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Paper

Evaluation of ELISA in raw milk for detection of antibodies to Mycobacterium avium subsp. paratuberculosis in dairy herd

Silvina Tieri[1*], Karina Cirone[1], Claudia Morsella[2], Laura Méndez[2], Silvia Mundo[3], Bárbara Fernández[3], Fernando Paolicchi[1]

[1]*Laboratorio de Bacteriología, Departamento de Producción Animal, EEA INTA, Balcarce, Buenos Aires, Argentina; CONICET. Buenos Aires, Argentina; Universidad Nacional de Mar del Plata, Facultad de Ciencias Agrarias, Balcarce, Buenos Aires, Argentina - AR*

[2]*Laboratorio de Bacteriología, Departamento de Producción Animal, EEA INTA, Balcarce, Buenos Aires, Argentina - AR*

[3]*CONICET-Universidad de Buenos Aires. Instituto de Investigaciones en Producción Animal (INPA). Av. Chorroarín 280, Ciudad Autónoma de Buenos Aires, C1427CWO, Argentina; Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Cátedra de Inmunología, Chorroarín 280, C1427CWO, Buenos Aires, Argentina - AR*

[*]*Corresponding author at: Laboratorio de Bacteriología, Departamento de Producción Animal, EEA INTA, Balcarce, Buenos Aires, Argentina; CONICET. Buenos Aires, Argentina; Universidad Nacional de Mar del Plata, Facultad de Ciencias Agrarias, Balcarce, Buenos Aires, Argentina - AR*

E-mail: silvinatieri@hotmail.com

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Abstract

Paratuberculosis (PTBC) is a chronic intestinal disease of animals caused by *Mycobacterium avium paratuberculosis* (MAP). MAP infection is diagnosed through indirect tests based on the immune response. The aims of this study were to compare the performance of two milk ELISA for the diagnosis of PTBC and to assess the bulk tank milk (BTM) MAP exposure in dairy cattle in Argentina. A total of 357 fecal, serum, and milk samples were collected. The fecal samples were processed by culture for MAP isolation, while both, serum and milk samples were used for the detection of antibodies by two different ELISA tests, "*in-house*" and commercial kit. MAP was isolated in 3.9% of fecal samples. For milk ELISA, poor concordances were obtained. Optimized cut-off points were calculated. The highest sensitivity and specificity values (64% and 80% respectively) were obtained with the combination of MAP isolation and commercial milk ELISA. The results indicate that the combination of different techniques to identify of dairy cattle infected with MAP increases the efficiency of diagnosis. In addition, BTM samples (n=98) were evaluated to determine herd status using the commercial kit during two seasons, identifying 33.3% of positive samples in autumn and 35.4% in spring.

Keywords

Bulk tank milk, ELISA, Mycobacterium avium subsp. paratubercolosis, Milk, Paratubercolosis

Introduction

Paratuberculosis (PTBC) or Johne disease is a chronic granulomatous enteritis disease of animals caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Collins and Morgan 1991, Ponnusamy *et al*. 2013). The clinical signs of PTBC are diarrhea, weight loss, emaciation, and eventually death (Mortier *et al*. 2015). The adverse effects on animal productivity are the decrease in milk production (McAloon *et al*. 2016), higher slaughter rates (Hendrick *et al*. 2005), and reduced value for slaughtered animals (Richardson and More 2009). The main route of MAP transmission in ruminants is fecal-oral, although infection can also occur *in utero*(Paolicchi and Romano 2007, Vasini Rosell *et al*. 2020), or, via ingestion of contaminated colostrum or milk (Cirone 2015). Most animals become infected within the first month of life but susceptibility to MAP infection decreases with age. MAP has also been implicated in a human disease called Crohn's disease (Sweeney *et al*. 2012). Possible sources of infection in humans

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are dairy products where the microorganism frequently resists pasteurization conditions, but also meat products from infected animals and water contaminated with MAP, representing a global concern for human health (Grant 2005).

The diagnosis of PTBC represents a challenge because the subclinical phase can last for years, and the disease development and the immune response are variable. Fecal culture for the MAP detection is considered the reference test but has a moderate sensitivity. Due to MAP is a slow-growing microorganism (5 to 24 weeks) and contaminant microorganisms present in the sample, it is required the use of decontaminants that affect the MAP viability (Ayele*et al*. 2001, Gilardoni *et al*. 2012). The ELISA test detects specific antibodies against MAP in serum or milk samples. It is widely used due to its speed and low cost.

Due to the high prevalence of PTBC in cattle in Argentina (Paolicchi 2004),the importance of the etiological agent for public health, and its diagnostic difficulty, it is necessary to have sensitive and specific techniques that allow the disease to be easily detected. It is hoped that the combination of the different tests for the identification of individual animal infection increases the efficiency of the diagnosis of PTBC.

The aims of this study were: a) to evaluate the performance of "*in-house*" and commercial tests for the detection of MAP antibodies in raw milk samples; b) to determine the concordance between "*in-house*" ELISA or commercial ELISA in milk and a serological "*in house*" ELISA using as reference "gold standard" the MAP fecal culture in bovine dairy herds; and c) evaluate the prevalence of MAP based on the detection of antibodies in bulk tank milk (BTM) using a commercial kit.

Materials and methods

Samples

The samples were obtained from three dairy herds belonging to the province of Buenos Aires, Argentina. A total of 357 samples of feces, serum, and milk from adult Holstein cows were selected for processing. All milk samples were analyzed by two indirect ELISA tests, one "*in house*" (using MAP protoplasmic antigen; PPA-3, Allied Monitor, USA) and the other using a commercial kit (ID Screen® Paratuberculosis Indirect-Test de Screening-IDVet, France). The serum samples were analyzed only by the "*in-house*" ELISA.

A total of 98 BTM paired samples from 49 dairy herds that belong to the Mar and Sierra Basin (MSB), were collected in May-June (autumn), and November-December (spring) 2019. Herds were selected based on a non-probabilistic convenience sampling design with a sampling fraction of approximately 32% (i.e., 49 out of approximately 150 dairy herds in the MBS). Fifty mL were taken directly from the bulk tank and were preserved with 0.1 g sodium azide medium at 4°C for transportation purpose. Samples were frozen at -20°C until use.

Sample Processing

Bacterial culture

The samples were grown according to Paolicchi and colleagues (Paolicchi *et al*. 2003). In short, 10 g of feces were decontaminated with 90 mL of 0.75% (w/v) hexadecilpyridinium (HPC-Sigma-Aldrich, USA), shaken and then, let sediment overnight. Forty mL of the supernatant were centrifuged at 3500 rpm for 15 minutes. The precipitate was resuspended in 500 µL phosphate buffered saline (PBS). A 100 µL were inoculated in each tube containing Herrold eggs yolk mycobactin (HEYM) without supplement, HEYM with supplements (2.0 mg/L mycobactin J (Allied Monitor, USA) and 4.1 g/L sodium pyruvate) and HEYM with supplements and antibiotics (2.0 mg/mL amphotericin B, 100 µg/mL vancomycin, 100 µg/mL nystatin and 3.0 mg/mL nalidixic acid) (Sigma-Aldrich, USA). The tubes were incubated at 37°C and observed weekly for four months. Colonies with MAP characteristics were identified and their positivity confirmed by Ziehl-Neelsen staining and PCR IS*900* (Green *et al*. 1989).

Serum and milk

Two different ELISA tests were used:

A) ELISA PPA-3 ("*in-house*"):The protocol described by Paolicchi and colleagues (Paolicchi *et al*. 2003), was used. The PPA-3 (Allied Monitor, USA) was diluted to a concentration of 7.2 µg/mL in carbonate buffer (pH 9.6), it was added into the plate wells and allowed overnight at 4°C. The wells were washed three times with Tween-80 saline solution. Each serum/milk was pre-treated by adsorption with a *Mycobacterium phlei* suspension. The samples were centrifuged, and the supernatant fraction was diluted 1:100 in a PBS-TG (phosphate buffered saline-tris glycerin). The duplicate samples were incubated for 2 hours at 15°C and, after being washed three times with PBS-TG, the

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conjugate anti-bovine IgG-peroxidase antibody produced in rabbit (Sigma-Aldrich, USA) (1/4000) was added and incubated during 1.5 hours at 15°C. The plates were washed with PBS-TG and ABTS (2,2'-azino-di-ethyl-benzy-thiazoline sulfate) diluted in citrate buffer (pH 4) was added. Positive and negative controls were included. The absorbance reading was performed at 405 nm in a Multiskan L-100 equipment (Finland) and the positivity percentage (%P) was calculated as follows:

$$\%P = \frac{OD^{*}\ (sample) - OD\ (negative\ control)}{OD\ (positive\ control) - OD\ (negative\ control)} \times 100$$

*OD: optical density

B) Commercial ELISA: ID Screen Paratuberculosis Indirect (Screening Test) IDVet: The protocol described by the manufacturer was used. In short, in a 96-well microplate, the milk samples were diluted 1:2 and the positive and negative controls 1:12 with diluent, and incubated for 45 minutes at 21°C. A hundred µL per well was transferred to sensitized ELISA microplates and incubated for 45 minutes at 21°C. The plates were washed and the 1x conjugate was prepared and 100 µL/well was added, incubating for 30 minutes at 21°C. The plates were washed and the developing solution was added incubating 15 minutes at 21°C in the dark. Finally, 100 µL/well of the stop solution was added and the absorbance at 450 nm was read, calculating the OD result as %P as before, when the value was greater than 15% the samples were considered positive, otherwise negative.

Statistical analysis

From the %P obtained for each ELISA "*in-house*" and commercial test, scatter diagrams of the milk values were generated using as true positives by the fecal culture and the corresponding ROC curves, plotting for each sensitivity (y-axis) of different cut-off points the value of 1 minus the specificity (false positives, x-axis). The analysis was performed using the MedCalc 4.16b program (Schoonjans *et al*. 1995).

The comparison and analysis of the concordance in pairs between the different techniques was carried out using a statistical analysis of Gwet's AC1 index. The interpretation of the coefficients was performed using the standardization procedure suggested by Gwet (Gwet 2014).

A one-way repeated measures ANOVA (ANOVA; "nlme" package v3.1- 130 142, R v3.5.1, R Core Team, 2018) was used to determine a difference between the %P bulk milk samples from the two sampling dates (autumn *vs* spring).

Results

MAP was isolated from 14/357 (3.9%) fecal cultures performed, confirmed by PCR IS*900*, while 343/357 samples (96.1%) were negative.

Figure 1 shows the distribution of the MAP fecal culture and the antibody detection in sera. The concordance between both tests is considerable with a Gwet's AC1 index of 0.65.

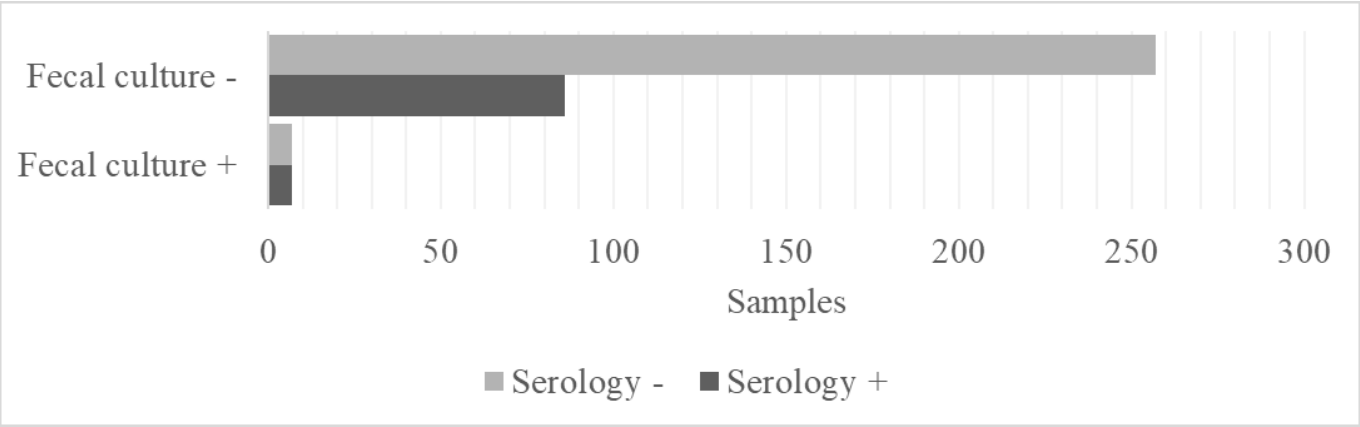

Figure 1 Isolation of MAP from fecal culture and ELISA in serum for antibodies detection.

For milk samples analyzed by the "*in-house*" ELISA, 279/357 (78.2%) were positive and 78/357 (21.9%) were negative. While using the commercial ELISA 96/357 (26.9%) were positives and 261/357 (73.1%) were negatives

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according to the cut-off recommended by the manufacturer (15 %P). The distribution of the %P for milk samples analyzed by "*in-house*" ELISA and commercial ELISA is observed in Figure 2. The correlation obtained was 0.1017.

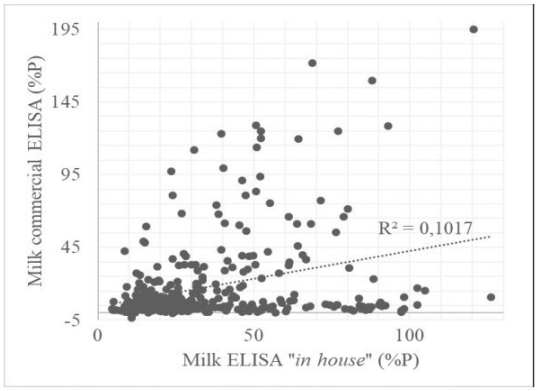

Figure 2 Correlation betweem the outcomes of milk "in house" ELISA and milk commercial ELISA

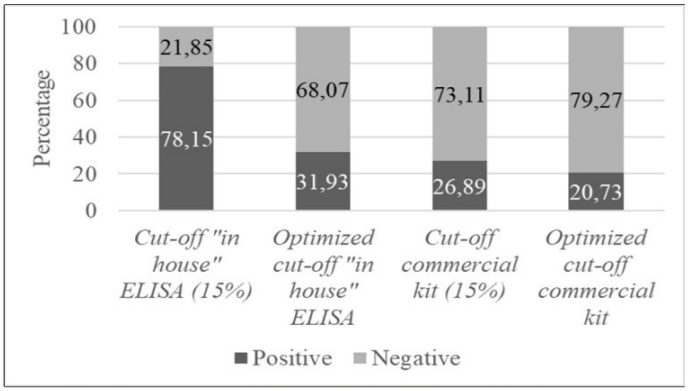

Figure 3 Percentage of positive and negative samples according to the cut-off point of each test

Table I shows the concordance between the different ELISAs using the cut-off point according to the manufacturer of the commercial kit. Negative Gwet's AC1 indices of -0.171 and -0.123 were obtained through the concordance analysis between serum and milk by the "*in-house*" ELISA and between the milk commercial ELISA and "*in-house*", respectively. Both values correspond to poor concordances according to the classification of Landis and Koch (Landis and Koch 1977). However, moderate concordance was obtained between serum and milk commercial ELISA.

ELISA Test	N*	Cut-off values (%)	Index Gwet's AC1	Standard error	CI 95%**	Concordance
Serum *vs* milk "*in house*" ELISA	357	15	-0.171	0.052	-0.277-0,071	Poor
Serum *vs* milk commercial ELISA	357	15	0.463	0.049	0.365-0.56	Moderate
Milk "*in house*" ELISA *vs* Milk commercial ELISA	357	15	-0.123	0.052	-0.227-0.019	Poor

Table I Concordance using the cut-off point according with commercial kit. *N: number of samples. **CI: confidence interval

To optimize techniques for this region of Argentina, new cut-off points were calculated using the fecal culture as the "gold standard". Table II shows the calculated cut-off points for the techniques and the sensitivity and specificity of the "*in-house*" and commercial ELISAs.

Variable	N*	Area down the curve	Standard error	CI 95%**	Cut-off values (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
"*in house*" ELISA	357	0.62	0.082	0.528-0.671	40.5	64	69	49	80
commercial ELISA	357	0.675	0.081	0.624-0.723	21.61	64	80	45	89

Table II Cut-off, sensitivity and specificity of milk "in house" ELISA and milk commercial ELISA (Reference Test (Gold Standard): fecal culture). *N: number of samples. **CI: confidence interval

The highest sensitivity and specificity values were 64% and 80% respectively, observed for the commercial ELISA. Using this cut-off point (21.61 %P), 74 and 283 out of 357 samples were positive (20.7%) and negative (79.3%), respectively. Similarly, with the calculation of the optimized cut-off point (40.5 %P) between the milk "*in-house*" ELISA and the fecal culture as a reference technique, a total of 114 positives (31.9%) and 243 negatives (68.1%) were obtained. Figure 3 shows the distribution of the percentage of positive and negative samples with the different cut-off values for each test.

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With the optimized cut-off points, the concordance values between the different techniques were higher than with cut-off values of 15 %P with indices AC1 between 0.39 (serology and milk "*in-house*" ELISA) and 0.54 (milk commercial and "*in-house*" ELISAs), which are translated as discrete and moderate values, respectively (Table III).

Techniques	N*	Cut-off values (%)	Index Gwet's AC1	Standard error	CI 95%**	Concordance
Serum *vs* milk "*in house*" ELISA	357	40.5	0.39	0.052	0.29-0.49	Discreet
Serum *vs* milk commercial ELISA	357	21.61	0.52	0.046	0.43-0.61	Moderate
Milk "*in house*" ELISA *vs* milk commercial ELISA	357	40.5/21.61	0.54	0.045	0.45-0.63	Moderate

Table III Concordance study using the optimized cut-off points. *N: number of samples. **CI: confidence interval

Table IV shows the classification of the animals according to the result of each ELISA. Comparisons between the "*in-house*" ELISA and the commercial ELISA with optimized cut-off values calculated from fecal culture as "gold standard" resulted in an index of 0.54, translated as moderate concordance.

"*in house*" ELISA	Commercial ELISA		
	Positive	Negative	Total
Positive	44	70	114
Negative	30	213	243
Total	74	283	357

Table IV Comparison of results between the "in house" ELISA and the commercial ELISA in milk samples with optimized cut-off point.

BTM samples were used to assess herd status based on the presence of specific antibodies. In the autumn sampling, 33.3% of the samples were positive, while in spring it was 35.4% (Figure 4). An ANOVA showed that there are no significant differences in %P over seasons ($p>0.05$). A significant linear correlation in the %P in BTM samples between both seasons was observed ($p<0.001$; Figure 5).

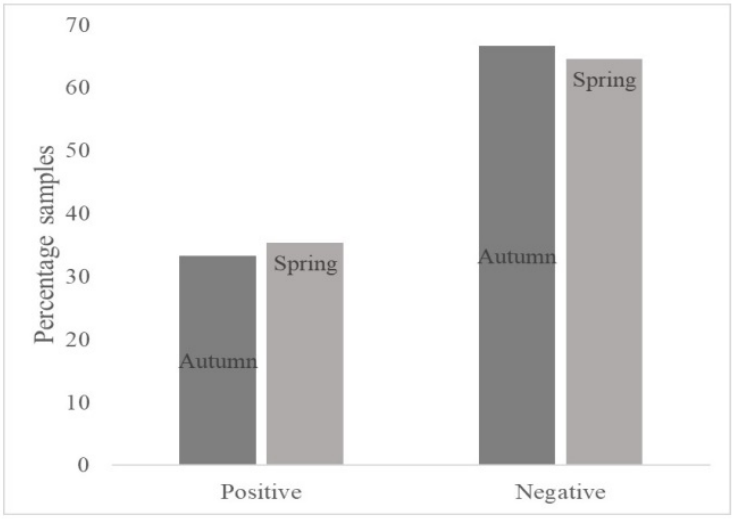

Figure4 Percentage of positive and negative samples in autumn and spring.

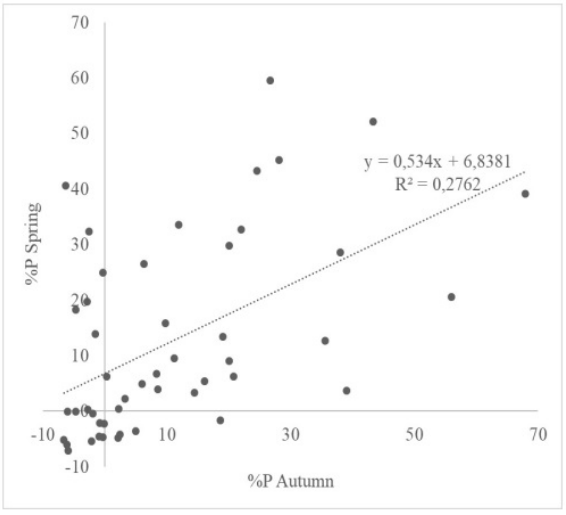

Figure 5 Correlation between the outcomesof bulk tankmilk in autumn vs spring

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Discussion

The bacterial culture has a specificity of 100% (Ayele *et al*. 2001, Gilardoni *et al*. 2012), and the sensitivity is dependent on the stage of infection of individual animals (Keller *et al*. 2014). In this study, MAP was isolated from 14/357 (3.9%) fecal cultures performed, confirmed by PCR IS*900*, while 343/357 samples (96.1%) were negative; the results are consistent with the infection development, where subclinically infected animals can shed bacteria at low levels (40-100 CFU/g of feces) and intermittently, while clinically infected cattle excrete MAP generally in high quantities ($1.3x10^5$-$5.9x10^6$ CFU/g of feces) and constantly (Mason *et al*. 1997). In addition, the reduction of MAP viability due to the decontamination procedure can also impact the isolation of MAP by culture (Khare *et al*. 2008, Nielsen and Toft 2008, Corbett *et al*. 2018). As subclinically infected cattle constitute the largest part of MAP-infected herds, the percentage of positive animals detected by fecal culture was low.

The correlation between the outcomes of milk "*in-house*" ELISA and milk commercial ELISA (Figure 2) indicates many samples with negative results in both tests, although there were cases in which one animal had an extremely low value with "*in-house*" ELISA and high value with commercial kit and *vice versa*. These results are consistent with McKenna and colleagues (McKenna *et al*. 2006), who demonstrate that different results could be obtained from a subclinical PTBC animal evaluated by different ELISA tests due to the low positive predictive values of the tests. This could make it difficult for disease control at the individual animal level, so it is necessary to have sensitive and specific diagnostic techniques to detect animals with PTBC. The identification of new highly immunogenic antigens (Eda *et al*. 2006, Speer *et al*. 2006, Scott *et al*. 2010, Mikkelsen *et al*. 2011), could be a solution to improve diagnosis and help make decisions to starting from the results obtained.

With a cut-off value of 15 %P according to the ELISA kit manufacturer's indications, low concordance was obtained between the "*in-house*" and commercial ELISA. To optimize techniques for this region of Argentina, new cut-off points were calculated using the fecal culture as a reference test. As demonstrated by Lombard and colleagues (Lombard *et al*. 2006), the variability between the different techniques could be due to the variation of antibody levels depending on the lactation stage or the infection stage. In early infection, the cellular-type immune response predominates and, with the progress of the infection, the immunological profile changes to the humoral response (Arsenault *et al*. 2014). In contrast, at the beginning and the end of lactation, the probability of obtaining positive results by the ELISA is increased (Nielsen *et al*. 2002, Lombard *et al*. 2006).

With the optimized cut-off points, the concordance values between the different techniques were higher than with cut-off values of 15 %P. These results show the importance of optimizing the cut-off values for different regions depending on the stage of the infection, the PTBC prevalence level of this region, the size and management of the different herds, and the proportion of animals eliminated due to clinical PTBC (Garcia and Shalloo 2015).

The %P in BTM were analogous between samples obtained in different seasons (autumn and spring). Similar results were described by Wilson and colleagues (Wilson *et al*. 2010), observing 39% BTM positives of dairy farms in Utah, USA, and adjacent areas, with a test sensitivity of 57%. Nielsen and colleagues (Nielsen *et al*. 2000), showed that the adaptation of the ELISA technique for the detection of antibodies in BMT samples can be used for the detection of herds with high prevalence. ELISA tests have the same performance applied on BTM and serum samples at herd level, with a sensitivity of 56 to 83% when fecal culture is used as a reference (Lombard *et al*. 2006, Wilson *et al*. 2010). According to Pesqueira and colleagues (Pesqueira *et al*. 2017), the sensitivity of analyzing BTM samples to detect positive or highly positive herds showed values ranging from 76.1% to 78.9% depending on the ELISA kit used. The BTM test for PTBC detection is affected by disease prevalence, and possible increased dilution of antibodies if uninfected cows produce more average milk than infected cows (Wilson *et al*. 2010).

The use of repeated tests, such as the collection of monthly samples in BTM is a practical and convenient way to monitor the presence of PTBC in dairy herds. However, more studies are needed on the use of individual milk tests to verify their accuracy, practicality, and effectiveness in reducing the prevalence of the disease in dairy herds (Wilson *et al*. 2010). The use of BTM would be a useful screening test for regional or national control programs (Van Weering *et al*. 2007).

According to the results observed in the present study, it can be suggested that the combination of the different tests for the identification of individual animal infection increases the efficiency of the diagnosis of PTBC. The best results of sensitivity, specificity, and concordance were obtained with the commercial ELISA, which uses optimized cut-off points and is suggested as a reference technique for the identification of anti-MAP antibodies in milk samples. Further studies are needed to determine the best time to take the sample for analysis and the cut-off for different regions and stage of PTBC infection.

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Declaration of animal rights

The institutional and national guidelines for the care and use of animals were followed.

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