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Genetic profiles of *Mycobacterium bovis* from a cattle herd in southernmost Brazil

Perfis genéticos de *Mycobacterium bovis* em um rebanho bovino no extremo sul do Brasil

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Abstract

Mycobacterium bovis is the agent of bovine tuberculosis, a disease endemic to all Brazilian states. Molecular typing techniques help to stratify and refine data, providing information that facilitates epidemiological research. In this study, MIRU-VNTR, targeting 24 loci, was employed to identify and characterize the genetic groups of *M. bovis* isolates obtained from an outbreak of bovine tuberculosis. Eighteen acid-fast bacilli isolates, obtained from bovine tissue samples, and reactive to the comparative cervical tuberculin test, were identified as species of the *M. tuberculosis* complex, and were genotyped by MIRU-VNTR with 24 primer pairs. Genotyping revealed three genetic profiles comprising one with 15 isolates (83.3%), one with two isolates (11.1%), and one profile with one unique isolate (5.6%). This distinction was achieved with the MIRU 31 primer, which resulted in clustering of two isolates into the same profile, and ETR A, B, and C, which discriminated the isolate with a unique profile. The occurrence of clustered isolates is indicative of recent transmission, whereas isolates with a unique profile suggest reactivation of latent infection. The presence of different *M. bovis* genotypes in the same herd suggests movement of infected animals or different sources of intra-herd infection. Use of the MIRU-VNTR molecular epidemiology technique in *M. bovis* isolates obtained from an outbreak of bovine tuberculosis in Rio Grande do Sul state demonstrated the genetic diversity of circulating strains, despite the presence of a predominant group.

Key words: Bovine tuberculosis. Genetic diversity. MIRU-VNTR. *Mycobacterium bovis.*

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Resumo

Mycobacterium bovis é o agente da tuberculose bovina, enfermidade endêmica diagnosticada em todos os estados brasileiros. As técnicas de tipagem molecular auxiliam a estratificar e refinar dados, fornecendo informações que facilitam as atividades epidemiológicas. Neste estudo aplicou-se a técnica MIRU-VNTR com 24 *loci* para caracterizar e identificar os agrupamentos genéticos de isolados de *M. bovis* oriundos de um foco de tuberculose bovina. Dezoito isolados de bacilos álcool-ácido resistentes, obtidos de amostras de tecido de bovinos reagentes ao teste cervical comparativo e identificados como espécies do complexo *M. tuberculosis*, foram genotipados por meio de MIRU-VNTR com 24 pares de iniciadores. A genotipagem revelou três perfis genéticos distintos: um para 15 isolados (83,3%); um para dois isolados (11,1%) e, por fim, um isolado com perfil único (5,6%). Os iniciadores responsáveis por essa distinção foram MIRU 31, que agrupou dois isolados em um perfil, e ETR A, B e C, que discriminaram o isolado de perfil único. A ocorrência de agrupamentos de isolados é indicativa de transmissão recente, ao passo que isolados de perfil único sugerem reativação de infecção latente. A presença de diferentes genótipos de *M. bovis* no mesmo rebanho sugere haver circulação de animais doentes ou existirem diferentes fontes de infecção intrarrebanho. A aplicação da técnica de epidemiologia molecular MIRU-VNTR a isolados de *M. bovis* obtidos a partir de um foco de tuberculose bovina no Rio Grande do Sul demonstrou haver diversidade genética entre as estirpes em circulação, embora exista um agrupamento predominante.

Palavras-chave: Diversidade genética. Tuberculose bovina. MIRU-VNTR. *Mycobacterium bovis*.

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis, an endemic disease in all Brazilian states. This agent has a broad spectrum of pathogenicity towards wild and domestic species, but mainly infects cattle and buffaloes. In addition to national and international trade burdens, the zoonotic nature of the disease is considered a public health problem (LAGE, 2006; OIE, 2009). In Brazil this disease is endemic and studies conducted in 13 Federative Units, which account for 75% of the Brazilian herd, showed the prevalence of infected herds to be between 0.36% and 9.0%, in the Federal District and São Paulo, respectively (BAHIENSE et al., 2016; BARBIERI et al., 2016; DIAS et al., 2016; GALVIS et al., 2016; GUEDES et al.; 2016; LIMA et al., 2016; NÉSPOLI et al., 2016; QUEIROZ et al., 2016; RIBEIRO et al., 2016; ROCHA et al., 2016; SILVA et al., 2016; VELOSO et al., 2016; VENDRAME et al., 2016).

Molecular typing techniques help to stratify and refine the data, providing information that facilitates epidemiological action, including disease surveillance and studies of outbreaks. This allows for the identification of transmission patterns and risk factors among apparently disparate cases (FOXMAN; RILEY, 2001), as well as identifying herds infected by more than one strain (TENOVER et al., 1997). The recent development of discriminatory molecular genotyping technology has clarified the transmission of *M. bovis* and facilitated the discrimination of individual isolates associated with an outbreak (GORMLEY et al., 2014).

The typing method called Mycobacterial Interspersed Repetitive Unit (MIRU) is fast and reproducible, allowing for the generation of genotypes based on the study of *loci* with a variable number tandem repeats (VNTRs) of the *M. tuberculosis* complex. The MIRU-VNTR method compares strains in different geographical areas, enabling the tracking of lineage dissemination, making it possible to analyze a large number of strains and identify dissemination foci within a population (GORTAZAR et al., 2011). This provides data for the control of the disease.

In this study, the MIRU-VNTR technique with 24 *loci* was used to characterize and identify genetic groupings of *Mycobacterium bovis* isolates obtained from a herd infected with bovine tuberculosis.

Materials and Methods

Twenty isolates of acid-fast bacilli (AFB), obtained in Stonebrink culture medium, were derived from samples of bovine tissues naturally infected and reactive to the comparative cervical test (CCT). Samples were obtained from a property intended for dairy farming located in the state of Rio Grande do Sul, Brazil, and were used for the extraction of genomic DNA by thermolysis (MAZARS et al., 2001). The obtained DNA was used as a template to amplify target sequences (RvD1-RV2031c) using the JB21 and JB22 primers for the identification of mycobacteria of the *M. tuberculosis* complex (ARAÚJO et al., 2014; RODRIGUEZ et al., 1995).

Samples identified as mycobacteria from the *M. tuberculosis* complex and the reference AN5 strain of *M. bovis* were genotyped by polymerase chain reaction (PCR) with 24 pairs of primers (SUPPLY, 2005; SUPPLY et al., 2006). In each reaction 6.4 µL of Go Taq Green Master Mix, 0.3 pmol of each primer, 100 ng of DNA and ultra-pure water to a final volume of 18 µL, were used. The strain H37Rv of *M. tuberculosis* and ultra-pure water was used as a positive and negative control, respectively.

The amplified products were analyzed by agarose gel electrophoresis in parallel to a 100 base pair standard. Imaging of the bands and the determination of the molecular weight of fragments of all samples for each locus were performed with the aid of Quantity One software. The number of alleles was determined according to Supply (2005), who lists the size of the fragment obtained with the number of copies of each allele. According to the number of alleles, each sample received a numeric code, which describes its genetic profile. The genetic profiles of the isolates in the study and of the reference strains, *M. bovis* AN5 and *M. tuberculosis* H37Rv, were used to generate a dendogram using the UPGMA method through the MIRU-VNTR plus application (http://www.miru-vntrplus.org/MIRU/index.faces). The test was considered valid when the genetic profile of the positive control (*M. tuberculosis* H37Rv) corresponded to the one obtained from Supply (2005). A cut-off point of 0.17 was adopted, which corresponds to a tolerance of up to four different loci, to analyze the similarity between the strains in the study.

Results and Discussion

Of the 20 AFB samples, 18 were confirmed as species of the *M. tuberculosis* complex by PCR. The study of genetic diversity of *M. bovis* strains, through the MIRU-VNTR technique using 24 primers, displayed three genetic profiles (Figure 1); one profile was comprised of 15 isolates (83.3%), one had two isolates (11.1%) and, one profile contained a single isolate (5.6%). Table 1 presents the genetic profile of the isolates.

This similarity is suggestive of exogenous infection with recent transmission and clonal nature, typical characteristics of epidemic outbreaks. Recent transmission would not confer sufficient genetic variability to differentiate the isolates into distinct groups (VAN SOOLINGEN, 2001). Moreover, the similarity found in the majority of *M. bovis* strains can arise from a typical profile of the region (PERUMAALLA et al., 1996). The profile with a single isolate did not present an epidemiological link to the other strains, but suggests disease from reactivation of a latent infection (VAN SOOLINGEN, 2001).

Figure 1. Dendrogram obtained by MIRU-VNTR plus analysis of 18 isolates of *Mycobacterium bovis* from an outbreak of bovine tuberculosis in Rio Grande do Sul, combined with AN5 *M. bovis* and H37Rv *M. tuberculosis* strains. It is possible to observe three distinct genetic profiles in the analyzed isolates: one comprised of 15 isolates, one comprised of two isolates and, one comprised of a single isolate.

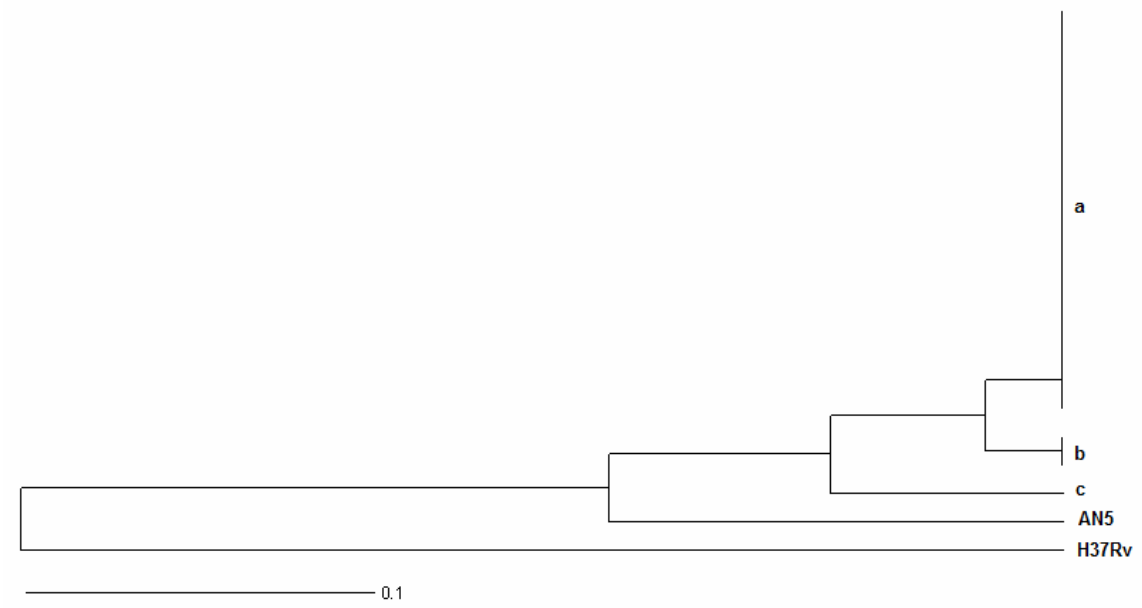

Table 1. Profiles of *Mycobacterium bovis* obtained by MIRU-VNTR analysis from an outbreak of bovine tuberculosis in Rio Grande do Sul, Brazil.

BOVINE	MIRU												ETR				VNTR						QUB		Profile
	02	04	10	16	20	23	24	26	27	31	39	40	A	B	C	42	46	47	49	52	53	1955	11b	26	
AN5	2	3	2	3	2	4	2	4	3	3	2	2	6	5	5	2	3	4	2	2	1	2	3	5	-
*1, 2, 4, 5, 6, 7, 8, 9, 11, 12**, 13, 14, 16**, 17**, 18*	2	3	2	3	2	4	2	5	3	3	2	2	3	2	5	2	3	4	3	2	1	3	2	5	a
10, 15	2	3	2	3	2	4	2	5	3	1	2	2	3	2	5	2	3	4	3	2	1	3	2	NA	b
3	2	3	2	3	2	4	2	5	3	3	2	2	6	4	3	2	3	4	3	2	1	3	2	5	c

*Bovines purchased from other properties three years before the herd was subjected to the comparative cervical test.
**Samples without amplification with the primers MIRU 16 and QUB 26.
NA: Non-amplified.

The presence of different genotypes of *M. bovis* in the same herd suggests the movement of infected animals (PERUMAALLA et al., 1996) or different sources of inter-herd infection (SALAMON et al., 2000). The identified genetic groups might be due to an active movement among herds, because of the 15 isolates belonging to the largest group, two came from bovines purchased from other properties three years earlier. Although the entry of animals into the property had been controlled by the CCT, it is known that some animals, even when infected, do not react to the tuberculin test due to immunosuppression (OIE, 2009) or being in the prodromal phase. The persistence of infection in herds might be due to

failure of the diagnostic tests or the presence of continuous risk of reinfection, which occurs when trading infected cattle or through contact with infected wild animals, in addition to inadequate sanitation measures (RODRIGUEZ-CAMPOS et al., 2013).

The primers responsible for this distinction were MIRU 31, which grouped two isolates into one profile, and ETR A, B, and C, which discriminated the unique profile of one isolate. Ramos et al. (2014) performed genotyping on *M. bovis* strains originating from the same geographical region, using 5 MIRU-VNTR loci (MIRU 26, ETR A, ETR B, QUB 1895, and QUB 3336). Of these loci, only ETR A appeared discriminatory in both studies. Figueiredo et al. (2012), using 15 loci from the DNA of strains isolated from a property in the state of Rio de Janeiro, observed that the MIRU 4, 26, and 40 loci, as well as ETR loci (A, B, C), allowed for the discrimination of the studied strains. This finding is similar to that of the present study, in which the ETR loci (A, B, C) allowed for the discrimination of the profile with a single isolate. MIRU 31, responsible for discrimination of the group with two isolates, has also been reported as capable of distinguishing strains (PARREIRAS et al., 2012), although it is considered to have low discriminatory power based on the work of other researchers (FIGUEIREDO et al., 2012; ROCHA et al., 2013). When comparing the samples characterized in other studies, similarity was observed among the isolates of profile A with isolates identified by Parreiras et al. (2012); however, these authors used only 12 MIRU loci. The profile (232324253322) revealed a higher frequency and greater distribution in the states of Amazonas, Distrito Federal, Minas Gerais, and Paraíba. Since this is the first study conducted with 24 MIRU-VNTR loci of *M. bovis* strains from Brazil to characterize the isolates, comparisons between isolates is difficult.

Five samples were not amplified, even with repeated attempts. The failed amplification of some loci might be due to mutations or DNA degradation, which prevented the annealing of primers. In these cases, according to Supply et al. (2006), the genotypes could still be compared in a reliable way based on the characterization of other loci, mainly because the analysis of similarity tolerates differences of up to four loci compared to the deposited strains.

Conclusions

This study forms the basis for future approaches to track infections caused by *M. bovis* in the extreme south of Brazil. The obtained data will be important to determine relationships among the strains, as additional studies are performed, and thus contribute to the understanding of the epidemiology of tuberculosis in the region and to advances in proposed control by the PNCEBT.

The application of the MIRU-VNTR molecular epidemiology technique to *M. bovis* isolates obtained from a herd infected with bovine tuberculosis in the state of Rio Grande do Sul demonstrates the genetic diversity among the strains in circulation, despite the existence of a predominant group. Moreover, it may be very useful as a discriminatory tool of *M. bovis* isolates for surveillance systems of bovine tuberculosis in the Brazilian states, where direct diagnostic methods have a prominent role.

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