



Indirect ELISA as a complementary diagnostic method of bovine tuberculosis¹

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Bovine tuberculosis is an economic and health problem, requiring precise diagnostic methods for its control and eradication. The aim of this study was to evaluate the performance of a commercial enzyme-linked immunosorbent assay (ELISA) test for the diagnosis of bovine tuberculosis. A total of 1,644 cattle from eight dairy herds were evaluated using the comparative cervical tuberculin test (CCTT). Three of the herds had no recent tuberculosis infection, and the other five had shown positive results in a previous tuberculin test. For the serological diagnosis of tuberculosis, a commercial ELISA antibody test kit for *Mycobacterium bovis* was used. Serum samples from 846 cattle from the eight herds were evaluated using ELISA for *M. bovis*. Animals that were positive based on either CCTT or ELISA for *M. bovis* or both were sent to slaughter. Samples of their lungs, livers, and lymph nodes were collected and stored under refrigeration for microbiological culture and subsequent confirmation by polymerase chain reaction. Samples from the same tissues were also fixed with 10% formaldehyde in bottles for histopathological examination and stained with hematoxylin and eosin (HE). Of the 1,644 cattle, 61 were considered positive and 65 inconclusive based on CCTT. Retesting of the inconclusive samples identified an additional 19 positive cases, totaling 80 (4.8%) CCTT-positive animals from five herds. ELISA for *M. bovis* identified 4.2% (36/846) positive cattle, of which 35 were considered negative and one inconclusive based on CCTT. Of the 36 positive cases identified by ELISA for *M. bovis*, 27 were euthanized, 11% (3/27) showed suggestive lesions of tuberculosis on macroscopic examination, and two were confirmed by histological, microbiological, and PCR methods. The weak association of ELISA for *M. bovis* with the results obtained by macroscopic, histological, and microbiological isolation indicates the fragility of ELISA performance in field conditions. Therefore, it is suggested that its use as a complementary method for herd sanitation be based on the local epidemiological situation.

INDEX TERMS: ELISA, bovine tuberculosis, *Mycobacterium bovis*, serologic diagnosis, anergy, tuberculin test, cattle.

RESUMO.- [ELISA indireto como método complementar de diagnóstico da tuberculose bovina.] A tuberculose bovina é um problema econômico mundial e de saúde, que requer métodos de diagnóstico precisos para controle e erradicação. Objetivou-se com este estudo avaliar o desempenho de um

teste comercial de ELISA no diagnóstico da tuberculose bovina. Foram avaliados pelo teste cervical comparativo (TCC) 1644 bovinos, provenientes de oito rebanhos de exploração leiteira, sendo três deles sem histórico recente da doença, e outros cinco com resultados positivos no último exame de tuberculinização. Para o diagnóstico sorológico da tuberculose utilizou-se um kit comercial de ELISA *Mycobacterium bovis* antibody test (ELISA *M. bovis*). Amostras de soro sanguíneo de 846 bovinos provenientes dos mesmos rebanhos foram também avaliadas no ELISA *M. bovis*. Os bovinos positivos no TCC e/ou ELISA *M. bovis* foram encaminhados para o abate sanitário. Dos positivos no ELISA para *M. bovis* foi realizado o exame macroscópico das carcaças e classificação das

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mesmas em: com presença ou ausência de lesões. Amostras de pulmão, fígado, e linfonodos, foram colhidas em duplicata para realização dos exames de cultivo microbiológico com posterior confirmação por PCR, e exame histopatológico com coloração de hematoxilina e eosina (HE). Dos 1,644 bovinos, 61 foram considerados positivos e 65 inconclusivos no TCC. O reteste dos inconclusivos identificou mais 19 positivos. No total, 80 (4,8%) bovinos positivos no TCC, provenientes de 5 rebanhos foram encaminhados para abate. O ELISA para *M. bovis* identificou 4,2% (36/846) de bovinos positivos, sendo 36 considerados negativos e um inconclusivo no exame de tuberculinização. Dos positivos no ELISA para *M. bovis*, 27 foram eutanasiados, e no exame macroscópico das carcaças 11% (3/27) dos animais apresentaram lesões sugestivas de tuberculose, e em apenas dois houve confirmação da doença pelos métodos histológico, microbiológico e PCR. A baixa associação dos resultados obtidos no ELISA para *M. bovis* com os exames macroscópico, histológico e isolamento microbiológico apontam para a fragilidade do desempenho do ELISA para o diagnóstico de tuberculose. Sugere-se assim que seu uso como método complementar para saneamento de rebanhos, seja adotado com cautela e considere a situação epidemiológica local.

TERMOS DE INDEXAÇÃO: ELISA, tuberculose bovina, *Mycobacterium bovis*, diagnóstico sorológico, anergia, tuberculinização, bovinos.

INTRODUCTION

Bovine tuberculosis (BT) is a chronic disease that is caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex, which comprises the causal agents of BT in different animal species (Casal et al. 2014). Because of its zoonotic character and the economic losses caused in the meat and milk supply chain, the disease is an object of control and eradication programs in several countries (Barbieri et al. 2016). The basic tool used in control programs is the adoption of routine diagnostic tests, which aim at identifying and eliminating sick animals. However, the quality of the tests used to detect animals infected by *M. bovis* has always been a great challenge for BT control (Álvarez et al. 2012, Bezos et al. 2014).

The results of the tests currently defined by the "Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose" (Brazilian National Program for the Control and Eradication of Brucellosis and Tuberculosis - PNCEBT) do not guarantee the complete identification of the disease in herds. According to Marassi et al. (2013), none of the possible tests that can be used in the diagnosis of BT can identify all animals at all stages of infection. Thus, a multivariate diagnostic approach is necessary to increase the efficiency and reliability of methods for the detection of infected animals.

The intradermal tuberculinization test, which uses purified protein derivatives (PPD), is the world's most commonly used standard test for the detection of BT in cattle (Seva et al. 2014). The test is based on the detection of cell-mediated immunity, which is predominant during the early and intermediate stages of infection, led mainly by Th1 lymphocytes (Casal et al. 2014). As the disease progresses, the Th1 response is replaced by Th2, which is associated with decreased cellular immune response and the development of a humoral immune response (Welsh et al. 2005, McNair et al. 2007, Schiller et al. 2010). Some studies indicated the

pre-allergic and advanced phases of BT as causes of false-negative reactions in the tuberculinization test (Lilenbaum et al. 1999, Pollock & Neill 2002). Thus, serological testing is an alternative for the screening of cattle infected with *M. bovis*. The combination of methods based on cellular immune response and serological assays could increase the level of detection of the agent, contributing to disease control (Schiller et al. 2010, Bezos et al. 2014).

Enzyme-linked immunosorbent assay (ELISA) tests for BT have shown good results as complementary tests for the identification of herds infected with *M. bovis* (Schiller et al. 2010, Waters et al. 2011). Although ELISA is not considered the first diagnostic choice, its advantage is its ability to identify anergic animals (McNair et al. 2001), which generally appear in the advanced stage of the disease (Pollock & Neill 2002, Welsh et al. 2005). However, there are some limitations in the applicability of ELISA as an eradication tool, particularly the type of antigen used in the test, due to the similarity of the *M. bovis* genome with that of nonpathogenic mycobacteria (Garnier et al. 2003) and to infection phases in which serological response cannot be detected (Welsh et al. 2005). When using tuberculinization and serological testing simultaneously, the animal is considered positive for BT when it is reagent in either or both tests and negative when there is no reaction in both tests (Seva et al. 2014).

Despite the effectiveness of the diagnosis based on the detection of cellular immune response to *M. bovis* antigens, the test must be conducted *in vivo*, which makes it difficult to use in high-density herds and in epidemiological studies. Moreover, animals with chronic infection may not be detected by intradermal tests. The possibility of using serological tests for BT has been suggested as a complementary tool to the tuberculinization test. Therefore, the objective of this study was to evaluate the performance of a commercial ELISA test for *M. bovis* detection in dairy cattle naturally infected with BT.

MATERIALS AND METHODS

Ethical assessment. This study was approved by the Ethics Committee on the Use of Animals of the "Universidade Federal de Uberlândia" (UFU) under protocol No. 066/14.

Study sites. A cross-sectional study was conducted in eight dairy herds located in the municipalities of Guimarães (A), Lagoa Formosa (B), Patos de Minas (C and D), Perdizes (E), Prata (F), Serra do Salitre (G), and Uberlândia (H), located in the regions of Triângulo Mineiro and Alto Paranaíba in the State of Minas Gerais, Brazil. The herds were selected for convenience – that is, those in which reactive animals were detected (n=5) or not (n=3) in the previous tuberculinization test. The herds consisted of crossbred cattle of various breeds, aged between six months and 20 years, and raised in an intensive or semi-intensive system, with cows submitted to mechanical or manual milking.

Tuberculinization. The results of CCTTs routinely performed by veterinarians officially accredited by the PNCEBT were kindly provided to this research. In total, 1,644 cattle were evaluated using CCTT, and the results of the differences ($\Delta B - \Delta A$) were interpreted according to the PNCEBT Technical Regulation (Brasil 2006). Animals with positive CCTT results were euthanized, and those with inconclusive results were retested within 60 days. Inconclusive results in two consecutive tests were considered positive.

ELISA for detection of *Mycobacterium bovis*. Of the 1,644 animals evaluated by tuberculinization, 846 from the eight selected

herds were also tested using ELISA for *M. bovis*. Blood samples were collected on the same day of inoculation with bovine and aviary PPD. For the serological diagnosis of BT, a commercial indirect ELISA antibody test kit for *M. bovis* was used. The examination was conducted according to the manufacturer's instructions.

Anatomical and histopathological diagnosis. The positive animals in ELISA for *M. bovis* were euthanized and necropsied. The carcasses and viscera were inspected, and the lesions were recorded and photographed. At necropsy, the animals were classified as either "lesion present," when at least one of the evaluated organs had lesions suggestive of tuberculosis, or "lesion absent."

Regardless of the presence of macroscopic lesions, lung, liver, retropharyngeal, submandibular, and cervical and mediastinal lymph node sections, approximately 1.5cm in size, were collected and stored in a sterile plastic vial and sent to the São Paulo Biological Institute for microbiological analysis. Samples of the same tissues were added to other vials containing 10% formaldehyde and sent to the "Laboratório de Patologia Animal" (Animal Pathology Laboratory) of UFU for histological examination. Routine dehydration, diaphanization, and paraffin inclusion techniques were performed. Four-micrometer-thick slices were obtained using a microtome (Leica 2125), and a histological slide of each block was obtained and stained with hematoxylin and eosin (HE).

Microbiological isolation. Microbiological tests were performed at the "Laboratório de Tuberculose" (Tuberculosis Laboratory) of the "Instituto Biológico de São Paulo". For sample decontamination, the classical Petroff method was used (Kantor & Ritacco 1988). The conventional egg-based Stonebrink medium was used for the isolation of mycobacteria. The tubes were incubated in an oven at 37°C, and growth was checked weekly until colonies suggestive of mycobacteria or contaminants appeared. The samples were observed up to 90 days after incubation.

Molecular identification of mycobacteria. The isolated colonies were identified using polymerase chain reaction (PCR) using the JB21 and JB22 primers described by Rodriguez et al. (1995) and modified by Harakava et al. (2010). The amplified product was then analyzed using electrophoresis in a horizontal gel. The 1.5% agarose gels containing 0.01% ethidium bromide were visualized under ultraviolet light and photographed using a photodocumenter.

RESULTS

Tuberculinization

At least one reactive animal was detected in five (62.5%) of the eight herds evaluated using CCTT. The frequency of

tuberculosis in animals per herd ranged from 0% to 29.2%. Of the 1,644 cattle evaluated, 61 (3.7%) were considered positive and 65 (4%) inconclusive. Retesting of the inconclusive animals identified 19 additional positive animals; therefore, the frequency in the animals was 4.8% (80/1,644). The size of reactions in reagent animals ranged from 4.2 to 12.2mm ($\Delta B - \Delta A$). Eighty positive cattle were sent to sanitary slaughter in refrigerated slaughterhouses in the region, of which 42.5% (34/80) tested positive for *Mycobacterium bovis* (Table 1).

According to the CCTT results and information on the herds' health history, no positive or inconclusive cattle were identified in the intradermal test at three sites (A, D, and F). These sites had no recent cases of infection, and one of them (F) had been certified by the PNCEBT as BT- and brucellosis-free. The other sites (B, C, E, G, and H) had a recent history of BT in the herd, which was confirmed in the previous evaluation by the presence of positive and inconclusive animals in CCTT.

ELISA for *M. bovis*

Of the 846 serum samples evaluated, ELISA for *M. bovis* identified 36 positive animals. The sample/positive ratio calculated for each sample ranged from 0.333 to 2.637. In all reactions, the mean optical density was greater than 0.3 for the positive control and lower than 0.20 for the negative control.

Of the 36 positive cattle identified using ELISA for *M. bovis*, only one animal was also inconclusive in CCTT, and the other 35 showed negative results. In two herds (A and B), all evaluated animals were considered negative in ELISA for *M. bovis* (Table 2). Based on the combination of the CCTT and ELISA results, the ELISA sensitivity was 1.25% and specificity was 95.4%. The proportion of concordant results between CCTT and ELISA was 86.4%, resulting in $\kappa = -0.03$ (Table 3). Of the 36 positive animals in ELISA for *M. bovis*, 27 were euthanized.

Necropsy findings of positive animals in ELISA for *M. bovis*

At least one animal was euthanized and necropsied in each herd in which reagent cattle were detected in ELISA for *M. bovis*. A total of 27 animals were either sent to slaughterhouses under sanitary inspection or euthanized on their own farm of origin. In the macroscopic inspection of the carcasses, it was observed that three animals (from herds C and E) presented lesions suggestive of tuberculosis. The most evident histopathological findings were granulomatous nodules

Table 1. Results of comparative cervical tuberculin tests (CCTT) and isolation of *Mycobacterium bovis* performed in cattle from eight dairy herds in the regions of Triângulo Mineiro and Alto Paranaíba, State of Minas Gerais, Brazil

Herd	CCTT tested cattle (N)	Positive CCTT (n)	Inconclusive CCTT	Retest of inconclusive (+)	Frequency CCTT % (n/N)	<i>M. bovis</i> isolation (n/N)
A	99	0	0	0	0 (0/99)	NP
B	50	1	1	1	4.0 (2/50)	(0/2)
C	92	2	6	1	3.2 (3/92)	(2/3)
D	155	0	0	0	0 (0/155)	NP
E	164	41	29	7	29.2 (48/164)	(16/48)
F	660	0	0	0	0 (0/660)	NP
G	90	8	12	3	12.2 (11/90)	(5/11)
H	334	9	17	7	4.8 (16/334)	(11/16)
TOTAL	1644	61	65	19	4.8 (80/1644)	(34/80)

(+) = Reagent in the CCTT retest; NP = test not performed.

Table 2. Results of ELISA tests for *Mycobacterium bovis*, macroscopic inspection, histopathological examination with hematoxylin and eosin (HE) staining, and isolation of *M. bovis* (culture and PCR) performed with cattle samples from eight dairy herds in the regions of Triângulo Mineiro and Alto Paranaíba, State of Minas Gerais, Brazil

Herd	Cattle tested with ELISA for <i>M. bovis</i> (N)	Positive ELISA test for <i>M. bovis</i> % (n/N)	Euthanized	Macroscopic lesions (n/N)	Histopathological HE staining (n/N)	Isolation of <i>M. bovis</i> (n/N)	
						Culture	PCR
A	40	0 (0/40)	0	NP	NP	NP	NP
B	50	0 (0/50)	0	NP	NP	NP	NP
C	92	3.2% (3/92)	3	(2/3)	(1/3)	(1/3)	(1/3)
D	50	4% (2/50)	1	(0/1)	(0/1)	(0/1)	(0/1)
E	92	6.5% (6/92)	2	(1/2)	(1/2)	(1/2)	(1/2)
F	148	3.3% (5/148)	2	(0/2)	(0/2)	(0/2)	(0/2)
G	40	7.5% (3/40)	2	(0/2)	(0/2)	(0/2)	(0/2)
H	334	5% (17/334)	17	(0/17)	(0/17)	(0/17)	(0/17)
TOTAL	846	4.2% (36/846)	27	(3/27)	(2/27)	(2/27)	(2/27)

NP = examination not performed.

Table 3. Results of ELISA tests for *Mycobacterium bovis* compared with the comparative cervical tuberculin test (CCTT) for the diagnosis of tuberculosis in cattle evaluated simultaneously in both tests from eight dairy herds in the regions of Triângulo Mineiro and Alto Paranaíba, State of Minas Gerais, Brazil

ELISA <i>M. bovis</i>	TCC		
	Positive	Negative	Total
Positive	1	35	36
Negative	79	731	810
TOTAL	80	766	846

Sensitivity: $1/80 \times 100 = 1.25\%$; Specificity: $731/766 \times 100 = 95.4\%$; Agreement: $1+731/846 = 86.4\%$; κ : -0.03; X²: 19.06; $p < 0.0001$; Positive predictive value: $1/35 \times 100 = 2.85\%$; Negative predictive value: $731/810 \times 100 = 90.2\%$.

with purulent to caseous aspect in the lungs (Fig.1) and in the mediastinal and retropharyngeal lymph nodes. Other carcasses (24) were classified as "lesion absent."

Histopathological diagnosis

In the histopathological examination, the lung and mediastinal lymph node samples from two of the three carcasses with lesions on macroscopic examination presented histological alterations characteristic of tuberculosis. A typical tuberculosis granuloma was observed in the mediastinal lymph node of the first animal, from herd E, with areas of necrosis and calcification surrounded by lymphocytes, macrophages, and Langhans giant cells. A granulomatous reaction was identified in the lung of the other animal, from herd C, which was characterized by caseous necrosis surrounded by predominantly mononuclear infiltrate and fibrous connective tissue (Fig.2) and pulmonary parenchyma with Langhans giant cells with broad cytoplasm and peripheral nuclei (Fig.3).

Culture and PCR

Only viscera samples from 2 of the 27 positive cattle in ELISA for *M. bovis* were also positive for *M. bovis* culture in Stonebrink medium and were confirmed using PCR with primers JB21 and JB22. These samples were also classified as "lesion present," with lesions suggestive of tuberculosis in the macroscopic evaluation and histological alterations

characteristic of tuberculous granuloma. As for the CCTT results of these two animals, one was considered positive and the other inconclusive. Among the reagent animals in ELISA for *M. bovis*, the expected proportion of lesions on macroscopic examination was 11.1% (95% CI: 2.35%-29.15%) and in the histological and isolation tests was 4.4% (95% CI: 0.91%-24.28%).

DISCUSSION

The frequency of 4.8% in animals and 62.5% in herds identified by CCTT in the present study demonstrates that BT infection was present in most of the dairy herds evaluated. The occurrence of CCTT-reactive animals was already expected, considering the selection of some herds due to their history of positivity in recent tuberculinization tests. In 2013, in the regions of the Triângulo Mineiro and Alto Paranaíba, which are important dairy producing regions in Brazil, prevalences of 4.24%-4.45% were observed in herds and 0.21%-0.47% in animals (Barbieri et al. 2016). Despite the relatively low prevalence of cattle with BT in these regions, the epidemiological situation of the disease remains practically stable. In countries with a longer active BT control program, the prevalence of infection is low, but only few areas have achieved total eradication of BT (Schiller et al. 2010). One of the factors possibly related to difficulties in eradicating BT is related to the technical limitations of diagnostic protocols (Schiller et al. 2010, Waters et al. 2011). According to Vidal (2013), the continued use of intradermal tuberculinization tests in isolation seems to be insufficient to eradicate the disease. With the evolution of BT control programs and the reduction of its prevalence, the use of methods complementary to intradermal tuberculinization is an important advance for the identification of foci and control of diseases (Schiller et al. 2010). In this sense, complementary tests, such as serological tests, could serve as additional tools to detect animals that do not react in tests based on cellular immune response, thereby assisting in the identification of cattle in the advanced stages of the disease, which produce false-negative results in tuberculinization (Welsh et al. 2005, Waters et al. 2011).

To evaluate the field performance of the commercial ELISA kit for *Mycobacterium bovis*, serum samples from herds naturally infected with tuberculosis (B, C, E, G, and H) were tested, along with others without a history of infection and with negative

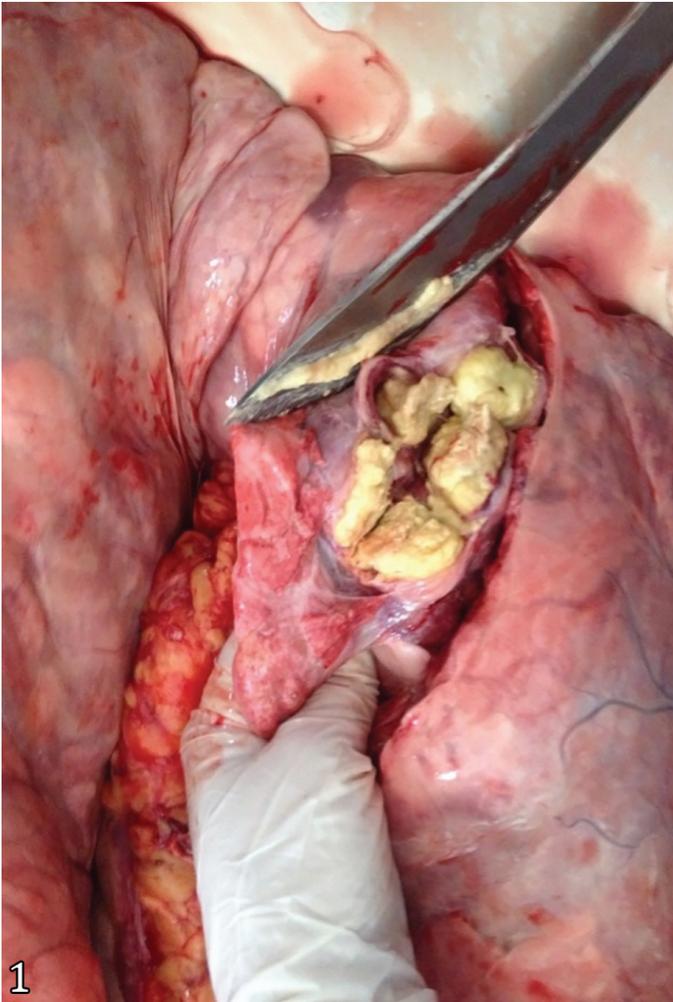


Fig.1. Lung observed during necropsy, with a granuloma containing a yellow caseous mass of pasty consistency, identified in a positive bovine (herd C) in ELISA for *Mycobacterium bovis*, State of Minas Gerais, Brazil.

results in tuberculinization tests (A, D, and F). In two herds (C and E), one negative animal and another inconclusive in CCTT were detected in ELISA for *M. bovis* and the viscera samples (lung and mediastinal lymph nodes) obtained in *M. bovis* isolation and positive PCR tests. In the original herds of these two animals (C and E), tuberculinization tests were already routinely adopted and frequently conducted by the owners, yet the exclusive use of CCTT did not result in the complete sanitation of the herds. These two positive animals in ELISA for *M. bovis* were cows aged five and eight years, and in CCTT, they presented $\Delta B=0.8$; $\Delta A=0.5$ and $\Delta B=2.8$; $\Delta A=0.8$, respectively. The complementary use of ELISA for *M. bovis* in these herds allowed the detection and elimination of animals that were possibly in energy, even if they did not present disseminated tuberculosis, and this was therefore fundamental for the good performance of the sanitary program implemented at the sites. It is possible that without the use of this strategy, the animals would have remained in the herds as reservoirs of the agent, spreading the infection to other animals. Other studies also demonstrated the ability of serological tests to identify negative animals in tuberculinization. Lilenbaum & Fonseca (2006) observed positivity in ELISA in two animals negative in tuberculinization, which was later confirmed using microbiological isolation.

Of the 36 positive cattle in ELISA for *M. bovis*, 35 were negative and one inconclusive in tuberculinization. CCTT is widely used to evaluate the performance of ELISA tests for bovine BT (Medeiros et al. 2010, Waters et al. 2011), but the inversion observed between cellular and humoral responses in different phases of the infection (Ritacco et al. 1991, Welsh et al. 2005) limit CCTT as an indicator for carrying out serological tests (Vidal 2013). This ability to identify animals in distinct phases of the disease is corroborated by very low or negative values for the κ index, indicating low agreement between the results of the two tests or even disagreement between them (Soares Filho et al. 2020). Considering the inverse relationship between cell-mediated and humoral immune responses against *M. bovis*, intradermal testing and

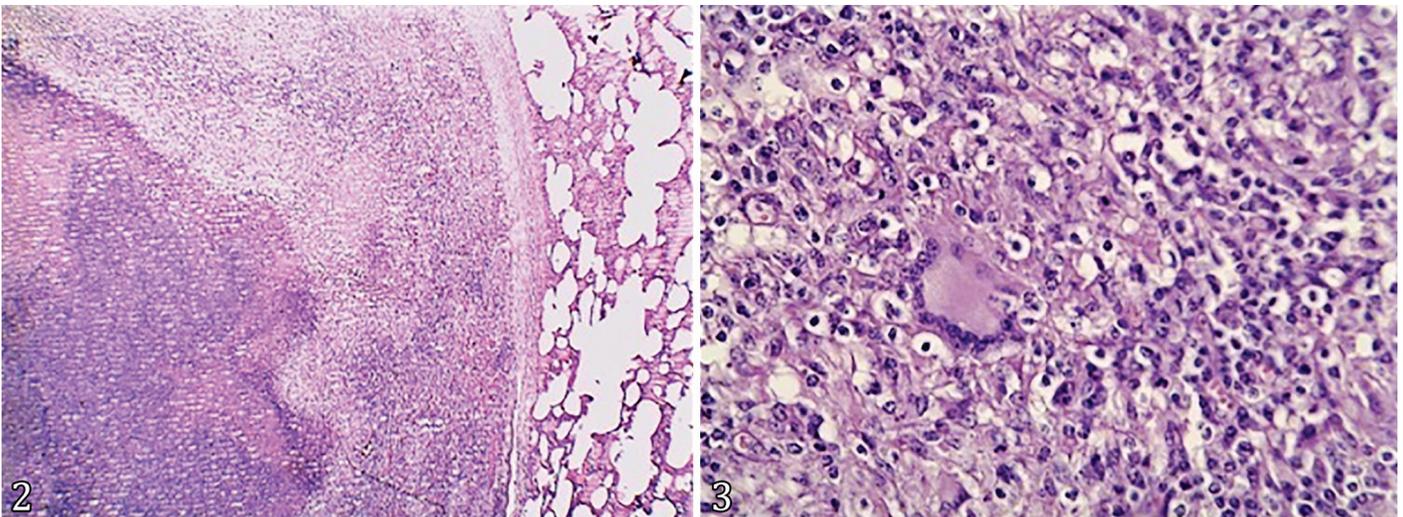


Fig.2-3. Microphotograph of lung identified in a positive bovine (herd C) in ELISA for *Mycobacterium bovis*, State of Minas Gerais, Brazil. (2) Granulomatous reaction characterized by central caseous necrosis surrounded by HE-stained fibrous connective tissue. HE, obj.10x. (3) Pulmonary parenchyma with mononuclear inflammatory cells and Langhans-type giant cells. Broad cytoplasm and nuclei arranged at the periphery of the cell. HE, obj.40x.

serological tests seek to measure different immunological responses, which develop at different stages of the infection (Pollock et al. 2001). In cattle, T lymphocytes are the first cells involved in the reaction to BT. As the disease progresses, the cellular immune response shifts to a humoral response. The cellular response decreases, whereas the humoral response based on IgG1 antibodies increases (Welsh et al. 2005, Schiller et al. 2010). However, the humoral response does not seem to be able to control infection, disease progression, and the increase in the bacterial load (Welsh et al. 2005).

Animals with antibodies against *M. bovis* were detected in three (A, D, and F) of the eight herds evaluated, despite these herds having no history of infection and no positive animals in the previous tuberculinization test; one herd (F) had even been certified brucellosis- and BT-free, yet animals with anti-*M. bovis* antibodies were identified within it. All the farms marketed milk and were therefore frequently evaluated through routine tests recommended by the PNCEBT. Consecutive tuberculinization tests can act as a booster for antibody production, thereby increasing the humoral response of cattle infected with BT (Harboe et al. 1990). Souza et al. (2012) suggest that the absorbance recorded in ELISA in cattle in BT-free herds was similar to that recorded in positive cattle in CCTT, probably due to the successive tuberculinizations necessary to acquire the BT-free status.

The main contribution indicated by studies with ELISA for BT is the complementary use of intradermal examinations to identify animals in anergy – that is, animals that although infected by *M. bovis* do not show a volume increase at the tuberculin inoculation site (Koo et al. 2005, Green et al. 2009, Whelan et al. 2010, Waters et al. 2011). Similarly, this study aimed to use ELISA for *M. bovis* in conjunction with CCTT in an attempt to identify infected animals not detected using the tuberculinization test. Thus, 36 positive animals from six herds (B, C, D, E, F, and G) were identified in ELISA for *M. bovis*, and 27 of them were euthanized. The most advanced stage of the disease, in which macroscopic lesions are present, is correlated with humoral immune response (Pollock & Neill 2002). Thus, given the results obtained in the macroscopic inspection of carcasses, culture, and PCR, a greater number of animals with lesions and positive isolation/PCR was expected. According to Pollock et al. (2006), primary BT lesions usually appear 7-11 days after infection, and they are small, pale yellow, with caseous cores. As these lesions progress, necrosis, mineralization, and fibrosis develop. Although the use of ELISA is recommended to detect animals in advanced stages of BT, in this study's field conditions, it was not possible to detect lesions in 24 of the 27 cattle with BT-positive serology. Several studies reported that the detection of anti-*M. bovis* antibodies occurs only in advanced stages of the disease or in cases of disseminated BT, whereas in the early stages, the humoral response is low or absent, increasing substantially with disease progression (Ritacco et al. 1991, Neill et al. 1994, Pollock et al. 2001, Pollock & Neill 2002, Welsh et al. 2005, McNair et al. 2007, Schiller et al. 2010).

Only three animals (11%) positive in ELISA for *M. bovis* presented lesions suggestive of BT at slaughter. The sensitivity seems to be dependent on the geographic region where the test is used and whether injury is present in the samples (Waters et al. 2011). The performance of ELISA for *M. bovis* evaluated in herds in Ireland showed that the sensitivity increased in

the presence of lesions, ranging from 90% in animals positive in CCTT or for interferon gamma to 20% in animals negative in both tests and without lesions at slaughter (Waters et al. 2011). The authors reported a sensitivity of 63% for the test (IDEXX ELISA for *M. bovis*) in the serum of animals reagent to intradermal or histopathological tuberculinization tests, histopathological examinations, or culture. According to Seva et al. (2014), all animals reagent in ELISA or tuberculinization tests, either alone or in combination, are considered positive for tuberculosis. Thus, 80 cattle positive in CCTT and 36 positive in ELISA for *M. bovis* would be infected, but the fact that the viscera samples from 24 of the 27 cattle euthanized with positive ELISA for *M. bovis* did not present lesions and presented negative results in *M. bovis* isolation and in CRP add to the uncertainty of the infection.

The use of serological diagnoses as complementary tools for tuberculinization tests is widely considered, especially due to the sensitivity variable (18%-73%) (Wood et al. 1992, Casal et al. 2014). The responsiveness of experimentally and naturally infected animals to various antigens of the *M. tuberculosis* complex has already been demonstrated, with extremely varied responses (Amadori et al. 2002). According to Buddle et al. (2013), changes in the diagnostic sensitivity of ELISA BT assays may be due to the stage of infection or to differences in virulence and antigenicity among *M. bovis* strains. The serological response varies according to the type of antigen used, and some antigens have already been described as potential diagnostic targets (ESAT-6, CFP-10, and MPB-70), although the response is mainly triggered by MPB-83 (McNair et al. 2001, Waters et al. 2011). MPB-83 was not detected in cattle infected with *M. avium* subsp. *avium* or *M. avium* subsp. *paratuberculosis*, but it has already been detected in cattle infected with *M. kansasii* (Waters et al. 2006, Green et al. 2009). MPB-83 is a constitutive protein of *M. bovis* that induces the production of antibodies at the onset of the disease. The response triggered against it is the highest, which suggests that it is a good antigen for serological tests (Waters et al. 2011, Bezos et al. 2014). On the other hand, antibodies against ESAT-6 and MPB-70 were detected 12 weeks (Lyashchenko et al. 1998) and 20 months after experimental infection (Fifis et al. 1994). The stage of infection is therefore a factor to be considered because the test employed in the present study detects antibodies against MPB-83 and MPB-70, which are proteins produced at different moments of infection.

The variations observed in the diagnostic sensitivity of ELISA BT trials are also influenced by the prevalence of the disease. The sensitivity of serological tests appears to be lower in countries where disease control programs have been successful, such as the United States and New Zealand, than in the countries with higher prevalence, such as Ireland and Great Britain. In countries such as New Zealand, where most infections are probably detected early due to herd monitoring and the advanced stage of disease control programs, the sensitivity of ELISA tests is lower (Buddle et al. 2013) because when infected animals are detected by routine monitoring tests, they are already eliminated from the herd. In contrast to these countries, the sanitary conditions observed in this study were herds with foci of the disease, verified by the presence of reactive animals in the tuberculinization test and lesions characteristic of BT at slaughter. The ELISA sensitivity

was evaluated using animals that were infected by *M. bovis*, and a 30%-90% variation was identified, depending on the geographical origin of the animals (Waters et al. 2011, Buddle et al. 2013). The reasons for these geographical differences in the sensitivity of the test were investigated by Trost et al. (2016). The hypothesis for these variations would be differences in the sequence of genes that encode and regulate the expression of proteins MPB70 and MPB84, but the results obtained suggest that this fact does not explain these geographical differences. Trost et al. (2016) also suggest that the stage of the disease, with a longer duration of infection, would be associated with a greater sensitivity of the test. The high frequency of infected animals observed in the present study through tuberculinization examination would probably be associated with a greater number of reactive animals in ELISA, even if they may be nonspecific for *M. bovis*.

The choice of the test and the cutoff point used to define an animal as infected is established by an inverse relationship between sensitivity and specificity within the local epidemiological context. High sensitivity increases the probability of detecting infected animals, but in situations of lower prevalence, high specificity is important to reduce the number of false positives and therefore the unnecessary slaughter of animals (Nuñez-García et al. 2017).

CONCLUSION

The low association of the results obtained in ELISA for *Mycobacterium bovis* with macroscopic, histological, and isolation examinations indicate the fragility of ELISA performance for the diagnosis of BT. It is thus suggested that its use as a complementary method for herd sanitation be adopted with caution, considering the local epidemiological situation.

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