JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Brief Original Article

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

Silvia Cristina da Silva Pedroso[1], Karla Valéria Batista Lima[2,3], Ismari Perini Furlaneto[3,4], Yan Corrêa Rodrigues[3], Darlene Kássia Saraiva Queiroz Pantoja[5], Alex Junior Souza de Souza[6], Washington Luiz Assunção Pereira[7]

[1] *Post-graduate Program in Animal Science, Federal University of Pará, Belém, Brazil*
[2] *Bacteriology and Mycology Section, Evandro Chagas Institute, Ananindeua, Brazil*
[3] *Post-graduate Program in Parasitic Biology in the Amazon, State University of Pará, Belém, Brazil*
[4] *Post-graduate Program in Health Education - Medical Education, University Center of Pará, Belém, Brazil*
[5] *Post-graduate Program in Animal Health and Production in the Amazon, Federal Rural University of Amazonia, Belém, Brazil*
[6] *Max Planck University Center, Indaiatuba, Brazil*
[7] *Institute of Animal Health and Production, Federal Rural University of Amazonia, Belém, Brazil*

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 *hsp*65, *IS*6110 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.

J Infect Dev Ctries 2021; 15(5):736-741. doi:10.3855/jidc.13558

(Received 27 July 2020 – Accepted 13 October 2020)

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.

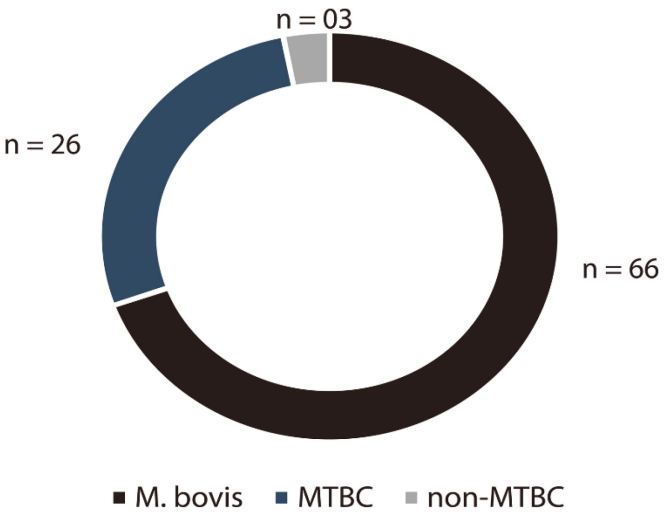
For histopathological analysis, fragments of lesions, approximately 0.5 cm thick were fixed in 10% neutral buffered formalin and processed by standard techniques for inclusion in paraffin and stained with hematoxylin and eosin. Histomorphological evaluation of tuberculoid granuloma was performed according 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 (-20ºC) 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 *hsp*65, 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 *IS*6110 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 (*k*) coefficient. Tests were performed in the BioEstat 5.4 software, and results presenting $p \leq 0.05$ were considered significant.

Figure 1. Distribution of samples presenting macroscopic lesions suggesting tuberculosis, according to positivity for molecular markers to *M. bovis, M. tuberculosis* Complex (negative for *M. bovis*) and non-tuberculous mycobacteria (NTM). Amapá, Brazil 2014-2015.



MTBC: *M. tuberculosis* Complex; $p < 0.0001$ (*Chi-square test* for Adherence); †Statistically 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.

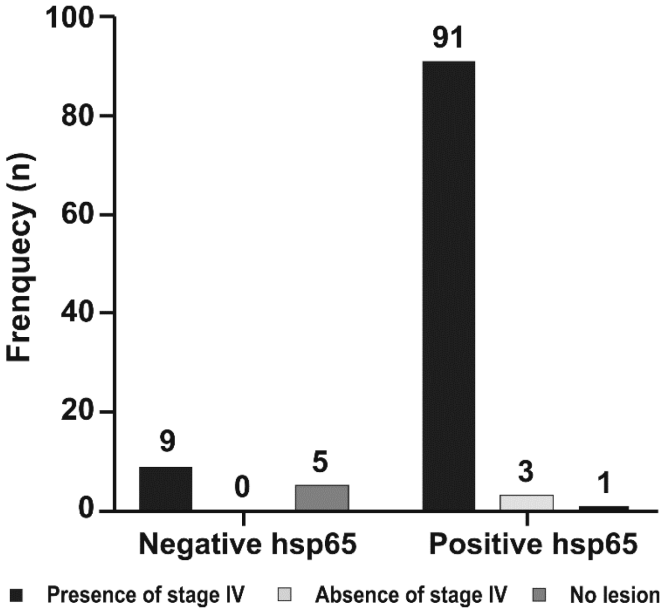
Ninety-five (87.2%) samples tested positive for *Mycobacterium* genus-specific marker *hsp65* gene. Most of them (66/95, 69.5%; $p < 0.0001$) tested positive exclusively for *M. bovis* specific marker; 27.4% (26/95) amplified the MTBC-specific fragment (but not amplified for *M. bovis*), and 3.1% (03/95) 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 *hsp*65) 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 *hsp*65 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 *hsp*65 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 *hsp*65 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).

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.



*hsp*65: *heat shock protein* 65kDa; G-Test / Chi-Square Residual Analysis $p = 0.0013$; †Statistically significant.

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

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* (*hsp*65)		Agreement (*k*) (interpretation)	*p* - value*
	Positive n (%)	Negative n (%)		
Histopathological				
Positive	94 (99.0)	9 (64.3)	0.4583 (good)	< 0.0001†
Negative	1 (1.0)	5 (35.7)		
Immunohistochemical				
Positive	83 (87.4)	7 (50)	0.3243 (poor)	0.0003†
Negative	12 (12.6)	7 (50)		

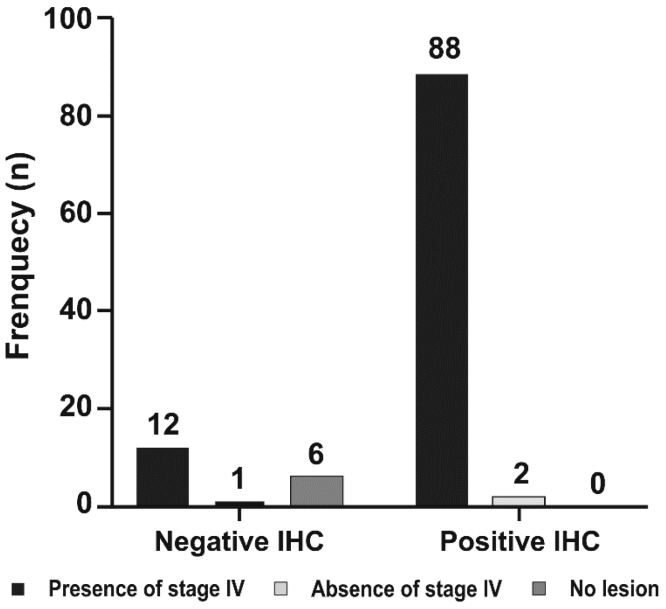
Source: research protocol. *: Kappa test; †: Statistically significant.

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].

Figure 3. Distribution of samples presenting macroscopic lesions suggesting tuberculosis based on the positivity of immunostaining for mycobacteria and on the presence of lesions at different development stages in the histopathological examination, Amapá, Brazil, 2014-2015.



IHC: immunohistochemistry; $p < 0.0001$ (G-Test / Chi-square residual analysis); †Statistically significant.

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]. Bahksi *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 *IS*6110 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 *IS*6110 target, showed *hsp*65 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 *hsp*65 gene were positive for *IS*6110. The *IS*6110 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 *IS*6110 in *M. tuberculosis,* whereas *M. bovis* usually 0 - 5 copies of the insertion sequence *IS*6110 [16,29]. However, the specificity of amplification techniques applied to *IS*6110 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.

Acknowledgements

This work was supported by the Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ministério da Saúde. Fundação de Amparo à Pesquisa do Pará/Universidade do Estado do Pará (FAPESPA/UEPA) [Cooperation grant Nº 004/2019].

References

1. El Debaky HA, Kutchy NA, Ul-Husna A, Indriastuti R, Akhter S, Purwantara B, Memili E (2019) Review: potential of water buffalo in world agriculture: challenges and opportunities. Appl Anim Sci 35: 255–268.
2. Minharro S, Alves CM, Mota PMPC, Dorneles EMS, Alencar AP, dos Santos RM, Leite BM, Lage AP (2016) Tuberculosis in water buffalo (*Bubalus bubalis*) in the Baixo Araguari region, Amapá, Brazil. Semin Ciênc Agrá 37: 885-890.
3. Instituto Brasileiro de Geografia e Estatística (IBGE) Censo agropecuário 2017, Amapá. Available: https://cidades.ibge.gov.br/brasil/ap/pesquisa/24/76693 Accessed 4 July 2020.
4. Ribeiro DLS, Santos LS, Cardoso DL, Souza OAF, Sousa KLM, Madeira RL, Santos HP, Melo FA (2011) Serological diagnosis of *Mycobacterium bovis* in buffaloes (*Bubalus bubalis*) in the region of Viana, Maranhão, Brazil. Vet Zootec 18: 839-841. [Article in Portuguese]
5. Barbosa JD, Da Silva JB, Rangel CP, Da Fonseca AH, Silva NS, Bomjardim HA, Freitas NF (2014) Tuberculosis prevalence and risk factors for water buffalo in Pará, Brazil. Trop Anim Health Pro 46: 513-517.
6. Albernaz TT, Oliveira CMC, da Silva Lima DH, Silva NDS, Cardoso DP, Lopes CTA, Brito MF, da Silva JB, Salvarani FM, Leite RC, Barbosa JD (2015) Comparison of the tuberculin test, histopathological examination, and bacterial culture for the diagnosis of tuberculosis (*Mycobacterium bovis*) in buffaloes (*Bubalus bubalis*) in Brazil. Trop Anim Health Pro 47: 1153-1159.
7. Pereira JDB, Cerqueira VD, Bezerra Junior PS, Bezerra DKO, Araújo FR, Dias ADCL, Araújo CP, Riet-Correa G (2017) Histopathological and molecular diagnosis of lesions suggestive of tuberculosis in buffaloes slaughtered in the municipalities of Macapá and Santana, Amapá state, Brazil. Pesq Vet Bras 37: 1198-1204. [Article in Portuguese]
8. Caswell JL, Williams KJ (2016) Respiratory system. In: Grant Maxie M, editor. Jubb, Kennedy, and Palmer's pathology of domestic animals. vol. 2., 6th edit. Missouri: Elsevier. 465-591.
9. Furlanetto LV, Figueiredo EE, Conte Júnior CA, Carvalho RC, Silva FG, Silva JT, Lilenbaum W, Paschoalin VM (2012) Use of complementary methods in post mortem inspection of carcasses with suspected bovine tuberculosis. Pesq Vet Bras 32: 1138-1144. [Article in Portuguese]
10. Alzamora Filho F, Vasconcellos SEG, Gomes HM, Cavalcante MP, Suffys PN, Costa JN (2014) Multiple strains of *Mycobacterium bovis* isolates identified by molecular typing of bovine animals slaughtered in slaughterhouse. Pesq Vet Bras 34: 103-108. [Article in Portuguese]
11. Conceição ML, Conceição EC, Furlaneto IP, da Silva SP, dos Santos Guimarães AE, Gomes P, Boschiroli ML, Michelet L, Kohl TA, Kranzer K, Francez LC, Lima LNGC, Portugal I, Perdigão J, Lima KVB (2020) Phylogenomic perspective on a unique *Mycobacterium bovis* clade dominating bovine tuberculosis infections among cattle and buffalos in Northern Brazil. Sci Rep 10: 1-13.
12. Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, Spencer Y, Hewinson G, Vordermeier M (2005) Advanced granulomatous lesions in *Mycobacterium bovis* - infected cattle are associated with increased expression of type I procollagen, gd (WC1+) T cells and CD 68+ cells. J Comp Pathol 133: 223-234.
13. Furlaneto IP, Sousa EB, Brito ML, Lima GLF, Lopes ML, Da Silva SHM, Lima KVB (2007) Evaluation of different methods of DNA extraction from baciloscopic tests of Ziehl-Neelsen staining. Cad Saude Colet 15: 401-414. [Article in Portuguese]
14. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 31: 175-178.
15. Sales ML, Fonseca Jr AA, Sales EB, Cottorello ACP, Issa MA, Hodon MA, Soares Filho PM, Ramalho AK, Silva MR, Lage AP, Heinemann MB (2014) Evaluation of molecular markers for the diagnosis of *Mycobacterium Bovis*. Folia Microbiol (Praha) 59: 433-438.
16. Thierry D, Brisson-Noël A, Vincent-Lévy-Frébault V, Nguyen S, Guesdon JL, Gicquel B (1990) Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. J Clin Microbiol 28: 2668-2673.
17. Fráguas SA, Cunha-Abreu MS, Ferreira AMR, Marassi CD, Oelemann W, Fonseca LS, Ferreira R, Lilenbaum W (2008) Comparative study of complementary diagnostic methods of bovine tuberculosis on skin-test reactive cattle. R Bras Ci Vet 15: 117-121. [Article in Portuguese]
18. França LR, Cruz JF, Neves VBF, Cerqueira RB (2013) Prevalence and histopathology of lesions suggestive of tuberculosis in cattle carcass slaughtered in Southwestern Bahia. Rev Bras Saúde Prod Anim 14: 721-733.
19. Shah KK, Pritt BS, Alexander MP (2017) Histopathologic review of granulomatous inflammation. J Clin Tuberc Other Mycobact Dis 7: 1-12.
20. Kohli R, Punia RS, Kaushik R, Kundu R, Mohan H (2014) Relative value of immunohistochemistry in detection of mycobacterial antigen in suspected cases of tuberculosis in tissue sections. Indian J Pathol Microbiol 57: 574-578.
21. Duarte MIS, Pagliari C (1999) Infectious diseases. In Alves VAF, Bacchi CE, Vassallo J, editors. Immunohistochemistry manual. Sao Paulo: Brazilian Society of Pathology. 195-207. [Book in Portuguese]

22. Fleischmann RD, Alland D, Eisen JA, Carpenter L, White O, Peterson J, DeBoy R, Dodson R, Gwinn M, Haft D, Hickey E, Kolonay JF, Nelson WC, Umayam LA, Ermolaeva M, Salzberg SL, Delcher A, Utterback T, Weidman J, Khouri H, Gill J, Mikula A, Bishai W, Jacobs Jr. WR, Venter JC, Fraser CM (2002) Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. J Bacteriol 184: 5479-5490.
23. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, Simon S, Harris B, Atkin R, Doggett J, Mayes R, Keating L, Wheeler PR, Parkhill J, Barrell BG, Cole ST, Gordon SV, Hewinson RG (2003). The complete genome sequence of *Mycobacterium bovis*. Proc Natl Acad Sci USA 100: 7877-7882.
24. Huard R, Fabre M, Haas P, Lazzarini LCO, van Soolingen D, Cousins D, Ho JL (2006) Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. J Bacteriol 188: 4271-4287.
25. Smith N, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, van Soolingen D, Hewinson RG, Smith JM (2006) Ecotypes of the *Mycobacterium tuberculosis* complex. J Theor Biol 239: 220-225.
26. Bakshi C, Shah DH, Verma R, Singh RK, Malik M (2005) Rapid differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7-kb fragment by a single tube multiplex-PCR. Vet Microbiol 109: 211-216.
27. Figueiredo EES, Silvestre FG, Campos WN, Furlanetto LV, Medeiros L, Lilenbaum W, Fonseca LS, Silva JT, Paschoalin VMF (2009) Identification of *Mycobacterium bovis* isolates by a multiplex PCR. Braz J Microbiol 40: 231-233.
28. Jordão Junior CM, Lopes FC, David S, Farache Filho A, Leite CQ (2009). Detection of nontuberculous mycobacteria from water buffalo raw milk in Brazil. Food Microbiol 26: 658-661.
29. Van Soolingen D (2001) Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med 249: 1-26.
30. Santos RMC, Ogusku MM, Miranda JM, Dos Santos MC, Salem JI (2006) Evaluation of polymerase chain reaction in the diagnosis of pulmonary tuberculosis in indigenous and non-indigenous patients. J Bras Pneumol 32: 234-240.

Corresponding author

Alex Junior Souza de Souza, VMD, MSc, PhD
Max Planck University Center, Rod. João Ceccon, Km 4, Jd. Altos do Bela Vista, 13.331-400, Indaiatuba, São Paulo, Brazil.
Phone: +55 (19) 3885-9902
Fax: +55 (19) 3885-9902
Email: souzajralex@gmail.com

Conflict of interests: No conflict of interests is declared.