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

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SUMMARY

This study investigates co-infections by *Leishmania (L.) chagasi, Trypanosoma evansi, Toxoplasma gondii* and *Neospora caninum* in dogs. Amastigotes of *Leishmania* spp. were detected by cytological examination of lymph nodes in 46.42% (39/84) of the dogs. The blood smears of an adult male mongrel dog, from rural area and positive for *Leishmania,* displayed the flagellated forms of *T. evansi*. By indirect immunofluorescence (IF), 5.95% (5/84) of dogs were considered reactive for *T. gondii,* with titers equal or higher than 1:64, whereas 3.57% (3/84) were positive for *N. caninum,* with titer ≥1:50. Among animals with visceral leishmaniasis, one had positive serological response to *T. gondii* and two for *N. caninum.* All dogs reactive to *N. caninum* were from rural areas, while dogs positive to *T. gondii* infection were 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 to the distemper virus. Thus, further studies are needed to assess the epidemiology of these infections in the canine population, especially with respect to *Trypanosoma* spp. reservoirs in rural areas.

KEY-WORDS: Leishmaniasis, Neosporosis, Serology, Toxoplasmosis, Trypanosomosis.

RESUMO

Foram investigadas coinfecções por *Leishmania (L.) chagasi*, *Trypanosoma evansi*, *Toxoplasma gondii* e *Neospora caninum* em cães. 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 imunofluorescência indireta (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.

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INTRODUCTION

Dogs can be naturally infected by a variety of etiological agents responsible for causing direct harm to them. According to Camargo et al. (2007), some of these pathogens cause serious public health problems since they are also able to infect humans. There are reports in the literature of various forms of co-infections in dogs. Associations of *Toxoplasma gondii, Neospora caninum, Leishmania* spp. (CRINGOLI et al., 2002; GENNARI et al., 2006; GUIMARÃES et al., 2009) and distemper (MORETTI et al., 2002) virus were observed, including co-infection with *Leishmania (L.) Chagasi* and *Trypanosoma evansi* (SAVANI et al., 2005).

Sousa & Almeida (2008) reported that parasitic co-infections can lead to serious clinical disorders in animals, and this becomes common when various diseases exist concurrently.

This study investigates the occurrence of co-infections by *L. chagasi, T. evansi, T. gondii* and *N. caninum,* in addition to the possible occurrence of other agents in domestic dogs of urban and rural areas in Andradina, São Paulo state, Brazil, since this area is endemic for a wide range of parasitic and viral diseases of dogs.

MATERIAL AND METHODS

The experimental group consisted of 84 dogs, of which 75 from urban and 9 from rural areas. Among these, 43 animals were male and 41 female, with 44 of defined/known breed (DB) and 40 mongrels (non-defined breed, NDB). The animals were classified regarding age, as 21 adult and 63 young adult dogs. This study was approved by the Ethics Committee on Animal Experimentation, Faculdade de Odontologia de Araçatuba (FOA), Universidade Estadual Paulista - UNESP, under the protocol number 2007-003276.

The 5-mL blood samples were collected by venipuncture in Vacutainer siliconized tubes without anticoagulant and centrifuged at 3000 rpm for five minutes. After separation, the serum samples were transferred to sterile plastic tubes and immediately frozen at -20°C.

Aspiration biopsy of lymph node and PCR were performed for the diagnosis of canine leishmaniasis. *Leishmania* sp. DNA was amplified from positive lymph node samples in microscopy, using (QIAamp Blood and Tissue, Qiagen®, CA, USA) DNA extraction kit, with amplification of DNA fragments (120 bp) of the kinetoplast minicircle (RODGERS et al., 1990).

Blood smears were prepared on microscope slides using peripheral blood smears and lymph node aspiration biopsy, via fine needle, to investigate the presence of hemoparasites in the dogs. These samples were stained with Quick Panoptic® kit and 300 microscopic fields were observed under 1000x magnification to determine the presence of *Leishmania* spp. amastigotes and flagellated forms of *Trypanosoma* spp. among other hemoparasites. *T. evansi* was identified through biometric measurements (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 by indirect immunofluorescence assay (IFA) for the presence of immunoglobulin G (IgG) against *T. gondii* and *N. caninum.* The used cutoff dilutions 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, the titers of 64 for *T. gondii* and 50 for *N. caninum.* Positive and negative serum samples were included in each reaction, and only samples with complete fluorescence around tachyzoites were considered positive.

The canine distemper virus (CDV) was investigated using the CDV Ag Rapid Test Kit® (Bioeasy) according to the manufacturer's recommendations.

Fisher exact and chi-square tests were used to assess associations between all categories (StatSoft, 2007), at significance level of $P<0.05$.

RESULTS AND DISCUSSION

Amastigotes of *Leishmania* spp., identified as *L. chagasi* by the PCR technique, were observed in 46.42% (39/84) of all dogs. Moreover, approximately 28 flagellated forms of *Trypanosoma* spp. were observed by field in blood smears of an adult male mongrel dog from a rural area that was positive for canine visceral leishmaniasis (CVL). These flagellated forms measured a total size of 26.4 microns (µm), free flagellum length of 7.59 µm, from the posterior end to the core center 11.02 µm and from the core center to the end of anterior extremity 10.22 µm. These findings are compatible with *T. evansi* (Figure 1).

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

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

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

Only one young male mongrel dog, from the rural area, was positive for the canine distemper virus, but did not present any other disease investigated in this study.

The variables age, race, sex and area did not influence significantly ($P \geq 0.05$) the occurrence of *T. evansi, T. gondii, Ehrlichia* spp. and canine distemper virus (Table 1). However, *L. chagasi* affected young dogs more significantly ($P \leq 0.05$) compared to adult animals while the other variables (race, sex, area) did not play a role. As for *N. caninum,* dogs from rural areas were significantly ($P \leq 0.05$) more affected by this infection compared to the animals from urban areas (Table 1).

Table 1 – Effect of race, sex, age and region on the occurrence of *Leishmania chagasi, Trypanosoma evansi, Toxoplasma gondii, Neospora caninum, Ehrlichia* spp. and canine distemper virus among 84 dogs from Andradina, SP, Brazil.

Variable	Category	*L. chagasi*		*T. evansi*		*T. gondii*		*N. caninum*		*Ehrlichia spp.*		Distemper Virus	
		(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
	Adults (n=21)	15[b]		1[a]		0[a]		1[a]		1[a]		1[a]	
Race	RD (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
	SRD (n = 62)	27[a]		1[a]		2[a]		1[a]		1[a]		1[a]	
Gender	Males (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 do not differ ($p \geq 0.05$).
1: Chi-square. 2: Fisher's exact test
Defined Breed (DB), Non-defined breed (NDB). Total number (n)

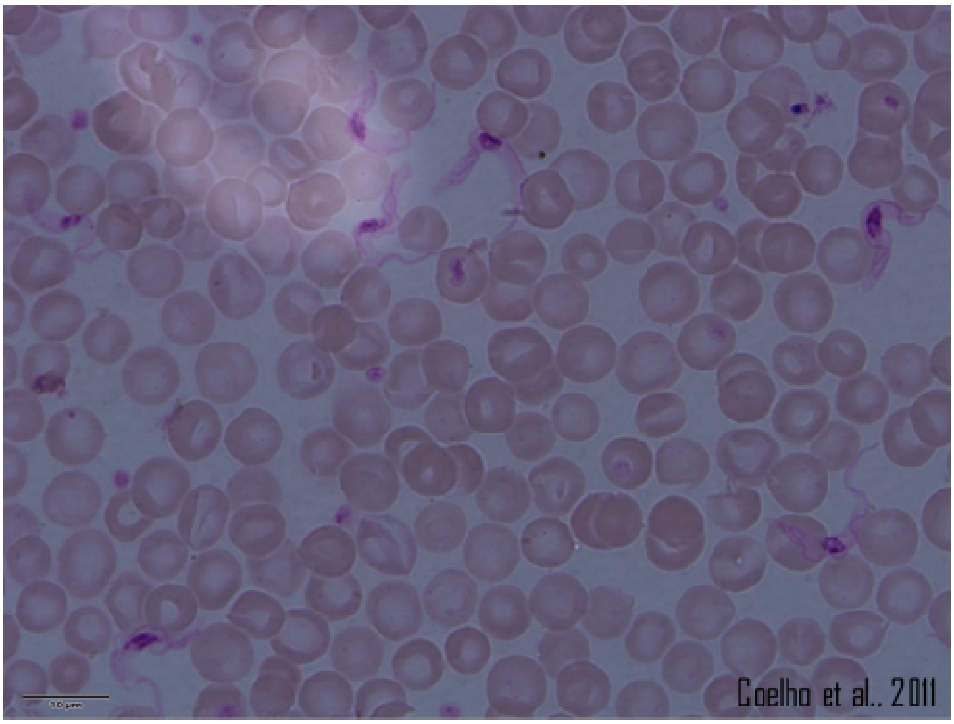

Figure 1 - *Trypanosoma evansi* in blood smear of dogs naturally infected by *Leishmania (L.) Chagasi.*

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

In Araçatuba, Gennari et al. (2006) used IFA to detect anti-*N. caninum* antibodies in 15.3% (15/98) and *anti-T gondii* in 23.4% (23/98) of 98 dogs with CVL, while sex of the animal had no influence on the occurrence of these parasites, confirming the data from this study, in which sex was not a determinant in the occurrence of these parasites.

Infections caused by *Ehrlichia canis* have been reported in dogs with visceral leishmaniasis (SOUSA

& ALMEIDA, 2008), which was not observed in the present study, where only one dog was simultaneously infected with *Ehrlichia* spp. and *T gondii*.

In Lavras, Minas Gerais, Brazil, Guimarães et al. (2009) also used the IFA to detect infection with *Babesia* spp. in 73.3% (220/300), *T. gondii* in 60.5% (132/218), *N. caninum* in 3.1% (7/228) and *Leishmania* spp. in 0.3% (1/300) of the dogs studied, with no co-infections diagnosed. On the other hand, this study diagnosed co-infections between *L. chagasi, T. gondii* and *N. caninum* and no co-infections between *T. gondii* and *N. caninum* in any of the dogs examined in agreement with the data reported by Guimaraes et al. (2009).

Azevedo et al. (2005) detected higher percentages of positivity for *N. caninum* and *T. gondii* in dogs from the state of Paraíba, Brazil: 45.1% (129/286) were positive for *T. gondii* and 8.4% (24/286) for *N. caninum.*

Unlike our work, these researchers found that 4.9% (14/286) of dogs had simultaneous occurrence of antibodies against both protozoa, which was reported by Mineo et al. (2004) and Romanelli et al. (2007). This difference can be explained by the fact that these researchers did not study dogs from urban and rural areas simultaneously. In this study, the isolated occurrence of *N. caninum* was observed in dogs from rural areas while the highest percentage of seropositivity for *T. gondii* was observed for dogs from urban areas. Similarly, Figueiredo et al. (2008) used IFA to analyze serum samples of dogs from Pernambuco and reported 28.3% (177/625) positivity for antibodies to *N. caninum* and from those samples, 57.6% were infected with *T. gondii.*

Co-infection with *T. gondii* and canine distemper virus has been reported by Moretti et al. (2002) in four dogs. In this study, the only animal positive for the virus was negative for all other agents studied, making it impossible to correlate this virus with any other.

This study found that the higher proportion of animals reactive for *N. caninum* were from rural areas. This is in agreement with the observations of Ploneczka et al. (2008) in Poland. Souza et al. (2003) also detected a higher percentage of seropositivity for *T. gondii* in dogs from rural areas in São Paulo and Paraná, differing from our findings. Cañón-Franco et al. (2006) similarly reported 8.3% (13/157) of dogs reactive for *N. caninum,* even in remote areas such as the state of Amazonas, Brazil.

Bresciani et al. (2007) used the IFAT to detect that 23.1% (25/108) of dogs from Araçatuba were seropositive for *T. gondii* and 15.7% (17/108) for *N. caninum* and concluded that dog environment played a relevant role in the infection. In this study, as reported by Silva et al. (2010), there was no correlation with race, sex or age, or statistical association between the occurrences of these agents. These results were similar to those obtained in our study, especially because Andradina belongs to the Araçatuba region and both cities are endemic to these enteric protozoa.

CONCLUSION

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

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