Genotypic characterization of *Mycobacterium bovis* isolates from dairy cattle

Elizabeth Melo[1], Harrison Gomes[2], Ícaro Santos[2], José Augusto Afonso[3], Marília Junqueira Franco[4], Helio Langoni[5], Antonio Paes[6], Philip Suffys [7], and Carla Mendonça[3]

[1]Federal Rural University of Pernambuco
[2]Instituto Oswaldo Cruz, FIOCRUZ
[3]Universidade Federal Rural de Pernambuco
[4]UNESP-Univ. Estadual Paulista
[5]UNESP Campus de Botucatu
[6]UNESP-Univ. Estadual Paulista, Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine and Animal Sciences, Botucatu, State of São Paulo, Brazil.
[7]Institute Oswaldo Cruz

October 2, 2020

Abstract

Molecular diagnosis of bovine tuberculosis plays an essential role in the epidemiological knowledge of the disease. Bovine tuberculosis caused by Mycobacterium bovis represents a risk to human health. This study aimed to perform the genotypic characterization of M. bovis isolated from bovines diagnosed as tuberculosis from dairy herds in the state of Pernambuco, Brazil. Granulomas from 30 bovines were sent for microbiological culture and colonies compatible with Mycobacterium spp were obtained in at least one culture from 17/30 granulomas. All isolates were confirmed to be M. tuberculosis bovis by spoligotyping and 24loci MIRU-VNTR typing. While spoligotyping characterized the isolates as SB0121, SB0295, SB0852, SB0120 and an unclassified genotype, 24loci MIRU-VNTR rendered two clusters of two isolates each and 13 unique profiles. Loci ETR-A showed higher discriminatory power, and loci (ETR-B, ETR-C, MIRU16, MIRU27 and QUB26) showed moderate allelic diversity. This is the first study on genetic variability of the infectious agent cause of bovine TB in Pernambuco and demonstrates variability of strains in the state. Thus, it corroborates the importance of this microorganism as agent of bovine tuberculosis and its zoonotic potential, being this epidemiological tool determinant in the rigor of the sanitary practices of disease control in dairy herds.

Genotypic characterization of *Mycobacterium bovis*isolates from dairy cattle

Elizabeth Hortêncio de Melo [1*], Harrison Magdinier Gomes[2], Ícaro Rodrigues dos Santos[2], José Augusto Bastos Afonso[3], Marília Masello Junqueira Franco[4], Hélio Langoni[4], Antonio Carlos Paes[4], Philip Noel Suffys[2], Carla Lopes de Mendonça[3]

Elizabeth Hortêncio de Melo [1]. Corresponding author: Elizabeth Hortêncio de Melo. Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, S/N, Dois Irmãos. 52171-900. Brasil, Pernambuco. E-mail: elishmelo@hotmail.com

Harrison Magdinier Gomes[2]. Laboratório de Biologia Molecular Aplicada à Micobactérias, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ, Brasil.

Ícaro Rodrigues dos Santos[2]. Laboratório de Biologia Molecular Aplicada à Micobactérias, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ, Brasil.

José Augusto Bastos Afonso[3]. Clínica de Bovinos, Campus Garanhuns/UFRPE, Pernambuco, Brasil.

Marília Masello Junqueira Franco[4]. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, UNESP, Botucatu, Brasil.

Helio Langoni[4]. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, UNESP, Botucatu, Brasil.

Antonio Carlos Paes[4]. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, UNESP, Botucatu, Brasil.

Philip Noel Suffys[2]. Laboratório de Biologia Molecular Aplicada à Micobactérias, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ, Brasil.

Carla Lopes de Mendonça[3]. Clínica de Bovinos, Campus Garanhuns/UFRPE, Pernambuco, Brasil.

ABSTRACT

Molecular diagnosis of bovine tuberculosis plays an essential role in the epidemiological knowledge of the disease. Bovine tuberculosis caused by *Mycobacterium bovis* represents a risk to human health. This study aimed to perform the genotypic characterization of *M. bovis*isolated from bovines diagnosed as tuberculosis from dairy herds in the state of Pernambuco, Brazil. Granulomas from 30 bovines were sent for microbiological culture and colonies compatible with*Mycobacterium* spp were obtained in at least one culture from 17/30 granulomas. All isolates were confirmed to be *M. tuberculosis bovis* by *spoligotyping* and 24*loci* MIRU-VNTR typing. While *spoligotyping* characterized the isolates as SB0121, SB0295, SB0852, SB0120 and an unclassified genotype, 24*loci*MIRU-VNTR rendered two clusters of two isolates each and 13 unique profiles. *Loci* ETR-A showed higher discriminatory power, and*loci* (ETR-B, ETR-C, MIRU16, MIRU27 and QUB26) showed moderate allelic diversity. This is the first study on genetic variability of the infectious agent cause of bovine TB in Pernambuco and demonstrates variability of strains in the state. Thus, it corroborates the importance of this microorganism as agent of bovine tuberculosis and its zoonotic potential, being this epidemiological tool determinant in the rigor of the sanitary practices of disease control in dairy herds.

Keywords : bovine tuberculosis, genotypic, *Mycobacterium bovis* .

INTRODUCTION

Bovine tuberculosis is a chronic progressive disease caused by*Mycobacterium tuberculosis var. bovis* which affects mainly cattle and buffalo but also infects other mammalian species of mammals, including humans (Cousins et al., 2003). The zoonotic potential of this disease is related to the consumption of raw milk and unpasteurized derivatives, representing the main route of transmission to humans, more pronounced in rural areas. In the state of Pernambuco, a prevalence of outbreaks of 2.87% and 0.62% of infected animals was reported in 2016, with a tendency to concentrate in the Agreste region of the state and with a predominance in dairy properties (Lima et al., 2016).

The interest in nucleic-acid based diagnostic procedures increased because of the limitations of conventional testing such as lack of sensitivity and specificity of the allergic-skin test and the long period for confirming the presence of the agent by bacteriological methods (Drewe and Smith, 2014). In addition, Molecular typing methods have provided a great impetus in the molecular epidemiology studies of the *M. tuberculosis* complex include comparing mycobacterial genome sequences. Among the most used genotyping techniques for the study of the *M. tuberculosis* complex are *Spoligotyping* and Variable Number of Interspersed Repetitive Units of Mycobacteria -MIRU-VNTR (Kamerbeek et al., 1997; Supply et al., 2006). The MIRU-VNTR has higher discriminatory power and has currently been the method of choice in the genotyping studies of *Mycobacterium* spp, and in particular related to *M. tuberculosis var. bovis,* allows identification of prevalent strains circulating in a herd or geographic regions (Kamerbeek et al., 1997; Supply et al., 2006).

M. tuberculosis var. bovis infection has an impact on both animal and human health, nonetheless, scarce are the studies in the region on molecular genotyping. Given the lack of data on the contribution and nature of *Mycobacterium tuberculosis complex* (MTBC) to bovine TB in the state of Pernambuco, we performed the genotypic characterization of Mycobacteria isolated from bovines from dairy herds in this region that were diagnosed clinically with tuberculosis, coming from dairy herds in the state of Pernambuco.

MATERIAL AND METHODS

The study included 28 bovines and two buffaloes that had been attended at the Bovine Clinic of Garanhuns/ UFRPE, presenting clinical symptoms suggestive for tuberculosis. The animals were submitted to clinical examination, being the information, including epidemiological, that was annotated in clinical records. Among the information present in the anamnesis provided by the owners, common to most animals, were progressive weight loss, dry cough, and decreased milk production.

According to the evolution/severity of the clinical cases and the result of the allergic-skin test, the animals were euthanized according to the current legislation (Brazil, Ministry of Agriculture, Livestock and Supply. Normative Instruction n. 19, 10 of October, 2016) and submitted to anatomopathological examination.

Fragments of organs with lesions characteristic of granulomas were collected for histopathological examination and lymph nodes with lesions for microbiological culture.

The samples for bacteriology were stored in a freezer (-80°C) for further processing while for histopathological evaluation, fragments were fixed in 10% buffered formaldehyde, processed, and stained with hematoxylin and eosin (HE). Granulomas from all 30 bovines were collected and sent for microbiological culture an sample processing and culture conditions favoring isolation of *M. tuberculosis var. bovis* were carried out following the recommendations of Franco et al. (2017). Samples were minced and decontaminated according to the Petroff method, inoculated on Lowenstein-Jensen and Stonebrink medium and incubated at 37°C for 90 days.

Nucleic acid obtained of the cells was performed by thermolysis. Molecular identification to the *Mycobacterium* species was performed by PCR amplification of a 1020 bp fragment of the *gyr* B gene as described by Chimara et al. (2004), Franco et al. (2017). In the reaction, 1 µL of DNA (20 ng), 47 µL of Master Mix (1x) were used (Thermo Scientific, Waltham, MA, USA) and 10 pMol of each primers MTUBf (5' TCGGACGCGTATGCGATATC 3') and MTUBr (5'ACATACAGTTCGGACTTGCG 3') [DNA Express Biotecnologia LTDA, Brazil]. The cycling profile consisted of denaturation at 95°C for 10 minutes, followed by 35 amplification cycles at 94°C for 1 minute, 65°C for 1 minute and 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The amplification and fragment size was confirmed by electrophoresis in agarose gel (1%) stained with GelRed (Biotium, Hayward, CA, USA) using a 100 bp molecular marker (DNA Express Biotecnologia LTDA). Then, 10 µL of the amplified product was submitted to Restriction Fragments Length Polymorphism (RFLP) through digestion by restriction enzymes*Rsa* I, *Taq* I and *Sac* II (Thermo Scientific, Waltham, MA, USA), following the manufacturer's recommendations. The generated fragments were separated on 2% agarose gel stained with GelRed using 50 bp and 100 bp molecular markers (DNA Express Biotecnologia LTDA). After electrophoresis, the gels were photographed in photo-documentation equipment (2UV Transilluminator UVP) and restriction patterns compared to those described by Chimara et al. (2004).

Spoligotyping was performed as described by Kamerbeek et al. (1997). For amplification of the DR region, 20 µM of each primer DRa 5´ GGTTTTGGGTCTGACGAC 3´ (5' biotinylated) and DRb (5´ CCGAGAGGGGACGGAAAC 3´), MyTaq Mix (12.5 µL, 1 µl (20 ng) genomic DNA and ultra-pure water (9.5 µL) were submitted to PCR in a final volume of 25 µL.

The MIRU-VNTR typing using a combination of 24 *loci* was performed according to Supply et al. (2006). In each PCR reaction 10 µL MyTaq Mix (BIOLINE®), 0.4 µL of each *primer* (20 mM), 2 µL of DNA (20 ng) and 7.2 µL of ultra-pure water were used in the final volume 20 µL. *Mycobacterium tuberculosis* H37Rv DNA and water were used as positive and negative controls, respectively.

The genetic profile based on *spoligotyping* of each isolate was compared to those present in the inter-

national database*http://www.mbovis.org/* and*http://www.pasteur-guadeloupe.fr:8081/SITVITONLINE*. The 24-MIRU-VNTR patterns were compared to those present in the MIRU-VNTRplus database deposited in the application*http://www.miru-vntrplus.org/MIRU/index.* The Hunter-Gaston discriminatory index (HGDI) was performed to evaluate the variability of the genotypes obtained by spoligotyping, and each of the alleles of 24-MIRU-VNTR typing.

RESULTS

The 17 animals from which *M. tuberculosis var. bovis* was isolated came from ten municipalities in the state of Pernambuco (Alagoinha, Bom Conselho, Chã Grande, Garanhuns, Ibirajuba, Jurema, Pedra, Pesqueira, Ribeirão and Venturosa), which were mostly raised in the semi-intensive management system. These municipalities belong to three geographic regions of the state, namely Southern Agreste, Central Agreste and South Agreste. Among the animals diagnosed with the disease, females were the most affected (16/17) and 64.7% (11/17) were older than five years; one calf of seven months old also yielded positive culture.

The clinical examination of cattle and buffaloes revealed apathy, lack of appetite, low body mass score, seromucous nasal discharge, dry cough, dyspnea, tachypnea, polyps, crackles, and areas of silence in the lung fields. Upon evaluation of the mammary gland, two (2/17) bovines were diagnosed with hypertrophied lymph nodes: one of these presented an enlarged posterior breast of firm consistency, hyperemia and hyperthermia and physical changes in milk in one of the teats (lumps with serum).The other bovine had an anterior breast of firm consistency but with no visible changes of the milk. During rectal examination, some animals presented nodular structures of varying sizes and hardened consistency in the region of the mesentery, serous in the rumen and uterus.

Macroscopic observation of lesions seen during *post-mortem*examination revealed that 12/17 animals (70.6%) had miliary or protruding tuberculosis, distributed mainly in the lungs, mediastinal and tracheobronchial lymph nodes, liver and mesenteric lymph nodes, and less frequently in the kidneys, spleen and greater omentum. Among the animals with generalized tuberculosis, two cattle also showed changes in the mammary gland and the uterus, characterized by granulomatous lesions with multifocal distribution and varied sizes, with areas of calcification and abscesses.

The granulomatous nodules observed in all animals were pleomorphic, had a caseous, thick, and yellowish content, with the formation of a fibrous capsule (Figure A). In buffaloes, granulomas had a more whitish color when compared to cattle (Figure B). In the young calf, in addition to lung lesions, small granulomas were observed in the central nervous system and lesions compatible with meningoencephalitis.

Histopathological analysis of the lesions revealed areas of central caseous necrosis and dystrophic calcification, intense inflammatory reaction in the regions adjacent to the necrosis areas, with a predominance of epithelioid macrophages and multinucleated giant cells, like *Langhans* .

Microbiological cultivation presented growth of colonies in 17/30 (57%) samples that were confirmed to be *Mycobacterium* spp and more specifically *M. tuberculosis var. bovis* by molecular techniques. In three samples, presence of *Trueperella pyogenes* and in a single animal*, Nocardia* spp was encountered. Of the 17 bacterial growths, 14 were classified by the enzymatic restriction analysis of the*gyr* B gene as *M. bovis* . However, due to the importance of bacterial isolation, recognized as a gold standard test, the 17 samples were submitted to molecular genotyping techniques by*Spoligotyping* and 24-*loci* MIRU-VNTR.

Spoligotyping revealed 17 patterns classified as belonging to*M. tuberculosis var. bovis* , including SB0121 (n=8), SB0295 (n=5), SB0852 (n=2), SB0120 (n=1) and a *spoligotype* that was not yet present in the Database (Table I).

The analysis of 24-*loci* MIRU-VNTR was identified 13 genetic profiles from the 17 isolates of *M. tuberculosis var. bovis* from 14 properties in the state of Pernambuco (Table II).

The analysis of the discriminatory power (HGDI) of MIRU-VNTR in this study was higher, as expected, than *Spoligotyping* , respectively (0.980) and (0.713). Distribution of the isolates according to the number

of alleles in each locus and the analysis of the allelic diversity of the 24 *loci* is summarized in (Table III). L*ocus* ETR A showed the highest discriminatory power ($h = 0.69$), while five *loci* (ETR B, MIRU 16, ETR C, MIRU 27 and QUB 26) were classified as moderately discriminatory with h between 0.33 to 0.58. Eight *loci* (MIRU 20, MIRU 26, Mtub 04, Mtub 29, QUB 11b, QUB 4156, Mtub 21, Mtub 39) presented low discriminatory power (h [?]0.27) while ten *loci* showed absence of allelic diversity.

Isolates 1 and 10 showed failures in the amplification of some*loci* that are generally attributed to possible DNA mutations or degradation (Supply et al., 2006), thus preventing the *primers*from ringing. Given these results, the respective isolates started to be analyzed only in *Spoligotyping,* obtaining significant results.

DISCUSSION

It should be noted that the state of Pernambuco occupies a prominent place in milk production in the Northeast region, and the municipality of Garanhuns and its microregion is recognized as the state's milk basin (Penaforte Júnior et al., 2009). Dairy cattle and buffaloes are considered more vulnerable to *M. tuberculosis var. bovis*infection, as they have a longer life expectancy, stay longer on the properties and are subjected to the rearing semi-intensive and intensive system, very common in the region. During milking and other common management practices, animals cohabit, therefore increasing their likelihood of contact and the transmission of tuberculosis (Lima et al., 2016; Veloso et al., 2016), considered endemic in the State of Pernambuco (Izael et al., 2009; Lima et al., 2016).The constant transit of animals between the properties within and between neighboring municipalities, the interstate cattle trade and the absence of an effective sanitary control of the herds are factors that contribute to the spread of the disease in the region (Lima et al., 2016; Veloso et al., 2016).

In the present study, all animals presented clinical symptoms of tuberculosis with predominating respiratory impairment. In dairy farms, female animals generally remain for longer periods depending on the reproductive period, and this could be the main reason for having observed in this study the predominance of females over the age of five years to be exposed to *M. tuberculosis var. bovis* when compared to young cattle (Veloso et al., 2016). Nonetheless, young animals also contract the infection and develop disease, as demonstrated by *M. tuberculosis var. bovis* isolation from a seven-month-old calf. The frequency of tuberculosis in cattle aged less than 12 months is generally associated with the ingestion of colostrum/milk from infected cows or transplacental infection (Konradt et al., 2016; Silveira et al., 2018). The most evident clinical signs were observed in the advanced stages of the disease, as described by Izael et al. (2009) and Waters (2015), except for the calf that manifested the disease earlier in the form of cerebral tuberculosis combined with depression and paresis of the limbs. In addition to the predominant respiratory impairment in the animals in this study, two animals showed clinical changes in the mammary gland result similar to described by Waters (2015). This observation reinforces the potential risk of the disease to public health due to the consumption of raw milk and non-pasteurized derivatives, mainly observed in inland cities and rural areas, such as Garanhuns and the microregion (Penaforte Júnior et al., 2009).

The generalized form of the disease was predominant both in cattle and the two buffaloes, with lesions that had disseminated to several organs. All animals had granulomatous injuries in the thoracic organs (lungs, pleura, tracheobronchial and mediastinal lymph nodes), causing respiratory impairment. This result is similar to those described by Ramos et al. (2018), who reported a higher prevalence of lesions compatible with tuberculosis in tracheobronchial, mediastinal lymph nodes and lungs; such typical predominance of lesions in the respiratory tract is indicative for air-borne transmission. On the other hand, Alzamora Filho et al. (2014) identified the most evident lesions in the lymph nodes of the head (retropharyngeal and parotid) with pulmonary parenchyma. These results corroborate with the findings of the present study, due to the typical predominance of lesions in the respiratory tract, suggesting the airway, as the main gateway for *M. tuberculosis var. bovis* in bovines. The lower occurrence of mesenteric lymph node involvement here observed was also described by Ramos et al. (2018) and justified by the fact that oral route infection is secondary to the respiratory route in adult cattle.

The granulomatous lesions observed in the mammary gland and uterus common to two animals in this study

reinforce the potential risk of transmission of *M. tuberculosis var. bovis* to humans due to the consumption of raw milk and its products (Cezar et al., 2016; Siala et al., 2019). On the other hand, the granulomatous lesions located in the central nervous system in young cattle is probably related to the ingestion of colostrum/milk from infected cows and can be justified by ascending infection via hematogenic route. This form of cerebral tuberculosis in cattle was also reported by Konradt et al. (2016) and Silveira et al. (2018).

The histopathological characterization of lesions present in granulomas was similar to the findings described by França et al. (2013) who found in some samples a marked process of calcification with mineralization, differing from the lesions observed by Ramos et al. (2018) and Silva et al. (2018) who presented a more caseous aspect, suggesting that the animals that had been slaughtered were suffering from a recent infection or disease development.

The frequency of isolation, of *M. tuberculosis var. bovis,* of 57% observed presently in animals, with clinical tuberculosis. It has been described that some factors can interfere with the success of mycobacterial isolation and in particular of *M. tuberculosis var. bovis* , including the rigorous decontamination process of samples and the chronic character of the disease that confers intense calcification of the lesions and leading to low concentration or absence of viable bacilli (Ambrosio et al., 2008). That might have been influenced the low isolation of *M. bovis* in the present sampling.

Besides *Mycobacterium spp* ., we also observed bacteria belonging to other genera such as *Trueperella pyogenes* and *Nocardia spp* . It is worth mentioning that some microorganisms besides these, such as *Actinomyces spp* and *Actinobacillus spp* , are also responsible for causing granulomatous lesions similar to tuberculosis lesions (Mendes et al., 2013).

In the present study, 17 isolates compatible with *Mycobacterium spp* . Were subjected to molecular diagnostics by RFLP of the *gyr* B gene. However, the analysis classified only 14/17 isolates as *M. tuberculosis var. bovis* , different from the study carried out by Franco et al. (2017) that obtained 100% compatibility between the isolation of*Mycobacterium spp* and the *gyr* B analysis. The result obtained in the RFLP is probably related to factors that interfere with molecular tests, such as the presence of inhibitors of PCR reactions, low amount of viable bacilli due to chronic lesions, contaminants in the samples, failures in extraction processing or DNA degradation (Carel et al., 2014).

Spoligotype SB0121, the most frequently encountered was described as the most prevalent in national territory with a frequency of 29.1% in a study conducted in Latin American countries (Zumárraga et al., 2013). The fact that we identified this *spoligotype* in the three defined geographical regions studies here could be caused by the constant movement of animals, due to the practice of interstate cattle trade and also strongly suggestive for recent infections (Rodríguez et al., 2010; Zumárraga et al., 2013).

The SB0295 profile was the second most prevalent *spoligotype* in this study (29%) and has been referenced in Brazil with a prevalence of 24% (Zumárraga et al., 2013). This is similar to that in the Midwest Region of the country, being identified in 16.2% of the total isolates (Carvalho et al., 2016). The two buffalo isolates in this study also presented *spoligotype* SB0295, a profile typical to those identified in buffaloes in the Amazon region (Carneiro et al., 2019), it is common to breed mixed buffalo and cattle under the same management conditions.

Spoligotype SB0852 was identified in two isolates. According to the international database, SB0852 has only been registered in Italy (Boniotti et al., 2009), suggesting a process of natural selection of these strains between geographic locations (Carvalho et al., 2016) or convergent evolution (Zumárraga et al., 2013).

Finally, two *spoligotypes* were observed in this study single isolates only, being the case for SB0120, similar to the low frequency of occurrence in other regions of the country (Parreiras et al. 2012; Zumárraga et al., 2013; Franco et al., 2017). The other was from a bovine that presented a *spoligotype* not present in the international database; this could be due to some microevolutionary events in the DR regions of a strain with an existing pattern (Adesokan et al., 2019).

In the region of development of the study, bovine tuberculosis is characterized as endemic, and the practice

of commercialization and consumption of milk and fresh products increases the risk of zoonotic transmission, increasing the risk of sharing *M. tuberculosis var. bovis* isolates common among dairy cattle and the human population of the region, as previously recorded in other studies in different areas of the world. Genomic diversity in the *M. tuberculosis* complex remains a significant factor in the pathogenesis of tuberculosis, which can affect virulence, transmissibility, host response, and drug resistance (Adesokan et al., 2019).

The genotyping performed in this study from the set of 24-*loci*MIRU-VNTR is recommended for the comparative study of *M. tuberculosis var. bovis* profiles world wide (Supply et al., 2006). Molecular genotyping identified 13 distinct genetic profiles, suggesting a diversity of *M. bovis* within and between the regions studied and considerable higher discriminatory power as compared to*Spoligotyping* . This is according to earlier results obtained both in Brazil (Carvalho et al., 2016) and in other countries. This demonstrated that although a large cluster was observed by*spoligotyping* alone, there exists genetic diversity among the strains of *M. tuberculosis var. bovis* in Pernambuco, probably due to the movement of animals between different regions, states and rural properties (Zumárraga et al., 2013; Carvalho et al., 2016).

The analysis of allelic diversity of the different MIRUs are similar to those found by Souza Filho et al. (2013) and Carvalho et al. (2016) and demonstrating that for this MTBC species, only six of 24 loci allowed good discrimination, different from *M . tuberculosis* (Hilty et al., 2005). The HGDI of 24-MIRU-VNTR and *spoligotyping* in this study was 0.980 and 0.713, respectively, close to that observed by Carvalho et al. (2016) with values 0.980 and 0.810 and the HGDI of 0.912 reported by Souza Filho et al. (2013). Therefore, it seems that simultaneous consideration of both genotyping techniques for clustering might be more accurate for *M. bovis* transmission studies, also in the present study. However, the association between these techniques has been considered the best strategy for the molecular typing of *M. tuberculosis var. bovis* because they present better reproducibility and reliability, aiming at the analysis of strains mycobacterial (Carvalho et al., 2016).

This study is of great importance for the region as it is the first work carried out on molecular genotyping through the association between*Spoligotyping* and MIRU-VNTR aiming at the molecular characterization of *M. tuberculosis var. bovis* isolates and identification of circulating genotypes in the state of Pernambuco. It is worth mentioning the importance of *M. bovis* as a cause of human tuberculosis, although sometimes neglected, especially in developing countries. The consumption of raw milk and dairy products and the constant exposure to reservoir animals are considered the main risk factors in the epidemiological chain of infection.

CONCLUSION

The genotypic characterization allowed the identification of different*M. tuberculosis var. bovis* genotypes circulating in the state of Pernambuco, presenting both two large clusters by *spoligotyping*but evidencing considerable heterogeneity when using 24-MIRU-VNTR. The consumption of raw milk and dairy products is still a frequent habit in the region endemic for bovine tuberculosis is endemic and together with our data on disease pathology and occurring of transmission between animals from a sampling not designed for detecting such an event warns us for an increased risk of zoonotic transmission. This should draw the attention of health authorities to the possibility of sharing common*M. tuberculosis var. bovis* isolates between dairy cattle and the population of the region. Having in mind the diversity of genotypes obtained by combining *spoligotyping* and 24-MIRU-VNTR in the present setting, this methodology could be additive during transmission studies.

ACKNOWLEDGEMENTS

We thank the Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE) for granting the scholarship (Process n. IBPG-1461-5.05 / 15). To Fernando José Paganini Listoni from the Department of Veterinary Hygiene and Public Health, Faculty of Veterinary Medicine and Animal Science - UNESP - Botucatu, São Paulo, for carrying out microbiological cultivation.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

The project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal Rural University of Pernambuco and recieved license no. 09/2017 CEPE/UFRPE, according to COBEA (Brazilian College of Animal Experiments) and National Institute of Health Guide for Care and Use of Laboratory Animals standards.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

AVAILABILITY OF DATA

The data that support the findings of this study are openly available in Eletronic publication system of Theses and Dissertations (TEDE) at http://www.tede2.ufrpe.br:8080/tede2/handle/tede2/8484. Digital Library. Federal Rural of Pernambuco.

REFERENCES

Adesokan, H.K., Streicher, E.M., Van Helden, P.D., Warren, R.M., Cadmus, S.I.B. 2019. Genetic diversity of *Mycobacterium tuberculosis*complex strains isolated from livestock workers and cattle in Nigeria.*PloS One* . 14, 1-13.

Alzamora Filho, F., Vasconcellos, S.E.G., Gomes, H.M., Cavalcante, M.P., Suffys, P.N., Costa, J.N. 2014. Múltiplas estirpes de isolados de*Mycobacterium bovis* identificados por tipagem molecular em bovinos abatidos em matadouros-frigoríficos. *Pesquisa Veterinária Brasileira.* 34, 103-108.

Ambrosio, S.R., Oliveira, E.M.D., Rodriguez, C.A.R., Ferreira Neto, J.S, Amaku, M. 2008. Comparison of three decontamination methods for*Mycobacterium bovis* isolation. Braz. *Brazilian Journal of Microbiology* . 39, 241-244.

Boniotti, M.B., Goria, M., Loda, D., Garrone, A., Benedetto, A., Mondo, A., Tisato, E., Zanoni, M., Zoppi, S., Dondo, A., Tagliabue, S., Bonora, S., Zanardi, G., Pacciarini, M.L. 2009. Molecular typing of*Mycobacterium bovis* strains isolated in Italy from 2000 to 2006 and evaluation of Variable Number Tandem Repeats for geographically optimized genotyping. *Journal of Clinical Microbiology* . 47, 636-644.

Carneiro, P.A.M., Pasquatti, T.N, Takatani, H., Zumárraga, M.J., Marfil, M.J., Barnard, C., Fitzgerald, S.D., Abramovitch, R.B., Araujo, F.R., Kaneene, J.B. 2019. Molecular characterization of *Mycobacterium bovis* infection in cattle and buffalo in Amazon Region, Brazil. *Veterinary Medicine and Science* . 1-9.

Carel, C., Nukdee, K., Cantaloube, S., Bonne, M., Diagne, C.T., Laval, F., Daffé, M., Zerbib, D. 2014. *Mycobacterium tuberculosis*proteins involved in mycolic acid synthesis and transport localize dynamically to the old growing pole and septum. *PloS One* . 9, 1-15.

Carvalho, R.C.T., Vasconcellos, S.E.G., Issa, M.A., Soares Filho, P.M., Mota, P.M.P.C., Araújo, F.R., Carvalho, A.C.S., Gomes, H.M., Suffys, P.N., Figueiredo, E.E.S., Paschoalin, V.M.F. 2016. Molecular Typing of*Mycobacterium bovis* from Cattle Reared in Midwest Brazil.*PloS One* . 11, 1-16.

Cezar, R.D.S., Lucena Silva, N., Borges, J.M., Santana, V.L.A., Pinheiro Júnior, J.W. 2016. Detection of *Mycobacterium bovis* in artesanal cheese in the state of Pernambuco, Brazil. *International Journal of Mycobacteriology* . 5, 269-272.

Chimara, E., Ferrazoli, L., Leão, S.C. 2004. *Mycobacterium tuberculosis* complex differentiation using *gyr* B-restriction fragment length polymorphismanalysis. *Memórias do Instituto Oswaldo Cruz* . 99, 745-748.

Cousins, D.V., Batisda, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., Duignan, P., Murray, A., Dupont, C., Ahmed, N., Collins, D.M., Butter, W.R., Dawson, D., Rodríguez, D., Loureiro, J., Romano, M.I., Alito, A., Zumarraga, M., Bernardelli, A. 2003. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex:*Mycobacterium pinnipedii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* . 53, 1305-1314.

Drewe, J.A., Smith, N.H. 2014. Molecular epidemiology of*Mycobacterium bovis* . In: Thoen, C.O., Steele, J.H., Kaneene, J.B., *Zoonotic Tuberculosis* : *Mycobacterium bovis* and other pathogenic mycobacteria. Chichester, UK: John Wiley, pp. 79-88.

França, L.R., Cruz, J.F., Neves, V.B.F., Cerqueira, R.B. 2013. Prevalência e histopatologia de lesões sugestivas de tuberculose em carcaça de bovinos abatidos no Sudoeste da Bahia. *Revista Brasileira de Saúde e Produção Animal* . 14, 721-733.

Franco, M,M,J., Ribeiro, M.G., Pavan, F.R., Miyata, M., Heinemann, M.B., Souza Filho, A.F., Cardoso, R.F., Almeida, A.L., Sakate, R.I., Paes, A.C. 2017. Genotyping and rifampicin and isoniazid resistance in*Mycobacterium bovis* strains isolated from the lymph nodes of slaughtered cattle. *Tuberculosis* . 104, 30-37.

Hilty, M., Digimbaye, C., Schelling, E., Baggi, F., Tanner, M., Zinsstag, J. 2005. Evaluation of the discriminatory power of variable number of tandem repeat (VNTR) typing of *Mycobacterium bovis*strains. *Veterinary Microbiology* . 109, 217-222.

Izael, M.A., Silva, S.T.G., Costa, N.A., Souza, J.C.A., Mendonca, C.L., Afonso, J.A.B. 2009. Estudo retrospectivo da ocorrencia dos casos de Tuberculose Bovina diagnosticados na Clinica de Bovinos de Garanhuns, PE de 2000 a 2009. *Ciencia Animal Brasileira* . 452-457.

Kamerbeek, J., Schouls, L., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., Van Embden J. 1997. Simultaneous detection and strain differentiation of*Mycobacterium tuberculosis* for diagnosis and epidemiology.*Journal of Clinical Microbiology* . 35, 907-914.

Konradt, G., Bassuino, D.M., Bianchil, M.V., Bandinelli, M.B., Driemeier, D., Pavarini, S.P. 2016. Neurotuberculosis in cattle in southern Brazil. *Tropical Animal Health Production* . 48, 1089-1094.

Lima, P.R B., Nascimento, D.L., Almeida, E.C., Pontual, K.A.Q., Amaku, M., Dias, R.A., Ferreira, F., Goncalves, V.S.P., Telles, E.O., Grisi-Filho, J.H.H., Heinemann, M.B., Silva, J.C.R., Ferreira Neto, J.S. 2016. Situacao epidemiologica da tuberculose bovina no estado de Pernambuco, Brasil. *Semina* . 37, 3601-3610.

Malama, S., Johansen, T.B., Muma, J.B., Munyeme, M., Mbulo, G., Muwonge, A., Djonne, B., Godfroid, J. 2014. Characterization of*Mycobacterium bovis* from Humans and Cattle in Namwala District, Zambia. *Veterinary Medicine International* . 1-8.

Mendes, R.E., Schneider, A.F., Werlich, D.E., Lucca, N.J., Lorenzett, M.P., Pilati, C. 2013. Estudo anatomopatologico em tecidos condenados pelo Servico de Inspecao Federal (SIF) por suspeita de tuberculose.*Ciencia Animal Brasileira* . 14, 448-453.

Parreiras, P.M., Andrade, G.I., Nascimento, T.F., Oelemann, M.C., Gomes, H.M., Alencar, A.P., Assis, R.A., Mota, P.M.P.C., Pereira, M.A.S., Lobato, F.C.F., Lage, A.P., Suffys, P.N. 2012. Spoligotyping and Variable Number Tandem Repeat analysis of *Mycobacterium bovis*isolates from cattle in Brazil. *Memorias do Instituto Oswaldo Cruz* . 107, 64-73.

Penaforte Junior, M.A., Borges, J.M., Azevedo, D.S., Borges-Filho, E.L. 2009. Perfil dos produtores de leite do municipio de Garanhuns.*In* : Jornada de ensino, pesquisa e extensao. pp. 1-3 Recife.*Anais eletronicos* Recife: UFRPE. Disponivel em: http://www.eventosufrpe.com.br/jepex2009/cd/resumos/R1002-2.pdf. Acesso em: 02 set. 2019.

Ramos, J.M., Heinemann, M.B., Ferreira Neto, J.S., Souza Filho, A.F., Cardenas, N.C., Dantas, A.F.M., Alves, C.J., Azevedo, S.S. 2018. Isolation and Identification of *Mycobacterium bovis* in bovines with positive reaction to the tuberculin test in the State of Paraiba, northeast Brazil. *Arquivo do Instituto Biologico* . 85, 1-7.

Rodriguez, S., Romero, B., Bezos, J., Juan, L., Alvarez, J., Castellanos, E., Moya, N., Lozano, F., Gonzalez, S., Saez-Llorente, J.L., Mateos, A., Dominguez, L., Aranaz, A. 2010. Hight spoligotyping diversity within

a *Mycobacterium bovis* population: Clues to understanding the demography of the pathogen in Europe. *Veterinary Microbiology* . 141, 89-95.

Siala, M., Cassan, C., Smaoui, S., Kammoun, S., Marouane, C., Godreuil, S., Hachicha, S., Mhiri, E., Slim, L., Gamara, D., Messadi-Akrout, F., Banuls, A.L. 2019. A first insight into genetic diversity of*Mycobacterium bovis* isolated from extrapulmonary tuberculosis patients in South Tunisia assessed by Spoligotyping and MIRU-VNTR*. PLoS Neglected Tropical Diseases* . 13, 1-19.

Silva, D.A.V., Siconelli, M.J.L., Burger, K.P., Keid, L.B. 2018. Comparison between tests for tuberculosis diagnosis in slaughtered bovines. *Arquivos do Instituto Biologico* . 85, 1-5.

Silveira, A.M., Nascimento, E.M., Konradt, G., Miranda Neto, E.G., Driemeier, D., Galiza, G.J.N., Dantas, A.F.M., Riet-Correa, F. 2018. Tuberculosis of the central nervous system in cattle in Paraiba, Brazil.*Pesquisa Veterinaria Brasileira* . 38, 2092-2098.

Souza-Filho AF. 2013. Genotipagem pela tecnica MIRU-VNTR de*Mycobacterium bovis* isolados de tecido bovino. *Dissertacao*(Mestrado em Ciencia Animal). Faculdade de Medicina Veterinaria e Zootecnia. Universidade Federal do Mato Grosso do Sul, Campo Grande. 73 pp.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., Haas, P., Van Deutekom, H., Roring, S., Bifani, P., Kurepina,N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S,, Skuce, R., Kremer, K., Locht, C., Van Soolingen, D. 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis* . *Journal of Clinical Microbiology* . 44, 4498-4510.

Veloso, F.P., Baumgarten, K.D., Mota, A.L.A.A., Ferreira, F., Ferreira Neto, J.S., Grisi-Filho, J.H.H., Dias, R.A., Amaku, M., Telles, E.O., Heinemann, M.B., Goncalves, V.S.P. 2016. Prevalence and herd-level risk factors of bovine tuberculosis in the State of Santa Catarina.*Semina* . 37, 3659-3672.

Waters, W.R. 2015. Diseases of the Respiratory System. In: Smith, B.P.*Large Animal Internal Medicine* . Elsevier. pp. 633-638.

Zumarraga, M.J., Arriaga, C., Barandiaran, S., Cobos-Marin, L., Waard, J., Estrada-Garcia, I., Figueiredo, T., Figueroa, A., Gimenez, F., Gomes, H.M., Gonzalez-y-Merchand, J.A., Macias, A., Milian-Suazo, F., Rodriguez, C.A.R., Santillan, M.A., Suffys, P.N., Trangoni, M.D., Zarraga, A.M., Cataldi, A. 2013. Understanding the relationship between*Mycobacterium bovis* spoligotypes from cattle in Latin American Countries. *Research in Veterinary Science* . 94, 9-21.

TABLES

Table I. Molecular characterization of *M. tuberculosis var. bovis*isolates from cattle in the state of Pernambuco by *Spoligotyping*and MIRU-VNTR

ID	Octal Spoligotype	Cluster Spoligotype	Profile of 24 MIRU-VNTR	Cluster MIRU-VNTR	SIT*	Mbovis.org**	Municipality
1	676773677777600	Cluster S1	2*63***313*343[illegible]2*12	[illegible] pattern	481	SB0121	Bom Conselho
5	676773677777600	[illegible]	22632233232441[illegible]2622	[illegible] pattern	481	SB0121	Garanhuns
6	676773677777600	[illegible]	22632233235443[illegible]2622	[illegible] pattern	481	SB0121	Chã Grande
7	676773677777600	[illegible]	22*32243236443[illegible]2632	[illegible] pattern	481	SB0121	Alagoinha
9	676773677777600	[illegible]	*2*32233236443[illegible]2312	[illegible] pattern	481	SB0121	Pedra

ID	Octal Spoligo-type	Cluster Spoligo-type	Profile of 24 MIRU-VNTR	Cluster MIRU-VNTR	SIT*	Mbovis.org**	Municipality
10	676773677777600	Orphan pattern	2*5******3*34*****3*2***	Orphan pattern	481	SB0121	Pesqueira
15	676773677777600	Orphan pattern	**5322*32353430253332*12	Orphan pattern	481	SB0121	Bom Conselho
26	676773677777600	Orphan pattern	22532222323534402533332512	Orphan pattern	481	SB0121	Pesqueira
29	676773677777200	Cluster S2	22432222323534402533332112	Orphan pattern	698	SB0295	Jurema
34	676773677777200	Cluster S2	22532222323534402533332512	Orphan pattern	698	SB0295	Jurema
38	676773677777200	Cluster S2	2*53223323434402533332612	Orphan pattern	698	SB0295	Ibirajuba
39	676773677777200	Cluster S2	2*43223323434402533332512	Cluster M1	698	SB0295	Ribeirão
40	676773677777200	Cluster S2	2*432233234*44402533332512	Cluster M1	698	SB0295	Ribeirão
35	676773777777200	Cluster S3	22532213232343402533333512	Cluster M2	797	SB0852	Bom Conselho
37	676773777777200	Cluster S3	2*53221323234340253333512	Cluster M2	797	SB0852	Bom Conselho
12	676773777777600	Orphan pattern	*1*32231342*43402533332512	Orphan pattern	482	SB0120	Garanhuns
2	New profile	Orphan pattern	23*32233155341024333332612	Orphan pattern	New	New	Venturosa

Cluster S – cluster Spoligotyping Cluster M – cluster MIRU-VNTR

Table II. Genetic profiles obtained from the 24-loci analysis of MIRU-VNTR

ISOLATES	MIRU 02 - 154	Mtub 04 – 424	ETR C - 577	MIRU 04 - 580	MIRU 40 - 802	MIRU 10 - 960	MIRU
15			5	3	2	2	
9		2		3	2	2	3
26	2	2	5	3	2	2	2
5	2	2	6	3	2	2	3
6	2	2	6	3	2	2	3
7	2	2		3	2	2	4
29	2	2	4	3	2	2	2
38	2		5	3	2	2	3
34	2	2	5	3	2	2	2
39	2		4	3	2	2	3
40	2		4	3	2	2	3
35	2	2	5	3	2	2	1
37	2		5	3	2	2	1
12		1		3	2	2	3
2	2	3		3	2	2	3

24 loci de MIRU-VNTR used in genotypic analysis

Table III. Distribution and allele diversity (HGDI) of the 24-*loci* MIRU-VNTR

Number of repetitions	**Number of repetitions**	**Number of repetitions**	**Number of repetitions**	**Number of repetitions**	**Number of repetitions**	**Number of repetitions**	**Number of repetitions**
Locus	**1**	**2**	**3**	**4**	**5**	**6**	**Allele diversity HGDI**
MIRU 02		14					0.000
Mtub 04	1	8	1				0.266
ETR C				3	7	3	0.570
MIRU 04			16				0.000
MIRU 40		15					0.000
MIRU 10		15					0.000
MIRU 16	2	3	8	1			0.571
Mtub 21	1		15				0.058
MIRU 20	2	13	1				0.275
QUB 11b			15	1	1		0.165
ETR A		4		3	6	2	0.690
Mtub 29			12	3			0.271
Mtub 30				17			0.000
ETR B	2		7	7			0.575
MIRU 23				16			0.000
MIRU 24		16					0.000
MIRU 26				1	15		0.058
MIRU 27	4		12				0.333
Mtub 34			17				0.000
MIRU 31			16				0.000
Mtub 39		15	2				0.158
QUB 26	1		1		7	5	0.582
QUB 4156	13	2	1				0.275
MIRU 39		16					0.000

FIGURES (Legends)

Figure A: Granulomatous lesions distributed in lung and mediastinal lymph nodes of bovines.

Figure B: Granulomatous lesions distributed in the liver of buffaloes