Molecular characterisation of *Mycobacterium bovis* isolated from African buffaloes (*Syncerus caffer*) in Hluhluwe-iMfolozi Park in KwaZulu-Natal, South Africa

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Bovine tuberculosis (BTB), a chronic disease of mammals caused by *Mycobacterium bovis*, is a threat to South African wildlife. It has been reported that African buffaloes (*Syncerus caffer*) are reservoir hosts of BTB in South African wildlife populations. This study reports on the molecular identification and typing of 31 *M. bovis* isolates collected between 1993 and 2008, mainly from buffaloes but also from two lions and a bush pig, in the Hluhluwe-iMfolozi Park (HiP) in KwaZulu-Natal. To study the dynamics of BTB in the buffalo populations, 28 *M. bovis* isolates from the HiP and epidemiologically related parks were characterised using regions of difference deletion analysis for species identification and spoligotyping, variable number of tandem repeats (VNTR), polymorphic G–C-rich sequences and IS*6110* restriction fragment length polymorphism (RFLP) genotyping methods. At least three distinct *M. bovis* genotypes were found amongst HiP samples. The combination of VNTR typing (using a 16-loci panel) and IS*6110* RFLP revealed the presence of three additional genetic profiles in individual buffaloes, demonstrating that the highest level of discrimination was achieved by these typing methods. One of the observed spoligotypes (SB0130) was dominant and represented 75% of isolates from buffaloes. A novel *M. bovis* spoligotype (SB1474), which is reported for the first time in this study, was observed in 14.3% of isolates from buffaloes. Based on the observed genetic relationships, the findings suggest independent introductions from at least three unrelated sources. These findings improve the knowledge regarding the diversity of circulating *M. bovis* strains in the HiP.

Introduction

Bovine tuberculosis (BTB) is a chronic, contagious disease caused by *Mycobacterium bovis*, a Gram-positive acid-fast bacterium. This pathogen is particularly noted for its diverse host tropism, which includes humans, livestock and several wildlife species. The first report of BTB in cattle in South Africa was made by Hutcheon (1880), whilst it was first diagnosed in African buffaloes (*Syncerus caffer*) in the Hluhluwe-iMfolozi Park (HiP) in 1986 (Jolles 2004). Spillover of BTB to other species has been documented (Michel *et al.* 2006; Michel *et al.* 2009). The HiP is situated in KwaZulu-Natal, South Africa and the area of 100 000 ha is almost entirely surrounded by communal farmland and livestock. It is the fourth largest game reserve in South Africa and has a buffalo population of approximately 3500 heads. The molecular characterisation of *M. bovis* is essential in understanding disease transmission between species and the spatial distribution of this infection in wildlife populations. In a previous study, molecular characterisation of *M. bovis* isolated from wildlife in the HiP was performed using a combination of three genetic tools, namely spoligotyping and restriction fragment length polymorphism (RFLP), including IS*6110* and polymorphic G–C-rich sequence (PGRS) typing (Michel *et al.* 2009). In that study, *M. bovis* isolates, the majority of which were derived from buffaloes, had been collected in the HiP and two epidemiologically related parks between 1993 and 2000. With one exception (TB 328), which had a shift in one of the bands (Michel *et al.* 2009), the isolates all displayed the same IS*6110* RFLP banding pattern. In addition, spoligotyping yielded only one type identical to SB0130 in the international *M. bovis* database (www.mbovis.org). This report also showed that epidemiologically unrelated strains are in circulation in the HiP and in the greater Kruger National Park (Michel *et al.* 2009).

Previous studies showed that variable number of tandem repeats (VNTR) typing is more discriminatory for *M. bovis* isolates than spoligotyping (Allix *et al.* 2006). In a study by Romero and co-workers (2008), both spoligotyping and VNTR were found to display similar discriminatory capacity. On the basis of the above information, the aim of this study was to investigate the circulating strains of *M. bovis* in the HiP further. Regions-of-difference (RD) deletion analysis was used for the identification of the isolates and spoligotyping, VNTR, PGRS and IS*6110* RFLP techniques were used for genotyping.

Methods

Sample collection

Tissue samples, collected from tuberculin skin tests and/or buffaloes that had tested positive for gamma interferon during BTB surveys between 1993 and 2008, were transferred to the Tuberculosis Laboratory at the Onderstepoort Veterinary Institute (OVI) for bacterial culture. A total of 28 buffaloes, from the HiP (*n* = 20), Munyawana Game Reserve (*n* = 4), St Lucia Wetland game parks (*n* = 3) and a private game reserve (*n* = 1), were included in the study. Buffaloes from Munyawana and St Lucia Wetland game parks (both in KwaZulu-Natal) were originally from the HiP, but were moved to the new locations between 1997 and 2000. A buffalo from a private game reserve (situated in Mpumalanga Province) was born to a dam originating from the HiP. Additional samples included in this study were collected during ad-hoc post-mortem examinations from two lions (*Panthera leo*) from, respectively, the HiP and Munyawana Game Reserve, and a bush pig (*Potamochoerus larvatus*) from the HiP.

Bacterial isolation and molecular identification

The tissue samples were processed at the Tuberculosis Laboratory, OVI, according to standard procedures (Alexander *et al.* 2002; Bengis *et al.* 1996). The samples were inoculated onto Löwenstein-Jensen media supplemented with pyruvate and incubated at 37 °C for up to 10 weeks. Acid-fast bacterial isolates were subjected to polymerase chain reaction (PCR) amplification using primers that target a sequence encoding the MPB 70 antigen to identify *Mycobacterium tuberculosis* complex bacteria (Alexander *et al.* 2002; Bengis *et al.* 1996; Michel *et al.* 2009). *M. tuberculosis* complex bacteria were identified following the presence of a 372-bp PCR product. Deletion analysis was performed as described by Warren *et al.* (2006) for species identification. The RD4 and RD9 genomic regions of difference were targeted using a multiplex PCR approach (Warren *et al.* 2006). The PCR products were separated on a 2–3% agarose gel to allow for good definition of the bands. *M. bovis* isolates were confirmed by the presence of two specific bands of 268 bp and 108 bp for RD4 and RD9, respectively.

Genomic DNA extraction

A PUREGENE™DNA extraction kit was used to extract genomic DNA from isolates according to the manufacturer's instructions (Gentra Systems, Minneapolis), with minor modifications. Cells treated overnight with glycine were heat killed at 94 °C for 10 minutes and allowed to cool. A volume of 500 µL extraction buffer (50 g/L monosodium glutamic acid; 6.06 g/L Tris-HCl (pH 7.4); 9.3 g/L EDTA) was added to the culture and cells were suspended by using an inoculating loop. Following centrifugation, 300 µL lysis solution and 5 µL RNase A solution (4 mg/mL) (Roche Diagnostics, Randburg) were added and the sample was then incubated at 37 °C for 30 min. A volume of 150 µL protein precipitation solution was added and samples were centrifuged at maximum speed for 2 min, followed by precipitation of the aqueous phase with isopropanol (Merck, Halfway House). The resulting pellet was dissolved in 40 µL TE buffer (10 mM Tris-HCl; 1 mM EDTA). The extracted DNA was stored at –20 °C until use.

Genotyping

Either pure genomic DNA (extracted using the PUREGENE™ DNA extraction kit) or cell lysates (prepared by boiling the *M. bovis* cells for 25 min and storing at –20 °C until used) were used for the PCR-based typing methods (i.e. spoligotyping and VNTR typing). For RFLP typing, pure genomic DNA was used.

Spoligotyping

Spoligotyping was done according to a standardised international method (Kamerbeek *et al.* 1997) using a commercially available kit (Isogen Biosciences BV, Maarsen). *M. tuberculosis* H37Rv, *M. bovis* BCG and sterile distilled water were used as controls. The spoligotype patterns were compared to those stored in the *M. bovis* spoligotype database (www.mbovis.org).

Variable number of tandem repeats typing

Cell lysates or genomic DNA was used for PCR amplification. Initially, VNTR analysis, also called multiple locus variable analysis, was carried out using primers to amplify the ETR-A to ETR-F loci (Frothingham & Meeker-O'Connell 1998). Additional VNTR typing was performed on 10 loci (Mtub 2, Mtub 12, Mtub 21, Mtub 29, Mtub 30, Mtub 39, MIRU 2, MIRU 10, MIRU 16 and Qub 11a) as described by Le Fleche *et al.* (2002). PCR was performed using an Eppendorf AG 22331 Hamburg thermocycler (Merck Eppendorf, Hamburg). The PCR products were separated on a 2% agarose gel at 85 V for 3 h and visualised under 312 nm UV light (Spectroline® model TC-312A transilluminator, Westbury). A 100-bp ladder (Inqaba Biotechnology, Pretoria) was included as a marker. The band sizes were determined using Quantity One® 1-D analysis software installed in the Gel Doc system (Bio-Rad Laboratories, Hercules, CA), converted to copy numbers (for exact tandem repeats [ETRs], see Frothingham & Meeker-O'Connell 1998; for additional loci, see Le Fleche *et al.* 2002) and saved in a spreadsheet. The genotyping data were exported to Bionumerics software (Applied Maths, Sint-Martens-Latem). The categorical coefficient option was used for cluster analysis to create a dendogram.

Polymorphic G–C-rich sequence and IS*6110* typing

PGRS and IS*6110* RFLP typing were performed as previously reported (Cousins *et al.* 1998; Michel *et al.* 2008). The GelCompar II system (Applied Maths, Sint-Martens-Latem) was used for analysis of the IS*6110* RFLP results, with the similarity coefficient of Dice and the unweighted pair group method with arithmetic averages for clustering maximum tolerance at 1.2%. PGRS RFLP typing patterns results were analysed manually.

Results

Mycobacterium bovis isolation and molecular identification

Bacterial growth was observed on Löwenstein-Jensen culture slopes after 6–10 weeks of incubation. The presence of acid-fast bacilli was confirmed by means of Ziehl–Neelsen stains.

Isolates were classified as members of the *M. tuberculosis* complex following detection of a 372-bp fragment by PCR using primers targeting the sequence that codes for the MPB 70 antigen of the *M. tuberculosis* complex. Deletion analysis using multiplex PCR targeting RD4 and RD9 (Warren *et al.* 2006) further confirmed the isolates as *M. bovis* owing to the amplification of DNA products with band sizes of 268 bp and 108 bp, respectively (results not shown).

Spoligotyping

Spoligotyping was performed on all 31 *M. bovis* isolates: 28 from buffaloes; two from lions and one from a bush pig. The results (Figure 1 and Figure 2) revealed four different spoligopatterns. The most prevalent spoligotype pattern was found in 24 of the 31 (77.4%) *M. bovis* isolates, including 21 isolates from buffaloes, two from lions and that of the bush pig. This spoligotype pattern was marked by the absence of spacers 3, 9, 11, 16 and 39–43, which is consistent with SB0130 (www.mbovis.org). The strain type was identified in isolates collected from the HiP during the study period (1993–2008) and has been reported earlier (Michel *et al.* 2009). A second spoligotype pattern was found in four buffaloes (TB 6452, TB 6453, TB 6459 and TB 6461) sampled in 2007. This *M. bovis* spoligopattern has not been described in the *M. bovis* database before and was allocated a new code, SB1474. It is characterised by the lack of spacers 3, 6, 8, 9, 10, 11, 12, 16, 22, 23 and 39–43. A third spoligotype pattern was found in two *M. bovis* isolates from buffaloes sampled in 2007 and 2008 (TB 6458 and TB 6696, respectively). The pattern, designated SB0121, is delineated by the absence of spacers 3, 9, 16, 21 and 39–43. This pattern is similar to that for *M. bovis* BCG except that spacer 21 is present in the latter. Another spoligotype pattern was isolated in 2000 from a buffalo (TB 1714) that was translocated from the HiP and was kept in a boma at Munyawana Game Reserve. This particular pattern was characterised by the absence of spacers 3, 6, 8, 9, 10, 11, 12, 16 and 39–43. It is identical to SB0140 in the international *M. bovis* database.

Analysis of variable number of tandem repeats

Results of the VNTR analyses on all 31 isolates, using a total of 16 loci, are summarised in Figure 1. Comparison of the copy numbers of the VNTR profile of each isolate revealed five profiles. The most prevalent VNTR profile was found in 23 of the 31 *M. bovis* isolates from buffaloes, lions and a bush pig. This VNTR profile was essentially shared by TB 328 (second profile) and TB 1714 (third profile), except for the copy numbers relating to locus Mtub 12 (in TB 328) and Mtub 29 (in TB 1714). The remaining two VNTR profiles were found in four (TB 6452, TB 6453, TB 6459, TB 6461) and two *M. bovis* isolates from buffaloes (TB 6458 and TB 6696) collected in 2007 and 2008, respectively). The genetic diversity observed in these isolates were attributed to the use of a combination of the loci ETR-A, ETR-B, ETR-C, ETR-E, Mtub 2, Mtub 12, Mtub 21, Mtub 29 and Qub 11a.

Polymorphic G–C-rich sequences

PGRS typing was performed on five *M. bovis* isolates cultured from buffaloes sampled in 2007 (Figure 1: TB 6452, TB 6453, TB 6459, TB 6461 and TB 6458). The PGRS profiles of 16 isolates have been analysed previously and were found to share a unique profile (Michel *et al.* 2009). In the present study, three PGRS DNA profiles were observed. One profile (HiP-2) was present in three of the five *M. bovis* isolates analysed, whilst the two remaining isolates each exhibited a genetically different profile (HiP-3 and E, respectively) as shown in Figure 1.

IS*6110* typing

The same five *M. bovis* isolates as analysed by PGRS typing were subjected to IS*6110* typing. Three IS*6110* patterns were identified, namely C17, C18 and C8v. *M. bovis* isolates with identical PGRS patterns also displayed identical IS*6110* patterns (Figure 1).

Discussion

This study presents data obtained from *M. bovis* isolates from the HiP and three epidemiologically related game reserves. PCR-based genotyping tools (i.e. spoligotyping and VNTR typing) were primarily applied to characterise 31 *M. bovis* isolates, complemented with PGRS and IS*6110* typing on some of the samples for added discriminatory power.

Most *M. bovis* infections examined in this study were caused by a unique genotype, which corresponded to a spoligopattern that had previously been identified as SB0130 (Michel *et al.* 2009). These results were in line with those from VNTR typing, except for VNTR typing singling out one isolate (TB 328) on the basis of a difference in locus Mtub 2. This finding was supported by previous IS*6110* typing results (Michel *et al.* 2009). Based on the high degree of homology between TB 328 and the dominant VNTR typing profile, it is unlikely that TB 328 resulted from a separate introduction of *M. bovis* into the HiP; rather, it may be linked to the dominant strain through an evolutionary process.

Spoligotyping and VNTR typing results were also found to correspond to a unique genotype isolated in buffaloes sampled in 2007 (TB 6452, TB 6253, TB 6459, TB 6461). The spoligopattern has not been described in the HiP previously nor included in the international *M. bovis* database and was therefore designated a new code, SB1474. However, when IS*6110* and PGRS typing data were considered for this group of isolates, both markers were able to discriminate between two profiles (e.g. C17 and C18). The genetic diversity of these strains collected in 2007 suggests that they did not evolve from the dominant HiP strain (SB0130) and therefore most likely represent an independent introduction of *M. bovis* into the HiP.

During the period prior to 2000 buffalo TB 1714 was translocated from the HiP to Munyawana Game Reserve where it was euthanised and sampled in 2000. Spoligotyping revealed a strain, SB0140, which is related to the novel SB1474. The novel strain (SB1474) differs from SB0140 by deletion of spacers 22 and 23 in addition to the loss of spacers 3, 6, 8, 9, 10, 11, 12 and 16, which is descriptive of the BOVIS2 family
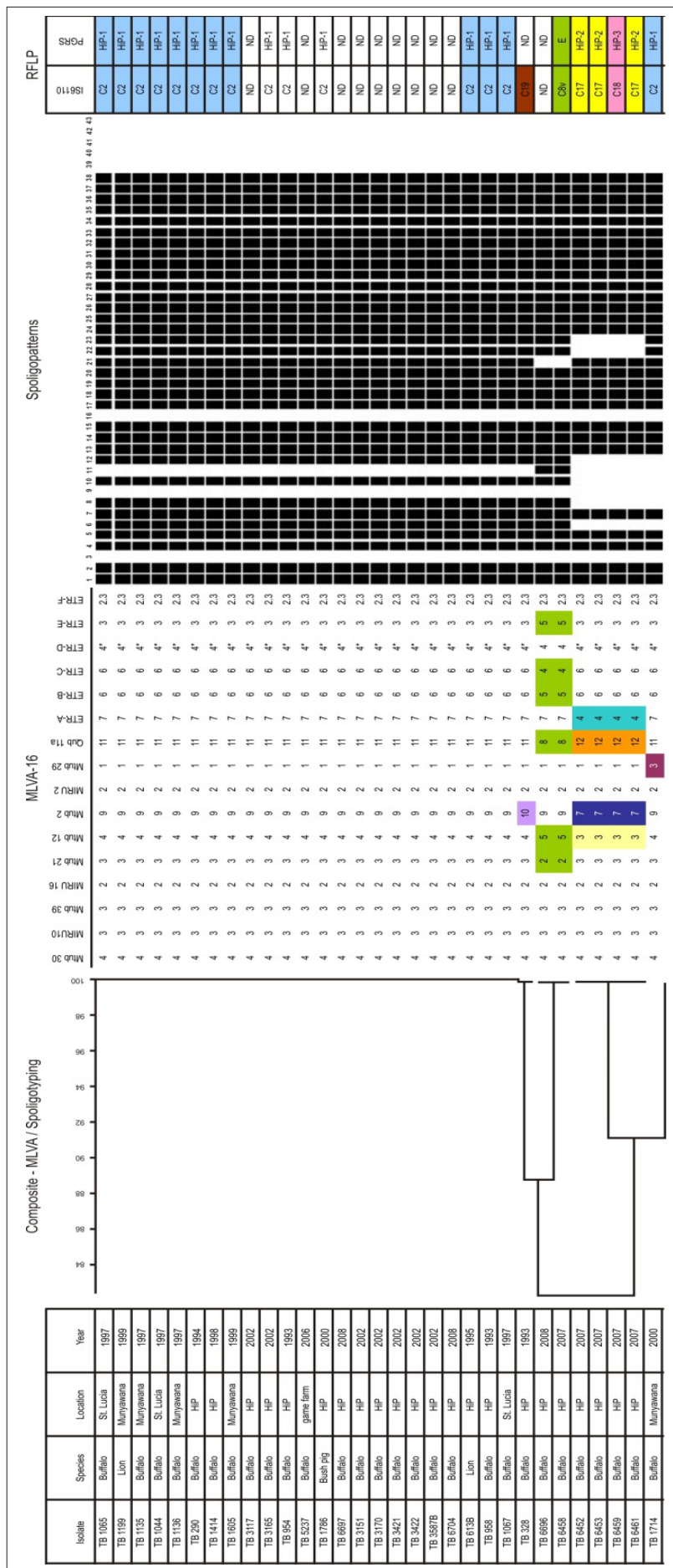
Note: Larger version of this image is availible as an online appendix. The different colours in the figure shows/highlights the difference between isolates.
HiP; Hluhluwe-iMfolozi Park, MLVA; Multiple Locus VNTR Analysis, RFLP; Restriction Fragment Length Polymorphism, PGRS; Polymorphic G-C rich Sequences, ETR; Exact Tandem Repeat, ND; not done.
*, indicates a 24-bp deletion in one of the tandem repeats in the VNTR-D locus.

FIGURE 1: Results from different typing methods applied to *Mycobacterium bovis* isolates from the Hluhluwe-iMfolozi Park and epidemiologically related game reserves.
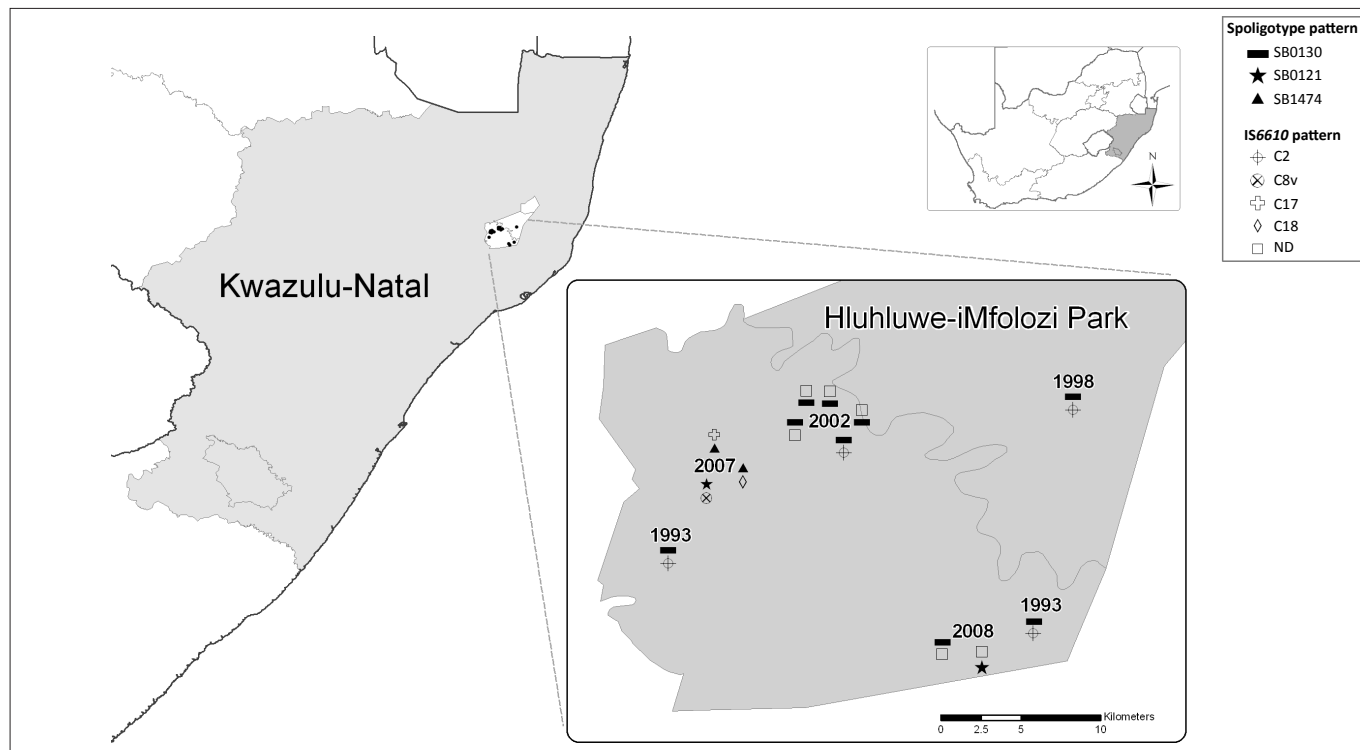
Locations of some isolates are not shown on the map owing to unknown coordinates.
ND, indicates that IS*6110* typing was not done.

FIGURE 2: Geographical distribution of spoligotype and IS*6110* patterns of *Mycobacterium bovis* isolates from the Hluhluwe-iMfolozi Park.

(Brudey *et al.* 2006). The origin of strain SB0140 remains elusive, despite identical PGRS and IS*6110* typing results with SB0130 as shown in Figure 1.

Evidence of another independent source of *M. bovis* in the HiP was found in two buffaloes (TB 6458 and TB 6696) sampled in 2007 and 2008, respectively, which both carried the spoligotype SB0121. This particular spoligotype pattern was previously isolated in cattle in Gauteng, Mpumalanga and the Limpopo provinces of South Africa (Michel *et al.* 2008), as well as in buffaloes and other wildlife in the Kruger National Park (Michel *et al.* 2009). Additional IS*6110* and PGRS typing (only on TB 6458) confirmed that this genotype was a member of the C8 variant strain complex, which is the cause of the BTB epidemic in the Kruger National Park (Michel *et al.* 2009). It is unclear whether the strain could have been introduced more recently from infected cattle or wildlife translocated from areas neighbouring the Kruger National Park to areas neighbouring the HiP. Alternatively, the C8 strain as found in the Kruger National Park may have been more widely spread in the eastern parts of the country than previously known and undergone similar evolutionary processes as described for the Kruger National Park (Michel *et al.* 2009). It is also possible that, at a particular time, strains present in buffaloes sampled in 2007 (western part of the HiP) and 2008 (eastern part of the HiP) may not have been present in buffaloes sampled before 2002 (eastern part of the HiP). Indeed, earlier behavioural studies indicate that HiP buffaloes have relatively stable and small home ranges (compared to those in the Kruger National Park) owing to the abundant year-round food and water availability in the HiP (Dora 2004)**.** Very little mixing, if any, of breeding herds

is known, with adult bulls implicated as the main source of infection among herds.

The use of molecular typing methods in the present study proved to be useful in confirming the origins of *M. bovis* strains translocated to, for example, St Lucia Wetland game parks in KwaZulu-Natal and a private game reserve in Mpumalanga. In all cases the *M. bovis* strain types were identical to the most prevalent type in the HiP. The methods show that the prevalent strain has spilled over to wildlife species other than buffaloes, as evidenced by the cases of two lions and a bush pig in the present study, and by IS*6110* typing in a previous study by Michel and co-workers (2009).

The identification of previously undetected *M. bovis* strains in the HiP is of great concern, since it indicates the possibility of multiple introductions in the past and a persistent risk for new *M. bovis* introductions into the park, as well as a risk of spillover to surrounding communal cattle farms. This presents a challenge for controlling the disease in livestock and has an impact on wildlife management and potentially also on public health (Michel *et al.* 2006; Romero *et al.* 2008).

Conclusion

The molecular typing methods described here have been useful in studying the epidemiology of BTB in the HiP and provide valuable information for future disease management strategies. This study further suggests that detection surveys for BTB are needed in communal cattle herds around the HiP to identify and efficiently manage sources of *M. bovis* infection.

Acknowledgements

Author's contributions

T.M.H. and A.O.J. contributed equally to the design of the study and preparation of the manuscript. E.M.S. performed spoligotyping of isolates. D.C. provided information relating to animals involved and their locations. A.L.M., J.G. and E.H.V. were involved in drafting the manuscript and revising it critically. All authors read and approved the final manuscript.

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