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Serologic immunoreactivity to *Neospora caninum* antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA

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Abstract

Neospora caninum, is a coccidian protozoan known as a major cause of bovine abortion and canine neuropathies. The aim of the present study was to develop a reliable and quick test to detect antibodies to *N. caninum* in dog sera. Sixty-five serum samples from dogs, including 35 positive and 30 negative for *N. caninum* antibodies were used for standardization of the test. In parallel, immunoreactivity of the sera to *Toxoplasma gondii* antigens was investigated using a passive agglutination test. A dot-ELISA test, using soluble extract of *N. caninum* tachyzoites on nitrocellulose ester membranes, was developed and standardized. SDS-PAGE and complementary analysis of reactivity by Western blotting were used for the characterization of the immunoreactive fractions of all tested sera. The sensitivity and specificity of the dot-ELISA were 94 and 73%, respectively, compared to IFAT at a cut-off of 1:50, and 87 and 100% compared to IFAT at a cut-off of 1:25. Among the sera that tested positively for both IFAT and dot-ELISA, only 8.6% were reactive to *T. gondii*. The most immunoreactive fractions in Western blots were the 14-, 33-, 42- and 55 kDa bands, with percentages of 42, 60, 42 and 37%, respectively. The 60 kDa band showed a non-specific reaction in 43% of neosporosis-negative animals by both dot-ELISA and IFAT. These results indicate that the dot-ELISA using *N. caninum* antigen present good sensitivity and specificity, and might be used as a screening test to detect antibodies to *N. caninum* in dogs.

Keywords: *Neospora caninum*; Immunodiagnosis; dot-ELISA; Dogs

1. Introduction

The coccidian protozoan *Neospora caninum* is a parasite of great veterinary importance, which infects different animal species; it has been extensively studied over the last years and has emerged as the main

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cause of bovine abortion in the United States, New Zealand and Holland (Dubey, 1999). In dogs, which the parasite undergoes sexual reproduction with the formation of oocysts (McAllister et al., 1998), the disease can cause severe neuromuscular alterations, especially in congenitally infected animals (Dubey, 2003).

Neosporosis is mainly identified by immunodiagnostic tests. Among these, the indirect immunofluorescence test (IFAT), enzyme-linked immunosorbent assays (ELISA), and the agglutination test, show high sensitivity and specificity (Björkman and Uggla, 1999), however, there is a lack of a quick and practical test for detection of *N. caninum* antibodies. The aim of the present study was to develop a dot-ELISA as a screening test for dog serum.

2. Material and methods

2.1. Serum samples

Sixty-five serum samples were obtained from dogs of both sexes and of different ages and breeds (53 sera were collected from municipalities of Salvador and Lauro de Freitas, Bahia, Brazil and 12 sera were acquired from University of Illinois, USA). Thirty-five samples were positive, 12 experimentally infected, with IFAT titers ranging from 1:50 to 1:800, and 30 samples were negative at an IFAT cut-off of 1:50.

2.2. N. caninum culture

Vero cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% heat-inactivated equine serum (Cultilab, Campinas, São Paulo, Brazil) at 37 °C in a 5% CO_2 atmosphere, with the medium being changed at regular intervals of 48 h. After formation of a confluent cell monolayer, cells were infected with *N. caninum* tachyzoites (NC-Bahia strain) (Gondim et al., 2001).

2.3. Purification and protein extraction from tachyzoites

Purified tachyzoites, obtained by a Sephadex G25 column, were adjusted to 1.5 to 6×10^4 cells/μL and lysed by ultrasound (GE-100 ultrasonic processor) on ice using six 60 Hz cycles for 30 s each. Lysis was monitored by microscopic observation of the tachyzoites in a Neubauer chamber. After refrigerated centrifugation at 15,000 × *g* for 30 min at 4 °C, the supernatant was recovered, protein concentration was determined by the method of Lowry (1951) (BioRad, USA), and small aliquots of the supernatant were stored at −70 °C until use.

2.4. IFAT for the detection of anti-N. caninum antibodies

Slide preparation and IFAT were performed as described by Dubey et al. (1988). Serum samples were first diluted 1:50 and then 1:25 in PBS. Positive serum samples were titrated. Positive and negative controls were included on each slide.

2.5. Standardization and use of dot-ELISA

To establish the best concentration of antigen, different amounts (0.1, 0.5, 5, 10 and 50 ng protein/well) of the soluble tachyzoite extract were applied to 0.45 μm nitrocellulose ester membranes (Millipore) under vacuum in a Bio-Dot apparatus (BioRad). The membranes were blocked with 5% powdered skim milk in 0.05% PBS-Tween (PBS-T) and tested at different dilutions (1:50; 1:100; 1:200 and 1:500) with *N. caninum*-positive sera previously diagnosed by IFAT and immunohistochemistry. After standardization, the membranes were sensitized with 0, 10, and 50 ng protein/well in a final volume of 25 μL. All serum samples were diluted in 1% skim milk in PBS-T at previously determined dilutions of 1:200 and 1:500, and incubated for 1 h at 37 °C. After six washes in PBS-T, the membranes were incubated with peroxidase-conjugated rabbit anti-dog IgG (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. The membranes were washed five times with PBS-T and once with 0.05 M Tris–HCl, pH 7.2, in 0.2 M saline (Tris-saline). The color reaction was carried out using a solution of 4-Cl-1-naphthol (0.3% 4-Cl-1-naphthol in methanol) diluted 1:5 in 0.05 M Tris-saline containing 30% 0.3 μL/mL H_2O_2. The membranes were incubated for 15 min at room temperature protected from light, and the reaction was stopped by washing the membranes in distilled water.