BOVINE TUBERCULOSIS IN THE NORTH OF BRAZIL

RAMOS, Daniela Fernandes [1, 3];
TAVARES, Lucas Alves [1];
NUNEZ, Cecília Veronica [2];
COSTA, Reinaldo Corrêa [2];
DELLAGOSTIN, Odir Antônio [1];
SILVA, Pedro Eduardo Almeida da [3].

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[1]Laboratório de Vacinologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brasil; [2]Laboratório de Bioprospecção e Biotecnologia, Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brasil; [3]Núcleo de Pesquisa em Microbiologia Médica, Laboratório de Micobacteriologia, Universidade Federal do Rio Grande, Rio Grande, RS, Brasil.

ABSTRACT

Bovine tuberculosis (BTB) is an infectious disease worldwide distributed, caused by *Mycobacterium bovis*, which affects cattle and other animals, including humans. In Brazil, BTB is endemic and causes economic losses by reducing the productivity of livestock and loss of carcasses in the slaughterhouses. Molecular epidemiology has been used as a tool for the investigation of *M. bovis* transmission and dynamic of this disease. Herein, we studied 99 samples of lymph nodes obtained from animals (with or without suggestive lesion) slaughtered in the Northern region of Brazil. We evaluated the presence of *M. bovis* through microscopy techniques, culture in Stonebrinck medium and molecular identification using polymerase chain reaction (PCR) testing. In addition, two genotyping methods (Spoligotyping and MIRU-VNTR) were used in order to identify the genotypic profile of these strains. Out of 99 retropharyngeal lymph nodes collected, only ten clinical samples were amplified using PCR technique, and were considered positive to *M. bovis*. These samples were further investigated using molecular analysis of the combination of spoligotyping and MIRU-VNTR, and it was possible to identify eight different patterns. Only one spoligotype, majority among the samples tested (40%), had already been identified in the database (SIT523). Through the epidemiological identification of these strains, it is possible to investigate the dynamic transmission of the disease, which is an essential part of more specific and effective control of diseases such as tuberculosis.

Keywords: *Mycobacterium bovis.* Molecular tools. Livestock. Spoligotyping. Genotyping.

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic bacterial disease caused mainly by *Mycobacterium bovis*, which is also the agent of major infectious diseases among other domesticated animals, and certain wildlife populations. In rare cases *M. bovis* can be aetiologic agent of tuberculosis in humans (ARANAZ et al., 1996; BRIONES et al., 2000; CORNER, 2006; MICHEL et al., 2006; RAMOS et al., 2014a; RODRÍGUEZ et al., 2010; ZUMÁRRAGA et al., 1999).

Despite the lack of data on bovine tuberculosis in Brazil, the Brazilian Department of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento - MAPA) released, in 2020, a Situational Diagnosis of the National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis - PNCEBT, reporting 4907 tuberculosis cases in 2018, nationwide. In addition, official data showed that the prevalence of this disease in different Brazilian regions, due to its continental extent and socio-economic diversity, is very variable, being nonexistent in the Northern region of the country, for example. Even so, it is estimated that, in the same year, 14 and 179 cases were identified in the states of Amazonas and Pará, respectively (BRASIL, 2020).

BTB is related with economic losses resulting from death of animals, decrease in weight gain and reduced milk production and quality, disposal and elimination of early high-value livestock and condemnation of carcasses at slaughterhouse (CARNEIRO; KANEENE, 2018). Prevention and control of zoonotic tuberculosis needs a cross-sectorial and multidisciplinary approach, linking animal, human, and environmental health approaches (BRASIL, 2017; OLEA-POPELKA et al., 2016).

Samples collected from tissue with suggestive lesions should be submitted to laboratory analysis. The identification is made by presumptive diagnosis, sample decontamination and primary culture of the bacillus, which needs to be confirmed based on physiological, biochemical characteristics or molecular identification (MEDEIROS et al., 2010). The sequence and availability of these techniques have an impact upon the sensivity of *M. bovis* diagnosis (ISSA et al., 2017).

Control of the transmission of *M. bovis* is often hampered by the lack of epidemiological data and early diagnosis. Molecular tools can be used to diagnose, monitor and control BTB, as well as track sources of infection (HILTY et al., 2005; OLEA-POPELKA et al., 2016).

Insertion sequence 6110 restriction fragment length polymorphism (IS6110 RFLP) analysis used to be considered as the gold standard and the most widely applied typing method for molecular epidemiology to *M. tuberculosis* complex (VAN SOOLINGEN, 2001). However, this method is not useful for *M. bovis* strains, because of the low power of RFLP-IS6110 discrimination in strains with a low number of IS6110 copies (CARVALHO et al., 2016; HILTY et al., 2005; KREMER et al., 1999; VAN SOOLINGEN, 2001).

PCR-based methods, such as spoligotyping (spacer oligonucleotide typing), variable-number tandem repeat (VNTR), mycobacterial interspersed repetitive unit (MIRU) and exact tandem repeats (ETRs), have become the preferred approach for genotyping *M. bovis* (CARVALHO et al., 2016; DEMAY et al., 2012, FIGUEIREDO et al., 2012; MCLERNON et al., 2010; RAMOS et al., 2014b).

The spoligotyping detect the presence or absence of 43 spacer DNA variables sequences among direct repeats (DR) region. Although this method does not present high discriminatory power, it is the largest worldwide database of genotyping markers for *M. tuberculosis* (MCLERNON et al., 2010; RAMOS et al., 2014b).

The MIRU-VNTR typing is a PCR-based method that like spoligotyping does not require large quantities of DNA. Specific sequences for *M. bovis* including ETR (FROTHINGHAM; MEEKER-O'CONNELL, 1998), MIRUs (MAZARS et al., 2001; SUPPLY et al., 2000) and two sets of Queen's University Belfast (QUB) VNTRs (RORING et al., 2002) have been described. The main advantages of VNTR typing are that this test is reproducible, fast and specific for *M. tuberculosis* complex isolates, therefore the results are easily comparable between laboratories (HILTY et al., 2005; MAZARS et al., 2001; SMITH et al., 2006; SUPPLY et al., 2000; ZANINI et al., 2005).

Herein, we aimed to identify, by genotyping, the molecular profile of BTB in the North of the Brazil using spoligotyping and MIRU-VNTR methods.

MATERIAL AND METHODS

Animals and bacterial isolates

Retropharyngeal, sublingual, scapular and/or pulmonary lymph nodes were obtained from 99 animals of different origins of the North region of Brazil in a slaughterhouse in Manaus - AM. The animals were originated from Amazonas and Pará states, located in the extreme north of Brazil. Most animals were from the state of Amazonas (64.5%), from Manicoré (46), Humaitá (14), Careiro da Várzea (3) and Apuí (1) cities. Thirty-five were from Pará (35.3%), from Alenquer (25) and Óbidos (10) cities. The distribution of the samples collected according geographic origin is present in Figure 1.

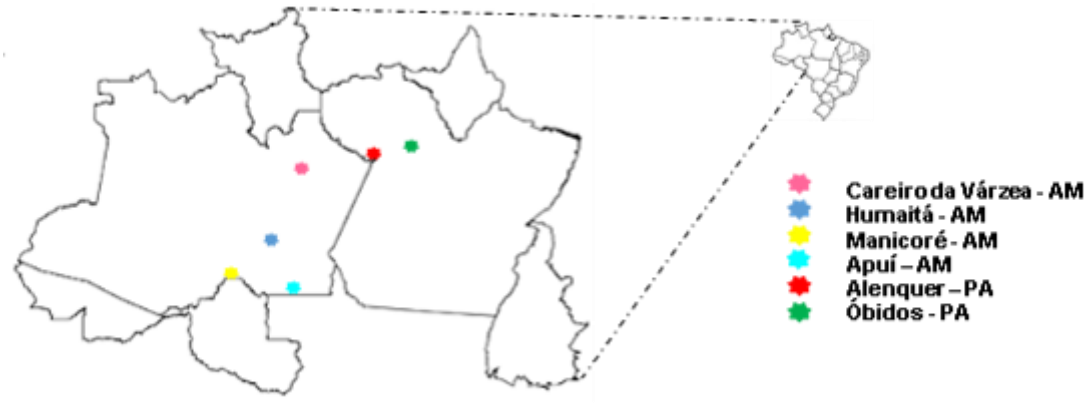

Figure 1 - Geographical distribution of the origin animals in the North Brazilian, indicated of the marker's color (Careiro da Várzea, Humaitá, Manicoré and Apuí of the Amazon state indicated with pink, yellow, cyan and blue, respectively; Alenquer and Óbidos of the Pará State marked with green and red, respectively).

All tissues (with or without suggestive lesion) were macerated and the Ziehl-Neelsen staining was performed. Furthermore, after maceration of lymph nodes, they were decontaminated by Petroff method, inoculated in Stonebrink medium, and incubated at 37 °C for up to 16 weeks. The colonies were evaluated by morphological analysis and DNA was extracted for further specie identification by molecular approach.

Bacterial DNA extraction

A loopful of bacterial growth was suspended in TE buffer (10 mM Tris–HCl; 1 mM EDTA [pH 8.0]), inactivated at 80 °C for 20 min, and lysed in ribolyser (Hybaid) with glass beads. DNA extraction was performed as previously described (SUPPLY et al., 2000).

Identification of *Mycobacterium bovis*

We amplified a region to differentiate between RD7 *M. bovis* and *M. tuberculosis* (ETCHECHOURY et al., 2010) using the primers described in the Table 1. The analysis was performed by the size of the fragments of 394 bp and 635 bp to *M. tuberculosis* and *M. bovis*, respectively.

Spoligotyping

Spoligotyping reactions were performed according Kamerbeek et al. (1997), using the primers described in the Table 1.

Table 1 - Primers used for the detection and genotyping of *Mycobacterium bovis*.

Gene	Primer	Reference
RD7 region	1566 up 5'-GCGTGGCGTGAATACCTACTT-3'; 14930 low 5'-CGGGTGTAGCTCGAGGATTTT-3'; 5'-TGAGAAACACCGAGCAAAAGA-3' 1960 low	Etchechoury et al., 2010
Direct repeat (DR) genomic region	Dra 5'-GGG TCT GGT TTT GAC GAC-3' biotinylated; DRb 5'-GCC GGG AGA GGA GAC AAC-3'	Kamerbeek et al*.*, 1997

MIRU-VNTR genotyping

The amplifications for the MIRU-VNTR were developed according Supply et al. (2000), using a set of 9 *loci*: 2996, 3007, 4348, 802, 2165, 2461, 154, 580, 960. The presence and size of each PCR product was determined by electrophoresis on 2% agarose gel in 5X TBE (Tris-borate-acid EDTA) buffer followed by staining with Gel Red.

Statistical and Clustering analysis

The discriminatory index (HGDI) described by Hunter and Gaston (1988) was used to calculate the allelic diversity at each *locus*. The diversity (*h*) was calculated using $h - 1 - \sum x^2_i [n / (n\text{-}1)]$ formula, being x_i the allele's frequency at the *locus* and *n* is the number of isolates. This index is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups (HUNTER; GASTON, 1988).

The spoligotypes were compared to SpoligoDatabase3 (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html). The construction of the dendrogram was performed on the website www.miru-vntrplus.org using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). We defined as a cluster when at least two *M. bovis* strains with identical patterns were isolated from different animals.

RESULTS AND DISCUSSION

Among the retropharyngeal lymph nodes from 99 animals collected, only one showed macroscopic characteristic TB lesion, and was positive in the acid-fast bacillus test. However, 33 samples (33/99) grew in stonebrink medium and out of those only in ten *M. bovis* isolates were identify by PCR method (10/33).

The main spoligotype found was SIT 523, which is represented in Cluster I (4/10), followed by Cluster II (2/10), as shown in Table 2, and other four strains identified as orphans. The clade SIT 523 was rarely found in others studies and had not been described in Brazil, previously, in other animals (ALEMU et al., 2016; CARNEIRO; KANEENE, 2018; CARVALHO et al., 2016; CAZOLA et al., 2015; FIGUEIREDO et al., 2012; JOJOA-JOJOA et al., 2016; KORO KORO et al., 2015; PARREIRAS et al., 2012; ROCHA et al., 2013; SOUZA-FILHO et al., 2016; ZUMÁRRAGA et al., 1999). On other continents, including Asia, Europe, Africa, and North America (KORO KORO et al., 2015; UZOEWULU et al., 2016), these clade, characterized by the presence of all 43 spacers, has been described in a small number of strains (one to four). In a study carried out in Colombia, only 3.8% of the cattle showed the SIT 523 genotype, a fact that could suggest the possible dissemination of *M. bovis* genotypes by transport of animals between regions or by the presence of a wildlife reservoir.

Table 2 - Classification of strains, genotypic profile and frequency of the spoligotypes.

Analysis of strains	**Spoligotype description[a]**	**Isolated n (%)**
Cluster I (as SITVIT: SIT523)		4 (40%)
Cluster II		2 (20%)
Orphan		1 (10%)
Orphan		1 (10%)
Orphan		1 (10%)
Orphan		1 (10%)

a: the black and white boxes indicate the presence and absence, respectively, of the specific spacer at position 1-43 in the direct repeat *locus.*

Three clusters were formed through genotyping by MIRU-VNTR analysis (Cluster I containing 30% of the isolates and Cluster II and III both with 20%) and three strains were defined as orphans, as shown in Table 3.

Table 3 - Frequency and genotypic profiles of mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) types.

Patterns classification	MIRU-VNTR genotypes[a]	Isolates n (%)
I	2-4'-3-6-3-7-7-8-7	3 (30%)
II	2-4'-2-6-4-7-7-8-7	2 (20%)
III	2-4'-2-6-4-7-7-8-5	2 (20%)
IV	2-4'-2-6-3-7-7-8-5	1 (10%)
V	2-4'-3-6-4-7-7-8-7	1 (10%)
VI	2-4'- 3-6-3-7-7-8-5	1 (10%)

a: The sequence corresponding the alleles of the MIRU 2, MIRU 4, MIRU 40, MIRU 10, ETRA, ETRB, MIRU 26, MIRU 27 and MIRU 39*,* respectively.

Combining the results of spoligotyping and MIRU-VNTR, the strains were grouped in a cluster (three isolates) and seven isolates orphans (Figure 2). According to Roring et al. (2002), although some individual *loci* have a high discriminatory power for both *M. tuberculosis* and *M. bovis* isolates, others seem to be more polymorphic for a particular species and thus, in general, fewer polymorphisms are observed in *M. bovis* isolates. Ramos et al. (2014b) conducted a study in the Southern region of Brazil and identified that MIRU-VNTR alone or associated with spoligotyping, demonstrated highest discriminatory power (HGDI 0.66 and 0.70, respectively). Analysis of allelic diversity of each *locus* (*h*) showed that MIRUs 39 and 40 and ETR-A have highest discriminatory power, with *h* value of the 0.5. This finding is in agreement with previously published data showing that ETR-A have the considerable discriminatory power (HLOKWE et al., 2013; ROCHA et al., 2013), while, unlike those found in our study, the *loci* 39 and 40 has been displayed with low discriminatory power (ALLIX et al., 2006; HLOKWE et al., 2013; PARREIRAS et al., 2012; RORING et al., 2002).

Besides the molecular epidemiology of *M. bovis* has been investigated in few regions of Brazil, some studies show that the spoligotypes SB0120 e SB0121 are the predominants in the country, mainly in the South, North, South-East and Center-West regions (ALZAMORA FILHO et al., 2014; CARNEIRO et al., 2020; CARVALHO et al., 2016; CAZOLA et al., 2015; FIGUEIREDO et al., 2012; PARREIRAS et al., 2012; RAMOS et al., 2014b; ROCHA et al., 2013; RODRÍGUEZ et al., 2010; SOUZA-FILHO et al., 2016; ZANINI et al., 2005). In this study, the profile of the *M. bovis* identified was characterized previously just in the North Brazil by Parreiras et al. (2012) and Carneiro et al. (2020).

The regional differences in the discriminatory power of genetic markers is important to define an optimal combination of genotyping markers to be used in a particular region or country (HILTY et al., 2005; RAMOS et al., 2014a; RORING et al., 2002).

Epidemiologically linked isolates have been matched through the use of spoligotyping and MIRU-VNTR typing. Strains in a cluster were obtained from animals from Manicoré – AM, however the other Manicoré - AM isolates were identified in four orphans' profiles in the

dendogram. The isolates from distinct geographic localities were found to have unique profiles, being two orphans from Alenquer - PA and the isolate 95 of Óbidos - PA (Figure 2).

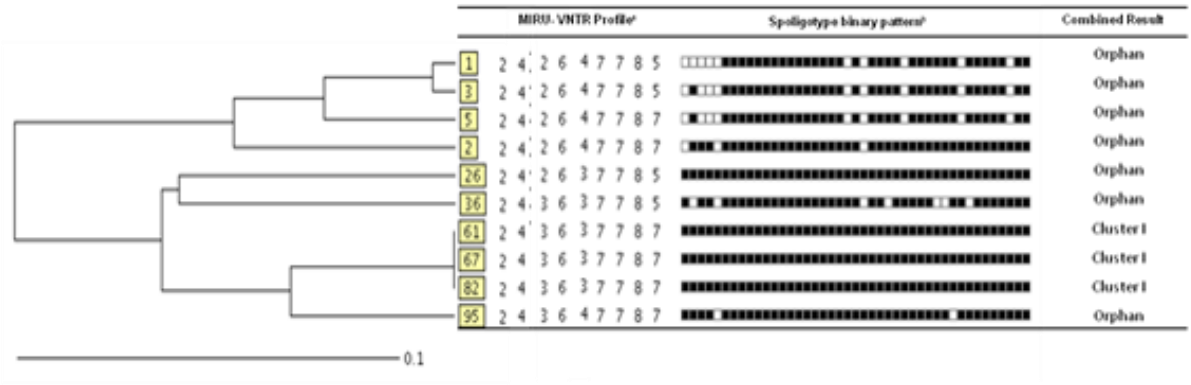

Figure 2 - Molecular characteristics of *Mycobacterium bovis* isolates obtained from a slaughterhouse in Manaus (AM), Brazil, in 2010.

This study revealed a high genetic diversity of *M. bovis* isolates obtained from cattle in the same slaughterhouse, probably due to different geographical origin among these animals. In our study using culture and PCR-based methods, the presence of *M. bovis* was identified in 10 lymph node samples that did not present any macroscopic lesions related to tuberculosis. This number exceeds the prevalence previously reported in cattle/buffalo with suggestive lesions slaughtered in the Northern region of the country (CARNEIRO et al., 2020). The results of this study suggest that tuberculosis infection of non-symptomatic animals can be a risk factor associated with the propagation of *M. bovis.*

Molecular typing of mycobacteria isolated from domestic and wild animals allows us to establish epidemiological links necessary for the development of successful control strategies. Thus, not only the potential risks of transmission between the animals are identified, but also the spread of ill animals in the meat trade. The epidemiological identification of these sources of infection and routes are a necessary part for a more specific and effective control of diseases such as tuberculosis.

In addition, the spoligotyping associated with MIRU-VNTR typing could serve as a tool to characterize the dynamics of bovine tuberculosis in this and other regions of Brazil. Therefore,

through epidemiological surveillance and knowledge of the spatial distribution of *M. bovis* strains, it is possible to monitor the cases, facilitating actions to control and eradicate this disease (CARNEIRO et al., 2019; CONCEIÇÃO et al., 2020).

CONCLUSION

Our results strongly suggest that strategies for BTB control must rely on continuous epidemiological studies, enhanced supervisory procedures, involvement of stakeholders and rigorous measures for avoiding contact between infected animals and other cattle herds. Furthermore, using molecular biology tools and molecular epidemiology analysis, we showed the importance of understanding the distribution and dynamics of *M. bovis* transmission in the Northern region of Brazil, mainly in dairy and beef cattle. Therefore, our data support the recommendation for improved and compulsory post-mortem inspections in animals in geographical areas where BTB risk is high and the trade in beef and milk is financially and socially an important activity.

TUBERCULOSE BOVINA NO NORTE DO BRASIL

RESUMO

A tuberculose bovina (TBB), causada pelo *Mycobacterium bovis*, é uma doença infecciosa distribuída mundialmente, afeta bovinos e outros animais, incluindo o homem. No Brasil, a TBB é endêmica e causa perdas econômicas pela redução da produtividade da pecuária e perda de carcaças durante o abate. A epidemiologia molecular tem sido usada como ferramenta para a investigação da transmissão do microrganismo e dinâmica da doença. Foram avaliadas 99 amostras de linfonodos obtidos de animais (com e sem lesão sugestiva) abatidos na região norte do Brasil, avaliando a presença de *M. bovis* através de técnicas de microscopia, cultura em meio Stonebrinck e identificação molecular usando o teste da reação da cadeia da polimerase (PCR). Além disso, dois métodos de genotipagem (*Spoligotyping* and MIRU-VNTR) foram utilizados para identificar o perfil genotípico dessas cepas. Dos 99 linfonodos retrofaríngeos coletados, apenas 10 amostras clínicas amplificaram usando a técnica de PCR, as quais foram consideradas positivas para *M. bovis* e, através das análises moleculares, combinando *spoligotyping* e MIRU-VNTR foi possível identificar oito diferentes padrões genotípicos. Apenas um *spoligotype*, majoritário entre as amostras testadas (40%), já foi identificado no banco de dados (SIT523). Pela identificação

epidemiológica destas cepas é possível investigar a dinâmica da transmissão da doença a qual é uma parte essencial do controle mais específico e efetivo de doenças tais como a tuberculose.

Palavras-chave: *Mycobacterium bovis*. Ferramentas moleculares. Gado. *Spoligotyping*. Genotipagem.

TUBERCULOSIS BOVINA EN EL NORTE DE BRASIL

RESUMEN

La tuberculosis bovina (TBB), causada por *Mycobacterium bovis*, es una enfermedad infecciosa de distribución mundial que afecta al ganado y otros animales, incluidos los humanos. En Brasil, TBB es endémica y causa pérdidas económicas al reducir la productividad del ganado y la pérdida de cadáveres en los mataderos. La epidemiología molecular se ha utilizado como herramientas para la investigación de la transmisión de microorganismos y la dinámica de esta enfermedad. Se estudiaron 99 muestras de ganglios linfáticos obtenidas de animales (con y sin lesión sugestiva) sacrificados en la región norte de Brasil evaluando la presencia de *M. bovis* mediante técnicas de microscopía, cultivo en medio Stonebrinck e identificación molecular mediante la prueba de reacción de cadena de la polimerasa (PCR). Además, se utilizaron dos métodos de genotipado (*Spoligotyping* y MIRU-VNTR) para identificar el perfil genotípico de estas cepas. De los 99 ganglios linfáticos retrofaríngeos recolectados, solo 10 muestras clínicas amplificadas mediante la técnica de PCR, que se consideraron positivas para *M. bovis* y, mediante el análisis molecular de la combinación de *spoligotyping* y MIRU-VNTR, fue posible identificar ocho patrones diferentes. Solo un *spoligotype*, la mayoría de las muestras analizadas (40%), ya había sido identificado en la base de datos (SIT523). A través de la identificación epidemiológica de estas cepas es posible investigar la transmisión dinámica de la enfermedad, que es una parte esencial del control más específico y efectivo de enfermedades como la tuberculosis.

Palabras clave: *Mycobacterium bovis.* Herramientas moleculares. Ganado. *Spoligotyping*. Genotipado.

CONFLICT OF INTEREST STATEMENT

All authors report no conflicts of interest relevant to this article.

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Corresponding author:
Daniela Fernandes Ramos.
Núcleo de Pesquisa em Microbiologia Médica, Laboratório de Micobacteriologia, Universidade Federal do Rio Grande/FURG. CEP 96015-000, Rio Grande, RS, Brasil.
daniferamos@gmail.com