CANINE TOXOPLASMOSIS: A COMPARATIVE EVALUATION OF THE DETECTION OF ANTI-*TOXOPLASMA GONDII* ANTIBODIES BY THE INDIRECT IMMUNOENZYMATIC ASSAY (ELISA) AND THE INDIRECT IMMUNOFLUORESCENCE REACTION (IIF).

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SUMMARY: An indirect immunoenzymatic assay (ELISA) was standardized and used for the detection of anti-*T. gondii* antibodies in 276 serum samples from captured dogs or from dogs taken at the Veterinary Hospital - FCAV-UNESP, Jaboticabal Campus. In the indirect ELISA the optimum antigen concentration was 10 µg/ml when a single dilution of 1/200 was used for reference sera (positive and negative). The sensitivity of indirect ELISA was compared to that of indirect immunofluorescence (IIF). The presence of anti-*T. gondii* antibodies was detected in 46.01% (n=127) of the sera tested by IIF. Of these, 31.5% (n=40) showed final reactivity at 1/40 dilution, 40.1% (n=51) at 1/80 dilution, 13.4% (n=17) at 1/160 dilution, 2.4% (n=3) at 1/320 dilution, 9.5% (n=12) at 1/1280 dilution, and 0.8% (n=1) at 1/10240 dilution. In contrast, the presence of anti-*T. gondii* antibodies was detected in 62.5% (n=169) of the sera tested by indirect ELISA. Serum reactivity was analyzed in terms of ELISA levels (0 to 9). Sera that presented reactivity starting from the ELISA 2 level were considered to be positive, and the ones of highest concentration with reactivity were located at levels 5 (10.14%, n=28), 6 (9.78%, n=27) and 7 (6.88%, n=19), for a total of 26.8% (n=74). Clinical cases of acute toxoplasmosis with serologic confirmation by IIF and indirect ELISA were diagnosed and in some cases indirect ELISA proved to be sensitive enough to detect the presence of antibodies. Statistical analysis by the exact Fisher test showed a significant difference at the 1% level between tests and demonstrated the higher sensitivity of indirect ELISA in the immunodiagnosis of canine toxoplasmosis.

KEY WORDS: *Toxoplasma gondii*, Indirect ELISA, Indirect immunofluorescence, dogs.

INTRODUCTION

Toxoplasmosis, a zoonosis caused by *Toxoplasma gondii* that is attracting increased attention of investigators due to the severity of its manifestations, especially in humans, in its congenital form of transmission. The definitive hosts of the disease are felids, domestic cats being the most important, although any mammal can become an intermediate host (DUBEY, 1986).

The first case of canine toxoplasmosis was recorded by MELLO (1910) in Italy and by CARINI (1911) in Brazil. Cases of canine toxoplasmosis have been reported since in several countries, suggesting a significant dissemination in the environment (DUBEY, 1985; VIDOTTO, 1992). More recently, FRENKEL *et alii* (1995) suggested that dogs play an important role in the mechanical transmission of *T. gondii* because of their habit of ingesting and rolling in cat feces.

The high infectivity and low pathogenicity of *T. gondii* justify the studies carried out simply to measure antibody levels in addition to clinical examination (ISHIZUKA *et alii*, 1974b). The variability of clinical signs and symptoms, easily confused with those of other diseases such as canine distemper kynomosis and canine viral hepatitis, also justifies the importance of serologic diagnosis (VIDOTTO, 1992).

The first serologic test used for the diagnosis of toxoplasmosis was the dye test of Sabin-Feldman in 1948. Comparative analyses of serologic tests have been performed by several investigators in an attempt to demonstrate the most

effective one in the diagnosis of toxoplasmosis (ISHIZUKA *et alii*, 1974a; DUBEY, 1985; LOÜGREN *et alii*, 1987; OPEL, 1987; ABATE *et alii*, 1989). Studies on the prevalence of the disease are also being conducted to better clarify the severity of the problem in Brazil (ISHIZUKA *et alii*, 1974b; MARTINEZ-MAYA, 1986; GERMANO *et alii*, 1989; FREIRE *et alii*, 1992; GUIMARAES *et alii*, 1992).

In view of the considerations above, the objective of the present study was to standardize the indirect ELISA for the diagnosis of canine toxoplasmosis and to compare the indirect immunofluorescence (IIF).

MATERIALS AND METHODS

***Toxoplasma gondii* strain:** The *T. gondii* strain (N strain) available at the Veterinary Parasitology laboratory of FCAV-UNESP was obtained from the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and is being maintained through successive passages in *Mus musculus* mice from the Central Animal House of UNESP. The tachyzoite suspension was obtained by the method of CAMARGO (1972) with minor modifications. A purified parasite suspension was obtained by the filtration of the tachyzoite suspension through a column packed with sterile nylon. This suspension used a substrate for IIF and for the preparation of a soluble antigen for indirect ELISA. The tachyzoite suspension (10^7-10^8/ml) in 0.85% saline was submitted to 11 freezing cycles at -70°C and thawing at 37°C on a water bath. The final suspension was centrifuged at 17000 g for one hour at 5°C and the supernatant was collected, aliquoted and stored at -20°C until use. The protein concentration of the soluble antigen was determined by the method of HARTREE (1972).

Hyperimmune, negative and test sera: A clinically healthy female adult mongrel dog, serologically negative for *T. gondii*, was experimentally infected with a tachyzoite suspension (1 x 10^7/ml) by two injections by the intraperitoneal route, 30 day apart. Clinical examination, full blood count and parasitemia were performed during the acute and chronic phase of experimental infection. Positive control serum samples were obtained from this animal (n=10). Negative control sera (n=10) were obtained from mongrel puppies from the kennel of the "Governador Laudo Natel" Veterinary Hospital (HVGLN), Jaboticabal, UNESP, raised free from *T. gondii* infection. Test sera (n=276) were obtained from dogs captured in different towns of the region and/or from dogs seen at the animal clinic of HVGLN-UNESP.

Indirect immunofluorescence test (IIF): Slides containing fixed tachyzoites were air dried and successive serum dilutions (1/40, 1/80, 1/160, 1/320, 1/640 etc.) were pipetted onto areas circumscribed with nail polish. The slides were incubated in a moist chamber at 37°C for 45 minutes and then washed 3 times by immersion in PBS for 5 minutes under gentle magnetic shaking. The delimited areas were covered with approximately 0.02 ml of rabbit IgG conjugate anti-dog IgG (Sigma) labelled with fluorescein isothiocyanate at 1/20 dilution in PBS containing 0.01% Evans blue. The slides were then incubated at 37°C for 45 minutes and washed 3 times in PBS for 5 min each time, dried, mounted with buffered glycerin, pH 9.5, and covered with a coverslip. The slides were examined under a fluorescence microscope (epiillumination system) using a 40X objective (Olympus CBB). The highest serum dilution at which fluorescence was detected in the entire periphery of the protozoa was taken as the positive titer for the reaction. In the negative reactions, the protozoa did not present fluorescence or fluorescence was located only at one of the extremities of the parasite (polar staining).

Indirect ELISA: Optimum antigen solution and positive, negative and conjugated sera were determined by en bloc titration, with various antigen solutions being allowed to react in the presence of different dilutions of positive and negative sera and in the presence of a single dilution of the conjugate.

One hundred µl of antigen (4 µg to 10 µg/ml) diluted in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6, were added to each microplate well and the plate was incubated at 4°C for 18 hours in a moist chamber.

Between the various reaction phases the microplates were submitted to five washings of one minute each with 0.01 M PBS, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). Test sera and positive and negative reference sera were assayed in duplicate at 1/100 and 1/3200 dilutions in diluent buffer (PBS-Tween with 5% normal rabbit serum added). After the addition of 100 µl of the diluted sera, the microplates were incubated at 37°C for 90 minutes in a moist chamber. One hundred µl rabbit IgG conjugate anti-dog IgG coupled to alkaline phosphatase (Sigma) and 100 µl of the reaction substrate were added to the microplates and allowed to react for 40 minutes at room temperature. The reaction was stopped by the addition of 25 µl 3 M NaOH solution and a reading was taken with a spectrophotometer (BT.10, Embrabio) equipped with a 405 nm filter.

The immunological activity of each serum in the ELISA was calculated by determining the sample to positive serum ratio (S/P) at each dilution, considering positive and negative sera as reference, using the following equation:

$$S/P = \frac{\text{Mean sample absorbance - Mean negative reference serum absorbance}}{\text{Mean positive reference serum absorbance - Mean negative reference serum absorbance}}$$

S/P values were divided into ELISA level (EL) groups. Zero level was determined by the mean S/P value of animals not immune to *T. gondii* + 2 SD. Starting from this limit, the intervals between the other ELISA levels were defined by the addition of 35%, as proposed by MACHADO *et alii* (1995) for the *B. bovis* system.

Statistical analysis: Indirect ELISA and IIF were compared by the exact Fisher test at the 1% level of probability.

RESULTS

Clinical evaluation of the *T. gondii*-infected dog: The dog experimentally infected with *T. gondii* presented the following clinical signs 6 to 10 days after inoculation: anorexia, intermittent diarrhea, hyperthermia and lethargy. Ophthalmologic examination (75 days after infection) showed eye fundus hemorrhage, congested vessels and optic papilledema, characterizing papillary chorioretinitis.

The *T. gondii* strain proved to be pathogenic, causing normochromic microcytic anemia with a 35% reduction in erythrocyte number on the 47th day of infection. Hemoglobin levels were reduced by 23% on the 47th day. Hematocrit and mean corpuscular volume (MCV) were markedly reduced by 51% and 28%, respectively, on the 53rd day of infection. Neutrophil rod and lymphocyte counts were significantly elevated on the 47th and 53rd day after infection. No other alterations in blood parameters were observed during the experimental period. The dog required chemotherapy at recommended doses 60 days after infection, with good recovery.

Standardization of indirect ELISA: The results of en bloc titration of antigen, serum and conjugate showed that in the standardization of indirect ELISA the optimum antigen concentration was 10 µg/ml. The single serum dilution of the positive and negative reference sera was 1/200 and the dilution of the conjugate was 1/9000 (recommended by the manufacturer, Sigma A-6024). Under these conditions, the highest mean absorbance of positive sera (1.005 ± 0.226) (n=10) and the lowest mean absorbance of negative sera (0.037 ± 0.024) (n=10) were obtained. Thus, the ELISA level scale (EL) was defined as follows:

EL	S/P ranges
0	0.000-0.122
1	0.123-0.164
2	0.165-0.221
3	0.222-0.298
4	0.299-0.402
5	0.403-0.542
6	0.543-0.731
7	0.732-0.987
8	0.988-1.332
9	>1.332

Reactivity of positive and negative sera submitted to IIF and to indirect ELISA: Test sera were diluted 1/40 (IIF) and 1/200 (indirect ELISA) with the appearance of fluorescent protozoa and of an ELISA level of 2 or more, respectively. The detection of anti-*T. gondii* antibodies in the serum of the dog experimentally infected with *T. gondii* was performed on the 10th and 644th days by IIF and by indirect ELISA.

The reactivity to IIF and indirect ELISA of the sera obtained from the experimentally infected dog on different days is illustrated in Figure 1. Antibodies started to be detected during the 2nd week at a final dilution of 1/80 and EL equal to 6 by IIF and indirect ELISA, respectively. Maximum reactivity was observed between the 58th and 72nd day of infection at a final dilution of 1/1280 (IIF) and EL=8 (ELISA), with negativity to IIF occurring at 644 days, with polar fluorescence. In contrast, in ELISA the EL=5 was maintained from the 325th to the 644th day of experimental infection.

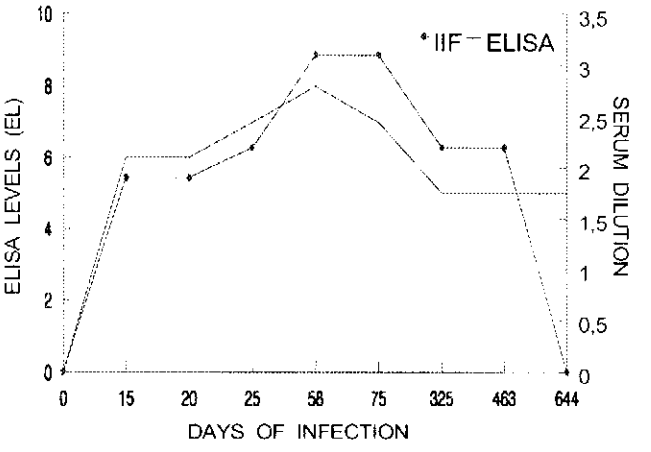

Fig. 1 - Titration of anti-*T.gondii* antibodies by IIF (log 2 dilution) and ELISA (EL) in an experimentally infected dog over a period of 0 to 644 days.

The test sera (n=276) were first divided into positive and negative groups by IIF. Positive sera were classified according to fluorescence intensity into weak reaction (1), medium reaction (2) and strong reaction (3) at the initial 1/40 dilution, so as to avoid nonspecific reactions. The presence of anti-toxoplasma antibodies was detected in 46.01% (127) of the 276 sera tested, with 14.1% (39) presenting a weak reaction (1), 26.1% (72) a medium reaction (2) and 5.8% (16) a strong reaction (3) (Figure 2). It should be pointed out that among the dog sera considered to be negative (53.9%; 149), 10% (15) presented a polar fluorescence pattern by IIF; however, 7 of these sera presented significant reactivity by indirect ELISA.

Figure 3 illustrates the number of positive sera previously classified by fluorescence intensity as a function of final

titration when diluted 1/80 to 1/10240. Of the 46.01% (127) reactive sera, 31.5% (40) presented final reactivity at 1/40 dilution, 40.1% (51) at 1/80 dilution, 13.4% (17) at 1/160 dilution, 2.4% (3) at 1/320 and 1/640 dilution, 9.5% (12) at 1/1280 dilution, and 0.8% (1) at 1/10240 dilution. Thus, the highest percentage of reactive sera was situated in the final titration range of 1/80. The reactivity of test sera (n=276) according to ELISA and divided by ELISA levels (EL) is illustrated in Figure 4, which shows serum reactivity at levels 2 (11.23% - 31), 3 (10.14% - 28), 4 (7.97% - 22), 5 (10.14% - 28), 6 (9.78% - 27), 7 (6.88% - 19), 8 (5.79% - 16) and 9 (1.81% - 5), for a total of 63.74% among the 276 sera tested in the presence of the *T. gondii* antigen. The sera that did not present reactivity to indirect ELISA belonged to levels 0 (27.89% - 77) and 1 (8.33% -23), representing 36.22% of all sera studied.

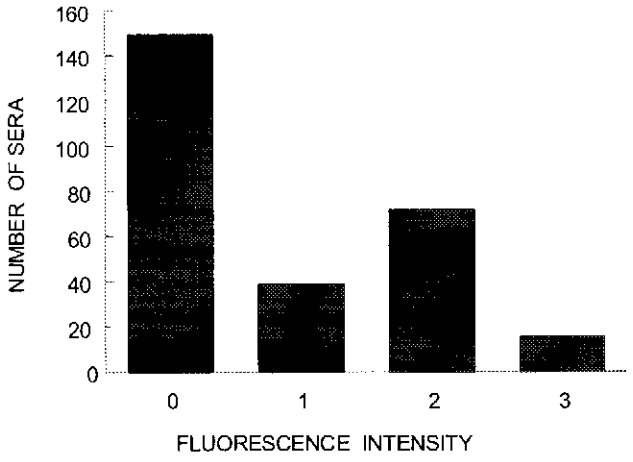

Fig. 2 - Distribution of the number of reactive test sera according to fluorescence intensity (1-weak; 2-medium; 3-strong) in the presence of *T. gondii* antigen.

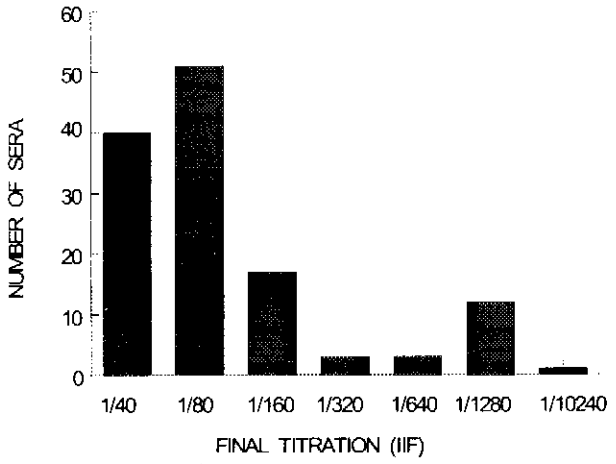

Fig. 3 - Distribution of the number of test sera (n=276) by reactivity considering the final dilution (1/40 to 1/10240) using IIF.

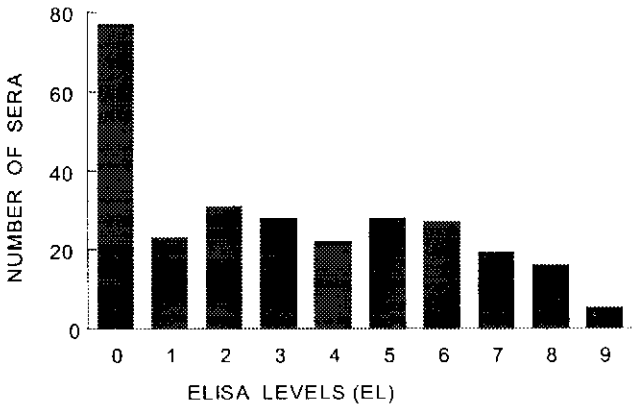

Fig. 4 - Distribution of the number of reactive test sera by ELISA levels (EL) in the presence of *T. gondii* antigen (10 mg/ml) using indirect ELISA.

Correlation between IIF and indirect ELISA: The maximum titration observed on the 58th day of experimental infection by IIF at 1/1280 dilution coincided with EL = 8 at the single 1/200 dilution, suggesting an intense immune humoral response provoked by the parasite.

The study of the correlation of the data obtained by IIF and indirect ELISA in the detection of anti-*T. gondii* antibodies (Table 1) showed 27.53% (76) co-negative sera and 36.59% (101) co-positive sera by the two tests. Comparative statistical analysis by the exact Fisher test showed a significant effect at the 1% level of probability, thus demonstrating the higher sensitivity of ELISA compared to IIF in the immunodiagnosis of canine toxoplasmosis.

Table 1 - Number of co-positive and co-negative sera in a total of 270 dog sera tested by indirect immunofluorescence (IIF) and by indirect ELISA at 1/40 and 1/200 dilution, respectively.

ELISA / IIF	Negative number	(%)	Positive number	(%)
Negative	76	(27.53)	72	(26.08)
Positive	24	(8.69)	101	(36.59)

Clinical cases of acute toxoplasmosis serologically confirmed by indirect immunofluorescence and indirect ELISA: Among the 170 sera (61.6% of the total) obtained from dogs seen at the HVGLN for the detection of anti-*T. gondii* antibodies, 12 (7.0%) were from animals with a clinical picture compatible with acute toxoplasmosis.

The clinical signs observed were apathy, anorexia, diarrhea, hyperthermia, oculonasal purulent secretion, sneezing, coughing, lack of motor coordination and, in some cases, convulsions. With respect to hematologic parameters, most dogs presented anemia, lymphocytosis, monocytosis and neutrophilia (data not shown).

Table 2 summarizes the clinical signs of dogs with acute toxoplasmosis (n=12) and the serologic results obtained by indirect ELISA and by IIF. Both the EL and the fluorescence intensity obtained by IIF were elevated in most animals, in agreement with the data obtained for the experimentally infected dog, i.e., indicating that the animals were in the acute phase of the disease. It should be pointed out that sera from 3 dogs (General HVGLN registration numbers 5986/2, 2586/0 and 6504/8) with EL 7, 8 and 6, respectively, did not react to IIF or presented only a weak fluorescence (1). However, these animals presented clinical signs suggestive of acute toxoplasmosis. Submitted to chemotherapy, these animals, presented good recovery.

Table 2: Clinical cases of acute toxoplasmosis among dogs seen at the "Governador Laudo Natel" Veterinary Hospital and relationship between clinical signs and serologic results obtained by indirect ELISA (EL) an IIF (fluorescence intensity) in 1993.

Animal registration number	Clinical signs	Serologic tests ELISA (EL)	IIF
5986/2	- Apathy - Hyperthermia - Increased volume of submandibular lymph nodes - Anemia	7	0
2586/0	- Apathy - Anorexia - Nasal secretion - Dry cough - Anemia	8	1
5141/1 3077/5	- Pale mucosae - Anemia - Diarrhea - Ocular secretion - Generalized tremors	7	3
6790/3	- Anorexia - Dry cough - Anemia - Ataxia - Oculonasal secretion	2	3
6516/1	- Lack of motor coordination - Tenesmus	8	2
6102/6	- Diarrhea - Wobbling gait - Salivation - Tremors - Auricular myoclonus - Anemia	9	3
6404/8	- Convulsions - Lack of motor coordination - Tremors	6	0
5923/4	- Tremors - Apathy	6	2

DISCUSSION

Infection by *T. gondii* is of wide geographic distribution both among humans and domestic or wild animals. The clinical signs of canine toxoplasmosis are associated with respiratory, neurologic and eventually digestive problems (BOURDEAU, 1993). Anorexia, intermittent diarrhea, hyperthermia and lethargy were the major signs observed in the dog experimentally infected with *T. gondii* and were compatible with those previously reported for dogs and cats (DUBEY, 1985; LAPPIN *et alii*, 1989b; BOURDEAU, 1993), and corresponded to the signs detected among dogs with confirmed acute toxoplasmosis (apathy, hyperthermia, anorexia, diarrhea, lack of motor coordination, oculonasal secretion, dry cough and convulsions). The presence of these signs and the serologic diagnosis reinforces the importance of this disease among dogs. The *T. gondii* strain (strain N) proved to be pathogenic, causing normochromic microcytic anemia, reduced hemoglobin levels, hematocrit and MCV and also neutrophilia and lymphocytosis.These data partially agree with those reported by DUBEY (1985) and BOURDEAU (1993). Another important finding reported in the literature (BOURDEAU, 1993) was the appearance of chorioretinitis 75 days after experimental infection in the dog.

The initial detection of anti- *T. gondii* antibodies by both IIF and indirect ELISA occurred starting from the 2nd week of experimental infection, in partial agreement with BOURDEAU (1993) who, however, observed a growing increase in serum IgG levels in cats, reaching a maximum titer between the 2nd and 3rd week of infection. However, the maximum IgG titer observed in the present study was reached between the 8th and 10th week after infection. Detection of anti- *T. gondii* antibodies remained at significant EL up to 644 days of experimental infection in the dog. In contrast, LAPPIN *et alii* (1989a,c) observed that the humoral immune response persists for several months since the detection of IgG in cat sera submitted to ELISA started at 21 days after infection, reaching elevated serum levels at 35 days and remaining elevated up to 365 days after infection. In the present study, *T. gondii* provoked an intense immune response, with antibody levels being demonstrated earlier and persisting (644 days).

The presence of anti-toxoplasma antibodies was detected by IIF in 46.1% (127) of test sera at 1/40 dilution. However, ISHIZUKA *et alii* (1974a) detected anti-toxoplasma antibodies in 72% of 210 sera tested by IIF at an initial dilution of 1/16. The possible occurrence of cross- or nonspecific reactions between antigens from protozoa that parasitize carnivores (BOURDEAU, 1993) led us to use an initial dilution of 1/40 of test sera submitted to IIF. On this basis, our results agree with those reported in the above papers even though the initial dilution was different. Titration of test sera at 1/40 and 1/10240 dilution or until they became negative demonstrated a higher percentage of reactive sera (40.2%) at a final titration of 1/80. However, ISHIZUKA *et alii* (1974b) and GUIMARAES *et alii* (1992), in a study of 210 sera from dogs in the municipality of São Paulo and 243 sera from dogs in Belo Horizonte, respectively, observed that the largest number of reacting sera

presented a final antibody titer of 1/256. In contrast, OPEL (1987), noted that 61% of the 100 sera of dogs tested by IIF were reactive to a final dilution of 1/32. It is interesting to note that GERMANO *et alii* (1989), using 657 sera from dogs submitted to anti-rabies vaccination, found that 598 of them (91%) were positive, with frequent titers of 1/1000 and 1/4000.

By comparing the results obtained in the detection of anti- *T. gondii* antibodies by IIF and indirect ELISA both in the experimentally infected dog and in the 276 samples tested, we may infer that indirect ELISA has higher sensitivity in the immunodiagnosis of canine toxoplasmosis, with 64% correlation between the two techniques, a statistically significant value by the exact Fisher test ($p \leq 0.01$).

SUMÁRIO

Um ensaio imunoenzimático indireto (ELISA) foi padronizado e utilizado para a detecção de anticorpos anti-Toxoplasma gondii em 276 amostras de soro, provenientes de cães capturados ou atendidos no Hospital Veterinário da Faculdade de Ciências Agrárias da UNESP, Campus de Jaboticabal. A concentração ótima de antígeno para o ELISA indireto foi de 10 µg/ml quando uma única diluição (1/200) foi utilizada para os soros de referência (positivo e negativo). A sensibilidade do ELISA foi comparada a do teste de Imunofluorescência Indireta (IIF). A presença de anticorpos anti-T. gondii foi detectada em 46,01% (n=127) dos soros testados por IIF. Entre estes, 31,5% (n=40) mostraram reatividade final na diluição de 1/40, 40,1% (n=51) na diluição de 1/80, 13,4% (n=17) na diluição de 1/160, 2,4% (n=3) na diluição de 1/320, 9,5% (n=12) na diluição de 1/1280, e 0,8% (n=1) na diluição de 1/10240. Em contraste, a presença de anticorpos anti-*T. gondii* foi detectada em 62,5% (n=169) dos soros testados pelo ELISA indireto. A reatividade dos soros foi analisada em termos de níveis ELISA (0 a 9). Os soros foram considerados positivos a partir do nível ELISA 2, e os de maiores concentrações com reatividade foram classificados nos níveis 5 (10,14%, n=28), 6 (9,78%, n=27) e 7 (6,88%, n=19), num total de 26,8% (n=74). Casos clínicos de toxoplasmose aguda com confirmação sorológica por IIF e ELISA foram diagnosticados, e em alguns casos somente o ELISA indireto provou ser sensível o bastante para detectar a presença de anticorpos. A análise estatística pelo teste de Fischer mostrou diferenças significativas (nível de 1%) entre os testes e demonstrou a maior sensibilidade do teste de ELISA indireto para o imunodiagnóstico da toxoplasmose canina.

PALAVRAS-CHAVE: Toxoplasma gondii, ELISA indireto, imunofluorescência indireta, cães.

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