Bovine tuberculosis due to *Mycobacterium bovis* and other mycobacteria among water buffalo (*Bubalus bubalis*) from the Brazilian Amazon

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**Abstract**

Introduction: Zoonotic tuberculosis is a disease of public health importance worldwide, especially in developing countries. The present study aimed to investigate the role played by *Mycobacterium bovis* and other mycobacteria as etiologic agents of bubaline tuberculosis (TB) in the Brazilian Amazon region.

Methodology: Granulomatous lesions suggestive of TB obtained from 109 buffaloes (n = 109) during sanitary inspection at slaughter were subjected to histopathological evaluation, immunohistochemical (IHC) detection of *Mycobacterium* antigens, and to molecular tests (PCR) to detect *hsp65*, *IS6110* and RD4 genes, which are specific to *Mycobacterium* spp., *Mycobacterium tuberculosis* Complex (MTBC) and *M. bovis*, respectively.

Results: PCR results indicated *Mycobacterium* infection in 87.2% of the cases, of which 69.5% were positive for *M. bovis*, 27.4% belonged to MTBC, and 3.1% were probably non-TB mycobacteria. There was good agreement between the genus-specific molecular technique and the histopathological analysis. This high frequency of TB cases caused by non-*M. bovis* suggests a diversified scenario of mycobacteria associated with bubaline TB in the Brazilian Amazon region.

Conclusions: The results reinforce the need of discussing the inclusion of more accurate techniques in examinations carried out by Inspection Services in Brazil.

**Key words:** Granuloma; mycobacteriosis; non-TB mycobacteria; sanitary inspection; zoonosis.

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**Introduction**

Buffalo breeding is a branch of livestock production of major economic importance in the Brazilian Amazon region, which has the largest herd in Brazil [1,2]. The water buffalo (*Bubalus bubalis*) presents characteristics of rusticity, adaptability to climatic and topographic factors and poor soils, factors that make these animals an excellent alternative for animal protein production in the Amazon region [1,2]. In 2017, the Amapá state, located in the eastern Brazilian Amazon, had an estimated number of 223,893 water buffalo heads created predominantly in extensive farming systems [3]. Water buffalo herd management presents advantages such as the animals’ strong resistance to diseases, however, several studies reported tuberculosis (TB) in buffaloes with a prevalence rate ranging from 3.5% to 8.11% in Brazilian Amazon [4-7].

Bovine TB (bTB) is enzootic in many countries and often caused by *Mycobacterium bovis*, being responsible for major economic losses and public health concerns [8]. Generally, bovine TB is diagnosed through anatomopathological post-mortem evaluation, but considerable limitations are observed, due to granulomatous inflammations presenting TB-like morphological features, including post-vaccination reactions [6-9].
M. bovis is reported as the main TB agent among cattle and buffaloes, nevertheless, routine diagnosis usually does not include identification of other species belonging to the Mycobacterium tuberculosis complex (MTBC) [4-11]. The present study aimed to investigate the main species of Mycobacterium causing tuberculosis among water buffaloes from the Brazilian Amazon, as well as to compare the results obtained through application of immunohistochemical (IHC), anatomopathological and molecular techniques.

Methodology
The study examined 109 tissue samples (n = 109) from water buffalos, which presented presumptive TB diagnosis at macroscopic examination conducted during sanitary inspection at an official slaughterhouse located in Santana, Amapá State, Brazil. The samples comprised of lymph nodes presenting minor changes such as white to yellowish color and increased size and granulomatous lesions with nodular tubercles composed of amorphous mass, full of encapsulated caseous material.

For histopathological analysis, fragments of lesions, approximately 0.5 cm thick were fixed in 10% neutral buffered formalin and processed by standard techniques for inclusion in paraffin and stained with hematoxylin and eosin. Histomorphological evaluation of tuberculoid granuloma was performed according to previously established classification by Wangoo et al. [12].

Histological sections of formalin-fixed paraffin embedded tissues in silanized slides (ImmunoSlide-EasyPath, Indaiatuba, Brazil) were used for immunohistochemistry (IHC) using the streptavidin-biotin method for Mycobacterium spp. antigen detection. The histological sections were incubated with rabbit polyclonal primary anti-M. bovis antibodies (B0124 Dako) (dilution 1:1000), followed by incubation with streptavidin-biotin complex (LSAB) with universal biotinylated secondary antibody and final revelation using diaminobenzidine chromogen (DAB).

Aliquots of samples with lesions suggestive of bTB were kept frozen (-20°C) until extraction of total DNA by the phenol-chloroform purification method with modifications [13]. Nested-PCR to amplify the gene encoding the 65-kDa heat shock protein hsp65, which is specific to the genus Mycobacterium, was performed as previously described [14]. Mycobacterium bovis-specific molecular marker was amplified by PCR according to methodology previously described [15]. The MTBC marker IS6110 was investigated to clarify whether the negative M. bovis cases would test positive for MTBC [16]. M. bovis BCG (Isogen, Bioscience, Maarsen, The Netherlands) and M. tuberculosis H37Rv DNA were used as positive controls.

The difference between the proportions of cases observed according to marker positivity was assessed through Chi-Square test (Adherence), whereas the association between categorical variables (positive or negative cases vs. lesion stage) was estimated through Chi-Square test (or G test, whenever it was necessary), complemented by the Chi-Square Residual Analysis in case of significant association. The agreement between results recorded through molecular analysis and the ones recorded through other techniques was estimated by Kappa (κ) coefficient. Tests were performed in the BioEstat 5.4 software, and results presenting p ≤ 0.05 were considered significant.

Results
One hundred and three (103/109; 94.5%) samples were defined as tuberculous granulomas by histopathological examination, and 97.1% (100/103) of them presented lesions on stage IV according to classification by Wangoo et al. [12], whereas 5.5% (6/109) presented negative results. According to IHC detection, 82.6% (90/109) tested positive for Mycobacterium antigens and 97.8% (88/90) of them presented stage IV lesions. In addition, 63.2% (12/19)
of the negative cases for *Mycobacterium* antigens by IHC presented lesions in the last development stage (IV), whereas 31.5% (06/19) did not present characteristics of tuberculoid granulomas in the microscopic examination.

Ninety-five (87.2%) samples tested positive for *Mycobacterium* genus-specific marker *hsp65* gene. Most of them (66/95, 69.5%; *p* < 0.0001) tested positive exclusively for *M. bovis* specific marker; 27.4% (26/95) amplified the MTBC-specific fragment (but not amplified for *M. bovis*), and 3.1% (03/95) did not belong to the MTBC, thus suggesting the presence of non-tuberculous mycobacteria (NTM) (Figure 1).

Nine (64.3%) of the 14 samples that did not show specific target amplification for the genus *Mycobacterium* (negative *hsp65*) were classified as tuberculoid granuloma in the histopathological examination; all of them presented lesions developed up to stage IV, and 50% (07/14) were positive upon immunostaining for *Mycobacterium* spp. in the IHC (Table 1). The five negative samples for the *hsp65* gene also did not present tuberculoid granuloma in histopathological analysis.

Good agreement between molecular and histopathological assays was observed (*k* = 0.4583, *p* < 0.0001), contrary to results obtained by comparing amplification of the *hsp65* marker with IHC (*k* = 0.3243, *p* = 0.0003) (Table 1).

A significant association was observed between cases presenting lesions at development stage IV and *hsp65* positivity (*p* = 0.0013) (Figure 2). Moreover, significant association between positive results obtained by IHC and the presence of chronic lesions (stage IV), as well as between the absence of immunostaining and cases histologically classified as “no lesions” (*p* < 0.0001) (Figure 3).

**Discussion**

The results obtained in the present and several other studies confirm the need for more accurate methods in examinations performed by inspection services [6,7,9,10,17]. The anatomopathological diagnosis conducted in slaughterhouses during post-mortem inspections presents several limitations since it does not confirm the presence of the agent; moreover, many granulomatous inflammatory processes are common to other comorbidities and agents other than *M. bovis* [6,7,8,12]. This limitation may lead to inaccurate diagnosis and carcass condemnation in slaughterhouses since the current legislation recommends condemning carcasses.

The present study considered at least one of the three conditions suggested by França *et al.* [18] for

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Table 1. Analysis of agreement between results according to molecular, histopathological and immunohistochemical techniques applied to detect mycobacteria in lesion suggesting tuberculous infection among water buffalo, Amapá, Brazil, 2014-2015.

| Technique               | Molecular identification for genus *Mycobacterium* (hsp65) | Agreement (k) (interpretation) | *p* - value* |
|-------------------------|-------------------------------------------------------------|-------------------------------|--------------|
|                         | Positive n (%) | Negative n (%) |                             |              |
| **Histopathological**   | 94 (99.0)      | 9 (64.3)       | 0.4583 (good)               | < 0.0001†    |
| Positive                | 1 (1.0)        | 5 (35.7)       |                             |              |
| Negative                |                |                |                             |              |
| **Immunohistochemical** | 83 (87.4)      | 7 (50)         | 0.3243 (poor)               | 0.0003†      |
| Positive                | 12 (12.6)      | 7 (50)         |                             |              |
| Negative                |                |                |                             |              |

Source: research protocol. *:* Kappa test; †: Statistically significant.

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*Figure 2.* Distribution of samples presenting macroscopic lesions suggesting tuberculosis based on the positivity for the molecular marker to genus *Mycobacterium* and on the presence of lesions at different development stages in the histopathological examination, Amapá, Brazil, 2014-2015.

*hsp65*: heat shock protein 65kDa; G-Test / Chi-Square Residual Analysis *p* = 0.0013; †Statistically significant.
tuberculoid granulomas: (I) the presence of caseous necrosis, with or without calcification, and chronic inflammation with Langhans cells; (II) the presence of necrosis, calcification and chronic inflammation; or (III) the presence of giant cells. The histopathological evaluation may be useful for the recognition of necrotizing or non-necrotizing granulomas; however, histopathological evaluation isolated from tuberculous granulomas does not allow the differentiation of mycobacterial species [19].

The difficulty in obtaining a more accurate diagnosis can be attributed to several complications in identifying the bTB agent. Immunohistochemical techniques may be used as an efficient diagnostic complement by Ziehl-Neelsen (ZN) staining in the diagnosis of tuberculoid granulomas since it is a simple, sensitive, and specific technique [20]. However, the immunostaining technique only identifies particulate antigenic material, mainly between epithelioid cells in the cytoplasm of macrophages and between necrotic material [21].

The MTBC presents microorganisms with great genetic homology. The genome of M. bovis is 99.95% similar to that of M. tuberculosis; the irreversible sequential genomic deletions (region of difference-RD) in the DNA are the main contributors to these differences [22,23]. The pattern of the presence or absence of these RDs in the genome of MTBC members provides a molecular signature that allows discrimination, as previously confirmed [24].

The M. bovis-specific markers used in the study flank the RD4 region, which is absent in M. bovis and in M. bovis BCG vaccine stains [25]. It enables the formation of a 400 bp amplicon, which is confirmed in in-silico tests [15]. Bahkshi et al. [26] analyzed the RD4 region and concluded that all M. bovis samples presented deletion. In the present study we used a specific marker for M. bovis, and the PCR for the IS6110 was used for research of M. bovis and MTBC co-infections which was not observed.

Ten samples presenting microscopic lesions compatible with TB and negative results for M. bovis and the IS6110 target, showed hsp65 gene amplification, suggesting the presence of NTM. NTM have been identified among mycobacterial cultures previously described as M. bovis [27] and in raw milk samples from buffaloes [28]. In this sense, although we have not determined the NTM species in the present study, additional studies for the characterization of NTM are necessary for a better understanding of the epidemiology and differential diagnosis of bubaline TB in the Amazon, since NTM are found in soil and water and infections by these agents may have several anatomopathological presentations, ranging from histiocytic responses to non-necrotizing or necrotizing granulomas [19].

Seven out of the ten samples that tested negative for M. bovis and positive for the hsp65 gene were positive for IS6110. The IS6110 is a 1350 bp genetic element found in different copy numbers and it is integrated to several genome sites of MTBC species. It is often possible to find 4 to 20 IS6110 in M. tuberculosis, whereas M. bovis usually 0 - 5 copies of the insertion sequence IS6110 [16,29]. However, the specificity of amplification techniques applied to IS6110 has been questioned because similar or identical elements have been described in NTM or even in species belonging to other genera [30].

Further studies should be conducted for NTM confirmation, to thoroughly investigate whether the presence of NTM in the biopsies is sufficient to define it as bubaline TB agent, as well as to assess its impact on human and animal infection cases.

**Conclusions**

We observed that M. bovis was the main causative agent of bubaline TB among the evaluated cases, however the occurrence of a high frequency of cases
caused by non-*M. bovis* points to the need for additional epidemiological investigations for a better understanding of bubaline TB in the Brazilian Amazon.

There was good agreement between histopathological analysis and molecular data results concerning the determination of the genus *Mycobacterium*; however, the results generated in this study reinforce the need for the discussion of the inclusion of more accurate techniques in examinations carried out by Inspection Services in Brazil, and also warn for the risk of occupational exposure to mycobacteria in slaughterhouses in the Amazon region.

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