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# Accuracy assessment and screening of a dairy herd with paratuberculosis by three different ELISAs

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ABSTRACT

Although the culture of *Mycobacterium avium* subsp. *paratuberculosisis* is the gold standard for the diagnosis of paratuberculosis, this bacterium is difficult to grow. In contrast, serological tests like ELISAs are inexpensive, rapid, and easy to perform. The aims of this study were to evaluate the accuracy of three different ELISAs: one with the commercial antigen PPA-3, another one with L5P (a recently described lipopentapeptide), and a third one with an in-house antigen whole cell lysates (WCL) of *M. avium* (MAA) strain *D4ER* (Study 1), and to compare them with other tests for paratuberculosis (PTB) diagnosis (Study 2). In Study 1, the sensitivities of the three ELISAs tested were 74.1%, 37% and 74.1%, respectively, whereas their specificities were 98.9%, 100% and 100%, respectively. In Study 2, we compared the three above-mentioned ELISAs with the intradermal reaction test using Avian PPD (PPDa) and fecal culture associated with Ziehl–Neelsen stain and PCR tests, in a dairy herd with 4.6% of cows with clinical signs of PTB. The results showed that fecal samples from 14 cows (16%) were culture-positive and that fecal samples from nine cows (10%) were PPDa-positive. Most of these animals (culture-positive and PPDa-positive) were detected as positive with any of the three ELISAs tested. Serological results showed that 31% of the animals were positive to ELISA-PPA-3, 17% to ELISA-L5P and 42.5% to ELISA-WCL. The combination of these three ELISAs identified 50.6% of the animals as positive in the infected herd. In particular, the results show that the locally developed ELISA seems to be useful for identifying many infected animals in a herd.

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## 1. Introduction

Paratuberculosis is caused by the infection of calves with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), resulting in chronic enteritis. PTB is characterized by an early Th1 response followed by a humoral response, characterized by the production of immunoglobulins, often associated with the emergence of clinical disease (Sigurðardóttir and Valheimb, 2004). The cellular response can be assessed by the determination of skin test with avian Purified Protein Derivative (PPDa) or production of IFN-g by blood cells stimulated with PPDa (Stabel et al., 2007; Robbe-Austerman et al., 2007). External factors could influence the specificity of these tests probably by the presence of environmental mycobacteria in some herds (Kalis et al., 2003). Fecal culture for *M. avium* subsp.

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*paratuberculosis* is the reference test for PTB diagnosis. However, diagnosis based on antibody detection using ELISA technology is frequently used because of the low cost and high-throughput potential through automation (Collins et al., 2005). Several commercial ELISA kits for bovine PTB are currently available, and multiple studies have compared their accuracy (Fry et al., 2008; Collins et al., 2005). The main limitation of these serological tests is their inability to accurately identify animals early in the course of the infection (Sugden et al., 1997). Also cross-reactive antibodies can be observed in cattle infected with mycobacteria other than MAP (Bannantine et al., 2008). Recently, it has been found that MAP strains produce L5P, a lipopentapeptide that is the target for highly specific humoral response in MAP-infected animals (Biet et al., 2008). Here, we evaluated this antigen with a panel of 114 sera, 27 of which were from animals with PTB confirmed by culture.

The objectives of this work were to evaluate three ELISAs based on different antigens for diagnosis of PTB (Study 1), and to compare them with the following tests: intradermal reaction test using PPDa and bacterial growth in culture, examined by Ziehl–Neelsen (ZN) stain and PCR (Study 2), in a herd with PTB.

## 2. Materials and methods

### 2.1. Study 1

A total of 114 serum samples were used to establish the sensitivity, specificity and performance of different ELISAs. Samples were from: (a) cows with isolation of MAP in feces ($n = 27$) and (b) cows from negative herds with no suspected cases of PTB and negative tests (fecal cultures, serology and INF-g) ($n = 87$). To evaluate whether any of the antigens of these different ELISAs was able to differentiate the animals infected with MAP from those infected or sensitized with other mycobacteria, we used 89 serum samples: 14 from MAP-infected animals, 18 from cows with bovine tuberculosis (bTB), 28 from healthy animals, and 29 from cows exposed to other mycobacteria. This latter group came from bTB- and PTB-free herds that tested positive by skin test with PPDa.

### 2.2. Study 2

Eighty-seven Holando-Argentino cows were randomly selected from a dairy herd that presented clinical cases of PTB. These animals were intradermally injected with 0.1 mL of 0.5 mg/mL PPDa, either in the inner right caudal fold or in the neck. Readings were made after 72 h.

Fecal cultures were carried out according to Whittington et al. (1999) in solid media, with some modifications. Then, colonies from culture-positive samples were picked up after 6 weeks of incubation, and examined by ZN stain and PCR. PCR assays were carried out to confirm presumptive mycobacteria. Briefly, colonies were resuspended in 250 μL of sterile distilled water, and subjected to 40 min at 90 °C. Then, 5 μL of this sample was examined by PCR with primers against: IS*900*, previously described by Collins et al. (1993) (218-bp amplification product and 65 °C annealing temperature), IS*1245*, previously described by Guerrero et al. (1995) (427-bp amplification product and 57 °C annealing temperature), and IS*6110*, previously described by Hermans et al. (1990) (245-bp-amplification product and 65 °C annealing temperature). Amplifications were performed in a final volume of 50 μL containing: 10 μL of 5×·PCR buffer (Green GoTaqBuffer, Promega, Madison, WI, USA), 4 μL of 2.5 mM dNTPs mix, 1 μL of 20 pmol of each primer, 5 μL of template sample, 1.25 U of GoTaq polymerase (Promega, Madison, WI, USA), and sterile double distilled water to reach the final volume. The conditions for amplification consisted of an initial denaturation step at 95 °C for 5 min and then 30 cycles: at 95 °C for 1 min (denaturation), annealing temperature for 1 min and 72 °C for 1 min (extension). In the final step, an additional extension at 72 °C for 1 min was carried out. Amplifications were performed in a Programmable Thermal Controller (Mj. Research Inc., Waltham, MA, USA). To visualize the amplified products, 10 μL of the PCR product was loaded in 1.5% agarose gels containing ethidium bromide 5% at 80 V for 1 h. In each case, DNA from MAP, *M. avium* subsp. *avium* (MAA) or *M. bovis* were included as positive control.

### 2.3. ELISA method (Study 1 and Study 2)

Three different antigens were used: PPA-3 (Allied Monitor), L5P (prepared as in Biet et al., 2008), and WCL. WCL was prepared from the *D4ER* strain of *M. avium*, cultured in Watson Reid liquid medium for 2 months. The culture was filtered, and the mycobacteria inactivated by gamma irradiation. The mycobacteria were then processed with a cell disrupter at a pressure of 40 ksi, the cell lysate centrifuged for 30 min, and the supernatant used as antigen. Microtiter plates were coated at 4 °C overnight with 100 μL of 40 μg/mL PPA-3 or 50 μg/mL WCL in carbonate buffer (pH: 9.6). L5P (5 μg) suspended in methanol was loaded into each well and air-dried. Then, the plates were saturated with 100 μL of PBS/0.5% (w/v) gelatin for 1 h at 37 °C, then washed five times with PBS/0.1% Tween 20 (PBS/T) and incubated for 1 h at 37 °C with 100 μL of 100-fold dilution of sera in PBS/T containing 0.5% (w/v) gelatin. The plates were then washed five times with PBS/T and incubated for 30 min at 37 °C with 100 μL of 1500-fold dilution of peroxidase-conjugated protein in PBS/T containing 0.5% (w/v) gelatin. Plates were washed five times with PBS/T, and 50 μL of peroxidase substrate was added. Optical density (OD) was measured at 405 nm.

## 3. Results

In Study 1, the sensitivity of the three ELISAs tested was 74.1% for ELISA-PPA-3, 37% for ELISA-L5P and 74.1% for ELISA-WCL, whereas the specificity was 98.9%, 100% and 100%, respectively (Fig. 1). As shown in Fig. 2(A)–(C), the three ELISAs were able to differentiate animals with PTB from animals with bTB, as well as from animals naturally exposed to other mycobacteria and healthy animals.

In Study 2, four out of 87 cows (4.6%) from this herd showed clinical signs of disease (diarrhea). Fecal samples

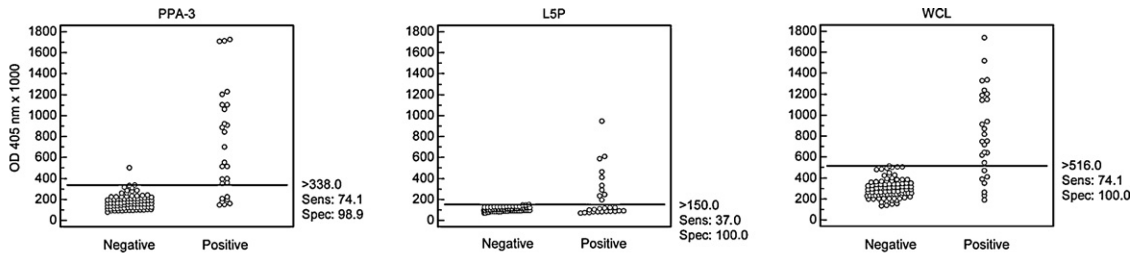


**Fig. 1.** Dot plot based on the ROC analysis to evaluate the sensitivity and specificity of the ELISAs based on different antigens. The PPA-3 antigen (Allied Monitor), the antigen L5P from MAP, and a WCL from the *Mycobacterium avium* strain *D4ER*. Each antigen was determined with: (a) sera from cows with isolations of MAP in feces (positive) ($n = 27$ serum samples), (b) negative sera (negative) from cows coming from a PTB-free herd and with negative fecal cultures ($n = 87$ serum samples). The data from ELISAs are expressed as optical density measured at 405 nm $\times$ 1000 and plotted in ROC Interactive dot diagrams, with MedCalc software.

**Fig. 2.** Differentiation between MAP-infected animals ($n = 14$) and, healthy ($n = 28$), naturally exposed to other mycobacteria ($n = 29$), *M. bovis*-infected animals ($n = 18$) by using (A) ELISA-PPA-3, (B) ELISA-L5P and (C) ELISA-WCL. Each antigen was coated to an ELISA plate and then incubated with sera as described in Section 2. Data were analyzed by one-way ANOVA with Dunnett's post test and represent mean $\pm$ S.D. of each group of cattle sera.

from 14 cows (16%) presented bacterial growth in culture and the colonies were ZN-positive. However, only six cultures were PCR-IS*900*-positive and two PCR-IS*1245*-positive. All the isolates were PCR IS*6110*-negative.

Nine out of 87 animals (10%) tested in this herd were classified as positive reactors to a single intradermal injection of PPDa. The serological analysis showed that 31% ($n = 27$) of the animals were positive to ELISA-PPA-3, 17% to ELISA-L5P ($n = 15$) and 42.5% to ELISA-WCL ($n = 37$). A total of 44 out of 87 animals (50.6%) were serologically positive to at least one of the three ELISAs tested (Table 1).

## 4. Discussion

Antibodies to MAP in milk or serum can be detected by different methods: agar gel immunodiffusion, complement fixation and ELISA. The last method showed the highest sensitivity of the serological tests for MAP (Harris and Barletta, 2001). However, the ELISA method depends on the immunoreactivity of antigen binding on the plastic surface of plates. Several proteins of MAP have been identified and tested for immunogenicity, but their diagnostic potential remains to be validated with appropriate sample sizes (Mikkelsen et al., 2011a). A mix of

**Table 1**
Comparative results from animals that were positive by at least one diagnostic tool (Study 2).

| Animal | Culture | PCR | ELISA | | | PPDa | Clinical signs |
|---|---|---|---|---|---|---|---|
| | | | PPA-3 | L5P | WLC | | |
| 5821 | ZN+ | Neg | + | + | + | – | – |
| 6237 | ZN+ | Neg | + | + | + | – | + |
| 6617 | ZN+ | IS900+ | + | + | + | – | – |
| 6605 | ZN+ | IS900+ | + | – | + | – | – |
| 6613 | ZN+ | IS900+ | + | + | + | – | + |
| 6636 | ZN+ | Neg | + | – | + | – | – |
| 6132 | ZN+ | IS1245+ | + | – | – | – | – |
| A015 | ZN+ | IS1245+ | – | – | – | – | – |
| 6678 | ZN+ | Neg | + | – | + | – | – |
| A005 | ZN+ | IS900+ | + | + | + | – | – |
| A013 | ZN+ | Neg | + | + | + | – | – |
| A028 | ZN+ | IS900+ | + | + | – | – | – |
| A031 | ZN+ | IS900+ | + | – | + | – | – |
| A032 | ZN+ | Neg | + | – | – | – | – |
| 6239 | ZN– | – | + | + | + | – | + |
| 6627 | ZN– | – | – | + | + | + | + |
| 6637 | ZN– | – | + | + | + | + | – |
| 6642 | ZN– | – | + | – | + | + | – |
| 6666 | ZN– | – | – | – | – | + | – |
| A000 | ZN– | – | + | – | + | + | – |
| A030 | ZN– | – | – | – | – | + | – |
| A043 | ZN– | – | – | – | – | + | – |
| A050 | ZN– | – | + | + | + | + | – |
| 6231 | ZN– | – | – | – | – | + | – |
| 6041 | ZN– | – | – | – | + | – | – |
| 6066 | ZN– | – | + | – | + | – | – |
| 6232 | ZN– | – | – | – | + | – | – |
| 6624 | ZN– | – | – | – | + | – | – |
| 6702 | ZN– | – | + | – | + | – | – |
| 6241 | ZN– | – | – | + | – | – | – |
| 6643 | ZN– | – | – | – | + | – | – |
| 6662 | ZN– | – | + | – | + | – | – |
| 6236 | ZN– | – | – | – | + | – | – |
| 6602 | ZN– | – | – | – | + | – | – |
| 6606 | ZN– | – | – | – | + | – | – |
| 6611 | ZN– | – | + | + | + | – | – |
| 6612 | ZN– | – | + | – | – | – | – |
| 6625 | ZN– | – | + | + | + | – | – |
| 6629 | ZN– | – | – | – | + | – | – |
| 6633 | ZN– | – | – | + | + | – | – |
| 6646 | ZN– | – | – | – | + | – | – |
| 6647 | ZN– | – | – | – | + | – | – |
| 6648 | ZN– | – | – | – | + | – | – |
| 6652 | ZN– | – | + | – | – | – | – |
| 6653 | ZN– | – | – | – | + | – | – |
| 6664 | ZN– | – | – | – | + | – | – |
| 6676 | ZN– | – | + | – | – | – | – |
| 6703 | ZN– | – | + | – | + | – | – |
| A003 | ZN– | – | – | – | + | – | – |

ZN: Ziehl–Neelsen; Neg: negatives results by direct colony PCR. These negative by direct colony PCR were contaminated cultures. Thirty-eight samples from 87 were negative for all tests.

proteins as the commercial protoplasmic antigen preparation (PPA-3) has been used in several reports on paratuberculosis diagnosis, and ELISA-PPA-3 has been recommended as a screening test to identify infected herds (Nielsen and Toft, 2008; Ferreira et al., 2002). Since the use of an antigen obtained in the laboratory can make this technique very appropriate at large scale due to its low cost, in the present work, we compared an ELISA based on an antigen prepared in the laboratory (WCL) with the commercial one (PPA-3). These antigens are prepared from *M. avium* D4ER and strain 18, respectively. The serology for the diagnosis of MAP using total antigens obtained from MAP-related bacteria can induce cross-reactivity due to the sensitization of the animals with these bacteria (Leroy et al., 2009). For this reason, we decided to evaluate an ELISA based on a MAP-specific antigen such as L5P (Biet et al., 2008), and to assess the cross-reactivity of the three ELISAs with sera from animals exposed to other mycobacteria.

In Study 1, the specificities of the three ELISAs (PPA-3, L5P and WCL) were similar (98.9%, 100% and 100%, respectively) and antibody response was higher in animals with PTB than in those exposed to other mycobacteria (Fig. 2(A)–(C)). However, while ELISA-PPA-3 and ELISA-WCL had a similar sensitivity (74.1%), L5P detected few animals (37%) (Fig. 1). Nielsen and Toft (2008) made a review about PTB diagnosis where ELISA based on PPA-3 antigen from Allied Monitor for detection of affected cattle had a sensitivity ranging from 0.40 to 0.80 with a specificity from 0.90 to 0.95 in most of the studies evaluated. The values obtained in the present study with WCL and PPA-3 are within the range reported by Nielsen and Toft (2008). However, improving humoral diagnosis with a 'golden' antigen able to discriminate infected animals regardless of their clinical stage and with a very small false-positive rate continues to be a challenge. Several researchers have attempted to search such antigen, and concluded that the most efficient diagnostic test will be obtained with a combination of purified antigens (Bannantine et al., 2008; Leroy et al., 2009). In this context, individual antigens, such as L5P (Biet et al., 2008), could be regarded as good candidates to formulate the antigen combination.

In Study 2 (Table 1), where the group of animals was randomly selected from a dairy herd with clinical signs of PTB, the application of the three ELISAs showed, as in study 1, that the ELISA PPA3 and ELISA WCL detected a higher number of animals than ELISA L5P (31%, 42.5% and 17%, respectively). Logically, the percentage of animals detected by ELISAs in these infected herds is lower than that detected in study 1, where the groups of infected animals tested were selected from animals with PTB confirmed by culture. In this infected herd we found that the animals with clinical symptoms or MAP-positive by culture and PCR were also serologically positive (Table 1). However, most cows that had tested positive with any of the ELISAs did not shed mycobacteria through the fecal matter (35.6%). Despite this, the positive results by ELISA followed by some positive culture of fecal matter allowed us to confirm the presence of bacteria in the herd. It is important to point out that we had serious problems of contamination with fungi in these cultures, which prevented us from correctly interpreting results. These contaminated cultures were negative by direct colony PCR. Clearly, DNA purification would improve PCR detection but would also increase the cost of the test. The problem of contamination of the culture makes the ELISA technique a more appropriate test to be used as an initial screening in a herd. In addition, all PPDa-positive animals from this study were culture-negative, but most of them were ELISA-positive (Table 1). These animals (PPDa-positive ELISA-positive) are probably recently infected because cell-mediated immune response can be measured at an early stage of infection (Mikkelsen et al., 2011b). Here, the antibody response produced in conjunction with cellular response in this group of animals suggests that the humoral immune response can be established relatively quickly after exposure to the bacteria. In this study, by combining the results obtained with the different antigens, the ELISAs tested reached a detection of 50.6% of the animals, but showed no full concordance between them (Table 1). Even so, the ELISAs demonstrated to be useful to identify a high number of PTB suspected animals in a PTB-positive herd.

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