First detection of *Mycobacterium bovis* Infection in Giraffe (*Giraffa camelopardalis*) in the Greater Kruger National Park Complex: Role and Implications

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Abstract

Bovine tuberculosis (bovine TB) caused by *Mycobacterium bovis* has become endemic in some wildlife populations in South Africa. The disease has been reported in 21 wildlife species in the country. In this study, we report *M. bovis* infection in two female giraffes (*Giraffa camelopardalis*) from two different nature reserves within the Greater Kruger National Park Complex (GKNPC). *M. bovis* was isolated from tissue lesions consistent with macroscopic appearance of tuberculosis (TB) and confirmed by polymerase chain reactions (PCR), targeting the RD4 region of difference on the genome of the isolates. Spoligotyping and variable number of tandem repeat (VNTR) typing revealed infection of one giraffe with a strain (SB0294) previously not detected in South Africa, while a resident *M. bovis* strain (SB0121) was detected from the other giraffe. Our work is first to report *M. bovis* infection in free ranging giraffes in South Africa. We have further demonstrated the existence of at least three genetically unrelated strains currently infecting wildlife species within the GKNPC. This finding suggests that the epidemiological situation of *M. bovis* within the GKNPC is not only driven by internal sources from its established endemic presence, but can be additionally fuelled by strains introduced from external sources. It further emphasizes that regular wildlife disease surveillance is an essential prerequisite for the timely identification of new pathogens or strains in ecospheres of high conservation value.

Keywords: Bovine tuberculosis, genotyping, giraffe (*Giraffa camelopardalis*), Greater Kruger National Park Complex, *Mycobacterium bovis*; wildlife

1 Introduction

The *Mycobacterium tuberculosis* complex (MTBC) consists of closely related organisms including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG (vaccine strain), *Mycobacterium microti, Mycobacterium canettii, Mycobacterium pinnipedii, Mycobacterium africanum* (of various subtypes and variants), *Mycobacterium caprae*, the rare dassie bacillus, *Mycobacterium orygis*, *Mycobacterium mungi* as well as *Mycobacterium suricattae* (Brosch et al. 2002; Mostowy et al. 2005; Parsons et al. 2013). Among members of the complex, *M. bovis* is known to have the widest host spectrum, infecting domesticated and undomesticated animals as well as humans (Brosch et al. 2002; Mostowy et al. 2005). Tuberculosis in free ranging wildlife is mainly caused by *M. bovis*. The disease has been reported in 21 wildlife species in the country (Michel et al. 2015; Hlokwe et al. 2014). In South Africa, African buffaloes (*Syncerus caffer*) have become wildlife maintenance hosts (De Vos et al. 2001), with greater kudu (*Tragelaphus strepsiceros*), lion (*Panthera leo*) and warthog (*Phacochoerus africanus*) showing reservoir potential (Michel et al. 2009). Wildlife species have been implicated as having a potential role to play in transmitting diseases to livestock (Michel and Bengis, 2012). In an earlier study, evidence of wildlife acting as the apparent source for *M. bovis* infection to neighbouring cattle was demonstrated (Musoke et. al. 2015). The translocation of infected wildlife in the absence of a suitable surveillance program was identified as one of the risk factors for the transmission of bovine TB amongst different wildlife reserves and farms (Hlokwe et al 2016). Evidence of further spill over of bovine TB from African buffaloes to a wide spectrum of wildlife species has long been reported within the Greater Kruger National Park Complex (GKNPC) (Michel et al. 2009; Hlokwe et al. 2014; Michel et al. 2015). The GKNPC consists of the KNP (Kruger National Park) and the adjoining nature reserves situated on its western border, where game fences have been removed to achieve enhanced free movements of wild animals and a larger connected habitat, which is

beneficial for nature conservation. It did facilitate more inter- and intra-species contact possibilities and as such, it has increased the potential for further transmission of diseases such as bovine TB.

In this study, we report the first described cases of bovine TB in free ranging (wild) giraffes (*Giraffe camelopardalis*) from the GKNPC and in South Africa, as well the pathological findings of the cases on macro- and microscopic levels. To investigate the disease epidemiology, we conducted spoligotyping and variable number of tandem repeat (VNTR) genotyping of *M. bovis* isolated from the giraffe. In one of the cases, results revealed detection of a genotype of *M. bovis* previously neither reported in the GKNPC nor in the country. These findings indicate not only the spread of bovine TB to giraffes as a new infected species within the GKNPC, but also a possible introduction of a new bovine TB strain within this ecosphere.

2 Materials and methods

2.1 Study area

The State Veterinary Office Bushbuckridge East (Orpen), of the Mpumalanga Veterinary Services under the DARDLEA (Department of Agriculture, Rural Development, Land and Environmental Affairs), encompasses private and provincial nature reserves to the west of the KNP and in the north-east of the Mpumalanga Province of South Africa. A number of these nature reserves and the KNP removed their perimeter fence lines in 1993 to form the GKNPC. Due to the natural occurrence of the Southern-African Territories serotypes of Foot-and Mouth Disease in the resident population of free-ranging African buffaloes, the area has animal disease control status, a dense network of veterinary services, as well as regular and extensive animal inspections and disease surveillance in the livestock farming communal areas and the neighbouring nature conservation areas. Land usage in the adjoining nature reserves is largely

for ecotourism with relatively higher intensity in some area, which can provide valuable reports on suspect or observed disease incidents in wildlife.

2.2 Animal history

The two cases presented in this report occurred in 2013 (case 1) and 2014 (case 2) in two nature reserves within the GKNPC. Both were reported by game viewers and field rangers via the reserve's managements to the local state veterinary services as part of routine disease surveillance and investigation in wildlife in disease-controlled area. The moderately fresh carcass of an adult female giraffe from one of the nature reserves was reported during midday in August 2013 and a field post mortem was subsequently performed in the afternoon. In May 2014, a carcass of an adult female giraffe, approximately two days after death, was reported in another adjoining nature reserve within the GKNPC. No further information or relevant observations of the animals prior to its death were available.

2.3 Necropsy examination

The post-mortem examination of the giraffe case from 2013 was undertaken about 12 hrs after the death of the animal. The carcass age of case 2 was about two days, and advanced autolysis was present. Post-mortem examinations in both cases were performed in the field, without the carcasses being moved or re-positioned. They were carried out by veterinary personnel of the local State Veterinary Office: Orpen BBR East (state veterinarian and animal health technician). In both cases, selected tissue samples were frozen and fixed in 10% formalin.

2.4 Histopathology examination

Routine processing and staining of formalin-fixed tissues from both cases was done at the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria according to standardized protocols. Selected lymph nodes such as head, bronchopulmonary,

sublumbar and mesenteric lymph nodes; lung and spleen (case 1) as well as a lung sample from case 2 were examined.

2.5 Tissue samples for *Mycobacterium* spp. culture

Nine tissue samples from the giraffe carcasses (case 1: n=8; case 2: n=1) with macroscopical findings indicative of TB, were received in the Tuberculosis laboratory of the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) for routine *Mycobacterium* spp. culture. These samples included a selection of different lymph nodes (head, prescapsular, retropharyngeal, lumbar and mammary), lung and kidney. They were kept on ice during transport to the Tuberculosis laboratory and stored at -20ºC until processing for *Mycobacterium* spp. isolation.

2.6 *Mycobacterium* spp. isolation

The tissue samples were processed at the Tuberculosis laboratory according to standard procedures (Hlokwe et al 2017). The samples were inoculated onto Löwenstein-Jensen (L-J) media supplemented with pyruvate and incubated at 37 °C for up to 10 weeks.

2.7 *Mycobacterium bovis* identification

Bacterial growth observed during weekly monitoring was subjected to Ziehl Neelsen (ZN) staining to check for acid-fast bacteria (AFB). DNA templates were prepared from acid-fast bacteria. Individual colonies or several small colonies were picked up from the L-J media and emulsified in 100 µl ultra-pure water. The suspension was heated at 100 °C for 25 min, then allowed to cool down at room temperature. DNA templates were subjected to polymerase chain reaction (PCR) amplification using primers that target a DNA sequence encoding the MPB 70 antigen to identify *Mycobacterium tuberculosis* complex bacteria (Michel *et al.* 2009; Hlokwe et al 2016). Deletion analysis was performed for species identification targeting the RD4 genomic region of difference (Warren *et al.* 2006).

2.8 Genotyping

2.8.1 Variable Number of Tandem Repeat (VNTR) typing

Polymerase chain reaction amplifications for VNTR typing of isolates were performed using a set of tandem repeat loci as described previously (Hlokwe et al. 2013; Le Fleche et al. 2002). These included Qub-11a, -11b, -18 and -26, MIRU 16, 23 and 26, Mtub 12 and 21 as well as ETR-A, -B, -C, and –E. *M. tuberculosis* H37Rv, *M. bovis* BCG and sterile distilled water were used as controls. VNTR typing PCR was performed in a 20µl reaction containing 2 µl of cell lysate (DNA template), 10 µl of the Qiagen master mix, 7 µl of DNA free water and 0.5 µl of each 20 pmol/ul primer. The cycling parameters were as follows: initial denaturation at 94 °C for 5 min, and 40 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, elongation at 72 °C for 1.5 min (40 cycles) and a final elongation step at 72 °C for 10 min. PCR was carried out using an Eppendorf AG 22331 Hamburg thermo cycler (Merck). The PCR products were separated on a 2 % agarose gel stained with 20 µl ethidium bromide (10 µg/ml) and electrophoresed at 80 V for 2 hours. A 100 bp ladder (Inqaba Biotechnical Industries) was included and used to estimate the sizes of the resulting PCR products. The band sizes were converted into number of tandem repeats at each locus based on the allele-naming table previously described (Le Fleche et al. 2002).

2.8.2 Spoligotyping

Spoligotyping of three of the nine isolates was done according to a standardized international method (Kamerbeek *et al.* 1997), using a commercially available kit (Ocimum Biosolutions, Indianapolis, IN, USA). *M. bovis* BCG and Ultra-pure water served as the test controls. The resulting spoligopatterns for the *M. bovis* isolates were compared to spoligopatterns found in the international *M. bovis* spoligotype database (www.mbovis.org).

3 Results

3.1 Necropsy findings

Large numbers of white to tan soft to firm discrete to coalescing nodules (0.5-5 cm in diameter) were present in the lung (case 1, Fig 1). The largest nodules were located in the cranial lobes, while miliary nodules were present in the caudal lobes. Bronchopulmonary, hepatic and lumbar lymph nodes, were variably enlarged, and firm, with an almost homogenous white parenchyma. The left prescapular lymph node was firm and contained one light tan nodule. (1 cm in diameter, Fig 2). The lateral and medial retropharyngeal lymph nodes were slightly enlarged and firm, and contained multiple granulomas (0.5- 1.5 cm in diameter, Fig 3). These described findings were consistent with death due to severe multisystemic fatal TB.

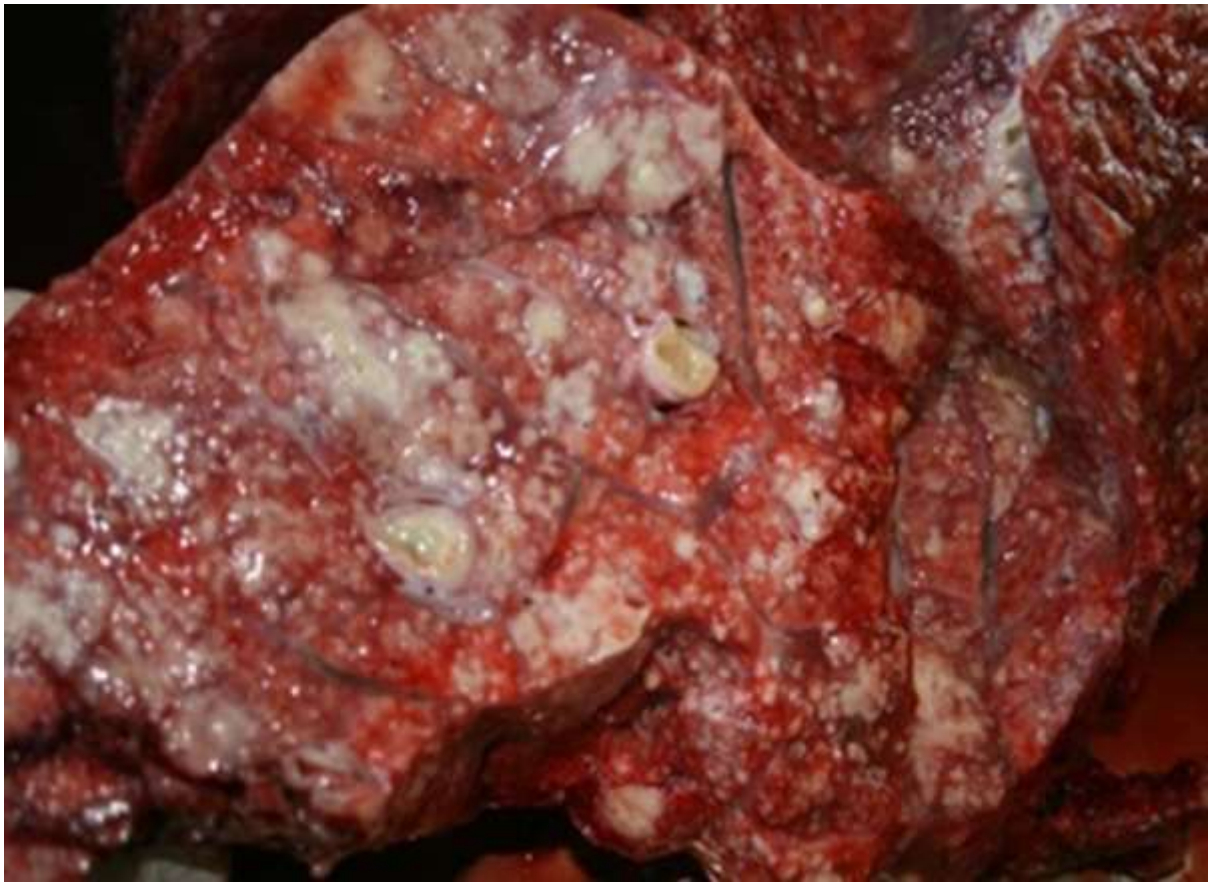

Figure 1. Disseminated discrete and coalescing TB granulomas in the lung from a giraffe (case 1)

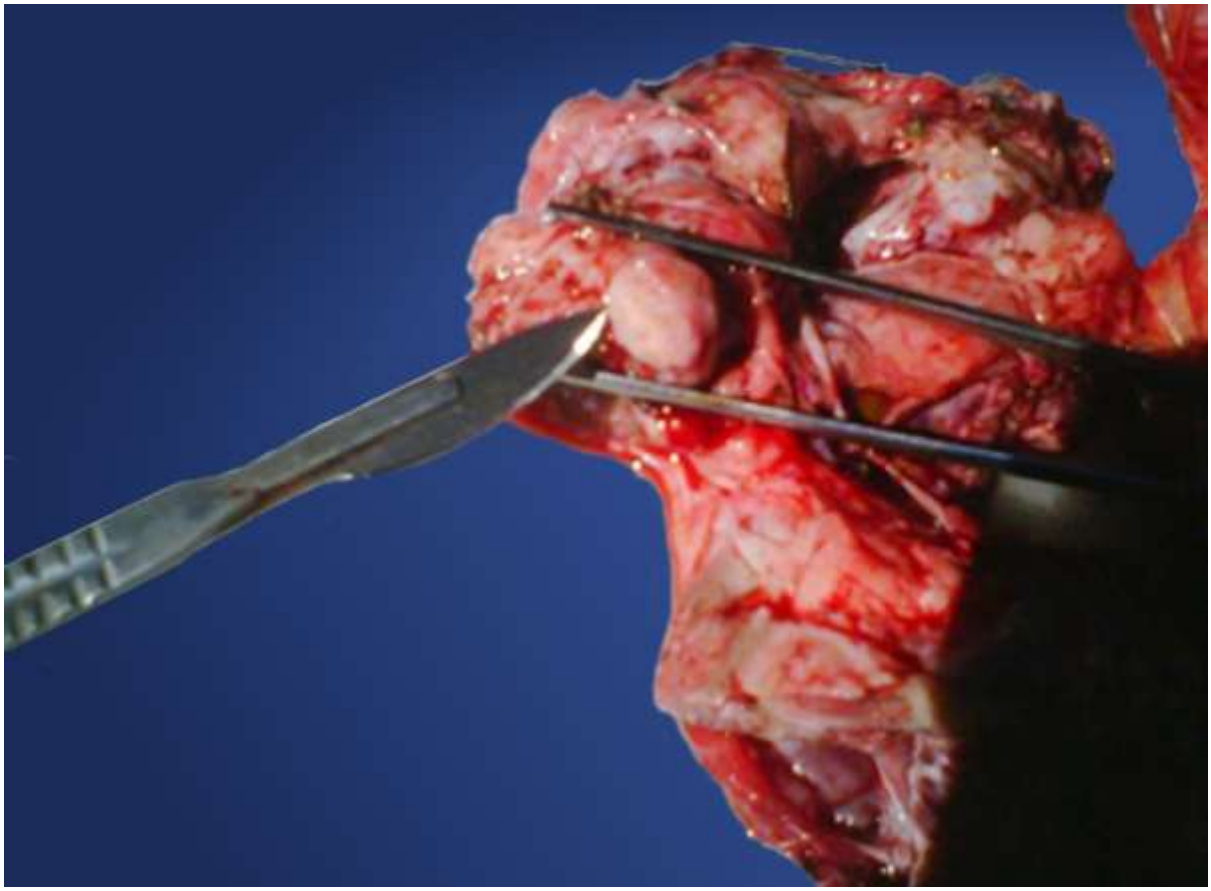

Figure 2. Single discrete TB granulomas in the left pre-scapular lymph nodes from a giraffe (case 1)

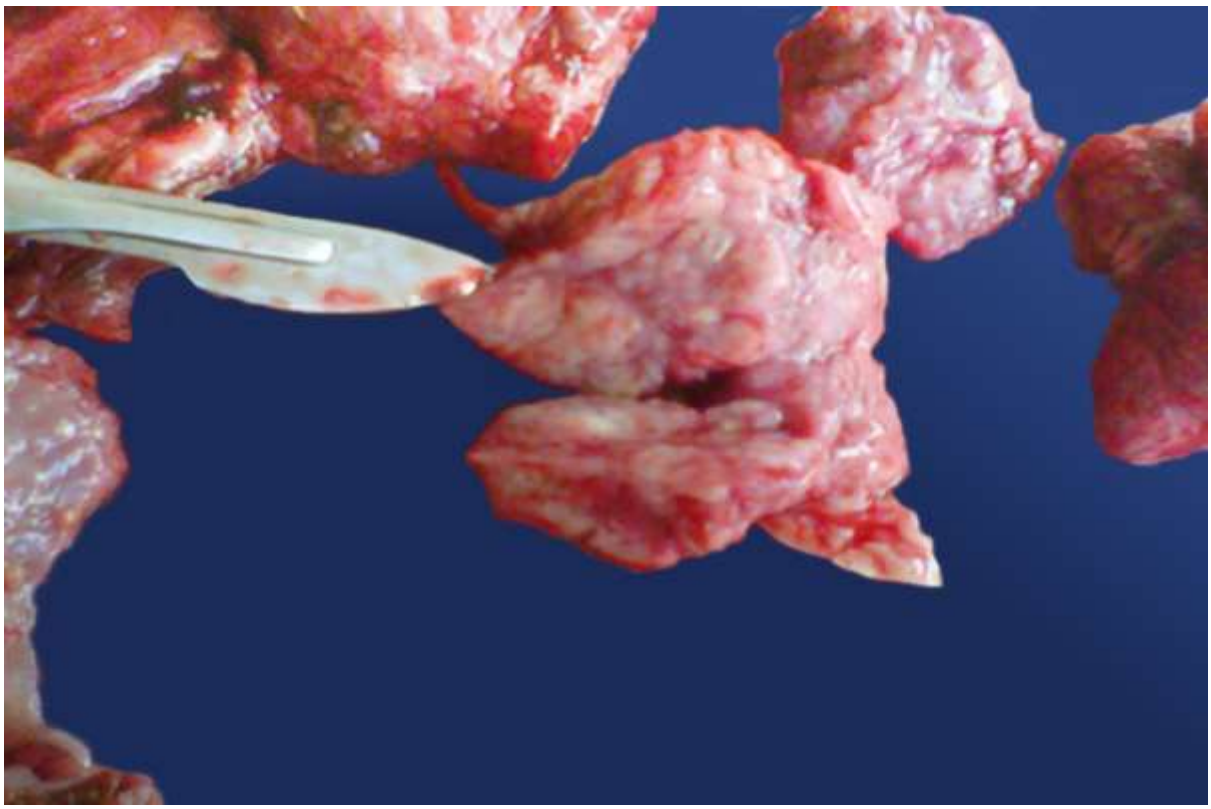

Figure 3. Multiple discrete and coalescing TB granulomas distort the cut surface of lateral retropharyngeal lymph nodes from a giraffe (case 1)

Only the lungs from case 2 were examined due to advanced autolysis and scavenging. They showed disseminated white to pale-yellow, firm, nodular lesions (Fig 4).

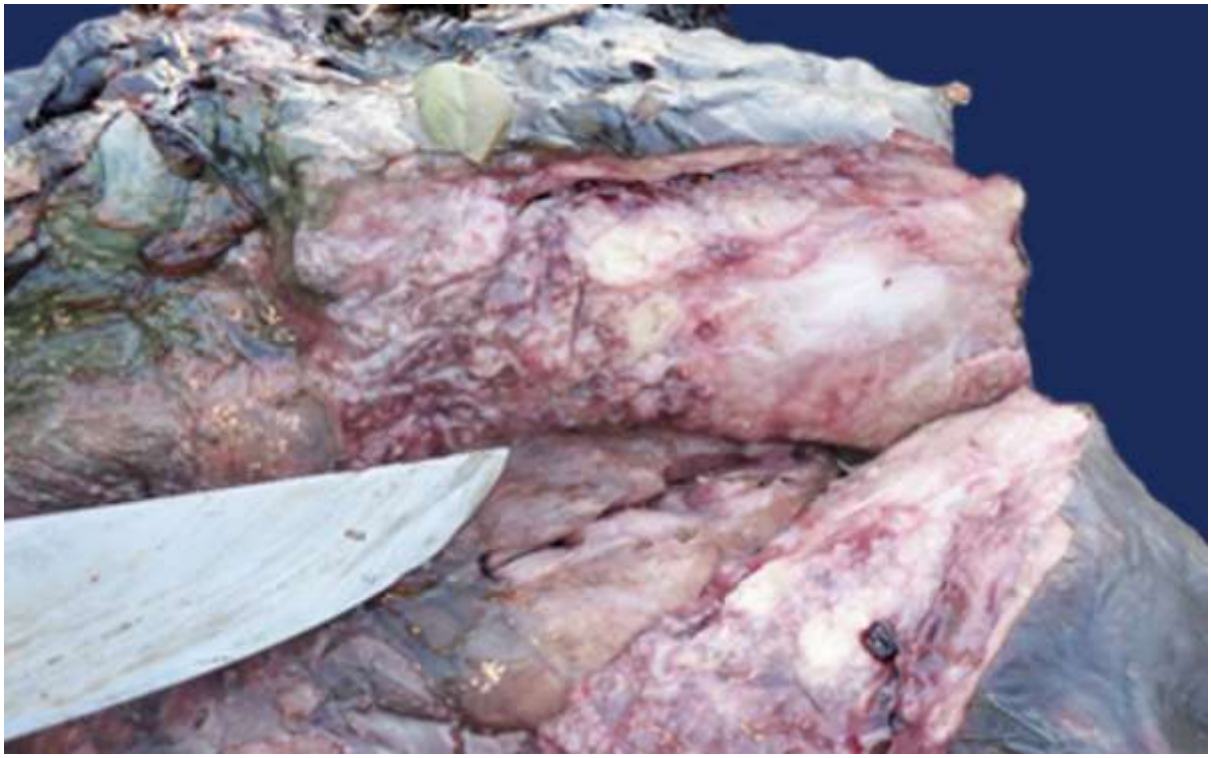

Figure 4. Disseminated bronchopulmonary TB with opened granuloma in autolysed lung tissue of carcass from giraffe (case 2)

3.2 Histopathology

The head, bronchopulmonary, sublumbar and mesenteric lymph nodes as well as lung and spleen of case 1 showed typical partially encapsulated coalescing necrogranulomas. Small numbers of acid-fast fine bacterial rods were present in macrophages and multinucleate giant cells of the head lymph nodes. Although tissue detail and identity were largely obscured by autolysis in case 2, multiple non-encapsulated necrogranulomas were present. Rare acid-fast fine bacterial rods were present mainly in multinucleate giant cells.

TABLE 1 Laboratory identification, sample type and genotyping data of *Mycobacterium bovis* isolated from two giraffes (*Giraffa camelopardalis*) originating within the Greater Kruger National Park Complex (GKNPC)

Giraffe identification/ Year of isolation[a]	Laboratory number	Sample type	Spoligopattern	VNTR profile												
				Qub 11a	Qub 11b	Qub 18	Qub 26	Miru 16	Miru 23	Miru 26	Mtub 12	Mtub 21	ETR-A	ETR-B	ETR-C	ETR-E
OR130803G/ 2013[a]/Case 1	TB 8289 A	Head LN[b]	SB0294	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 B	Lung[b]	SB0294	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 C	Retropharyngeal LN[b]	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 D	Kidney[b]	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 E	Lumbar LN[b]	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 F	Pre-scapular LN	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 G	Pre-scapular LN[b]	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
	TB 8289 H	Mammary LN[b]	N/D	8	2	4	4	3	4	5	4	2	5	3	3	4
OPR140506G/ 2014[a]/Case 2	TB 8409	Lung[b]	SB0121	8	2	4	4	3	4	5	4	2	6	4	3	4

Note: Abbreviations: N/D, not done; VNTR, variable number of tandem repeat.

[a]Year of isolation.

[b]*Mycobacterium bovis* was isolated.

3.3 *Mycobacterium* spp. isolation and identification of *Mycobacterium bovis*

Dysgonic colony shaped bacterial growth was observed on Löwenstein-Jensen media supplemented with pyruvate. Culture results and the tissue samples from which *M. bovis* was isolated are summarised in table 1. Acid-fast bacilli were identified following Ziehl Neelsen staining and microscopic examinations of the bacterial smears. *M. bovis* was confirmed by deletion analysis PCR (amplification of a 268 bp product) as previously described (Warren et al 2006).

3.4 Genotyping

3.4.1 Variable number of tandem repeat (VNTR) typing

Variable number of tandem repeat (VNTR) typing based on a 13-locus panel revealed VNTR profiles 8244345425334 (case 1) and 8244345426434 (case 2) from giraffes sampled in 2013 and 2014, respectively (table 1).

3.4.2 Spoligotyping

Spoligotype pattern SB0294 was detected from *M. bovis* isolated from the giraffe sampled in 2013 (case 1). This pattern was identified by lack of spacers 3, 9, 12, 16, 21, 39-43 (www.mbovis.org). Spoligopattern SB0121 was detected from the giraffe sampled in 2014 (case 2). The spoligopattern was characterised by lack of spacers 3, 9, 16, 21, 39-43 (www.mbovis.org). Spoligopatterns detected are shown in table 1.

4 Discussion

There are few reports of TB in giraffes, both in captive and free ranging individuals (Fowler 1978; Goble 1994; Schliesser, 1978). In this study, *M. bovis* was detected in samples from two adult giraffes in the GKNPC. No other life-threatening lesions were present in the giraffe

sampled in 2013 (case 1), and *M. bovis* was isolated from the lung and kidney, as well as from the prescapsular, head, lumbar, retropharyngeal and mammary lymph nodes, therefore suggesting that disseminated tuberculosis was present and the giraffe may have died of the disease. Case 2 was too autolysed to determine the cause of death. However, given the extent of the lesions in the lungs, extensive bronchopulmonary TB could be attributed as the cause of death for this animal. Genotyping of isolates from the adult female giraffe sampled in 2013 (Table 1) revealed a VNTR profile (8244345425334) and a spoligopattern (SB0294) previously neither detected in the GKNPC nor in other known infected wildlife and livestock areas in South Africa. The resident *M. bovis* strain (SB0121) was detected from the lung sample with granulomas in case 2. This genotype is the parental *M. bovis* strain and single cause of the TB epidemic which underwent clonal expansion, with clonal variants identified by IS*6110* (Michel et al 2009) and VNTR genotyping (Hlokwe et al 2013). The identification of the SB0121 strain in the giraffe from 2014 is in line with the evidence of active spread of the parental strain and its clonal variants among different wildlife species and subpopulations in the GKNPC (Hlokwe et al 2014).

In the present study, we detected both SB0121 and SB0294; which had one-spacer difference between them. The difference in strain type was also supported by their VNTR typing outcome, which is more discriminatory than spoligotyping. VNTR allele variants in loci ETR A and ETR B as observed in the case of *M. bovis* SB0294 has never been observed in the genotyping history of all variant strains of the GKNPC parental strain (SB0121). This suggests that these loci are highly stable and reliable to be utilized in discriminating amongst different *M. bovis* strains. Given the slow 'molecular clock' of mutations in *M. bovis*, it is likely that evolution from SB0121 could have taken place outside the GKNPC and spilled over to wildlife or alternatively, the strain could have mutated within the GKNPC since there are multiple host species. This latter scenario is probably less likely given the many opportunities for strain introduction by

game translocations in several private reserves of the GKNPC. The nature reserve described in this report is known to have a history of introducing wildlife animals of unknown TB status, hence potential sources of new strains. In 2013, a novel *M. bovis* strain (SB2200) was detected in a blue wildebeest (*Connochaetes taurinus*) in the GKNPC representing only the second known introduction of *M. bovis* into this ecosystem. This *M. bovis* strain was suspected to have been introduced into this reserve during repeated translocations of blue wildebeest of unknown BTB status (Hlokwe et al 2014). The adult female giraffe, which was found to be infected with SB0294 in this study (case 1), lived in the same nature reserve as the blue wildebeest infected with SB2200. Although it is unknown whether an increase in the number of circulating outbreak strains has any effect on the bovine TB disease dynamics in an already infected ecosystem, strain-associated differences in virulence have been reported (Garbaccio et al 2014). Therefore, enhanced spill over to other, especially endangered, wildlife species, or neighbouring domestic animals cannot be ruled out as a possible consequence.

Giraffes are the tallest land animals and largest ruminants. They are usually found in African savannahs grasslands and open woodlands. They feed on leaves from various tree species, including higher canopy regions, which cannot be reached by other browsing species (science.com/giraffes-adaptation-grasslands-820851.html). *M. bovis* has been previously found in zoo giraffes in Silesia. It is unclear how these animals would have been infected, however, post mortem examinations confirmed generalized tuberculosis (Krajewska-Wędzina et al 2018). In the current two cases described, *M. bovis* was isolated from the lungs, which showed typical necrogranulomatous inflammation, suggesting that this organ may be a predilection site in this species, and therefore indicating a transmission pathway possibly via aerosols and inhalation. This appears to be a plausible and realistic mechanism for intra-species transmission between different giraffes, given their social behaviour, and resulting contact options and rates. However, other transmission routes such as ingestion of contaminated water

or browse cannot be ruled out. In addition, the isolation of *M. bovis* from the mammary lymph node and kidney suggests additional possible and important dissemination via milk and urine.

The role of giraffe in the epidemiology of *M. bovis* in the GKNPC is currently not known. Their social behaviour in combination with the extensive lesions and the range of target organs found to be affected in the two cases provide support for transmissibility of *M. bovis*, a requirement for maintenance hosts. However, the generally small herd sizes (10 to 20 individuals) may not reach the required population density nor the critical community size needed for pathogen maintenance (Hayden et al. 2002). Within the maintenance host community, greater kudu may be considered a potential source of infection to giraffe through infected browse (Michel et al 2009).

This is the first report of *M. bovis* infection in free ranging giraffes from South Africa, and provides further proof of the ongoing spread of bovine TB between species within the GKNPC. Infection in these two cases proved that despite their anatomy and ecological adaptation, giraffes are susceptible to infection with *M. bovis*. The current findings have dire implications for animal translocations, into the GKNPC or out of this ecosystem, through untested wildlife, especially since movement control of wildlife in this country is only applied to buffaloes. Financial gain is speculated to be at the forefront of these introductions, with little or no assessment of the disease consequences. On the other hand, validated diagnostic tests are available for only a limited number of wildlife species. A good example of this disease threat was recently demonstrated in a previously bovine TB free game reserve in South Africa where the disease was introduced through infected plains game species, transmitted to buffaloes in the reserve and led to the infection of private buffalo ranches, which had bought presumably bovine TB negative buffalo (Hlokwe et al 2016).

In conclusion, our findings have shown for the first time that *M. bovis* can cause overt TB in free-ranging giraffe, which poses the question of their role in the *M. bovis* epidemic in the GKNPC. Secondly, *M. bovis* strain introductions into wildlife in the GKNPC and other conservation areas are a risk. They contribute to the continuous intra and inter-species transmission of *M. bovis* is a leading to an expanding range of infected host species, including endangered species. Therefore, any new introductions of wild animals into larger natural ecosystems and conservation areas should only be done after a thorough risk assessment, especially with regard to establishing the disease status of the place of origin, and of the animals targeted for relocation. This work also demonstrates the value and high importance of regular animal disease surveillance in wildlife, especially in areas with disease control and/or protected status, as well as on the interface between livestock and wildlife, and the significant findings that can be obtained even by passive surveillance.

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Conflict of interest statement

The authors declare no conflict of interest.

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