**Interference of anti-*M. bovis* antibodies in serological tests for paratuberculosis**

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**ABSTRACT**

ELISA assays are broadly used as a diagnostic tool of paratuberculosis. Nevertheless, problems of specificity are due to the great number of shared antigens between MAP and other Mycobacteria, especially the closely related *M. avium*. An *M. phlei* preabsorption step in the ELISA eliminates much of the cross-reacting antibody due to environmental bacteria. Despite this, other infections such as bovine tuberculosis, a frequent occurring disease in Brazil and in many other developing countries, may interfere with the specificity of the test. In this study, 97 sera from *M. bovis* infected cows that were skin-test reactive and confirmed as infected by histopathology. Sera were tested in a PPA (paratuberculosis protoplasmatic antigen – Allied Monitor, USA) ELISA that is routinely used for the diagnosis of paratuberculosis. From the 97 sera 25 (26.59%) presented an OD >0.60; the cut-off point for this test was standardized at OD 0.35. From these, twenty sera were randomly chosen for immunoblot analysis. Eighteen sera (90%) strongly reacted with four PPA bands: one 32-36KDa, one 42KDa and two others between 77 and 100KDa. An ELISA-positive control serum sample from a confirmed case of Johne’s disease from a region free of tuberculosis also recognized these same four bands. The findings indicate that the proteins are shared between *M. bovis* and MAP. In conclusion, although ELISA tests may be used as valuable tools for the detection of paratuberculosis, interpretation of results in herds known to be infected with *M. bovis* should be done carefully.

**INTRODUCTION**

The most common immunological tests to identify MAP (*Mycobacterium avium* subsp. *paratuberculosis*) infection are the complement fixation test (CFT), agarose gel immunodiffusion (AGID), and ELISA. ELISA-based methods show the highest sensitivity of serological tests for paratuberculosis (PTB), since these assays are capable of detecting small amounts of antibodies. On the other hand, ELISA is less specific than fecal culture (Collins, 1996) and, therefore, not recommended as the sole diagnostic tool. In Brazil, Marassi et al. (2005) reported a sensitivity of 76.7% and a specificity of 70.5% in an improvement of a previously described ELISA recommended as screening test for herds (Ferreira et al., 2002).

MAP shares several antigens with other mycobacteria, including *M. bovis*. Reports show that paratuberculosis can compromise the specificity of bovine tuberculosis (TB) diagnostic tests, and the influence of MAP co-infection on the diagnosis of bovine TB stills needs investigation (Vordemeier et al., 1999). Although the interference of bovine TB on the efficacy of Johne’s disease diagnostic tests has not been widely evaluated (Olsen et al., 2001), natural infection with MAP was demonstrated to lead to false-positive reactions in TB skin tests (Buddle et al., 2003).

Since most commercial tests for the serological diagnosis of PTB have been developed in countries where bovine TB is eradicated or at least controlled, minimal effort in wasa taken to evaluate the interference of anti-*M. bovis* antibodies in those tests. However, in various developing countries where both mycobacterial infections occur, the potential for interference of bovine TB in PTB-ELISAs must be understood before recommendations can be made for Johne’s disease herd surveillance.

In order to reduce interference and increase ELISA specificity, recent studies focused on the development of tests using new purified immunogenic and species-specific antigens. AhpC, AhpD and 14kDa proteins were used in an ELISA assay in order to discriminate MAP infected from *M. bovis* infected-cattle (Olsen et
al., 2001). Recently, another ELISA assay was tested using the ESAT-6:CFP-10 fusion protein with the same purpose and promising results (Waters et al., 2004). Although these antigens could augment specificity levels of immunological assays, the assays were less sensitive and could misdiagnose animals in the early stages of the disease. Regardless of this, new antigens could be helpful to diminish cross reactions between antibodies due to MAP vs. *M. bovis*. (Olsen et al., 2001; El-Zaatari et al., 2002; Waters et al., 2004).

The aim of this study was to evaluate the performance of a PTB-ELISA in an *M. bovis* infected herd without symptoms of paratuberculosis.

**MATERIALS AND METHODS**

**Serum samples**

Ninety-seven adult cows with TB from five dairy herds with a previous history of bovine tuberculosis (including clinical cases and recovery of *M. bovis* from slaughtered animals) were studied. At the moment of sample collection, no cattle in the herds had chronic diarrhoea, weight loss or any suspicion or history of paratuberculosis. Infection with *M. bovis* was diagnosed by intradermal tuberculin test. The test consists 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 hours. The interpretation of the results was performed according to the recommendations of the Department of Agriculture in Brazil, i.e., a test-positive animal has more than 4.0 mm of swelling at the site of inoculation. All test-positive animals were slaughtered and cases were confirmed by histopathology and visualization of acid-fast bacilli on Ziehl-Neelsen staining of lesions consistent with tuberculosis, mainly located in lymph nodes and lungs. Serum from a confirmed case of bovine paratuberculosis with a strong ELISA result located in a TB-free region was used as a control (kindly provided by the Johne’s Testing Center, U. Wisconsin, USA).

**Study design**

The study was conducted in two steps. First the 97 sera were tested by PPA-ELISA as follows. From those, twenty test-positive sera were randomly chosen for the western blot analysis.

**PPA-ELISA protocol**

ELISA was conducted as previously described (Marassi et al., 2005). Briefly, Paratuberculosis Protoplasmic Antigen (Allied Monitor, USA) was used as a capture antigen. It was diluted in a carbonate buffer (9.6 pH) and adsorbed in a 96-well plate overnight at 8°C. Each well was coated with 100 μL of antigen solution (0.07 mg/mL) and blocked with 2% casein in TBST (Tris 10mM, 0.9% NaCl, 0.2% Tween 20). *M. phlei* extract (5 mg/mL) was mixed with an equal volume of serum for 60 minutes with constant agitation at 37°C. After that, the *M. phlei* – serum suspension was incubated overnight at 8°C., the pre-adsorbed sera were added to the wells (100 μL/well) and incubated for one hour at 37°C. Monoclonal bovine IgG linked to alkaline phosphatase was used as a conjugate. Immune complexes were detected by addition of p-NPP substrate (1 mg/mL) with incubation for 30 minutes at room temperature in the dark. Three wash steps with TBST were included after incubation with the first and second antibody. Sera were tested at the dilution 1:100 and all assays were run in duplicate. Any assay with a between-well coefficient of variation of >15% was repeated. The sample’s optical density (OD) was measured by a wavelength of 405nm. Final results were expressed as the ratio S/P, obtained by dividing the mean OD value (S) of a given serum divided by the mean OD (P) of the positive control. Thus, sera were classified as positive when their S/P was equal or higher than 0.35.

**Western-blot**

Twenty micrograms of PPA were applied to a 12% SDS-PAGE gel and protein electro-transferred onto nitrocellulose membranes using a mini-Protean II system (Bio-Rad laboratories, USA), according to the manufacturer’s protocol. Membranes were blocked in a solution of 5% of casein (Sigma-USA) in TBST (Tris 10mM, 0.9% NaCl, 0.2% Tween 20) overnight at 8°C. *M. phlei* was mixed with an equal volume of serum for 60 minutes with constant agitation at 37°C. After that, the *M. phlei* – serum suspension was incubated overnight at 8°C. Serum samples were used at a dilution of 1/100 and incubated for one hour. A 1/5000 IgG bovine conjugated with alkaline-phosphatase (Sigma-Aldrich laboratories, USA) was added and the
membrane was incubated for one hour at room temperature. Three wash steps of five minutes with TBST were included after incubation with the first and second antibody. The antibody-antigen complex was detected using a solution of 4mL of BCIP/NBT (Sigma-Aldrich laboratories, USA) for 10 minutes. The reaction was stopped with distilled water.

RESULTS

From the 97 tested sera, 39 (40.2%) were test-positive with PPA-ELISA. From those, fourteen presented weak reactions bordering the 0.35 cut-off of the test and twenty-five (25.6%) presented SP > 0.60. Those animals were considered as strong positives and their samples were tested twice. As stated, none of these cattle presented symptoms characteristic of paratuberculosis nor had any herd a previous history of Johne’s disease (Fig. 1).

![Fig. 1. Performance of 20 selected sera at ELISA. Cut-off value: 0.35.](image)

From those strongly ELISA-positive sera, twenty were randomly chosen for western blot analysis in order to identify the immunodominant bands that reacted with these sera. This analysis demonstrated that 18 sera (90%) reacted strongly with four PPA bands: one 32-36kDa, one 42kDa and two others between 77-90kDa. The positive control serum also recognized these same four bands, indicating the bands are shared by *M. bovis* and MAP (Fig. 2).

![Fig. 2. Western blot analysis of 20 sera from *M. bovis* infected cattle. Arrow indicates the control sera.](image)

DISCUSSION

Tuberculosis is an endemic bovine infection in many developing countries which is controlled through periodic PPD testing and culling of the reactive animals. The usefulness of serologic surveillance of mycobacterial infections is influenced by several variables, such as the nature of the capture antigen used,
the evolution of the disease with the switch from cellular to humoral immunological response, the genetic of the exposed herd, and the epidemiologic conditions of each country (Köhler et al., 2001).

Cross-reacting antibody responses due to infection by different mycobacterial species can interfere with diagnosis (Waters et al., 2004). Immunological assays for paratuberculosis diagnosis were developed to identify the infection in a herd earlier than with the culture test. Research efforts have been made to develop serological assays employing purified and species-specific antigens in order to identify subclinical paratuberculosis free of interference due to other mycobacterial infections (El-Zaatari et al., 1997).

Commercial PTB-ELISAs have a supposedly high specificity as a consequence of the preadsorption step with *M. phlei* which is believed to remove most of the cross-reactive antibodies formed during infection with related environmental mycobacteria. Nevertheless, few studies have evaluated the anti-*M. bovis* antibody interference in those tests (El-Zaatari et al., 1997, Olsen et al., 2001).

Olsen et al. (2001) reported that the specificity of commercial assays for paratuberculosis may be very low and noted that commercial ELISAs may be unable to distinguish between PTB and TB. Those results are in agreement with the present study. We studied only naturally *M. bovis* infected animals and found that sera from these animals strongly reacted to several PPA antigen proteins. These findings lead to the supposition that some proteins of PPA are shared between MAP and *M. bovis*, which could confound the diagnosis of both mycobacterial diseases.

In the present study, twenty-five (26.6%) of the animals with tuberculosis were strongly reactive to PPA. All of these animals were confirmed as cases of tuberculosis based on typical lesions and the presence of acid-fast organisms; these findings also indicate that they were in an advanced phase of the disease. As in many other mycobacterial infections, it is expected that animals in advanced stages of tuberculosis produce antibodies against the infectious agent, *M. bovis*. Many researchers have demonstrated the presence of several proteins shared between mycobacterial species (Mutharia et al., 1997; Olsen et al., 2001; Waters et al., 2004). Therefore, antibody cross-reaction to shared antigens with these sera was not an unexpected. The most likely reason for this finding is that commercial antigen-based diagnostic tests for PTB use as antigens a crude mixture of whole-cell proteins that may not be completely specific for MAP (Buddle et al., 2003; Huntley et al., 2005).

Sera of animals in different stages of PTB have been reported to react to a 34kDa protein (Gilot et al., 1994), while a 32kDa molecule was identified in MAP as well as in *M. bovis* that produced cross-reactive responses. (Amadori et al., 2002). We believe those proteins may be related to the 32-36kDa band observed on this study’s Western Blot analysis.

In the present study, sera also reacted with a 42kDa component of PPA. This band was also identified as an intracellular molecule of MAP which cross-reacted with *M. bovis* and the *M. avium* complex (Harris & Barletta, 2001). Further studies are being conducted to obtain a better definition of the nature of the two bands between 77-90kDa.

**CONCLUSION**

Serologic tests are valuable tools for control and diagnosis of paratuberculosis and tuberculosis infection. Therefore, further studies using specific antigens to reliably differentiate between anti-*M. bovis* and anti-MAP antibodies are imperative for the control of the two diseases in developing countries where both infections may occur simultaneously.
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