Case Report

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An atypical *Toxoplasma gondii* genotype in a rural Brazilian dog co-infected with *Leishmania (Viannia) braziliensis*

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ABSTRACT

Toxoplasmosis and leishmaniasis are two worldwide zoonoses caused by the protozoan parasites *Toxoplasma gondii* and *Leishmania* spp., respectively. This report describes the clinical and laboratorial findings of a co-infection with both parasites in a 4-year-old female dog suspected of ehrlichiosis that presented anemia, thrombocytopenia, hypoalbuminemia, hyperglobulinemia, tachyzoite-like structures to the lung imprints, and polymerase chain reaction (PCR) results positive for *T. gondii* (kidney, lung, and liver) and *Leishmania* spp. Co-infection with *Toxoplasma gondii* and *Leishmania braziliensis* was confirmed by sequencing; restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) confirmed an atypical *T. gondii* genotype circulating in dogs that has been reported to cause human congenital toxoplasmosis.

Keywords: Immunosuppression. American visceral leishmaniasis. Toxoplasmosis.

INTRODUCTION

Toxoplasmosis is a worldwide anthropozoonosis caused by a coccidian protozoan, *Toxoplasma gondii*. Felids are the definitive hosts, while most warm-blooded animals are intermediate hosts[1]. Dogs are considered sentinels for human infection; they are infected by ingesting raw or poorly cooked meat containing tissue cysts and by ingesting sporulated oocysts present in the contaminated feces of cats[2] [3] [4].

Leishmaniasis is a zoonotic disease caused by *Leishmania* spp., and manifested in South America as American visceral leishmaniasis (AVL, *Leishmania infantum chagasi*) and American tegumentary leishmaniasis (ATL, mainly *Leishmania braziliensis*)[1]. *L. braziliensis* can cause visceral disease as a consequence of host immunosuppression, and is considered an opportunistic disease transmitted by phlebotomine bites after blood feeding from infected dogs, the main urban reservoir.

Hence, we report a *T. gondii* and *L. braziliensis* co-infection in a Brazilian dog suspected to have an *Ehrlichia canis* infection, and an atypical *T. gondii* genotype circulating in dogs.

CASE REPORT

A mixed breed female dog weighing 15.3kg and approximately 4 years of age from the rural area of Botucatu, SP, Brazil (22°53'08"S; 48°26'42"W) was brought to the Veterinary Hospital, School of Veterinary Medicine and Animal Science [*Faculdade de Medicina Veterinária e Zootecnia* (FMVZ)], São Paulo State University [*Universidade Estadual Paulista* (UNESP)] in May 2013. The animal had a previous history of fever and apathy starting 1 year prior. During that time, the animal was examined at a private veterinary clinic, clinically diagnosed, and treated for ehrlichiosis. One year later, the animal presented hyporexia, diarrhea with mucous, tachypnea, a reactive left submandibular lymph node, rectal temperature of 39.9°C, and was suspected to have bronchopneumonia.

Blood samples were collected for a complete blood cell count, serum biochemical analyses, and testing for *T. gondii*, *Neospora caninum*, and *Leishmania* spp. antibodies by the indirect fluorescent antibody test (IFAT) method. The IFAT was assayed with antigens produced in-house (tachyzoites of *T. gondii*, RH strain; tachyzoites of *N. caninum*, NC-1 strain;

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and promastigotes of *Leishmania major*, MHOM/SU/1973/5-ASKH); the cut-off titers were 16, 40, and 50, respectively. IFAT was carried out using a goat anti-dog immunoglobulin G [IgG (H&L)] commercial conjugate (A40-123F; Bethyl Laboratories Inc., Montgomery, TX, USA). The serology results were positive for *N. caninum* (titer 100) and *T. gondii* (titer 16), but negative for *Leishmania* spp.

In addition, the animal presented pulmonary fields with diffuse opacification on a thoracic radiographic image with bronchial, interstitial, and alveolar patterns, and enlargement of the bronchial wall, suggesting bronchopneumonia. The heart presented a discrete hogback with respect to the right atrium. Three days later, the previous findings had worsened with accentuated diffuse opacification. The heart limits of the right atrium and pulmonary trunk were still curved. After 3 days, impairment was observed with a mixed pattern that tended to be micronodular in structure, suggestive of a fungal infection.

An erythrogram demonstrated normocytic normochromic anemia [mean corpuscular volume (MCV) = 70.5%; mean corpuscular hemoglobin concentration (MCHC) = 33.1%) and thrombocytopenia (28,000 platelets/μL^{-1}) **(Table 1)**; lymphopenia (290 cells/µL) and eosinopenia (0.0 cells/µL) were observed on a leukogram; and hypoalbuminemia (1.5g/dL) and hyperglobulinemia (5.1g/dL) were found in the biochemical analyses[5]. Rouleaux was still observed. Chloramphenicol sodium succinate (50mg/kg three times a day; Ariston, São Paulo, SP, Brazil) was used to treat the ehrlichiosis and bronchopneumonia. Furosemide (4mg/kg twice a day; Sanofi, São Paulo, SP, Brazil), bromhexine (3mg/kg once a day, Boehringer-Ingelheim, São Paulo, SP, Brazil), and oxygen therapy were used for pulmonary complications; fluid therapy with lactated Ringer's solution plus glucose for dehydration; and a multivitamin (5mL twice a day, Clusivol®; Wyeth Consumer, São Paulo, SP, Brazil) to stimulate the appetite. The animal died 10 days later, and pneumonia associated with renal and hepatic lesions was confirmed by necropsy. Lung, kidney, and liver samples were finely chopped, imprinted in slides, and stained with Giemsa; they showed negative results for *Ehrlichia* spp., but presented tachyzoite-like structures in the lung tissue.

The samples were tested for *T. gondii*[6], *N. caninum*[7], *E. canis*[8], and *Leishmania* spp. deoxyribonucleic acid (DNA)[9] by the PCR **(Table 2)**. Positive results for *T. gondii* were observed in the lung, kidney, and liver samples; *Leishmania* spp. was found in the lung and liver. All samples yielded negative results for *E. canis*, *N. caninum*, and *L. infantum chagasi*.

Samples positive for *Leishmania* spp. were typed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) using one marker for species identification and the restriction enzyme *Hae*III[9], and showed *L. braziliensis*. In the same way, samples positive for *T. gondii* were also typed by RFLP-PCR using 11 markers for genotype identification[10]. All three tissue samples were confirmed to contain *T. gondii* by genotyping, and demonstrated an atypical genotype **(Table 3)** reported in the scientific literature to cause human congenital toxoplasmosis, TgCTBr5[4]. All amplifications were performed in a MasterCycler ep gradient (Eppendorf, USA). Sequencing of the purified PCR products (Tg18s58F and Tg18s348R for *T. gondii*; LITSR and L5.8S for *Leishmania* spp.) was performed, and the results searched in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST). All *T. gondii* and *Leishmania* spp. products matched the *T. gondii* 18S ribosomal ribonucleic acid (rRNA) gene [L37415.1] and *L. braziliensis* kinetoplast minicircle gene [EU370877.1], respectively.

TABLE 1 - The hematological and biochemical parameters of the dog sent to FMVZ-UNESP Veterinary Hospital*.

Hematological parameters	Reference		Leukogram	Reference	
Erythrogram					
Erythrocytes ($\times 10^6$ μL^{-1})	3.69	5.5 – 8.5	Leukocytes ($\times 10^3$ μL^{-1})	9.50	6 – 17
Hemoglobin (g/dL^{-1})	8.6	12 – 18	Neutrophils ($\times 10^3$ μL^{-1})	8.93	3 – 11.5
PCV (%)	26.0	37 – 55	Lymphocytes ($\times 10^3$ μL^{-1})	0.29	1 – 4.8
MCV (fL)	70.5	60 – 77	Eosinophils ($\times 10^3$ μL^{-1})	0.00	0.15 – 1.35
MCHC (%)	33.1	32 – 36	Monocytes ($\times 10^3$ μL^{-1})	0.29	0.1 – 1.25
Biochemical parameters	Reference		Leukogram	Reference	
Analyses					
Albumin (g/dL^{-1})	1.5	2.6 – 3.3	ALT (g/dL^{-1})	41.0	21.0 – 73.0
Serum total protein (g/dL^{-1})	6.6	5.4 – 7.1	Creatinine (g/dL^{-1})	0.6	0.5 – 1.5
GGT (g/dL^{-1})	5.1	1.2 – 6.4	Urea (g/dL^{-1})	22.0	21.4 – 59.92
ALP (g/dL^{-1})	123.0	20.0 – 156.0	Globulin (g/dL^{-1})	5.1	2.7 – 4.4

FMVZ-UNESP: *Faculdade de Medicina Veterinária e Zootecnia - Universidade Estadual Paulista*; PCV: packed cell volume; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; GGT: γ-glutamyl-transferase; ALP: alkaline phosphatase; ALT: alanine aminotransferase. *Hematological and biochemical reference parameters for healthy dogs (Feldman et al., 2000).

TABLE 2 - The set of primers used in the present case report.

Agent	Target	Primer name	Primer design (5'-3')	Size (bp)	Reference
Toxoplasma gondii	18S rRNA gene (external)	Tg18s58F	CTAAGTATAAGCTTTTATACGGC	290	Da Silva et al.[6]
		Tg18s348R	TGCCACGGTAGTCCAATAC		
	18S rRNA gene (internal)	Tg18s48F	CCATGCATGTCTAAGTATAAGC		
		Tg18s359R	GTTACCCGTCACTGCCAC		
Leishmania spp.	ITS1 gene	LITSR	CTGGATCATTTTCCGATG	300-350	El Tai et al.[9]
		L5.8S	ACACTCAGGTCTGTAAAC		
Leishmania infantum chagasi	mkDNA gene	LC14	CGCACGTTATATCTACAGGTTGAG	167	FMVZ-UNESP
		LC15	TGTTTGGGATTGAGGTAATAGTGA		
Neospora caninum	Nc5 region	Np6	CAGTCAACCTACGTCTTC	330	Yamage et al.[7]
		Np21	GTGCGTCCAATCCTGTAAC		
Ehrlichia canis	*dsb* gene	Dsb-330	GATGATGTCTGAAGATATGAAACAAAT	409	Doyle et al.[8]
		Dsb-729	CTGCTCGTCTATTTTACTTCTTAAAGT		

rRNA: ribossomal RNA; ITS: Internal Transcribed spacer; mkDNA: minicircle kinetoplast DNA; Nc: *Neospora caninum*; Bp: base pair; FMVZ-UNESP: *Faculdade de Medicina Veterinária e Zootecnia - Universidade Estadual Paulista.*

TABLE 3 - Genotypic profiles of the *Toxoplasma gondii* samples from the dog tissues.

	Genetic markers												
Samples	SAG1	5'-3'SAG2	alt-SAG2	SAG3	β-TUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype	Reference
Lung	I	III	III	III	III	III	I	I	I	u-1	I	TgCTBr5	Carneiro et al.[11]
Kidney	I	III	III	III	III	III	I	I	I	u-1	I	TgCTBr5	Carneiro et al.[11]
Liver	I	III	III	III	III	III	I	I	I	u-1	I	TgCTBr5	Carneiro et al.[11]

DISCUSSION

In this study, a dog undergoing routine treatment at the FMVZ-UNESP Veterinary Hospital presented a *T. gondii* and *L. braziliensis* co-infection. This type of co-infection has already been reported in Brazil in 17/66 (25.8%) cats by molecular methods[11], but was caused by *L. infantum chagasi*, not *L. braziliensis*. Both parasites have been reported to be opportunistic. Moretti et al.[2] reported a case of a dog co-infected with *T. gondii*, distemper virus, and ehrlichiosis. Immunosuppressive factors decrease host immunity and cause the rupture of pre-existing cysts, reactivating latent infections[1] [3] [2]. In this case, even with a negative result for *E. canis,* ehrlichiosis was not ruled out because the animal could have been a carrier. Additionally, an atypical *T. gondii* genotype was identified from the Brazilian dog; curiously, the same genotype has been isolated only in Brazil from cats, chickens, capybaras, sheep, rabbits, and mice, and was recently reported by Carneiro et al.[4] to cause human congenital toxoplasmosis in the State of Minas Gerais, Brazil, but with no clinical signs. The authors reported that it was confirmed as an avirulent genotype, among others, by mouse bioassay. The present study emphasizes the occurrence of this genotype and disease with pulmonary complications probably caused by the opportunistic multiplication of *T. gondii* as a consequence of immunosuppression. Even under these conditions, an avirulent parasite can multiply, infect other hosts, and cause disease. The findings observed by Moretti et al.[2] were similar, but they observed a virulent type I genotype (SAG2 *locus*) rather than the type III genotype (SAG2 and alt-SAG2 *loci*) found in this study. This report emphasizes the importance of *T. gondii*, the circulation of the same genotypes in animals and humans, and supports dogs as perfect sentinels for this infection and genotype, as observed in other species.

The hematological, biochemical, and imaging analyses suggested a *T. gondii* and *Leishmania* spp. co-infection with an additional immunosuppression factor causing a visceral form of the disease by *L. braziliensis*. *L. braziliensis* causes ATL in humans and dogs; however, both here and in the scientific literature, it has been reported to be an opportunistic parasite in immunosuppressed individuals[12].

The low titer for the *T. gondii* antibody findings associated with the hematological, biochemical, and imaging results

suggests a possible acute infection with a low parasite load producing a low humoral immune response dependent on a cellular response. Alternatively, it could suggest a reactivation of a chronic *T. gondii* infection caused by any immunosuppressive factor, for example, ehrlichiosis[2]; the same was observed for leishmaniasis. Immunosuppression caused by an immunosuppressive factor changed the cutaneous form of leishmaniasis caused by *L. braziliensis* into a visceral pattern, as observed by Hernandez et al.[12]. The positive result from *N. caninum* antibody testing suggests an old infection with a low parasite load.

Thus, this report demonstrates a *T. gondii* and *L. braziliensis* co-infection in a dog presumed to have ehrlichiosis and a cutaneous *Leishmania* species causing a visceral form of the disease, as well as the circulation of the same *T. gondii* atypical genotype in dog and human populations, causing congenital toxoplasmosis in humans and other domestic animals. These findings highlight the importance of molecular techniques as epidemiological and diagnostic tools and adjuncts to standard tests to determine the causative agent(s), and supports dogs as the perfect sentinel model for this infection.

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