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Genotyping and rifampicin and isoniazid resistance in *Mycobacterium bovis* strains isolated from the lymph nodes of slaughtered cattle

Marília Masello Junqueira Franco [a, *], Márcio Garcia Ribeiro [a], Fernando Rogério Pavan [b], Marcelo Miyata [b, 1], Marcos Bryan Heinemann [c], Antonio Francisco de Souza Filho [c], Rosilene Fressatti Cardoso [d], Aryadne Larissa de Almeida [d], Ricardo Ichiro Sakate [e], Antonio Carlos Paes [a]

[a] *Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine and Animal Sciences, UNESP-Univ. Estadual Paulista, Zip Code: 18618-681, P.O. Box: 560, Prof. Dr. Walter Mauricio Correa Street, s/n, Rubiao Junior district, Botucatu, SP, Brazil*

[b] *Laboratory of Tuberculosis Research, School of Pharmaceutical Sciences, UNESP- Univ. Estadual Paulista, Zip Code: 14800-903, Araraquara-Jau, km 01, Araraquara, SP, Brazil*

[c] *Department of Preventive Veterinary Medicine and Animal Health, USP-Univ. de Sao Paulo, Zip Code: 05508-270, Prof. Dr. Orlando Marques de Paiva Avenue, 87, Sao Paulo, SP, Brazil*

[d] *Department of Clinical Analysis and Biomedicine, UEM-Univ. Estadual de Maringá, Zip Code: 87020-000, Colombo Avenue, 5790, Maringa, PR, Brazil*

[e] *Federal Veterinary Inspection Service, State of Sao Paulo, Zip Code: 18681-030, Dr. Gabriel de Oliveira Rocha Street, 704, Lencois Paulista, SP, Brazil*

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ABSTRACT

In developing nations, 10–20% of the human cases of tuberculosis are caused by *Mycobacterium bovis*. However, this percentage may be underestimated because most laboratories in developing countries do not routinely perform mycobacterial cultures, and only a few have the systems in place to identify *M. bovis*. There are few studies investigating genotypic diversity and drug resistance in *M. bovis* from animal and/or human infections.

The genotypic diversity of *M. bovis* strains obtained from bovine lymph nodes were investigated by spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive unit–variable-number tandem repeat typing (MIRU-VNTR). The phenotypic resistance to isoniazid and rifampicin and MIC values of the isolates were determined using the resazurin microtiter assay plate method (REMA). The evaluation of the possible genetic basis for such resistance was performed with GenoType MTBDR*plus*. Sixty-seven isolates were obtained, of which 11 (16%) were MDR-TB, 8 (12%) were isoniazid-resistant, and 2 (3%) were rifampicin-resistant. Mutations associated with drug resistance were not found. Genotyping techniques enabled the grouping of the strains into 12 clusters and 21 isolates with unique profiles.

The high frequency of *M. bovis* reinforces the impact of the pathogen as a major causal agent of bovine tuberculosis in the study area. The resistance of the strains to drugs used for first-line treatment of human tuberculosis raises public health concerns. Further studies are required to elucidate the basis of drug resistance and genotypic diversity in *M. bovis*.

1. Introduction

Tuberculosis caused by *Mycobacterium bovis* (bTB) is a zoonosis with worldwide distribution and a broad host range. Although the disease primarily affects cattle, it can be transmitted to humans by the consumption of milk and raw, unpasteurized, or non-heat-treated dairy products. It may also be transmitted by inhalation of infectious aerosols, mainly among immunosuppressed individuals, such as people living with HIV/AIDS [1]. There is significant

* Corresponding author. Rua Primo Paganini, 755, Jardim Panorama, CEP: 18608-190, Botucatu, SP, Brazil.

E-mail addresses: marilia_gu@uol.com.br (M.M.J. Franco), mgribeiro@fmvz.unesp.br (M.G. Ribeiro), fernandopavan@fcfar.unesp.br (F.R. Pavan), marcelo_miyatabr@yahoo.com.br (M. Miyata), marcosbryan@usp.br (M.B. Heinemann), antoniosouzafilho@gmail.com (A.F. de Souza Filho), rfressatticardoso@gmail.com (R.F. Cardoso), aryadne_almeida@hotmail.com (A.L. de Almeida), ricardo.sakate@agricultura.gov.br (R.I. Sakate), paesacmi@fmvz.unesp.br (A.C. Paes).

[1] Present address: Department of Pathophysiology and Host Defense, JATA-Japan Anti-tuberculosis Association, RIT-Research Institute of Tuberculosis, Zip Code: 204-8533, 3-1-24 Matsuyama, Kiyose, Tokyo, Japan.

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variation in how different microorganisms of the *Mycobacterium tuberculosis* complex (MTBC) affect specific hosts, including humans. However, there are also important intersections between animals and humans regarding tuberculosis epidemiology. The best example is the occurrence of bTB in humans, domestic animals, and wildlife [2].

Currently, bTB corresponds to approximately 1–2% of tuberculosis cases in humans in developed countries [1,3]. In contrast, in developing nations, it is estimated that, on average, 10–20% cases of human tuberculosis are caused by *M. bovis* [3]. In particular, this is the case in the poorest regions of Africa where bTB remains prevalent and milk pasteurization and slaughter surveillance activities are not extensively practiced. Studies in Mexico have reported a greater prevalence, with annual rates of up to 40% [4]. In Brazil, published studies on this issue are rare, and some of them did not find *M. bovis* strains in samples of human tuberculosis [5,6]. In contrast, a study in the state of Minas Gerais showed that *M. bovis* accounted for 1.6% of the mycobacterial isolates obtained from patients with tuberculosis [7].

M. bovis infection in humans is worrisome, as its chronic clinical course is indistinguishable from tuberculosis caused by *M. tuberculosis* [8]. Humans are accidental hosts of *M. bovis* [9]. However, bTB in humans is as severe as the disease caused *M. tuberculosis* and can be even more lethal [10,11]. Historically, since Carmichael's observations in 1810, the predominant form of *M. bovis* infection in humans included pediatric scrofula (cervical lymph node infection) [12]. Most laboratories in developing countries do not routinely perform mycobacterial cultures, and only a few laboratories have systems in place to identify *M. bovis*. Indeed, it is believed that the number of human cases of *M. bovis* infection is underestimated and unreported [4]. Often, the mycobacterial species responsible for pulmonary tuberculosis are not properly differentiated. The use of inappropriate diagnostic protocols and laboratory techniques (e.g., the use of culture media or substances, such as glycerol, that inhibit *M. bovis* growth), or the lack of additional testing to identify the causative agents at the species level, contribute to the underreporting of human bTB [13].

Notwithstanding, the great spread of tuberculosis and the rise and dissemination of multidrug-resistant strains (MDR-TB), extensively drug-resistant (XDR-TB), and totally drug-resistant lineages (TDR-TB) are emergent human health threats [14]. *M. bovis* is naturally resistant to pyrazinamide (PZA), which is classified as a first-line anti-tuberculosis drug [13]. The common side effects of PZA treatment are hyperuricemia, hepatotoxicity, dysuria, arthralgia, and sideroblastic anemia. Therefore, the complete identification of MTBC isolates at the species level is required for the appropriate treatment of the patient and for appropriate public health and epidemiological measures to be taken [15]. Furthermore, all over the world, few studies have investigated the resistance to isoniazid (INH) and rifampicin (RMP) in *M. bovis* strains from cattle, wildlife reservoirs and human cases of tuberculosis [4,16,17].

An investigation on the genetic diversity of *M. bovis* isolates can improve our understanding of the structure and population dynamics of the pathogen, as well as its transmission patterns. Moreover, it will contribute to the success of programs for bTB control and eradication [18]. To address these questions, spoligotyping (Spacer Oligonucleotide Typing) and MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit–Variable-Number Tandem Repeat Typing) have been combined for the characterization of *M. bovis* in most laboratories, as these techniques are fast, cost-effective, and have already been standardized [19,20]. More recently, whole genome sequencing (WGS) has gained a prominent role in answering research questions such as the rate of transmission within cattle herds and between hosts, the history of movement of *M. bovis* throughout the world, and routine surveillance activities [21].

In addition to the impact on public health, bTB has a high economic importance in the agricultural context worldwide. It directly affects animal productivity, may influence the international trade of animal products, and, depending on regional laws, may require the mandatory slaughter of infected animals [22,23]. In Brazil, an official study conducted in 2014 revealed that the prevalence of bTB outbreaks in cattle herds in the states of Rondonia, Bahia, Mato Grosso, Parana, and Sao Paulo was 2.3%, 1% 1.2%, 2.3%, and 8.6%, respectively. In the same study, the number of positive animals in these states was 0.1%, 0.1%, 0.1%, 0.4%, and 1.6%, respectively [24].

The aims of the current study were to investigate the genotypic diversity of *M. bovis* strains isolated from the lymph nodes of slaughtered cattle using spoligotyping and 12-*loci* MIRU-VNTR. Additionally, this study aimed to determine the INH and RMP phenotypic resistance profile of these lineages and the MIC values and the possible genetic basis for such resistance.

2. Methods

2.1. Sample collection

One hundred cattle lymph nodes with gross lymphadenitis lesions (granulomas, caseum, lymphadenopathy) were randomly collected from different carcasses from different herds of cattle in southeastern Brazil. Each sample corresponded to a different animal. Sample collection occurred between August 2014 and May 2015, under the approval of the Brazilian Federal Inspection Service (SIF), which was given by the veterinarian responsible for the official inspection of the carcasses in the slaughterhouse before collection. Sample collection was carried out during the official veterinary postmortem examinations in a slaughterhouse inspected by the SIF in the central region of the state of Sao Paulo, Brazil. Slaughtered animals came from herds in 26 different cities of the state of Sao Paulo and one city in the state of Minas Gerais. The entire lymph node was collected and each lymph node was individually placed in a sterile stomacher bag (Twirl'EM™, Labplas, Ste-Julie, Canada). The samples were immediately placed in isothermal boxes with ice (4–8 °C) where they were stored until their arrival at the laboratory. Then, they were frozen (−20 °C) until the moment of culture.

2.2. Mycobacterial cultures

A 1-g aliquot of the foci-presenting lesion for each lymph node was selected for microbiological examination. After maceration, Petroff's sodium hydroxide decontamination method [25] was carried out in each sample prior to culture on Löwenstein-Jensen and Stonebrink media. Cultures were incubated aerobically at 37 °C for 90 days and examined weekly. All colonies exhibiting phenotypes suggestive of mycobacteria were stained using the Ziehl-Neelsen method. Acid-fast bacilli (AFB) positive colonies were confirmed to be *M. bovis* by polymerase chain reaction (PCR), as described previously [26].

2.3. Species identification by PCR

Genomic DNA extraction was performed by thermolysis [27]. Molecular identification of the species of the isolates was performed by *gyr*B-RFLP [26]. For the PCR, 1 μL of DNA (0.02 μg/μL), 47 μL of PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA), and 10 pMol of each primer, MTUBf (5′–TCGGACGCGTATGCGATATC–3′) and MTUBr (5′–ACATACAGTTCGGACTTGCG–3′), were combined [26]. A positive control with *M. bovis* AN5 DNA and a negative

control consisting of the PCR solution without DNA were included in each set of reactions.

The amplicons were further digested using the restriction enzymes *Rsa*I, *Taq*I and *Sac*II (Thermo Scientific, Waltham, MA, USA). The restriction digests were separated on a 2% agarose gel (Sigma Aldrich, Saint Louis, MO, USA) stained with GelRed™ (Biotium, Hayward, CA, USA) using 50- and 100-bp molecular weight markers (DNA Express, Guarulhos, SP, Brazil). The results were compared with the patterns described previously [26]. Samples identified as *M. bovis* were submitted for genotyping and drug-susceptibility testing.

2.4. MIRU-VNTR typing

MIRU-VNTR genotyping was performed using the primer sets for the 12 MIRU *loci* 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 [20]. For the amplification of each *locus*, one pair of primers in one PCR reaction was used. The PCR mixture consisted of 1 μL of the extracted genomic DNA (0.02 μg/μL), 18 μL of PCR Master Mix (2×), and 10 pMol of each primer. The PCR product was subjected to electrophoresis on a 1.5% agarose gel stained with GelRed™, using 50- and 100-bp molecular weight markers. The amplicon sizes for each sample were analyzed with AlphaEase™FC software version 6.0 (Alpha Innotech, San Leandro, CA, USA) and classified as alleles as previously described [20]. The sample profiles were analyzed using the international database SITVIT WEB [28] to find the corresponding MIT (MIRU International Type) number for each isolate.

2.5. Spoligotyping

For the PCR amplification of the DR *locus*, we combined 1 μL of the extracted genomic DNA (0.02 μg/μL), 22 μL of PCR Master Mix (2×) and 10 pMol of each primer, DRa (5′-GGTTTTGGGTCTGACGA-3′, 5′ biotinylated) and DRb (5′-CCGAGAGGGGACGGAAAC-3′) [29]. The PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to a spoligo membrane prepared in-house, according to prior standardization [30].

Detection of the hybridized DNA was done by chemiluminescence with an Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences, Little Chalfont, UK), followed by exposure on X-ray film (Kodak, Rochester, NY, USA) for 30 min. The autoradiograms were obtained using standard photochemical products for X-ray film processing (Kodak, Rochester, NY, USA) in accordance with the manufacturer's instructions. Spoligotypes in binary and octal formats were compared with the online SITVIT WEB [28] and Mbovis.org [31] databases, thus ensuring the identification of spoligotyping international types (SIT) and *M. bovis* spoligotype patterns (SB) of the lineages.

2.6. Drug susceptibility testing

The drug susceptibility testing of the *M. bovis* strains was performed using resazurin microtiter assay plate (REMA) method [32]. The technique was carried out using standardized bacterial inoculums, 96-well plates (Techno Plastic Products, Trasadingen, Switzerland), with *M. tuberculosis* H37Rv ATCC 27294 as the reference strain and sodic resazurin (Sigma Aldrich, Steinheim, Germany) to reveal bacterial viability and growth. Each test was carried out in triplicate, with one isolate tested per plate. The minimum inhibitory concentration (MIC) of the antimicrobial agents was defined as the lowest concentration that inhibited the *in vitro* growth of 90% of the bacterial population [33,34]. Qualitative classification of the isolates as sensitive or resistant was based on the cut-off points established by the Clinical and Laboratory Standards Institute (CLSI) and were equal to 0.1 μg/mL for INH and 1.0 μg/mL for RMP [35].

Antimicrobial solutions were prepared in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with oleic acid, albumin, dextrose, and catalase (OADC) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), as well as the bacterial inoculums, which were prepared by adjusting the turbidity to the McFarland standard tube number 1 and then diluted 1:20 (100 μL were used as inoculum). The test concentrations for both antibiotics ranged from 25.0 to 0.098 μg/mL. Growth controls (containing no antibiotics) and sterility controls (without inoculum) were also included in each plate. Assessment of the changes in color were carried out by visual and quantitative fluorescence inspection of the plates, using a Cytation™ 3 cell imaging multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA). A color change from blue to pink indicated resazurin reduction and, therefore, bacterial growth. The MIC_{90} was defined as the lowest drug concentration that prevented this change in color.

2.7. Detection of genetic mutations

In all the phenotypically resistant *M. bovis* lineages, the possible INH- and RMP-resistance-associated genetic mutations were assessed using the GenoType MTBDR*plus* version 2.0 commercial kit (Hain Lifescience, Nehren Tubingen, Germany) according to the manufacturer's instructions. The identification of RMP resistance was enabled by the detection of the most significant mutations within the *rpo*B gene (encoding the β-subunit of RNA polymerase). For INH resistance, the *kat*G gene (encoding catalase-peroxidase), and the promoter region of the *inh*A gene (encoding enoil-ACP-reductase) were assessed.

3. Results

Among the 100 bovine lymph nodes with caseous lesions that were analyzed, there were 67 (67%) isolates compatible with *Mycobacterium* sp. The *gyr*B-RFLP analysis revealed that all isolates were *M. bovis* and thus, the 67 lineages were subjected to REMA, spoligotyping, and MIRU-VNTR techniques.

3.1. Genotyping data

Spoligotyping of the 67 strains resulted in 16 different spoligotype patterns, of which 14 were classified as a known SIT (corresponding to 63 isolates) and 2 as unknown or “orphan” profiles (corresponding to 4 isolates). One of the profiles without a known SIT had a known SB pattern, as shown in Table 1.

The clade BOV_1 predominated, containing 77.6% (52/67) of the isolates. The clade BOV_2 was represented by 11.9% (8/67) of the isolates and 7.4% (5/67) of the isolates were included in the BOV clade. Two “orphan” isolates belonged to an unregistered profile in the online SITVIT WEB [28] and Mbovis.org [31] databases and were thus not classified into clades. In BOV_1, the following SITs (and SBs) were identified: 481 (SB0121), 482 (SB0120), 594 (SB0131), 665 (SB0134), 691 (SB0130), 698 (SB0295), 1021 (SB0881), 1667 (SB01055), 1852 (SB1139), and 2141 (SB1093), as well as 2 isolates without a known SIT (SB1144). BOV_2 was the second most frequent clade, with isolates belonging to SIT 683 (SB0140). In addition, SITs 982 (SB0875), 1851 (SB0996), and 1853 (SB1137) were identified in the BOV family.

The MIRU-VNTR analysis using the 12 classical MIRU *loci* identified 31 isolates belonging to MIT 49, 1 isolate belonging to MIT 5, and 35 isolates that were considered “orphans” or unknown MITs (Table 1). The combination of spoligotyping and MIRU-VNTR analysis enabled the grouping of the isolates into 12 clusters (defined as two or more isolates having identical genotypes) containing 68.5%

Table 1
Genotypic information of 67 *M. bovis* lineages obtained from slaughtered bovine lymph nodes, clustered by Spoligotyping and 12-*loci* MIRU-VNTR typing. Brazil, 2014 to 2015.

Ref.	octal Spoligotype43	SIT (SB)	Clade	Spoligotying groups	MIRU 12*	12-MIT	MIRU 12 subgroups
4	676773677777600	481 (SB0121)	BOV_1	**A**	232324253322	49	**A1**
6	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
14	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
24	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
52	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
77	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
78	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
79	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
81	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
84	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
85	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
88	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
97	676773677777600	481 (SB0121)	BOV_1		232324253322	49	
7	676773677777600	481 (SB0121)	BOV_1		232222253321	Orphan	Ungrouped
13	676773677777600	481 (SB0121)	BOV_1		232224133322	Orphan	
21	676773677777600	481 (SB0121)	BOV_1		2_2324253_22	Orphan	
22	676773677777600	481 (SB0121)	BOV_1		222324253222	Orphan	
39	676773777777600	482 (SB0120)	BOV_1	Ungrouped	232324252322	Orphan	Ungrouped
66	656573777777600	594 (SB0131)	BOV_1	**B**	232224233322	Orphan	**B1**
67	656573777777600	594 (SB0131)	BOV_1		232224233322	Orphan	
16	616773777777600	665 (SB0134)	BOV_1	Ungrouped	232324252422	Orphan	Ungrouped
69	664073777777600	683 (SB0140)	BOV_2	**C**	222324253322	5	Ungrouped
12	664073777777600	683 (SB0140)	BOV_2		232324253322	49	**C1**
19	664073777777600	683 (SB0140)	BOV_2		232324253322	49	
53	664073777777600	683 (SB0140)	BOV_2		232324253322	49	
42	664073777777600	683 (SB0140)	BOV_2		232324251322	Orphan	Ungrouped
86	664073777777600	683 (SB0140)	BOV_2		232224243322	Orphan	
93	664073777777600	683 (SB0140)	BOV_2		232324153322	Orphan	**C2**
96	664073777777600	683 (SB0140)	BOV_2		232324153322	Orphan	
57	676573777777600	691 (SB0130)	BOV_1	**D**	222222263322	Orphan	**D1**
73	676573777777600	691 (SB0130)	BOV_1		222222263322	Orphan	
63	676573777777600	691 (SB0130)	BOV_1		232224233222	Orphan	Ungrouped
5	676773677777200	698 (SB0295)	BOV_1	**E**	232324253322	49	**E1**
26	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
45	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
48	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
50	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
51	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
72	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
83	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
91	676773677777200	698 (SB0295)	BOV_1		232324253322	49	
8	676773677777200	698 (SB0295)	BOV_1		232324253222	Orphan	**E2**
31	676773677777200	698 (SB0295)	BOV_1		232324253222	Orphan	
29	676773677777200	698 (SB0295)	BOV_1		232324243322	Orphan	**E3**
59	676773677777200	698 (SB0295)	BOV_1		232324243322	Orphan	
64	676773677777200	698 (SB0295)	BOV_1		232324243322	Orphan	
74	676773677777200	698 (SB0295)	BOV_1		232324243322	Orphan	
76	676773677777200	698 (SB0295)	BOV_1		232324243322	Orphan	
11	676773677777200	698 (SB0295)	BOV_1		232424253322	Orphan	Ungrouped
82	676773677777200	698 (SB0295)	BOV_1		232324253321	Orphan	
15	416773777777600	982 (SB0875)	BOV	Ungrouped	232324263322	Orphan	Ungrouped
28	676773674037600	1021 (SB0881)	BOV_1	**F**	232324251322	Orphan	Ungrouped
30	676773674037600	1021 (SB0881)	BOV_1		332314253322	Orphan	
90	616773777777200	1667 (SB1055)	BOV_1	Ungrouped	232324253322	49	Ungrouped
18	464073777777600	1851 (SB0996)	BOV	Ungrouped	232324221222	Orphan	Ungrouped
9	676773770003600	1852 (SB1139)	BOV_1	**G**	232324253322	49	Ungrouped
10	676773770003600	1852 (SB1139)	BOV_1		232324251222	Orphan	
27	674773674377200	1853 (SB1137)	BOV	**H**	232324253322	49	Ungrouped
25	674773674377200	1853 (SB1137)	BOV		232324253222	Orphan	**H1**
60	674773674377200	1853 (SB1137)	BOV		232324253222	Orphan	
55	636773677777600	2141 (SB1093)	BOV_1	**I**	232324253322	49	Ungrouped
56	636773677777600	2141 (SB1093)	BOV_1		242324263322	Orphan	**I1**
62	636773677777600	2141 (SB1093)	BOV_1		242324263322	Orphan	
20	636773676777600	Orphan		**J**	232324253322	49	**J1**
23	636773676777600	Orphan			232324253322	49	
17	676773677600600	Orphan (SB1144)	BOV_1	**K**	232324251322	Orphan	**K1**
41	676773677600600	Orphan (SB1144)	BOV_1		232324251322	Orphan	

Ref. = reference number of the strains SIT = Spoligotyping International Type SB = *M. bovis* Spoligotype Pattern MIRU = Mycobacterial Interspersed Repetitive Unit MIT= MIRU International Type *order of MIRU analysis: *loci* 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40.

of the strains (46 isolates in total), whereas 21 isolates showed unique profiles.

3.2. Phenotypic resistance to isoniazid and rifampicin

The REMA plate method revealed 21 isolates (31% of total) with phenotypic resistance to the tested drugs. Among these, 11 (16%) were classified as MDR-TB. Eight other isolates (12%) were resistant to INH and 2 (3%) were resistant to RMP. MIC_{90} values and qualitative classification of the *in vitro* sensibility profile to INH and RMP of the *M. bovis* lineages are shown in Table 2.

3.3. Detection of genetic mutations

The search for genetic mutations related to INH- and RMP-resistance using the GenoType MTBDR*plus* version 2.0 did not reveal the presence of mutations in any of the phenotypically resistant isolates.

4. Discussion

Among the 67 isolates of *M. bovis* obtained here, 21 (31.3%) showed some resistance to the tested drugs. Among these, 11 (16%) were classified as MDR-TB, 8 (12%) were resistant to INH, and 2 (3%) were resistant to RMP. These data contrast with those reported in studies on *M. bovis* strains obtained from Michigan's wild white-tailed deer (considered as the primary tuberculosis reservoirs for domestic cattle in Michigan), which did not find drug-resistance in the microorganisms using the proportions method and the Bactec™ system [17]. In contrast, in Italy, a study reported 22.7% of *M. bovis* as multidrug-resistant, as well as 22.7% RMP-resistant and 18.9% INH-resistant in strains from cattle herds [16].

In Brazil in particular, drug-resistance in 7 *M. bovis* strains out of 200 samples of human tuberculosis was already reported as early as 1974, with 3 of them being MDR-TB [36]. Another study found no phenotypic resistance to INH and RMP in *M. bovis* strains isolated from caseous lesions in cattle using qualitative assessment on solid culture media with pre-established concentrations of antimicrobial agents [37]. However, other authors reported 2.4% INH-resistance in *M. bovis* lineages from the same type of lesion in cattle [38].

In our study, the identification of *M. bovis* strains isolated from cattle with mono- and multidrug-resistance to first-line drugs used in human tuberculosis treatment raises public health concerns, and addresses the need for the intensification of epidemiological surveillance of bovine tuberculosis in the Brazilian territory. It is important to note that the implementation of the Brazilian Program for Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) in 2001 by the Brazilian Ministry of Agriculture (MAPA) prohibited the treatment of infected cattle [39].

Although it may be influenced by multiple factors, *Mycobacterium* sp. resistance to antimicrobials is often associated with mutations in target-encoding or related genes [40]. None of the 21 tested strains presented mutations covered by the commercial kit MTBDR*plus* version 2.0. However, the resistant strains were not

Table 2
Qualitative classification (with respective cut-off points) and minimum inhibitory concentrations (MIC, expressed in μg/mL) for INH and RMP sensibility testing by REMA plate method in *M. bovis* strains isolated from slaughtered bovine lymph nodes. Brazil, 2014 to 2015.

Ref.	Classification		MIC (μg/mL)		Ref.	Classification		MIC (μg/mL)	
	(0.1 μg/mL) INH	(1.0 μg/mL) RMP	INH	RMP		(0.1 μg/mL) INH	(1.0 μg/mL) RMP	INH	RMP
4	S	S	<0,098	<0,098	51	**R**	**R**	**>25**	**3125**
5	S	S	<0,098	<0,098	52	S	S	<0,098	<0,098
6	**R**	S	**>25**	0,156	53	S	S	<0,098	<0,098
7	S	S	<0,098	<0,098	55	S	S	<0,098	<0,098
8	**R**	S	**0,246**	<0,098	56	S	S	<0,098	<0,098
9	**R**	S	**0,850**	<0,098	57	S	S	<0,098	<0,098
10	**R**	S	**0,420**	<0,098	59	S	S	<0,098	<0,098
11	**R**	S	**>25**	<0,098	60	S	S	<0,098	<0,098
12	**R**	**R**	**>25**	**3125**	62	S	S	<0,098	0171
13	S	S	<0,098	<0,098	63	**R**	S	**0,194**	<0,098
14	S	S	<0,098	0185	64	S	S	<0,098	<0,098
15	S	S	<0,098	0354	66	S	S	<0,098	0117
16	S	S	<0,098	0413	67	**R**	S	**0,156**	<0,098
17	**R**	**R**	**0,296**	**1219**	69	S	S	<0,098	<0,098
18	S	S	<0,098	0702	72	S	S	<0,098	<0,098
19	S	S	<0,098	<0,098	73	**R**	**R**	**6,25**	**>25**
20	S	S	<0,098	<0,098	74	S	S	<0,098	0164
21	S	S	<0,098	0562	76	S	S	<0,098	<0,098
22	S	S	<0,098	<0,098	77	**R**	**R**	**>25**	**3125**
23	S	S	<0,098	<0,098	78	S	S	<0,098	<0,098
24	S	S	<0,098	<0,098	79	**R**	**R**	**>25**	**3125**
25	**R**	**R**	**0,203**	**1389**	81	S	S	<0,098	<0,098
26	S	S	<0,098	<0,098	82	**R**	**R**	**>25**	**3125**
27	S	S	<0,098	<0,098	83	S	S	<0,098	<0,098
28	**R**	**R**	**>25**	**5624**	84	S	S	<0,098	<0,098
29	S	**R**	<0,098	**1259**	85	**R**	S	**0,120**	0099
30	S	S	<0,098	<0,098	86	S	S	<0,098	<0,098
31	S	S	<0,098	<0,098	88	S	S	<0,098	<0,098
39	S	S	<0,098	<0,098	90	S	**R**	<0,098	**2759**
41	**R**	**R**	**>25**	**3125**	91	**R**	**R**	**>25**	**5771**
42	S	S	<0,098	<0,098	93	S	S	<0,098	<0,098
45	S	S	<0,098	0141	96	S	S	<0,098	0727
48	S	S	<0,098	0150	97	S	S	<0,098	<0,098
50	S	S	<0,098	0419					

Ref. = reference number of the strains; MIC = Minimal Inhibitory Concentration; INH = isoniazid; S = sensible; RMP = rifampicin; R = resistant. Bold lines show resistant strains.

subjected to sequencing of *rpo*B, *kat*G or the promoter region of *inh*A gene.

Chromosomal mutations do not elucidate all cases of mycobacterial resistance. In fact, approximately 20–30% of INH-resistant *M. tuberculosis* isolates do not have mutations in any of the genes associated with INH resistance [41]. In contrast, for RMP, the occurrence of genetic mutations has been most commonly associated with resistance, especially in the hot spot region of the *rpo*B gene. However, rare cases, which correspond to 2–4% of the clinical isolates, do not have these mutations [42]. Moreover, it is unclear whether the current understanding of the genetic basis of antimicrobial resistance in *M. tuberculosis* is sufficiently comprehensive [43]. Recent studies have discovered new genes and/or intergenic regions associated with antimicrobial resistance in clinical isolates of *M. tuberculosis* and have identified various single-nucleotide polymorphisms consistently associated with drug resistance in comparison with the *M. tuberculosis* H37Rv reference genome [43].

Data are still scarce regarding the genetic basis of drug-resistance in *M. bovis*, especially in strains of animal origin. Bovine tuberculosis remains one of the biggest infectious threats to cattle worldwide, making it important to control and prevent outbreaks of MDR-*M. bovis* and its transmission to humans [16]. Given the emergence of multidrug resistant *M. bovis*, similar approaches comparing the genome of drug-resistant *M. bovis* clinical strains to the *M. bovis* BCG (bacillus Calmette-Guérin) reference genome are essential in order to elucidate whether there are differences in the mutation patterns associated with drug resistance in *M. tuberculosis* and *M. bovis* strains. These potential differences could explain, at least in part, the absence of the identification of genetic mutations in drug-resistant *M. bovis* lineages in our study.

Recent data suggests that the emergence of drug resistance and the transmissibility patterns of mycobacteria are strongly influenced by MTBC genetic and evolutionary scenarios [44]. In our study, the MIRU-VNTR technique, with its classical 12-*loci* format, identified a cluster of 31 isolates belonging to MIT 49, one isolate belonging to MIT 5, and 35 isolates considered “orphans” or unknown MIT. These data fit the classification described in the largest currently available international database for the study of genetic diversity and molecular epidemiology of MTBC, the SITVIT WEB [28]. To date, MITs 49 and 5 have not been reported in Brazil. MIRU-VNTR combined with spoligotyping results enabled the grouping of the isolates into 12 clusters and 21 unique profiles.

Spoligotyping and MIRU-VNTR showed a high rate of clustering of clinical isolates of *M. bovis* in the studied population (46/67 isolates, representing 68.5% of total). BOV_1 was the main genotypic lineage observed and comprised BOV_1/SIT698 (SB0295) (26.8%) and BOV_1/SIT481 (SB0121) (25.3%), followed by the BOV_2/SIT683 (SB0140) (11.9%) genotypes. These data are similar to those already described in the same region, which referred to the same three genotypes at the same order of frequency [22]. The SIT 698 (SB0295) genotype is highly prevalent in Brazil (72.5%) and has been identified in Argentina, France, Spain, Mexico, the Netherlands, the United States, Costa Rica, and Belgium [28]. SIT 481 (SB0121) (the second most prevalent in our study) is ubiquitous and was described in some countries in the Americas and Europe, and in South Africa [28]. In the BOV_2 subfamily, SIT 683 (SB0140) has a higher prevalence in Ireland and the United Kingdom. It was described as the most common genotype in Argentina and was also present in Paraguay and Uruguay, whereas it was uncommon in Brazil [28,45].

These 3 genotypic profiles were also reported in Portugal, as genotypes shared among cattle (*Bos* spp.) and wild species, such as deer (*Cervus elaphus*) and wild boars (*Sus scrofa*), indicating that interspecific tuberculosis transmission may occur [18]. Similar evidence has been described in Italy and Spain [46–48]. Analogous situations may be occurring in Brazil, where little is known about the impact of wild animal species as tuberculosis reservoirs or the role of sick cattle as sources of infection for wildlife.

Among the other identified profiles, it is worth mentioning that genotypes BOV/SIT982 (SB0875) and BOV/SIT1851 (SB0996) have not been described in Brazil to date. SIT 982 (SB0875) was identified in Spain and France, and SIT 1851 (SB0996) was described in Argentina and the United Kingdom [28].

The SIT 665 (SB0134) genotype was predominantly found in France and was previously identified in Brazil, as along with SIT 1021 (SB0881) and SIT 2141 (SB1093). SIT 482 (SB0120) was ubiquitously distributed and was identified in all continents, except in Oceania [22,28]. SITs 1852 (SB1139) and 1853 (SB1137) have few records in the SITVIT WEB database and were obtained in Argentina and Brazil. These countries also reported SIT 691 (SB0130), SIT 594 (SB0131), and SIT 1667 (SB1055) [28].

A spoligotype that included 2 isolates in the present study with no known SIT (octal pattern 676773677600600, SB1144) was included in the BOV_1 clade, and a single report of its isolation in Brazil was presented [28]. Another “orphan” pattern (octal pattern 636773676777600) comprising 2 isolates in our study had not been referred to in SITVIT WEB or Mbovis.org databases. Therefore, it was not classified into clades and may represent a new spoligotype profile.

The genetic diversity of *M. bovis* lineages in cattle may be attributed to the indiscriminate movement of animals between herds, which occurs in Brazil mainly due to trade, agricultural exhibitions, and auctions. Indeed, this transit complicates the establishment of the geographical specificity for *M. bovis* strains [49]. In addition, our study covered only part of a Brazilian state, which is a small area compared to the territory of the country. This study design did not allow the investigation of associations with different spoligotype patterns and regions of Brazil.

However, it should be noted that deletions of spacers in the DR *locus*, which generate new spoligotypes, are very common. In fact, similar spoligotype patterns can occur independently in unrelated lineages (genetic homoplasy). Thus, spoligotyping can be a poor indicator for the establishment of phylogenetic relationships between strains [50]. In addition, the genotypic classification of *M. bovis* strains have some limitations due to the overrepresentation of *M. bovis* data entries in MTBC genotypic diversity databases from Europe and South America (approximately 30 and 25%, respectively). Although this fact limits the interpretation of *M. bovis* molecular epidemiology on a global level, it reflects the economic importance of cattle breeding in South America (Brazil and Argentina) and in Europe [51].

5. Conclusions

The high prevalence of *M. bovis* in bovine lymph nodes with gross pathology suggestive of tuberculosis reinforces the importance of the pathogen as a causal agent of lymphadenitis and tuberculosis in cattle herds in the study area. The occurrence of *M. bovis* lineages resistant to first-line treatment drugs for human tuberculosis raises public health concerns and reinforces the need to intensify the activities of the Brazilian Program for Control and Eradication of Brucellosis and Tuberculosis. Antimicrobial resistance is a complex and multifaceted phenomenon, particularly in MTBC species. Comprehensive studies in different geographical areas around the world are necessary to elucidate the basis of drug resistance in *M. bovis* strains isolated from animals.

Molecular epidemiology in *M. bovis* lineages enables possible inferences about their geographical origins, sources of infection for hosts, and the spread of the pathogen from animals to humans. However, there are some limitations in interpreting global *M. bovis*

genetic diversity, reflecting the need for more studies in different geographical areas involving epidemiological surveillance and molecular typing of *M. bovis* strains.

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Conflict of interests

None.

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