Evaluation of the discriminatory power of variable number of tandem repeat (VNTR) typing of Mycobacterium bovis isolates from southern Africa

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Abstract

The usefulness of variable number tandem repeat (VNTR) typing based on limited numbers of loci has previously proven inferior compared to IS*6110*-RFLP typing when applied to the study of the molecular epidemiology of bovine tuberculosis in both livestock and wildlife in southern Africa. In this study, the discriminatory power of 29 published VNTR loci in the characterization of 131 *Mycobacterium bovis* strains isolated predominantly from wildlife and a smaller number from livestock in southern Africa was assessed. Allelic diversities calculated when loci were evaluated on a selected panel of 23 *M. bovis* isolates with identified varying degrees of genetic relatedness from different geographic origins as well as *M. bovis* BCG ranged from 0.00-0.63. Of the 29 loci tested, 13 were polymorphic (Qub-11a,

Qub-11 b, Qub-18, ETR-B and C, Mtub21, MIRU16 and 26, ETR-E, Qub- 26, MIRU 23, ETR-A, and Mtub 12). In addition, a comparative evaluation of the 13 loci on a panel of 65 isolates previously characterized by IS*6110* Restriction Fragment Length Polymorphism (RFLP) typing and further evaluation on 41 isolates with no typing history from Kruger National Park highlighted that *M. bovis* from epidemiologically unrelated cases of bovine tuberculosis in different geographic regions can be adequately distinguished. However, there is a need for improvement of the method to fully discriminate between the parental Kruger National Park strain and its clones to allow the detection of evolutionary events causing transmission between and within wildlife species.

Keywords: Mycobacterium bovis, VNTR typing, Bovine tuberculosis, Wildlife, Kruger National Park

1. Introduction

Bovine tuberculosis (BTB) was first diagnosed in the Kruger National Park (KNP) in African buffaloes in 1990. Over the past twenty years, the disease has spread throughout the park and there is evidence showing that it has spilled over into at least 12 other small and large mammalian species (Michel and Bengis 2012). The establishment of the Greater Limpopo Transfrontier Park (GLTFP), which already links South Africa's KNP with the Limpopo National Park in Mozambique and a future link is envisaged with the Gonarezhou National Park in Zimbabwe, might increase the risk of the disease spreading within the GLTP and out of the park to livestock in neighbouring farming areas (Michel et al, 2006). In 2008, *M. bovis* was isolated from two African buffaloes in the Gonarezhou National Park, less than 45 km from the unfenced northern boundary of the KNP, raising questions regarding the origin of these cases (De Garine-Wichatitsky et al, 2010).

Bovine tuberculosis is a serious threat to livestock and wildlife species and is therefore controlled in many countries. A better understanding of transmission patterns and distribution of the disease will permit more precise targeting of control measures that will potentially benefit both wildlife and livestock (Aranaz et al, 2004, Michel et al, 2009). Molecular typing techniques are now useful tools that can be applied to M. bovis epidemiology in order to underpin control of bovine tuberculosis (Hilty et al, 2005; Allix et al, 2006, Boniotti et al, 2009, Hlokwe et al, 2011).

Restriction fragment length polymorphism (RFLP) typing techniques using IS*6110* and PGRS (Polymorphic G-C Rich Sequences) as probes as well as spoligotyping were applied to establish diversity of M. bovis strains within the KNP. The study revealed that the BTB epidemic was most likely caused by a single outbreak strain designated C8 (cattle strain). Due to a clonal expansion of the C8 strain in the wildlife population and accompanying changes in its genome, at least 21 C8 variant strains could be identified by IS*6110* RFLP typing. As expected, some variant strains of C8 were found to occur in spatial clusters and in wildlife species within these territories (Michel et. al., 2009).

Although IS*6110* RFLP typing provided useful insight into the epidemiology of bovine tuberculosis in the KNP, this technique is time consuming, technically demanding and requires large amounts of pure genomic DNA and complex data analysis (Allix et al 2006). Spoligotyping on the other hand, though PCR based and therefore rapid, could not differentiate the parent C8 strain from its variant strains because of slow evolutionary rate of the direct repeat region (Smith et al, 2003). Spoligotyping is therefore considered unsuitable to study recent M. bovis transmission events within and between wildlife species in the KNP.

More PCR (Polymerase Chain Reaction) based genotyping methods have become available for rapid molecular epidemiology investigations. Initially, six variable number of tandem repeat (VNTR) loci/ markers described as exact tandem repeats A to F (ETR-A, -B, -C, D, -E, and -F) were reported to be more discriminatory than spoligotyping (Frothingham and Meeker-O'Connell ,1998). A novel class of genetic markers collectively known as MIRU-VNTR was later described. This includes the ETRs, mycobacterial interspersed repetitive units (MIRUs) (Supply et al, 2000 and 2006) and VNTRs (LeFleche et al, 2002, Roring et al, 2002 and Skuce et al, 2002). Most of these differently named loci have received the reference names according to their position in the M. tuberculosis H37Rv genome (Supply 2006). VNTR typing is highly discriminative for M. tuberculosis isolates and therefore has the potential to be a method of choice for typing M. bovis (Hilty et al, 2005, Allix et al, 2006).

In the current study, we have assessed VNTR typing using 29 loci for molecular epidemiological characterization of *M. bovis* isolates from southern Africa, with special emphasis on isolates from the Kruger National Park. The aim was to select a set of loci that provides sufficient discriminatory power to detect evolutionary events indicative of recent transmissions.

2. Materials and methods

2.1 Bacterial isolation and identification

Frozen Mycobacterium bovis isolates previously typed by RFLP and/or spoligotyping methods were used. These M. bovis isolates had been collected between 1993 and 2008 from different wildlife species and cattle from different regions in South Africa (Table 1). The isolates were sub-cultured on Löwenstein-Jensen medium supplemented with pyruvate and

incubated at 37°C until confluent growth was observed. Additional M. bovis isolates were isolated from tissue samples received in the Tuberculosis Laboratory of the Onderstepoort Veterinary Institute for routine diagnostic purposes. These isolates were collected between the years 2004 and 2010 and no previous genetic analysis was done. Processing and isolation was performed according to standard procedures (Alexander et al, 2002). For identification, acid fast isolates were subjected to PCR amplification using primers that target a sequence encoding the MPB 70 antigen to identify Mycobacterium tuberculosis complex bacteria (Michel et al, 2009 and Hlokwe et al, 2011). M. bovis was confirmed by deletion analysis PCR as described by Warren and co-workers (Warren et al. 2006). The PCR products were separated in 1.5-2% agarose gels to allow a good separation of fragments. M. bovis isolates were confirmed by the presence of two specific bands of 268 bp and 108 bp for RD4 and RD9, respectively.

2.2 Bacterial DNA extraction

Depending on the amount of culture available, DNA from M. bovis isolates was extracted by either using a PUREGENE^{TM} DNA purification kit (Gentra Systems, Minneapolis, USA) or a crude preparation made by boiling M. bovis cells at 100°C for 25 minutes and storage at -20°C until use as previously reported (Hlokwe et al, 2011).

2.3 Variable number of tandem repeat (VNTR) typing of M. bovis isolates

The performance of available VNTR loci in the genetic characterisation of South African M. bovis isolates was evaluated in two stages. Initially, a total of 29 loci/markers were assessed for their ability to amplify the expected DNA product as reported by previous investigators, the repeatability of the amplification as well as for polymorphism within a selected panel of

M. bovis strains with high genetic diversity. Markers which failed on any of these criteria were excluded from further evaluation. Markers considered suitable were then compared based on their allelic diversity and combined discriminatory power among wildlife isolates from the KNP. IS*6110* RFLP profiles were available for the majority of the isolates and have assisted in the overall evaluation of the VNTR typing method.

Stage 1

A set of 29 loci, including the Exact Tandem Repeats (ETR-A, B, C, D, E and F), the Mycobacterial Interspersed Repetitive Units (MIRU02, 10, 16, 23, 26, 27, 39, 40), the Queen's University of Belfast VNTRs (Qub11a, b, 18, 26, 3232 and 3336) as well as Mtub 01,02, 12, 21, 29, 30, 31, 38 and 39 were applied as described by other authors (Frothingham and Meeker-O' Connell, 1998; Supply et al, 2000; Skuce et al, 2002; Le Fleche et al, 2002; Roring et al, 2004 and Supply et al, 2006). The PCR primer sequences are described in Table 2. The suitability of the loci was assessed on a genetically highly diverse panel (Panel 1) of 23 isolates from wildlife and livestock from southern Africa that were carefully selected based on profiling by RFLP typing, spoligotyping as well as traditional disease outbreak investigations (Hlokwe, T., unpublished results; Michel et al, 2008; Michel et al, 2009; and Hlokwe et al, 2011). The panel comprised of epidemiologically related and unrelated strains from different geographical regions and host species (Table 1). *Stage 2*

A set of 13 loci described in stage 1 (i.e. Qub11a, b, 18, and 26; Mtub 12 and 21, MIRU16, 23 and 26, and ETR-A, -B, -C and -E) were further subjected to a panel of 65 M. bovis isolates (Panel 2) from different wildlife hosts in the southern part of the KNP previously characterized by IS*6110* RFLP typing. . The M. bovis isolates included the parent C8 type (cattle strain) and 12 of its unique variant strains (Michel et al, 2009).

The same 13 loci were used to analyse 43 M. bovis isolates (Panel 3) from different wildlife species (i.e. Forty one of these cases were from the northern part of the park and isolates were never characterized before, whereas 2 cases were from the Gonarezhou National Park in Zimbabwe (Table 1)

2.4 PCR amplification of VNTR loci

Primer sets as outlined previously (Frothingham and Meeker-O'Connell, 1998 and Le Fleche et al, 2002) for the different loci were used for amplification. VNTR typing PCR was performed in a 20 µl reaction containing 2 µl of template DNA, 10 µl of the Qiagen mastermix, 7 µl of DNA free water and 0.5 µl of each 20 pM primer. The cycling parameters were as follows: initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 62 °C for 1 minute, elongation at 72 °C for 1.5 minutes and a final elongation step at 72 °C for 10 minute**.** PCR was done using an Eppendorf AG 22331 Hamburg thermo cycler (Merck Eppendorf, Hamburg, Germany).

2.5 Estimation of molecular size of the amplified DNA fragments and analysis of VNTR profiles

The PCR products were separated electrophoretically as outlined previously. The resulting fragment band sizes were estimated by comparison with the size marker (A 100 bp or 100 bp plus DNA ladder, Fermentas Life Sciences, Vilnus, Lithuania) and/or by use of the Quantity One 1-D analysis software installed in the Gel doc system (Bi-Rad Laboratories, Milan, Italy) (Hlokwe et al, 2011). The sizes were converted to copy numbers (Le Fleche et al, 2002) and the resulting VNTR profiles were saved in a spread sheet and analysed using the Bionumerics software package (Applied Maths, St-Martin-Latem, Belgium).

2.6 Calculation of allelic diversity and construction of phylogenetic trees

Allelic diversity of each locus was calculated using the Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat (Hunter and Gaston, 1988). The genetic relationships of M. bovis isolates were deduced by construction of an UPGMA (Unweighted Pair Group Mean Average) tree as well as the maximum parsimony tree using the Bionumerics software package version 6.6 (Applied Maths, St-Martin-Latem, Belgium).

2.7 Spoligotyping

All isolates not spoligotyped before (n=116) were subjected to spoligotyping for additional comparative evaluation of VNTR loci. Spoligotyping was done according to a standardized international method described by Kamerbeek et al, (1997) using a commercially available kit (Ocimum Biosolutions, Indianapolis, United States of America). 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).

3. Results

3.1 M. bovis isolates

In total, DNA from 131 M. bovis isolates was available for use in the study (Table 1). This group included 77 IS*6110* RFLP typed isolates of wildlife species from the southern part of KNP, 11 isolates from wildlife species (n=5) outside the KNP and cattle (n=6) from different geographical regions of South Africa with varying degrees of genetic relatedness (as determined either by IS*6110* RFLP typing or a combination of IS*6110* RFLP typing, PGRS typing, spoligotyping or traditional outbreak investigation), as well as a panel of 41 isolates

from different wildlife species from the northern part of KNP not characterized before, and 2 from Gonorezhou National Park which were only partially characterized using VNTR loci ETR-A to F (De Garine-Wichatitsky et al, 2010).

3.2 Variable number of tandem repeat (VNTR) analysis of M. bovis *isolates*

Two-stage evaluation of VNTR loci:

Stage 1

Twenty nine loci (Table 2) were assessed individually and comparatively on a test panel of 23 genetically diverse M. bovis isolates (Panel 1: Figure 1). Of the 29 loci studied, 13 were found to be stable and polymorphic (Qub11a, Qub11b, Qub18, ETR-B, ETR-C, Mtub21, MIRU26, MIRU16, ETR-E, Qub26, MIRU23, ETR-A, Mtub12) and 14 were monomorphic (Mtub1, Mtub2, Mtub29, Mtub30, Mtub 31, Mtub38, Mtub 39, MIRU2, MIRU10, MIRU27, MIRU39, ETR-D, ETR-F,Qub23). Two loci (Qub3232 and Qub 3336) could not be studied, since we either could not amplify DNA or unreliable results with multiple DNA fragments were produced during repeat runs (Table 3). All monomorphic loci as well as Qub3232 and Qub 3336 were regarded as unsuitable and therefore excluded from the evaluation.

The allelic diversity of individual loci was calculated and results are shown in Table 3. Ten loci (Qub-11a, Qub-11 b, Qub-18, ETR-B, ETR-C, Mtub21, MIRU16, MIRU26, ETR-E and Qub-26) were regarded as highly discriminating, with allelic diversity ranging from 0.42 to 0.63 and three loci (MIRU23, ETR-A and Mtub12) were grouped as moderately discriminating (allelic diversity ranging from 0.16-0.33). Lastly, 14 loci (Mtub1, Mtub2, Mtub29, Mtub30, Mtub31, Mtub38, Mtub39, MIRU2, MIRU10, MIRU27, MIRU39, ETR-D, ETR-F, and Qub 23) were regarded as poorly discriminating, with allelic diversity of 0.0 per locus and results

were discarded. Of the 13 polymorphic loci, only Qub26 correctly identified isolates KNP 70 and KNP 171 as C8 variant strains (from a total of 9 variants strains), with 2 and 3 copies of tandem repeats, respectively, at this locus, while the other KNP isolates harboured 4 copies. Isolate TB 1464, which was previously classified as a C8 strain, was identified by ETR-C locus as a C8 variant strain (Figure 1). Other loci were useful in discriminating between isolates from different geographical regions, as well as identifying isolates with known epidemiological linkage. Isolates from distinct geographic localities were found to have unique VNTR profiles, whereas epidemiologically related isolates harboured identical VNTR profiles (Figure 1 and 2).

Stage 2

The thirteen loci described above as stable and polymorphic were further evaluated on a panel of 65 IS*6110* RFLP typed isolates (Panel 2: Figure 1) from the southern part of the KNP, with the aim to compare the power of resolution of the combined loci to IS*6110* RFLP typing and spoligotyping. Among the 48 *M. bovis* isolates (73.8%) previously classified as representatives of the C8 parent strain by IS*6110* RFLP typing (Michel et al. 2009), 11 VNTR profiles were identified (VNTR 1, 2, 3, 4, 5, 6, 7, 10, 11, 12 and 14). On the other hand, within the 17 M. bovis isolates (26.2%) which were previously classified as representatives of the 12 unique C8 variant strains (C8V1-12) by IS*6110* RFLP typing (Figure 1), only four VNTR profiles (VNTR 1, 2, 3 and 13) were identified, three (VNTR 1, 2 and 3) of which matched profiles in the previous C8 group. In summary, 12 VNTR profiles were generated from this panel of isolates (Figure 1). Two most dominant profiles in this panel were found in 22 (33.8 %=VNTR2) and 15 (23%=VNTR1) of the isolates respectively. A combination of only 5 of the 13 loci contributed to profile variation. ETR-E had the highest allelic diversity

within this group of isolates (h=0.49), followed by ETRC (h=0.38), MIRU 26 (h=0.33), Qub 26 (h=0.09) and ETR-B (h=0.03) (results not shown).

The 13 loci were further applied to 43 isolates (Panel 3: Figure 1) collected from different wildlife species from the northern part of KNP including 2 isolates from Gonarezhou National Park. Two VNTR profiles (VNTR 1 and 5) were detected in this group and both matched profiles detected within the C8 group of isolates. The most frequent profile (VNTR 1) was found in 41 (95.3%) of the isolates. Qub 26 identified two isolates, i.e. TB 7118A and TB 7298F as C8 variants (Figure 1).

3.3 Spoligotyping

In this study, 116 M. bovis isolates were analyzed by spoligotyping. All isolates, those analysed in this study including those previously characterized, from Greater Kruger National Park complex (GKNPC) and the Gonarezhou National Park yielded one spoligopattern, identified as SB0121, while M. bovis from buffalo and cattle in other locations revealed diverse spoligotypes (i.e. SB0130, SB0267,SB0140,SB1235 and SB1474)

4. Discussion

The importance of bovine tuberculosis in wildlife as a potential source of infection for domestic animals as well as a threat to valuable wildlife species is well documented (de Lisle, 2002; Aranaz, 2004, Michel et al, 2009, de Garine-Wichatitsky et al, 2010, Hlokwe et al 2011). Knowledge of the epidemiology of bovine tuberculosis in free-ranging wildlife is an important factor in understanding the challenges associated with the management of the disease in this group of animals (de Lisle, 2002). The current study was aimed at evaluating the usefulness of the currently available loci used in VNTR typing for studying the

epidemiology of bovine tuberculosis in South and Southern Africa. A correct analysis of the spatial and inter-species spread of bovine tuberculosis in the KNP is a particular challenge because of the clonal nature of the epidemic and typing tools have to be highly discriminatory to allow detection of recently mutated variants of the parent outbreak strain.

Several studies have indicated that increasing the number of VNTR loci increases the chances of detecting possible genetic variation and thereby improves the resolution of VNTR typing (Ojo et al, 2008 and Millet et al, 2012). It is also cautioned that variability of the loci should be assessed as loci that proved to be discriminatory differ when tested in isolates from one country to another. Based on this information, a panel of 29 previously identified loci were evaluated individually and comparatively on a panel of 23 M. bovis isolates from South African wildlife species and cattle. The isolates were carefully selected and represented a broad spectrum of different levels of genetic relatedness. Of the 29 loci assessed, 13 loci were found to be polymorphic, with Qub11a having the highest allelic diversity (0.63). Qub11a was also found to be most discriminatory in studies conducted in Portugal (Duarte et al, 2010 and Matos et al, 2010), Republic of Ireland (Mc Lernon et al 2010), North America (Martinez et al, 2008), and also improved strain discrimination of M. bovis isolates from humans in Southwest Ireland (Ojo et al, 2008). In contrast, allelic diversity of this locus was found to be low in studies carried out in Spain (Romero et al, 2008). As in this study, Qub 11b, ETR-A, MIRU 26 and Qub 26 were also among the loci which provided good differentiation of strains in the Republic of Ireland (Mc Lernon et al, 2010) and Northern Ireland (Roring, 2004). ETR-B had the highest allelic diversity in M. bovis isolates from Chad (Hilty et al, 2005). ETR-D, MIRU02, MIRU10, MIRU39 and ETR-F were amongst the 14 loci that were not informative in this study. Similar findings were obtained

for M. bovis isolates from Chad. ETR-D, ETR-F, MIRU39 and MIRU 40 were also not polymorphic for 41 North American M. bovis isolates (Martinez et al., 2008). DNA amplification of Qub 3232 and Qub 3336 (also named VNTR 3232 and VNTR 3336 in other studies) was not possible in this study. Either several non-specific PCR products were obtained or no product was generated in some of the isolates, making it difficult to interpret results. These findings are in consensus with findings by other researchers. In some of these studies, PCR amplifications had to be repeated in order to obtain interpretable amplification products (Martinez et al, 2008, Boniotti et al, 2009). In contrast to our findings, a study done on 68 M. bovis isolates from Belgium found Qub 3232 to be the most discriminative locus, with allelic diversity of 0.76. On the other hand, the same study found Qub 3336 to be difficult to amplify and was therefore not recommended for routine use, even though it was found to be the second most discriminative marker, with allelic diversity of 0.74. In Spain, Qub 3232 was also found to be the most discriminatory locus of M. bovis isolates from alpacas (Rodriguez-Campos et al., 2012).

In this study, a comparative evaluation of the 13 polymorphic loci identified on isolates from the south of the Kruger National Park which were previously characterized by IS*6110* typing had highlighted the discriminatory superiority of VNTR typing over IS*6110* typing within the isolates designated C8 type. The isolates were further resolved into 11 VNTR profiles. The ability to recognize recent evolutionary events is a powerful tool for analysing population structure and history (Smith et al., 2003). The resolution power of VNTR typing using the 13 loci was, however, lower for the C8 variant strains group, with the approach detecting only 4 of the 12 unique C8 variants identified by IS*6110* RFLP (Figure 1). A total of 12 VNTR profiles were generated on a panel of 65 isolates (panel 2), with only Qub 26, MIRU26, ETR-

B, ETR-C and ETR-E contributing to the variation seen amongst isolates. The fact that locus ETR-E demonstrated the highest allelic diversity (h=49) within this panel of isolates highlighted its potential not only in discriminating genetically diverse isolates (panel 1) but also in discriminating M. bovis strains linked through clonal expansion. In contrast, Qub 11a was the most discriminatory locus within genetically diverse isolates, however, it was found to be monomorphic in strains linked through clonal expansion, and so were loci Qub 11b, Qub 18, ETRA, MIRU 16 and 23, Mtub 12 and 21.

Previous typing reports of isolates from Greater Kruger National Park Complex (GKNPC) have indicated that there were more IS*6110* C8 variants in the central and northern regions than in the southern regions (Michel et al, 2009). This finding suggested that the IS*6110* C8 strain was transmitted progressively from the high prevalence southern region into previously uninfected buffalo herds further north which was accompanied by potentially cumulative mutational events. In the current study, analysis on a panel of 41 isolates from different host species from the northern part of the park collected over a period of 6 years, as well as 2 isolates from Gonarezhou National Park did not yield this expectation, as no new additional profiles were detected.

Overall, our results showed that isolates from the Kruger National Park and Gonarezhou National Park (120 isolates) can be classified into 14 VNTR profiles, with two most prevalent profiles detected in 54.2% (VNTR-1) and 18.3% (VNTR-2) respectively, of the total isolates analysed. These two strains seem to spread actively throughout the park infecting different wildlife species while individually undergoing some evolutionary changes as clearly illustrated by the maximum parsimony tree (Figure 2).

All VNTR types in the Kruger National park have one locus difference compared to their neighbour VNTR types and this entire clonal complex corresponds to spoligotype SB0121. As reported previously, spoligotyping on its own lacked the ability to discriminate between strains within the GKNPC but successfully differentiated epidemiologically unrelated isolates from buffalo and cattle (Figure 1).

The value of the 13 VNTR loci was greatly highlighted when local isolates from different geographical areas within South Africa were characterized. All isolates with known genetic variations and those with identified epidemiological link were discriminated as such (Figure 1 and 2), making this typing approach an appropriate tool for molecular epidemiological studies of M. bovis isolates in South Africa.

In a recent study carried out on M. bovis isolates from Hluhluwe-iMfolozi Park, ETR-B, ETR-C and ETR-E were amongst the 9 loci which contributed to the genetic diversity observed amongst the 31 isolates analyzed, and there was no indication of the occurrence of mutational events (Hlokwe et al, 2011). These loci are amongst the loci described in this study as highly discriminative, highlighting their potential as tools to distinguish M. bovis isolates from South Africa.

With the limited sample size of isolates unrelated to the KNP epidemic included in this study, it was confirmed that a KNP strain and a non KNP strain can be clearly distinguished by application of the 13 loci (Figure 1 and 2). So far, our results confirm previous findings indicating that bovine tuberculosis in the KNP is a result of a single strain which seems to undergo evolutionary processes. In addition, this study further supports the emphasis on the importance of evaluating the different loci for each country or geographical setting. Although the composition of panels 1 and 2 was intentionally biased towards genetically

diverse strains to allow best possible evaluation of the discriminatory power of VNTR loci, panel 3 was included for comparison of the marker performance in randomly selected, previously uncharacterised *M. bovis* from KNP.

5. Conclusion

The outcome of this assessment highlighted that molecular typing using 13 loci seems to be the best selection we can use far across regional South African M. bovis isolates. This combination of loci is good enough for application in epidemiological studies but not adequate for Kruger National Park, where active clonal expansion is observed. There is still room for further improvement of the VNTR typing method to be able to fully discriminate between the parent KNP strain and its variants to detect recent transmissions between and within wildlife species after evolutionary events. For this purpose, we recommend a search for new additional VNTR loci from whole genome sequence data of local M. bovis isolates.

6. Acknowledgements

This work was funded by the Department of Agriculture and the Department of Science and Technology. The authors would like to thank Dr Claude Sabeta for critically reviewing the manuscript.

Table 1. Animal species and numbers of isolates from different regions analysed in this study

Animal species	Location							
	KNP	**Gonarezhou**	**SNR**	**MP***	**KZN**	**NW**	**HiP**	**Total**
Cattle				4	1	1		6
Buffalo	51	2	1				4	58
Lion	42							42
Kudu	12							12
Baboon	6							6
Leopard	4							4
Cheetah	1							1
Warthog	1							1
Bushbuck	1							1
Total	118	2	1	4	1	1	4	**131**

KNP, Kruger National Park; HiP, Hluhluwe-iMfolozi Park, SNR, Spionkop Nature reserve;

*MP, Different regions in Mpumalanga Province; KZN, KwaZulu Natal Province; NW, North west Province

Table 2. Variable number of tandem repeat (VNTR) loci and PCR primer sequences used in this study

VNTR locus	VNTR alias	VNTR locus size (bp)	Forward primer	Reverse Primer
2163a	QUB 11a	69	CCCATCCCGCTTAGCACATTCGTA	TTCAGGGGGGATCCGGGA
2163b	QUB 11b	69	CGTAAGGGGGATGCGGGAAATAGG	CGAAGTGAATGGTGGCAT
1982	QUB 18	78	ATCGTCAGCTGCGGAATAGT	AATACCGGGGATATCGGTTC
2461	ETR-B	57	GCGAACACCAGGACAGCATCATG	GGCATGCCGGTGATCGAGTGG
0577	ETR-C	58	GACTTCAATGCGTTGTTGGA	GTCTTGACCTCCACGAGTGC
1955	Mtub 21	57	AGATCCCAGTTGTCGTCGTC	CAACATCGCCTGGTTCTGTA
2996	MIRU 26	51	CCCGCCTTCGAAACGTCGCT	TGGACATAGGCGACCAGGCGAATA
1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA	CCCGTCGTGCAGCCCTGGTAC
3192	ETR-E	53	ACTGATTGGCTTCATACGGCTTTA	GTGCCGACGTGGTCTTGAT
4052	QUB 26	111	AACGCTCAGCTGTCGGAT	GGCCAGGTCCTTCCCGAT
2531	MIRU 23	53	CAGCGAAACGAACTGTGCTATCAC	CGTGTCCGAGCAGAAAAGGGTAT
2165	ETR-A	75	ATTTCGATCGGGATGTTGAT	TCGGTCCCATCACCTTCTTA
1121	Mtub 12	15	CTCCCACACCCAGGACAC	CGGCCTACCCAACATTCC
0024	Mtub 1	18	GACAAACAGGAGGGCGTTG	TATTACGACGACCGCTATGC
0079	Mtub 2	9	CGTGCACAGTTGGGTGTTTA	TTCGTTCAGGAACTCCAAGG
2347	Mtub 12	57	AACCCATGTCAGCCAGGTTA	ATGATGGCACACCGAAGAAC
2401	Mtub 30	58	AGTCACCTTTCCTACCACTCGTAAC	ATTAGTAGGGCACTAGCACCTCAAG
2990	Mtub 31	55	GTGACGTTTACCGTGCTCTATTTC	GTCGTCGGACAGTTCTAGCTTT
3663	Mtub 38	63	GCCCAAAAAGCATGGGAACGTGCCCCT	GGTTGTCCCCGCAGTATCTC
3690	Mtub 39	58	AATCACGGTAACTTGGGTTGTTT	GATGCATGTTCGACCCGTAG
0154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT	TACTCGGACGCCGGCTCAAAAT
0959	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC	GCCACCTTGGTGATCAGCTACCT
3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA	GCGATGTGAGCGTGCCACTCAA
4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC	CGGAAACGTCTACGCCCCACACAT
0577	ETR-D	77	CAGGTCACAACGAGAGGAAGAGC	GCGGATCGGCCAGCGACTCCTC
1612	QUB 23	21	GCTGCACCGGTGCCCATC	CACCGGAGCCGGAACGGC
3239	ETR-F	79	CTCGGTGATGGTCCGGCCGGTCAC	GGAAGTGCTCGACAACGCCATGCC
3232	QUB 3232	56	CAGACCCGGCGTCATCAAC	CCAAGGGCGGCATTGTGTT
3336	QUB 3336	59	ATCCCCGCGGTACCCATC	GCCAGCGGTGTCGACTATCC

Table 3. Determination of heterogeneity at each of the loci among the 23 genetically diverse M. bovis from different geographic regions in southern Africa (Panel 1) and M. bovis BCG.

Locus	No. of isolates and Copy number													Allelic Diversity
	1	2	3	4	5	6	7	8	9	10	11	12	15	
Qub 11a								14		4	3	2	1	0.63
Qub 11b		14	5	5										0.60
Qub 18		6	4	14										0.60
ETR-B				14	10									0.50
ETR-C			14		10									0.50
Mtub 21		14	10											0.50
MIRU 26			1		16	7								0.49
MIRU 16		8	16											0.46
ETR-E			8	16										0.46
Qub 26		3	3	18										0.42
MIRU 23		5		19										0.33
ETR-A				1	1	20	2							0.30
Mtub 12			2	22										0.16
Mtub 1									24					0.00
Mtub 2							24							0.00
Mtub 29			24											0.00
Mtub 30				24										0.00
Mtub 31			24											0.00
Mtub 38			24											0.00
Mtub 39			24											0.00
MIRU 2		24												0.00
MIRU 10		24												0.00
MIRU 27			24											0.00
MIRU 39		24												0.00
ETR-D			24											0.00
Qub 23						24								0.00
ETR-F	24													0.00
Qub 3232	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Qub 3336	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: - indicates that no interpretable results were obtained

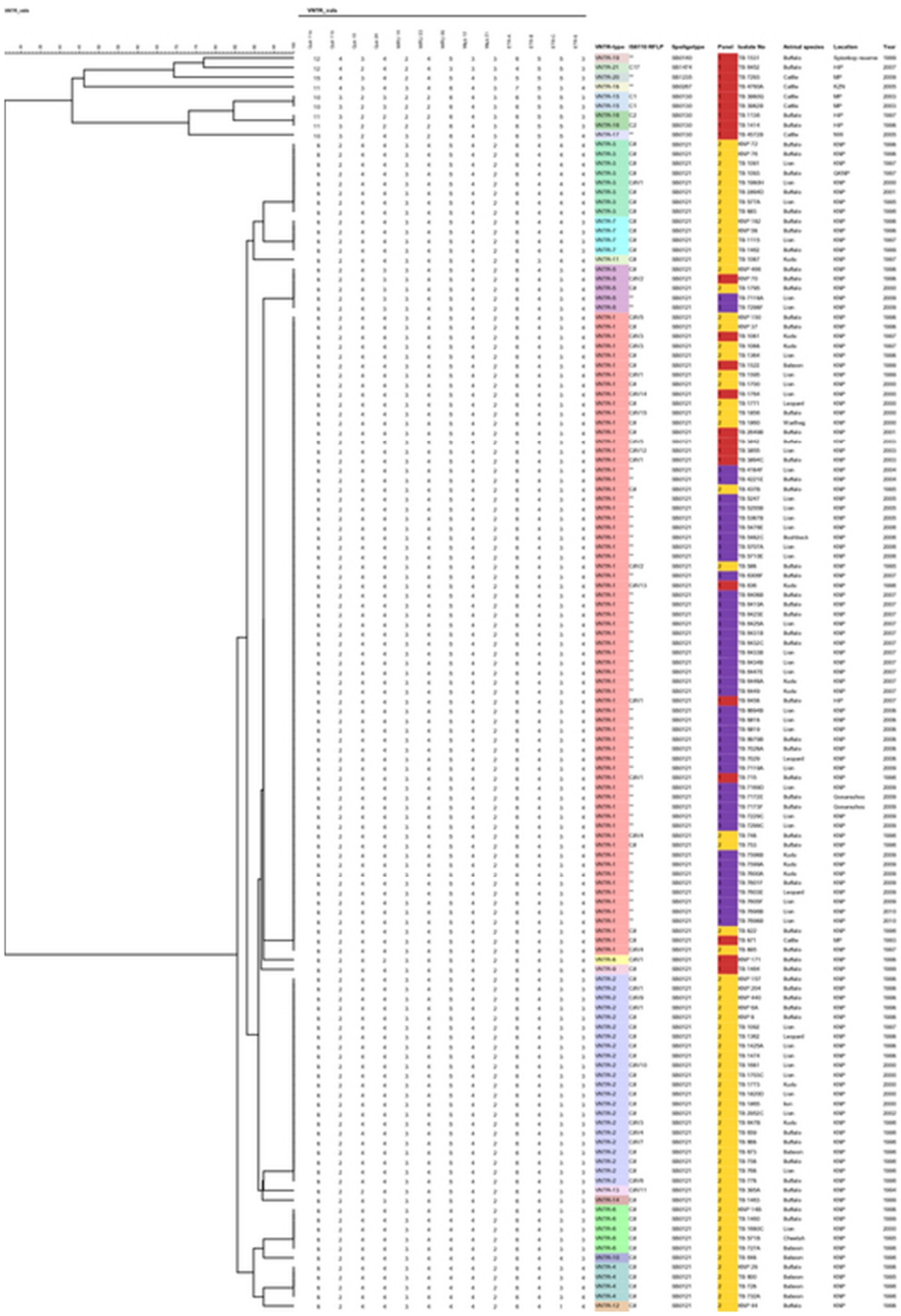

Figure 1. Dendogram and schematic representation of 131 M. bovis isolates typed for 13 VNTR loci as well as IS*6110* RFLP typing and spoligotypes of the isolates. The dendogram was generated as described in the Materials and Methods. Please note: KNP; Kruger National Park, HiP; Hluhluwe-iMfolozi Park, MP; Mpumalanga Province, NW; Northwest Province, KZN; KwaZulu Natal Province, C8V; C8 variant, VNTR; variable number tandem repeat, ETR; Exact tandem repeat, RFLP; Restriction Fragment Length Polymorphism and asterisk denotes no IS*6110* RFLP typing was done

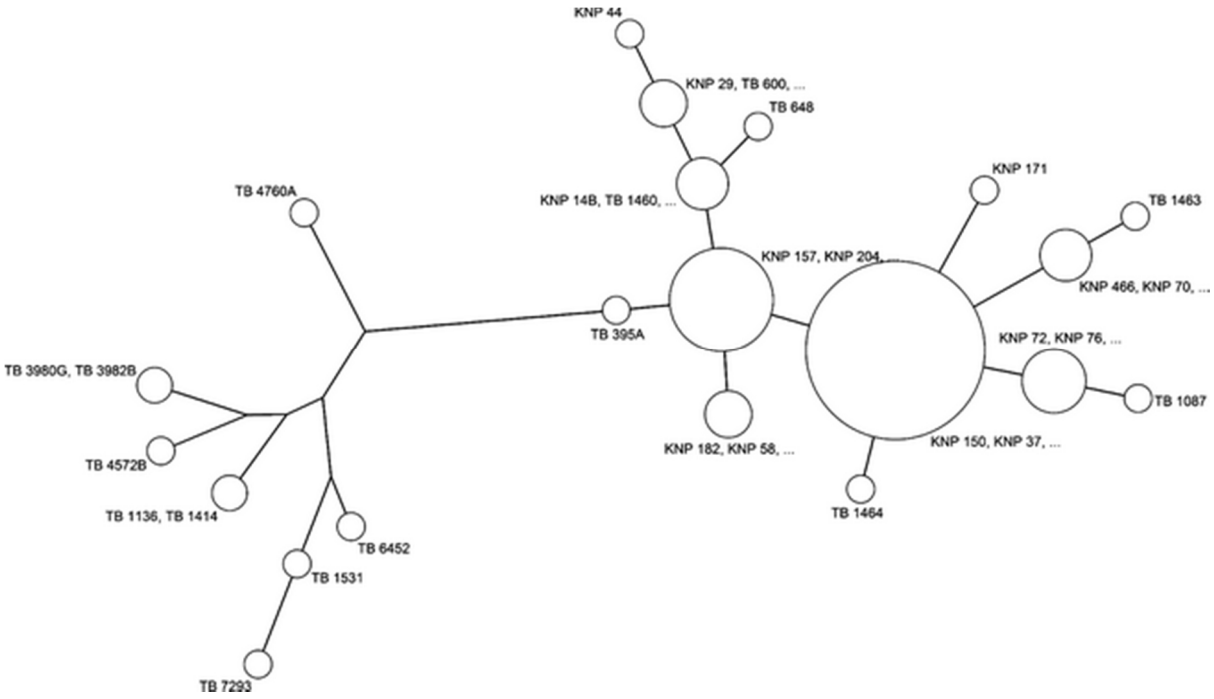

Figure 2 Maximum parsimony tree for the 131 isolates based on 13 polymorphic VNTR loci. The sizes of the circles differ according to the number of isolates represented.

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