DETECTION OF CO-INFECTIONS BY *Leishmania* (*l.*) *chagasi*, *Trypanosoma evansi*, *Toxoplasma gondii* AND *Neospora caninum* IN DOGS

(*DETECÇÃO DE CO-INFECÇÕES POR Leishmania (l.) chagasi, Trypanosoma evansi, Toxoplasma gondii E Neospora caninum EM CÃES*

ABSTRACT – Co-infections by *Leishmania* (*L.*) *chagasi*, *Trypanosoma evansi*, *Toxoplasma gondii* and *Neospora caninum* in dogs were investigated. Amastigote forms of *Leishmania* spp. were detected by cytopathological analysis of lymph nodes in 46.42% (39/84) of the dogs. In a male adult without defined breed dog from a rural area, positive for *Leishmania*, flagellated forms of *T. evansi* were observed in blood smear. Using the immunofluorescence antibody test, 5.95% (5/84) of dogs were considered reactive to *T. gondii*, with titers equal to 64, while 3.57% (3/84) were reactive to *N. caninum*, with titers of 50. Among the animals with visceral leishmaniasis, one showed positive serological response to *T. gondii* and two for *N. caninum*. All dogs reactive to *N. caninum* were from rural areas and the predominance of infection by *T. gondii* was mainly in dogs from urban areas. A young male dog from a rural area, seropositive for *T. gondii*, showed *Ehrlichia* spp. morulae in the cytology and positive reaction for canine distemper virus. Thus, further studies are needed in order to assess the epidemiology of these infections in canine population, especially with respect to the reservoirs of *Trypanosoma* spp. in rural areas.

Keywords: Leishmaniasis, Neosporosis, Serology, Toxoplasmosis, Trypanosomiasis.

RESUMO – Coinfecções por *Leishmania (L.) chagasi*, *Trypanosoma evansi*, *Toxoplasma gondii* e *Neospora caninum* em cães foram investigadas. Formas amastigotas de *Leishmania* spp. foram detectadas pela análise citopatológica de linfonodos em 46.42% (39/84) dos cães. Em um cão macho, adulto, sem raça definida, proveniente de área rural e positivo para *Leishmania*, foram observadas formas flageladas de *T. evansi* em esfregaço sanguíneo. Pela RIFI, 5.95% (5/84) dos cães foram considerados reagentes para *T. gondii*, com titulação igual a 64, enquanto que 3.57% (3/84) foram reagentes para *N. caninum*, com título 50. Entre os animais com leishmaniose visceral, um apresentou resposta sorológica positiva para *T. gondii* e dois para *N. caninum*. Todos os cães reagentes para *N. caninum* eram de área rural e, o predomínio da infecção pelo *T. gondii* ocorreu em cães da área urbana. Um cão macho, jovem, da zona rural e soropositivo para *T. gondii*, apresentou mórulas de *Ehrlichia* spp. na citologia e reação positiva para o vírus da cinomose. Deste modo, mais estudos são necessários para avaliar a epidemiologia dessas infecções na população canina, principalmente com relação aos reservatórios de *Trypanosoma* spp. nas zonas rurais.

Palavras-chave: Leishmaniose, Neosporose, Sorologia, Toxoplasmose, Tripanosomose.

INTRODUCTION

Dogs can be naturally infected by a wide variety of etiologic agents, responsible for causing direct damage to animals. According to Camargo et al. (2007), some of these pathogens contribute to a serious public health problem, since they are also capable of infecting humans.

The scientific literature contains reports of various forms of co-infections in dogs. The association of *Toxoplasma gondii* and *Neospora caninum* with leishmaniasis (CRINGOLI et al., 2002; GENNARI et al., 2006; GUIMARÃES et al., 2009) and canine distemper virus (MORETTI et al., 2002) have been reported, including the co-infection by *Leishmania* (*L.*) *chagasi* and *Trypanosoma evansi* (SAVANI et al., 2005).

Sousa & Almeida, (2008) state that parasitic co-infections can lead to clinical aggravations in animals, being a common fact where the occurrence of several diseases exists concomitantly.

Due to the fact that the city of Andradina, in the state of São Paulo, Brazil, is considered an endemic area for a wide range of parasitic and viral diseases in dogs, the present study focused on investigating the occurrence of co-infections by *L. chagasi, T. evansi, T. gondii* and

N. caninum, as well as the eventual occurrence of other agents on household dogs from the urban and rural areas of this city.

MATERIALS AND METHODS

The experimental group consisted of 84 dogs, 75 from urban and 9 from rural areas, respectively. Amongst these, 43 animals were males and 41 females; 44 had defined breed (DB) and 40 without defined breed (WDB). The animals were classified according to age in groups composed of 21 adults and 63 young dogs. This work was performed with approval of the Animal Experimentation Ethics Committee of "Faculdade de Odontologia de Araçatuba (FOA), Universidade Estadual Paulista" - UNESP, Brazil, under the protocol number 2007-003276.

Blood (5 mL) was collected by venipuncture into siliconized vacutainer tubes without anticoagulant and centrifuged at 3000 rpm for 5 minutes. The obtained sera was transferred to sterile plastic tubes and immediately frozen at -20^{o}C.

Canine leishmaniasis was diagnosed by aspirative biopsy of popliteal lymph nodes and PCR. The amplification of DNA from *Leishmania* sp. was made from samples of positive lymph nodes in microscopy, using a DNA extraction kit (QIAamp Blood and Tissue; Quiagen®, CA, USA), with the amplification of DNA fragments (120 bp) of the minicircle kinetoplast (RODGERS et al., 1990).

Hemoparasites were investigated with the aid of peripheral blood smears and fine-needle aspiration biopsies of lymph nodes in microscope slides. These samples were stained with the Quick Panoptic Method (Hematocor®, Biolog®) to visualize amastigote forms of *Leishmania* spp., flagellated forms of *Trypanosoma* spp. and hemoparasites, analyzing 300 fields in 1000x magnification. The identification of *T. evansi* was conducted by analyzing their biometric measures (RAMIREZ et al., 1997; AQUINO et al., 1999) and absence of kinetoplast (NUNES et al., 1993; SANTOS SILVA et al., 2002).

Serum samples were analyzed through the immunofluorescence antibody test (IFAT) in order to investigate the presence of immunoglobulin G (IgG) against *T. gondii* and *N. caninum.* The dilutions used as cutoffs were 1:64 and 1:50 for *T. gondii* (DA SILVA et al., 2002) and *N. caninum* (KIM et al., 2003), respectively, considering, as positive results, titles of 64 for *T.gondii* and 50 for *N. caninum*. Positive and negative control sera were included in each slide. Only samples presenting complete fluorescence around tachyzoites were considered positive.

Canine distemper virus (CDV) was detected by Rapid CDV Ag Test Kit (Bioeasy), following the recommendations of the manufacturer.

The chi-square and Fisher's exact test was used to test for associations between all of the categories (StatSoft, 2007). Differences were considered significant for P-values < 0.05.

RESULTS AND DISCUSSION

Amastigote forms of *Leishmania* spp. were observed in 46.42% (39/84) of the total group of dogs. By PCR, they were identified as *L. chagasi*.

Approximately 28 slenders trypomatigotes, acinetoplastics with tapered rear end of *T. evansi* were observed, by field, in blood smears of a male dog, adult, without defined breed, from a rural area and positive for *Leishmania.* The biometric measurements of flagellated forms correspond to a maximum total size of 26.4 micrometres (µm), length of free flagellum 7.59 µm, away from the posterior half of the nucleus of 2.11 µm, away from the middle of the nucleus and the anterior end of 10.22 µm (Figure 1).

From all examined canine serum samples, 5.95% (5/84) were considered positive for *T. gondii*, with titers equal to 64, and 3.57% (3/84) were positive for *N. caninum*, with IFAT equal to 50.

Co-infection by *Leishmania* spp. was detected in 20% (1/5) of seropositive animals for *T. gondii* and 66.6% (2/3) reactive to *N. caninum.*

A young male dog, WDB, from the rural area and seropositive for *T. gondii*, showed *Ehrlichia* spp morulae in monocytes and inclusion bodies (Lentz corpuscles) in erythrocytes.

In the CDV detection test conducted, only one young, male, WDB animal was diagnosed as positive, proveninent from a rural area. This animal was not positive for any other illness analyzed in this study.

The age, breed, sex and area variables showed no significative influence ($p \geq 0.05$) on the occurrence of *T. evansi*, *T. gondii*, *Erlichia* spp. and CDV (Table 1).

Young dogs were significantly ($P \leq 0,05$) more prevalent for *L. chagasi* than adults, and this infection was not influenced by any of the reamining variables analyzed (breed, sex, area). Regarding *N. caninum* occurrence, dogs originated from rural áreas were significantly ($P \leq 0,05$) more prevalent than dogs from urban áreas (Table 1).

Several studies have reported the occurrence of co-infections in dogs positive for leishmaniasis. As described in this study, the simultaneous presence of *T. evansi* and *L. chagasi* was also observed in one dog by Savani et al. (2005). The morphometric parameters and the absence of kinetoplast verified in flagellated forms found in this study are similar to patterns observed in infections with *T. evansi* in dogs in Brazil (RAMIREZ et al. 1997; AQUINO et al., 1999; SANTOS SILVA et al., 2002).

In the city of Araçatuba, Gennari et al. (2006) showed anti-*N. caninum* antibodies by IFAT in 15.3% (15/98) and anti-*T. gondii* in 23.4% (23/98) out of 98 dogs with visceral leishmaniasis, with no influence of the animal's sex in the occurrence of these parasites, corroborating with data from the present study, where gender was not a determinating cause in the occurrence of these parasites.

Infections caused by *Ehrlichia canis* were observed in dogs with visceral leishmaniasis (SOUSA & ALMEIDA, 2008), result that was not observed in the present study, where only one dog presented a co-infection by *Ehrlichia spp.* and *T. gondii*, simutaneously.

In Lavras city, state of Minas Gerais, Brazil, Guimarães et al. (2009) detected infection by *Babesia* in 73.3% (220/300), *Toxoplasma* in 60.5% (132/218), *Neospora* in 3.1% (7/228) and *Leishmania* in 0.3% (1/300) of investigated dogs through IFAT, and co-infections were not found, diverging from the present study, that diagnosed co-infections between *L. chagasi*, *T. gondii* and *N. caninum*. However, in conformity with data obtained by Guimarães et al. (2009), this study did not diagnose co-infections between *T. gondii* and *N. caninum* in none of the examined dogs.

Azevedo et al. (2005) detected higher positivity percentages for *N. caninum* and *T. gondii* in dogs from the Paraíba state, Brazil: 45.1% (129/286) were seroreactive to *T. gondii* and 8.4% (24/286) to *N. caninum.* Differently from this study, those researchers observed that 4.9% (14/286) of dogs presented simultaneous occurrence of antibodies against both protozoans, which was cited by Mineo et al. (2004) and Romanelli et al. (2007). This difference can be explained by the fact that these researchers did not use dogs of urban and rural areas, simultaneously, in their studies and also because, in this present work, isolated occurrence of dogs seropositive for *N. caninum* in rural areas was found, with the highest percentage of seropositivity for *T. gondii* in the urban area.

Similarly, Figueiredo et al. (2008) evaluated serum samples of dogs from the state of Pernambuco, Brazil, by IFAT and detected 28.3% (177/625) positivity for anti-*N. caninum* antibodies*.* Among these samples, 57.6% occurrence was speculated for *T. gondii*.

Co-infection by *T. gondii* and canine distemper virus was reported by Moretti et al. (2002) in four dogs. In this study, only one animal was positive for CDV, and negative for the remaining investigated agents, making it impossible to correlate CDV with any of them.

In the present study, the largest proportions of animals reactive to *N. caninum* were from rural areas, which corroborate the reports of Ploneczka and Mazurkiewicz (2008) in Poland. In the states of São Paulo and Paraná, Brazil, Souza et al. (2003) also detected higher percentage of *T. gondii* positivity for dogs from rural areas, differing from our findings. Similarly, Cañón-Franco et al. (2003) reported a percentage of 8.3% (13/157) of dogs seropositive for *N. caninum*, even in remote areas such as the Amazonas state, Brazil.

Bresciani et al. (2007) detected, by IFAT, that 23.1% (25/108) of the investigated dogs from Araçatuba city, São Paulo, Brazil, were seropositive for *T. gondii* and 15.7% (17/108), for *N. caninum*. In addition, the environment where those animals were raised was highly relevant to the infection. In the present study, there was neither correlation with breed, sex or age, which was observed by Silva et al. (2010), non statistical association between the occurrences of these agents. These findings were similar to those obtained in our study, especially because Andradina belongs to the mesoregion of Araçatuba and both cities are endemic to those protozooses.

CONCLUSIONS

Simultaneous infections by *Leishmania* (*L.*) *chagasi* and *Trypanosoma evansi* were detected in one dog from a rural area of Andradina. This is the first description of *T. evansi* in dogs in this region. Some animals with visceral leishmaniasis were seropositive to *Toxoplasma gondii* and *Neospora caninum.* Although with low prevalence, toxoplasmosis had higher occurrence among dogs from the urban area, while neosporosis was more prevalent in the countryside. Thus, further studies are needed to assess the epidemiology of these infections in canine population, especially with respect to the reservoirs of *Trypanosoma* in rural areas.

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Table 1 - Influence of breed, sex, age and area on the occurrence of *Leishmania chagasi*, *Trypanosoma evansi*, *Toxoplasma gondii*, *Neospora caninum*, *Ehrlichia* spp. and distemper virus among 84 dogs in Andradina Municipality, São Paulo State, Brazil.

Variable	Category	*L. chagasi*		*T. evansi*		*T. gondii*		*N. caninum*		*Ehrlichia spp.*		Distemper vírus	
		(n=39)*	p value[1]	(n=1)*	p value[2]	(n=5)*	p value[2]	(n=3)*	p value[2]	(n=1)*	p value[2]	(n=1)*	p value[2]
Age	Young (n=63)	24^{a}	0,0080	0^{a}	0,2500	5^{a}	0,2277	2^{a}	0,5832	0^{a}	0,7500	0^{a}	0,7500
	Adult (n=21)	15^{b}		1^{a}		0^{a}		1^{a}		1^{a}		1^{a}	
Breed	DB (n=22)	12^{a}	0,3742	0^{a}	0,7381	3^{a}	0,1099	2^{a}	0,1665	0^{a}	0,7381	0^{a}	0,7381
	WDB (n=62)	27^{a}		1^{a}		2^{a}		1^{a}		1^{a}		1^{a}	
Sex	Male (n=43)	23^{a}	0,1840	0^{a}	0,5119	4^{a}	0,1951	2^{a}	0,5181	0^{a}	0,5119	0^{a}	0,5119
	Female (n=41)	16^{a}		1^{a}		1^{a}		1^{a}		1^{a}		1^{a}	
Area	Urban (n=75)	34^{a}	0,5612	0^{a}	0,1071	4^{a}	0,4409	0^{b}	0,0009	0^{a}	0,1071	0^{a}	0,1071
	Rural (n=9)	5^{a}		1^{a}		1^{a}		3^{a}		1^{a}		1^{a}	

*: Values followed by the same letter in the column for each variable do not differ ($p \geq 0.05$)

1: Chi-square. 2: Fisher's exact test

Defined breed (DB), Without defined breed (WDB). Total number (n)

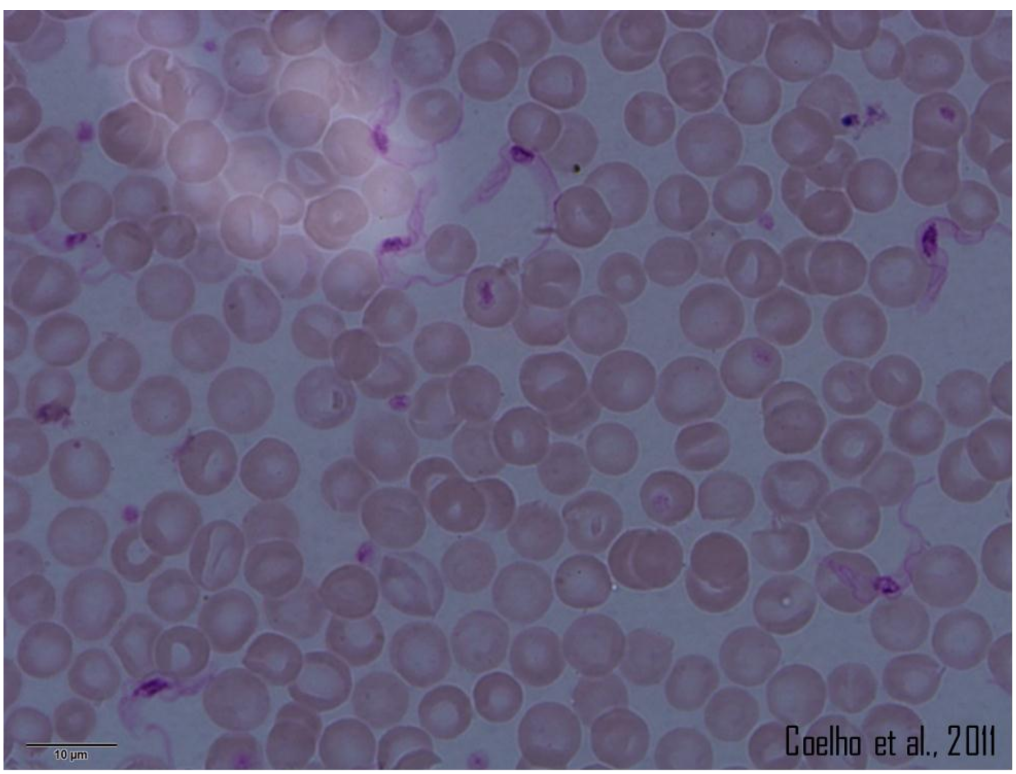

Figure 1 - Acinetoplastics forms of *Trypanosomaevansi* in blood smear of dog naturally infected by *Leishmania (L.) chagasi.*

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