Histopathological and molecular methods as complementary diagnostic in case of lymphadenopathies suggestive of bovine tuberculosis

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Bovine tuberculosis,
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Summary
Bovine tuberculosis (TB) is a chronically evolving zoonotic infectious disease caused by *Mycobacterium bovis*. Anatomopathological examination during *post mortem* inspection in bovines is the main resource engaged in sanitary slaughter; however, it is very troublesome since many granulomatous inflammatory processes have similar morphological characteristics. Thus, this study aims to use complementary diagnosis methods (histopathological and polymerase chain reaction – PCR assays) to confirm the macroscopic assessment of lymphadenopathies indicative of tuberculosis in bovines slaughtered in a refrigerated slaughterhouse in Tailândia city, PA, Brazil. Fifty-one samples were collected from lesions indicative of tuberculosis in pre-scapular and pre-pectoral lymph nodes (or different lymphadenitis) in condemned carcasses. Histological processing employed routine techniques carried out at the Laboratory of Animal Pathology of the Federal Rural University of the Amazon, while the PCR assay was performed at the Bacteriology Laboratory of the Evandro Chagas Institute. Results showed that 1.96% of the histopathology samples corresponded to inflammatory processes typical of TB and that, in PCR, 4.25% of the samples had the amplification profile of the *M. bovis* species. These results indicate the importance of adding complementary methods to assist the sanitary inspection line and make inspection more efficient in its decisions.

Introduction

Bovine tuberculosis (TB) is an infectious disease with chronic evolution and zoonotic character (Radostits *et al.*, 2006). It is caused by *Mycobacterium bovis* but other *Mycobacterium* species can infect cattle. The classic gross lesion is the "tubercle", consisting in well-demarcated, and often encapsulated, foci of granulomatous inflammation with central caseous necrosis and / or mineralization (Jubb and Palmer, 2016).

Despite being present worldwide, some countries have managed to reduce TB frequency through control programs (Wadhwa and Mahajan, 2006), although it is still prevalent in Africa, Asia and Americas (Renwick *et al.*, 2007). The disease has a major economic impact as it results in direct losses because of animal death, reduced weight gain, decreased milk production, early disposal of high zootechnical value animals and carcasses and organs condemnation in slaughter (Pacheco *et al.*, 2009). The Ministry of Agriculture, Livestock and Food Supply (MAPA), in 2001, established the National Program for the Control and Eradication of Animal Brucellosis and

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Tuberculosis, to combat these diseases, reducing their incidence and prevalence through compulsory sanitary measures and also, adherence actions, such as certification and monitoring (Brasil, 2006).

TB transmission occurs through inhalation of infectious aerosols released from animals by coughing or sneezing, and infected dust particles. Infection spreads more rapidly in intensive animal farming (Cousins *et al.*, 2004; Sabedot *et al.*, 2009). In humans, it is considered an occupational disease. Apart from this risk group, people may be contaminated by the consumption of raw or undercooked meat, milk and contaminated derivatives (Veronesi and Focaccia, 2004).

As *in vivo* diagnostic methods, recommended by the *Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal* (PNCEBT), clinical examination has a relative value, since symptoms occur in advanced stage of the disease. Another test employed in the field is the intradermal tuberculin (IDT) test. The test evaluates a delayed-type hypersensitivity reaction in previously sensitized animals after intradermal injection of purified protein derivate (PPD) tuberculin from mycobacteria (De la Rua-Domenech *et al.*, 2006). It is a non-immediate cutaneous hypersensitivity testing that may reveal incipient infections from 3 to 8 weeks after exposure to Mycobacterium so that positive animals can be eliminated. IDT test is considered as a reference technique by the WOAH (World Organization for Animal Health), because of its sensitivity, simplicity and practicality (Brasil, 2006; Ruggiero *et al.*, 2007). Direct diagnostic methods, based on visualization or isolation of the etiologic agent from lesions of diseased animals, are reliable but not feasible in routine due to the difficulty in obtaining samples by bronchoalveolar lavage of cattle (Lilenbaum, 2000).

Despite the wide availability of tests for the identification of *M. bovis* infection at herd level, diagnosis of TB is often difficult due to the scarcity of diagnostic tests that fulfills all the essential criteria necessary for the identification of infected animals. Noteworthy, *post mortem* inspection allows for confirmation of TB in herd test reactors but often provides additional data on infected animals that did not react in field tests (Abbate *et al.*, 2020). The importance of anatomo-pathological examination in *post mortem* inspection in cattle is emphasized. According to the rules of the industrial and sanitary inspection of animal products - RIISPOA (Brasil, 2006), it is the main resource used in sanitary inspection, in slaughterhouses (Corner *et al.*, 1990). However, it presents great difficulty, as many granulomatous inflammatory processes have morphological characteristics similar to those of tuberculosis. In TB, lesions generally constitute nodules of 1 to 3 cm in diameter or more, purulent or caseous (with a fibrous capsule, which may be caseified or calcified) (Brasil, 2006; Corner, 1995; Reis *et al.*, 1995).

Among the direct diagnostic methods, PCR demonstrates efficacy in detecting *M. bovis* in samples of lesions suggestive of TB by detecting a genus-specific DNA fragment or *M. tuberculosis* complex (Brasil, 2006; Furlanetto, Figueiredo, Conte Junior, *et al.*, 2012). Thus, this study aims to use complementary diagnostic methods (histopathology and PCR) to confirm the macroscopic judgment of lymphadenopathy suggestive of tuberculosis in cattle slaughtered in Tailândia, Pará, Brasil, and to offer comparative support for future diagnoses.

Materials and methods

Fifty-one samples of lesions located in prescapular and prepectoral lymph nodes suggestive of tuberculosis or miscellaneous lymphadenitis were collected from condemned carcasses, only by visual examination and without tuberculization test. This procedure was conducted in the slaughterhouse under municipal inspection, between February and July 2014, in Tailândia, Pará. The slaughtered animals were from Tailândia municipality and neighboring municipalities, and they were males and females, most aged over 36 months.

The samples, collected in duplicates, were fragments of lesions, about 0.5 cm of diameter from the areas with the most evident alterations. They were packed in sterile and identified plastic vials. For the histopathological analysis, the samples were put in 10% buffered formalin (100 ml 37% formaldehyde, 900 ml distilled water, 4 g sodium chloride and 3.6 g sodium hydroxide), while for the PCR analyzes they were immersed in physiological solution (10%)and then chilled.

The histological processing adopted the routine techniques, with dehydration, diaphanization, impregnation and inclusion in paraffin, with cuts and routine staining by Hematoxylin-Eosin (HE). The technical procedures for histopathological processing were performed at the Animal Pathology Laboratory (Laboratório de Patologia Animal -LABOPAT) of the Institute of Health and Animal Production (Instituto da Saúde e Produção Animal - ISPA), at the Federal Rural University of Amazonia (Universidade Federal Rural da Amazônia - UFRA).

Mycobacterial DNA was extracted directly from the lymph node, treated with proteinase K and purified by the phenol-chloroform method (Sambrook and Russell, 2001). The biopsy was macerated and 150 µl homogenization buffer (1M tris-HCL, pH 8.0; 1M NaCl; 0.5 M EDTA, pH 8.0; sucrose), 150 µl lysis buffer (1M tris-HCL, pH 9.0, 10mM EDTA, pH 8.0, 20% sucrose, 10% SDS) and 10 µl proteinase K (10 mg / ml)

were added to it. This mixture was incubated at 56 °C for 12 hours. At environmental temperature, 300 µl buffered phenol (PA) was added, then homogenized for 10 minutes and centrifuged at 13,000 rpm /10 minutes. The extraction was repeated with 300 µl chloroform, followed by 300 µl chloroform-isoamyl alcohol (24:1), interspersed by homogenizing step for 10 minutes and then centrifugation (13,000 rpm / 10 minutes).

The supernatant was transferred to another tube and 100 µl sodium acetate (3 M, pH 4.8) and 500 µl isopropanol were added for DNA precipitation. The DNA was washed with 500 µl of 70% ethanol, dried at 37 °C and resuspended in ultrapure water. PCR reactions for each target sequence (RvD1-Rv2031C) were performed in 25 µl volume with 2.5 mM $MgCl_2$, 0.12 mM each deoxynucleotide, 10 pg from each primer (JB21 - TCG TCC GCT GAT GCA AGT GC, JB22 - CGT CCG CTG ACC TCA AGA AG), Q® solution, 1X *Taq* buffer, 0.5 U *Taq* DNA polymerase enzyme (Qiagen, The Netherlands) and 20 to 100 ng of target DNA (Rodriguez *et al.*, 1995).

Thermocycling conditions (Veriti®, Applied Biosystems) covered an initial stage of 95 °C/10 minutes, followed by 40 cycles of 94 °C /1 minute, 64 °C /1 minute, 72 °C /1 minute, and a final stage of 72 ° C/7 minutes.

The amplified products were submitted to 1% agarose gel electrophoresis, stained with SYBRsafe (Invitrogen) and visualized in ultraviolet light transilluminator. PCR analyzes were performed at the Bacteriology Laboratory located in the Evandro Chagas Institute (Instituto Evandro Chagas - IEC), Ananindeua, Pará, Brasil.

Results

Histological examination of lymph nodes revealed that in 75% of them, lesions were related to fat-soluble injectables and probable contamination.

They presented microscopic characteristics of multiple areas with tissue reaction characterized by infiltration of macrophages, some foamy and mainly polymorphonuclear inflammatory cells, such as granulocyte neutrophils.

This change has the particularity of displaying the center with large bubble (negative image), devoid of content (Figure 1A, 1B and 1C). 1.96% of the samples revealed under microscopy a tuberculosis-like inflammatory processe, not shown in the original tissue.

The samples had multiple and extensive areas of caseous necrosis, with mineralization on the margins of this macrophage cell reaction. Most of them were foamy and epithelioid, with presence of giant cells, peculiar to the tuberculoid process.

Through PCR, *Mycobacterium bovis* was identified in 2/47 (4.25%) samples, (Figure 2), while the other samples did not present amplifications that characterize this species.

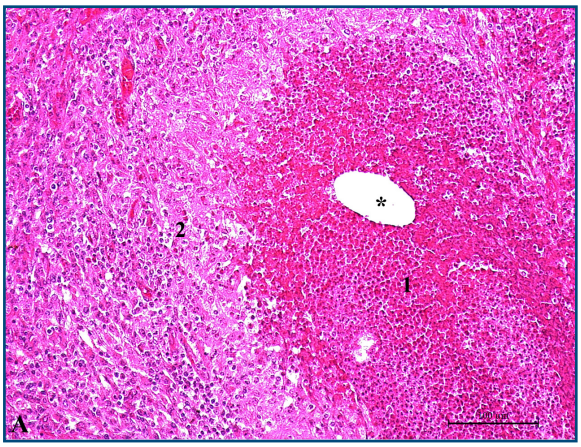

Figure 1A. *Lymph node; in the center, negative image of lipoid substance (*). Detail of a pyogranuloma with accumulation of pyocytes (1); surrounded by macrophagepithelioid cells (2). HE. Bar = 100µm.*

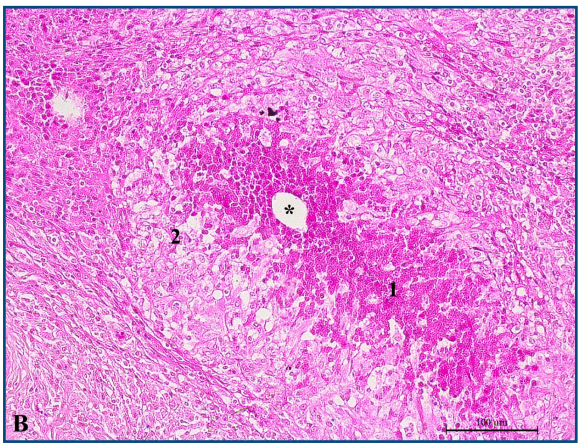

Figure 1B. *Lymph node; in the center, negative image of lipoid substance (*). Detail of a pyogranuloma with accumulation of pyocytes (1); surrounded by macrophagepithelioid cells (2). HE. Bar = 100µm.*

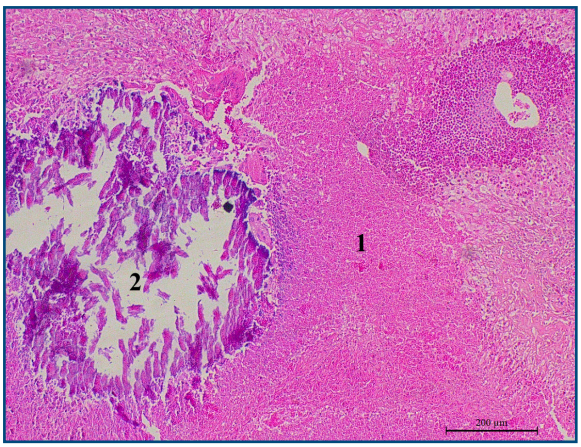

Figure 1C. *Detail of a pyogranuloma with accumulation of pyocytes (1); surrounded by macrophagepithelioid cells (2). HE. Bar = 100µm.*

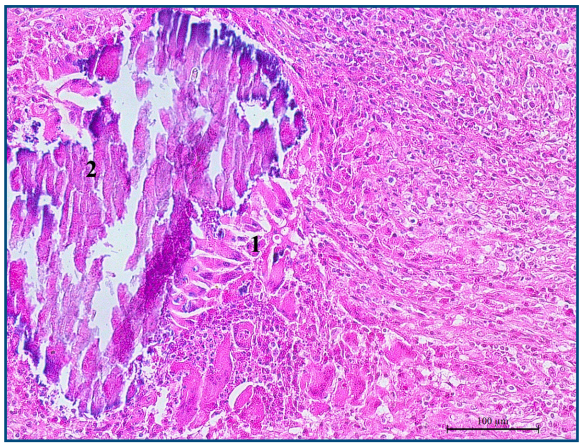

Figure 1D. *Detail of a pyogranuloma with accumulation of pyocytes (1); surrounded by macrophagepithelioid cells (2). HE. Bar = 100µm.*

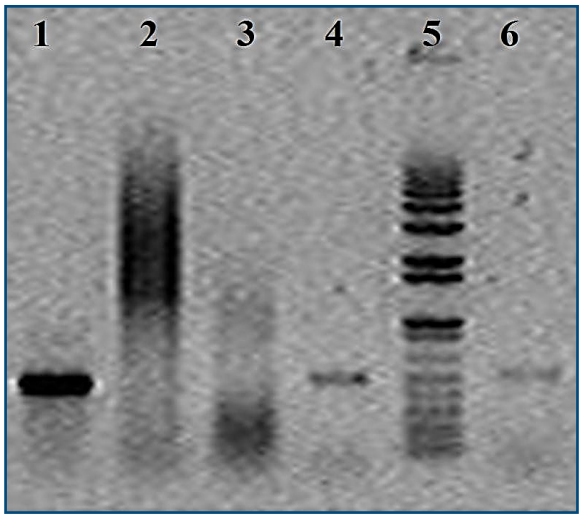

Figure 2. *PCR amplification profile of M. bovis from isolates of bovine lymph node lesions.*

Discussion

Leal *et al*. (2014) in histopathological analysis and bacterial culture of lesions collected at the slaughter of lateral neck muscles of cattle, found that 100% of cases were by vaccine reaction. The findings from this study are related to the application of vaccines and drugs to the neck and palette. The characteristics of oily adjuvants when drained to regional lymph nodes can cause macroscopically similar lesions to TB (França *et al*., 2013; Tizard, 2002).

The observed tuberculosis-like inflammatory processes were similar to those observed by Mendes *et al*., (2013), in 90.16%, Souza (2013), in 100%, and França *et al*. (2013), in 60.66%, of their samples. In the remaining samples (23.04%), the tissue showed neither inflammatory process nor lymphoproliferation. The detection of *M. bovis* DNA, similar to this research, was also found by Furlanetto *et al.* (2012a), who identified *M. bovis* DNA in 7%, and Araújo *et al.* (2005) in 13% of the analyzed samples. On the other hand, Souza (2013) observed 63.3% positivity, and Alzamora Filho *et al.* (2014) identified 56% of *M. bovis* by PCR in isolates of lymph node lesions located in the head and neck, as well as in the lung and thorax, liver and peritoneum. Bermudez *et al.* (2010) analyzed 298 samples from a slaughterhouse in Baja, California, and reported that 53 of them were PCR positive. Costa *et al.* (2010) analyzed by the spolygotyping molecular technique samples of 43 carcasses condemned for tuberculosis or pulmonary alterations of macroscopic diagnosis and seven of them were identified as *M. bovis*. Through the same molecular method, Muller *et al.* (2008) reported 33.33% in slaughtered animals in Sahel, Africa. In the present study, the TB positive results were found in different samples using different methods. Costa (2008) observed 11.62% positivity for TB in histopathological, microbiological and molecular methods.

The impossibility of identifying the agent in typical mycobacterial lesions may occur due to the presence of scarce number of bacteria in the sample and difficulties inherent to isolation, due to the high degree of contamination in the collected material. This fact may result in non-visualization of *M. bovis*, which does not mean its absence in the lesions (Fráguas *et al.*, 2008). The idea of including more accurate techniques for macroscopic examination to avoid misdiagnosis and condemnation in slaughterhouses is reported in the literature (Alzamora Filho *et al.*, 2014; Bermudez *et al.*, 2010; Fráguas *et al.*, 2008; Furlanetto, Figueiredo, Conte Júnior, *et al.*, 2012). In the post mortem inspection, the anatomopathological diagnosis in refrigerators presents great difficulty, since several granulomatous inflammatory processes, as well as some neoplastic ones, have morphological characteristics similar to those described for tuberculosis. This fact results in the condemnation of carcasses and organs by TB, without presenting microscopic characteristics compatible with such pathology, since the current legislation recommends the condemnation of carcasses or part of them, when lesions are suggestive of tuberculosis.

The histopathological diagnosis identified lesions characteristic of the tuberculoid process in 1.96% of the samples whereas PCR was able to detect *M. bovis* in 4.25% of the samples. However, it should be taken into account that in Pará state rearing systems, this disease is not endemic, although it is associated with lymph node damage to organs that may be affected, such as lungs, liver and head. The results confirm the importance of adding microscopic methods of complementary diagnostics to assist the examinations in sanitary inspection lines and make the inspection service more efficient in its decisions.

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