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Short communication

Improvement of an enzyme immunosorbent assay for detecting antibodies against *Dioctophyma renale*

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ABSTRACT

An available enzyme-linked immunosorbent assay (ELISA) was studied for the detection of anti-*Dioctophyma renale* antibodies in the sera of dogs using, detection of parasite eggs in urine sediment as a reference test. ELISA uses a soluble antigenic preparation of esophagus of *D. renale* and the optimal dilutions of the antigen, serum and conjugate were determined by means of checker board titration, using positive ($n = 13$) and negative ($n = 27$) reference serum. The specificity and sensitivity of the ELISA were 93.8% and 92.3% respectively and the kappa index was good (0.76). These results suggest that ELISA described may prove to be an effective serological test for detecting dogs infected and exposed to this parasite mainly dogs that are not eliminating parasite eggs through their urine.

1. Introduction

Dioctophyma renale is a renal nematode parasite of mustelids, canids and even humans (who eat raw fish) (Measures, 2001; Ignjatovic et al., 2003).

The adults are found in the definitive host's kidneys, and mature females lay eggs that are eliminated with the host's urine. Larval forms develop in the aquatic oligochaete *Lumbriculus variegatus*, which acts as the intermediate host. Third-stage larvae present in oligochaetes are infective for the definitive host and also paratenic hosts fish and frogs (Measures, 2001).

Despite having wide geographical distribution, this helminth shows prevalence, which differs according to the region evaluated, being higher in cold climate and with abundant water sources. In Brazil, there are prevalence reports among dogs ranging from 0.5% to 3.6% in different states (Kommers et al., 1999; Pereira et al., 2006).

Dioctophymosis is difficult to diagnose before it utterly destroys the kidneys. *D. renale*-infected dogs usually show no clinical signs, but dysuria (especially at the end of urination) and low back pain may be present (Nakagawa et al., 2007; Ferreira et al., 2010).

The main diagnostic are based on identifying parasite eggs in urine samples (gold standard) or radiography and ultrasonography (Monteiro et al., 2002; Costa et al., 2004). In cases of infection only involving male nematodes, immature parasites or ectopic locations, parasitism will not be detectable through examination of urinary sediment. On the other hand, contrast radiography only demonstrates the degree of renal function impairment, without demonstrating the presence of the parasite. The sonography suggests the parasitism by viewing cross-sections of the nematode (Costa et al., 2004) being the suggestive image when the parasites are already adults and located in the kidney.

Currently, there is no standardized immunodiagnostic method for identifying dogs infected and exposed to *D. renale* and the currents methods may lead to false-negative diagnoses. Immunological methods for identifying exposed or infected dogs without clinical signs or without presence of eggs in urinary sediment may be an important tool for diagnosing dioctophymosis.

In the São Cristóvão district of the municipality of Três Barras, state of Santa Catarina, southern Brazil, high prevalence of *D. renale* infection was found in dogs between 2000 and 2003, comprising 30% among dogs that were necropsied (Pedrassani and Camargo, 2004). Furthermore, in that same municipality, third-stage larvae of the parasite were found in the stomach of 5.1% of frogs (*Chaunus ictericus*) (Pedrassani et al., 2009). These occurrences motivated a search for new techniques for diagnosing dioctophymosis.

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For these reasons, the aim of the present study was to evaluate the diagnostic performance of soluble antigen from the esophagus of adult *D. renale* helminths, in an indirect ELISA using, detection of parasite eggs in urine sediment as a reference test.

2. Materials and methods

2.1. Preparation of soluble antigen of D. renale

Four adult helminths (one male and three females) were obtained from naturally parasitized dogs that died due to other causes. They were washed in 0.9% saline solution and their esophagi were removed. These were washed five times in phosphate-buffered saline (PBS 1X; pH 7.2), added to ethylenediaminetetraacetic acid (1 mM) and macerated. This antigen solution was disrupted by seven freezing (−70 °C) / thawing (37 °C) cycles, with sonication (Branson Sonifier 250; 20 kHz, 1 mA), after centrifuged at 12,000 × g for 30 min, at 4 °C. The protein concentration was determined by means of the bicinchoninic acid method (Pierce Chemical Company) and the solution was stored at −70 °C until use.

2.1. Sedimentation technique for eggs in urine

Urine samples were examined by means of the sedimentation technique (Sloss et al., 1999).

2.1.1. Dog serum samples

The procedures used were approved by the Animal Ethics Committee of Universidade do Contestado (no. 67/2006).

Serum samples collected from dogs in an area endemic for dioctophymosis were used, as follows. Thirteen samples were obtained from dogs parasitized by *D. renale*, which had been confirmed through necropsy or the presence of eggs in urine (positive controls). Negative control serum samples were obtained from dogs showing negative results in both urinalysis and kidney and abdominal ultrasound. Among these, 25 samples were from dogs in Três Barras (longitude 50°22′53″W; latitude 26°8′42″S) and two samples were obtained from 3 days of age puppies in Jaboticabal, state of São Paulo (longitude 48°17′31″W; latitude 21°14′41″S), which is a locality that is not endemic for dioctophymosis. Furthermore, 54 serum samples from dogs in Três Barras (an area that is endemic for this parasitosis), which had been found to be negative in the urine sediment examination, were also used.

2.1.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method used was essentially as described in detail by Machado et al. (1997), with modifications. Briefly, 100 μl of antigen diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, was added to each well of a micro-ELISA plate (Nunc, Denmark) and protein concentrations was adjusted between 2.5 μg mL^{-1} and 20 μg mL^{-1}. The plates were sealed and incubated overnight at 4 °C. Plates were blocked for 90 min at 37 °C in a humid chamber with 2%, 6% or 10% skimmed fat-free milk powder in carbonate-bicarbonate buffer. After three washes with buffer phosphate buffered saline, pH 7.2, and 0.05% Tween 20 or Tween 80 (PBST), 100 μl of diluted canine sera (1:50–1:3,200) in PBST were added in duplicate to the ELISA plate. Plates were incubated at 37 °C in a humid chamber for 90 min and then washed as described above. A 100 μl aliquot of a 1:5,000 and 1:7,000 dilution (in PBST, with 6% skimmed milk powder) of peroxidase conjugated anti-dog IgG (Sigma) was added to each well and the plates were incubated at 37 °C under the same conditions for 90 min. After the same washing procedure, a volume of 100 μL of the substrate ortho-phenylenediamine was added to a concentration of 20 μg, along with 0.02% hydrogen peroxide in citrate buffer (pH 5.0). The plate was incubated in the dark at room temperature for 15 min, and 50 μL of stop buffer (2% H_2SO_4) was then added and the plates were then read at 490 nm wavelength on an ELISA reader.

2.1.3. Statistical analysis

The cutoff point was determined through analysis on the receiver operating curve (ROC) (Solano-Gallego et al., 2014) using the SPSS software. After defining the cutoff, the results were expressed as a percentage of the positive-control serum (PP) using the formula: (mean OD of duplicate test serum/mean OD of positive-control serum) × 100, where OD is optical density.

Sensitivity, specificity, accuracy, the positive predictive value (PPV), negative predictive value (NPV) and kappa index were made using the indirect ELISA results obtained from the 94 serum samples tested, in relation to the finding of eggs in the urine.

3. Results and discussion

Checkerboard titrations showed that antigen concentrations of 5 μg mL^{-1} and 10 μg mL^{-1} did not present any marked differences in absorbance among the control serum samples, and this made it possible to differentiate between the positive and negative samples (Fig. 1).

Serum dilution of 1:50 resulted in the following high absorbance values for the positive controls (0.731 and 0.835) and for the negative controls (0.138 and 0.271 (control 1)). At the dilution of 1:100, the absorbance of the positive controls were between 0.637 and 0.493, and the value of the negative control 1 was of 0.130. From the dilution of 1:200 onwards, the absorbance decreased greatly and therefore the serum dilution was standardized at 1:100 (Fig. 1).

In relation to using substances for blocking and washing plates, the combination of 6% skimmed milk powder and PBST-80 provided adequate differentiation according to the absorbance values, between the positive and negative serum samples. Serum diluted at 1:100, conjugate diluted at 1:5,000 and antigen at a concentration of 5 μg mL^{-1}, when tested with blocking using 2% skimmed milk powder and PBST-20, produced mean absorbance in the negative controls of 0.717 and in the positive controls, 0.873. Under the same initial conditions, but with blocking using 6% and 10% skimmed milk powder and PBST-80, the means absorbance in the negative controls were 0.096 and 0.080 and in the positive controls, 0.583 and 0.301, respectively.

The peroxidase conjugate tested with 6% skimmed milk powder and PBST-80 presented similar absorbance values for positive and negative controls regardless of the dilution tested and antigen protein concentration. For the positive controls, the absorbances were 0.498 and 0.667 with the conjugate diluted at 1:5,000 and these became 0.493 and 0.637 with the dilution of 1:7,000 (Fig. 1).

The mean absorbance, for the negative control serum samples was 0.132 ± 0.0372 and for the positive animals was 0.480 ± 0.097. The absorbance values most frequently observed for all the serum samples tested were in the range of 0.150–0.299. Dogs with *D. renale* generally present chronic infection with small numbers of helminthes (Monteiro et al., 2002; Nakagawa et al., 2007) and therefore can be expected to present low titers of specific antibodies. Dogs used in standardizing the test presented natural infection and this may explain the low level of antibodies. A serum sample (used as a positive control) from a necropsied dog with a female nematode in its kidney, presents the absorbance value 0.637 and it was one of the highest absorbance values.

For the ROC curve, the best cutoff was 54.67PP. The area under the ROC curve was 0.967 (95% CI: 0.898–1.000) with a standard error of 0.035 (Fig. 2a). The specificity and sensitivity of the ELISA proposed were respectively 93.8% (95%CI: 85.9–100.0) and 92.3% (95%CI: 64.2–100.0). The kappa index was 0.76 (good) and the