COMPARISON OF AN IN-HOUSE AND A COMMERCIAL ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DIAGNOSIS OF PARATUBERCULOSIS

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SHORT COMMUNICATION

ABSTRACT

An in-house PPA-ELISA was compared to a commercial ELISA using a panel of 108 serum samples. In relation to commercial test, in-house assay presented 100% of sensitivity and 83.5% of specificity and a high concordance ($\kappa > 0.5$), which demonstrates that the in-house assay is a valuable tool for use in developing countries.

Key words: Cattle, *Mycobacterium avium* subsp. *paratuberculosis*, serology

Paratuberculosis is a chronic enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is characterized by chronic intermittent diarrhea with bacillary excretion. It progresses through several stages and, in the majority of cases, takes several years to manifest itself with clinical signs (11).

The diagnosis of paratuberculosis in ruminants is based on serological tests, detecting antibodies. Complement fixation test or enzyme-linked immunosorbent assay are routine laboratory methods, while the cultivation of feces and the isolation of Map is the gold standard diagnostic method (9). Problems of specificity are due to the high degree of the similarity that exists between *Map* and environmental Mycobacteria, especially the closely related *Mycobacterium avium* subsp. *avium.* Despite a *M. phlei* preadsorption step, which is intended to eliminate cross-reactions with environmental Mycobacteria, other infections such as bovine tuberculosis may interfere with the specificity of the test (10).

An in-house ELISA protocol using PPA (paratuberculosis protoplasmic antigen – Allied Monitors, USA) as capture antigen evaluated a selected panel of serum samples, presenting an overall sensitivity of 76.9% and specificity of 70.0% (8). This assay achieved the sensitivity and specificity index after some modifications of the original ELISA-PPA developed before in our laboratory (5). The most valuable alterations, which improved the results, were the antigen sonication and the overnight coating of the plate at 8ºC (8). These results have encouraged us to compare the in-house protocol with a commercial accredited test for the diagnosis of paratuberculosis. The aim of this study was to compare the performance of the in-house PPA-ELISA (ELISAinh) with a commercial ELISA (ELISAcom - Pourquier-France).

A panel of serum samples obtained from 108 dairy adult animals from herds kept in Rio de Janeiro, Brazil, was used. Sera were divided into two categories and four groups. Category 1 comprised sera of infected animals and was used to determine the sensitivity of the test. This category comprised Group A, which included 12 sera from animals with confirmed diagnosis trough positive fecal culture and/or histhopatological findings but none of them were tested by serological assays; eight of

which were asymptomatic (12). The Group B comprising sera from 14 asymptomatic animals serologically positive. From those animals, bacteriological culturing was not attempted, but they were considered as infected since their sera were reactive in three serological assays: two different commercial ELISAs (Parachek-CSL, HerdCheck-Idexx) and the old version of our in-house ELISA, which also uses PPA antigen (5). Category 2 consisted of sera from uninfected animals and was used to determine the specificity of the test. This category was composed of Group C, which included sera of 40 asymptomatic animals from infected herds with negative fecal culture (12) and Group D, which included sera from 42 animals from herds PTB-free and non-reactive in the same three ELISAs used to characterize Group B.

Tests with the commercial assay (ELISAcom) were performed according to the manufacturer's instructions (Pourquier Institute). The in-house ELISA protocol (ELISAinh) was performed as previously described (8). Briefly, PPA was diluted in carbonate buffer and sonicated for one minute with a 75-W cell disruptor (VCX 400W). The plates were coated overnight at 8ºC. Each well was blocked with 2% Casein (Sigma) in TBST (Tris (Sigma) 10 mM, 0.9% NaCl, 0.2% Tween 20), 100 μL/well. *M. phlei* (whole lyophilized *M.phlei* – Allied Monitor- USA) was resuspended at a concentration of 5 mg/mL and mixed with an equal volume to the serum samples for 60 minutes in constant agitation at 37ºC. After that, *M. phlei*- serum suspension was incubated overnight at 8ºC. Monoclonal IgG bovine (Sigma) linked to alkaline phosphatase was used as a conjugate. Immune complexes were detected by addition of p-NPP substrate (Sigma) (1 mg/mL). After incubation for 30 minutes, the reactions were stopped with NaOH 2N. Three wash steps with TBST were included after incubation with first and second antibody. All the sera were tested in duplicate at a dilution 1:100. The optical density (OD) at a wavelength of 405 nm was recorded. 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. Sera was classified as positive when their S/P ≥ 0.35.

In cattle from category 1 with positive culture results (Group A), six sera were reactive with ELISAcom, while seven were reactive with ELISAinh. These findings were expected and are in agreement with other investigators, who demonstrated that commercial ELISA employing sera pre-adsorbed with *M. phlei* can detect only 50% of infected cattle, with a sensitivity of 49% when compared to culture (6). Considering the presence of clinical signs of disease, both ELISAs correctly identified all of the four symptomatic animals. The commercial test detected two asymptomatic cows, while the in-house assay detected the same two cows plus one other. The other culture-positive asymptomatic five cows were not identified by either of the serological assays. This finding is consistent with the reports that some infected animals seroconvert after becoming shedders,

while other animals first shed the bacteria and then undergo seroconversion (4).

In the fourteen seroreactive animals without culture results (Group B) only two (14.3%) were reactive in ELISAcom, while the in-house protocol detected these two plus seven others (64.3%) animals. In paratuberculosis, the humoral response tends to increase mainly during the late stages of the infection, when clinical signs appear (3). Since those animals were asymptomatic, the failure of some serological tests in detecting them is not unexpected.

Sensitivity was estimated using the two groups of positive samples (culture for Group A and three concordant serological results for Group B). From the 12 animals of Group A, seven were reactive to ELISAinh, as well as 9/14 from Group B. Therefore, the overall sensitivity of ELISAinh was 61.5%. The commercial ELISA correctly identified six reactive animals from Group A and two from the Group B, with an overall sensitivity of 34.6%.

Specificity was estimated using the two groups of negative samples. In the 40 animals of Group C, ELISAcom and ELISAinh correctly identified as non-reactive 37 and 34 sera, respectively. From the 42 sera of Group D, 39 were identified as non-reactive by ELISAinh, while none of them were reactive in ELISAcom. The overall specificity of the in-house assay was 89%, which means that 73/82 negative animals were correctly identified. The commercial ELISA correctly identified 79/82 animals and presented an overall specificity of 96%.

A detailed examination of each group revealed that main differences between the two assays were observed to the Group B samples. For these samples, ELISAinh presented 64.3% of sensitivity, while ELISAcom correctly identified only 14.3% of the sera. Since Group B samples are composed of serologically reactive animals without culture results, the most probable explanation for this discrepancy should be found in the nature of the used antigens. ELISAinh uses a different antigen than the commercial test and, therefore, probably detects distinct subsets of infected animals at different moments of evolution of the infection. This phenomenon has been well characterized and discussed before (2,5,7). Therefore, in a more restrict point of view, when only animals with bacteriological culture are studied (Groups A and C), then sensitivity and specificity of both assays become very similar (50%-58.3%; 92.5-87.5%, respectively), with a non-significant difference ($\chi^2 = 0.7$, $p < 1$) and a high concordance ($\kappa > 0.5$) between them.

When ELISAcom is considered as standard test, since it is an accredited test worldwide used, then ELISAinh presented 100% of sensitivity and 83.5% of specificity in relation to the standard and a high concordance ($\kappa > 0.5$) between the two tests. ELISAinh presented some false-positive results, which may be a disadvantage. Nevertheless, in the first phases of the control program of the disease, a highly sensitive test is preferable as a screening test for diagnosis over a highly specific one, and some false-positive results may be acceptable (1).

Paratuberculosis is a chronic disease, which its first peculiar symptoms appear only after two or three years after Map infection. At the first stages of the disease, cattle shed intermittently Map with feces, so a negative fecal culture of an animal came from an infected herd does not rule paratuberculosis out. As shedding of bacilli at levels detectable by fecal culture is irregular and does not occur during the early stages of infection, serological assays could be used as an early diagnostic test for its high sensitivity and capability to identify infected herd at this stage. The antibody levels against Map became detectable before shedding of the microorganism in the majority of animals (11).

Our results demonstrated the usefulness of the in-house assay, since it is easy to perform and presents lower costs than the commercial test, which may be very important in developing countries. Due to its sensitivity, the in house protocol described here can be recommended as a screening test for identification of paratuberculosis foci and is a valuable tool for diagnosis and control of the disease, with results comparable to the commercial accredited tests.

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RESUMO

Comparação de um teste in-house e um teste ELISA comercial para Diagnóstico de Paratuberculose

Um teste ELISA *in-house* foi comparado a um teste comercial utilizando um painel de 108 soros. Em relação ao teste comercial, o ensaio *in-house* apresentou sensibilidade de 100%, especificidade de 83,5% e alta concordância ($\kappa > 0,5$), o que demonstra seu valor para uso em países em desenvolvimento.

Palavras-chave: Bovinos, *Mycobacterium avium* subsp. p*aratuberculosis*, sorologia

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