Spoligotyping, genotyping, and spatial distribution of *Mycobacterium bovis* in cattle in the state of Bahia, Brazil[1]

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ABSTRACT.- Santos E.S.V., Alzamora Filho F., Santos B.R., Silva A.V., Gomes H.M., Suffys P.N. & Costa J.N. 2021. **Spoligotyping, genotyping, and spatial distribution of *Mycobacterium bovis* in cattle in the state of Bahia, Brazil**. *Pesquisa Veterinária Brasileira 41:e06729, 2021*. Universidade Federal da Bahia, Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: evelin_vet@hotmail.com

Mycobacterium bovis is responsible for bovine and buffalo tuberculosis, an important zoonotic disease with global distribution. The knowledge of the distribution and the precise identification of this disease, including advanced diagnoses such as spoligotyping, allows choosing the best strategies to fight the disease's progress. The present work aimed to investigate mycobacteria's presence, genotype their strains, and evaluate tuberculosis cases' spatial distribution from suggestive lesions in carcasses of bovine and buffalo inspected in slaughterhouses under an official inspection regime in the state of Bahia, Brazil. The study investigated 453,417 animals. Among these, 31 (0.007%) from 17 municipalities were suspected of tuberculosis. Among the culture medium growth, 95% of these were categorized as alcohol-acid resistant bacilli (BAAR). All isolates were subjected to spoligotyping and 95% were confirmed as *M. bovis* (SB0120, SB0121, SB0852, SB0828, SB0295, SB0881, SB1648, SB6119, SB0140, SB1055). The strain SB0120 was the most prevalent, and this profile has been described in cases of human tuberculosis by *M. bovis,* highlighting the zoonotic potential of this profile. This study also identified strains never reported in Bahia, highlighting a distinctive pattern from other parts of Brazil, besides mixed infections. Besides, to identify strains never before described in the state, highlighting a distinctive pattern in Brazil (SB6119 and SB0852, respectively). An unpublished profile was identified and inserted in the international database (Mbovis.org), named SB2715.

INDEX TERMS: Spoligotyping, genotyping, spatial distribution, *Mycobacterium bovis*, cattle, Brazil.

RESUMO.- [**Genotipagem por *spoligotyping* e distribuição espacial de *Mycobacterium bovis* em bovinos no estado da Bahia, Brasil**]. O *Mycobacterium bovis* é o responsável pela tuberculose bovina e bubalina, doença zoonótica importante e com distribuição global. O conhecimento da distribuição e a identificação precisa dessa enfermidade, incluindo diagnósticos mais avançados como o *spoligotyping*, permite escolher as melhores estratégias de combate ao avanço da doença. O presente trabalho objetivou investigar a presença de micobactérias, genotipar suas estirpes e avaliar a distribuição espacial dos casos de tuberculose a partir de lesões sugestivas nas carcaças de bovinos e bubalinos inspecionadas em frigoríficos sob regime de inspeção oficial no estado da Bahia. Foram investigados 453.417 animais dentre os quais 31 (0,007%) foram suspeitos de doença e provenientes de 17 municípios. Após o crescimento em meio de cultura, 95% foram categorizados como bacilos álcool-ácido resistentes (BAAR). Todos os isolados foram submetidos à *spoligotyping* e 95% foram confirmados *M. bovis* (SB0120, SB0121, SB0852, SB0828, SB0295, SB0881, SB1648, SB6119, SB0140, SB1055). A cepa SB0120 foi a mais prevalente e este perfil vem sendo descrito na literatura com casos de tuberculose humana por *M. bovis* ressaltando o potencial zoonótico deste perfil. Este estudo também identificou cepas nunca relatadas no estado

da Bahia, destacando um padrão distinto de outras partes do Brasil, além da existência de infecções mistas. Permitiu ainda relatar linhagens nunca antes descritas no estado com destaque para um padrão novo no Brasil (SB6119 e SB0852 respectivamente). Um perfil inédito identificado foi identificado e inserido no banco de dados internacional (Mbovis.org), nomeado SB2715.

TERMOS DE INDEXAÇÃO: Genotipagem, *spoligotyping*, distribuição espacial, *Mycobacterium bovis*, bovinos, Brasil.

INTRODUCTION

The sector agribusiness faces considerable health challenges, including animal tuberculosis (Embrapa 2014), a neglected disease. Epidemiological studies are of crucial importance (Ghavidel et al. 2018). Human tuberculosis is a significant cause of death from infection worldwide. Annually it causes illness in 10 million people, leading to the death of 1.5 million (WHO 2020). In some countries, 10% of human tuberculosis cases are caused by *Mycobacterium bovis*. It is worth remembering that there are reasons for the *Mycobacterium* species differentiation, such as the resistance of *M. bovis* to pyrazinamide (1st line antituberculosis drug) and reintroduction of the disease by this species in countries in which it was practically eradicated (OIE 2020).

The disease's genetic and demographic characterization contributes to a better understanding of its distribution. Thus, molecular genotyping using the spoligotyping technique contributed to improving the understanding of *M. bovis* infections, distribution of strains in a given area, and how multiple outbreaks occur, providing an increase in the efficiency of disease control programs (Cazola et al. 2015).

The spoligotyping technique is based on PCR (polymerase chain reaction) amplification of DNA in the locus of the direct repeat region (DR) exclusive to the mycobacterial genome of the CMT (*Mycobacterium tuberculosis* complex). The spacers' order is approximately the same in all isolates to detect the presence or absence of spacers in this locus, identifying the polymorphism. Detection is carried out by hybridizing the spacers to a membrane using a minibloter (Kamerbeek et al. 1997). The spoligotype determination is essential to find the infection source and control the disease (Ghavidel et al. 2018). Ramos et al. (2014) reported that the technique reveals possible links between sick animals, detecting outbreaks, and still allows a greater understanding of the evolution and population structure of *M. bovis*.

Given the context presented, the combination of conducting epidemiological surveys from slaughterhouses with *M. bovis* genotyping works as an essential indicator of bovine tuberculosis (BT) frequency. This association can contribute to a better understanding of the infection dynamics, an essential fact to improve the effective management of control systems to the referred disease, subsidizing the "Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal" (PNCEBT; National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis). The present study aimed to investigate mycobacterias presence, genotype their strains using the spoligotyping technique, and evaluate BT cases spatial distribution from suggestive lesions in the bovine and buffalo carcasses inspected in slaughterhouses under official inspection in the state of Bahia.

MATERIALS AND METHODS

From January 2016 to October 2018, the researchers monitored the slaughter and post-mortem inspection of 453,417 carcasses, including bovines (n=452,619) and buffalo (n=798), both dairy and beef cattle. Included animals were assessed as healthy on the antemortem examination performed by the official inspection service (state and federal) and slaughtered in 11 slaughterhouses.

The research addressed suspected bovine tuberculosis (BT) lesions, circumscribed lesions, pale yellow, cheesy and/or mineralized appearance observed in cattle from 17 municipalities. Samples from suspected cases were collected and submitted for microbiological culture, which was performed in duplicated at the "Laboratório de Micobacterioses" of the "Universidade Estadual de Santa Cruz", Ilhéus (LAMVET-UESC). The samples were decontaminated by the Hexadecyl Pyridinium chloride 1.5% (HPC) method with subsequent sowing in *Lowenstein-Jensen* (LJ) and *Stonebrink-Leslie* (SL) media and kept frozen until analysis. The tubes were observed weekly during their incubation in a BOD (biochemical oxygen demand) incubator at 37°C for 90 days (Brasil 2008).

The isolates obtained from culture were stained using the Ziehl-Neelsen (ZN) method in those strains displaying the expected growth of *Mycobacterium* sp., and the DNA extracted by thermal lysis (Shi et al. 2018). The resulting DNA was then taken to the "Laboratório de Biologia Molecular Aplicada a Micobactérias" of the "Instituto Oswaldo Cruz" (LABMAM-Fiocruz) in Rio de Janeiro for molecular analyzes by spoligotyping.

Typing by spoligotyping was performed as described by Kamerbeek et al. (1997), and the amplified products underwent membrane hybridization with 43 oligonucleotides. The nomenclature adopted for the spoligotypes obtained was as available on MBovis.org[7] and the profiles compared to those available on the same site and SITVIT-WEB[8]. The new profile identified in this study was inserted in the international database[7], and the new spoligotype was accepted.

The maps were generated using the QGIS software, version 2.14.3 - Essen, based on data on the location of the animals' origin and cartographic bases (*shapefiles*) provided by "*Instituto Brasileiro de Geografia e Estatistica*" (IBGE). The Coordinate Reference System used was EPSG: 31999 - SIRGAS/UTM zone 24S. The geographic distribution was generated by the interpolation method Inverse Distance Weighting (IDW), which used the indexes found in each region for mapping.

RESULTS AND DISCUSSION

Thirty-one of the 452,619 bovine carcasses analyzed were identified by the inspection as suspected of bovine tuberculosis (BT) and had characteristic macroscopic lesions, representing a frequency of 0.007% for the analyzed period. Buffalo carcasses did not present any suspect lesion of tuberculosis.

In four of the suspect cattle, it was not possible to perform the microbiological analysis. Thus, 27 animals were considered under analysis. Suspected BT cases represented a 74.1% growth rate in mycobacterial selective culture medium (20/27). The observed cultures were submitted to ZN staining, and 95% (19/20) showed acid-alcohol resistant bacillus (BAAR).

Among BAAR positive isolates, 95% (18/19) were confirmed as *Mycobacterium bovis* using spoligotyping. (Table 1). The

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[7] Available at <http://www.mBovis.org/>

[8] Available at <HTTP://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/>

typing of the profiles showed deletions in the spacers 3, 9, 16, and 39-43, characterizing the referred bacteria (Kamerbeek et al. 1997). This result indicates that tuberculosis is present in the herds investigated and may represent a public health risk.

In cases where more than one sample was collected, the analysis was performed on all of them (32 samples), which allowed the detection of infections caused by different spoligotypes in the same animal (identified by the code-numbers 3, 7, 8, 10, 16, and 17). In some cases, the same granuloma revealed different BT strains (the animal 10, as an example, housed three different profiles) (Table 1). Mixed infections can reveal reinfection, especially in immunity failure (Warren et al. 2004). The variety of profiles can also be explained as microevolution of strains, occurrence of small evolutionary changes, as reported by Navarro et al. (2016), who claim that such events do not exclusively occur in infection periods.

Animal 2 presented a previously undescribed strain pattern. This result enabled the discovery of a new spoligotype profile not yet known in international databases. The spoligotype was named SB2715 and derived from the municipality of Vera Cruz. Only one sample, 3.2% (1/32), revealed a doubtful pattern that was impossible to elucidate. Although spoligotyping is a very enlightening typing method, its association with other molecular techniques is more elucidative, such as typing by MIRU-VNTR, considering that it increases discriminatory power (Supply et al. 2006). The decision of which of the techniques to use relies on factors such as available infrastructure, technical human resources, lineage to be identified, and the discriminatory power required for the study's aim (Shi et al. 2018).

It was possible to identify ten spoligotypes that had already been described in the international literature (SB0120, SB0121, SB0852, SB0828, SB0295, SB0881, SB1648, SB6119, SB0140, SB1055) and an unprecedented genotype allocated as SB2715, and three monophyletic groups of the studied microorganism: BOV-1, BOV, and BOV-2.

The variability of spoligotypes found in the present study demonstrates a probable movement of strains between the five continents. Among the identified strains, the most prevalent were SB0120 and SB0121. These data corroborate with a broad survey performed by Ghavidel et al. (2018), reporting these two strains as the most common in animals worldwide. The authors claimed, these two strains were the most common

Table 1. Molecular characterization by Spoligotyping of isolates obtained from lesions in cattle suspected of having tuberculosis from carcasses from a slaughterhouse in the state of Bahia, Brazil

Animal	Sample	Spoligotype profile - spacers 1-43*
1	AD01	■■□■■■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□
2	AD02	■■□■■■■■□■■■■□□■■■■□■■■■■■■■■■■■■■■■□□□□□**
3	AD06	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■■■■□□□□□
3	AD06LJ	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■■■■□□□□□
3	AD07	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■■■■□□□□□
3	AD07LJ	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■■■■□□□□□
6	AD16LJ	■■□■■■■■□■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□□
7	AD18	■■□■■■■■□■■■■■□■■■□■■■■■■■■■□■■□■■■■□□□□□
7	AD20	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■□■■■■□□□□□
8	AD21	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□■□□□□□□
8	AD21LJ	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■■■■□□□□□
9	AD27	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■□■□□□□□
10	AD23	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□□
10	AD24	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□
10	AD24LJ	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□
10	AD25	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□
10	AD25LJ	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□
14	AD31	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■□□□□□
15	AD32	■■□■■■■■□■■■■□■■■□■■■□■■■■■■■■■■□□□□□
16	AD33	■■□□□■■■■■□■■■■□■■■□■■■■■■■■■■■□■□□□□□
16	AD33LJ	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
17	AD34	■■□■■■□■■□■■■□■■■□■■■■■■■■■■■■■□□□□□
17	AD34LJ	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
18	AD35	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
18	AD35LJ	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
19	AD36	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
19	AD36LJ	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
21	AD38	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
22	AD39	■■□■■■■■□■■■■■■■■■□■■■□■■■■■■■■■■□■□□□□□
23	AD40	Inconclusive
27	AD44	■■□■■■■■□■■■■□■■■□■■■■■■■■■■■■■□□□□□
28	AD45	■■□■■■■■□■■■■□■■■■■□■■■□□□□□□■■■■■□□□□□

* Black and white frames indicate the presence and absence of the spacer in the direct repeat locus, respectively, ** new *Mycobacterium bovis* profile found.

in animals worldwide. These authors also suggested the high transmission and aggressive potential of these two profiles as crucial factors for their wide distribution.

SB0120 was the most prevalent spoligotype: 32.2% (10/31) with a cluster of 10 isolates, and distribution among five municipalities (Jequié, Pedro Alexandre, Colonel João de Sá, Ipirá, and São Filipe). This spoligotype had been previously described in Brazil in the states of Paraíba, Distrito Federal (Parreiras et al. 2012), São Paulo (Rocha et al. 2013, Rodriguez 2005), and Bahia (Costa et al. 2010) being and also found in other parts of the world. This strain has a great diversity of hosts, besides being disseminated globally, especially in European countries. Haddad et al. (2001) claimed a 26% prevalence of this spoligotype. Several authors correlate this spoligotype to human tuberculosis cases by *M. bovis* (Gibson et al. 2004, Amato et al. 2018, Siala et al. 2019), suggesting the zoonotic potential of this profile. A study by Alzamora Filho et al. (2014) in Bahia did not identify the above-quoted strain.

Spoligotype SB0121 formed the other cluster in this study with nine isolates and a frequency of 29% (9/31). Although it is not the one with the highest prevalence, it is more widespread being present in the municipalities of Mutuípe, Serrinha, Santa Bárbara, Jequié, Pedro Alexandre, Colonel João de Sá, and Santa Luz. Alzamora Filho et al. (2014) claimed this was the most prevalent strain in the Bahia state. The present research agrees with the study results, describing the spoligotype SB0121 as the second most prevalent spoligotype in the state. The authors detected this profile in the municipalities of Euclides da Cunha, Ipirá, Pojuca, and Serrinha. Other studies described the spoligotype SB0121 as the most prevalent in other Brazilian states, such as Minas Gerais (Parreira et al. 2012), São Paulo (Rocha et al. 2013), Rio Grande do Sul (Ramos et al. 2014), and throughout the whole area of the Midwest region (Carvalho et al. 2016). Still, in the American continent, it is the most frequent in Mexico (Zumárraga et al. 2013). It is widely reported in Europe and less frequently in African countries (Ghavidel et al. 2018).

The profiles SB6119 (Mutuípe), SB0852 (Santa Bárbara and Jequié) and SB0295 (Santa Bárbara and Coronel João Sá) had two isolates each, corresponding to a prevalence of 6.4% (2/31) of the findings individually. The first spoligotype had previously been mentioned only in Brasília/DF (Sitvit Web). SB0852 was first described in Brazil by the present work and had previously been reported in France and the United States (Sitvit Web). SB0295 has been described as the second most frequent in the states of Mato Grosso and Goiás (Carvalho et al. 2016). The profile had also been previously reported in Paraíba, Federal District, Minas Gerais, Mato Grosso do Sul, Santa Catarina (Parreiras et al. 2012), São Paulo (Rocha et al. 2013). In Bahia, it had been previously reported in Alagoinhas and Glória (Alzamora Filho et al. 2014). In addition to the states mentioned, it was reported on the European continent (Sitvit Web). Findings from the present study indicate that animal trades between states or different countries may contribute to the spread of different isolates.

The spoligotypes SB0140 (Serrinha), SB0828 (Araci), SB1648 (Araci), SB0881 (Dom Macêdo Costa), SB1055 (Colonel João Sá) and EG-BA13 (Vera Cruz) were identified in a unique sample each 3.2% (1 / 31). In Bahia, Alzamora Filho et al. (2014) had already reported the first spoligotype in Santo Antônio de Jesus, and Costa et al. (2010) described the SB1055

as the most frequent isolate in the state. SB0881 is the third most found in Brazil, although this is the first record in Bahia and cattle. Zumárraga et al. (2013) claimed the spoligotype SB0140 was the most representative in Argentina and Chile. In Mexico, it occupies the third position (Razo et al. 2018) and has been reported worldwide but in Asia. The spoligotypes SB0828 and SB1648 had only been reported in Europe and one report in Brazil.

Limited information on the spatial epidemiology of a disease can significantly hinder the study of its prevalence and occurrence. The present work identified the areas with the highest concentration of BT cases through interpolation, using the IDW method easing the tracking of outbreaks, thus allowing targeted sanitary actions. The region that displayed the highest prevalence of BT was the Northeast region. The municipalities of Serrinha, Vera Cruz, Mutuípe, Araci, Santa Bárbara, Jequié, Pedro Alexandre, Colonel João Sá, Ipirá, São Felipe, Santa Luz, and Dom Macêdo Costa were described as the focus of BT in the studied area. They should deserve special attention to the control of the disease (Fig.1). In Bahia, the defense agency works in partnership with slaughterhouses to monitor suspicious injuries as part of the evolution of the PNCEBT. This action aims beyond animal health, but also surveillance of human health.

Ávila et al. (2013) carried out a temporal space characterization study of BT in the State of Bahia. The authors could not find any significant evidence of spatial agglomeration of the disease in the state was detected in a study based on tuberculization. The authors focused on outbreaks' geographical identification. In the present study, Coronel João Sá displayed a greater variety of strains (four of them - SB1055, SB0120, SB0121 and SB0295), followed by Santa Bárbara, Mutuípe, and Jequié with three profiles each (Fig.2). It is worth remembering that Colonel João Sá is very close to the border with the state of Sergipe, where little has been revealed about BT and enabling a possible transit route for infected animals.

These results demonstrate that distant municipalities in Bahia have similar spoligotypes found in other Brazilian states, and different continents. The origin of the animal
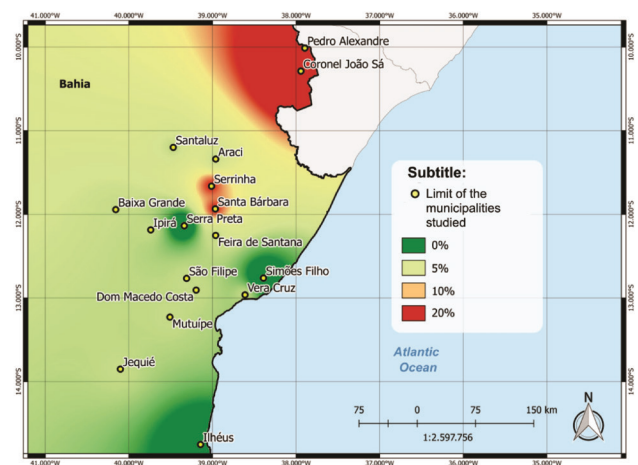

Fig.1. Geographic distribution of bovine tuberculosis confirmed by spoligotyping through interpolation using the Inverse Distance Weighting (IDW) method, emphasizing the intensity of the disease in the analyzed regions.
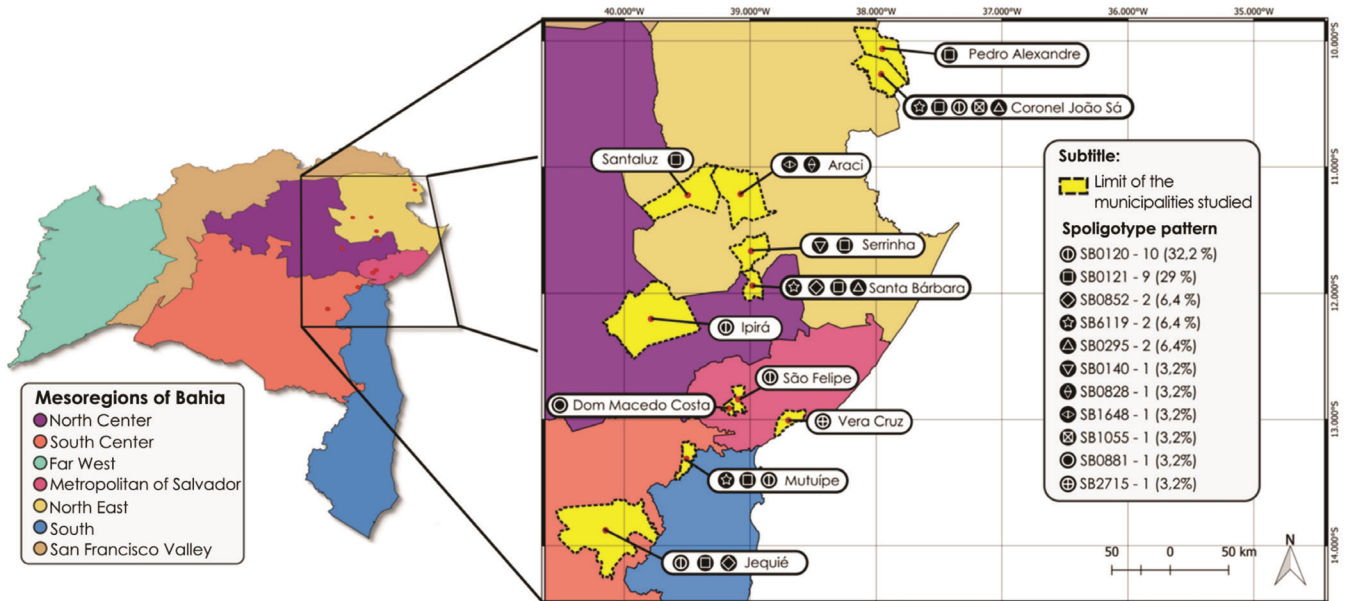
Fig.2. Genetic diversity of *Mycobacterium bovis* isolates distributed by the municipality in the state of Bahia.

(Mckinley et al. 2018) and the transit from properties, regions, or countries with BT represent a potentially critical factor for transmitting the disease (Skuce et al. 2012). Only one of the profiles found (SB1055) was not described in Europe, revealing a close relationship between the study's strains and the European continent. This fact can perhaps be explained due to the introduction of European races in Latin America. For strains located very distant geographically where there may have been no commercial relationships, it is possible to speculate the possibility of homoplasy giving rise to new genetic lines, with spoligotypes similar to those observed in another location without an epidemiological relationship (Zumárraga et al. 2013).

In the epidemiological context, Ramos et al. (2014) claim that it is essential to perform a molecular differentiation of the isolates. The genetic diversity of *M. bovis* isolates from bovine carcasses in Bahia suggests the probable movement of infected animals. The movement of the animals can be an essential factor for the transmission of BT. This study described the highest diversity of profiles in the state of Bahia so far reported. The combination with spatial visualization highlights *M. bovis* strains' geographic distribution, thus contributing to the BT control and eradication program in the studied area.

CONCLUSIONS

The spoligotyping technique verified the genetic diversity among the bovine tuberculosis (BT) strains of the Bahia state, the occurrence of mixed infections, and the predominance of the SB0120 profile. This investigation provides important information that contributes to the disease's molecular epidemiology, including the report of a new genomic profile.

The analysis of the spatial epidemiology in the study region proved to be an essential tool for directing more specific BT control actions, hence contributing to the disease control and eradication program in the state of Bahia.

Conflict of interest statement.- The authors declare that there is no conflict of interest regarding the publication of this article.

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