

EVALUATION OF CROSS-REACTIVITY OF *TOXOPLASMA GONDII* AND *NEOSPORA CANINUM* ANTIGENS IN DOGS SERA

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SUMMARY: *Neospora caninum* is a protozoan parasite, recently recognized, intimately related with *Toxoplasma gondii*, but different in its ultrastructural and antigenic capacity. The serological diagnostic can be realized by Indirect ELISA-test and Indirect Immunofluorescent antibody test (IFAT), observing cross-reactivity between the parasites when the ELISA-test is used, what wasn't observed with IFAT. Sera of 203 dogs examined at the Veterinary Hospital were tested, with the objective of verify the prevalence of canine neosporosis and cross reactivity between *T. gondii* and *N. caninum*, besides to research the oocysts elimination in feces of seropositive dogs to *N. caninum*. We observed that 16,36% (27 samples) of sera were positive by ELISA for the soluble antigen of *T. gondii* and at the same time positive to IFAT in the detection of antibodies anti-*N. caninum*, what shows the occurrence of cross reactivity. It called the attention that 4,84% (8 sera) showed positivity at the same time in the detection of antibodies anti-*T. gondii* and anti-*N. caninum* through IFAT concluding that the animals were infected with the two parasites.

KEY WORDS: *Toxoplasma gondii*, *Neospora caninum*, Indirect ELISA-test, Indirect Immunofluorescent antibody test, dogs.

INTRODUCTION

Toxoplasmosis is a zoonosis caused by an obligatory intracellular protozoan *Toxoplasma gondii*, wich belongs to the Apicomplexa subphylo, Coccidia class, Sarcocystidae family and Toxoplasmatinae subfamily. The definitive hosts are the felids, although any mammal can become an intermediate host (DUBEY, 1986). The common clinical signs described in dogs are paresis of hind limbs, paralysis, difficulty to swallow, flaccidity and muscles atrophy (DUBEY & LINDSAY, 1996).

Firstly, the serological diagnostic of toxoplasmosis was realized with "dye test" (SABIN-FELDMAN, 1948) and because of the difficulties in the execution, it was substituted by the HI (JACOBS & LUNDE, 1957) Indirect Immunofluorescent antibody test (IFAT) (KELEN *et alii*, 1962), Indirect ELISA-test (VOLLER *et alii*, 1976) and Dot-ELISA.

Researches made in Brazil, using IFAT and Indirect ELISA-test (ISHIZUKA, *et alii*, 1981; FREIRE *et alii*, 1992.; GUIMARÃES *et alii*, 1992) have shown high prevalence of canine toxoplasmosis. In Jaboticabal/SP/Brazil, DOMINGUES

et alii (1998) showed high prevalence of canine toxoplasmosis (62,5%) by Indirect ELISA-test and still made evidence bigger sensibility of this assay, when compared to IFAT, in the detection of antibodies anti-*T. gondii*, obtaining 64% of correlation between the two tests.

The *N. caninum* is a protozoan parasite, closely related to *T. gondii*, only different in his ultrastructural and antigenic capacity (LINDSAY *et alii*, 1993). The presence of the infection by *N. caninum*, in dogs, has been already described in many countries, among them the United States (DUBEY *et alii*, 1988), England (TRESS *et alii*, 1993), Sweden (WORKMAN *et alii*, 1994), Denmark (RASMUSSEN & JENSEN, 1996) and in Spain (PUMAROLA, *et alii*, 1996), with a variation of prevalence between 0,5 and 16,6% (RASMUSSEN *et alii*, 1-996).

The complete life cycle of *Neospora caninum* in nature is still unknown. The vertical transmission in natural infections of dogs, have been documented and the only known life cycle stages of *N. caninum* identified are the tachyzoites and the tissue cysts with bradyzoites (COLE *et alii*, 1995) and oocysts, identified by McALLISTER *et alii* (1998) in dogs feces experimentally infected.

The serological diagnosis and the study of neosporosis prevalence in dogs can be done using IFAT (TREES *et alii*, 1993; WORKMAN, 1994; PUMAROLA *et alii*, 1996; RASMUSSEN & JENSEN, 1996; DUBEY & LINDSAY, 1996) and the ELISA-test (WORKMAN *et alii*, 1994; DUBEY & LINDSAY, 1996); but cross reactivity was observed between the *N. caninum* and the *T. gondii*, when using ELISA-test, what wasn't observed when the IFAT was used (DUBEY & LINDSAY, 1996).

Based on the literature and on the studies developed in our laboratory, which made evident high prevalence of toxoplasmosis among the canine population, our objective is to verify the prevalence of canine neosporosis and the cross reactivity between *T. gondii* and *N. caninum* added to research the oocysts elimination in the seropositive dogs feces to *N. caninum*.

MATERIALS AND METHODS

Experimental samples:

203 dogs sera with neurologic symptoms, carriers of other diseases or asymptomatic, obtained from the Medical Clinical Center of HVGLN-UNESP/Jaboticabal were used. From the corresponding promptuaries date about anamnese, phisical exams and auxiliary exams were compiled with the purpose of have individual and clinical information of the patients.

Positives and negatives control sera were obtained from the sera collection of the Immunoparasitology Laboratory of the Veterinary Patology Department and then used in IFAT and ELISA-test to detection of antibodies anti-*T. gondii*. The positive and negative control sera used in IFAT for the detection of antibodies anti-*N. caninum* were obtained commercially from VRMD (Inc. Pullman, WA).

Antigens:

The *T. gondii* antigen used in the serological tests of IFAT and ELISA-test was produced from purified tachyzoites obtained through peritoneal wash in mice previously infected, according to DOMINGUES *et alii* (1998). The *N. caninum* antigen used in IFAT was obtained commercially from VMRD (Inc. Pullman, WA).

Imunofluorescent Antibody Test (IFAT):

The IFAT for the detection of antibodies anti-*T. gondii* in dogs sera was processed as described by DOMINGUES *et alii* (1998). The conjugate used was a sheep IgG anti-dog IgG, labelled with fluorescein isothiocyanate (affinity purified antibody to dog IgG (γ). N 02-19-02. Kirkegaard and Perry Laboratories Inc.), at 1:20 dilution in PBS, containing 0,01% Evans Blue. To the analyzed sera in single dilution of 1:40 were attributed scores of intensity of fluorescence, divided at following way: weak positive (1), medium (2) and strong (3), and the no reagent or with polar fluorescence (0).

The method used to the detection of antibodies anti-*N. caninum* was proposed by the manufacturer (VMRD, Inc. Pullman, WA) and the sera were diluted at 1:50.

Indirect Elisa-test:

The method used for prepare the plates, sera dilution (1:200), washing and incubation was the same described by DOMINGUES *et alii* (1998). A rabbit γ globulin anti-dog IgG coupled to alkaline phosphatase (Sigma Immuno Chemicals Anti-dog IgG A-6042), at 1:9000 dilution was used as conjugate. The reactivity of the tested sera through this essay was expressed in ELISA level, which vary from 0 to 9, being consider positives the sera that were between level 2 to 9, and the negative sera between the level 0 and 1.

Research of oocysts in the feces:

For the detection of *N. caninum* oocysts in the feces of seropositive dogs, the method used was the same described by DUBEY (1995) and developed to cats feces and after adjusted by McALLISTER *et alii* (1998) to dogs feces.

Statistical Analysis:

The results obtained in Indirect ELISA-test and IFAT were compared by the Exact Fisher test at 1 % level of probability.

RESULTS

Reactivity of the sera to the *Toxoplasma gondii* antigens, through the Indirect ELISA-test and IFAT: Of the 203 sera tested through the Indirect ELISA-test, 165 (81,28%) showed antibodies anti-*T. gondii* and were divided within the ELISA levels (EL): 19 sera were in EL 2 (11,52%); 22 sera were in EL 3 (13,33%), 27 sera in EL 4 (16,36%), 23 sera in EL 5 (13,94%), 29 sera in EL 6 (17,58%), 32 sera in EL 7 (19,39%) and 13 sera in EL 8 (7,88%). Thirty eight sera (18,72%) didn't present reactivity to Indirect ELISA-test, being 25 situated in EL 0 (65,79%) and 13 in EL 1 (34,21 %). The reactivity of the 203 sera tested face to *T. gondii* antigens it's expressed in ELISA levels (EL) and it's represented in Figure 1.

Figure 2 illustrates the intensities of fluorescence of the sera (n=203) diluted at 1/40 and classified in negative reaction (0),

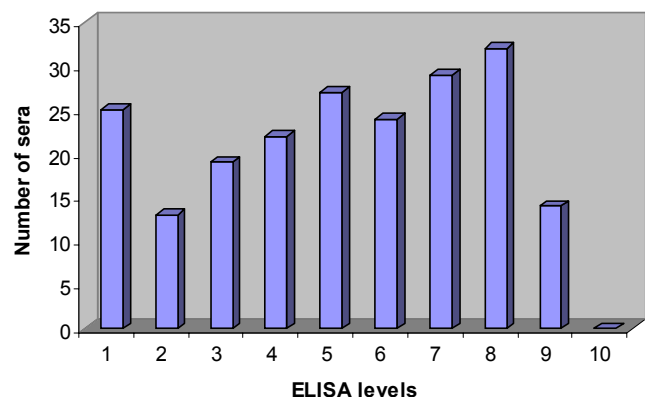


Fig. 1 – Distribution of the reactive sera in the presence of *T. gondii* antigens, through Indirect ELISA-test, expressed in ELISA levels (EL).

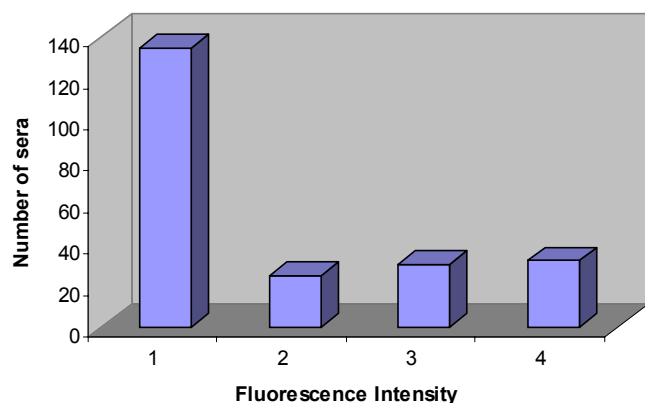


Fig. 2 – Distribution of the tested sera (n=203) in the presence of *T. gondii* antigens through IFAT and divided according to fluorescence intensity.

weak reaction (1), medium reaction (2) and strong reaction (3). The antibodies anti-*T. gondii* detected through IFAT were identified in 73 (35,96%) of the 203 sera tested, being 20 sera (27,40%) showed weak reaction, 26 (35,61%) medium reaction and 27 (36,99%) strong reaction, while 130 (64,04%) didn't show reactivity through IFAT.

Correlation between the IFAT and Indirect ELISA-test results:

The analysis of the correlation of the results of the 203 sera submitted to IFAT and Indirect ELISA-test in the presence of *T. gondii* antigens showed that 22 sera (10,84%) were co-negative and 57 sera (28,08%) were co-positive in both assays. The comparative statistical analysis by the exact Fisher test showed a significant effect at the 1% level of probability ($p \leq 0,01$).

Reactivity of dogs sera in the presence of *Neospora caninum* by IFAT:

The reactivity of the tested sera diluted at 1/40 was classified according to the intensity of fluorescence. From 203 sera tested

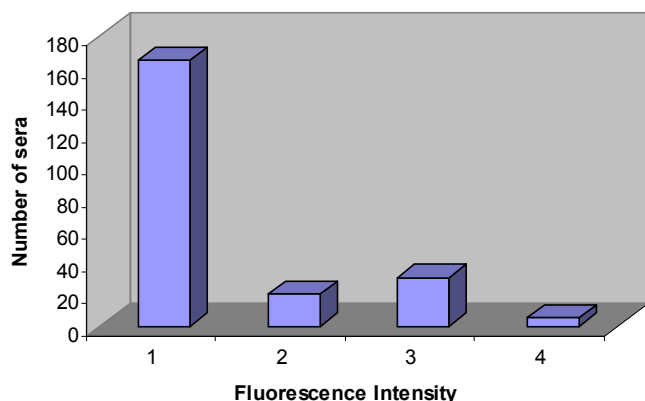


Fig. 3 – Distribution of the number of reagent sera, according to the intensity of fluorescence, to *Neospora caninum* antigens, by IFAT.

Table 1 – Number of dogs sera (n=203) tested by Indirect ELISA-test and IFAT at 1/200 and 1/40 dilution, respectively, co-positive or co-negative in the presence of *T. gondii* antigens.

IFAT	ELISA-test	
	Positive	Negative
Positive	57 (28,08%)	16 (7,88%)
Negative	108 (53,20%)	22 (10,84%)

Table 2 – Cross reactivity of positive sera (165) and negative sera (38) to the *T. gondii* antigens by ELISA-test, comparatively analyzed to the results obtained in the detection of antibodies anti-*T. gondii* and anti-*N. caninum* by IFAT.

<i>T. gondii</i> (IFAT)	<i>N. caninum</i> (IFAT)		
	Positive	Negative	Total
1*			
Positive	3 (7,90%)	13 (34,21%)	16 (42,11%)
Negative	6 (15,79%)	16 (42,10%)	22 (57,89%)
Total	9 (23,69%)	29 (76,31%)	38 (100%)
2**			
Positive	8 (4,84%)	49 (29,70%)	57 (34,54%)
Negative	27 (16,36%)	81 (49,10%)	108 (65,45%)
Total	35 (21,21%)	130 (78,79%)	165 (100%)

1* - negative sera by ELISA-test

2** - positive sera by ELISA-test

159 (78,33%) didn't react and the other 44 sera (21,67%) showed reactivity, 16 sera (36,36%) showed weak reaction, 25 (56,82%) medium reaction and three sera (6,82%) strong reaction (Figure 3).

Cross reactivity of the tested sera by Indirect ELISA-test and submitted to the detection of antibodies anti-*N. caninum* and anti-*T. gondii* by IFAT:

The cross reactivity of the sera tested by Indirect ELISA-test to *T. gondii* was noticed when these same sera were submitted to IFAT for the detection of *T. gondii* and *N. caninum* antigens individually (table 2). Among 165 sera reagent to Indirect ELISA-test, 8 sera (4,84%) showed co-positivity to both antigens, 81 (49,10%) showed co-negativity and 27 (16,36%) reacted only with the *N. caninum* antigens, showing a low frequency of cross reaction.

In opposition, the negative sera to ELISA-test in the detection of antibodies anti-*T. gondii* (38) were submitted to IFAT, where were detected 16 sera (42,10%) Go-negatives, 3 (7,90%) co-positives, 6 (15,79%) positives only to *N. caninum* antigens, while 13 (34,21%) showed reactivity only to *T. gondii* antigens.

Analysis of the promptuaries of dogs serologically reagents to *T. gondii* and/or *N. caninum* antigens:

Among the clinical promptuaries studied (203), 160 showed one or more clinical signs consistent with the ones described in

Table 3 – Clinical cases of neosporosis and/or toxoplasmosis in dogs examined at HVGLN-UNESP, correlating the signs/symptoms and the serologic results to *T. gondii* and *N. caninum* antigens.

Animals	Clinical Signs	<i>T. gondii</i> <i>N. caninum</i>		
		ELISA*	IFAT**	IFAT**
Mixed Breed Female 2 years	Piodermatitis ataxia myoclonia opisthotonus	7	0	2
Mixed Female 15 years	Anorexy Walk in circles vocalizations paresis of hind limb and foreleg	6	3	2
Toy Fox Terrier Male 5 years	anorexy seborrhea paresis of hind limb tense locomotion	7	3	2
Rottweiler Male 2 years	muscle atrophy of hind limbs ataxia generalized hyporeflexion anorexia	3	0	3

* ELISA levels (EL)

** Fluorescence intensity

literature to canine toxoplasmosis, such as convulsion, sialorrhea, ataxia, paresis, muscle atrophy, and others. The neurologic signs were more frequently detected in 44 seropositive animals to *N. caninum*, inasmuch 12 dogs showed convulsions and 15 dogs showed paresis or paralysis of the hind limbs. It's important to stand out that 4 animals showed dermatologic problems (no hair and piodermatitis) and other 2 dogs showed changes in cervical musculature. In Table 3, four clinical cases are presented, relating the main signs to the results of the antibodies detection anti-*N. caninum* and anti-*T. gondii* through FAT and ELISA, respectively.

Although 38 dogs showed compatible signs with canine neosporosis and with positive seroreactivity to *N. caninum*, no oocysts were detected in the samples of feces analyzed from each animal.

DISCUSSION

The detection of the infection by *T. gondii* in dogs, evaluated by Indirect Immunofluorescence and Indirect ELISA-test, in this study, showed that there is a high frequency of occurrence of the disease in the northeast area of São Paulo State, when compared to related prevalences in other areas of the country (ISHIZUKA *et alii*, 1981; FREIRE *et alii*, 1992; GUIMARAES *et alii*, 1992). The results of this study agree with those reported by DOMINGUES *et alii* (1998), which detected a frequency of canine toxoplasmosis occurrence of 46,01% by IFAT and 63,74% by ELISA-test, at the same region of State.

In this study, such as in the one reported by DOMINGUES *et alii* (1998), was showed a high sensibility of ELISA-test, when compared to IFAT, in the detection of anti-*T. gondii* antibodies in dogs sera. Furthermore, high prevalence of antibodies anti-*Neospora caninum* (21,67%) was also identified when compared to the prevalence estimate, already mentioned in many countries, which changes between 0,5% to 16,6% (RASMUSSEN *et alii*, 1996), however oocysts were not found in the seropositive dogs feces. The fact that the oocysts were not detected suggests the possibility of existence of a negative period of detection or a short period of oocysts elimination, like happens with *T. gondii* in cats, beyond the fact that there is no confirmation of *N. caninum* oocysts elimination in natural infections of carnivorous. Another fact that might have contributed is the low number of oocysts spread in feces, as detected by McALISTER *et alii* (1998), after administration of big quantities of tecdial cysts.

We observed 16,36% of cross-reactivity, inasmuch the sera showed reactivity to ELISA-test to *T. gondii* soluble antigen and by IFAT to *N. caninum* antigen. DUBEY & LINDSAY (1996) detected higher incidence of cross-reactivity when the pure extract of *Neospora caninum* is used as antigen in ELISA-test. However, 4,84% of tested sera were reagent to *N. caninum* and *T. gondii* by IFAT, result that, according to DUBEY & LINDSAY (1996), the animals are infected by both protozoans, because IFAT is more specific when compared to the Indirect ELISA-test.

The relation of sintomatology with the positive serologic results for toxoplasmosis showed that paresis of hindlimb, convulsions, ataxia and some ophthalmic signs (uveitis and chorioretinitis causing visual deficit) were the most frequent clinical signs, also evident in positive animals to neosporosis, showing the resemblance between the two diseases. However in canine neosporosis may occur involvement of all organs, including the skin, and the dermatitis may be severe if the infestation by the protozoan is massive (DUBEY & LINDSAY, 1996). POLI *et alii* (1998) described a severe nodular lesion with epidermic ulceration in an adult dog with neosporosis. It's important to point out that also in this study, beyond the clinical symptoms already mentioned, four seropositive animals for neosporosis showed skin lesions (no hair and piodermatitis). Other two seropositive animals showed changes in cervical muscles (myoclonia and harshness) that agree with reports of paralysis and dysphagia (DUBEY & LINDSAY, 1996) and cervical harshness (BARBER & TREES, 1996).

Based on these results, we can conclude about the presence of *Neospora caninum* in dogs in the Northeast region of São Paulo State, proving the importance of the differential diagnostic of neurological diseases with compatible symptoms to toxoplasma and/or canine neosporosis.

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