Characterizing epidemiological and genotypic features of *Mycobacterium bovis* infection in wild dogs (*Lycaon pictus*)

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ABSTRACT

Mycobacterium bovis (M. bovis) infects a wide range of wildlife species and has recently been discovered in the endangered African wild dog (*Lycaon pictus*). This study aimed to characterize the epidemiology of tuberculosis (TB) in wild dogs in endemic areas of South Africa. We describe 12 TB cases in wild dogs from Kruger National Park (KNP), Hluhluwe–iMfolozi Park (HiP) and a private facility in Hoedspruit from 2015 to 2017. Spoligotyping was used to identify the disease-causing *M. bovis* strain in these cases, and whole-genome sequencing was performed on 5 M. *bovis* isolates (KNP = 2 and HiP = 3) to investigate genomic diversity as well as the relationship to other isolates found in these geographical areas. Three distinct strain types were responsible for the *M. bovis* infections in this species. The SB0121 strain was observed in wild dogs from KNP, whereas SB0130 was responsible for infection in wild dogs from HiP. A novel strain, SB2681, was also identified in the HiP wild dogs. Whole-genome sequence analysis suggests that different infection sources exist among these wild dogs and that inter-species transmission most likely occurred between wildlife predators and prey located within shared geographical areas. This study highlights the importance of regular disease surveillance to identify and characterize potential threats for successful control of infection and protection of endangered species.

Keywords
African wild dog, bovine tuberculosis, Mycobacterium bovis, spoligotyping, whole-genome sequencing

1 INTRODUCTION

Tuberculosis (TB) in wildlife, a chronic bacterial disease caused primarily by infection with *Mycobacterium bovis* (*M. bovis*), has been documented in more than 20 wildlife species in key conservation areas in South Africa, such as the Kruger National Park (KNP) and Hluhluwe–iMfolozi Park (HiP) (Gormley & Corner, 2018b; Hlokwe et al., 2014; Michel et al., 2006; Miller, 2015). The existence of multiple susceptible hosts poses a challenge to successful disease control as well as protection and management of priority or endangered species (Garine-Wichatitsky et al., 2013). For example, *M. bovis* infection in South Africa's endangered carnivore, the African wild dog (*Lycaon pictus*), had been undocumented until cases were reported in 2015 (Higgitt et al., 2019). This is problematic since the largest and most stable wild dog populations in South Africa reside within game reserves that are endemic for TB, including KNP and HiP (Davies-Mostert et al., 2012; Lindsey et al., 2005). Specifically, the presence of controlled infectious diseases within wild dog populations may restrict translocations and subsequently affect metapopulation management intended to ensure maintenance of genetic diversity.

Wild dogs have been classified as endangered by the International Union for Conservation of Nature (IUCN) since 1990, with an estimated 6,600 individuals remaining globally (Woodroffe & Sillero-Zubiri, 2012). African wild dogs are under threat from habitat destruction, human–wildlife conflict (persecution and snaring) and infectious diseases, causing viable populations to become fragmented (Campana et al., 2016; Woodroffe et al., 2007). Rabies and canine distemper (CD) have already contributed to the decline of several wild dog populations in Africa, increasing the risk of future local extinction events (Woodroffe & Donnelly, 2011). Furthermore, although *M. bovis* infection in wild dogs had not been reported prior to 2019, mortalities associated with TB have recently been observed in wild dogs in KNP and HiP (Higgitt et al., 2019).

The recent discovery of TB morbidity and mortality in an endangered species warrants further investigation, including characterization of *M. bovis* isolated from wild dogs. It is unknown whether the strain of *M. bovis* or host immune response characteristics affect the pathogenesis of TB in this species. Since variable virulence of different strains of *M. bovis* has been documented in cattle, deer, wild boar and badgers (Abdelaal et al., 2019; Fuente et al., 2015; Garbaccio et al., 2014; Gormley & Corner, 2018a; Joshi et al., 2012; Vargas-Romero et al., 2016; Wright et al., 2013), it may be important to describe the strains in circulation in wild dog populations, as well as other species within the system, and monitor for changes over time.

In order to investigate the source of *M. bovis* and determine the strains in different populations of wild dogs, isolates from naturally occurring cases need to be described. Spoligotyping and whole-genome sequencing (WGS) are techniques in which organisms are genetically characterized at different levels of resolution (Guimaraes & Zimpel, 2020). Although spoligotyping has been used in molecular epidemiological investigations of *M. bovis* outbreaks, WGS is able to further differentiate *M. bovis* strains with identical genotypes by identifying variant differences between and among the strains (Guimaraes & Zimpel, 2020; Price-Carter et al., 2018; Zimpel et al., 2020). These differences in strain diversity can be used to identify transmission networks and sources of infection based on previous studies characterizing *M. bovis* in wildlife and livestock in South Africa. This study describes a case series of *M. bovis* infection in wild dogs using mycobacterial culture and speciation, as well as characterizing *M. bovis* isolates from different wild dog populations in

South Africa using spoligotyping and WGS, which improves understanding of disease epidemiology in this species.

2 MATERIALS AND METHODS

2.1 Sample population and post-mortem examinations

African wild dogs found dead or euthanized between 2015 and 2017, in KNP, HiP, and a private facility in Hoedspruit, South Africa, underwent post-mortem examination by veterinary staff. In total, 19 wild dogs were found during this period and were subjected to post-mortem examination. Of the 19 wild dogs, 12 had *M. bovis* culture-positive results and were selected for further investigation (Table 1). Samples were collected from lungs, liver, kidneys and lymph nodes (LN) and examined for the presence of gross lesions suggestive of TB. If no detectable lesions were present, samples were pooled according to anatomical site, that is lung tissue, head, thoracic, peripheral or abdominal lymph node pools. Sections of samples were frozen at −20°C until processed for mycobacterial culture. Additional sections were also fixed in 10% buffered formalin and processed for histopathological examination and Ziehl–Neelsen (ZN) staining. Ethical approval for this study was received from Stellenbosch University Animal Care and Use Committee (SU-ACUD16-00076). Approval was also obtained from Department of Agriculture, Forest, and Fisheries (DAFF) in terms of Section 20 of the Animal Diseases Act (Act no. 35 of 1984) with reference number: 12/11/1/7/2.

2.2 Mycobacterial culture and speciation

Tissue processing for mycobacterial culture was performed in a Biosafety Level 3 (BSL3) laboratory using the BACTEC™ Mycobacteria Growth Indicator Tube (MGIT™) 960 Mycobacterial Detection System (BD Biosciences, Franklin Lakes, New Jersey, USA) as previously described (Goosen et al., 2014). If positive growth was observed in the MGIT culture, an aliquot was boiled and inspected for the presence of acid-fast bacilli using the Ziehl–Neelsen (ZN) staining technique (Global Laboratory Initiative, 2014). Additionally, 250 µl of positive MGIT™ samples was streaked out on 2% blood agar plates (National Health Laboratory Service; Cape Town, South Africa) to test for fungal and bacterial contamination. Plates were incubated at 37°C for at least two days before visually inspecting them for contamination. All ZN stain-positive bacterial cultures were speciated using genetic region of difference (RD) analysis and 16S rRNA sequencing (Harmsen et al., 2003; Warren, Gey van Pittius, et al., 2006) to confirm the presence of *M. bovis*. *Mycobacterium bovis* isolates were inoculated in duplicate onto Middlebrook® 7H11 (BD Biosciences) agar plates, supplemented with 0.5% sodium pyruvate (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 37°C for 6–8 weeks. Subsequently, bacterial colonies were harvested and duplicate plates were pooled for DNA extraction (Warren, de Kock, et al., 2006). A small sample of each isolate was boiled and subjected to spacer oligonucleotide typing (spoligotyping) to identify the *M. bovis* strain (Kamerbeek et al., 1997). The resulting binary spoligotype patterns were compared to those in the *M. bovis* spoligotype database (https://www.mbovis.org/database.php). Novel spoligotype patterns were submitted to the database in order to generate new SB numbers.

TABLE 1. Summary of African wild dog samples used in this study, including collected tissue samples and spoligotypes of *Mycobacterium bovis*-positive samples

Case, Year	Wild dog ID[a]	Location	Sex	Age[c]	Samples collected for mycobacterial culture	*M. bovis* culture-positive samples	Spoligotype SB number	Samples selected for WGS
1 2015	15/740	Tshokwane, KNP[b]	Female	Juvenile (6 months)	Head, thoracic, abdominal, peripheral LN[c]; lung and liver	All collected samples	NA	NA
2 2016	16/241 LVS201605-002	Sand River, Tshokwane, KNP	Female	Young adult	Head, thoracic, abdominal and peripheral LN; lung	Peripheral LN	SB0121	NA
3 2016	16/253 LVS201605-007	Sand River, Tshokwane KNP	Female	Young adult	Head, abdominal peripheral and thoracic LN; lung	Head, abdominal and thoracic LN	SB0121	NA
4 2016	LVS201605-008	Sand River, Tshokwane KNP	Male	Young adult	Head, thoracic, peripheral and abdominal LN; lung	Abdominal LN	SB0121	Abdominal LN
5 2016	16/255 LVS201605-010	Sand River, Tshokwane KNP	Male	Young adult	Head, thoracic, abdominal and peripheral LN; lung	Abdominal LN	SB0121	Abdominal LN
6 2016	16/256 LVS201605-011	Sand River, Tshokwane KNP	Male	Adult	Head, thoracic, abdominal and peripheral LN; lung	Abdominal LN	SB0121	NA
7 2016	16/413 LVS201607-004	Talamati, KNP	Female	Adult	Head, peripheral, bronchial, and abdominal LN; lung and kidney	All collected samples	SB0121	NA
8 2017	17/123 LVS201703-001	Delaporte, Skukuza, KNP	Female	Juvenile (9–10 months)	Head, thoracic, abdominal, peripheral LN; lung and abdominal lesion	Head, thoracic, abdominal, peripheral LN; abdominal lesion	SB0121	NA
9 2017	#1	Hoedspruit (captive)	Male	Adult (3-year-old)	Mesenteric, tracheobronchial LN; lung	Tracheobronchial LN	SB0121	NA

10 **2017**	#2	Hoedspruit (captive)	Male	Adult (3-year-old)	Mediastinal, mesenteric LN; lung	Mediastinal, mesenteric LN; lung	SB0121	NA
11 **2017**	HiP4	HiP[d]	Not recorded	Young adult	Tracheobronchial, mesenteric and left retropharyngeal LN	Tracheobronchial, mesenteric and left retropharyngeal LN	SB2681	Left retropharyngeal LN
12 **2017**	HiP5	HiP	Female	Adult (3-year-old)	Axillary, mesenteric and tracheobronchial LN; lung	Axillary, mesenteric and tracheobronchial LN; lung	SB2681(lung); SB0130 (axillary, mesenteric, tracheobronchial LN)	Axillary and mesenteric LN

Note: Wild dog age ranges: Juvenile: 0–1 year; Young adult: 1–2 years; Adult: 3–5 years.

[a] Identification.

[b] Kruger National Park.

[c] Lymph node.

[d] Hluhluwe–iMfolozi Park.

[e] Age was not recorded for every wild dog.

2.3 Whole-genome sequencing (WGS)

Based on isolate availability and spoligotype results, two SB0121 isolates (cases 4 and 5), one SB2681 (case 11), and two SB0130 isolates (case 12) were selected for DNA extraction and WGS (Table 1). WGS was performed using the Illumina NextSeq 550 platform (Illumina, Inc., San Diego, California, USA) at the Centre for Disease Control and Prevention (Atlanta, Georgia, USA). A paired-end approach was used with approximately 600 base pair fragment sizes. The raw sequence data were deposited to the European Nucleotide Archive under project accession number PRJEB39449. One microgram of DNA was used for library preparation for sequencing per the manufacturer's instructions using the Illumina NEBNext sample preparation kit (Illumina, Inc.). The Illumina paired-end reads generated for the purpose of this study (five genomes) and 108 genomes published previously or available in public databases (Table S1), were analysed with open source software as previously described (Black et al., 2015; Dippenaar et al., 2015). Briefly, low-quality bases and reads were trimmed using Trimmomatic (Bolger et al., 2014) after which they were aligned to the reference genome *M. tuberculosis* H37Rv (GenBank NC_000962.2). Three tools were used for the alignment, namely Novoalign (Novocraft), Burrows–Wheeler Aligner (BWA) (Li & Durbin, 2009) and SMALT (Ponstingl & Ning, 2010). An average depth of coverage of >60x was obtained for *M. bovis* isolates sequenced for this study. The Genome Analysis Tool Kit (GATK) (McKenna et al., 2010) and SAMtools (Li et al., 2009) were both used to identify single-nucleotide variants (SNVs) from each of the three alignments. SNVs identified by GATK and SAMtools in all three alignments that overlapped in both position and base identity were further filtered to exclude SNVs in the *pe/ppe* family regions, repeat regions, insertion sequences and bacteriophages, as previously described (Dippenaar et al., 2019).

2.4 Phylogenetic analysis

Concatenated sequences of 113 M. *tuberculosis* complex (MTBC) isolates containing 31,027 high-confidence variable sites were used to construct a maximum-likelihood phylogeny of the isolates included in this analysis with IQ-TREE and RaxML with 1,000 bootstrap pseudo-replicates (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015; Stamatakis, 2006, 2015). The general time reversal nucleotide substitution model was used for phylogenetic inference with RaxML, whereas IQ-TREE employs an ultrafast and automatic model selection method (ModelFinder) for phylogenetic estimates (Kalyaanamoorthy et al., 2017). The phylogenetic trees were annotated using the Interactive Tree Of Life (iTOL) software (Letunic & Bork, 2019).

3 RESULTS

The first case of TB described in this series was a free-ranging juvenile (six month old) female African wild dog (case 1; Table 1) from KNP, identified in 2015. *Mycobacterium bovis* was isolated from lesions consistent with TB in lung and liver tissue, as well as head, thoracic, abdominal and peripheral lymph nodes, which was confirmed by RD analysis. Histopathological examination confirmed systemic tuberculosis. At the time, spoligotyping was not conducted and a stored sample was not available.

In May 2016, a young adult female wild dog (case 2) was found ataxic, lethargic and straining, but still alive in KNP. This animal was euthanized, and a post-mortem examination was conducted. Histopathological examination revealed pneumonia accompanied by inclusion bodies that were suggestive of canine distemper virus (CDV). Inclusion bodies

could however not be identified in the brain tissue, although CDV was confirmed by positive immunoreactivity to viral antigens and PCR. Mycobacterial culture of peripheral lymph nodes and spoligotyping confirmed *M. bovis* infection with the strain SB0121, although cultures of other tissues (lung tissue, head, thoracic and abdominal lymph node pools) were negative. This wild dog was a member of a pack of 12 wild dogs, 7 of which were found dead (6 confirmed to have CDV by pathological analysis). Three of the four remaining wild dogs in the pack died shortly after capture, and the fourth wild dog was euthanized due to progressive signs of CDV infection and subjected to post-mortem examination. Mycobacterial culture and spoligotyping were conducted on the entire pack, and the results indicated that an additional 4 wild dogs (cases 3–6; Table 1) from this pack (total of 5 out of 12) were also infected with the SB0121 *M. bovis* strain. Although case 3 had *M. bovis* isolated from head, thoracic and abdominal lymph node pools, only the abdominal lymph nodes were culture positive in cases 4–6.

In July 2016, KNP rangers reported a recumbent adult female wild dog (case 7) with wounds to the axilla and thorax, accompanied by laboured breathing. This animal died while being immobilized for examination. Histopathological examination identified pyogranulomatous inflammation in the lung, and identification of acid-fast bacilli within lesions of the lung, lymph nodes and intestines. The pattern and presence of bacteria in multiple organs suggested that this wild dog had systemic TB disease. *Mycobacterium bovis* was isolated from peripheral, bronchial, head and abdominal lymph nodes, as well as lung and kidney tissues which were confirmed by RD analysis (Warren, Gey van Pittius, et al., 2006). Spoligotype analysis revealed SB0121 as the infecting *M. bovis* strain.

In March 2017, a juvenile (9–10 months) wild dog (case 8) died, likely as a result of TB. Post-mortem analysis revealed a ruptured intestine due to extensive TB lesions. Mycobacterial culture and RD analysis confirmed *M. bovis* infection in the head, thoracic, abdominal, and peripheral lymph nodes, as well as a lesion isolated from the small intestine. Spoligotyping results indicated that this wild dog was also infected with the SB0121 *M. bovis* strain.

In December 2017, two captive wild dogs with positive tuberculin skin test results were euthanized and necropsied at a private facility in Hoedspruit, South Africa. Mycobacterial culture, in combination with RD analysis, confirmed *M. bovis* infection in a tracheobronchial lymph node of one wild dog (case 9), and mediastinal and mesenteric lymph nodes as well as lung tissue in the second wild dog (case 10). Spoligotyping indicated that the same strain of *M. bovis* as that found in KNP, SB0121, infected both wild dogs.

Two young adult members of a wild dog pack from HiP were kept under observation after the rest of the pack succumbed to CDV infection in 2016. Case 11 (a young adult, sex not recorded) eventually also succumbed to CDV. Case 12 (3-year-old female) was observed to rapidly develop signs of nasal and ocular discharge, loss of body condition, head twitching and laboured breathing. This animal was euthanized due to evidence of disease progression (CDV). *M. bovis* was isolated from lymph node samples from both wild dogs (cases 11 and 12). Two spoligotypes were found, which differed from those isolated from wild dogs in KNP. Wild dog case 11 was infected with a novel, unpublished *M. bovis* strain that had not been previously described in the *M. bovis* database (https://www.mbovis.org/database.php) and was allocated a new SB number, SB2681. This strain was isolated from tracheobronchial, mesenteric and retropharyngeal lymph nodes of this wild dog. A pack mate, wild dog case 12, was also infected with the novel strain (SB2681) which was isolated from lung tissue.

Additionally, a different strain, SB0130, was also isolated from axillary, mesenteric and tracheobronchial lymph nodes of wild dog case 12.

Five isolates from KNP and HiP wild dogs (one sample each from cases 4, 5 and 11, and two samples from case 12; Table 1) were available for WGS and, together with 108 previously published *M. bovis* isolates and other isolates belonging to the *Mycobacterium* genus, were included in a maximum-likelihood phylogenetic reconstruction (Figure 1; Table S1). The phylogeny produced by IQ-TREE confirmed that *M. bovis* isolates cultured from the KNP wild dogs (cases 4 and 5) clustered with *M. bovis* isolates sampled from different animal hosts in KNP. The analysis also showed that the HiP wild dog isolates (cases 11 and 12) clustered with those cultured from animals in Mpumalanga, Madikwe Game Reserve (MGR) in North West province, and St. Lucia in KZN (Figure 1). The variant distance between isolates from case 4 and case 5 (both SB0121) was 25. The *M. bovis* isolate cultured from case 11 (SB2861) had 6 unique SNVs when compared to both isolates from case 12 (SB0130), and the two isolates from case 12 had no unique SNVs when compared to each other. The phylogenetic tree produced by RaxML showed similar topology to that produced by IQ-TREE (see Figure S1).

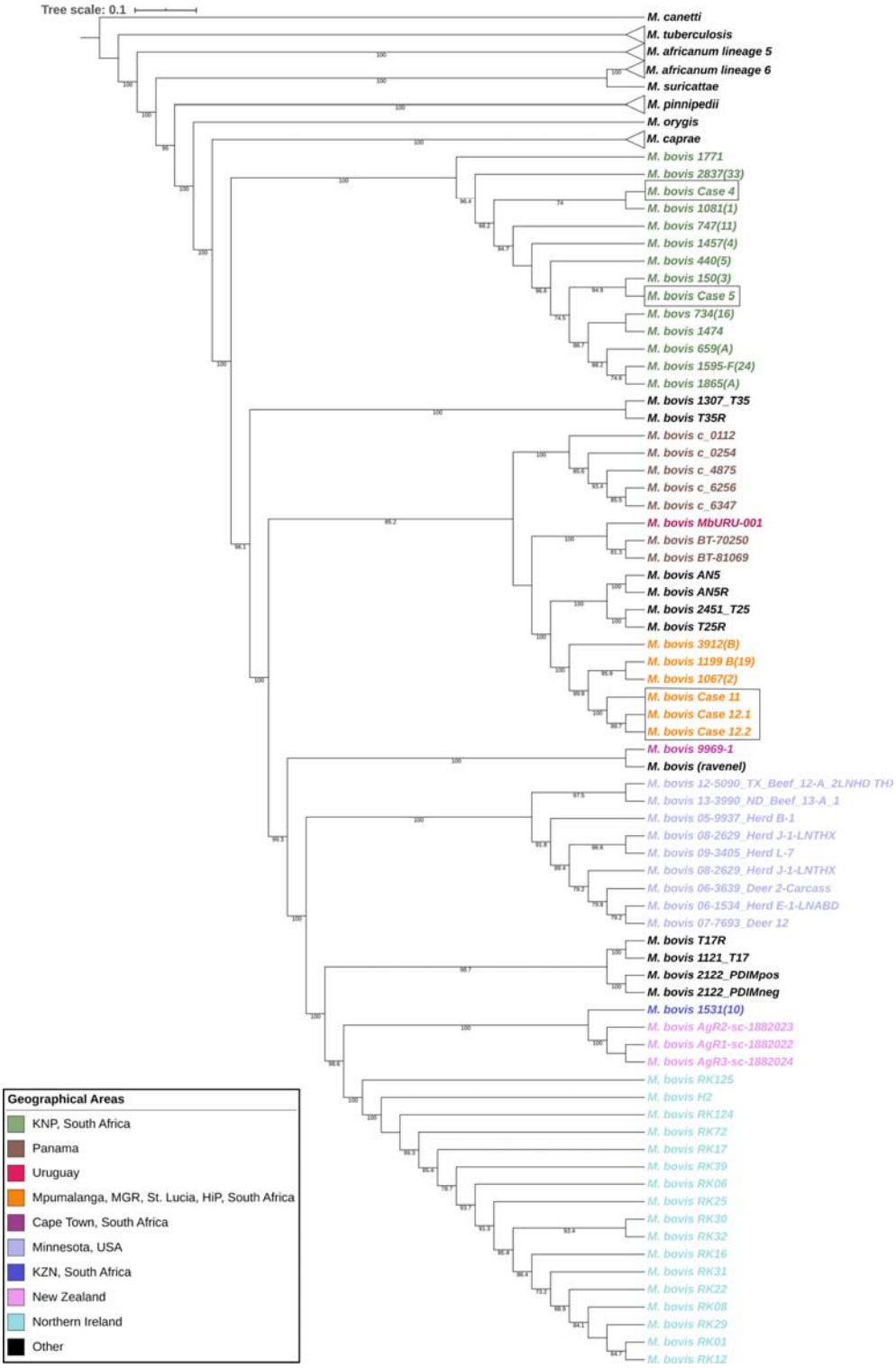

FIGURE 1. Molecular phylogenetic analysis by maximum-likelihood method with 1,000 bootstrap replicates showing the relationship of the five wild dog *Mycobacterium bovis* isolates to the 108 other *M. bovis* isolates from different animal hosts. The bootstrap support values are shown next to the nodes. The phylogenetic tree was produced by IQ-TREE which was based on variable sites identified when compared to the *M. tuberculosis* H37Rv reference sequence (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). Kruger National Park (KNP) wild dog isolates (cases 4 and 5) clustered with other *M. bovis* isolates within KNP and Hluhluwe–iMfolozi Park (HiP) wild dog isolates (cases 11, 12.1 and 12.2) with *M. bovis* isolates from Mpumalanga, Madikwe Game Reserve (MGR), and St. Lucia (Dippenaar et al., 2019)

4 DISCUSSION

This study describes cases of *M. bovis* infection in wild dogs and characterizes the *M. bovis* isolates using spoligotyping and whole-genome sequencing to gain insight into the epidemiology of TB in wild dogs. The results provide evidence of the spread of TB between wildlife species within conservation areas in South Africa through phylogenetic relatedness of *M. bovis* isolates. The cases of TB reported here were identified opportunistically; in KNP and HiP following a CDV outbreak and in Hoedspruit following the suspicious death of one adult in the captive pack. However, there were also several animals that died or were euthanized which were not related to CDV (cases 1, 7, 8, 9 and 10). The observation of systemic pathological changes and mortality linked with TB in two wild dogs younger than one year of age (cases 1 and 8) is of concern and warrants further investigation of pathogenesis in this species.

In some cases, multiple members of a pack were found to be infected with *M. bovis* (cases 2–6, 9 and 10, 11 and 12). However, the only pack that was completely eliminated was the original pack for cases 2–6; therefore, it is possible that other members of the packs to which cases 1, 7 and 8 belonged may be infected but this is unknown since they were not tested. The most likely route of infection in wild dogs is ingestion, similar to observations in other carnivores such as lions and leopards (Michel et al., 2006; Renwick et al., 2007). Free-ranging wild dogs hunt as a pack and may be exposed to a high infection load of bacilli when feeding on infected prey species. All the *M. bovis* cases in free-ranging individuals were from TB-endemic parks, and the captive wild dogs had the same spoligotype as the KNP animals. It is likely that affected individuals had multiple exposure events and were in contact with the same sources.

The route of *M. bovis* infection may also be inferred by the pattern of lesions seen at post-mortem examination (Biet et al., 2005; de Lisle et al., 2002), with cases in which lesions were confined to the thoracic cavity suggesting aerosol transmission, while lesions in mesenteric lymph nodes thought to indicate infection via ingestion (Pollock & Neill, 2002). In the 12 cases in this study, *M. bovis* was isolated from tissues from gastrointestinal system (mesenteric lymph nodes, liver, intestines) in 9 cases, with 3 wild dogs only having culture-positive abdominal lymph nodes. This finding highlights ingestion as an important route of infection in wild dogs. However, the presence of *M. bovis* in multiple tissues (7 cases) suggests disease can become disseminated. Both juvenile wild dogs had generalized disease, indicating that infection may progress rapidly in some individuals. This may be a result of lymphatic and haematogenous spread, or transmission by both ingestion and respiratory aerosols since there were 8 cases in which *M. bovis* was isolated from the lung or thoracic lymph nodes. Multiple routes of infection (oral and respiratory) have been suggested in other carnivores such as lions and leopards (Maas, 2013; Renwick et al., 2007). However, little is known about the infection dynamics within this species, although epidemiological studies may provide insight.

In order to investigate potential sources of infection, spoligotyping was performed on *M. bovis* isolated from wild dogs in this study. Previous research has shown that different strains are associated with different geographical areas; wildlife in KNP typically are infected with SB0121, and SB0130 in HiP (Hlokwe et al., 2014). As expected, all *M. bovis* infections in wild dogs from KNP were caused by the SB0121 strain. However, in HiP, there has been greater diversity in the strains reported, with more than one genotype being present in a population, as was the case in this report. Two different spoligotypes were found in the wild

dogs in this region: SB0130 and the novel SB2681 strain. Similar to previous reports in other wildlife species, the strains isolated from wild dogs in KNP and HiP were epidemiologically distinct, although shared with other wildlife in the respective regions, suggesting that sources of infection were likely infected prey within the system (Hlokwe et al., 2014).

This study also found *M. bovis* infection in two captive wild dogs, supporting the likelihood that ingestion is an important route of transmission, even in those individuals that do not hunt prey. These wild dogs were fed with local livestock and wildlife carcasses (which did not undergo inspection), which was most likely the source of their infection. This finding highlights that captive wild dogs are still at risk of infection in areas where TB may exist in other species. A single *M. bovis* strain was isolated from these wild dogs (SB0121) which is the local circulating strain found in KNP and cattle on surrounding farms, supporting inter-species transmission through infected meat (Musoke et al., 2015).

Five *M. bovis* isolates from wild dogs were available for higher resolution phylogenetic analysis by whole-genome sequencing. The sequenced *M. bovis* isolates from KNP wild dogs (cases 4, 5) revealed clustering within the KNP clade which includes *M. bovis* isolates obtained from buffaloes, lions, a baboon, a leopard and a kudu (Dippenaar et al., 2017). This provides additional evidence that *M. bovis* infection was most likely transmitted from wildlife hosts within KNP. Even though these two wild dogs were infected with the same *M. bovis* strain (SB0121) as other KNP hosts, WGS revealed a variant distance of 25 SNVs between the isolates, which is considered a large SNV distance. This indicates that different transmission events might have occurred and that these individuals may have been infected at separate times (Guimaraes & Zimpel, 2020). Wild dogs hunt on a regular basis and multiple kills per hunt are not uncommon (Creel & Creel, 2002; Hayward et al., 2006), which could explain why wild dog pack members may be infected by different prey sources with slightly varying *M. bovis* sequences. Isolates of *M. bovis* in the KNP clade have been reported to have 5–25 SNVs, suggesting clonal expansion in this geographical location (Dippenaar et al., 2017). In addition, this study demonstrated that the SNVs clustered by location rather than host species, which can provide a tool for tracking movement of disease within the country (Dippenaar et al., 2017), and especially in wild dogs that are translocated as part of the South African metapopulation.

Interestingly, two different strains (SB0130, SB2681) were responsible for the *M. bovis* infections in the wild dogs from HiP. These strains also clustered with local *M. bovis* isolates (Dippenaar et al., 2017). This emphasizes that *M. bovis* infection is not host-specific, which may create challenges when trying to identify possible transmission networks and sources of infection. Additionally, one wild dog was co-infected with two different strains (case 12; SB0130 isolated from axillary, mesenteric and tracheobronchial lymph nodes and SB2681 isolated from lung tissue). Since there were few genomic differences (6 SNVs) between SB0130 (case 12) and SB2681 (case 11), the novel strain is closely related to SB0130. When such a small variant distance is identified among isolates, it can be assumed that the cases are epidemiologically linked and that the cases are part of the same chain of transmission. One possible scenario is that the animals had been infected with these two closely related strains on separate occasions and possibly from separate sources due to the strain types circulating in the area. Alternatively, the same *M. bovis* strain could have been transmitted to both wild dogs from a single source, and the SNVs observed were due to within-host evolution (Guimaraes & Zimpel, 2020). An evolutionary event might have occurred in the genomic direct repeat (DR) locus of the strain (SB0130) which altered the composition of the spacer sequences, ultimately causing a change in the genotype (SB2681; Table S2). However,

further investigation is required to confirm this. Since SB0130 is commonly found in wildlife and cattle in the area in and around HiP, this finding supports local transmission of *M. bovis* in the wild dogs. The greater resolution of WGS supports its use as a tool to investigate epidemiology of TB in wild dogs.

5 CONCLUSION

This study is the first to characterize *M. bovis* isolated from free-ranging wild dogs in South Africa. The cases described in this series highlight the knowledge gaps around the epidemiology of *M. bovis* in wild dogs. Evidence of generalized infection and mortality in several cases suggests that although the discovery of TB in wild dogs was incidental, further research is needed to determine pathogenesis of disease and whether it has an impact on populations. Regardless, the presence of TB in wild dogs has a significant conservation impact since as a controlled disease, it can limit the use of translocations between populations for genetic management. Therefore, additional studies are needed to determine potential routes of transmission and the risks associated with ingestion of infected carcasses. In TB-endemic areas, frequent exposure to infected prey introduces numerous transmission opportunities for free-ranging wild dogs; however, captive wild dogs may also be at risk of infection by feeding on uninspected carcasses that are infected with *M. bovis*. The different strains isolated from the free-ranging wild dogs clustered with the disease-causing strains previously identified within KNP and HiP. WGS data enabled the inference of potential chains of transmission by combining information on variant distance, epidemiological data and phylogeny. This revealed a relatively high level of variation between isolates that were identical by spoligotyping (case 4: SB0121 and case 5: SB0121; genetic distance of 25 SNVs). In contrast, it showed that two different strains (case 11; SB2681 and case 12 SB0130) were separated by only 6 SNVs, which could be an example of within-host diversity, possibly arising from mixed infection or microevolution (i.e. bacterial mutations occurring during an extended period of co-existence between the host and pathogen) (Guimaraes & Zimpel, 2020; Hatherell et al., 2016). However, challenges in data interpretation still exist even with the high resolution offered by WGS, especially in TB where time of infection and onset of disease are difficult to establish. As increasing numbers of isolates are sequenced, the knowledge of *M. bovis* genomic diversity and evolution will improve and facilitate interpretation of TB epidemiology based on WGS data (Zimpel et al., 2020). This study demonstrates the value of characterizing *M. bovis* strains to gain insight into disease transmission networks which could be crucial for wild dog translocation decisions and metapopulation management which is currently limited due to infectious diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The Stellenbosch research ethics committee's guidelines for the Care and Use of Animals were followed.

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