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ABSTRACT: In South Africa, African buffaloes (*Syncerus caffer*) are one of the wildlife maintenance hosts for bovine tuberculosis (BTB) and play a key role in the spread of the disease to other wildlife species and potentially back to cattle. We report a trace-back investigation following the diagnosis of BTB in a previously BTB-free provincial game reserve, founded in the early 1990s in the North West Province of South Africa (SA). Using the intradermal tuberculin and interferon gamma tests, we detected *Mycobacterium bovis* infection in captured African buffaloes intended for sale. Detection of *M. bovis* was confirmed by culture and PCR. Molecular typing of *M. bovis* isolates from three African buffaloes revealed spoligotype SB0140 and a variable number of tandem repeat genotypes which had been previously isolated from wildlife in the KwaZulu-Natal Province of SA. Diagnosis of BTB in a previously uninfected buffalo population provides evidence that the disease can be introduced into an ecosystem through the translocation of untested plains game species. We further illustrate how BTB can remain unnoticed for considerable periods of time in free-ranging wildlife populations and emphasize the need for validated diagnostic tests for application in suitable and practical monitoring programs. This is especially important for species with maintenance host potential and those in high demand at game auctions.
   Key words: African buffaloes, bovine tuberculosis, molecular typing, wildlife translocation.

INTRODUCTION

Bovine tuberculosis (BTB) has affected wildlife in South Africa (SA) for almost a century (Paine and Martinaglia 1929; Jolles et al. 2005) and the disease is now enzootic in the Greater Kruger National Park Complex and Hluhluwe-iMfolozi Park (Michel et al. 2009; Hlokwe et al. 2011). The disease status of animals in private game parks, reserves, and farms in the country is largely unknown, but sporadic disease reports indicate a number of BTB cases involving different wildlife species (Hlokwe et al. 2014).

Wildlife maintenance hosts of tuberculosis pose major difficulties to controlling infection in cattle and other wildlife species with which they interact (Michel et al. 2006). African buffaloes (*Syncerus caffer*) are maintenance hosts of BTB (Michel and Bengis 2012), and there is growing evidence that greater kudu (*Tragelaphus strepsiceros*) have maintenance

host potential (Keet et al. 2001; Michel et al. 2009). Movement of infected livestock and wildlife species is one of the most important risk factors in the transmission of BTB (Skuce et al. 2012). In SA, movement control measures in the wildlife industry apply only to African buffaloes. Outside the foot-and-mouth disease control zones, there are no restrictions for other wildlife species that may play a role in the spread of several high-impact livestock diseases such as BTB (Woodford and Rossiter 1993; De Vos et al. 2001). The wildlife industry is the fastest growing agricultural sector in SA and provides sources of income not only through ecotourism but also game sales, hunting, and consumption (van der Merwe and Saayman 2003).

Our aim was to unravel the descriptive and molecular epidemiology underlying the BTB scenario that led to the infection of African buffaloes in the Madikwe Game Reserve (GR)

with *Mycobacterium bovis*, detected during routine testing of buffaloes for auction.

MATERIALS AND METHODS

Study area and animals

The Madikwe GR covers 63,000 ha (24°81′S; 26°21′E) and harbors approximately 1,000 African buffaloes. The 53 founder buffaloes were introduced into Madikwe GR in 1992 from European and American zoos and from Addo Elephant National Park, via Willem Pretorius GR or Pilanesberg GR. In 1996, the second and last introduction of 13 buffaloes took place from Pilanesberg GR. Between 2006–11, at least 6,000 animals, mostly various antelope species including greater kudu, were introduced into the Madikwe GR from various regions in SA.

BTB history

Due to import and movement regulation requirements for disease control, all buffaloes had been obtained from BTB-negative populations (nationally and internationally). In addition, all buffaloes had been tested for BTB using the intradermal tuberculin test (IDT) and their BTB-negative status was established upon arrival. The BTB status of all other animal species was unknown at the time of their introduction and it remained undetermined. Madikwe GR held buffalo auctions every year at which between three and 34 live buffaloes were sold per annum between 2002–10, following BTB testing using the interferon-gamma (IFN-γ) assay (Michel et al. 2011) or the IDT. No BTB-positive animals were detected.

Intradermal tuberculin testing

Single intradermal tuberculin testing: In June 2012, the management of Madikwe GR randomly selected and captured 51 free-ranging buffaloes and placed them in a boma for testing for BTB in preparation for auction. The local state veterinarian performed BTB testing by using one IDT (using only bovine purified protein derivative [PPD] according to guidelines; World Organisation for Animal Health [OIE] 2009). Buffaloes with skinfold thickness ≥6 mm were classified as positive while those with skinfold thickness ≥4 mm but <6 mm were regarded as inconclusive skin test reactors.

Comparative intradermal tuberculin testing: Three months after the first IDT (September 2012), the local state veterinarian conducted a comparative IDT using bovine and avian PPDs (OIE 2009). This testing was repeated 12 mo later (June 2013). Briefly, we classified buffaloes as BTB-positive if the increase in skinfold thickness at the bovine PPD injection site exceeded the increase at the avian PPD injection site by 4 mm. We considered differential increases of between 2 mm and 4 mm to be inconclusive.

Interferon gamma testing

Fresh blood samples in heparin tubes were collected from 11 buffaloes with a positive or inconclusive test outcome in the single IDT for IFN-γ (July 2012) using a modified Bovigam® assay (Prionics AG, Wagistrasse, Schlieren, Switzerland) (Michel et al. 2011). All 51 selected buffaloes were subjected to an IFN-γ test 11 mo later (June 2013).

Tissue sample collection

Six buffaloes (PH713; 4-256762; 32-252380; 33-257215; 38-240440; and 39-221392) found positive by IDT or IFN-γ were euthanized and necropsied. Lymph nodes were collected for mycobacterial culture at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI).

Mycobacterium isolation and identification

Collected lymph nodes were processed as described (Bengis et al. 1996). We identified *Mycobacterium tuberculosis* complex bacteria by PCR using primers targeting a sequence encoding the MPB70 antigen as described by Cousins et al. (1992). Deletion analysis was performed using primers targeting the RD4 and RD9 regions of difference as described for *M. bovis* identification (Warren et al. 2006).

Molecular typing and construction of a dendrogram

We genotyped *M. bovis* isolates from tissue samples using spoligotyping (Kamerbeek et al. 1997) and variable number tandem repeat (VNTR) typing (Le Fléche et al. 2002; Hlokwe et al. 2013). The VNTR types were compared to those already available in the database at the tuberculosis laboratory at ARC-OVI. A dendrogram was constructed using the Bionumerics software version 7.1 (Applied Maths, Saint-Martens-Latum, Belgium) to determine genetic relationships of the strains. The genetic profiles of the *M. bovis* strains from Madikwe GR were compared to the recently established database at ARC-OVI, which contains genetic profiles of approximately 631 animals (livestock and wildlife species) from regions of SA.

TABLE 1.   Culture, skin test, and interferon gamma results of 2012–13 testing for bovine tuberculosis (TB; *Mycobacterium bovis*) of euthanized African buffaloes (*Syncerus caffer*) captured in Madikwe Game Reserve, South Africa.

Buffalo identification	Laboratory identification	Culture	Tuberculin skin test (single and/or comparative IDT)[a]	Interferon gamma test (2012/2013 testing)[b]
PH713	TB 8146	*M. bovis*	Positive	Positive/Positive
4-256762	TB 8277	*M. bovis*	Positive	ND/Positive
32 252380	TB 8274	Negative	Positive	ND/Positive
33 257215	TB 8275	Negative	Positive	ND/Negative
38-240440	TB 8278	*M. bovis*	Positive	ND/Negative
39-221392	TB 8276	Negative	Positive	ND/Negative

[a] IDT = intradermal tuberculin testing.

[b] ND = not done.

RESULTS

Intradermal tuberculin testing

Single intradermal tuberculin testing: Two buffaloes reacted to bovine PPD with increases in skinfold thickness ≥6 mm (including PH713; Table 1). Nine buffaloes had an inconclusive skin reaction (increase in skinfold thickness ≥4 mm).

Comparative intradermal tuberculin testing (3 mo after the single IDT): Three of 51 buffaloes were BTB-positive based on the reaction to bovine PPD of >4 mm of the reaction to avian PPD. Five buffaloes were regarded as suspect based on an increase in bovine skinfold thickness of 1–4 mm greater than the avian reaction (including 39-221392 and 4-256762; Table 1).

Comparative intradermal tuberculin testing (twelve months after the single IDT): Eight buffaloes (including 33-257215, 38-240440, 32-252380, 39-221392, and 4-256762; Table 1) were BTB positive. These included two positive animals detected in the initial comparative IDT. Results for seven animals were inconclusive.

Gamma interferon testing

Of the 11 buffaloes tested in July 2012, two were BTB-positive (PH713 and a temporarily tagged buffalo). In June 2013, two of the 51 buffaloes were also BTB-positive (4-256762 and 32-252380; Table 1).

Mycobacterium isolation and identification

We cultured 35 tissue samples from six buffaloes that were selected due to their positive skin test reactions. We confirmed BTB in three buffaloes (PH713, 4-256762, and 38-240440) through isolation of *M. bovis* from tonsil (PH713), prescapular lymph node (4-256762), and lung, bronchial, mediastinal, right and left retropharyngeal, and mesenteric and parotid lymph nodes (38-240440). Acid fast bacterial isolates were confirmed as *M. tuberculosis* complex species through amplification of a 372-base pair (bp) PCR product from the MPB70 gene, and amplification of two specific DNA fragments indicative of *M. bovis* by the presence of a 268-bp DNA product for RD4, and by the absence of *M. tuberculosis* by the detection of a 108-bp product for RD9.

Genotyping

Spoligotyping of the isolates from different tissue samples from all three buffaloes yielded a pattern corresponding to SB0140 in the international *M. bovis* database (www.mbovis. org). We found two VNTR profiles with 90% relatedness differing only at one VNTR locus (M tub 12). We detected one VNTR profile (Fig. 1; TB 8146) in animal PH713 and another in isolates from animals 4-256762 and 38-240440 (Fig. 1; TB 8277C and TB 8278A).
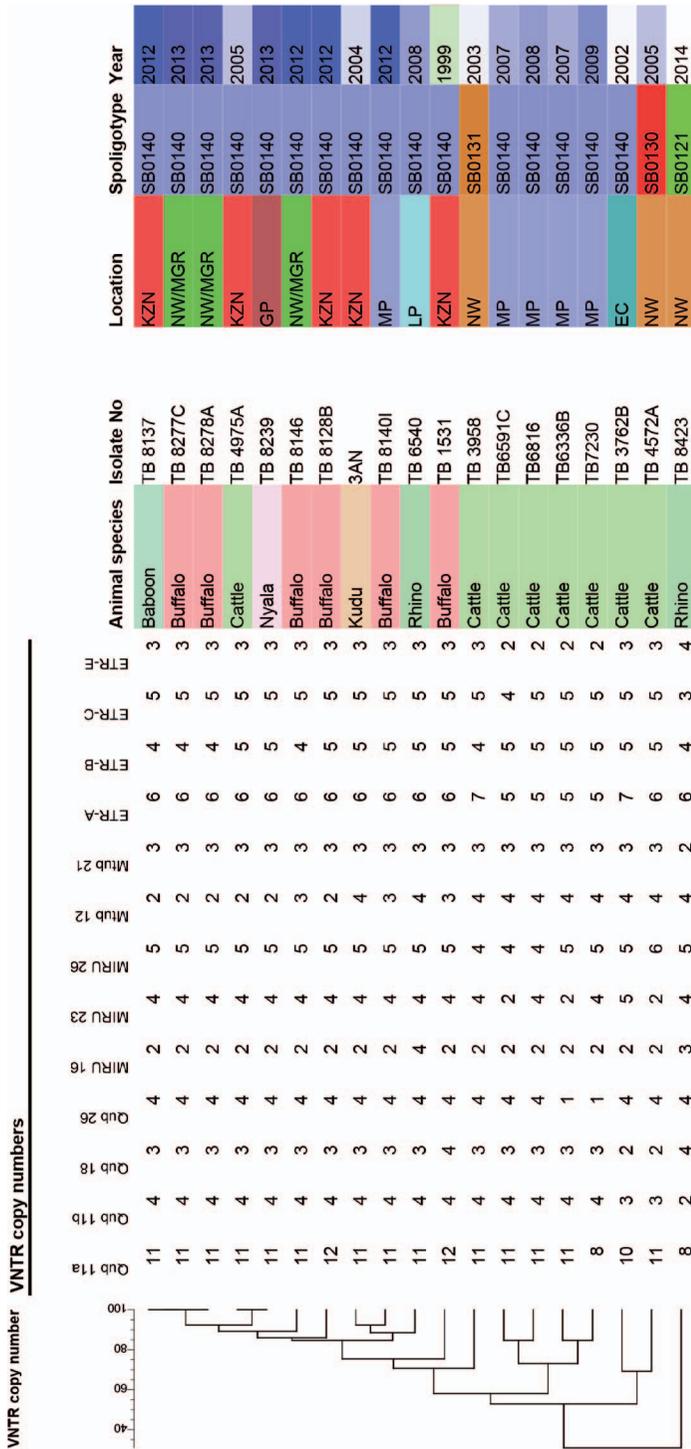
FIGURE 1.   Dendrogram based on variable number of tandem repeat (VNTR) typing data illustrating the genetic relationships of *Mycobacterium bovis* isolates from African buffaloes (*Syncerus caffer*) in South Africa. KZN=KwaZulu-Natal Province; NW=North West Province; MGR=Madikwe Game Reserve; GP=Gauteng Province; MP=Mpumalanga Province; LP=Limpopo Province; EC=Eastern Cape Province; Baboon=*Papio ursinus*; Buffalo=*Syncerus caffer*; Cattle=*Bos taurus*; Nyala=*Tragelaphus angasii*; greater Kudu=*Tragelaphus strepsiceros*; Black rhinoceros=*Diceros bicornis*.

Trace-back

To trace back the geographic source of infection, the two genetic profiles of the *M. bovis* strain from Madikwe GR were compared to those in the ARC-OVI database. The specific VNTR profile detected in Madikwe GR was identical to an *M. bovis* VNTR genotype identified in a moribund chacma baboon (*Papio ursinus*) sampled in 2012 from Mkuze GR in KwaZulu-Natal (KZN) Province (Fig. 1). The genotype from the Madikwe GR also had a close genetic relationship (≥85% similarity) with genotypes isolated from the following animal species between 2004 and 2013: 1) African buffalo (TB 8128B; isolated in 2012), KZN Province; 2) greater kudu (3AN; isolated in 2004), Spioenkop Nature Reserve, KZN; 3) nyala (*Tragelaphus angasii*, TB 8239, isolated in 2013) recently translocated from unknown origin to Gauteng Province, where it died; 4) cattle (*Bos taurus*; TB 4975A; isolated in 2005), KZN; 5) African buffalo (TB 8140I, isolated in 2012), Mpumalanga Province via KZN; and 6) black rhinoceros (*Diceros bicornis*; TB 6540, isolated in 2008) from the National Zoological Gardens in Limpopo Province via Mkuze GR (Fig. 1). The Madikwe GR genotype was genetically unrelated to *M. bovis* genotypes isolated from cattle and a black rhinoceros (translocated via Mpumalanga Province) in other regions of the North West Province (Fig. 1).

DISCUSSION

We have used traditional outbreak investigations and molecular typing to confirm and characterize *M. bovis* isolated from African buffaloes in a reserve previously free of *M. bovis* infection in the North West Province of SA. The source and time of introduction of this *M. bovis* strain into the game reserve are unknown. We investigated several possible sources. The Madikwe GR is bordered by three cattle farms on the southern border and by communal villages in the west. The *M. bovis* strain we detected could not be linked to any of the cattle strains isolated in the North West Province (Fig. 1); the prevalence of BTB in the province is very low. It is unlikely that buffaloes in this particular game reserve could have contracted the disease from cattle on the neighboring farms. In addition, the game reserve had a well maintained electric fence bordering the communal grazing land to prevent wild animals from escaping and causing conflict with cattle owners. This measure is considered acceptably effective in preventing cattle from straying into the reserve. Alternatively, the disease could have been introduced with the founder buffalo between 1992 and 1996. This is, however, unlikely because of their negative skin test status and the negative tuberculosis history of the herds of origin. The introduction of the plains game species in large numbers and from different sources during the 8 yr prior to the confirmation of the index case is, in retrospect, considered a high-risk management intervention which most-likely facilitated the establishment of BTB in the resident buffalo population.

Spoligotype SB0140 was detected in the Madikwe GR buffaloes and also in cattle and wildlife species in other parts of the country (Hlokwe et al. 2014). Strains bearing the same spoligotype pattern are assumed to be a set of individuals derived relatively recently by clonal replication from a single ancestral cell (Smith et al. 2006). On the other hand, VNTR analysis allows for higher resolution of genetic patterns within the SB0140 clone and therefore leads to improved discrimination between isolates associated with individual, epidemiologically unrelated outbreaks. With further tracing, we found that the VNTR genotype of the *M. bovis* strain from Madikwe GR was identical to the genotype isolated from a moribund chacma baboon associated with the Mkuze GR in KZN Province. As shown in Figure 1, all isolates with more than 85% genetic similarity with the *M. bovis* strain in the Madikwe GR stemmed from KZN, with or without intermediate translocation. These molecular findings are strongly suggestive of an epidemiologic link between BTB detected in KZN and the Madikwe GR. The VNTR genotypes from the buffaloes in Madikwe GR,

the chacma baboon, greater kudu, and nyala are suggested to have evolved from the most-recent common ancestor, which is a genotype isolated from cattle (Fig. 1). These findings further support our knowledge that cattle historically served as source of BTB for wildlife species (Thorburn and Thomas 1940).

According to official premovement testing records, no *M. bovis* reactors were detected in any of the groups of buffalo sold in auctions during the previous 2 decades. However, this testing was sporadic and selective for prime animals. It is therefore plausible that, in the absence of a sound surveillance program for the entire African buffalo population, the presence of the BTB remained undetected until it had reached a prevalence that made it more likely for infected buffalo in prime body condition to be selected for preauction testing. This is supported by the observation that none of the three culture-positive buffaloes showed an indication of advanced or generalized tuberculosis. We speculated that the introduction of BTB in the game reserve could have been a recent event. The diagnosis of BTB in this game reserve suggests that control measures previously applied were inadequate due to the insidious nature of the disease. In SA, the mandatory test for BTB in African buffalo is the IDT test, and IFNγ is only recommended as ancillary. Overall, our findings emphasize that caution should be taken not to rely solely on ad hoc testing for the diagnosis of BTB in wild animals because the time of infection is unknown in most cases.

Knowledge of the fact that BTB-infected African buffaloes may have been sold to both national and international buyers further complicates the situation, as BTB may have been translocated with auctioned buffaloes to several properties free of *M. bovis* infection. Madikwe GR is on the national border with Botswana. There is also a concern of the disease spreading into Botswana, whose national cattle population is officially free of BTB (Jori et al. 2013). Detection of *M. bovis* in the buffalo population in Madikwe GR has already had negative financial implications for the reserve's income generation because the land remains under quarantine and no further buffalo sales have been allowed. Our study highlights the significance of *M. bovis* as a silent, growing, disease risk factor in game translocation in the absence of herd or property-based surveillance.

We highlight the importance of suitable long-term surveillance programs for early detection of BTB at the herd level. In SA, movement control measures in wildlife apply only to African buffaloes. The current rate of BTB spread among wildlife properties, however, suggests expansion of testing requirements to at least those species that previously have been diagnosed with BTB (Hlokwe et al. 2014) and those in high demand for sale at game auctions. In view of the limitations of available tests, we need to develop and validate diagnostic tests for wildlife species other than buffalo.

ACKNOWLEDGMENTS

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