

## INTERFERENCE OF TUBERCULOSIS ON THE PERFORMANCE OF ELISAS USED IN THE DIAGNOSIS OF PARATUBERCULOSIS IN CATTLE

Walter Lilenbaum<sup>1,\*</sup>; Rachel Ferreira<sup>1</sup>; Carla Dray Marassi<sup>2</sup>; Paula Ristow<sup>2</sup>; Walter Martins Roland Oelemann<sup>2</sup>; Leila de Souza Fonseca<sup>2</sup>

<sup>1</sup>Laboratório de Bacteriologia Veterinária, Universidade Federal Fluminense, Niterói, RJ, Brasil; <sup>2</sup>Laboratório de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil.

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### ABSTRACT

Forty-four cows from five herds infected with tuberculosis (TB) and without paratuberculosis (PTB), and 21 cows from a herd without either infection were studied. The cattle presented concordant results in both the skin test and  $\gamma$ -interferon assay for TB and two commercial ELISAs for PTB. Animals were divided according to TB test results into Group A with 28 TB-infected animals, Group B with 16 TB-negative animals from infected herds, and Group C with 21 TB-negative cows from a tuberculosis-free herd (which were used as controls). Twenty of 28 animals from Group A (71.4%), 6/16 from Group B (37.5%) and none from Group C were reactive to PTB ELISAs, suggesting that these commercial kits were unable to distinguish between PTB and TB. It is proposed that natural occurring TB strongly interferes in the diagnosis of PTB and that commercial ELISAs cannot be considered reliable tools in the diagnosis of paratuberculosis in tuberculosis-infected herds.

**Key words:** Paratuberculosis, Tuberculosis, *Mycobacterium bovis*, *Mycobacterium avium paratuberculosis*

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### INTRODUCTION

Paratuberculosis (PTB, Johne's disease) is characterised by chronic enteritis as consequence of *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection in cattle. The disease occurs worldwide and represents a significant financial burden (9). It begins asymptotically and evolves to a clinical form where characteristic malabsorption signs can be observed, such as chronic diarrhoea, emaciation, cachexia and occasionally death.

The diagnostic 'gold standard' for PTB is the isolation of its agent from faeces, tissues or milk. The fastidious growth of Map, together with an estimated sensitivity of 50% for culture (3), has led to the necessity of using other diagnostic serological tests such as the complement-fixation (CF) test, agar gel immunodiffusion test (AGID), and enzyme-linked immunosorbent assay (ELISA). Cross-reactions with environmental mycobacteria, which result in a low specificity

of the test, are diminished through pre-adsorption of sera with *Mycobacterium phlei* (2).

Map shares several antigens with other mycobacteria, including *M. bovis*. Reports show that paratuberculosis can compromise the specificity of bovine tuberculosis (TB) diagnostic tests, but the real influence of Map co-infection on the diagnosis of bovine TB remains to be elucidated (14). Diagnosis of PTB is even more difficult than TB, especially in sub-clinically infected animals. The interference of other mycobacterial infections, such as bovine TB, on the efficacy of diagnostic tests has been suggested but not yet widely evaluated (11).

In spite of several studies that evaluated the specificity of PTB-ELISAs (3,12), few studies have focused on the evaluation of the interference of TB infection in PTB tests. Also, vaccination of cattle with a live modified Map vaccine has been shown to impair the diagnosis of bovine TB, due to the close antigenic relationship between the two *Mycobacterium* species (7), as

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\*Corresponding Author. Mailing address: Laboratório de Bacteriologia Veterinária, Universidade Federal Fluminense, Rua Ernani Mello 101, Niterói, 24210-130, Brasil. Tel.: (21) 2629-2435 ou (21) 2629-2433. E-mail: mipwalt@vm.uff.br

well as natural infection with Map, which leads to false-positive reactions in TB skin tests (1).

Since most commercial tests for the serological diagnosis of PTB have been developed in countries where bovine TB has been eradicated or at least controlled, there has not been much effort expended in quantifying and reducing the interference of anti-*M. bovis* antibodies in those tests. However, in many developing countries there is a need to understand the real interference of bovine TB in PTB-ELISAs before recommending the PTB-assays for large scale screening in herds that live in regions where both mycobacterial infections occur. Therefore, in order to correctly diagnose PTB a better understanding of the influence of bovine TB on the results obtained with the PTB-ELISAs is required and could be of value in regions where both mycobacterial infections occur simultaneously.

The purpose of this study was, through a retrospective study, to suggest the hypothesis that the naturally occurring tuberculosis interferes on the performance of commercial ELISAs used for the diagnosis of PTB in cattle.

## MATERIALS AND METHODS

**Cattle** – Forty-four adult cows from five dairy herds with a previous history of bovine TB, including clinical cases and recovery of *M. bovis* from slaughtered animals, were studied. When samples were collected, none of the herds presented animals with chronic diarrhoea, leanness or any suspicion or history of PTB. For the following three years those herds have been accompanied and no clinical case of suspect of PTB was observed. One herd known to be TB-free for >5 years, confirmed by regular intradermal testing each six months, was selected as the control group. Twenty-one blood samples from the 147 animals tested by the single cervical tuberculin test (SCTT) of that herd were randomly collected. Therefore, a total of 65 cows were studied, originated from six herds. Herds have been accompanied for three years after the collection of samples. Characterisation of the studied herds is shown in Table 1.

### Intradermal Tests

SCTT consisted of the injection of 0.1 mL of bovine PPD (*M. bovis* strain AN5, 1 mg protein/mL), corresponding to 5000 international units (IU) per dose and examination of the site after 72 h. The interpretation of the results was performed according to the recommendations of the Department of Agriculture in Brazil, i.e., a positive animal with >4.0 mm swelling at the site of inoculation.

### Gamma-interferon ( $\gamma$ -IFN) assay

Blood samples were collected in heparin tubes and transported to the laboratory without refrigeration in <8 h, as previously described (Lilenbaum *et al.*, 1999). Stimulation of whole blood was performed in 24-well tissue culture trays with

**Table 1.** Characterisation of the six studied herds.

Herd	Number of cows	Number (%) of TB cases	Signs of PTB <sup>b</sup>	Number of studied cows
1	175	12 (6.9%)	-	10
2	262	18 (6.9%)	-	10
3	30	16 (53.3%)	-	7
4	182	5 (2.7%)	-	10
5	49	8 (16.3%)	-	7
6 <sup>c</sup>	147	-	-	21
Total	845	59 (6.9%)	-	65

<sup>a</sup> According to Single Cervical Tuberculin Test - SCTT; <sup>b</sup> Considered as animals presenting leanness and/or chronic diarrhoea in the past five years; <sup>c</sup> Control herd.

no antigen, bovine PPD, or avian PPD, and incubated at 37°C in a humid atmosphere without CO<sub>2</sub> for 16-24 h. Sandwich enzyme immunosorbent assay (EIA) for bovine  $\gamma$ -IFN was performed using a commercial bovine  $\gamma$ -IFN EIA kit (Bovigam). Test performance was validated by the mean absorbance of negative and positive controls and the interpretation of the results was conducted as suggested by the manufacturer.

### Commercial PTB ELISAs

The commercial kit Parachek (CSL) was used according to the manufacturer's instructions. Capture antigen is a sonicated extract of *M. avium paratuberculosis* strain VRI 316/102-2 and kits are also supplied with adsorbing *Mycobacterium phlei* antigen. Another commercial PTB ELISA kit, HerdChek Mpt (IDEXX) was also used according to the manufacturer's instructions. The capture antigen was Map strain VRI 316/102-2 crude protoplasmic antigen and, as in the first kit employed, was supplied with adsorbing *Mycobacterium phlei* antigen. All sera were tested for paratuberculosis by the two commercial ELISAs based on pre-absorption of the sera with *M. phlei*.

### Study design

This is a retrospective study since the hypothesis of the interference of anti-*M. bovis* antibodies in the PTB commercial tests raised after the slaughtering of the animals. Most common available tests were used in this study, i.e. two commercial ELISA kits for PTB and SCTT and  $\gamma$ -IFN assay for TB. Cows were considered as TB-infected when reactive by both SCTT and  $\gamma$ -IFN test. After the slaughtering, tissues and lymph nodes samples were macroscopically examined and cultured for *M. bovis*, using standard bacteriological procedures. Samples were considered as PTB-reactive when they presented concordant positive results for both commercial ELISA kits employed. For both TB and PTB, cows with positive result by only one of the employed assays were not considered for this study, since it

was not our purpose to evaluate the performance of each test separately. Although Map culturing of the slaughtered animals was not conducted, faeces samples were randomly collected from adult cows (20%) on the same herds and processed for Map culturing. Animals were bled for  $\gamma$ -IFN assay and for PTB-ELISAs on the same day of tuberculin inoculation, moments before the injection. Results were statistically analyzed using a chi-square ( $\chi^2$ ) test.

## RESULTS

In the light of the results of the SCTT and the  $\gamma$ -IFN assays, 28/65 cows were considered TB-infected. From those, 17 yielded *M. bovis* at the culturing procedures. The other eleven animals, although presenting negative culture results for *M. bovis*, presented lesions with typical macroscopical aspect of TB and were also considered as TB-positive. Sixteen cows of the TB infected herds plus 21 from the control herd were not TB-infected and showed negative results by both SCTT and  $\gamma$ -IFN tests. According to these results, the cattle were categorised into three groups; Group A with 28 TB-infected animals, Group B with 16 TB-negative animals but from herds where TB occurred, and Group C, with 21 TB-negative cows from a TB-free herd. None of the samples presented a high optical density (OD) reading in the  $\gamma$ -IFN assay when avian tuberculin was used as stimulating antigen. Culturing for Map from other animals from the same herds were all negative, what reinforces that those herds are PTB-free.

From the forty-four cows from TB-infected herds, 26 (59.1%) presented positive results for tests for both *M. bovis* and Map

**Table 2.** Results obtained by two commercial ELISA-based kits for the diagnosis of paratuberculosis of forty-four cows from tuberculosis-infected herds

PTB-ELISAs TB-status	Reactive	Non-Reactive	Total
Positive	20 (71.4%)	8 (28.6%)	28
Negative	6 (37.5%)	10 (62.5%)	16
Total	26	18	44

**Table 3.** Optical density (OD) ratios obtained by two commercial ELISA-based kits for the diagnosis of paratuberculosis of 26 reactive cows from tuberculosis-infected herds

PTB-ELISAs TB-status	N	Pcheck			Herd Check		
		MinOD	MaxOD	Mean OD	MinOD	MaxOD	Mean OD
Positive (Group A)	20	0.167	2.829	(0.573)	0.147	2.155	(0.638)
Negative (Group B)	6	0.239	1.322	(0.689)	0.157	1.243	(0.524)

infection tests. When evaluated according to their TB status, 20/28 (71.4%) of the animals from Group A (TB-infected) were reactive in PTB-ELISAs, while 6/16 (37.5%) cows of group B (TB-negative cows from TB-infected herds) were positive for PTB. No animal from Group C, which comprised the cows from the TB-free herd, was reactive in PTB-ELISAs (Table 2). OD-readings obtained for different groups in the two commercial ELISAs are listed in Table 3. Differences in seroreactivity of animals from Group A and B, Group A and C and Group B and C were all significant ( $P < 0.05$ ).

## DISCUSSION

We assumed that the status of TB-infection of the animals was reliable as international standards of diagnosis were used for its definition. The use of results where IFN and the skin test agreed increased reliability, since the possibility of occasional errors was diminished considerably. Additionally, positive culturing and gross examination of the lesions obtained at the abattoir contributed to confirm the TB-positive status of those animals. The tuberculin skin reaction is the standard test in Brazil and is widely used in many other countries, whereas the  $\gamma$ -IFN assay is based on the detection of  $\gamma$ -IFN in plasma supernatants from tuberculin stimulated whole blood cell cultures (14). In a previous study using this assay in Brazil (8), a relative sensitivity of 100% was found under field conditions, in agreement with several studies that evaluated its efficacy in relation to intradermal tests elsewhere (16).

The  $\gamma$ -IFN assay is also considered to be more sensitive and capable of detecting infected animals earlier than other tests, including skin tuberculin tests (8). Therefore, even animals in the initial phases of the TB-infection would probably be identified, and Group B animals are very probably uninfected, although members of an infected herd. In order to investigate the interference of TB in PTB assays at a herd level, the uninfected animals from positive herds (Group B) and those from the TB-free herd (Group C) were studied in separate groups.

PTB is very rarely described in Brazil. Few studies have reported the isolation of the agent (13). In spite of the absence of negative faecal culturing for Map on those animals, other animals from the same herds were later tested by culturing for Map and no evidence of infection in those herds was observed.

It is important to bear in mind that the studied animals had never presented signs of PTB and belonged to closed herds where clinical signs of PTB was unknown. Therefore, the possibility that the animals were infected with Map in a sub-clinical form, although not impossible, is highly improbable. The absence of history of PTB in those herds, not only at the moment of the collection of samples but also for the following three years also reinforces the PTB-negative status that we have assumed for those herds.

Olsen *et al.* (10) employed a commercial ELISA for PTB on sera from four cattle with natural bovine TB infection and eight experimentally infected animals. The eight experimentally infected animals showed a strong positive reaction with a mean OD ratio of 1.04 (range 0.41-1.49). One of the naturally infected animals was also positive using the commercial kit. The authors concluded that several of the bovine TB sera had high levels of cross-reactive antibodies and that the commercial ELISA was unable to distinguish between paratuberculosis and bovine TB. Those findings are in agreement with the present study.

We studied only naturally infected animals and, in order to improve reliability of the diagnosis, only animals with two agreeing results for TB and PTB were considered. Even then, an enormous number of cross-reactions were observed, which finding was of considerable concern. Since TB still occurs in many countries, the available commercial kits for PTB cannot be considered to be reliable tools unless a complete investigation on the incidence of TB in each herd is undertaken.

Although a concomitant infection of TB and PTB has already been described in dairy sheep in Argentina (5), co-infection in all animals in our study would appear to be very unlikely. Humoral response in Map-infected cattle and, consequently, sensitivity of the ELISA varies according to the clinical stage of the disease. It has been stated that the development of humoral responses in mycobacterial infections indicates bacterial multiplication and that ELISAs become more sensitive with the evolution of the disease and the appearance of clinical signs (2). Collins *et al.* (3) observed that cattle with high ELISA results, called "strong-positive" results, have a post-test probability of concurrent Map fecal shedding of >90%. If Map infection was really present in those herds, animals presenting OD ratios as high as 1.467 or 2.829 (Paracheck), as observed in some of the studied cows, were expected to present clinical symptoms. The herd should have a history and previously presented some animals with characteristic signs of the disease, such as chronic diarrhoea, emaciation, cachexia and even death. It is important to reinforce that the five herds investigated had never presented a history of paratuberculosis or any animals with a suspected infection, and that characteristic lesions of the disease, such as thickness of intestines or enlargement of intestinal lymph nodes had not been reported for slaughtered cows coming from those herds. It is also important to emphasize that later culture testing of other cows from those herds did not present Map positive results.

Bacteriological diagnosis of PTB is very slow, time-consuming and expensive and cannot be considered as a screening test for diagnosis in developing countries. Since the skin test with the Johnin antigen has been shown to be highly unspecific (10), serological tests, especially ELISA-based methods, offer the highest sensitivity testing for PTB due to their capacity to detect small amounts of antibodies. They have been widely recommended for the routine diagnosis of the infection, mainly as screening tests (9).

Nevertheless, in spite of the supposedly high specificity of commercial PTB-ELISA as a consequence of the pre-adsorption step with *M. phlei*, which is believed to remove most of the cross-reactive antibodies formed during infection with related environmental mycobacteria, few studies have evaluated anti-*M. bovis* antibody interference (11). It is currently known that the specificity of those tests may be poor due to antigenic cross-reactivity between Map and other mycobacterial species (4). The most likely reason for this finding is that all available antigen-based diagnostic tests for PTB use an undefined mixture of proteins as antigens, and these may not be specific for Map (1,6).

Thus, it is extremely important to characterise the chemical and antigenic composition of the antigenic proteins which could potentially be used as antigens for more specific tests. It is however expected that the use of purified specific proteins as antigens for diagnostic tests to distinguish between TB and PTB may reduce the sensitivity of those tests. Olsen *et al.* (11) reported encouraging results using as antigens the 14-kDa antigen, AhpC and AhpD, and obtained a good discrimination between the two mycobacterial diseases and an increase in specificity when compared to the commercial ELISA for PTB. More recently, Waters *et al.* (15) suggested that rESAT-6: CFP-10 should be useful for the differentiation of infection by *M. bovis* and by Map.

One important and unexpected finding in our study was the occurrence of 35.7% cross-reactivity in PTB-ELISAs of TB negative animals from three herds where TB occurs (Group B). One possible explanation for this finding is that these animals could have had previous contact with antigens from *M. bovis*, but did not develop infection. Therefore, the use of commercial ELISA kits for the diagnostic of PTB should always be decided at the level of the entire herd.

Sera from animals from TB-free herds did not present false-positive reactions that could be attributed to cross-reactions, as expected. Those animals had a comparable immune status to animals in countries where bovine TB has been eradicated, and have probably never been in contact with *M. bovis* antigens. Therefore, we can conclude that the pre-adsorption step with *M. phlei* was adequate to eliminate cross-reaction with antigens from environmental mycobacteria. This finding also reinforces the idea that the observed false-positive reactions in PTB-ELISAs were due to anti-*M. bovis* antibodies.

In a strict point of view, the absence of a definitive confirmation of the PTB-negative status of the tested animals, i.e. by histopathology or bacterial culturing, would compromise the reliability of the results. Nevertheless, we are convinced that epidemiological evidence on those animals is strong enough to allow this conclusion. In our opinion, the low prevalence of the disease in Brazilian herds, the absence of clinical signs or history of PTB in the herds at the moment of the study or in the following three years, associated to the high reading observed at the PTB-ELISAs, which should be associated to advanced and clinical disease, are sufficient to demonstrate the PTB-negative status of those animals and then support our conclusions.

In conclusion, we suggest that naturally occurring bovine TB strongly interferes in the performance of commercial ELISAs for bovine PTB diagnosis. Commercial ELISAs could not differentiate between these two infections and cannot be applied in a reliable way to the diagnosis of PTB in TB-infected herds. Further studies should investigate the inclusion of supplementary pre-adsorption steps or the use of different purified proteins as antigens, in order to improve commercial ELISAs for PTB diagnosis, making them recommendable for developing countries.

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#### RESUMO

##### **Interferência da tuberculose no desempenho de ELISAs comerciais utilizados para o diagnóstico de paratuberculose**

Quarenta e quatro animais provenientes de cinco rebanhos infectados com tuberculose e livres de paratuberculose, e 21 animais provenientes de rebanhos livres de ambas as infecções foram analisados. Todos os animais foram testados para tuberculose pelo teste intradérmico de PPD-bovino e pelo ensaio comercial de Interferon-gama (IFN- $\gamma$ ). Para o diagnóstico de paratuberculose, dois ELISAs comerciais foram usados neste estudo. Os animais foram divididos em três grupos, de acordo com os resultados obtidos pelos testes diagnósticos para tuberculose. O grupo A foi composto por 28 animais com resultados positivos para tuberculose; o grupo B foi formado por 16 animais provenientes de rebanhos infectados com tuberculose, porém com resultados negativos nos testes diagnósticos para esta infecção. O grupo C foi composto por 21 animais provenientes de propriedades livres de ambas as

doenças e que foram utilizados como grupo controle deste estudo. Vinte dos 28 animais do grupo A (71,4%), 6/16 do grupo B (37,5%) e nenhum animal do grupo C foram reativos aos ELISAs para diagnóstico de paratuberculose, o que demonstrou que os kits comerciais utilizados não são capazes de diferenciar entre ambas as doenças. Os resultados aqui obtidos sugerem que a ocorrência natural de tuberculose pode interferir no diagnóstico da paratuberculose e os ELISAs comerciais disponíveis não são seguros para utilização em rebanhos onde há tuberculose.

**Palavras-chave:** paratuberculose, tuberculose, *Mycobacterium bovis*, *Mycobacterium avium paratuberculosis*

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