Research Note

Toxoplasma gondii, Neospora caninum and Leishmania spp. serology and Leishmania spp. PCR in dogs from Pirassununga, SP

Sorologia para *Toxoplasma gondii*, *Neospora caninum* e *Leishmania* spp. e PCR para *Leishmania* spp. em cães de Pirassununga, SP

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Abstract

We examined the presence of antibodies against the parasites *Toxoplasma gondii*, *Neospora caninum*, and *Leishmania* spp., as well the presence of DNA from *Leishmania* spp., in dogs from Pirassununga - SP. The seropositivity rate was compared with the animals' originating location. Three hundred seventy-three blood samples from the county's kennel and local veterinary clinics were collected and analyzed. A total of 300 samples were tested for *T. gondii* and *N. caninum* using an indirect immunofluorescence antibody test (IFAT); 45% (135/300) were positive for *T. gondii* and 24.3% (73/300) were positive for *N. caninum*. Three hundred seventy-three samples were tested for *Leishmania* spp. using the IFAT. Of these, 4.6% (17/373) were positive. Additionally, 145 samples were tested using a polymerase chain reaction (PCR); of these samples, 0.7% (1/145) was positive. Considering the results, we conclude that these parasites are present in the city of Pirassununga - SP and that the animals have contact with the protozoan. It is therefore necessary to create methods for disease prevention to maintain both animal and human health in regard to leishmaniasis and toxoplasmosis.

Keywords: IFAT, PCR, dogs, epidemiology, *Leishmania* spp., *Neospora caninum*, *Toxoplasma gondii*.

Resumo

Avaliou-se a presença de anticorpos contra *Toxoplasma gondii*, *Neospora caninum* e *Leishmania* spp.; assim como a presença de DNA de *Leishmania* spp. em cães de Pirassununga-SP, e associou-se sua soropositividade ao local de origem dos animais. Foram coletadas 373 amostras de sangue do canil municipal e de clínicas veterinárias locais, que foram analisadas pelo teste de Imunofluorescência Indireta (RIFI). Do total, 300 amostras foram testadas para *T. gondii* e *N. caninum*, das quais 45% (135/300) foram positivas para *T. gondii* e 24,3% (73/300) para *N. caninum*. Para *Leishmania* spp. foram avaliadas 373 amostras pela RIFI, sendo 4,6% (17/373) positivas. Adicionalmente, 145 amostras foram testadas utilizando-se a PCR e, dessas amostras, 0,7% (1/145) foi positiva. Considerando-se os resultados, pode-se concluir que esses parasitos estão presentes na cidade de Pirassununga - SP e que os animais tiveram contato com os protozoários. Faz-se, dessa forma, necessária a divulgação de meios de prevenção às doenças, com o intuito de manter o controle sobre as mesmas, tanto na saúde animal quanto na saúde humana, em relação à leishmaniose e à toxoplasmose.

Palavras-chave: RIFI, PCR, cães, epidemiologia, *Leishmania* spp., *Neospora caninum*, *Toxoplasma gondii*.

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Introduction

Neosporosis and toxoplasmosis are diseases caused by Apicomplexa obligate intracellular parasites (DUBEY et al., 2002). Toxoplasmosis is important to both animal and human health, and neosporosis is very important to animal health, as both diseases cause respiratory, gastrointestinal, neurological and muscular symptoms (MINEO et al., 2001). Through the excretion of the oocysts eliminated in its feces, the domestic dog is the definitive host and source of infection for *Neospora caninum*. This parasite also negatively impacts cattle, causing abortion and a decline in reproductive performance resulting in economic loss (DE MORAES et al., 2008). The domestic dog is a toxoplasmosis intermediate host, infected by the ingestion of oocysts from cat feces and contaminated water, food, and soil or by carnivorism (TAYLOR et al., 2007).

Visceral leishmaniasis (VL) is a zoonotic disease caused by the protozoan *Leishmania infantum* (Syn. *L. chagasi*), which is considered to be one of the six most important tropical diseases in the world (WHO, 2010). VL is transmitted to humans and other animals by a vector's bite, such as *Lutzomyia longipalpis* and *L. cruzi* sandflies in Brazil. The sandfly acquires the parasite after hematophagism of infected animals (SHAW, 2006). Domestic dogs are considered the primary animal reservoir hosts of the disease and perform an important role in the transfer of this disease to humans (MONTEIRO et al., 2005).

This study aimed to evaluate the presence of antibodies against *T. gondii* and *N. caninum* and the presence of both, antibodies against *Leishmania* spp. and DNA from this parasite in dogs from Pirassununga-SP, and compared with the locations where the animals originated; county's kennel or local veterinary clinics. With the expansion of VL and the increase in the number of cases and because of the significance of these parasites to public health and their associated economic losses, evaluating the presence of these parasites is of great importance.

Materials and Methods

Study area

Pirassununga is located at a latitude of 21º59'46" South and a longitude of 47º25'33" West in São Paulo State, Brazil, and its altitude is 627 meters. The city has a high-altitude tropical climate. The rainy season occurs from October to March, and the city currently has 70,138 inhabitants (IBGE, 2013). The neighboring cities are Descalvado, Porto Ferreira, Santa Cruz das Palmeiras, Analândia, Santa Cruz da Conceição and Leme.

Animals

From August 2010 to July 2013, a total of 373 blood samples were collected from dogs. A convenience sampling method was used because the study aimed to describe the main characteristics of the studied groups. Three hundred animals were tested for the presence of anti-*N. caninum* and anti-*T. gondii*. Three hundred and seventy three were tested against anti-*Leishmania* spp. antibodies by IFAT and 145 dogs were tested for leishmaniasis by PCR. These samples were obtained at Pirassununga's kennel and the city's veterinary clinics; 240 and 133 blood samples were obtained from the kennel and the clinics, respectively. All of the samples were collected according to the rules of the Animal Ethics Committee from the University of São Paulo's faculty of Animal Science and Food Engineering, process number 12.1.1301.74.1.

Immunofluorescence Antibody Test (IFAT)

The IFAT was performed according to previously described methodology for *Toxoplasma gondii* (GARCIA et al., 2008), *Neospora caninum* (HIGA et al., 2000) and for *Leishmania* spp (OLIVEIRA et al., 2008). Antigen slides previously prepared of *N. caninum* (Imunoteste®, *Neospora caninum*, Imunodot, Jaboticabal, SP, Brasil), *T. gondii* (Imunoteste®, *Toxoplasma gondii*, Imunodot, Jaboticabal, SP, Brasil) and *Leishmania chagasi* (Imunoteste®, *Leishmania chagasi*, Imunodot, Jaboticabal, SP, Brasil) were used. Positive and negative controls were added in each test slide. Serial dilutions of each serum specimen were performed. The IFAT used a canine anti-IgG (immune globulin G) conjugated to fluorescein isothiocyanate (Sigma-Aldrich, Bellefonte, PA, USA, catalog n-F7884), diluted according to the manufacturer's recommendations. The dog sera were considered positive when parasites exhibited fluorescent staining. A cutoff dilutions of 1:16, 1:40 and 1:25 was used for *T. gondii*, *Leishmania* spp. and *N. caninum*, respectively, according to references cited above.

Polymerase Chain Reaction (PCR) for Leishmania spp.

DNA Extraction

DNA purification from blood was performed using the salting out technique described by Lahiri & Nurnberger (1991). After extraction, the DNA was stored at -20 °C until evaluation.

DNA Amplification.

DNA amplification was performed using a pair of oligonucleotides for the *Leishmania* spp. genre previously reported (RODGERS et al., 1990): 13A 5′-dGTG GGG GAG GGG CGT TCT-3′ and 13B 5′-dATT TTA CAC CAA CCC CCA GTT-3′; based on these oligos, 120 base pairs (bp) DNA amplification product was anticipated. The reactions were composed of 1 unit (U) of DNA polymerase Platinum® Taq (Invitrogen, Carlsbad, CA, USA), 15.25 μL of ultrapure water, 1X PCR buffer, 1.5 mM MgCl2, 0.31 mM each of dNTP (dATP, dCTP, dGTP e dTTP), 0.26 μM each of the forward and reverse primers and 2.5 μL of DNA extracted from blood. Each reaction was subjected to 34 cycles through a thermo cycler, with the quantity of DNA ranging from 50 to 300 ng, in a final volume of 25 μL. The positive controls were *Leishmania* DNA extracted from *L. infantum* cultured in vitro. The negative controls were DNA extracted from blood samples known to be negative. The amplified products were subjected to

electrophoresis in a 2% agarose gel, ethidium bromide colored and photographed with a Sony cybershot DSC-W70 7.2 megapixel digital camera.

Sequencing

After observation through electrophoresis on a 2% agarose gel, the PCR product was excised from the gel and purified by using GE Healthcare kit (Illustra, GFX PCR DNA and GEL Band purification Kit), according to the manufacturer`s instructions. DNA sequencing was performed at DNA Sequencing Service of the Research Center on the Human Genome and Stem Cells-Biologic Institute (IB)-University of Sao Paulo (USP).

Statistical analysis

Chi-square tests were used to compare the seropositive percentages, including the positivity levels among categories of the same independent variable (i.e., type of housing) and the total occurrence of antibodies to each one of the three agents, with a probability (p) value of <0.05 regarded as statistically significant.

Results

Within the group of 300 evaluated dogs, 45% (135/300) of the animals were seropositive for *T. gondii* and 24.3% (73/300) for *N. caninum*. In addition to the animals tested for *T. gondii* and *N. caninum*, another 73 dogs were tested for *Leishmania* spp., of which 4.6% (17/373) were seropositive on IFAT. Blood PCRs for *Leishmania* spp. were performed in 145 of these samples, and 0.7% (1/145) of the animals tested positive, despite the fact that this one was IFAT-negative. Sequencing results was not god to characterization of the *Leishmania* species. We conducted tests to evaluate the association of seropositivity with the location of the origin of the dogs; the results are displayed below (Table 1).

Discussion

The results found for *T. gondii* were similar to those of the study conducted by Azevedo et al. (2005) in Campinas-SP, which found 45.1% of the dogs were seropositive for this parasite. Both higher and lower occurrence rates of *T. gondii* have been reported in different regions of Brazil. A study conducted by

Guimarães et al. (2009) in Lavras-MG found that 60.5% of the dogs were seropositive for *T. gondii,* whereas a study conducted in Brotas-SP by Langoni et al. (2013) found 26.9% of its dogs were seropositive for *T. gondii*. This study and the studies just mentioned above had cutoff titer ≥16. Figueredo et al. (2008) had a seropositivity of 56.7% by IFAT, with the cutoff titer ≥64, in Pernambuco state. Despite being a cosmopolitan coccidia, *T. gondii* is found in very diverse geographic regions at quite variable prevalence rates (DE MOURA et al., 2009).

Some studies have reported that stray dogs are more susceptible to *T. gondii* infection because of their increased likelihood to come into contact with rodents and the oocysts eliminated by cats (MINEO et al., 2004). However, in our study, we found no association between animals in the kennel and seropositivity to *T. gondii* (p>0.05), which is similar to the results observed by Azevedo et al. (2005). Yet, in the studies by Ali et al. (2003) and Cañón-Franco et al. (2004) a significant increase in the seropositivity against *T. gondii* in stray dogs was observed when compared with pet dogs.

The prevalence of canine toxoplasmosis within the studied regions may be an indicator of whether the studied location offers the appropriate ecological conditions for maintaining the parasite in its infective form and is therefore useful as a sentinel for human toxoplasmosis (TENTER, 1999).

For *N. caninum*, 24.3% (73/300) of the dogs were seropositive using IFAT. These results were higher than those of studies performed by Gennari et al. (2002) in São Paulo-SP, Guimarães et al. (2009) in Lavras-MG and Fernandes et al. (2004) in Uberlândia-MG; these studies detected seropositivities of 10%, 3.1% and 10.7%, respectively. Nevertheless, the results were similar to those found by Figueredo et al. (2008), who obtained a 28.3% of seropositivity rate. This wide range of results can be explained by the different serological tests used, cutoff point adopted, population sampled and the sampling type (GUIMARÃES et al., 2009).

Some studies have found an increased prevalence of antibodies against *N. caninum* in dogs from rural areas, suggesting a greater exposure to horizontal transmission from contact with aborted fetuses and fetal membranes (BRUHN et al., 2013; NOGUEIRA et al., 2013). However, De Sousa et al. (2012) obtained a seroprevalence of 4.2%, with half of the dogs' population from urban areas and half from rural areas with no significant difference observed between the groups.

In this study, the *N. caninum* seropositive occurrence rates between the pet and stray dogs (local clinic or kennel) were

Table1. Prevalence of anti-*Neospora caninum*, anti-*T. gondii*, and anti-*Leishmania* spp. antibodies detected by indirect fluorescent antibody test (IFAT) in dogs from kenel and clinic, from august 2010 to July 2012, from Pirassununga, São Paulo, Brazil.

Origin	Protozoan					
	N. caninum		*T. gondii*		*Leishmania* spp.	
	%	N(total)	%	N(total)	%	N(total)
Kenel	22.1	37/167	45.5	76/167	6.2[a]	15/240
Clinic	27	36/133	44.3	59/133	1.5	2/133
Total	24.3	73/300	45	135/300	4.6	17/373

N = Number of dogs positive. total = Number of dogs according to their place of origin. [a] = *Leishmania* spp.-positive serology percentage with superscripts are significantly different (p<0.05).

approximately equivalent, with no statistically significant difference (p>0.05). It is noteworthy that the prevalence of neosporosis in the dogs from the urban areas can be associated with what the animals are fed. Those who have a homemade diet that includes raw meat may be at higher risk of contamination by ingesting tissue cysts (KRAMER et al., 2004).

The samples evaluated for *Leishmania* spp. showed 4.5% (17/373) positivity on IFAT, with only 0.7% (1/145) positive using PCR, despite the fact that this one was IFAT-negative. This disagreement between the tests most likely reflects the limitations inherent in both tests in detecting different stages of infection, suggesting that they do not have the same diagnostic value and may be complementary.

IFAT was used on large-scale in epidemiological surveys to detect canine leishmaniasis until recently, in the endemic areas of Brazil, where the prevalence of visceral leishmaniasis is the greatest in the Americas (BRASIL, 2006; DIETZE, 2006), whereas PCR has been used primarily in research (DE ASSIS et al., 2010).

De Assis et al. (2010) showed a low agreement between PCR and other tests, with PCR reporting more positive detection results than the other diagnostic methods for *L. chagasi*. Savani et al. (2011) reported the first autochthonous *L. chagasi* case in Campinas-SP using PCR. Silva et al. (2008) discovered 3% of *Leishmania* spp. seropositive dogs in Bom Sucesso-MG, which is an area that is non-endemic for visceral leishmaniasis. Moreover, in endemic areas, such as Belo Horizonte-MG, a seroprevalence of 15.9% was reported by ELISA, whereas PCR-RFLP revealed that 24.7% of dogs positive for *L. infantum* DNA (COURA-VITAL et al., 2011).

Some authors suggests that common serological tests to *Leishmania* can cross-react with other infections than *L. infantum* (Zanette et al., 2014). Herein we agree with the results reported by Oliveira et al. (2008) and Guimarães et al. (2009), that there is no cross reaction between *Leishmania* spp and other protozoan that can infect dogs. Probably they can cause co-infections.

There was a significant association (p<0.05) between seropositivity for leishmaniasis and dogs from the kennel, based on place of origin. This demonstrates that these animals may have more susceptibility to acquiring the disease. Naveda et al. (2006) found that the origin of the dog is a great risk factor because a stray animal is more likely to be exposed to the agents of the disease than a domiciled animal that does not wander free.

The expansion of leishmaniasis in the non-endemic areas in Brazil, such as Pirassununga-SP, can be associated with several factors, including movement of infected animals, failure of early detection of human and canine cases, the vectors' adaptability to diverse environments and the possibility of involvement of other reservoirs in the cycle. Hence, epidemiological surveillance is of the utmost importance (Desjeux, 2004; Oliveira et al., 2008).

Conclusion

As definitive hosts and source of neosporosis, the presence of seropositive dogs indicates the spread of this disease in the county, which can be problematic due to its abortive effect on cattle. Toxoplasmosis is a widespread zoonosis in our country, and the high seropositivity rate in dogs from Pirassununga demonstrates

the disease dissemination in the studied environments. With respect to leishmaniasis, the presence of infected animals suggests the importance of the epidemiological surveillance of these diseases, especially visceral leishmaniasis, which poses serious risks to human and canine health.

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