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Chapter 9

Molecular Diagnostic Testing on Post Mortem Inspection and Rulings on Bovine Tuberculosis — An Experience Report in Brazil

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1. Introduction

Bovine tuberculosis (BTB) is an infectious disease of chronic evolution and debilitating effects. The etiologic agent of this disease is *Mycobacterium bovis*, which, alongside *M. tuberculosis, M. bovis* BCG, *M. africanum, M. caprae, M. canettii* and *M. microti*, form the *Mycobacterium tuberculosis* complex (MTC) [1]. Members belonging to this complex exhibit high homology between gene sequences present in their genomes. The *M. bovis* genome AF2122/97 (4.345.492-bp) was sequenced in 2003 by Garnier [2], who detected a 99.95% genetic similarity between *M. bovis* and *M. tuberculosis*.

Cattle are the primary hosts for *M. bovis*. Several mammalian species, however, including humans, are also susceptible to this bacillus [3, 4]. This zoonosis is of global importance and shows a high prevalence in developing countries, due to lack of or ineffectiveness of tuberculosis control and eradication programs. BTB can be considered a socioeconomic disease, since it causes decreases in herd productivity, which lead to significant economic losses in the global agriculture industry, estimated at approximately 3 billion dollars a year [5, 6]. Whereas more than 94% of the world population lives in countries where BTB control is limited or absent [7], there is a consensus regarding the imminent risks to human health, especially immunossupressed or convalescent individuals, such as patients infected with the human immunodefi-

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ciency virus (HIV) or patients undergoing cancer chemotherapy treatments [8] which present greater risk of infection by BTB [9, 10].

Infection by *M. bovis* in humans is typically caused by the consumption of animal products contaminated with the bovine bacillus, usually unpasteurized milk and milk derivatives [11], leading to disease development in extrapulmonary form [12]. According to the Centers for Disease Control and Prevention (CDC, USA), 35 cases of *M. bovis* infection were reported in the city of New York from 2000 to 2004, and some of these cases were associated with the consumption of cheeses made with unpasteurized milk, imported from countries where BTB is endemic [13]. Another form of *M. bovis* infection in humans is through airborne transmission [14, 15]. Infections caused in this manner are clinically and pathologically indistinguishable from tuberculosis caused *by M. tuberculosis* [5, 12]. It is suspected that infections caused by *M. bovis* are responsible for more than 4000 cases among the 100,000 cases of human tuberculosis described annually in Brazil [10, 16]. However, according to the World Organisation for Animal Health (OIE), the number of human TB cases caused by *M. bovis* in Brazil cannot be estimated [17], since bacteriological culture tests and biochemical identification tests to diagnose whether the infection is caused by *M. bovis* or *M. tuberculosis* are not performed in most tuberculosis cases [18]. The absence of a specific diagnosis to identify the etiological agent of tuberculosis in humans is very detrimental, as patients infected with *M. bovis* require special treatment since the antibiotic pyrazinamide, used as the standard treatment for human tuberculosis, is not effective in infections caused by *M. bovis*. This results in incorrect treatment and subjects resistant to *M. bovis*, that in turn become potential transmitters of resistant strains to other people and animals [19].

Concerning *M. bovis* transmission to cattle, approximately 80-90% of the animals are infected by airborne transmission through inhalation of bacillus-contaminated aerosols [20, 21]. It is important to note that cattle with chronic or recent infections may excrete viable bacilli, thus causing infections in other animals [22]. In calves, airborne transmission of *M. bovis* is also regarded as the most important transmission route [20], although healthy calves can also become infected by ingestion of bacillus-contaminated milk [20, 23]. In countries where no effective measures to control and eradicate the disease exist, the morbidity rates in BTB-infected cattle range at about 8-10%, and fatality rates can reach up to 50% [23, 24]. It should be emphasized that BTB is a disease that mainly affects stabled cattle (dairy cattle), spreading rapidly due to the proximity of the animals to each other. In beef cattle, the opposite occurs, due to the fact that the animals are raised extensively [23, 25].

An important feature of mycobacterial infections is the cell-mediated immune response developed by the infected host, due to the intracellular location of mycobacteria. This leads to the development of granulomatous inflammations in the host, resulting in tuberous lesions [26, 27]. These lesions often occur in organs rich in reticuloendothelial tissue, especially in the head, neck, mediastinal and mesenteric lymph nodes, but also in the lungs, intestines, liver, spleen, pleura and peritoneum [28, 29]. Although tuberous lesions are not considered pathognomonic for BTB in cattle, their presence is closely linked to the appearance of clinical signs of BTB in animals [4, 5].

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In developed countries, where TB control programs have been established longer and executed with rigor, BTB control is accomplished through mandatory procedures such as pasteurization of cow milk and its derivatives and sanitary inspection of cattle during slaughter, thereby drastically reducing cases of the disease in humans and animals [30]. Although a tuberculosis control and eradication plan exists in Brazil, illegal sales of meat, milk and dairy products not inspected by sanitary control agents still occur and constitute a risk to public health [19].

The detection of the pathogen responsible for BTB is crucial for the control and eradication of the disease and should be performed as recommended by the OIE [31], by late hypersensitivity reactions in cattle (intradermal tuberculin tests), sanitary inspection in slaughterhouses, tracing the origin of diseased animals and disease sanitation [31].

With the aim of reducing the prevalence and incidence of new BTB outbreaks, the Brazilian national program for control and eradication of brucellosis and tuberculosis (PNCEBT) was instituted in 2001. This program is based on the performance of intradermal tuberculin tests and the slaughter of reactive animals (test and slaughter), associated with the health inspections carried out in slaughterhouses [15]. Although the intradermal tuberculin test is widely used worldwide for BTB diagnosis, this test presents sensitivity and specificity problems, generating false-positive or false-negative results. These flaws are important, since the reference microbiological methods for BTB diagnosis also exhibit low sensitivity and are effective for pathogen detection only when the number of viable bacilli is higher than 100 bacilli/mL. In addition, microbiological testing procedures are laborious and time consuming, taking from 1 to 3 months for bacilli isolation and a further two or three weeks for the biochemical identification of the isolates [32].

Despite the occurrence of BTB, there is no official data on the current prevalence of the disease in Brazil. Data from official reports from 1989-1998 indicate that the national average prevalence was of 1.3% of infected cattle [15]. Since the beginning of the PNCEBT program, however, few studies have been conducted to determine the prevalence of the disease, and estimates vary from 0.7% to 3.3% [33, 34-35, 36]. According to the data obtained by Roxo and Kantor [37, 38], the estimated national prevalence was of 0.83% and the region with the lowest prevalence of BTB was the Brazilian Midwest (0.37 %), where beef cattle in its majority is raised. In studies conducted by Salazar and Furlanetto [6, 39], in slaughterhouses in the state of Mato Grosso, located in the Midwest region of the country, a very low BTB prevalence was detected, of only 0.007%.

One of the main economic activities in the state of Mato Grosso is cattle production. This state is prominently the largest producer of beef cattle, with around 28 million cattle heads, and the second largest beef exporter the country [40], increasing beef exports to EU countries each year. However, countries that buy Brazilian beef are increasing pressure to implant effective, quick and definitive BTB diagnosis methods to identify tuberculosis-suspected lesions. In 2012, the Ministry of Agriculture, Livestock and Supply (MAPA), determined that farms in which suspected cases of BTB had been detected could no longer export beef to the Customs Union of Belarus, Kazakhstan and Russia, and that all lots of animals of the property must be sequestered during slaughter, until confirmation of the diagnosis of the BTB-suspected lesions by official MAPA laboratories [41, 42].

Due to the demands imposed by countries that import Brazilian beef and the difficulties in achieving quick and specific BTB diagnoses, molecular tests based on PCR assays and its variations (*multiplex*-PCR, *nested*-PCR, real-time PCR and *nested* real-time PCR) [42,43 – 44], have been considered the most promising alternatives to accomplish BTB identification quickly and effectively in both live animals [45, 46] and in fragments of tissue samples presenting BTB-suspected lesions [5, 47 – 48, 49, 50], nasal exudates [5, 46 – 51, 52] and milk [53, 54 – 55].

In the present study, different BTB diagnosis tests, used singly or in combination with each other, were evaluated. Different methods, i.e. macroscopic analyses, histopathological examinations and *multiplex*-PCR, were evaluated for the rapid and specific detection of BTB (*M. bovis* and *M. tuberculosis* complex) directly from BTB-suspected lesions, with the aim of accelerating and adding specificity to the diagnosis of the disease, and, consequently, supporting the rulings of the health inspection service (SIF) performed in slaughterhouses, as stipulated by the tuberculosis control and eradication program in Brazil. The apparent prevalence of BTB among animals slaughtered in the state of Mato Grosso was also re-estimated and discussed, due to the great importance that this region has in the production of exported meat to different consumer countries worldwide, including the European Community.

2. Geographic region and study conditions

The study of the prevalence of BTB in animals slaughtered in the state of Mato Grosso, Brazil, was carried out by monitoring cattle slaughter and by *post-mortem* inspection of 41.193 carcasses. The duration of sample collections in each slaughterhouse was of approximately 10 days, between May and October 2009. The inspected carcasses belonged to 492 herds, from 85 (60%) municipalities in the state of Mato Grosso. Most of the slaughtered cattle was male (76.2%), from 1 to 2 years old (2.4%), 2 to 3 (54.2%) and> 3 years old (43.4%). A total of 77.8% (32.048/41.193) of the animals originated form herds monitored by the PNCEBT Program. The sample size (n) was calculated using the standard formula for simple random sampling, considering a degree of confidence of 95%, level of absolute accuracy of ± 0.022% [56] and expected prevalence of 0.05%, considering the results previously described by Salazar [39].

Seven slaughterhouses inspected by the SIF were monitored, located in six different cities in the state of Mato Grosso (Figure 1). As mentioned above, this region is considered the largest producer and second largest beef exporter in the country [40]. For the sampling to be considered representative with regard to cattle herds in this geographical area, slaughterhouses in four areas of Mato Grosso which have significant cattle herd production were selected: Southeast, south central, southwest and north. No sampling was conducted in the northeast area due to the unavailability of establishments with SIF inspection. However, animals from that region were slaughtered at the Paranatinga municipality, an area fortunately covered by this study. The selected sampling sites covered the four Mato Grosso cattle-producing circuits, divided according to Negreiros [57], in: Pantanal-represented by the Cáceres (16º 04' 14" S, 57º 40' 44" W) and Várzea Grande (15º 38' 48" S, 56º 07' 57" W) municipalities; Milk-represented by

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the Rondonópolis municipality (16º 28' 15" S, 54º 38' 08" W); Fattening-represented by the Paranatinga municipality (14º 25' 54" S, 54º 03' 04" W); and Reproduction-represented by the Juara (11º 15' 18" S, 57º 31' 11" W) and Tangará da Serra (14º 37' 10" S, 57º 29' 09" W) municipalities. Cattle slaughter at Juara was only monitored in two slaughterhouses.

During carcass inspections, all fragments of lesions classified by SIF as lymphadenitis or tuberculosis lesions located in the head, neck, chest cavity or cervical area lymph nodes (areas frequently affected by BTB) were sampled, according to official standards [58]. Once identified, the lesions were photographed, divided into samples and properly packaged. Information on body condition score, age and sex, origin (municipality and property where the cattle were raised) and health status of animals (participation or non-participation in the PNCEBT program), were obtained and recorded during sampling by means of the Animal Traffic Guide (GTA) of each lot.

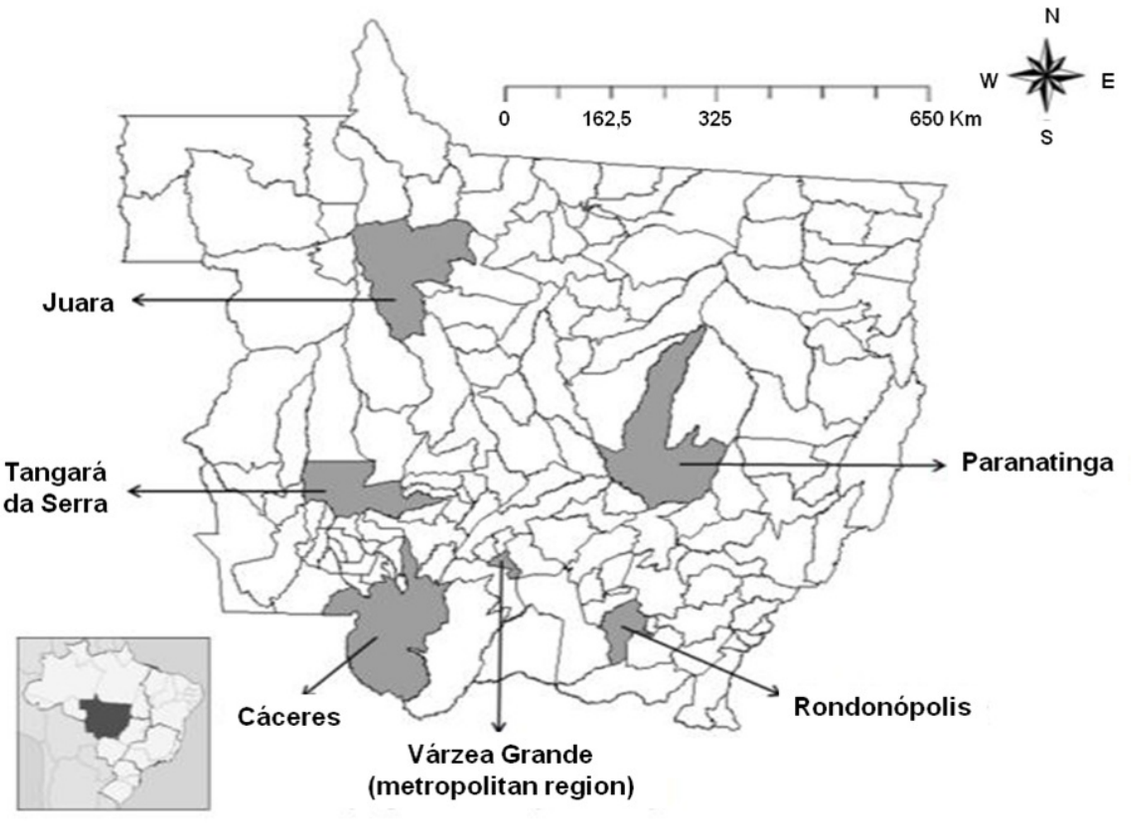


Figure 1. Map of the state of Mato Grosso, Brazil. Municipalities in gray indicate where samplings suggestive of BTB were taken during *post-mortem* inspections carried out in slaughterhouses in the region.

2.1. Prevalence of bovine tuberculosis in herds slaughtered in 2009 in the state of Mato Grosso, Brazil, determined using conventional tests [6]

The inspected carcasses 41.193 carcasses belonged to 492 herds, from 85 (60%) municipalities in the state of Mato Grosso (Figure 2). From the 41.193 carcasses assessed during the *post-*

mortem inspection, 198 (0.48%) showed lesions suggestive of BTB or lymphadenitis located in the front portion of the carcass (BTB-suspected lesions) (Table 1), according to the official standards for *post-mortem* examinations [58]. The decision to sample all lymphadenitis lesions from the head, neck and chest cavity lymph nodes was adopted to avoid losing potentially positive samples due to errors during the evaluations of the macroscopic lesions, since BTB lesions, lymphosarcomas or nonspecific lymphadenitis have very similar features and are difficult to be distinguished by the naked eye [59]. In addition, previous studies report that 86% of BTB lesions are present in the lymph nodes of the head and chest cavity (superior portion of the carcass) [28, 60 – 61].

After the *post-mortem* inspections, the collected lesions were photographed, divided into samples and either preserved in 10% buffered formalin for the histopathology analyses or frozen at-20°C for the bacteriology analyses and subsequent molecular technique applications. All samples fixed in 10% buffered formalin were cleaved so that each fragment of the lesion covered all layers of the granuloma (the necrotic material, capsule and transition area between the lesion and normal tissue). They were then subjected to dehydration techniques, diafanization clarification, embedding in paraffin and microtomizaion of the paraffin block at 4 μm, thus obtaining two histological slides of each sample for hematoxylin and eosin (HE) staining, with the purpose of observing histopathological changes, and Ziehl-Neelsen (ZN) staining, used for the detection of alcohol-acid resistant bacilli (AARB or BAAR) [62].

The HE histopathological examination performed on 198 samples of BTB-suspected lesions, indicated that 83.8% of the lesions were granulomatous, 8.1% were pyogranulomatous, 6.1% were suppurative, and 2.0% were lesions characteristic of interstitial pneumonia. The ZN histopathological examination indicated no AARB in the samples. The absence of AARB in BTB-suspected lesions has been reported by Salazar [39], and may occur due to the low bacilli concentrations in the examined lesions (paucibacillary lesions) [63].

Although granulomas are a classic BTB lesion, they cannot be considered pathognomonic of the disease [20, 32 – 59]. This statement was confirmed in the present study, where 91.9% (182/198) of the samples were granulomatous or piogranulomatose lesions (classic BTB lesions) and only 1.64% (3/182) of the lesions were affected by *M. bovis*. Therefore, the presence of the granulomas observed during the histopathological examinations is not conclusive and cannot be considered a supportive BTB diagnosis.

Samples stored at-20°C were processed for bacteriological analyses within three months after their collection. Approximately 3g of each sample were macerated with ground glass, and subjected to the hexadecylpyridinium chloride (HPC) 0.75% decontamination method and an adapted Petroff method (4% NaOH). The 0.75% HPC decontamination method was performed as described by Ambrosio [64], and the Petroff method [64] was adapted for the simultaneous processing of up to five samples, respecting the collection order and slaughterhouse of origin. When colony growths were observed, samples were reprocessed individually to identify the infected sample. After decontamination, the samples were plated in duplicate in Stonebrink and Lowenstein-Jensen (LJ) culture media, and incubated at 37°C. The samples were observed weekly during the first month and subsequently every two weeks until 90 days of culture. After isolate growth, the samples were stained by ZN to indicate the presence of AARB [62],

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as recommended in the National Manual of tuberculosis and other mycobacteria laboratory surveillance, by the Ministry of Health [65].

Sampling municipality	Number of slaughtered cattle	Number of carcasses presenting lesions	
		n	%
Cáceres	4,328	77	1.78
Juara	6,591	17	0.26
Paranatinga	8,068	23	0.29
Rondonópolis	5,914	03	0.05
Tangará da Serra	9,689	20	0.21
Várzea Grande	6,603	58	0.80
Total	41,193	198	0.48

Table 1. Occurrence of BTB-suggestive lesions in lymph nodes in the front portion of carcasses during post mortem inspection in 41,193 cattle in slaughterhouses from different municipalities in the state of Mato Grosso, between May and October 2009.

The bacteriology analyses isolated *M. bovis* in three animals from distinct herds, from the cities of Guarantã do Norte and Juína (upstate) and Pontes e Lacerda (southwest region), located in the Mato Grosso cattle reproduction circuit (Figure 2), according to Negreiros [57]. Their lesions were all located in the retropharyngeal lymph nodes and had been classified during HE histopathological examinations as granulomatous or pyogranulomatose.

Because of the characteristics of the present study, in which the purpose was to estimate the prevalence of BTB through animals destined for slaughter without possessing knowledge of the number of cattle specimens from each sampled property, it was not possible to calculate the actual prevalence of BTB in the state. Therefore, we calculated only the simple apparent prevalence of BTB in cattle and herds slaughtered in the state of Mato Grosso under SIF supervision, according to the method described by Martin [66]. BTB prevalence was calculated as 0.007% [CI 95%=-0.001%; 0.016%] for cattle and 0.61% [CI 95%=-0.08%; 1.30%] for herds. These results were similar to those found by Salazar [39], 0.007%, when surveying 57.641 cattle slaughtered in the state of Mato Grosso under supervision of the state sanitary inspection (SSI) service, from November 2004 to August 2005, during the PNCEBT program deployment by bacteriological analyses [15]. Of the total inspected cattle, 0.05% (27/57.641) showed BTB-suggestive lesions according to SISE, with four of these animals (14.8%) confirmed as BTB-positive by the bacteriological analyses. Similar results observed in the present study, four years after the start of the PNCEBT program, indicate a slow progress of the BTB eradication program in Mato Grosso, with the need for greater involvement of all the public and private links involved in this process.

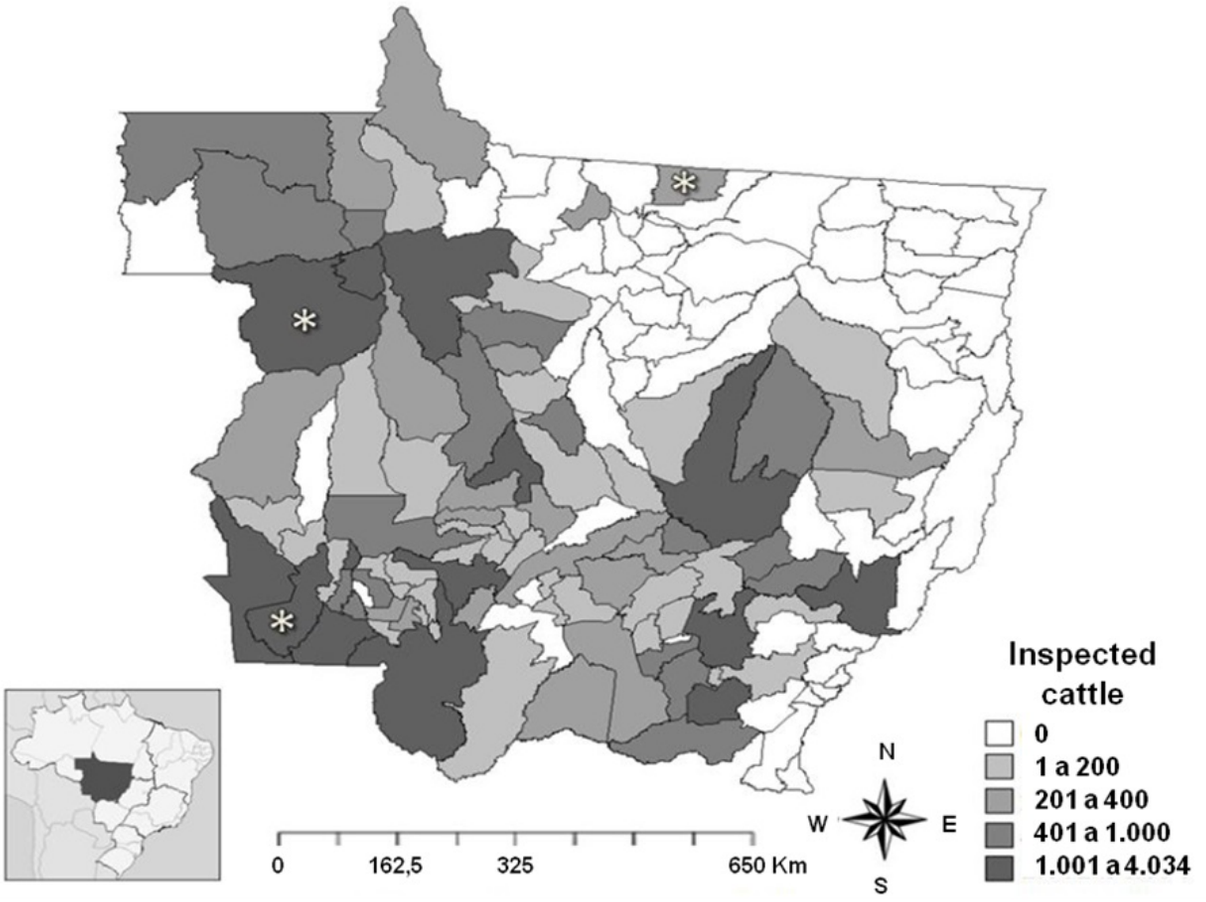


Figure 2. Mato Grosso municipalities, indicating total inspected cattle properties. *Municipalities where 03 animals with BTB were found.

Between 1993 and 1997, the prevalence of BTB in cattle slaughtered at ten slaughterhouses in the state of Minas Gerais (southeastern region) under SIF supervision, was of 0.08%, considering only official macroscopic findings [34]. Meanwhile, estimates on the apparent prevalence of infected animals in the same state, considering the results of the comparative cervical tuberculin test (CTT) (official *in vivo* examination) were 10 times higher (0.8%), according to the 1999 survey conducted on 22.990 animals from 1586 properties [67]. These results confirmed that the estimated prevalence of BTB is higher when applying *in vivo* diagnoses (bovine herds) when compared to *post-mortem* inspection diagnoses. Given this reasoning, it is expected that the prevalence of BTB in cattle herds in the state of Mato Grosso is higher than estimated by the present study, since the macroscopic inspections performed routinely in slaughterhouses are not able to identify all of the infected animals [32]. Other extrinsic factors can also interfere extensively in these estimates, such as the lack of random sampling in the slaughter groups, possible disposal of cattle shipments to state, municipal or clandestine plants, and the elimination of BTB-positive cattle in the breeding areas themselves. Therefore, the low prevalence observed in the present study, should be considered only as an indicative of the real cattle BTB situation in the state of Mato Grosso.

According to Kantor [38], estimates lower than 0.1% suggest areas considered low-prevalence or virtually tuberculosis-free. Therefore, the results of the present study may be underestimating up to 14 times the total number of infected animals and yet, even with this underestimation, Mato Grosso would still be considered a low prevalence or virtually tuberculosis-free area. To provide confirmatory estimates of the disease in Mato Grosso, it would be necessary to conduct a representative BTB sampling survey of the main cattle raising properties in the region [34].

The low prevalence status found in Mato Grosso was expected, since the area presents certain characteristics that hinder the spread of BTB, including a tropical climate, cattle raised predominantly by the extensive system, aimed at beef exports, low pasture stocking, and early slaughter of the animals. Because of this, the animals end up having less contact with each other and, consequently, shorter exposure to possibly infected animals [68].

As a result of the low prevalence status observed in this study, the state of Mato Grosso may advance to the stage of BTB eradication, using strategies such as the implementation of an efficient monitoring system, performed alongside inspection officers and the health defense service, so that, together, they are able to detect remaining BTB foci in the region, the application of *post-mortem* inspection routines in slaughterhouses and the use of additional BTB-diagnostic techniques, such as molecular techniques applied directly to BTB-suggestive lesions. Consequently, these suggested strategies can contribute to accelerate the process of bovine tuberculosis eradication in the state of Mato Grosso, Brazil.

2.2. Use of complementary tests in the *post-mortem* inspection of suspected bovine tuberculosis infections [69]

The association of molecular tests and conventional tests was evaluated to contribute to the choice of additional tests in order to reach the BTB-eradication stage in Mato Grosso, identifying the limitations and benefits of each approach regarding their use in *post-mortem* cattle inspection. The same 198 lymph node samples were evaluated by macroscopic examinations, histopathology and *multiplex*-PCR assay using DNA fragments arrays obtained directly from BTB-suggestive lesions.

DNA extraction was performed using the commercial Qiagen extraction and purification kit (DNeasy® Blood & Tissue kit), with modifications in the protocol as described by Figueiredo [5]. Five microliters of template DNA-about 100 ng-were used for the m-PCR test based on the method described by Figueiredo [50] using a reaction mixture of 5 μL reaction buffer (Invitrogen, USA), 0.2 mM dNTPs (Fermentas, USA), 1.5 U of recombinant Taq polymerase (Platinum® Taq – Invitrogen, USA), 5 mM $MgCl_2$ (Invitrogen, USA) and 20 pmols of each primer (Invitrogen, USA) for the amplification of IS6110 genomic sequences (245 bp) Ixlink: (5′-CGTGAGGGCATCGAGGTGGC-3′) and INS2: (5′-GCGTAGGCGTCGGTGACAAA-3′) [70] present only in MBC members, and *RvD1Rv2031c* (500 pb) Jb21: (5′-TCGTCCGCTGATGCAAGTGC-3′) and Jb22: (5′-CGTGAACGTAGTCGCCTGC-3′) [71], present only in *M. bovis*, with a final volume of 50 μL. Amplification of the target sequence was performed in a thermocycler GeneAmp 9700 PCR System (Applied Biosystems, USA) according to the following parameters: 94°C for 5 min, followed by 37 1 min cycles at 94°C, 1 min at 68°C and

1 min at 72°C with a final extension at 72°C for 7 min. The resulting PCR products were analyzed by 1.5%, ultrapure agarose gel electrophoresis (Invitrogen, USA) stained with ethidium bromide (10 mg/mL) and visualized/documented in a MiniBIS pro system (DNR Bio-Imaging Systems, USA).

M. bovis was detectable in 7.0% of the samples when performing the m-PCR technique directly on the fragments of BTB-suspicious lesions (Figure 3) (14/198), including lesions from the same three strains in which *M. bovis* was isolated. The results obtained without applying *M. bovis* cultivation and isolation steps, allowed the detection of the pathogen in 14 samples, representing an increment of almost 5 times in method efficiency, consistent with other studies conducted by Meikle, Cardoso and Figueiredo [46, 49-72]. Thus, according to the results, it is suggested that the m-PCR method may be useful in monitoring BTB in slaughterhouses, reducing the diagnosis time from 90 days to only 2 working days in addition to increasing pathogen detection sensitivity.

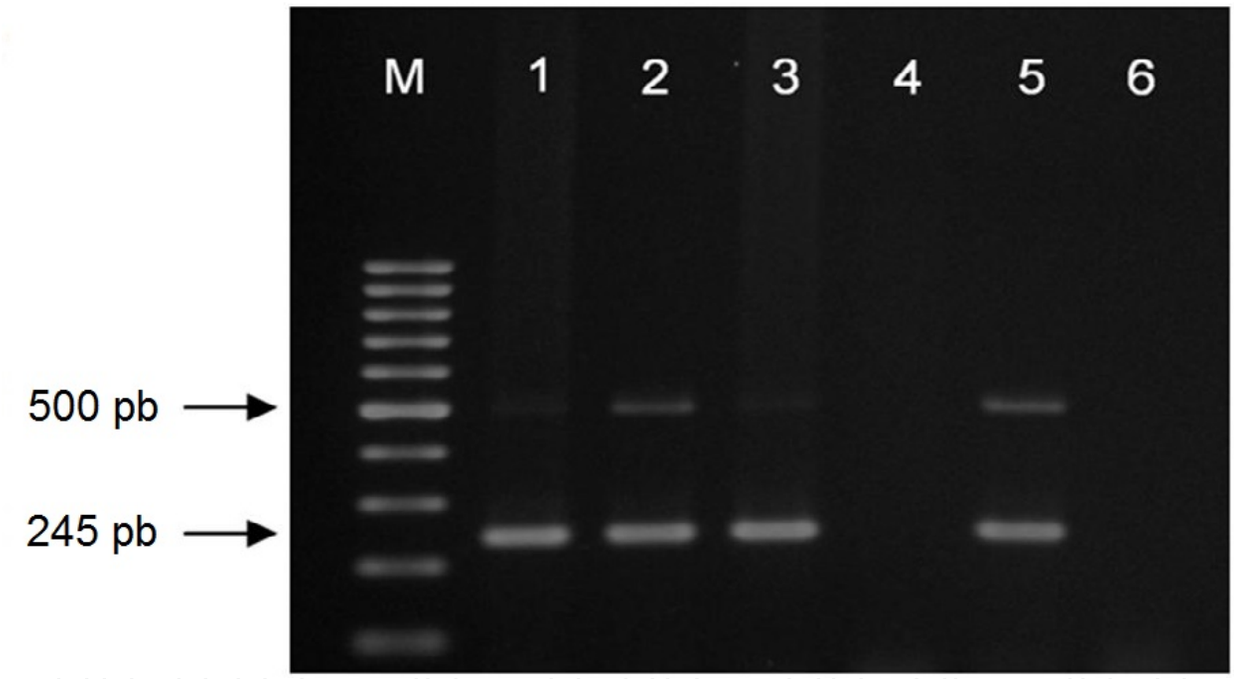


Figure 3. Detection of *M. bovis* directly from fragments of BTB-suspicious lesions using the m-PCR technique. Template DNA extracted directly from the lesions was used to amplify the *RvD1Rv2031c* (500 bp) sequences specific to *M. bovis* and *IS6110* (245 bp) specific to the MTC. Lane M: molecular marker (DNA ladder-100 bp); Lanes 1, 2 and 3: positive reaction to m-PCR, originating from three of the 14 carcasses with BTB-suggestive lesions, inspected in slaughterhouses in the state of Mato Grosso, Brazil.; Lane 4: negative for m-PCR reaction, injury not affected by *M. bovis*; Lane 5: reference strain of *M. bovis* (ATCC 19210), used as positive control reaction; Lane 6: Negative control reaction.

On the revaluation of the macroscopic analyses of the carcasses inspected in the present study, a high incidence of lesions in the pre-scapular and pre-pectoral lymph nodes, of approximately 73.2% (145/198) was observed (Table 2). Despite *M. bovis* having already been detected in these lymph nodes [28, 32], these lesions could be attributed to vaccine reactions (Figure 4A), since the HE histopathological examinations showed that 84.5% (126/145) of the lesions exhibited

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granulomatous reactivity with intralesional vacuoles (Figure 4B), that indicate the presence of mineral oil drained from the vaccine application site (shoulder or neck), to the cervical lymph nodes (pre-scapular and pre-pectoral lymph nodes), triggering an immunostimulatory effect by vaccine adjuvants [73]. This fact becomes more relevant, since mycobacteria were not isolated from these lesions. Thus, macroscopic findings in these nodes should be considered only alongside the presence of BTB-suggestive lesions in several areas of the carcass and/or in animals from herds showing a history of bovine tuberculosis.

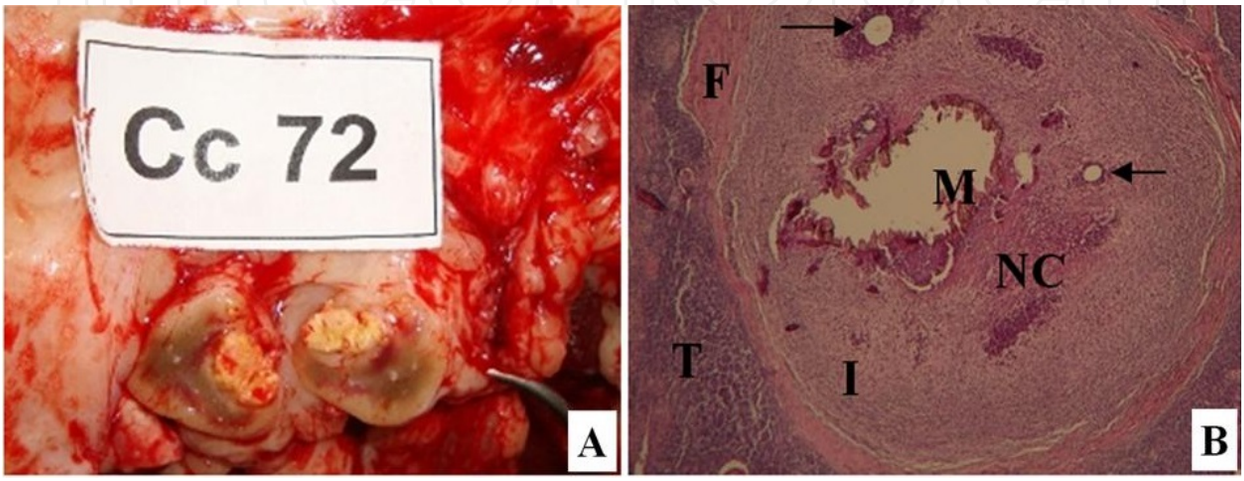


Figure 4. (A). Pre-pectoral lymph node containing a granuloma with a caseous mass of pasty, yellow and calcified consistency surrounded by a capsule of approximately 1 cm of connective tissue; (B). Lesion visualized on a 5X objective during HE histopathological examination, showing a granulomatous reaction characterized by central caseous necrosis (CN) with intense mineralization (M) surrounded by a predominantly mononuclear infiltrate (I), containing occasional intralesional vacuoles (arrow). The lesion is surrounded by fibrous tissue (F) and well-defined when compared to whole tissue (T).

The affected retrofaringeal lymph nodes showed increases in size and number of lesions (Figure 5-A, B and C). However, the lesions were localized (restricted to the retropharyngeal node) with no BTB-suggestive lesions in other areas of the carcasses.



Figure 5. Bovine tuberculosis lesions collected during *post-mortem* examination in slaughterhouses in the state of Mato Grosso, Brazil; (A). retropharyngeal lymph node affected by *M. bovis*, containing purulent exudate; (B-C). retropharyngeal lymph nodes affected by *M. bovis*.

According to these results, it is advisable that *post-mortem* inspections be carried out carefully, especially with regard to the head and thoracic cavity lymph nodes, especially in the retropharyngeal node, since the number of retropharyngeal node samples containing *M. bovis* was higher (Table 2). These results have already been described by other authors, who also found high percentages (22.9 to 49.2%) of BTB lesions in retropharyngeal lymph nodes [28, 74].

	Number and percentage of lesions					
Animal body parts	***Post mortem evaluation***		**Culture**		**m-PCR**	
	N	**%**	**N**	**%**	**N**	**%**
Respiratory apparatus	**29**	**14.6**	**0**	**0**	**4**	**2**
Lung	6	3	0	0	1	0.5
Apical lymph node	4	2	0	0	1	0.5
Esophageal lymph node	7	3.5	0	0	0	0
Mediastinal lymph node	4	2	0	0	0	0
Tracheo-bronchial lymph node	6	3	0	0	2	1
Thoracic cavity	2	1	0	0	0	0
Head	**22**	**11.1**	**3**	**1.5**	**5**	**2.5**
Retropharyngeal lymph node	19	9.5	3	1.5	5	2.5
Parotid lymph node	1	0.5	0	0	0	0
Sublingual lymph node	2	1	0	0	0	0
Carcass	**147**	**74.2**	**0**	**0**	**5**	**2.5**
Pre-pectoral lymph node	108	54.5	0	0	4	2
Pre-scapular lymph node	37	18.6	0	0	1	0.5
Ischiatic lymph node	2	1	0	0	0	0
Total	**198**	**100**	**3**	**1.5**	**14**	**7.0**

Table 2. Diagnosis of bovine tuberculosis in samples with BTB-suspected, collected from inspected and slaughtered cattle at slaughterhouses in the state of Mato Grosso, Brazil.

When comparing macroscopic analyses, bacteriological cultures and m-PCR, the results indicate that the macroscopic analyses correctly identified 93% (184/198) of the samples, considering these lesions as common lymphadenitis (non-tuberculosis) samples. However, *M. bovis* was identified in 7.0% (14/198) of samples that were also considered as non-tuberculosis samples. Despite the mistakes made by SIF inspection when ruling positive BTB lesions as common lymphadenitis, the destination of the carcasses adopted by the inspection service (partial condemnation) was consistent with the standards established in Article 196 of the Regulation for Industrial and Sanitary Inspection Products of Animal Origin-RIISPOA [58], with regard to the presence of BTB-suggestive lesions in only one part or area of the carcass.

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These mistakes in the rulings of BTB-suspected lesions may be due to the occurrence of, mainly, paucibacillary lesions similar to those observed in the present study, since they did not present classic tuberculosis features, probably because the animals displayed recent infection and were slaughtered early (2-3 years old). This factor can distort BTB estimations in slaughterhouses, and, consequently, hinder the success of the PNCEBT program, making it difficult to eradicate the disease in areas such as in Mato Grosso, where the prevalence of BTB is low. In this case, the Federal Inspection plays a very important role in evaluations of the carcasses in slaughterhouses for BTB diagnoses, assisting in the detection of remaining disease foci and in tracing infected herds. This role is confirmed by the results of disease control programs implemented in high prevalence areas [32, 75]. However, as noted above, as the prevalence decreases, the identification of remaining BTB infected livestock becomes increasingly difficult. Although at present there is no diagnosis method (*ante* or *post* mortem) able to identify all animals infected with *M. bovis*, the detection is more efficient when more than one diagnostic method is used [60]. Thus, in areas where the disease prevalence is very low, such as in the state of Mato Grosso, [6, 39], awareness should be raised regarding the increased difficulty of detecting BTB during *post-mortem* inspections. In addition, the use of complementary tests that result in rapid diagnoses should be adopted, such as the m-PCR in this study, which showed the versatility of combining sensitivity and specificity for rapid diagnoses (approximately 2 working days), since this technique can be used to detect *M. bovis* directly from fragments of BTB-suspected lesions and may contribute to the success of the PNCEBT program with regard to tracking remaining BTB foci.

3. Discussion

The state of Mato Grosso has emerged in the Brazilian national scene as the largest beef cattle producer and second largest beef exporter in the country [40], leading to annual increases in the amount of meat exported to EU countries. Consequently, the pressure on Brazil by countries that buy Brazilian products to implant an effective, rapid and definitive diagnosis of BTB in tuberculosis-suspected lesions has also increased.

In 2012, the Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento-MAPA) determined that farms where the detection of BTB cases took place can no longer export beef to the Customs Union of Belarus, Kazakhstan and Russia, recalling all lots from these animal farms until the diagnosis of suspicious lesions in samples collected after slaughter could be conducted at an official MAPA laboratory [41, 42].

In view of these commercial and sanitary restrictions, the difficulties in tuberculosis diagnosis must be overcome. Diagnosis alternatives for the quick and specific identification of BTB in clinical samples or isolated colonies have emerged, such as molecular methods based on PCR and its variants (simplex PCR, *multiplex*-PCR, *nested* PCR, real-time PCR and real-time *nested* PCR) [42, 43-44].

In this context, the purpose of the present study was to evaluate the performance of diagnostic tests, such as m-PCR, culture and histopathology, on the detection of MTC species directly

from suspected BTB lesions. The apparent prevalence of BTB among animals slaughtered in the state of Mato Grosso was also re-evaluated and discussed, due to the great importance of this geographic region in meat production and export to several consumer countries worldwide, including the European Community.

When comparing macroscopic analyses, bacteriological cultures and m-PCR, the results indicate that the macroscopic analyses correctly identified 93% (184/198) of the samples, categorizing these lesions as common lymphadenitis (non-tuberculosis) samples. However, *M. bovis* was identified by m-PCR tests in 7.0% (14/198) of samples previously considered as non-tuberculosis samples.

As a result of the low prevalence status established in this study, BTB in the state of Mato Grosso may advance to the stage of eradication, using strategies such as the implementation of an efficient monitoring system, performed alongside inspection officers and the health defense service. Alongside the application of *post-mortem* inspection routines in slaughterhouses and the use of additional BTB-diagnostic tests, such as molecular methods applied directly to BTB-suggestive lesions, these strategies should be efficient in detecting any remaining BTB foci in this geographic region, contributing to accelerate the process of bovine tuberculosis eradication in the state of Mato Grosso, Brazil.

Currently, there is no diagnosis test (*ante* or *post* mortem) able to identify all animals infected by *M. bovis.* Detection of contaminated animals is more efficient if two or more diagnostic tests are combined [60]. In geographic regions where the disease shows low prevalence, as the observed in the state of Mato Grosso [6, 39], awareness should be raised regarding the augmented difficulty of detecting BTB during *post-mortem* inspections. The use of complementary tests that result in rapid diagnoses should be adopted, such as the m-PCR described in this study, which demonstrated the versatility of both sensitivity and specificity in the rapid detection of *M. bovis* (approximately 2 working days), directly from fragments of BTB-suspected lesions. The guidelines proposed herein may contribute to the success of the PNCEBT program with regard to tracking remaining BTB foci.

4. Conclusions

The results of the present study indicate that mistakes can occur during rulings of suspected bovine tuberculosis lesions in cattle, particularly those presenting paucibacillary lesions. These mistakes cause a distortion in BTB estimates in slaughterhouses, with harmful consequences to the success of the Brazilian Tuberculosis Control Program (PNCEBT). The results point to the use of complementary molecular assays for rapid diagnoses of lesions situated in frequently BTB-affected carcass areas, thus minimizing mistakes in judging the disease in slaughterhouses. m-PCR was the most sensitive, rapid and specific method among the complementary methods tested in the present study when compared to conventional methods for BTB-diagnosis. It is, therefore, a promising alternative in disease surveillance to be used by the federal inspection service to contribute to the bovine tuberculosis control and eradication program, for disease surveillance in slaughterhouses and for tracking remaining BTB foci

in the state of Mato Grosso, as well as in other regions of the country, contributing even further to the success of the PNCEBT program.

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