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Original Article

Clinical-dermatological, histological abnormalities and prevalence of *Trypanosoma caninum* and *Leishmania infantum* in dogs from Midwest region of Brazil

Anormalidades clínico-dermatológicas, histológicas e prevalência de Trypanosoma caninum e Leishmania infantum em cães da região Centro-Oeste do Brasil

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Abstract

Leishmania infantum is a trypanosomatid that causes parasitic dermatopathy in dogs. *Trypanosoma caninum* is another trypanosomatid, which infects the skin of dogs, although cutaneous abnormalities are absent. This study aimed to investigate the occurrence of *T. caninum* infection and its associated cutaneous and histological changes and compare it with the occurrence of *L. infantum* infection in dogs. The study included 150 dogs, of which *T. caninum* infection was identified in 3 (2%) and *L. infantum* infection in 15 (10%) of them, with no association ($p>0.05$) of these infections with the breed, gender, age, or cutaneous abnormalities. The cutaneous abnormalities were based on 1 (4.8%) and 12 (57.1%) dogs infected by *T. caninum* and *L. infantum,* respectively. The dermatohistopathological abnormalities in the dogs infected with *T. caninum* included mild perivascular lymphohistioplasmacytic infiltrates in the clinically asymptomatic ones, while in those with dermatological abnormalities, acanthosis, epidermal orthokeratotic hyperkeratosis, melanomacrophages, and co-infection with *Microsporum* sp. and *Trichophyton* sp. were observed. In *L. infantum* infected, the histopathological findings included chronic granulomatous inflammatory infiltrates and structures compatible with amastigotes. Despite the low frequency of *T. caninum* infection, our findings suggest that this trypanosomatid, unlike *L. infantum*, does not cause any macroscopic skin abnormalities.

Keywords: Trypanosomatids, dermatopathy, zoonosis, canine, skin.

Resumo

Leishmania infantum é um tripanosomatídeo que causa dermatopatia parasitária em cães. *Trypanosoma caninum* é outro tripanosomatídeo, que infecta a pele de cães, embora anormalidades cutâneas sejam ausentes. Este estudo teve como objetivo investigar a ocorrência da infecção por

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T. caninum e suas alterações cutâneas e histológicas associadas e compará-las com a ocorrência da infecção por *L. infantum* em cães. O estudo incluiu 150 cães, dos quais a infecção por *T. caninum* foi identificada em 3 (2%) e a infecção por *L. infantum* em 15 (10%) deles, sem associação (p>0,05) dessas infecções com a raça, sexo, idade ou anormalidades cutâneas. As alterações cutâneas foram observadas em 1 (4,8%) e 12 (57,1%) cães infectados por *T. caninum* e *L. infantum*, respectivamente. As anormalidades dermato-histopatológicas nos cães infectados por *T. caninum* incluíram infiltrados linfo-histioplasmocitários perivasculares leves nos clinicamente assintomáticos, enquanto naqueles com anormalidades dermatológicas, foram observados acantose, hiperqueratose ortoqueratótica epidermal e melanomacrófagos e co-infecção por *Microsporum* sp. e *Trichophyton* sp. Nos cães infectados por *L. infantum*, os achados histopatológicos incluíram infiltrados inflamatórios granulomatosos crônicos e estruturas compatíveis com amastigotas. A despeito da baixa frequência da infecção por *T. caninum*, nossos achados sugerem que esse tripanosomatídeo, diferentemente de *L. infantum*, não causa anormalidades macroscópicas na pele.

Palavras-chave: Tripanosomatideos, dermatopatia, zoonose, canino, pele.

Introduction

Among parasitic dermatoses, those caused by the protozoa of the Trypanosomatidae family, especially of the *Leishmania* genus, are relevant to dogs residing in endemic areas of Brazil (Gasparetto et al., 2013). Although many *Trypanosoma* species affecting dogs have been described (Hoare, 1972), there is no description of the cutaneous changes caused by these protozoa. However, in 2009, a new *Trypanosoma* species, named *Trypanosoma caninum*, parasitizing the skin of a dog co-infected with *Leishmania braziliensis* was described (Madeira et al., 2009), suggesting that dermatological changes may be associated with this parasite.

Little is known about the development cycle, tropism, and pathogenicity of *T. caninum* (Madeira et al., 2009). *T. caninum* has been isolated only from the skin so far, a factor that is unusual of the *Trypanosoma* genus, and it shares the same reservoir with *L. infantum*, the infectious agent of visceral leishmaniasis (VL). It has been isolated from several regions of Brazil (Alves et al., 2012; Oliveira et al., 2015). These aspects raise some public health concerns, especially the risk of erroneous diagnoses of LVC in areas of sympatry of *L. infantum* and *T. caninum* (Almeida et al., 2011; Alves et al., 2012).

The aspects described above, and the limited information available related to the clinical aspects and the possible dermatological alterations associated with *T. caninum* infection in dogs motivated this study. We aimed to investigate the frequency of *T. caninum* infection in dogs selected at the University Veterinary Hospital of Cuiabá, Mato Grosso, Brazil, and examine possible dermatological and histological abnormalities in *T. caninum*-infected dogs and compare it to those seen in dogs infected with *L. infantum*.

Materials and Methods

Dogs

This study included 150 dogs, selected while attending clinic and dermatological at the Veterinary Hospital (HOVET) of the Federal University of Mato Grosso (UFMT) campus Cuiabá, Mato Grosso, from May 2015 to May 2016. As inclusion criteria, dogs with a history of dermatological alterations (symptomatic) and healthy dogs (asymptomatic) submitted to elective castration were selected. The cohort size was determined based on the number of dogs presented to the HOVET-UFMT annually, taking into account a *T. caninum* infection prevalence of 10.9% (Pinto et al., 2014), and error margin ≤ 0.05.

This study was approved by the Committee for Ethics in Animal Use (CEUA) of UFMT under nº 23108.102289/2015-92 and executed according to the ethical principles. Informed consent was signed by the owner of each dog.

Clinical analysis: dermatology and complementary examinations

Dermatological, epidemiological, and general clinical evaluations were performed on the dogs. The data related to the dermatological changes included macroscopic morphological characteristics (location, shape, and the intensity of changes) (Ginn et al., 1972; Scott et al., 2001). Based on the cutaneous changes found in the dogs, complementary tests including direct parasitological examination of samples acquired via skin scrapings or adhesive tape (Pereira et al., 2015); wood lamp examination; direct microscopic hair examination, after imbibition in 10% potassium hydroxide, fungal culture, and cytological examination of fine-needle aspirates of skin samples (Bond, 2010) were performed for a definitive diagnosis of the dermatopathy.

Detection of *Trypanosoma caninum* and *Leishmania infantum* infection

Blood samples (5 mL) were collected by cephalic or jugular venipuncture using the aseptic technique into 10% sodium EDTA tubes for parasitological culture and molecular tests. In addition, five skin biopsies of dermatologically asymptomatic dogs and five skin biopsies from cutaneous lesions of dermatologically symptomatic dogs were collected. Three-millimetre tissue biopsies were obtained after trichotomy, antisepsis, and 2% lidocaine local anaesthesia (Almeida et al., 2010) for parasitological culture, molecular testing, and histopathology.

Parasitic isolation in culture medium

Skin biopsies were immersed in sterile buffered saline containing penicillin, streptomycin, and fluorocytosine, and preserved at 4 °C for 24 hours. The samples were then seeded into screw-cap tubes containing blood-agar slants biphasic medium (Novy, MacNeal, Nicole - NNN - Sigma-Aldrich®) overlaid with 2 mL of Schneider's *Drosophila* medium (Sigma-Aldrich®) plus 10% fetal bovine serum (Sigma-Aldrich®). Peripheral blood collected in tubes containing anticoagulants were seeded directly into the culture medium (400 µL). The cultures were seeded in duplicates, placed in an incubator at 26-28 °C, and examined weekly for 40-50 days by fresh tests to detect flagellate forms (Almeida et al., 2011).

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

DNA extraction from the skin and blood samples were performed using the phenol/chloroform method (Sambrook & Russell, 2001). For the detection of *T. caninum* DNA, nested PCR was used with the outer primers TRY927F and TRY927R during the first round and the inner primers SSU561F and SSU561R, which amplify a conserved region of the 18S rDNA (ribosomal DNA) gene of all trypanosomatids (Smith et al., 2008), during the second round. For the detection of an *L. infantum* infection, RV1 and RV2 primers were used to amplify a 145 bp region of kDNA (kinetoplast DNA) specific for this species (Lachaud et al., 2002). For both sets of primers, the *L. infantum* DNA (MHOM/BR/1974/PP75) was used as the positive control and a corresponding negative control (DNA free reaction) was included in every reaction.

The amplification products of the nested PCR and *L. infantum* specific PCR were fractionated by 2% agarose gel electrophoresis, stained with Red gel (Biotium®) and viewed with ChemiDoc XRS using the Image Lab Software. A 100 bp molecular weight marker (Fermentas®) was used. The products obtained by the 18S rDNA nested PCR were purified using the GFX PCR DNA kit and sequenced using Sanger sequencing (Applies Biosystems® Genetic Analysis) for etiological characterization. The sequences obtained were deposited and compared to sequences of different species of trypanosomatids using GenBank.

Histopathological examination

Skin sections were fixed in a 10% formalin solution, embedded in paraffin, cut into 5-µm-thick sections, and stained with hematoxylin-eosin (HE) (Prophet et al., 1992).

Statistical analysis

The data were tabulated and analysed descriptively. The association of sex, breed and age with the presence of dermatological changes and *T. caninum* or *L. infantum* infection was examined using chi-square or Fisher Exact tests, using statistical software (Epi Info 3.3.2 program, CDC, Atlanta, GA). A P-value < 0.05 was considered significant.

Results

The study included 150 dogs (males, n = 64, 42.7%; females, n = 86, 57.3%) of which 77 (51.3%) were of mixed breed and 73 (48.7%) were pure-breed dogs, with an overall median age of 4 years (range 2 months - 13 years). Dermatological abnormalities were noted in 117 dogs (78%), including pruritus (62.7%), alopecia or hypotrichosis (60%), desquamation (60%), hyperpigmentation (8%), cutaneous ulceration (4.7%), meliceric crusts (4.7%), onychogriphosis (2.7%) and lichenification (0.7%).

In the parasitological culture, flagellar forms were isolated from two skin biopsies from two different dogs. By molecular analysis, trypanosomatid DNA was detected in 18 (12%) dogs, independent of the samples and primers used (Table 1). *T. caninum* infection was detected in three dogs (2.0%), all from skin samples, and identified using nested PCR. Sequencing of the amplification revealed 99.45% and 99.46% homology with *T. caninum* isolate 118 and *T. caninum* isolate MT604, respectively (GenBank accession number GU385825.1 and JF907512.1). *L. infantum* was detected in 15 (10%) dogs in blood and/or skin samples, and revealed 99.62%, 99.23% and 99.81% homology with *L. infantum* isolate LL1678 LN, *L. chagasi* isolate LL2264 LN and *L. infantum* isolate LL1884 MO, respectively (GenBank accession number KU948456.1, KU948484.1 and KU948467.1%, respectively). The positivity values for the agents, targets, and samples investigated are included in Table 1.

Table 1. Positivity to *Trypanosoma caninum* and *Leishmania infantum* in blood and skin samples in molecular analysis using the 18S rDNA and *Leishmania* kDNA targets.

Agent	Dogs n = 150	18S rDNA		kDNA	
	Positive	Blood	Skin	Blood	Skin
Asymptomatic dogs					
Trypanosoma caninum	2	0	2	0	0
Leishmania infantum	3	0	1	2	0
Symptomatic dogs					
Trypanosoma caninum	1	0	1	0	0
Leishmania infantum	12	2	5	4	7

T. caninum and *L. infantum* infections were not significantly associated (p>0.05) with the breed, sex, age, or the presence of dermatopathies. *T. caninum* infection was detected in two (1.3%) asymptomatic dogs and one (0.7%) dog which presented skin pruritus with alopecia and desquamation. *L. infantum* infection was found in three (9.1%) asymptomatic dogs and twelve (10.2%) dogs which showed the following clinical findings: alopecia (75%), desquamation (75%), pruritus (67%), cutaneous ulceration (33%), and onychogriphosis (25%).

Of the 117 dermatologically symptomatic dogs studied, a dermatopathic presumptive diagnosis was made based on laboratory and histopathological examination in 82% of the cases, while results were inconclusive in 18%. The presumptive diagnoses included allergic

(16%), parasitic (15.3%), fungal (14.7%), bacterial (2.0%), endocrine (1.3%), immune-mediated (0.7%) and hereditary (0.7%) dermatopathies, as well as keratinization defects (3.3%).

One hundred of the fungal cultures (66.7%) were positive for *Microsporum* spp. or *Trichophyton* spp., of which, twelve dogs (12%) were positive for *L. infantum* and three (3.0%) were positive for *T. caninum*. *Demodex* or *Sarcoptes* mites were detected by skin scrapings or adhesive tape in eleven dogs (7.3%). One (0.7%) of the dogs detected with demodicosis was positive for *L. infantum*.

The skin histopathology results of the infected dogs are presented in Table 2. In three *L. infantum* infected dogs (20%), no histopathological abnormalities were observed. In one dog, the changes were suggestive of endocrinopathy, characterized by acanthosis, epidermal and follicular infundibulum orthokeratotic hyperkeratosis, and hair follicle and sebaceous gland atrophy.

Table 2. Skin histopathological findings of dogs infected with *Trypanosoma caninum* or *Leishmania infantum*.

Histopathological changes	*T. caninum* infection (n=3)	*L. infantum* infection (n=15)
Epidermis		
Acanthosis	33% (1)	7% (1)
Orthokeratotic hyperkeratosis	33% (1)	7% (1)
Dermis		
Lymphohistioplasmacytic perivascular infiltrate	67% (2)	47% (7)
Lymphohistioplasmacytic perivascular/perionxys infiltrate	0% (0)	13% (2)
Perivascular/perionxys pyogranulomatous infiltrate	0% (0)	7% (1)
Melanomacrophages	33% (1)	0% (0)
Atrophy of the hair follicles and sebaceous glands	0% (0)	7% (1)
L. infantum amastigotes	0% (0)	7% (1)

Discussion

The present study conducted in an endemic area of *T. caninum* and *L. infantum* showed infection occurrence rates of 2% and 8% for these parasites, respectively, in agreement with previous findings (Gasparetto et al., 2013; Pinto et al., 2014). Infections by different protozoa of the Trypanosomatidae family in canines have been described previously (Nwoha, 2013). In this scenario, *L. infantum* infection has important public health implications (Madeira et al., 2006) due to the relevance of dogs as a reservoir of zoonotic visceral leishmaniasis. The recent isolation of *T. caninum* from dogs' skin samples in endemic areas for visceral leishmaniasis has called attention to this protozoan (Barros et al., 2012; Oliveira et al., 2015).

In addition, *T. caninum* infection was identified only in skin samples, even when parasitological culture and molecular analysis of blood samples were utilized (Pinto et al., 2014; Madeira et al., 2014). Skin was also the biological sample with the highest detection rate of *L. infantum* in symptomatic and asymptomatic dogs, independent of the target gene sequences used. Despite the low number of *T. caninum* positive dogs, the present results reinforce the finding that *T. caninum* infection is restricted to the skin (Madeira et al., 2014). Conventional PCR and parasitological culture, presently utilized for identifying *T. caninum* infection, might not be sensitive enough to detect very low parasite burdens, possibly limiting the detection of this parasite, especially since little is known of its pathogenesis and biological cycle (Almeida et al., 2011; Madeira et al., 2014). However, the importance of dogs in canine visceral leishmaniasis (CVL) reinforces the need to promote the distinction of these agents (Barros et al., 2012).

Although the 18S rDNA gene detects *Leishmania* DNA, the use of the primers with a conserved region of *L. infantum* kDNA employed in this research obtained a higher positivity for this region, which makes it a good diagnostic method for visceral leishmaniasis, especially in endemic and sympatric areas. These results are consistent with those obtained by Pinto et al. (2014). However, regarding *L. infantum*, it is important to submit several biological samples for specific PCR and parasitological cultures, in order to have higher chances of detecting this agent (Almeida et al., 2011, 2013). The use of specific *T. caninum* primers might be more effective for its detection, as described for LVC. In this study, there was no association of *T. caninum* or *L. infantum* infection with age, breed, and sex, which is in agreement with previous findings in cases of visceral leishmaniasis (Almeida et al., 2009; Oliveira et al., 2010), while *T. caninum* has been detected in asymptomatic dogs aged 3 to 5 years (Oliveira et al., 2015). Nevertheless, the present *T. caninum* infection rate should be accepted cautiously, due to the low number of infected dogs.

Dermatological changes are frequently reported in *L. infantum* infections in canines (Queiroz et al., 2011; Silva et al., 2017). In this study, the main dermatological signs in dogs that were positive for this agent were alopecia, desquamation, pruritus, skin ulcers, and onychogriphosis. These findings are consistent with those of other authors (Almeida et al., 2009; Solano-Gallego et al., 2009). The occurrence of cutaneous coinfections, mainly fungal are the result of opportunist agents (Gasparetto et al., 2013), but concomitant dermatological diseases can be associated with LVC. Nevertheless, in cases of LVC, many dogs may be asymptomatic (Almeida et al., 2009; Barros et al., 2012), as was observed herein.

The isolation and detection of *T. caninum* DNA exclusively in intact skin has been considered a peculiar aspect of this agent (Madeira et al., 2014). According to Alves et al. (2012), *T. caninum* can provoke an insufficient mild humoral response to cause clinical lesions, however, no significant histopathological changes related to this infection have been described yet. In this study, only one dog positive for *T. caninum* infection presented dermatological abnormalities, characterized by multifocal alopecia, meliceric crusts, and generalized desquamation. Nevertheless, the present results should be interpreted cautiously, since this dog was also positive for dermatophytosis based on fungal culture (Mattei et al., 2014), which affects keratinized tissues (Nweze, 2011), and leads to similar morphological changes as described in the presented dog (Bond, 2010).

The main histopathological change seen in asymptomatic animals with *T. caninum* infection was a mild lymphoplasmacytic inflammatory infiltrate (Figure 1A). However, the significance of this mild infiltrate might be questionable, because lymphocytes and plasma cells in small numbers are also present in the dermis of healthy dogs (Hargis & Ginn, 2007). The finding of acanthosis, epidermal orthokeratotic hyperkeratosis, and presence of melanomacrophages in the dermis of the dog with dermatological changes could not be associated with *T. caninum* infection due to the fungal co-infection with *Microsporum* sp. and *Trichophyton* sp., which produce similar changes (Bond, 2010).

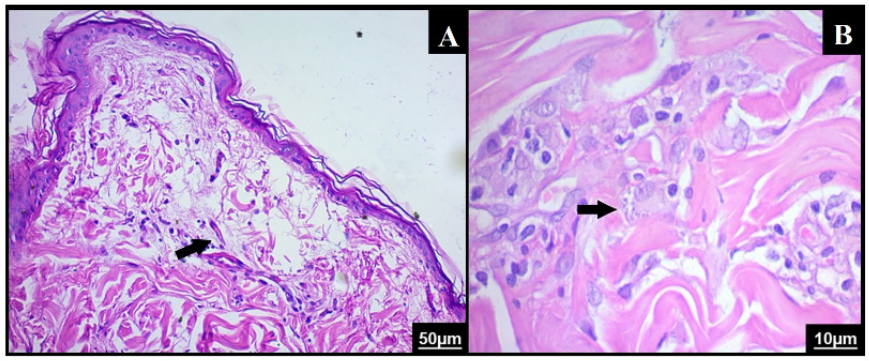

Figure 1. (A) The skin of a dog positive for *Trypanosoma caninum* presenting with lymphohistioplasmacytic perivascular inflammatory infiltrate (arrow). Magnification: 20X (HE); (B) The skin of a dog positive for *Leishmania infantum* with perivascular and perifollicular infiltrate, lymphohistioplasmocitary, and amastigotes of *Leishmania* sp (arrow). Magnification: 60X (HE).

Histopathological changes consistent with dermatitis have been described in CVL (Queiroz et al., 2011). Perivascular and perifollicular changes are the most common, even in absence of the *Leishmania* sp. amastigotes (Figure 1B). However, similar to the present study, chronic dermatitis, manifested by mononuclear cell, including plasma cells, macrophages and lymphocytes, infiltration was also described in dogs with and without CVL, and in those with the presence or absence of amastigotes (Figueiredo et al., 2010). The presence of mild lymphohistioplasmacytic inflammatory infiltrate that is commonly found in healthy tissue was also observed in dogs with leishmaniasis without clinical symptoms as well as in dogs infected with *T. caninum*. However, in animals with visceral leishmaniasis, an asymptomatic infection may persist for years before the development of clinical symptoms (Baneth et al., 2008); whereas, in a *T. caninum* infection, it is not yet known what the incubation period is or if this parasite is capable of producing clinical signs in infected dogs throughout the course of the disease. The presence of more than one inflammatory pattern, mixed inflammatory patterns, or an overlapping of histological patterns may be observed in *L. infantum* infections in dogs (Torres et al., 2008). CVL leads to diverse clinical and histological manifestations and the latter mostly demonstrates mild to marked inflammatory infiltrates, depending on host resistance variations (Calabrese et al., 2010). These findings may change due to cutaneous coinfections and concomitant diseases (Gross et al., 2005), such as dermatophytosis fungal, observed in 64.7% of *L. infantum* positive dogs herein, or hypothyroidism, where the characteristics were more suggestive of atrophic dermatoses than that of parasitic dermatoses.

Conclusion

The presence of nonspecific changes found in the histology of dogs with and without dermatopathies leads us to infer that *T. caninum* may not induce a cutaneous inflammatory reaction. However, more studies are necessary to better characterize the clinical and histological changes induced by this agent.

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