibody detection. The DPP VetTB Assay uses serum, plasma or whole blood. The sample is applied to the SAMPLE+BUFFER well with the buffer. After the sample and buffer have migrated onto the test strip additional buffer is added to the BUFFER well. The buffer facilitates the lateral flow of the released products and promotes the binding of antibodies to the antigens. If present, the antibodies bind to the gold conjugated antibody binding protein. In a reactive sample, the dye conjugated-immune complex migrates on the nitrocellulose membrane and is captured by the antigens immobilized in the TEST (1 2) area producing a pink/purple line. In the absence of detectable antibody, no specific immune complex would be formed on the test line, and, therefore, no pink/purple line would appear in the TEST (1 2) area. Unbound conjugated gold particles continue to migrate along the membrane and produce a pink/purple line in the CONTROL (C) area. This procedural control serves to demonstrate that the reagents have been properly applied and have migrated through the device.

MATERIALS PROVIDED

Each kit contains the following items:

- 5 DPP VetTB test devices
- 1 DPP VetTB buffer vial (6mL)
- 1 Product insert

Additional Material Required But Not Provided

- Clock, watch or other timing device
- Disposable gloves
- Biohazard disposal container
- Collection devices for specimens
- Pipettor capable of delivering 5µL of sample

10-6294-1 Rev 2
November 2012

STORAGE AND STABILITY

The DPP VetTB Assay should be stored at 2 to 30°C in the original sealed pouch. The diluent should be stored in the original vial at 2 to 30°C. The kit is stable until the date printed on the box label and/or pouch.
NOTE: Do not use expired test kits.
CAUTION: Do not freeze test kits.

PRECAUTIONS

1. The test is designed FOR *IN VITRO* DIAGNOSTIC USE only. Use the test only in accordance with instructions supplied with the kit.
2. Handle all specimens as recommended for any potentially infectious serum or blood specimen in the CDC-NIH manual, *Biosafety in Microbiological and Biomedical Laboratories, 4th ed., 1999.*
3. Use suitable protective clothing (gloves, lab coat, safety glasses) when handling samples or test devices after samples have been applied. Avoid any contact between hands, eyes, nose or mouth during specimen collection and testing.
4. Do not pipette any material by mouth. Do not smoke, eat or drink in areas where specimens or kit material are kept.
5. All testing should be performed at a temperature of 18 to 30°C.
6. After the completion of the assay, carefully dispose of materials treating them as biohazardous waste.
7. Do not use expired test kits. Do not freeze test kits.
8. Do not mix reagents from different kit lots.

SPECIMEN COLLECTION

The DPP VetTB Assay can be performed on whole blood, serum or plasma.

Whole Blood: Collect whole blood into tubes containing heparin or EDTA. Be sure to thoroughly mix whole blood by inverting capped tube several times just prior to testing. Follow test procedure instructions.

Serum: Serum is obtained from whole blood collected aseptically by venipuncture into a clean tube without anticoagulant. Allow the blood to clot at room temperature, centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and separate the serum from the clot within 24 hours of blood collection..

Plasma: Collect whole blood with anticoagulant (heparin, EDTA or sodium citrate), centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and isolate the plasma supernatant.

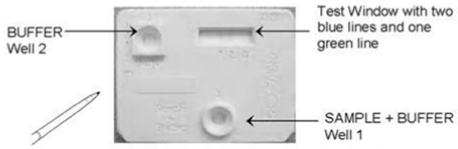
Samples perform best when tested immediately after collection. Specimens should be immediately refrigerated at 2 to 8°C following collection and can be used up to 3 days. If testing within 3 days is not possible, the specimens should be frozen at -20°C or colder until use. Avoid repeated freezing and thawing. DO NOT FREEZE WHOLE BLOOD.

NOTE: If specimens are to be shipped, they should be packed in compliance with regulations covering the transportation of etiologic agents. Venous whole blood, serum and plasma specimens should be shipped refrigerated with cold packs or wet ice.

TEST PROCEDURE

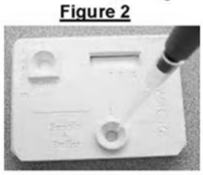
1. If test samples are refrigerated, remove them from the refrigerator and allow them to come to a temperature of 18 to 30°C before testing.
2. Remove the required number of DPP VetTB Assay devices from their pouches and place the devices on a flat surface area. It is not necessary to remove the desiccant from the package.
   NOTE: If desiccant packet is missing, DO NOT USE, discard the test device and a new test device should be used.
3. Label test units with sample names and/or identification numbers. (see Figure 1 below)

Figure 1



4. Using a 10µl disposable pipette (for whole blood) or a laboratory pipettor (for 5 µl of serum or plasma), release the specimen carefully to the center of the round SAMPLE+BUFFER Well 1. (See Figure 2 below)

Figure 2



5. Once the specimen has been applied to the SAMPLE+BUFFER Well 1, remove the cap, invert the buffer bottle, hold it vertically over the SAMPLE+BUFFER Well 1, and add **2 drops (~65 µl)** of the buffer slowly into SAMPLE+BUFFER well. (See Figure 3)

Figure 3

6. **Wait 5 minutes**, and then add 4 drops of the buffer to the square BUFFER Well 2. (See Figure 4 below.)
   NOTE: The blue and green colored lines should have disappeared from the rectangular TEST and CONTROL window. If not, discard the test device and repeat the procedure with a new DPP test device.

Figure 4

7. Read the test result 15 minutes after the addition of the buffer into the BUFFER Well 2. In some cases a test line may appear in less than 15 minutes; however, 15 minutes are needed to report a non-reactive result. **Do not read results after 25 minutes from addition of Sample+Buffer to Well 1**.
8. After reading and recording test results, discard the used test devices and any other test materials into a biohazard waste container.

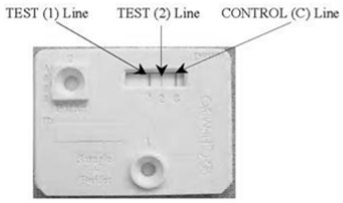
QUALITY CONTROL

A pink/purple colored line should always appear in CONTROL (C) area if the test has been performed correctly and the device is working properly. It serves as an internal test procedural control.

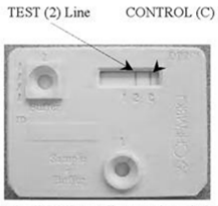
INTERPRETATION OF RESULTS

Reactive Result

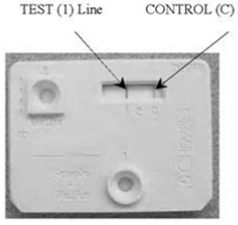
1. Three pink/purple lines, one line in the CONTROL area, one line in the TEST (1) area and one line in the TEST (2) area indicates a reactive result. This suggests that the sample is reactive for TB.



2. A pink/purple TEST (2) line and a pink/purple CONTROL line are visible. This suggests that the sample is reactive for TB.



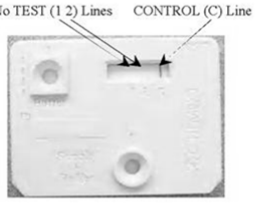
3. A pink/purple TEST (1) line and a pink/purple CONTROL line are visible. This suggests that the sample is reactive for TB or mycobacteriosis.



NOTE: Intensities of the TEST and CONTROL lines may vary. Test lines are considered reactive regardless of intensity.

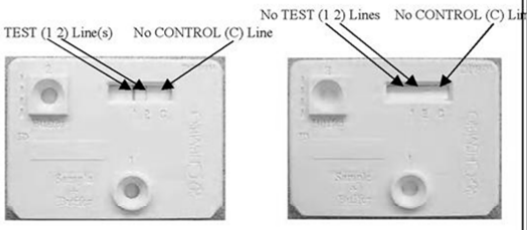
Nonreactive Result

Only a pink/purple CONTROL (C) line is visible. The sample contains no detectable antibody to both TB and mycobacteriosis antigens. A nonreactive result does not preclude the possibility of TB infection.



Invalid Result

A pink/purple line should always appear in the CONTROL (C) area, whether or not a line appears in the TEST area. If there is no distinct pink/purple line in the CONTROL (C) area, the test is invalid and should be repeated using a new device.



LIMITATIONS OF THE PROCEDURE

1. The Chembio DPP VetTB Assay is designed for detecting antibodies against *M. tuberculosis* and *M. bovis* only from elephant plasma, serum or whole blood. Any other body fluids or pooled samples or specimens from other than elephant species should not be used.
2. Test results must be read between 15-20 minutes after the addition of the buffer to the square BUFFER Well 2.
3. Do not use hemolyzed blood samples.
4. Blood specimens must be thoroughly mixed just prior to testing.
5. Do not open the sealed foil pouch until just prior to use.
6. Do not use kit contents beyond labeled expiration date.
7. Read results in a well-lit area.
8. A reactive result using the Chembio DPP VetTB Assay suggests the presence of antibodies to *M. tuberculosis* and/or *M. bovis*. The Chembio DPP VetTB Assay is intended as an aid in the diagnosis and treatment of TB in elephants.
9. For a reactive result, the intensity of the test line does not necessarily correlate with the titer of antibody in the specimen.
10. A non-reactive result does not preclude the possibility of exposure to TB or infection with TB. An antibody response to recent exposure may take several months to reach detectable levels.
11. In treated elephants, interpret results with caution:
    a. Treatment against TB may reduce antibody responses, thus resulting in non-reactive results in some cases.

b. A reactive result may persist in infected and treated elephants for months and years even if an elephant is considered cured.

PERFORMANCE CHARACTERISTICS

Highly specific and sensitive antibody binding antigens are used in the DPP VetTB Assay. The diagnostic performance was compared to the standard USDA-recommended method, trunk wash culture, and the DPP VetTB Assay diagnostic performance was found to be superior [4-5].

Further, it was shown that both Asian and African elephants infected with *M. tuberculosis* or *M. bovis* could be detected by DPP VetTB Assay up to several years prior to finding positive culture in trunk washes [4-5].

Sensitivity and Specificity

Sensitivity of the DPP VetTB Assay was determined by testing 40 culture positive elephants. All 40 samples were reactive (Table 1).

The specificity of the DPP VetTB Assay was determined by testing serum, plasma, and/or whole blood samples collected from 147 trunk-wash culture negative elephants without history of TB. All 147 samples were non-reactive (Table 2).

Table 1.
Diagnostic sensitivity of DPP VetTB Assay

Elephant species	Mycobacterial species isolated	DPP VetTB reactive
African	*M. tuberculosis*	6/6
African	*M. bovis*	1/1
Asian	*M. tuberculosis*	33/33

Table 2.
Specificity studies of DPP VetTB Assay

Elephant species	DPP VetTB Assay non-reactive	Trunk wash culture negative
African	79/79	79/79
Asian	68/68	68/68

REPRODUCIBILITY STUDIES

Reproducibility was evaluated at three independent laboratories using two serials of DPP VetTB Assay. A reference panel of 40 blindly-coded samples representing negative, weakly reactive and strongly reactive sera were tested 3 times on 3 different days. The compiled results from 3 laboratories demonstrated 98.6% accuracy.

REFERENCES

1. Mikota, S.K., Peddie, L., Peddie, J., Isaza, R., Dunker, F., West, G., Lindsay, W., Larsen, R.S., Salman, M.D., Chatterjee, D., Payeur, J., Whipple, D., Thoen, C., Davis, D. S., Sedgwick, C., Montali, R.J., Ziccardi, M., and Maslow, J. (2001) Epidemiology and diagnosis *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). *J. Zoo Wildl. Med.* 32: 1-16.
2. Lewerin, S.S., Olsson, S-L., Eld, K., Röken, B., Ghebremichael, S., Koivula, T., Källenius, G., and Bölske, G. (2005) Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. *Vet. Rec.* 156: 171-175.Lyashchenko, K.P., et al., (2006) Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical And Vaccine Immunology* 13: 722-732.
3. Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J.H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J.B., Harris, B., Sofranko, D., Waters, W.R., and Lyashchenko, K.P. (2009) Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clinical And Vaccine Immunology* 16:605-612.
4. Lyashchenko K.P., Greenwald R., Esfandiari J., Mikota S., Miller M., Moller T., Vogelnest L., Gairhe K.P., Robbe-Austerman S., Gai J., and Waters W.R. (2012) Field application of serodiagnostics to identify elephants with tuberculosis prior to case confirmation by culture. *Clin. Vaccine Immunol.* 19(8):1269-75.

FOR MORE INFORMATION, CONTACT:

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Fax: (631) 924-6033
Email: info@chembio.com
Web Site: www.chembio.com

ORDERING INFORMATION

Cat #	Product
65-9110-0	DPP® VetTB 2 Test Kit
65-9111-0	DPP® VetTB 5 Test Kit
65-9112-0	DPP® VetTB 20 Test Kit

Antigens licensed from
Statens Serum Institut
www.ssi.dk

Appendix 7: Project Approvals

To acquire and manage a system of national parks which represents the Indigenous wildlife, vegetation, landscapes and significant cultural assets of South Africa for the pride and benefit of the nation.

South African NATIONAL PARKS

23 February 2012

To Whom It May Concern

SANParks Animal Use and Care Committee: Confirmation of Approval of Research Project

This is to confirm that the SANParks AUCC approved the project: "Screening of small predators in the Kruger National Park for mycobacterial infection and disease: A pilot study in the banded mongoose (*Mungos mungo*)" by Dr Angela Brüns.

Sincerely,

Dr Peter Novellie
Convenor: SANParks AUCC

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ANIMAL USE AND CARE COMMITTEE
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0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: V085-11

24 April 2012

Prof A Michel
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(anita.michel@up.ac.za)

Dear Prof Michel

V085-11 : Screening of small predators in the Kruger National Park for mycobacterial infection and disease: A pilot study in the banded mongoose*(Mungos mungo) (AC Bruns)*

The application for ethical approval, dated 20 February 2012 was approved by the Animal Use and Committee at its meeting held on 23 April 2012.

You are however requested to submit a copy of DAFF, Section 20 approval for the project.

Kind regards

E Mostert

Elmarie Mostert

AUCC Coordinator

Copy Dr AC Burns

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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From: Directorate Animal Health
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Fax: 012 319 7470
E-mail: HerryG@daff.gov.za
Enquiries: Mr. Herry Gololo
Our Ref: 12/11/1/7/2
Your Ref No :

Dr. Angela Brüns
Faculty of Health Science, Stellenbosch University
PO Box 19063
Tygerberg
7505
Dear Dr. Brüns

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 14 May 2012 requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa.
2. Export of samples to France is subject to compliance with requirements set by the importing country.
3. Importation of the STAT-PAK is subject to obtaining a veterinary import permit.
4. Laboratory diagnostics may only be conducted at the DAFF approved, Molecular Biology and Human Genetics Laboratory, Faculty of Health Science, University of Stellenbosch.

Title of research/study: Screening of small predators in the Kruger National Park for mycobacterial infection and disease: A pilot study in the banded mongoose *(Mungos mungo)*.
Researcher (s): Dr. A.C. Brüns
Institution: Stellenbosch University, Faculty of Health Science
Your Ref./ Project Number:
Our ref Number: 12/11/1/7/

Kind regards

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for **DIRECTOR: ANIMAL HEALTH**

DATE: 07-01-2013