Usefulness of serological ELISA assay for *Taenia saginata* to detect naturally infected bovines

Utilização de teste sorológico ELISA para a detecção de bovinos naturalmente infectados por *Taenia saginata*

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Abstract

Bovine cysticercosis, a cosmopolitan disease caused by *Taenia saginata*, leads to economic losses due to carcass devaluation at slaughter. Sanitary inspection at slaughterhouses, the routine diagnostic method in Brazil, lacks the necessary sensitivity to detect the mildly infected cattle that are typically encountered in Brazil. In this study we have tested cattle sera from animals diagnosed as positive and negative by veterinary inspection for (1) anti-parasite antibodies using metacestodes antigens (*T. solium* vesicular fluid and *T. saginata* secretions) and (2) the HP10 secreted antigen of viable metacestodes. The cut-off values were calculated by ROC curve for intense and mild infections conditions, and by the classical method (X + 2DP for negative samples). The sensitivity and specificity of these diagnostic tests were different depending on the assumed cut-off value and, importantly, whether the infection was mild or intense. In spite of these observations, however, such ELISA assays for serum antibodies and parasite antigens constitute an important tool for epidemiological purposes, and in establishing priorities for the control of bovine cysticercosis.

Keywords: Sanitary inspection, HP10 antigen assay, ELISA, vesicular fluid, excretion-secretion.

Résumé

La cisticercose bovina, une maladie cosmopolite causée par *Taenia saginata*, entraîne des pertes économiques en raison de la désvalorisation des carcasses au moment de l’abattage. L’inspection sanitaire dans les abattoirs est le diagnostic standard au Brésil, il ne possède pas la sensibilité nécessaire pour détecter les bœufs légèrement infectés, qui sont généralement rencontrées au Brésil. Dans cette étude nous avons testé le sérum de bœufs provenant de bœufs diagnostiqués comme positifs et négatifs par inspection vétérinaire pour (1) anticorps anti-parasite en utilisant des antigènes de métacystodes (*T. solium* fluide vesiculaire et *T. saginata* sécrétions) et (2) l’antigène HP10 sécrété des métacystodes viables. Les valeurs de seuil étaient calculées par la courbe ROC, considérant les conditions de forte et modérée infections, et par le méthode classique (X + 2DP des échantillons négatifs). La sensibilité et l’exactitude de ces tests diagnostiques étaient différentes selon la valeur de seuil présumé et, notamment, selon que l’infection était légère ou intense. Malgré ces observations, cependant, ces assisses ELISA pour les anticorps sériques et les antigènes parasites constituent un outil important pour des fins épidémiologiques et dans l’établissement de priorités concernant le contrôle de la cisticercose bovine.

Introduction

Cattle are intermediate hosts of the cestode parasite *Taenia saginata*, developing bovine cysticercosis, a cosmopolitan disease characterized by the localization of the parasitic larval form (metacestode) in the muscles of infected animals. *T. saginata* infected carcasses have reduced value due to the treatment necessary to kill the parasite (freezing, heat or brine) or to its condemnation when the intensity of infection makes the carcasses unfit for human consumption (Brasil, 1952; Ferrer et al., 2007a; Yamane et al., 2012).

Bovine cysticercosis is endemic in Brazil with a prevalence that ranges from 0.7% to 7.4% (Ungar; Germano, 1992; Marques et al., 2008). Thus, with an infection rate of 5%, in the approximately 209 million Brazilian cattle, there would be an economic loss equivalent to about 10 million infected animals (SOUZA et al., 2007; ABIEC, 2012).

There are two factors that play a negative role in the control of this disease in Brazil. First, infections are typically mild, raising the risk that some infected animals will escape veterinary inspection at the slaughterhouses. Second, ranches supplying the slaughterhouses usually do not breed the animals; these are supplied at about three months of age from other farms without any testing. After their arrival at the cattle ranches, the animals then spend the following twelve to eighteen months on pasture, and their final three months prior to slaughter in confinement feeding on silage. This is the typical scenario in the major beef producing States of Brazil like São Paulo and Mato Grosso do Sul.

Diagnosis of bovine cysticercosis in Brazil is based on meat inspection of the carcasses during slaughter by cuts in the predicted sites of infection such as masseter, heart and diaphragm, and tongue if palpation indicates the possible presence of metacestodes (Brasil, 1952). However, this method presents low sensitivity, especially in mild infection where metacestodes can occur in other than the predicted sites, for example, shoulder clod, knuckle and back ribs (Lopes et al., 2010).

ELISA immunoassays for anti-parasite antibodies using *T. saginata* vesicular fluid (Onyango-Abuje et al., 1996b), total soluble metacestode antigens (Minozzo et al., 2004), recombinant peptides (FerreR et al., 2007b) and excretion-secretion antigens (Ogunremi; Benjamim, 2010) have been developed. Homology between *Taenia* species has been also explored and used in favor of antigen production for diagnostic purposes (GONZÁLEZ et al., 2007; OLIVEIRA et al., 2007; GONÇALVES et al., 2010). Such tests only demonstrate exposure of the animals to the infection and not necessarily a concomitant infection (HARRISON et al., 1989). Evidence for viable metacestodes through HP10 ELISA test detecting a selected parasite molecule in contrast, does identify cattle harbouring living and thus transmissible parasites. Taken together, ELISA assays identifying anti-parasite antibodies and the HP10 metacestode antigen provide useful information relevant for epidemiological studies or for the selection of animals to be treated before slaughter.

In this study we have tested cattle sera from animals diagnose as positive and negative by veterinary inspection for (1) anti-parasite antibodies using metacestode antigens and (2) the secreted HP10 antigen of viable metacestodes. The results illustrate the problem of diagnosing mild infections, but do reinforce the usefulness of such serological ELISA assays as tool for epidemiological studies and as an adjunct to meat inspection.

Materials and Methods

1. Biological samples

This research was approved by the Ethics Committee on Animal Experiments (CEUA 02017-2011).

Serum samples were obtained from 200 bovines during sanitary inspection divided in two groups: positive (n = 100) and negative bovines (n = 100) and conserved at –20 °C, until processing.

In all ELISA tests, serum from two *T. saginata* experimentally infected 8-month-old calves harboring 3.072 and 3.762 metacestodes each, and a 60-day-old calf non-cysticercotic from a non-endemic area were included as positive and negative controls, respectively, in order to normalize any day to day variation.

2. Antigens

*Taenia solium* cysticerci were dissected from skeletal muscles of naturally infected pigs during slaughter procedures and collected in phosphate buffered saline (PBS, pH 7.4). The metacestodes were ruptured with fine needle and vesicular fluid was collected. Calcium was removed by adding 50 μL of ammonium oxalate (0.3 M) and 25 μL ammonia (1:3, v/v, in water) to each mL of vesicular fluid, centrifuging (2,000 × g, 4 min, at 20 °C), and discarding all precipitated material. The supernatant was then collected and stored in liquid nitrogen (Larralde et al., 1986).

*T. saginata* metacestodes were excised from the skeletal muscle of naturally infected cattle from São Paulo State, Brazil, during slaughter procedures. After dissection, pooled metacestodes were washed in saline (NaCl 0.15 M) at 4 °C, in the presence of a protease inhibitor (PMSF 25 mM). The metacestodes were ruptured with fine needle and vesicular fluid was collected and centrifuged at 15,000 × g for 60 minutes at 4 °C. The supernatant was collected and a protease inhibitor (PMSF 25 mM 10%) was added. Afterwards, the fluid was centrifuged at 15,000 × g for 30 minutes at 4 °C and the supernatant was collected and stored at –80 °C (VAZ et al., 1997).

*T. saginata* excretory-secretory antigen (ES) was prepared with modifications according to Ogunremi and Benjamim (2010): groups of twenty viable metacestodes were washed in phosphate-buffered saline (NaCl 0.15 M) at 4 °C were cultured in RPMI 1640 medium (GIBCO®) supplemented with penicillin (500 units/mL) and streptomycin (500 μg/mL) at 37 °C and 5% CO2. After three days, RPMI 1640 supplemented with L-glutamine was substituted for the DMEM medium. Metacestodes were cultured up to 15 days, the supernatant was collected every 48 hours and stored at –20 °C until used. Pooled supernatant containing the ES antigen was centrifuged then dialyzed against PBS at 4 °C and lyophilized for concentration.
3. Detection of anti-parasitic antibodies

Indirect ELISA was performed according to Ferrer et al. (2007b) with slight modifications. MaxiSorp (Nunc-Immuno™ Plates) polystyrene microplates were coated with antigens extracts, at 10 µg/mL, diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6), at 4 °C, overnight. Blocking was done by 5% nonfat milk diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6), at 4 °C, for 2 hours. Assays were performed with bovine serum samples diluted 1:100 in phosphate solution (PBS 0.01M, pH 7.2, Tween 20® 0.05%) plus 5% nonfat milk, followed by incubation for 1 hour, at 37 °C. Peroxidase-labelled rabbit IgG antrovine conjugate (Sigma®-A5295), diluted 1:8000 in PBS/Tween 20® 0.05% / 5% nonfat milk, was added and incubated at 37 °C for 1 hour.

The 3, 3’, 5, 5’ tetramethyl benzidine (TMB) in citrate-phosphate buffer (pH 5.0) (Invitrogen® cat.00-2023) was used as substrate and the reaction was stopped by adding 3N HCl 20 minutes after its addition. Microplates were read by Multiskan EX (Labsystems®) photometer at 450 nm.

The test was performed in duplicate with a final volume of 100 µL/well and the microplates were washed 3 times, for 3 minutes with PBS-Tween 20® 0.05% between each immunoassay stages.

4. Detection of HP10 antigen

Sandwich ELISA was performed according to Harrison et al. (1989) with slight modification, using HP10 monoclonal antibody. MaxiSorp (Nunc-Immuno™ Plates) polystyrene microplates were coated with monoclonal antibody HP10 (10 µg/mL) diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6), at 4 °C, for 18 hours. Microplates were blocked with 1% bovine serum albumin (BSA) (Sigma – A7906) diluted in phosphate buffer (PBS, 0.01M, pH 7.2) and incubated at 37 °C, for 1 hour.

Bovine serum samples were added undiluted and incubated for 1 hour, at 37 °C, followed by biotinylated monoclonal antibody HP10 (2,5 µg/mL in PBS 0.01M, pH 7.2), 1 hour, at 37 °C. As detection system, streptavidin labeled with horseradish peroxidase (Sigma®-S2438) was added (1:40.000) diluted in PBS (0.01M, pH 7.2) and incubated for 1 hour, at 37 °C. Samples were tested in duplicate with a final volume of 100 µL well.

Microplates were washed 4 times, for 3 minutes each, with PBS-Tween 20® 0.05%, between each immunoassay stage, except after incubation with biotinylated HP10 and before chromogen solution addition, when washing was done for 5 minutes each time.

The 3, 3’, 5, 5’ tetramethyl benzidine (TMB) in citrate-phosphate buffer, pH 5.0 (Invitrogen® cat. n. 00-2023) was used as substrate. The color reaction was allowed to proceed for 15 minutes at 37 °C in the dark, before stopping by adding 100 µL of 0.2 M H₂SO₄ (Baker). Absorbance values were measured in a Multiskan EX (Labsystems®) photometer at 450 nm.

5. Statistical analysis

Optical density (O.D.) values were standardized for indirect ELISA by the formula described by Ogunremini and Benjamim (2010) modified to:

\[
\text{Sample/Positive} = \frac{\text{Mean O.D. sample}}{\text{Mean O.D. negative sample control}}
\]

\[
= \frac{\text{Mean O.D. positive sample control}}{\text{Mean O.D. negative sample control}}
\]

The ELISA cut-off for each antigen preparation and under intense and mild T. saginata infection was established based on the classical method, using the mean S/P values plus two standard deviations of the negative samples as well as by the Receiver Operating Characteristic Curve (ROC Curve). Tests performances were compared through the area under the curve (AUC) with its respective confidence interval of 95%, using GraphPad® Prism4 software.

In order to verify the difference among the antigens preparation for the indirect ELISA assay Cochran Q test (α = 0.05) was performed by the BioEstat® 5.0 program.

To control for day to day variations, standard negative and positive control bovine sera were included, and the values obtained for the experimental samples were accordingly normalised.

Results

Cut-off values were calculated by the classical way (X + 2SD) using 100 samples negative by inspection. Values were 0.655 for HP10 ELISA and 0.653, 0.628 and 1.559 for T. solium vesicular fluid, T. saginata vesicular fluid and T. saginata ES antigens in indirect ELISA, respectively.

As the majority of cases of bovine cysticercosis in Brazil are light infections with few metacestodes, cut-off values were also calculated by the ROC curve considering both, intense and mild infection condition. Thus for bovine harboring up to two viable metacestodes (n = 95) the cut-off values were 0.171 for HP10 ELISA and 0.240, 0.256 and 0.682 for T. solium vesicular fluid, T. saginata vesicular fluid and T. saginata ES indirect ELISA, respectively. For animals harboring more than 50 viable metacestodes (n = 95), the cut-off values were 0.349 for HP10 ELISA and 0.528, 0.615 and 1.560 for T. solium vesicular fluid, T. saginata vesicular fluid and T. saginata ES indirect ELISA, respectively.

The various assays and their established cut-off values were then applied to sera from bovines diagnosed as negative (n = 100) and positive (n = 100) by sanitary inspection. As can be seen, there were significant difference between the veterinary inspection and the four different serological (ELISA) analysis (Figure 1).

Sensitivity and specificity of HP10 ELISA and indirect ELISA varied according to the antigen preparations, the way cut-off values were established and due to intensity of infection (Table 1). Values of the area under the curve (AUC) for intense and mild infection conditions were 0.905 and 0.681 for HP10 ELISA; 0.872 and 0.684 for T. solium vesicular fluid; 0.834 and 0.567 for T. saginata vesicular fluid; 0.794 and 0.530 for T. saginata ES indirect ELISA. The AUC performed best for both mild and intense infections using T. solium vesicular fluid antibody assay.
The Q Cochran test showed no significant difference between antigens preparations used in this study (P > 0.05) in indirect ELISA. Best sensitivity was achieved by indirect ELISA using *T. solium* vesicular fluid in mild infection condition. Specificity was better with indirect ELISA using either *T. saginata* vesicular fluid or excretion-secretion antigen in intense infection conditions.

**Discussion**

In this study the possibility of serological identification of naturally *T. saginata* infected bovines in Brazil was explored by ELISA assays detecting (1) anti-metacestodes antibodies using three different target antigens and (2) viable metacestodes through detecting of HP10 antigen, a secreted product of viable metacestodes.

Reliable diagnosis of bovine cysticercosis in Brazil, either through veterinary inspection or serological procedures, is complicated by the fact that most of the bovines harbor few metacestodes.

A particular problem for serological diagnosis is that the negative control cut-off value is often close to the infected group in the ELISA assays. For example, Smith et al. (1991) performing indirect ELISA for bovines experimentally infected with a low dose of *T. saginata* eggs encountered precisely this problem.

As control of bovine cysticercosis is both a veterinary and public health problem, high sensitivity is essential, and this ultimately depends on how the cut-off is established. In this study, cut-off values were established through the classical way ($\bar{X} + 2SD$) and

![Graph showing S/P values for different antigens](image)

**Table 1.** Sensitivity and specificity for bovine cysticercosis by indirect ELISA, using *T. solium* and *T. saginata* vesicular fluid and *T. saginata* excretion-secretion antigen, and sandwich ELISA using HP10 monoclonal antigen.

<table>
<thead>
<tr>
<th></th>
<th><em>T. solium</em> vesicular fluid</th>
<th><em>T. saginata</em> vesicular fluid</th>
<th><em>T. saginata</em> excretion-secretion</th>
<th>HP10</th>
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<tbody>
<tr>
<td><strong>Sensitivity (%)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Classical cut-off$^a$</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>11</td>
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<tr>
<td>Intense infection$^b$</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td>16</td>
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<tr>
<td>Mild infection$^b$</td>
<td>64</td>
<td>49</td>
<td>52</td>
<td>31</td>
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<tr>
<td><strong>Specificity (%)</strong></td>
<td></td>
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<tr>
<td>Classical cut-off$^a$</td>
<td>93</td>
<td>96</td>
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<td>94</td>
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<tr>
<td>Intense infection$^b$</td>
<td>89</td>
<td>96</td>
<td>96</td>
<td>92</td>
</tr>
<tr>
<td>Mild infection$^b$</td>
<td>70</td>
<td>30</td>
<td>57</td>
<td>83</td>
</tr>
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</table>

$^a$Cut-off calculated by $\bar{X} + 2SD$ of negative samples. $^b$Cut-off calculated by ROC curve.
for conditions of mild and intense infection, calculated by the ROC curve. The latter has the advantage of permitting changes in the cut-off value depending on the purpose of the testing (GARDNER et al., 2010).

Although the antibody detection measures previous exposure and not necessarily a concomitant infection it does serve to identify infected herds. Applying the cut-off calculated by the ROC curve and considering mild infection condition, good sensitivity was observed for antibody detection using T. solium vesicular fluid (64%). Minozzo et al. (2004) also reported higher sensitivity using T. solium vesicular fluid antigen to detect infected bovines diagnosed as negative by sanitary inspection during slaughter in Brazil. T. saginata excretion-secretion antigens also showed good sensitivity (52%), although lower than the observed by Ogunremi and Benjamin (2010) that identified experimentally infected bovines with mild infection, using the same antigen and calculating the cut-off value by the ROC curve. Usually, the identification of positives is easier in experimental condition. Monteiro et al. (2006) observed higher number of false negative reactions on sera of naturally infected bovines compared to the experimentally infected animals, confirming the difficulties while testing field samples.

In contrast to antibody detection, the HP10 assay detects viable metacestodes and thus cattle with the potential to transmit taeniasis to humans. Clearly, the sensitivity in the HP10 assay depends on the number of viable metacestodes in an infected animal; the lower the number, the lower the signal, as has been pointed out in studies conducted in Spain Allepuz et al. (2012). Using the HP10 assay, better results were observed considering cut-off under mild infection condition (Table 1). One explanation is the higher possibility of antigen-antibody complexes in conditions of intense infection (DORNY et al., 2012). Using the HP10 assay, better results were observed considering cut-off under mild infection condition (Table 1). Monteiro et al. (2006) observed higher number of false negative reactions on sera of naturally infected bovines compared to the experimentally infected animals, confirming the difficulties while testing field samples.

Thus, low infection condition as well as the stage of infection can influence the amount of excretion-secretion circulating antigen making more difficult the detection of a naturally infected bovine.

**Conclusion**

Measurement of anti-parasite antibodies, particularly with T. solium vesicular fluid, would be a recommended strategy for epidemiological purposes, whereas measurement of parasite antigens by HP10 sandwich ELISA would be a recommended strategy for identifying infected animals at slaughter. Overall, there is a need of improving the sensitivity of immunodiagnosis to identify infected cows with low level infections.

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