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BRIEF REPORT

Comparison of two serological diagnosis tests for bovine paratuberculosis

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causal agent of paratuberculosis (PTBC), a chronic infectious granulomatous enteritis of ruminants. The PTBC diagnosis with commercial ELISA has limitations in sensitivity and specificity, and its results depend on the state of progress of the disease. This research aimed to evaluate two different ELISAs: (a) an "*in-house*" ELISA with a sonicated antigen obtained from a MAP I47 strain, and (b) a commercial ELISA. In total, the evaluated sample consisted of 394 bovine serum samples from 12 farms in Argentina with high (5–9%) and low (≤0.05%) prevalence of PTBC. The evaluation of the new antigen (2.5 µg/mL) was against a 1:50 dilution of the *M. phlei* faced sera. The cut-off point, sensitivity, and specificity determinations of both techniques were by ROC curve analysis. The area under the curve for the I47 ELISA was 0.9 (CI 95%, 0.93–0.97). With a cut-off point of 8.8%, the sensitivity was 84.3% and the specificity 96.6%. The agreement between both techniques was 0.7 (CI 95%, 0.6–0.8). These results indicate a high discriminative capacity to differentiate positive and negative bovine sera of MAP infection with the I47 ELISA. This result would represent an advantage to dispense with the imported kit.

Keywords *Mycobacterium avium* subsp. *paratuberculosis* · Bovine paratuberculosis · ELISA · Sonicated antigen

Introduction

Paratuberculosis (PTBC) is a chronic, progressive, infectious, granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This disease affects mainly domestic ruminants (Gopi et al. 2022) and a wide variety of wild species. The main sources of infection are both clinically sick animals and those with subclinical signs.

PTBC diagnosis represents a challenge because the effectiveness of the tests depends directly on the clinical stage of the animal. The most widely diagnostic technique used for detecting the humoral response against MAP is the indirect enzyme-linked immunosorbent assay (ELISA), which uses antigens to detect the presence MAP specific antibodies in blood or milk samples (Nielsen 2020).

Between 30% and 40% of bovines that were positive for MAP culture using fecal samples also tested positive for ELISA (Whitlock et al. 2000). The sensitivity of the ELISA in serum is lower than in fecal culture or fecal PCR (Nielsen and Toft 2008). In the early stages of the disease, the sensitivity can vary between 7% and 15%, but it increases as the infection progresses (Nielsen and Toft 2008). According to Nielsen and Toft (2008), the specificity of the ELISA varies between 40% and 100% depending on the reagents used and the categories of the animals. For this reason, various authors raised the need to identify new antigens to improve PTBC diagnosis.

Diagnostic tests are essential to detect infected animals to establish the control measures for MAP infection. This research aimed to evaluate and compare an "*in-house*" ELISA with a validated commercial kit for the diagnosis

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of PTBC in cattle herds, including infected and uninfected animals.

Materials and methods

Serum and fecal samples

We examined 394 samples of blood serum and feces collected from adult dairy cattle and breeding stock of Aberdeen Angus, Hereford and Argentine Holstein breeds, aged ≥ 3 years, from 12 establishments across Argentina.

The uninfected population compromised 324 cattle from four establishments. One of these establishments is located in Buenos Aires province, an endemic area for PTBC with a prevalence is 5–9%, while the remaining three are located in the province of Tierra del Fuego, an area with sporadic PTBC, with a prevalence ≤ 0.05%. None of the herds had a history of animals with clinical signs of PTBC in the decade leading up to the study or animals with positive test results.

To identify cases of bovine PTBC, we utilized 70 cattle (presenting clinical signs compatible with the disease and confirmed by PCR) from eight establishments in Buenos Aires. All herds had a history of clinical PTBC, with a prevalence of 5–9%.

Both, infected and uninfected animals belonged to the same age range.

Blood samples were taken from the jugular vein using a 16G needle attached to a 20 mL syringe, collecting a volume of 20 mL. Serum was isolated by centrifugation at 1000 g for 10 min and stored at -20 °C until analysis by ELISA. Fecal samples were obtained directly from the rectum and stored at -20 °C until processed for fecal culture and PCR.

Bacterial culture

The fecal samples were cultured according to Paolicchi et al. (2003). Each fecal sample (10 g) was decontaminated with 90 mL of 0.75% (w/v) hexadecyl pyridinium chloride solution (HPC) (Sigma Aldrich, USA), shaken for 30 min and then left still at room temperature. The suspension (40 mL) was centrifuged at 3500 rpm for 15 min and subsequently the precipitate was resuspended in 1 mL of phosphate buffered saline (PBS) solution. It was inoculated 100 µL into each tube containing Herrold eggs yolk agar medium (HEYM) supplemented with mycobactin (HEYM without supplement or mycobactin; HEYM with supplements: 2 mg/L mycobactin J (Allied Monitor, USA) and sodium pyruvate (4.1 g/L); HEYM with supplements and antibiotics (amphotericin B 2.0 mg/mL; vancomycin 100 µg/mL; nystatin 100 µg/mL; nalidixic acid 3.0 mg/mL). The tubes were incubated at 37 °C for 120 days.

Reference MAP strain

The sequenced strain I47 (Vasini et al. 2022) was selected from the EEA INTA Balcarce Strain Bank. This strain was isolated from cattle intestine that showed PTBC clinical signs. The MAP strain was cultured on Herrold's egg yolk medium supplemented with mycobactin and pyruvate at 37 °C for three months. Grown cultures were resuspended in formalin (0.05%) in PBS (pH 7). Sonication was performed in an ice bath using a probe sonicator (Sonics & Materials, Inc, Newtown, USA) with two cycles of 15 min each one. Residues were removed by centrifugation at 14,000 rpm for 20 min at 8–10 °C. The supernatant was collected, and the obtained proteins were quantified using the Bradford technique (Bradford 1976).

I47 ELISA

The protocol described by Paolicchi (Paolicchi et al. 2003) was used for the ELISA assay. Hundred µL of the I47 antigen (2.5 µg/mL in carbonate buffer; pH 9.6) was added into each well (Immulon I B, Dynatech Lab, USA) and incubated overnight at 4 °C. The wells were washed with Tween-80 saline solution 0.05%. Each serum was diluted in equal parts with a *Mycobacterium phlei* suspension (4 g/L 0.85% NaCl; SENASA Central Laboratory, Argentina, WOAH reference), shaken every 10 min for one hour. Samples were centrifuged at 3500 rpm for 15 min, and the supernatant was diluted 1/50 in phosphate buffered saline-tris glycerin (PBS-TG). Hundred µL was added and the plates were incubated for 2 h at 15 °C and subsequently washed with PBS-TG. A protein G (1/2000) (Affinity Purified Protein G-HRP Conjugate; Blotting Grade Protein G - Horseradish Peroxidase Conjugate, Catalog Number 170–6425, BIO-RAD, USA) was added and then incubated for 1.5 h at 15 °C. The plates were washed with PBS-TG and then 2,2'-azino-di-ethyl-benzy-thiazoline sulfate (ABTS) diluted in citrate buffer (pH 4) was added into the wells. The absorbance reading was performed at 405 nm in a Multiskan L-100 equipment (Finland) and the optical density (OD) result was calculated as the positivity percentage (%P):

$$\%P = \frac{\text{OD (sample)} - \text{(OD negative control)}}{\text{OD (positive control)} - \text{OD (negative control)}} \text{x } 100$$

Commercial ELISA

The assay of the commercial ELISA was performed following the manufacturer's indications (ID Screen® Paratuberculosis Indirect (Screening Test) IDVet, France). In a predilution microplates, the samples and positive and

Table 1 Results of the Receiver Operating Characteristic (ROC) curves analysis for I47 ELISA and commercial ELISA.

Test	AUC[a]	Standard error	Confidence interval 95%	Cut-off points %	Se[b] %	Sp[c] %	*p*-value*
I47 ELISA	0.96	0.02	0.93–0.97	8.85	84.3	96.6	0.2
Commercial ELISA	0.92	0.02	0.89–0.94	24.52	85.7	95.1	0.2

[a]AUC=Area under the curve. [b]Se=Sensitivity. [c]Sp=Specificity. *$P < 0.05$, significant differences.

negative controls were diluted to 1/12 and incubated during 45 min at 21 °C. Then, 100 µL of the mix was transferred to the wells on sensitized ELISA microplates and incubated for 45 min at 21 °C. The plates were then washed with wash solution to subsequently add 100 µL of 1x conjugate to each well. After an incubation for 30 min at 21 °C, the plates were washed and 100 µL of the developing solution was added to each well and incubated for 15 min at 21 °C in the dark. Finally, the reaction was stopped by adding 100 µL of a stop solution. The absorbance reading was performed at 450 nm and the optical density (OD) was calculated as %P.

Statistical analysis

The fecal culture results were chosen as the gold standard for comparing serum samples. The cut-off point that maximizes the sensitivity and specificity of the ELISA techniques was determined using the receiver operating characteristic (ROC) curves. The %P obtained was used to plot scatter diagrams with the values of pairs of variables (positive and negative sera). The analysis was performed using the MedCalc 4.16b program (MedCalc®, Mariakerke, Belgium). The area under the ROC curve (AUC) was used to compare the combination of sensitivity and specificity between the different categories of the study subjects.

A kappa (*k*) statistical analysis with $\alpha = 0.05$ and the WinEpi program (WinEpi 2.0, Zaragoza, Spain) was performed to assess the level of concordance by pairs between the different techniques. The interpretation of the *k*-coefficient values was carried out using the standardized procedure suggested by Landis and Koch (1977).

The association between the categorical results (positive/negative) and the tests (I47 ELISA, commercial ELISA and fecal culture) was determined with the Chi-square X^2 test with $\alpha = 0.05$ using the WinEpi program (WinEpi 2.0, Zaragoza, Spain).

Results

All animals with clinical signs from MAP infected herds were culture positive (70/70) and as confirmed by PCR IS*900*. While MAP was not isolated from samples of animals from herds with no history of PTB in the last 10 years.

An analysis of the ROC curves was performed to compare both ELISA techniques (I47 ELISA and commercial ELISA) using the fecal culture as the gold standard technique. For the I47 ELISA yielded a cut-off value of 8.8%P to best discriminate between positive and negative samples. The sensitivity was 84.3% and the specificity 96.6%. On the other hand, the cut-off value for the commercial kit was 24.5%P, with a sensitivity and specificity of 85.7% and 95.1%, respectively (Table 1). Thus, 70 (17.8%) of the sera tested were positive using the I47 ELISA, and 76 (19.3%) were positive with the commercial ELISA (Table 2).

Table 2 Isolation of MAP from fecal culture and results of serological tests

		Fecal culture		Total
		Positive	Negative	
I47 ELISA	**Positive**	59	11	70
	Negative	11	313	324
Commercial ELISA	**Positive**	60	16	76
	Negative	10	308	318

Figure 1 shows the %P distribution of serum samples analyzed by the I47 ELISA and the commercial ELISA. In addition, the correlation between the bivariate results of the ELISA tests was 73% ($P = 2.2e^{-16}$).

The two ELISA tests did not show different discriminatory ability ($p = 0.2$), as evidenced by the absence of significant differences between the AUC.

We next determined the degree of concordance between the I47 ELISA and the commercial ELISA by using the optimized cut-off values and the analysis of the kappa index (*k*-index of 0.7). The *k*-index indicated a substantial concordance between the two tests according to the classification of Landis and Koch (1977).

An analysis of the concordance between the fecal culture and the two ELISA tests yielded a higher *k*-index using the I47 ELISA ($k = 0.8$, almost perfect concordance), although the use of the commercial ELISA also gave a substantial *k*-index ($k = 0.7$). The two techniques are significantly associated (Chi-square $p < 0.0001$).

Discussion

This study evaluates the monitoring of MAP infection in cattle by specific antibody detection. To optimize the techniques applied in this region of Argentina, we first calculated new cut-off points using fecal cultures as the gold standard for the diagnosis.

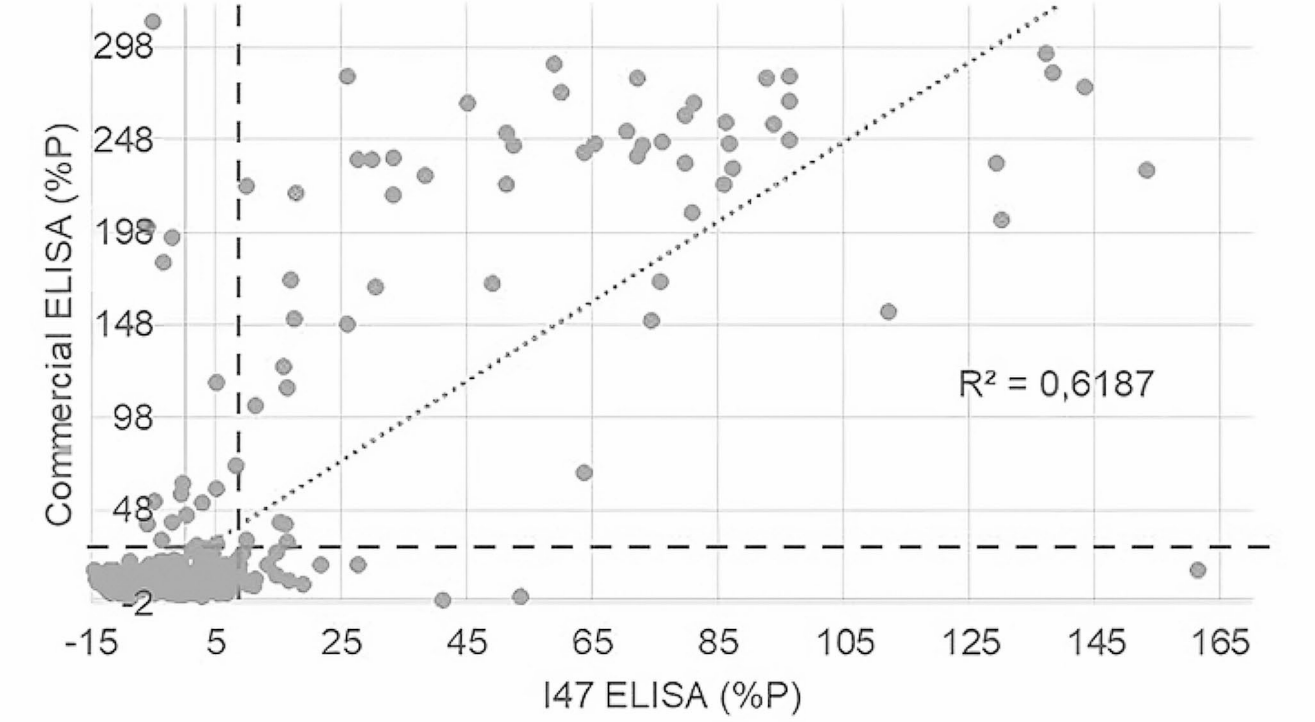

Fig. 1 Scatterplot indicating the relationship between the positivity percentage (%P) of the I47 ELISA and the commercial ELISA in serum samples. The horizontal and vertical striped lines indicate the cut-off values calculated for each ELISA test, whereas the diagonal line indicates the trend line

The two ELISA techniques showed no significant differences regarding MAP detection ($p = 0.2$). The correlation between the results of I47 ELISA and commercial ELISA (Fig. 1) was 73% and the coefficient of determination of 0.6 indicates that many samples gave negative with both ELISA tests. This relationship is consistent with previous studies (Whitlock et al. 2000; Winterhoff et al. 2002), probably because of the high specificity of the technique. McKenna et al. (2006) have reported that different ELISA tests can yield different results in animals with subclinical signs of PTBC in herds with low prevalence due to the low positive predictive values of this test.

The I47 ELISA slightly improved its specificity, although not its sensitivity, when compared to the commercial ELISA. The comparison between both techniques showed a high level of concordance. The *k*-index of 0.7 was similar to the *k*-indices reported by Collins et al. (2005) in a study comparing five different ELISA tests ($k = 0.6–0.8$). In that study, the researchers have also obtained similar determination coefficients ($R^2 = 0.4–0.8$). However, other previous studies have shown lower *k*-indices (0.09–0.2) when comparing four ELISA techniques (Diéguez et al. 2009) and (0.2–0.3) using three different tests (McKenna et al. 2006). The discrepancies in serological testing results may be due to inherent characteristics of the antigens used in the procedures.

Collins et al. (2005) obtained variable sensitivities, between 27.9% and 44.5%, when comparing five ELISA tests with fecal culture. However, in another study, the sensitivity of ELISA was 88% for infected cattle with clinical signs, and 48.8% and 47.3% for two groups of cattle with subclinical infection (Ridge et al. 1991).

In this work, the results of the X^2 test indicated a significant association between the conventional fecal culture analysis and ELISA techniques. Both ELISA techniques detected more than 80% of the infected cattle that were positive by the fecal culture. The *k* statistic was high, suggesting both tests detected infection in the same animal groups.

The difference in the results between fecal culture analysis and ELISA may be due to the higher resistance of adult animals to infection after MAP exposure. Therefore, these animals developed antibodies but did not excrete a detectable number of MAP through the feces.

In our work, 100% of the animals with clinical signs from MAP infected herds were positive for MAP by fecal culture. The bacterial culture is the gold standard technique for the diagnosis of PTBC (Whittington 2020), its specificity is between 95% and 100% (Singh et al. 2018; Gilardoni et al. 2012). Regarding the sensitivity of the gold standard, it changes depending on the stage of infection between high (up to 91% in the advanced stages of the disease), medium

(45–72% in animals with clinical stages), and relatively low (16–53% in subclinical stage of infection) (Whitlock et al. 2000; Nielsen and Toft 2008).

The results are consistent with the disease development, where subclinical infected animals can excrete bacteria intermittently at low levels (Li et al. 2017), while animals with clinical signs constantly excrete large amounts of MAP (Faruk et al. 2020). Likewise, culture preparation requires using decontaminants. These decontaminants negatively modify MAP viability (Dane et al. 2023) and this, in turn, affects the number of viable culturable cells. The considerably increased recorded prevalence in this research is due to the type of animals included here (animals with clinical signs and fecal MAP culture confirmation). The long incubation period before the manifestation of the disease makes it difficult to rely on the negative results of fecal cultures as a gold standard technique for MAP detection in animals residing in infected herds (Collins et al. 2005). For example, Whitlock and Buergelt (1996) have suggested that for each animal with clinical signs and positive diagnosis in an establishment, 15 to 25 herd animals could be suffering an asymptomatic infection.

The present research shows high sensitivity and specificity of the I47 ELISA test in PTBC diagnosis, with a similar AUC to that of the imported commercial kit. Therefore, the production of an antigen from a native strain selected in the region represents an advantage by reducing diagnostic costs and avoiding imported commercial kits.

The I47 ELISA could be used as a routine diagnosis for PTBC control in cattle improving the detection of infected animals. This implementation could contribute to controlling this important infectious disease of cattle.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare that they have no conflict of interests.

Research with animals This study involved a survey of producers and blood and fecal samples were taken from their animals. The study protocol was evaluated and approved by INTA Balcarce. The producers gave their consent for sampling. Sample collection was carried out by veterinarians respecting the rules and guidelines on animal welfare.

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