Short Communication

Molecular typing of *Mycobacterium bovis* isolated in the south of Brazil

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Abstract

Bovine tuberculosis is a major infectious disease of the cattle. In this study, 85 *M. bovis* isolates from 162 lymph nodes, obtained from a herd of cattle on a farm in southern Brazil, were evaluated using spoligotyping and VNTR. The strains were grouped into five clusters and five orphans, showing a heterogenic genetic profile, what could represent diverse geographic origins of the introduced cows and/or the frequent movement of cattle between different properties.

Key words: bovine tuberculosis, *Mycobacterium bovis*, genotyping.

Bovine tuberculosis is an endemic disease responsible for significant economic losses related to decreases in the production of meat and milk and reductions in the export of beef products. *Mycobacterium bovis*, the main causative agent of the disease, has a broad host range, infecting different wild and domestic animals, and occasionally humans (Mignard *et al.*, 2006).

An important tool for the control of bovine tuberculosis is the application of "tracking" epidemiology. By understanding the source and mode of transmission of the bacilli, more effective control measures can be implemented. The advent of molecular typing techniques has greatly increased epidemiological knowledge, which is advantageous for the study of outbreaks of tuberculosis (TB) and for understanding the dynamics of the disease (Roring *et al.*, 2002).

Analysis of variable number tandem repeat (VNTR) sequences and direct repeat sequences (spoligotyping) is emerging as a valuable tool for genotyping bacterial species and several members of the *Mycobacterium tuberculosis* complex. Spoligotyping is a technique based on the polymorphism of the direct repeat (DR) locus present in *Mycobacterium tuberculosis* complex DNA. The DR sequences are composed of multiple 36 bp copies, interspersed by short non-repetitive sequences. The presence or absence of each non-repetitive sequence creates a pattern for each strain when analyzed by spoligotyping. Spoligotyping and VNTR patterns are now considered standard techniques for the molecular typing of *M. bovis* (Mignard *et al.*, 2006).

This study surveys 162 lymph node samples collected from cattle in a slaughterhouse in Capão do Leão, RS, Brazil. We describe the spoligotype diversity and the VNTR patterns associated with the main circulating strains of *M. bovis* in the study region.

The 162 lymph node samples, were collected from adult Holstein dairy cattle in a sanitary slaughter on a single farm in 2003 in Capão do Leão, RS, Brazil; 136 animals presented typical lesions. Each sample was decontaminated using the Petroff method (Meikle *et al.*, 2007), inoculated on Ogawa-Kudoh medium with pyruvate, and incubated for 3 months at 37 °C. After growth, all AFB-positive samples were preliminarily identified as *M. bovis* based on growth in the presence of pyruvate and colony morphology (Skuce *et al.*, 2002). Mycobacterial DNA was extracted as described by Van Embden *et al.* (1993).

The *M. bovis* isolates were typed by spoligotyping following procedures described by Kamerbeek *et al.* (1997). The PCR products were hybridized by reversed-line blot hybridization to a membrane (Isogen®, The Neth-

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erlands) containing 43 immobilized oligonucleotides of known spacer sequences. Data were compared to the *M. bovis Spoligotyping Database* (www.Mbovis.org).

VNTR analysis was performed according to the method published by Supply *et al.* (2001) by determining the copy number ETR-A, ERT-B (Frothingham and Meeker-O'Connell, 1998), QUB 1895, QUB 3336 (Skuce *et al.*, 2002), and MIRU 26 (Supply *et al.*, 2001), chosen by greater discriminatory power (Supply *et al.*, 2002). The fragment size of the PCR products was estimated by electrophoresis on a 2% agarose gel, staining with ethidium bromide (0.5 µg/mL), and comparison to a 100-bp DNA ladder (Invitrogen®, Life Technologies, Carlsbad, CA).

The Hunter-Gaston discriminatory index (HGDI) was used as a numerical index to calculate the discriminatory power. HGDI was calculated using the following formula

$$HGDI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1) \right]$$

where N is the total number of strains in the typing scheme, s is the total number of different MIRU-VNTR or spoligotyping patterns, and n_j is the number of strains belonging to the jth pattern (Hunter and Gaston, 1988).

Of the 162 lymph nodes of the cattle slaughtered in southern Brazil, 123 were purified protein derivative (PPD)-positive, and 136 had macroscopic lesions compatible with TB. Thirty-nine animals were not reactive to PPD, and 26 had no lesions. Of the 162 samples plated, 85 were positively identified as *M. bovis* (Table 1). Of the 39 cattle that were not reactive to PPD, 12 were culture-positive. Of the 26 that did not have characteristic lesions, 3 were culture positive.

Four spoligotype patterns were identified among the 85 *M. bovis* isolates. One isolate showed a unique pattern, whereas the remaining 84 isolates were grouped into 3 clusters. One particular spoligotype (SB0121) contained 79 isolates, and the other 2 clusters contained 3 and 2 isolates each. Two spoligotypes (SB0121 and SB0119) were present in the *M. bovis Spoligotyping Database* (www.Mbovis.org). SB0121, accounting for more than 92% of the strains, has been previously reported in Brazil (Costa *et al.*, 2010; Zanini *et al.*, 2001). SB0119 occurs frequently in Spain and in other European countries (Matos *et al.*, 2010; Rodriguez-Campos *et al.*, 2011).

The 2 most common patterns, SB0121 and SB0119 (Kubica *et al.*, 2003; Milian-Suazo *et al.*, 2002), differ by a

single spacer and together account for 94.12% of the spoligotypes found. These spoligotypes could represent strains with selective genetic advantages such as increased pathogenesis, transmissibility, or poor immunogenicity, which are generally not detected by conventional serological or skin tests (Skuce *et al.;*, 2002; Van Embden *et al.*, 2002).

Haddad *et al.* (2004) suggested the existence of 2 dominant groups of spoligotypes. The first group is the BCG-like group, represented by SB0121 and the second one is the ancestor BCG-like (SB0120), which has a high frequency in countries such as France, Italy, Belgium, Spain, and Portugal, as well as in countries that are heavily involved in trading cattle with these countries (Haddad *et al.*, 2004). Two other novel spoligotypes (SB1177 and SB1178), which have not been previously reported, were deposited in the database by our research group.

The VNTR analysis revealed 5 different profiles: 1 unique and 4 clustered. ETR-B and MIRU26 were poorly discriminative (Cluster I: 41 strains; Cluster II: 21 strains, Cluster III: 19 strains; and Cluster IV: 3 strains). After combining the spoligotyping and VNTR results, the strains were grouped into 5 clusters (Cluster A: 39 strains; Cluster B: 19 strains; Cluster C: 17 strains; Cluster D: 3 strains; and Cluster E: 2 strains) and 5 orphan profiles (Table 2).

For the analysis of *M. bovis*, MIRU-VNTR has been shown to have more discriminatory power than spoligotyping when a limited number of isolates from Europe and Africa were used (Hilty *et al.*, 2005; Roring *et al.*, 2002). However, the epidemiological relevance of the MIRU-VNTR method has been difficult to appreciate, mainly because of the lack of detailed epidemiological information about the isolates involved (Allix *et al.*, 2006). For our isolates, from the same farm and therefore, epidemiologically related, MIRU-VNTR typing provided a higher discriminatory power (0.66) using HGDI, whereas spoligotyping

Table 1 - Results of the presence the macroscopic lesions and PPD analysis of the cattle slaughtered.

	PPD	Lesion
Positive	123	136
Negative	39	26

Table 2 - Molecular characteristics of *Mycobacterium bovis* isolates obtained from a slaughterhouse in Capão do Leão, RS, Brazil.

Spoligotype pattern[a]	VNTR profile[a]	Isolates n (%)	Combined result
SB0121	5-6-6-8-13	39 (45.88%)	Cluster A
SB0121	5-6-6-8-12	19 (22.35%)	Cluster B
SB0121	5-6-6-8-9	17 (20.0%)	Cluster C
SB0121	5-6-3-8-9	3 (3.53%)	Cluster D
SB0119	5-6-6-8-13	2 (2.35%)	Cluster E
SB0121	5-6-6-7-13	1 (1.18%)	Orphan
SB1178	5-6-6-8-12	1 (1.18%)	Orphan
SB1177	5-6-6-8-9	1 (1.18%)	Orphan
SB1178	5-6-6-8-9	1 (1.18%)	Orphan
SB0119	5-6-6-8-12	1 (1.18%)	Orphan

a: international name assigned by Mbovis.org and *b*: The sequence corresponding the alleles of the MIRU 26, ETRB, Qub 1895, ETRA and Qub 3336 *locus*, respectively.

showed a lower discriminatory power (0.14). A recent study showed that analysis of six VNTR loci provided adequate differentiation of strains (McLernon *et al.*, 2010). However the combination of spoligotyping and MIRU-VNTR typing showed a higher HGDI (0.70) when used in combination than they did when used individually.

In addition, spoligotyping and MIRU-VNTR typing were convenient, rapid, and reproducible techniques for the molecular characterization of isolates in southern Brazil. Advances in molecular characterization allow us to increase our understanding of the spread of *M. bovis* and TB control.

Genotyping techniques such as PCR-based spoligotyping and VNTR have been adopted in various laboratories for typing *M. bovis* with the advantage that the results are qualitative (presence or absence, or numerical form) (Romero *et al.*, 2006). The approach used in this study for the genetic analysis of *M. bovis* isolates, combining spoligotyping and VNTR analysis, is still limited in Latin America. Epidemiologically related isolates are derived from the clonal expansion of a single precursor and, as a result, have common characteristics that differ from those of epidemiologically unrelated isolates (Mignard *et al.*, 2006).

Isolates obtained from the same herd are expected to be genetically closer than strains obtained from different regions (Perumaalla *et al.*, 1999). Therefore, we infer that the genetic heterogeneity observed in the strains from this herd most probably reflects the wide and diverse geographic origins of the introduced cows and the frequent movement of cattle between different properties.

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