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High *Mycobacterium bovis* genetic diversity in a low prevalence setting

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Abstract

The genetic diversity among South African *Mycobacterium bovis* isolates from cattle was determined by genetic fingerprinting. The restriction fragment length polymorphism (RFLP) markers IS*6110* and polymorphic GC-rich sequence (PGRS) as well as spoligotyping and determination of variable number of tandem repeats (VNTR) were used to characterize sub samples of 91 *M. bovis* field isolates. PGRS RFLP was the single most discriminatory method and combinations of typing methods, which included IS*6110* and/or PGRS had the highest discriminatory power, able to reveal 29 distinct genotypes among 35 farms with no epidemiological link. Three of the farms were co-infected with two genetically unrelated strains.

In contrast to reports from European and also other colonised countries on the African continent our findings are suggestive of a high genetic diversity of *M. bovis* in South Africa's cattle population, implying a variety of unrelated ancestor strains. Despite effective intervention through test-and-slaughter campaigns no indication of a 'founder effect' was apparent in the panel of isolates derived from all infected provinces.
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Keywords: *Mycobacterium bovis*; Cattle; RFLP; IS*6110*; PGRS; Spoligotyping; VNTR typing; Genetic diversity

1. Introduction

Bovine tuberculosis is believed to have been introduced into South Africa and possibly the sub region by European settlers (Hutcheon, 1880). In addition, cattle imports from Australia, Argentina and Madagascar in the early 20th century were reported to often include infected animals (Cousins

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et al., 2004). The introduction of a National Tuberculosis Scheme in 1969 resulted in the reduction of infected commercial cattle herds from 11.85% in 1971 to 0.39% in 1995, but ever since sporadic outbreaks continued to occur. Between 1993 and 2005 a total of 209 outbreaks were reported, with a peak of 20 outbreaks in 2003 (Animal disease statistics, National Department of Agriculture). Presently, the control of bovine tuberculosis is based on intradermal tuberculin testing and slaughter as well as on abattoir surveillance. Genetic typing of *Mycobacterium bovis* has contributed to a greatly improved knowledge of inter-bovine and interspecies transmission of bovine tuberculosis (Durr et al., 2000). This understanding is crucial to the effective management of bovine tuberculosis control schemes and the wildlife–livestock interface in countries where wildlife reservoirs for *M. bovis* have been identified, including South Africa (Haddad et al., 2004; Skuce and Neill, 2001). In the absence of a 'best' technique, the most widely used DNA typing techniques for *M. bovis* include IS*6110* and PGRS restriction fragment length polymorphism (RFLP) typing, spoligotyping and variable number of tandem repeat (VNTR) typing (Van Soolingen, 2001). In this study these techniques were used to conduct the first comparative genetic analysis of *M. bovis* isolates from South African cattle. We aimed at determining the genetic diversity of *M. bovis* among domestic cattle in South Africa using established typing methods with proven reproducibility in our setting.

2. Materials and methods

2.1. Sample collection

A total of 90 *M. bovis* isolates from 42 South African cattle herds in six provinces and one additional isolate from Swaziland were used in this study. Thirty-nine isolates were derived from a field investigation in the year 2003 into a multiple farm outbreak assumed to involve 12 farms (Mpekwane et al., 2004). Fifty-two *M. bovis* isolates from 35 infected farms in all six provinces with known occurrence of bovine tuberculosis had been collected as a result of routine sample submissions by state veterinary officials between 1993 and 2000. This represents 23% of the 148 outbreaks reported in South Africa during this period. No selection took place but all viable *M. bovis* isolates available in the laboratory were used for a retrospective characterization study involving combinations of RFLP typing, spoligotyping and VNTR typing. It was postulated that the typing method or combination of methods distinguishing the highest number of known unrelated outbreak strains would be considered the most discriminative approach. To facilitate this interpretation within the epidemiological context, isolates were classified into the following panels and evaluated against their specific background:

2.1.1. Isolates from epidemiologically related herds

2.1.1.1. Isolates from the same herd. Between two and four isolates were analysed from each of 15 farms (farms 3, 4, 7–11, 19, 22, 25, 26, and 36–39). In addition, 28 isolates from farm 40 were examined.

2.1.1.2. Isolates from different but epidemiologically linked herds. Several months after a dispersal sale of an infected cattle herd in the Mpumalanga Province a back tracing investigation by the veterinary field officials indicated the possible spread of *M. bovis* to 12 different farms in various districts, and another province. Following slaughter of tuberculin reactors *M. bovis* was isolated from 39 cattle from eight of the suspected farms (farms 36–43) (Fig. 1).

An epidemiological link had also been suspected between two other farms. Bovine tuberculosis had been diagnosed on farm 26 which had introduced cattle from farm 27 several months before. Subsequent skin testing confirmed the presence of bovine tuberculosis infection on farm 27.

2.1.2. Isolates from epidemiologically unrelated herds

Thirty-three of the 35 farms sampled between 1993 and 2000 were, to the best of our knowledge, epidemiologically unrelated. Farms 26 and 27 were linked to each other but not any other farm. The eight herds described under Section 2.1.1.2 were considered as one epidemiological unit and unrelated to the other 34 unlinked herds. The total number of outbreaks considered unrelated was 35.

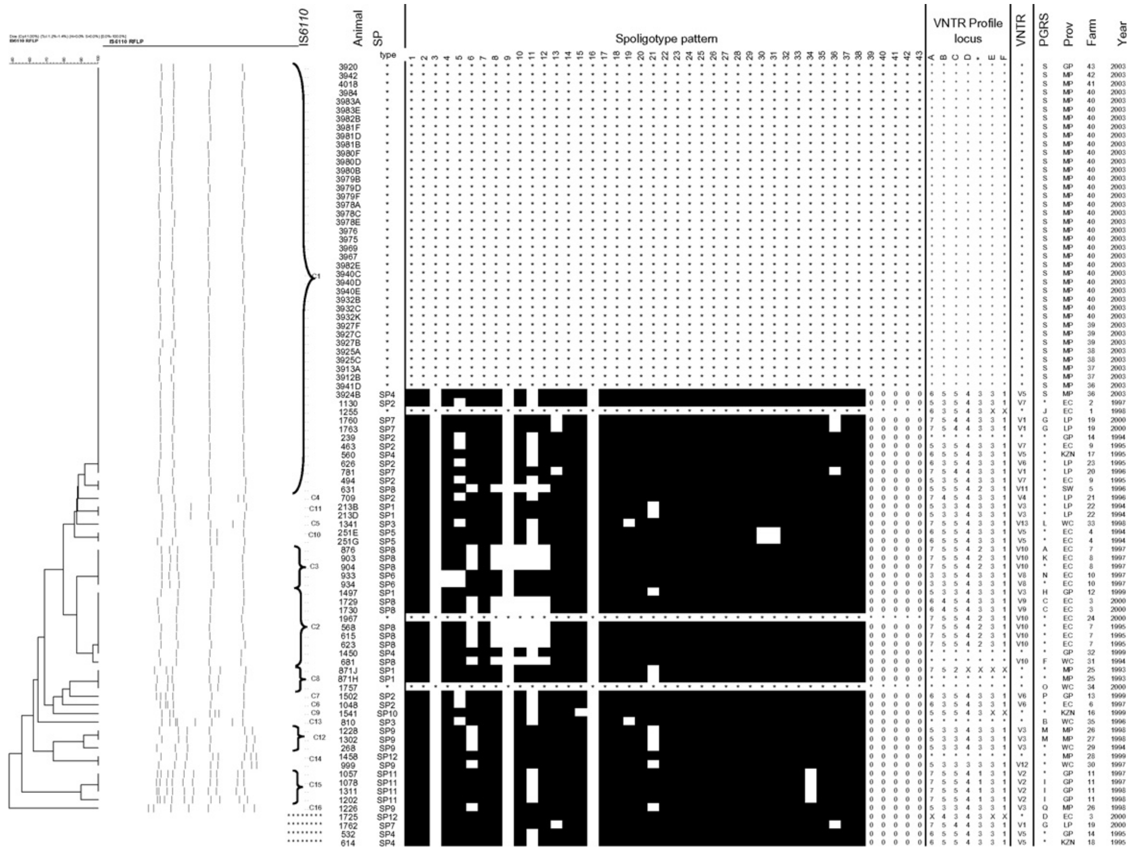

Fig. 1. DNA fingerprint results of 91 *M. bovis* isolates from cattle ordered by IS*6110* RFLP similarity, followed by spoligopatterns, VNTR profiles and PGRS RFLP types as well as farm identifications, province of origin, year of collection. *Data not available. MP: Mpumalanga Province; EC: Eastern Cape Province; GP: Gauteng Province; LP: Limpopo Province; KZN: Kwazulu/Natal Province; and WC: Western Cape Province.

2.2. Bacterial isolation

All samples were processed in the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute for culture according to standard procedures (Bengis et al., 1996; Alexander et al., 2002). Pure subcultures from all *M. bovis* isolates on Löwenstein–Jensen medium containing pyruvate were routinely stored at −20 °C.

2.3. DNA extraction

DNA extraction from *M. bovis* isolates was accomplished from colonies of either fresh or revived subcultures on Löwenstein–Jensen medium with pyruvate. Following heat-inactivation at 80 °C for 60 min, the colonies were scraped off and suspended in 5 ml of extraction buffer (50 g/l mono sodium glutamic acid; 6.06 g/l Tris–HCl (pH 7.4); 9.3 g/l EDTA) (Warren, personal communication), to which lysozyme (50 mg/ml) and RNAseA (10 mg/ml) were added. The suspensions were incubated for 2 h at 37 °C after which proteinase K at a final concentration of 0.5 mg/ml was added. After incubation at 45 °C overnight phenol/chloroform extraction was performed. The final pellet was resuspended in 40 μl TE (1 mM Tris–HCl (pH 7.6); 0.1 mM EDTA).

2.4. Genetic typing

2.4.1. IS6110 RFLP typing

For IS*6110* RFLP typing approximately 1.5 μg of mycobacterial DNA was digested overnight with 1.5 units of PvuII. Subsequently, the resulting frag-

ments were separated by electrophoresis on a 0.8% agarose gel. DIG-labelled molecular weight size marker VII (Roche) was loaded in the first, middle and last lane of the gel. Southern blot transfer was performed as described by Skuce et al. (1994). IS*6110*-containing DNA fragments were detected through hybridization with the entire IS*6110* sequence as a probe, according to the manufacturer's instructions (Roche Molecular Biochemicals-The DIG System User's Guide, 1995).

2.4.1.1. Analysis of IS6110 RFLP patterns. The GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to determine the level of similarity between the IS*6110* RFLP patterns by using the similarity coefficient of Dice and the unweighted pair group method with arithmetic averages (UPGMA) for clustering maximum tolerance 1.2%). DIG-labelled Molecular Weight Marker VII (Roche Diagnostics) was used as external marker for normalisation. Strains with a similarity coefficient of greater than 90% were considered identical provided they contained the same number of bands.

2.4.2. PGRS RFLP typing

For PGRS RFLP typing 1.5 μg of *M. bovis* DNA was digested with AluI and electrophoresis of DNA fragments was performed on a 1.2% agarose gel (Cousins et al., 1998). Southern blotting and detection after hybridization at 55 °C with a DIG-labelled PGRS oligonucleotide probe (5′-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3′) were performed according to the manufacturer's instructions (Roche Diagnostics). Banding patterns were analysed manually.

2.4.3. Spoligotyping

Spoligotyping was performed according to the protocol of Kamerbeek et al. (1997). With this method, the direct repeat region of the isolates was amplified by PCR, and the PCR products were hybridized to 43 oligonucleotides of known spacer sequences by reversed-line blot hybridization. Because one of the primers for PCR was biotin labelled, and hence the PCR product, the presence of spacers was detected after incubation with streptavidin-peroxidase and enhanced chemiluminescence detection (ECL Detection kit, Amersham Biosciences). The spoligopatterns were compared and assigned a *M. bovis* spoligotype number (SP number). The spoligopatterns were also compared to the international database on http://www.mbovis.org and new patterns were reported and assigned a unique SB code.

2.4.4. VNTR typing

VNTR typing was performed according to the method of Frothingham and Meeker-O'Connell (1998), at the Veterinary Laboratory Agencies Weybridge, United Kingdom. Primer pairs for ETR loci A to F were similar to those published previously (Frothingham and Meeker-O'Connell, 1998), except for the following minor changes: the ETR-B forward primer had a GGTT extension and the ETR-B reverse primer had a CTATA extension to improve annealing. The ETR-F forward and reverse primers were shortened by 3 and 4 bp, respectively, at the 5′ side of the probes (ETR–B forward: 5′-GCGAACACCAGGACAGCATCATGG-GTT-3′, ETR-B reverse: 5′-GGCATGCCGGTGATC-GAGTGGCTATA-3′, ETR-F forward: 5′-GGTG-ATGGT CCGGCCGGTCAC-3′ and ETR-F reverse 5′-GTGCTCGACAACGCCATGCC-3′). Each PCR reaction consisted of 10 μl of Qiagen Hotstar Mastermix (Qiagen), 0.5 μl of each of the primers for each locus (at 10 pmol/μl for all primers except for ETR-A primers which were used at 20 pmol/μl) and 2 μl of heat-killed cell supernatant in a final volume of 20 μl. Following an initial denaturation at 94 °C for 15 min each sample was subjected to 30 cycles of 94 °C for 30 s, 68 °C for 60 s, and 72 °C for 2 min. Followed by an extended annealing temperature of 72 °C for 10 min. Heat-killed cell supernatants of *M. tuberculosis* H37RV were used in each set of reactions as a positive control. PCR products were separated on a ABI 377 Sequencer, and analysed using ABI Prism Genescan software. The sizes of VNTR loci alleles were estimated by comparison to a ROX size standard (Applied Biosystems). The PCR products were compared to size standards and converted to repeat numbers at each locus using standard allele naming tables (ABI Genotyper software).

The VNTR genotype of a strain, representing the number of repeat elements at each locus, is presented as a series of integers representing the A to F VNTR loci, respectively. The ETR-D locus contains a 24 bp deletion in one of the repeats and the naming

convention indicates the presence of this deletion by a * i.e. 4* (=3 × 77 bp repeats and one 53 bp repeat). The ETR-F locus contains 79 bp tandem repeats and 55 bp tandem repeats. The naming convention indicates the number of 79 bp repeats followed by 55 bp repeats separated by a period.

3. Results

3.1. Identification of bacterial isolates

All isolates from cattle tissues were identified as *M. bovis* by either confirming the biochemical characteristics of microaerophilic growth, lack of niacin production and nitratase reductase, and pyrazinamide resistance or, alternatively, by PCR amplification of targets specific for the *M. tuberculosis* complex and *M. bovis*, in particular.

3.2. Genetic typing

IS*6110* and PGRS RFLP analysis, spoligotyping and VNTR typing were applied to different subsamples of 92 *M. bovis* isolates. For most isolates typing procedures could not be synchronized but had to be performed partially on frozen and revived cultures. This is considered the most likely cause of failures to obtain sufficient DNA of good quality for typing. Complete analysis involving all four typing methods was carried out on 17 isolates from 12 farms, while only three or less typing methods could be applied to the remaining isolates. Results are summarized in Fig. 1.

3.2.1. Isolates from epidemiologically related herds

3.2.1.1. Isolates from the same herd. Genetic typing rendered identical typing patterns for multiple isolates from the same farm in 15 of the 16 herds in this panel. With the exception of two farms this was true for all ‘‘same herd’’ isolates. Both in herds 3 and 7 one strain was isolated which had a genotype that did not match the genotype of the isolates of the herd mates, suggesting the co-existence of two *M. bovis* strains in those herds. In the case of farm 3, the PGRS RFLP pattern as well as spoligotyping data of isolate 1725 differed from those of the other isolates from this farm. In the case of farm 7, isolate 876 could be distinguished by an additional band in the IS*6110* RFLP pattern, but not by spoligotyping nor VNTR typing (PGRS RFLP typing data lacked). In contrast, the only two isolates from farm 26 (1228 and 1226) were found to be unrelated regarding both the PGRS and IS*6110* RFLP patterns (similarity coefficient of 0.60). Unique DNA fingerprints remained unchanged for the period between samplings from the same herd, which was on average 2 years (see Fig. 1).

3.2.1.2. Isolates from different but epidemiologically linked herds. The genetic relatedness of 39 isolates from eight infected herds, assumed to be linked through a dispersal sale of an infected cattle herd, was investigated. Initial IS*6110* RFLP analysis grouped all samples in the genotype C1, which contains only two IS copies (Fig. 1, farms 36–43). In subsequent PGRS RFLP typing all isolates again displayed an identical PGRS banding pattern, hence supporting the outcome of the epidemiological field investigation of a common source of infection for all eight farms.

Because of cattle movement from farms 27–26, an epidemiological link was also suspected between these two farms. Indeed, the PGRS and IS*6110* RFLP patterns of one of the isolates from farm 26 (isolate 1228) were identical to those of the isolate of farm 27 (isolate 1302), confirming the epidemiological link (Fig. 1).

3.2.2. Genetic profiles among epidemiologically unrelated M. bovis isolates

M. bovis strains isolated from herds with no known link are assumed to be genetically different. This section therefore served to evaluate the results for the various typing methods against this hypothesis. The study identified 29 genetically distinct *M. bovis* strains among 35 herds with no known epidemiological link (Table 1). The highest level of discrimination was achieved when RFLP typing with IS*6110* and/or PGRS was used.

3.3. IS6110 RFLP

Among the 49 *M. bovis* isolates from 34 unrelated farms subjected to IS*6110* RFLP analysis, 16 distinct banding patterns were identified. These patterns comprised four to ten bands, which, due to the use

Table 1
Comparison of the discriminatory power of different genetic markers used individually and in combination to characterize *M. bovis* isolates from epidemiologically unrelated farms

Typing method(s) used in parallel	Number of unrelated outbreaks (isolates) analysed	No. of genotypes identified
IS*6110* + PGRS + spoligotyping + VNTR	11 (17)	12
IS*6110* + PGRS + spoligotyping	14 (22)	15
PGRS + spoligotyping + VNTR	11 (18)	12
IS*6110* + spoligotyping + VNTR	23 (39)	21
IS*6110* + PGRS + VNTR	11 (17)	12
Spoligotyping + PGRS	14 (23)	16
Spoligotyping + VNTR	25 (41)	15
IS*6110* + spoligotyping	30 (45)	24
IS*6110* + PGRS	16 (24)	17
IS*6110* + VNTR	24 (39)	21
IS*6110*	34 (49)	16
VNTR	26 (42)	13
PGRS	16 (5)	18
Spoligotyping	33 (50)	12
Total (IS*6110* and/or PGRS and/or spoligotyping and/or VNTR)	35 (53)[a]	29

[a] One isolate from the multiple farm outbreak (Section 2.1.1.2) was included in the analysis.

of the entire sequence of IS*6110* probe, as described previously (Skuce et al., 1994), relates to two to five copies of IS*6110*. Unique IS*6110* RFLP types, not shared with any other herd, were found for 10 infected farms (C4–C7, C9–C11, C13, C15, and C16), four IS*6110* RFLP types (C3, C8, C12, C14) were shared between two or three farms, and two patterns, both resembling *M. bovis* strains with two copies of IS*6110*, were found in ten and six unrelated outbreaks, respectively (C1 and C2) (Fig. 1).

3.4. PGRS RFLP

PGRS RFLP typing was only applied to 25 isolates from 16 unrelated farms which yielded a total of 18 unique patterns (Fig. 1 and Table 1). All PGRS types were unique and were not found in more than one epidemiologically unlinked herd. Among the isolates of two farms (farms 3 and 26), two distinct PGRS RFLP types were observed.

3.5. Spoligotyping

Spoligotyping was performed on 50 isolates from 33 farms resulting in the identification of 12 spoligotypes, all of which lacked spacers 3, 9, 16, and 40–43 (Fig. 1). Seven types were each associated with several unrelated herds (SP1, SP3, SP4, SP7–SP9, and SP12), while four spoligotypes were each associated with one outbreak only (SP5, SP6, SP10, and SP11). Spoligotype SP8 was found along with SP12 on the same farm (farm 3) (see also Section 2.1).

Two of the 12 spoligopatterns identified (SP7 and SP11) had not been reported in the international *M. bovis* database before and were assigned the SB codes 1163 and 1164 (Table 2).

3.6. VNTR typing

VNTR typing was performed on 43 isolates resulting in 13 distinct patterns relating to 26 epidemiologically unrelated outbreaks (Table 1). As a result, seven farms revealed unique VNTR types (V2, V4, V8, V9, and V11–V13) while other types were shared by between two and five farms. Further discrimination of the more common patterns was possible if either PGRS RFLP patterns were available (V3 and V10), or if IS*6110* banding patterns comprised at least six bands (V3, V5, and V6). For VNTR types V1 and V7, both detected in two herds, no further discrimination was possible due to a lack of PGRS data and corresponding low copy number IS*6110* patterns. With the exception of VNTR types V3 and V5, VNTR typing revealed superior or equal differentiation between strains compared to spoligotyping.

Table 2
M bovis spoligotype and VNTR patterns frequencies among isolates in 35 unrelated cattle herds

Spoligotype	Frequency	SB code[a]	VNTR	Frequency	SP/VNTR	Frequency
SP1	3	SB0121	V1	2	SP4/V5	4
SP2	7	SB0131	V2	1	SP2/V6	3
SP3	2	SB0267	V3	4	SP7/V1	2
SP4	5	SB0130	V4	1	SP9/V3	3
SP5	1	SB0163	V5	5	SP8/V10	2
SP6	1	SB0134	V6	3	SP1/V3	2
SP7	2	SB1163	V7	2	SP2/V4	1
SP8	5	SB0140	V8	1	SP2/V7	2
SP9	3	SB0265	V9	1	SP8/V11	1
SP10	1	SB0678	V10	2	SP9/V12	1
SP11	1	SB1164	V11	1	SP3/V13	1
SP12	2	BCG	V12	1	SP11/V2	1
[b]	[b]	[b]	V13	1	SP5/V5	1
[b]	[b]	[b]	[b]	[b]	SP6/V8	1

[a] http://www.mbovis.org.
[b] End of table.

4. Discussion

The present study is the first to investigate DNA polymorphism among *M. bovis* isolates from cattle in South Africa. Four of the most commonly used genetic markers (IS*6110* RFLP, PGRS RFLP, spoligotyping and VNTR typing) provided high levels of both, reproducibility and genetic diversity in our setting. Although the study did not permit a true comparative evaluation of the methods due to incomplete typing data for several of the isolates, we are of the opinion that the study allows first conclusions regarding the genetic diversity among South African *M. bovis* isolates.

We found IS*6110* RFLP to be highly discriminatory for all *M. bovis* strains which contained more than three copies of the IS sequence. However, 44% of the outbreaks examined in this study were caused by a strain comprising only two copies of IS*6110* (C1 or C2), hence limiting the value of this probe. PGRS RFLP was the single most discriminatory method as it was able to distinguish between all 16 epidemiologically unrelated outbreaks subjected to this method. Furthermore two of the outbreaks (farms 3 and 26) were found to be associated with two genetically different *M. bovis* strains, bringing the total of PGRS types identified to 18. Previous investigators reported a similar superior performance of PGRS (Cousins et al., 1998; Van Soolingen et al., 1994). The technically much less demanding spoligotyping provided the lowest level of differentiation between strains in our study. It was possible to increase the level of discrimination of spoligotyping by second stage IS*6110* RFLP, PGRS RFLP or VNTR typing, as suggested previously for *M. tuberculosis* isolates with low copy numbers of IS*6110* (Rasolofo-Razanamparany et al., 2001). Both the IS*6110* and PGRS RFLP typing methods confirmed transmission of infection between farms 26 and 27 as well as the co-infection of farm 26 with two genetically distinct *M. bovis* strains.

VNTR typing appeared to be less discriminatory than PGRS and IS*6110* RFLP typing in our study but comparable to spoligotyping. However, certain spoligotypes and IS*6110* RFLP types with three or less insertion elements could be subdivided by VNTR typing. In conclusion, the current VNTR typing protocol can provide a valuable first stage screening tool as recently suggested for *M. tuberculosis* strains (Kremer et al., 2005b). Strains with different VNTR patterns will most likely represent genetically distinct strains, but strains with the same VNTR type should be sub-typed with IS*6110* RFLP typing or even PGRS RFLP typing to determine whether they represent the same clone or not. Alternatively, the resolution of VNTR typing can be increased by the number and configuration of loci most appropriate for the locally prevalent strains.

Three of the herds examined were found to be co-infected with two distinct genotypes (3, 7, and 26) as demonstrated by various of the typing methods

employed. Multi-genotype infections may not be a rare event, especially in countries where bovine tuberculosis occurs at a prevalence of >1% (Serraino et al., 1999). Costello et al. found that 10% of cattle herds examined in Ireland harboured more than one strain (Costello et al., 1999). We were unable to reliably estimate the percentage of herds with multiple strain involvement due to the small number of outbreaks analysed with all four markers. However, the fact that such events were detected in the small sample analysed in this study may either suggest a relatively high frequency of outbreaks with multiple sources of infection probably due to purchase of infected animals (Skuce et al., 1994; Neill et al., 1994) or, alternatively, persistence and evolution of "old" *M. bovis* strains within the country's cattle population (Milian-Suazo et al., 2002). Both scenarios appear plausible in the South African context. Despite an initial sharp decline in the bovine tuberculosis herd prevalence to below 0.4%, the disease was never eradicated from the country but continued to occur and more recently the spread of the disease to all nine provinces of South Africa has been confirmed (Michel, unpublished data).

The genetic diversity detected among the *M. bovis* isolates in this study appears to be high compared to studies conducted in European countries where test-and-slaughter is enforced more strictly. Genotyping of 233 *M. bovis* isolates from cattle in Ireland yielded 17 spoligotypes (Costello et al., 1999), Skuce et al. (2005) found 14 spoligotypes among 461 isolates of *M. bovis* in Northern Ireland and spoligotyping of 1349 *M. bovis* isolates in France identified 161 spoligotypes (Haddad et al., 2001). It has recently been reported that clonal expansion following a bovine population bottleneck is a major determinant of the reduced strain diversity of *M. bovis* in Great Britain (Smith et al., 2006). In the central African region the degree of heterogeneity appears to be low despite the absence of eradication programmes. Possible explanations are limited cattle imports from Europe and fairly recent introduction of the disease (Njanpop-Lafourcade et al., 2001; Cadmus et al., 2006; Diguimbaye-Djaibé et al., 2006). The findings of our study do not fit any of these epidemiological scenarios for South Africa. During colonial times and into the 20th century cattle imports from different European countries and other continents were responsible for multiple introductions of *M. bovis* and most probably a high strain heterogeneity. On the other hand, the rinderpest pandemic, killing 66% of South African cattle (Rossiter, 2004), as well as an effective 'test-and-slaughter' scheme should be considered powerful population and diversity reducing factors, commonly facilitating a founder effect characterized by the establishment of a successful genotype in a geographical region (Smith et al., 2006). Our study did not present any indicators of such an effect and it may be speculated that many genotypes were only introduced in the 20th century and that incomplete eradication of outbreaks during 'test-and-slaughter' campaigns may have allowed survival of 'old' strains. In addition, it cannot be ruled out that undetected spillover of strains into wildlife occurred at the wildlife/livestock interface, possibly re-infecting cattle at a later stage.

In conclusion, the data obtained in this retrospective study show that IS*6110* and PGRS RFLP represented powerful markers in revealing a high genetic diversity among cattle strains in South Africa, where the overall bovine tuberculosis prevalence is low compared to countries with a more limited strain diversity.

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