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Comparison between a commercial and an in-house ELISA for anti-*M. avium paratuberculosis* antibodies detection in dairy herds in Rio de Janeiro, Brazil

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ABSTRACT. Paratuberculosis (Johne's disease) is a chronic enteritis that affects ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is worldwide spread and causes important economic losses. In Brazil, *Map* has already been isolated, but there are no enough epidemiological studies about its prevalence. In this study, 179 sera of dairy cows originated from Rio de Janeiro State, Brazil, were tested for the presence of anti-*Map* antibodies using two ELISAs. First one was an "in house" ELISA using a protoplasmic paratuberculosis antigen (PPA - Allied Monitor) as capture antigen and a monoclonal anti-bovine IgG conjugated to alkaline phosphatase (Sigma) and the other was "HerdChek *Mpt*" (IDEXX), used as standard method in this study. Fifty-nine (32.9%) samples were reactive in the PPA-ELISA. Sensitivity of ELISA-PPA in relation to HerdCheck was 47.5% and specificity was 86.8%, with 54/103 of false negatives and 10/76 of false positives. Positive predictive value of PPA-ELISA was calculated as 86% and efficiency as 64.2%. PPA-ELISA and HerdChek results were also compared by kappa test (κ) and concordance index was 0.320. PPA-ELISA can be useful as a collective herd test and can be recommended as a screening test for identification of infected herds and foci of Johne's disease.

Key words: Paratuberculosis, mycobacteria, diagnostic, serology, bovine.

RESUMEN. La Paratuberculosis (enfermedad de Johne) es una enteritis crónica que afecta a los rumiantes y es causada por el *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). La enfermedad está presente en todo el mundo, y causa importantes pérdidas económicas. En Brasil, el *Map* ya fue aislado, pero no existe una caracterización epidemiológica de su prevalencia. En éste estudio, 179 sueros de vacas provenientes de establos lecheros del Estado del Río de Janeiro, Brasil, fueron analizados para la presencia de anticuerpos específicos para *Map* usando dos ELISAs. Se elaboró un ELISA "in house" que utilizó el "protoplasmic paratuberculosis antigen" (PPA - Allied Monitor) como antígeno de captura y el conjugado monoclonal anti-IgG bovina/fosfatasa alcalina (Sigma). Se consideró el kit comercial "HerdChek *Mpt*" (IDEXX) como el método patrón. El 32.9% (59) de los sueros resultaron positivos al PPA-ELISA. La sensibilidad del PPA-ELISA con relación al HerdCheck *Mpt* fue 47.5% y la especificidad fue 86.8%, con 54/103 de falsos negativos y 10/76 de falsos positivos. El valor predictivo positivo del PPA-ELISA fue 86% y la eficiencia fue 64.2%. Los resultados de PPA-ELISA y HerdChek fueron comparados, sin encontrarse diferencias significativas (Kappa test, valor 0.320). El PPA-ELISA puede ser útil como prueba colectiva, recomendándose su uso como método para la identificación de rodeos infectados y focos de la enfermedad de Johne.

Palabras clave: Paratuberculosis, micobacterias, diagnóstico, serología, bovinos.

INTRODUCTION

Paratuberculosis, also called Johne's disease, is a chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that affects ruminants. During the first stage the disease is asymptomatic and nonexcretory, and gradually develops towards an excretory disease where bacteria are shed in the faeces, thus spreading the infection within the herds. During the final stage, the animal shows chronic diarrhoea, weight loss and reduction in milk production. Bacte-

ria are also found in the milk of infected animals, representing therefore another form of spreading of disease.²

It is a worldwide spread disease and has been frequently detected in many countries. In the United States, Johne's disease is considered one of the most prevalent infectious diseases in dairy herds and a cause of important economic losses calculated in US\$1.5 billions/year.²⁰ In Brazil, some sporadic cases of Paratuberculosis have been described during past years. Recently, a serological screening was performed in 20 dairy farms in São Paulo State, and ELISA showed reactivity in 37.9% of 403 animals and in 95% of the herds.⁹ In Rio de Janeiro, 45 herds were evaluated by another commercial ELISA kit, showing reactivity in 18% of animals and 82% of the herds.⁷

The existing diagnostic tests for Johne's disease are not very accurate, thus limiting the diagnostic of the disease and the reservoirs detection. Faecal culture of *Map* is the

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most sensitive method to identify shedding animals.²⁵ However, despite of many efforts to reduce culture contamination and time, 6 to 16 weeks of incubation are still needed.⁶

Serologic tests have been evaluated for rapid detection of clinically and subclinically infected animals, such as complement fixation (CF), agarose gel immunodiffusion (AGID) and pre-adsorbed ELISA. Although AGID can be used as a confirmatory method, mainly for clinical cases of paratuberculosis with good results in Brazil,⁸ the latter is currently the most recommended method, because of its acceptable sensitivity and good specificity, mainly in animals with a heavier bacterial load.^{13,25} Although humoral immune responses tend to increase only on late stages of the infection, it can be observed before the appearance of clinical signs.²⁰

The aim of this study was to compare an "in house" and a commercial standardized ELISA for the detection of anti-*Map* antibodies in dairy cattle under field trials in Brazil.

MATERIALS AND METHODS

Cattle - Serum samples from 179 adult (older than one year of age) cows originated from 12 dairy herds in Rio de Janeiro State were used. Cows that had been calved less than six months prior to this study were excluded. Those animals were bled and serum samples were separated by centrifugation and stored in 2 ml aliquots on criotubes at -20°C to be tested as a batch. One herd known to be paratuberculosis-free for more than 5 years was chosen to be the control group of the study, and 14 blood samples were collected from this herd.

Commercial ELISA - HerdChek *Mpt* (IDEXX) was used in this study according to the manufacturer's instructions. The capture antigen (*Map* strain VRI 316/102-2 crude protoplasmic antigen) is adsorbed within the wells of 96 well polystyrene plates, which are blocked and air-dried. The kits are also supplied with serum diluents that contain adsorbing *Mycobacterium phlei* antigen, anti-bovine IgG conjugated to horseradish peroxidase, enzyme substrate solution with hydrogen peroxide/tetramethyl benzidine and stop solution.

PPA-ELISA: Protoplasmic Paratuberculosis Antigen (PPA), a sterile-filtered and lyophilized protoplasmic cell extract of *Mycobacterium* Strain 18 (Allied Monitor Lab), was used as capture antigen in concentration of 0.72 mg/ml in carbonate buffer. For pre-adsorption of unspecific antibodies, a whole-cell, heat-killed, lyophilized *M. phlei* (Allied Monitor Lab) was used, diluted in saline solution at 4 g/l. After incubation, *M. phlei* was removed by centrifugation at 2000 'g for 3 min. Test sera were diluted 1/50 in Tris-HCl-Tween containing 1% gelatin. A positive control

serum from Allied Monitor was diluted 1/100. A positive serum sample from an animal with clinical disease and bacteriological isolation of *Map* was also used as positive control. All sera were used in duplicate, including positive controls. Monoclonal anti-bovine IgG conjugated to alkaline phosphatase (Sigma) was diluted 1:5000 in Tris-HCl-Tween. Substrate solution was *p*-nitrophenylphosphate diluted in diethanolamine buffer. Results were read after 30 min. at a wavelength of 405 nm. The mean absorbance values for each serum were calculated from the replicate wells, and for samples showing a discrepancy of more than 20% the test was repeated. All incubations were at room temperature for 1 h, except the last.

Statistical Analysis - Several cut-off values were tested for PPA-ELISA, and the O.D. 0.400 was the value giving the most accurate division between positive and negative sera. HerdChek *Mpt* (IDEXX) was considered as the standard test. The Student's T-test compared mean O.D. of PPA results between "true-positive" and "true-negative" (for HerdChek *Mpt*) samples. Sensitivity and specificity of PPA-ELISA were determined following Galen & Gambino¹⁰. The correlation between HerdChek *Mpt* and PPA-ELISA were compared, using the Kappa-test (*k*).

RESULTS AND DISCUSSION

From 179 studied samples, 59 (32.9%) were considered as reactive in the PPA-ELISA. The standard method, HerdChek *Mpt*, showed 103 positive and 76 negative samples, considered in this study as "true-positive" and "true-negative" results, respectively (table 1). According to the Student's T-test, differences between PPA-mean OD of true-positive and true-negative samples were highly significant ($p < 0.01$), but between the PPA-mean OD of true-negative samples and negative controls were not ($p = 0.122$). The PPA-ELISA cut-off was determined as the OD 0.400, since this was the best value to distinguish between positive and negative sera.

According to these results, we believe that the PPA-ELISA can be useful as a screening test. The significant dif-

Table 1. Detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in bovine serum samples obtained by two ELISA.

	PPA-ELISA		Total (%)	
	Positive (%)	Negative (%)		
True Positive ¹	49 (27.4%)	54 (30.2%)	103	(57.6%)
True Negative ²	10 (5.6%)	66 (36.8%)	76	(42.4%)
Total (%)	59 (33%)	120 (66%)	179	(100%)

1. HerdChek *Mpt* (IDEXX) positive samples (standard in this study)

2. HerdChek *Mpt* negative samples

ference between mean OD observed in the comparison of true-positive and true-negative groups of sera shows the possibility of distinguishing between infected and uninfected herds and demonstrates that this assay can be used as a collective herd test. It is feasible and can be useful as a diagnostic tool for identification of paratuberculosis foci and for the periodical control of supposedly infection-free herds.

Faecal culture and identification of *Map* is the gold standard for the diagnosis of Johne's disease, and is the most sensitive method to identify shedding animals.²⁵ Unfortunately, several of those animals were culled after collecting blood samples, and we were unable to collect their faeces or tissues for culturing. Therefore, it was not possible to determinate the real level of shedding *Map* in faeces confirming by culture the results of serologic tests. For that reason HerdChek *Mpt* was used as standard method in this study. It is a commercially available method for screening Johne's disease in herds that has been extensively tested and approved in many countries. This method was used for government screening in USA,¹⁷ Belgium¹ and Netherlands,¹⁶ and has also been well evaluated by many researchers worldwide. Based on HerdChek *Mpt* results, sensitivity of the PPA-ELISA was 0.47% with 42/103 of false negative results, and specificity was 0.86% with 18/76 of samples incorrectly diagnosed as positives.

Many different ELISAs have been used as screening method. A LAM-ELISA (with lipoarabinomannan polysaccharide as antigen) was used for a screening survey in Costa Rica,⁵ and has recently been evaluated using serum samples from animals infected with *Map*, *M. bovis* and *M. phlei*, showing cross-reactive responses.²⁴ A paratuberculosis control program in Australia used a commercial ELISA as diagnostic method, and reduced the level of prevalence of the disease.¹⁴ Another ELISA Paratuberculosis commercial kit (Institute Pourquier) has been used in Slovenia for two years, showing a high prevalence of infected herds.¹⁸ PPA antigen was also tested in an in-house ELISA in Austria, and revealed two to three times more positive reactors when compared to another in-house assay.¹¹

Sensitivity and specificity rates of diagnostic methods may be influenced by different criteria adopted for the definition of the cut-off point. In ELISA tests, we can increase sensitivity/specificity just by altering the cut-off point. Depending on the veterinarian decision and the point of development of the eradication program, the cut-off point can be altered in order to obtain greater sensitivity or specificity of the test. In the first steps of a control program, a method with good sensitivity is required, and a little reduction in specificity can be acceptable. Due to the nature of the immune response to mycobacterial infections, mainly cell-mediated, ELISAs generally present low sensitivity when compared to bacteriological culture.

The low sensitivity of PPA-ELISA in the present study, although not satisfactory, was similar to those reported for other ELISA. Collins *et al.*³ reported sensitivity of ELISA as 47.3% and Sockett *et al.*,¹⁹ evaluated the sensitivity of two different kits, finding 58.8% of sensitivity for ELISA-PPA and 43.4% for another commercial kit, Paracheck (CSL) in relation to culture findings. In two studies with different populations of cattle, sensitivities were $24.6 \pm 10.2\%$ for low shedders and $88.2 \pm 5.5\%$ for high shedders⁴ and 15% for low shedders and 75% for high shedders.²⁵

However, the high level of discordant results between the two tests was an unexpected finding. ELISA, as well as other serologic tests, is considered to be very specific, with false-positive results occurring in very a low frequency.¹³ In other reports, specificity was much higher: $99.7 \pm 0.3\%$,⁴ 99.0% ³ and 95.4% to ELISA-PPA and 99.0% to a commercial-ELISA.¹⁹ Recently, a study comparing five different ELISAs was performed, and clear differences in overall sensitivity, specificity and diagnostic performance were found. Also, for subpanels of different origin differences in specificity were found, possibly reflecting regionally relevant cross-reactions.²³

Also Kappa-test was used to compare results from PPA-ELISA and HerdChek *Mpt*, with a low value ($k = 0.320$), characterizing a weak correlation between the two tests. These results were also confirmed by a previous study using the same panel of samples that showed discordance between PPA-ELISA and Paracheck (CSL) (data not showed). However, results from Paracheck observed in that study and HerdChek *Mpt* were also discordant ($k = 0.505$). Nevertheless, we have to be awarded that comparison of different systems using distinct antigens and conjugates is not simple. PPA-ELISA uses protoplasmic cell extract of *M. avium* strain 18, while the commercial tests, despite uses the same *Map* strain (VRI 316/102-2) as antigen source, prepared different commercial extracts from it.

It is known that mycobacterial infections, such as paratuberculosis, cause various changes in the host immune response during the development of disease. The classical paratuberculosis immune pattern is the strong cell-mediated immune response during the early, subclinical stages of infection and strong humoral responses during the late clinical stages of the disease.²⁰ Humoral response to antigens is not constant during the infection. A recent study compared specific Ig-isotype responses to different *Map* antigens in different stages of infection. The response patterns found for all combinations tested were different, indicating that there is no uniform association between increased antibody responses and the progression of the disease.¹⁵

In addition, an inhibition study was performed with three ELISA using different antigens, in which the PPA and LAM cross-reacted among themselves but the crude

protoplasmic VRI 316/102-2 extract (used in HerdChek *Mpt*) and LAM did not. The lack of cross-reactivity among those assays suggests the absence of shared antigens in these preparations. Therefore, it is possible that these ELISA, individually, can identify only a subset of an infected population of animals.²² This is in agreement to our findings and could explain the high number of discordant results, despite both presented acceptable results in positive and negative controls.

Therefore, we suggest that the most probable hypothesis to explain this high occurrence of false-positive results is that, since they use different antigens, the two tests used in this study has detected distinct subsets of infected animals and different moments in the evolution of the infection, as well as in our previous study.

In conclusion, although it was not concordant in all samples, sensitivity and specificity levels were comparable to PPA-ELISA, PARACHEK and HerdChek *Mpt*. Due to the great number of discordant results, PPA-ELISA cannot be used as an individual and definitive diagnostic method. Nevertheless, because of its feasible execution and low cost, it can be recommended as a screening test for identification of infected herds and foci of paratuberculosis in Brazil.

REFERENCES

- Boelaert, F.; Walravens, K.; Biront, P.; Vermeersch, J.P.; Berkvens, D. and Godfroid, J. 2000. Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. *Vet. Microbiol.* 77:269-281.
- Cocito, C.; Gilot, P.; Coene, M.; De Kesel, M.; Poupard, P and Vennuffel, P. 1994. Paratuberculosis. *Clin. Microbiol. Rev.* 7(3):328-345.
- Collins, M.T.; Sockett, D.C.; Ridge, S. and Cox, J.C. 1991. Evaluation of a commercial enzyme-linked immunosorbent assay for Johne's disease. *J. Clin. Microbiol.* 29(2):272-276.
- Collins, M.T. and Sockett, D.C. 1993. Accuracy and economics of the USDA-licensed enzyme-linked immunosorbent assay for bovine paratuberculosis. *J. Am. Vet. Med. Ass.* 203 (10):1456-1463.
- Dolz, G.; Araya, L.N.; Suárez, J. and Jiménez, C. 1999. Prevalence of antibodies to bovine paratuberculosis detected by a LAM-ELISA in Costa Rica. *Vet. Rec.* 144:322-323.
- Eamens, G.J.; Whittington, R.J.; Marsh, I.B.; Turner, M.J.; Saunders, V.; Kemsley, P.D. and Rayward, D. 2000. Comparative sensitivity of various faecal culture methods and ELISA in dairy herds with endemic Johne's disease. *Vet. Microbiol.* 77:357-367.
- Ferreira, R.; Fonseca, L.S. and Lilenbaum, W. 2001. Detecção de anticorpos contra *Mycobacterium paratuberculosis* em rebanhos bovinos do Estado do Rio de Janeiro, Brasil. *Rev. Bras. Med. Vet.* 23:166-171.
- Ferreira, R.; Fonseca, L.S. and Lilenbaum, W. 2002. Agar Gel Immunodiffusion test (AGID) evaluation for detection of bovine paratuberculosis in Rio de Janeiro, Brazil. *Lett. Appl. Microbiol.* 35: 163-175.
- Fonseca, L.F.L.; Santos, M.V.; Pereira, C.C.; Olival, A.A.; Hiene-mann, M.B. and Richtzenhain, L.J. 1999. Identificação da presença de anticorpos contra *Mycobacterium paratuberculosis* em rebanhos leiteiros do Estado de São Paulo. *Arq. Inst. Biol. São Paulo*, 66:122.
- Galen, R.S. and Gambino, S.R., 1975. Beyond Normality: The Predictive Value and Efficiency of Medical Diagnosis, (John Wiley and Sons, New York).
- Gasteiner, J.; Awad-Masalmeh, M. and Baumgartner, W. 2000. *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle in Austria, diagnosis with culture, PCR and ELISA. *Vet. Microbiol.* 77:339-349.
- Goldberger, M. 1997. Regulatory issues for new devices. WHO Tuberculosis Diagnostic Workshop, Product Development Guidelines. WHO. Cleveland, Ohio.
- Harris, N.B. and Barletts, R.G. 2001. *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. *Clin Microbiol. Rev.* 14 (3):489-512.
- Jubb, T.F. and Galvin, J.W. 2002. Testing to control Johne's disease in dairy herds in Victoria. 7th. Int. Coll. Paratuberculosis. Bilbao, 11-14th June 2002. p. 126.
- Koets, A.P.; Retten, V.P.M.G.; De Boer, M.; Bakker, D.; Valentin-Weigand, P. and Van Eden, W. 2001. Differential Changes in Heat Shock Protein-, Lipoarabinomannan-, and Purified Protein Derivative-Specific Immunoglobulin G1 and G2 Isotype Responses during Bovine *Mycobacterium avium* subsp. *paratuberculosis* Infection. *Infect. Immun.* 69 (3):1492-1498.
- Muskens, J.; Barkema, H.W.; Russchen, E.; Van Maanen, K.; Schukken, Y.H. and Bakker, D. 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. *Vet. Microbiol.* 77:253-261.
- NAHMS, 1997. Johne's Disease on U.S. Dairy Operations. USDA: APHIS: VS. CEAH, National Animal Health Monitoring System. Fort Collins, CO. #N245.1097.18. Ocepek, M.; Krt, B. and Pogacnik, M. 2002. Seroprevalence of paratuberculosis in bull mothers in Eslovenia. 7th. Int. Coll. Paratuberculosis. Bilbao, 11-14th June 2002. p. 113.
- Socket, D.C.; Conrad, T.A.; Thomas, C.B. and Collins, M.T. 1992. Evaluation of four serological tests for bovine paratuberculosis. *J. Clin. Microbiol.* 30:1134-1139.
- Stabel, J.R. (2000). Transitions in immune response to *Mycobacterium paratuberculosis*. *Vet. Microbiol.* 77:465-473.
- Stabel, J.R. 1998. Johne's disease: a hidden threat. *J. Dairy Sci.* 81:283-288.
- Sugden, E.A.; Stilwell, K. and Michaelides, A. 1997. A Comparison of lipoarabinomannan with other antigens used in absorbed enzyme immunoassays for the serological detection of cattle infected with *Mycobacterium paratuberculosis*. *J. Vet. Diagn. Investig.* 9:413-417.
- Van Maanen, C.; Koster, C.; van Veen, B.; Kalis, C.H.J. and Collins, M.T. 2002. Validation of *Mycobacterium paratuberculosis* antibody detecting ELISAs. 7th. Int. Coll. Paratuberculosis. Bilbao, 11-14th June 2002. p. 61.
- Walravens, K.; Marché, S.; Malbrecq, S.; Rosseels, V.; Huygen, K. and Godfroid, J. 2002. Evaluation of a (lipo)polysaccharides absorbed ELISA for the serological diagnostic of *M. paratuberculosis*. 7th. Int. Coll. Paratuberculosis. Bilbao, 11-14th June 2002. p. 65.
- Whitlock, R.H.; Wells, S.J.; Sweeney, R. Wand and Van Tiem, J. 2000. ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Vet. Microbiol.* 77:387-398.

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