Assessing the histopathology to depict the different stages of bovine tuberculosis infection in a naturally infected herd[1]

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ABSTRACT.- Medeiros L., Marassi C.D., Figueiredo E.E.S., Leite J., Ferreira A.M.R. & Lilenbaum W. 2012. [**Assessing the histopathology to depict the different stages of bovine tuberculosis infection in a naturally infected herd].** *Pesquisa Veterinária Brasileira 32(2):135-139.* Laboratório de Bacteriologia Veterinária, Faculdade de Veterinária, Universidade Federal Fluminense, Rua Hernani Mello 101, sala 309, Niterói, RJ 24210-130, Brazil. E-mail: lusmedeiros@yahoo.com.br

The standard method for detection of bovine tuberculosis (TB) is the single intradermal tuberculin test (SITT). Nevertheless, current studies suggest that a single test is not enough to detect all cattle infected by TB, particularly when animals present different stages of infection. A dairy herd comprised of 270 cows was studied and 15 were reactive to SITT plus nine inconclusive animals. Blood samples (for IFN and ELISA) were collected from these 24 cows. At 30 days after injection of PPD, all the cows that were reactive to any of the employed tests were slaughtered, and tissues were processed by Bacteriology, Histopathology (HP) and PCR. According to HP 33.4% of the animals were positive, 45.8% inconclusive and 20.8% were negative. The inconclusive samples came from IFN positive animals, signalizing recent infection. Regarding the animals that were negative to HP, all of them were identified by IFN while ELISA was negative. Immune responses are different in recent and advanced infections, what supports the identification between chronically or recently infected animals. This multidisciplinary approach is mandatory for the interpretation of the various tools that are frequently employed for the diagnosis of TB and mainly to identify all infected animals.

INDEX TERMS: Bovine tuberculosis, diagnosis, multidisciplinary approach, histopathology.

RESUMO.- [**Avaliação da histopatologia na descrição dos diferentes estágios da tuberculose bovina em rebanhos naturalmente infectados.**] O método padrão para detecção de tuberculose bovina (TB) é o Teste Cervical Simples (TCS). No entanto, estudos atuais sugerem que um único teste não é suficiente para detectar todos os bovinos infectados por TB, particularmente quando os animais de uma rebanho apresentam diferentes estágios de infecção. Um rebanho leiteiro composto de 270 vacas foi estudado e no TCS 15 animais foram reagentes e nove animais inconclusivos. Amostras de sangue (para IFN e ELISA) foram coletadas destas 24 vacas. Trinta dias após a injeção do PPD, todas as vacas que foram reativas a qualquer um dos testes utilizados foram abatidas e os tecidos foram processados por bacteriologia, histopatologia (HP) e PCR. De acordo com a HP 33,4% dos animais foram positivos, 45,8% inconclusivos e 20,8% foram negativos. As amostras classificadas como inconclusivas foram provenientes de animais IFN positivo, sinalizando infecção recente. Em relação aos animais negativos na HP, todos eles foram identificados por IFN enquanto no ELISA apresentaram resultados negativos. Respostas imunes são diferentes em infecções recentes e avançadas, o que suporta a identificação entre os animais cronicamente ou recentemente infectados. Esta abordagem multidisciplinar é obrigatória para a interpretação das várias ferramentas que são freqüentemente empregadas para o diagnóstico da TB e, principalmente, para identificar todos os animais infectados em um rebanho.

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TERMOS DE INDEXAÇÃO: Tuberculose bovina, diagnóstico, abordagem multidisciplinar, histopatologia.

INTRODUCTION

Bovine tuberculosis (TB) is a major infectious disease among cattle in several countries. Immune responses against mycobacterial infections are predominantly cellular, at least initially (Wood & Jones 2001). Therefore, diagnostic techniques should be based preferentially on the measurement of the lymphocytes T responses (Wood & Rothel 1994). The standard method for detection of bovine tuberculosis is the intradermal tuberculin skin test (Monaghan et al. 1994) which assesses the cellular immune response.

Several methods have been employed for the in vivo diagnostic of TB, regarding both cellular and humoral responses. The Gamma-Interferon assay (IFN) is based on the release of IFN from previous *M. bovis*-sensitized blood cells cultured in vitro, and detects an early cell-mediated response (Wood & Jones 2001). It has been evaluated in Brazil with encouraging results (Lilenbaum et al. 1999, Marassi et al. 2010), as well as in many other countries (Wood & Jones 2001). In regards to humoral responses, it has been stated that B lymphocytes are stimulated to induce antibodies production only in advanced stages of bovine tuberculosis (Pollock & Neill 2002). Therefore, serological tests showed to be less efficient to identify cattle in the early stages of tuberculosis infection (Wood & Rothel 1994) but have been recommended for diagnostic of anergic animals (Lilenbaum & Fonseca 2006) and as a complementary diagnostic herd tool (Lilenbaum et al. 1999, Welsh et al. 2005, de la Rua Domenech et al. 2006).

Nevertheless, due to the limitations of all those methods, the definitive diagnosis that a herd is infected requires a clear evidence of the agent, based on bacteriological culture, histopathology (HP) or molecular methods (Thoen et al. 2009, Medeiros et al. 2010). The histopathology of lesions, in addition of being a diagnostic tool, also provides information on the immune responses of the host (Pollock et al. 2005).

The purpose of the present study was to assess the histopathology, in conjunction with IFN, ELISA, Bacteriology and PCR, depicting the different stages of infection in a naturally infected herd and measuring the cellular differences among recent and advanced infections.

MATERIALS AND METHODS

Study design. A dairy herd comprised of 270 adult crossbred Holstein and Gir cows was studied. In a routine testing (Single Intradermal Tuberculin Test - SITT) 15 cows had shown to be reactive. Those cows were kept in quarantine for 90 days, waiting for confirmatory tests to be conducted. After 90 days, a Comparative Intradermal Tuberculin Test (CITT) was performed in these 15 cows, plus nine cows that were inconclusive at the first SITT testing. Blood samples (for IFN and ELISA) were collected from these 24 cows, at the time of the injection of PPD for the CITT. At 30 days after injection of PPD, all the cows that were reactive to any of the employed tests (CITT, IFN or ELISA) were slaughtered in accordance with Brazilian laws and regulations. Necropsies were performed and tissues were collected and subjected to, histophatology, bacteriological culture and PCR.

Intradermal tests. Intradermal tests (CITT) were performed on all 24 cows, in accordance with the regulations of the Brazilian Department of Agriculture. For CITT, 0.1mL of bovine PPD (bovPPD - *M. bovis* strain AN5, 1mg protein/mL; Instituto Biologico, São Paulo, Brazil) plus an inoculation of 0.1mL of avian PPD (avPPD - *Mycobacterium avium* strain D4, 0.5mg protein/mL; Instituto Biologico, Brazil) were inoculated in the cervical area. After 72 hours, the site of inoculation was measured with calipers and the animal was considered reactive if the difference between the thicknesses of both sites of inoculation were >4.0mm.

Interferon-gamma assay (IFN). Heparinized blood samples from the 24 cows were collected for IFN testing just prior to injection of PPDs. The assay was performed according to the manufacturer's instructions (Bovigam, Prionics, Zürich, Switzerland) and as previously conducted by our group (Lilenbaum et al. 1999).

MPB70/MPB83 - ELISA. Serum samples from the 24 cows were collected for ELISA prior to injection of PPD. The recombinant proteins MPB70 and MPB83 were gently donated by Professor Jim McNair (Agri-Food and BioSciences Institute, Ireland) and performed as previously described (Marassi et al. 2010). Briefly, each antigen was used separately as a capture antigen (1μg/mL) in an ELISA and bovine sera were added to wells. An alkaline-phosphatase conjugated anti-bovine IgG was used (1:5,000). Cut-off points based on OD readings were calculated using Receiver Operator Characteristic Curves (ROC) analysis and the under ROC area for each antigen using a 95% confidence interval was calculated and compared. Positive and negative sera controls were also tested by this assay.

Histopathology. At 30 days after injection of PPD, all the cows that were positive to any of the employed tests (CITT, IFN or ELISA) were slaughtered and necropsies were performed. Lung samples, with or without characteristic lesions, were collected and fixed in 10% neutral buffered formalin, processed by standard paraffin wax techniques and stained using the standard HE method. An estimate of granuloma area in a lung section was determined by taking the average of approximately 5 microscopic (x100) fields examined in one plane of section. Despite been lung samples instead lymph nodes, samples were categorized according to Varello et al. (2008), as follows: Positive: tubercular granuloma displaying central necrosis with or without mineralization surrounded by macrophages, lymphocytes, plasma cells, neutrophils, epithelioid cells, and Langerhan's giant cells, and enclosed partly or completely by a thin capsule. Inconclusive: lesion characterized by irregular with no capsulated clusters of epithelioid macrophages; associated with a not Langerhan's-type multinucleated giant cells and necrosis. Negative: features not consistent with tubercular granuloma, including significant eosinophilic infiltrates and lymphoid hyperplasia.

Evidence of *Mycobacterium bovis.* Lung samples were collected from all the slaughtered cows. All samples were processed for both bacteriology and PCR. For bacteriologic culture to detect *M. bovis*, samples were divided in three portions and decontaminated according to three different standard methods. One portion was treated with 0.75% hexadecylpyridinium chloride (HPC), another with 4% sodium hydroxide, and the third with 6% sulfuric acid. After decontamination, samples were inoculated on two slopes of solid, egg-based media Lowenstein-Jensen with 0.5% pyruvate, and two slopes of Stonebrink media, which were incubated at 37°C and observed once weekly for 12 weeks (OIE 2010). For evidence of *M. bovis*-specific DNA, a multiplex PCR targeting the RvD1Rv2031c genomic sequence was employed on the tissue samples before decontamination (Figueiredo et al. 2009).

RESULTS

The Comparative Intradermal Tuberculin Test (CITT) confirmed the first 15 cows that were previously reactive, plus six out of nine that had been inconclusive at the first SITT testing, totaling 21 skin-test reactive cows. The IFN confirmed all the 21 CITT reactive cows, and identified as reactive plus two cows that were negative to both SITT and CITT. MPB70 and MPB83-ELISAs presented identical results and were studied as one. They could identify four animals which were reactive to CITT plus one cow that was negative to SITT, CITT and IFN. Therefore, the study group was composed by 24 cows that were positive to any of the employed tests (21 CITT, 2 IFN and 1 ELISAs). Those cows were slaughtered and fragments of lungs submitted to culture and histopathology. *M. bovis* was recovered by culture from tissues of 13 cows, while its DNA was evidenced on tissue of 17 samples (Table 1).

According to HP, in nine cows (37.5%) macroscopic lesions positive for bovine pulmonary tuberculosis were observed. At microscopy, lesions presented typical tubercular granuloma displaying central necrosis with mineralization surrounded by macrophages, lymphocytes, plasma cells, neutrophils, epithelioid cells, and Langerhan's giant cells, and enclosed partly or completely by a thin capsule. From those cows, eigth (88.9%) were IFN-positive and three (33.3%) reactive to ELISAs. *Mycobacterium bovis* was recovered from tissues of eight of these cows, while its DNA was evidenced on seven samples.

Ten cows presented macroscopic lesions suggestive of bovine pulmonary tuberculosis but were considered as inconclusive at HP (45.8%). At microscopy three animals (27.3%) presented lesions with diffuse neutrophilic infiltrate, and seven animals (63.3%) presented lesions with clusters of epithelioid macrophages, two of them with fibrosis. From those cows, all the ten (100%) were IFN-positive and two (18.2%) were reactive to ELISAs. *M. bovis* was recovered from tissues of three cows, while its DNA was evidenced on eight tissue samples.

Five animals were negative at HP (20.8%), although one of them presented macroscopic lesions suggestive of bovine pulmonary tuberculosis. From those cows, anyone was reactive to ELISAs while all the five (100%) were IFN--positive. *M. bovis* was recovered from tissues of two cows, while its DNA was also evidenced on two samples.

DISCUSSION

The present study assessed the histopathology, in conjunction with IFN, ELISA, Bacteriology and PCR, depicting the different stages of infection in a naturally infected herd and measuring the cellular differences among recent and advanced infections.

Status of the herd was well defined since not only the herd was positive at the first intradermal tests (single tuberculinization) but also was confirmed by CITT. Additionally, *M. bovis* was recovered from the lesions and its DNA was evidenced by PCR.

It is not surprising that more animals (21) were detected by the CITT than in the first SITT testing (15). There was an interval of 90 days between the two skin tests, and it is possible that during that interval some cows that had been recently infected developed cell-mediated response and could only be detected at the second testing. In relation to the accuracy of IFN, this test has been extensively described to be more sensitive than skin tests (Lilenbaum et al. 1999, Wood & Jones 2001), and therefore the identification of one supplementary cow that was skin-test negative is

Table 1. Results of Intradermal Tuberculin Test (SITT), Comparative Tuberculin Test (CITT), Interferon-gama Assay (IFN), MPB70/MPB83- ELISA (ELISA), Culture, Polymerase Chain Reaction (PCR) and Histopathology (HP) for the diagnosis of bovine tuberculosis in the naturally infected herd

Nº of Cow	SITT	CITT	IFN	ELISA	CULTURE	PCR	HP
1	Inconclusive	Reactive	Reactive	Negative	Negative	Negative	Negative
2	Inconclusive	Negative	Reactive	Negative	Negative	Negative	Negative
3	Reactive	Reactive	Reactive	Negative	Positive	Negative	Negative
4	Reactive	Reactive	Reactive	Negative	Negative	Positive	Negative
5	Reactive	Reactive	Reactive	Negative	Positive	Positive	Negative
6	Inconclusive	Negative	Negative	Reactive	Negative	Negative	Positive
7	Inconclusive	Reactive	Reactive	Reactive	Positive	Positive	Positive
8	Inconclusive	Reactive	Reactive	Reactive	Positive	Positive	Positive
9	Reactive	Reactive	Reactive	Negative	Positive	Negative	Positive
10	Reactive	Reactive	Reactive	Negative	Positive	Positive	Positive
11	Reactive	Reactive	Reactive	Negative	Positive	Positive	Positive
12	Reactive	Reactive	Reactive	Negative	Positive	Positive	Positive
13	Reactive	Reactive	Reactive	Negative	Positive	Positive	Positive
14	Reactive	Reactive	Reactive	Negative	Positive	Positive	Positive
15	Inconclusive	Reactive	Reactive	Negative	Negative	Negative	Inconclusive
16	Inconclusive	Negative	Reactive	Negative	Negative	Negative	Inconclusive
17	Inconclusive	Reactive	Reactive	Negative	Positive	Positive	Inconclusive
18	Inconclusive	Reactive	Reactive	Negative	Negative	Positive	Inconclusive
19	Reactive	Reactive	Reactive	Negative	Negative	Positive	Inconclusive
20	Reactive	Reactive	Reactive	Negative	Negative	Positive	Inconclusive
21	Reactive	Reactive	Reactive	Negative	Positive	Positive	Inconclusive
22	Reactive	Reactive	Reactive	Negative	Negative	Positive	Inconclusive
23	Reactive	Reactive	Reactive	Reactive	Positive	Positive	Inconclusive
24	Reactive	Reactive	Reactive	Reactive	Negative	Positive	Inconclusive

not surprising. Additionally, the fact that MPB70 and 83 are very similar between themselves and presented identical results when used as capture antigens in an ELISA has already been described (McNair et al. 2001), as well as the role of ELISA in the detection of anergic cows (Silva et al. 2001, Pollock & Neill 2002, Lilenbaum & Fonseca 2006).

The positive microscopy results (9/24) were in accordance with the suggestive macroscopic lesions found in the lungs. These animals were probably in a more advanced stage of the disease, presenting classic tubercular granuloma, which is frequently observed with the progress of the disease (Welsh et al. 2005). In those animals, one was identified exclusively by ELISA. Additionally, eight out of nine were positive at the culture, the greatest proportion among the groups, suggesting a higher bacillary burden.

According to current knowledge regarding both IFN and ELISA, in this herd these cows may have been in different stages of the disease, what could justify the high percentage of inconclusive samples (45.8%), as well as the negative results at histopathology. Initial immune responses against mycobacterial infections are expected to be predominantly cellular, while humoral responses become detectable later (Wood & Jones 2001). In that regard, IFN signalizes recent infection, since it has been described that this test detects infected cattle in very early stages of infection (Lilenbaum et al. 1999), as soon as 14 days after inoculation (Buddle et al. 1995). Similarly, ELISA has been described to be more useful for detecting animals with classic, advanced lesions, when bacterial load is higher and humoral response is evident (Silva et al. 2001, Pollock & Neill 2002, Lilenbaum & Fonseca 2006).

In relation to the animals that presented mild lesions, considered as inconclusive to pulmonary tuberculosis, we assume that they had a recent infection. Three animals presented lesions with diffuse neutrophilic infiltrate, and other seven animals presented lesions with clusters of epithelioid macrophages, what is consistent to initial stages of infection. Cassidy et al. (1998) identified neutrophils as one of the earliest cells associated with the developing granuloma, and Pollock and Neill (2002) described the initial interplay between macrophage and mycobacteria. Additionally, IFN identified as reactive all of those animals, while only two of them were reactive to ELISA. In relation to the direct evidenciation of the agent on those samples, an interesting finding was observed. Although only three samples were positive by bacterial culturing, eight of them were positive by PCR. Molecular evidence of *Mycobacterium bovis* has been described as more sensitive than bacteriology since it requires a low bacterial load (Parra et al. 2008). Therefore, all the histopathological, bacteriological, molecular and immunological findings support the hypothesis that those animals were recently infected.

Regarding the five animals that were negative to HP, all of them were identified by IFN while ELISA was negative (Table 1). Therefore, there are evidences to support that those animals had been recently infected and, for that reason, they did not present visible lesions at HP. Tuberculin-reactive cattle with no visible lesions (NVL) have been reported when cows are in a very early stage of the disease, leading to small and/or only few lesions (Corner 1994), what agrees with our results.

Concluding, histopathology was employed in the present study as a tool to identify the different stages that are observed in the development of TB pathophisiology. Considering a multidisciplinary approach, when histopathological, bacteriological, molecular and immunological findings were analyzed altogether, it was possible to identify all the infected animals and to determine the stage of the infection in different cows from the same herd. This approach is mandatory for the interpretation of the various tools that are frequently employed for the diagnosis of TB, since it has been demonstrated that not all the cows forming the same herd are in the same stage of the infection, and therefore a multidisciplinary approach is required for detecting all the infected animals.

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