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ORIGINAL PAPER

Identification of *Leishmania* spp. promastigotes in the intestines, ovaries, and salivary glands of *Rhipicephalus sanguineus* actively infesting dogs

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Abstract Sand flies are recognized as the major vector of canine visceral leishmaniasis. However, in some areas of Brazil where sand flies do not occur, this disease is found in humans and dogs. There has been speculation that ticks might play a role in transmission of canine visceral leishmaniasis and the DNA of *Leishmania* spp. has been reported in whole ticks. We investigated the presence of *Leishmania* spp. promastigotes in the intestines, ovaries, and salivary glands of *Rhipicephalus sanguineus* ticks collected from tick-infested dogs in two cities of Brazil. We used 66 dogs that tested positive and 33 that tested negative for *Leishmania* spp. according to direct cytological examination assays. Ten ticks were collected from each dog and dissected to collect the intestines, ovaries, and salivary glands for immunohistochemistry (IHC) and diagnostic real-time polymerase chain reaction (RT-PCR). IHC results showed *Leishmania* spp. in 98, 14, and 8 % of the intestines, ovaries, and salivary glands, respectively. Real-time PCR showed that 89, 41, and 33 % of the tick intestine, ovary, and salivary glands, respectively, were positive for *Leishmania* spp. The verification of promastigotes of *Leishmania* spp. by two independent techniques in ticks collected from these urban region dogs showed that there is need for clarification of the role of ticks in the transmission of canine visceral leishmaniasis in Brazil.

Keywords Ixodids · Leishmaniasis · Canine · Immunohistochemistry · Real-time PCR

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Introduction

The dog is the main urban reservoir of visceral leishmaniasis, a zoonosis of considerable relevance to public health. The causal agent, *Leishmania* spp., is transmitted by the bite of sand flies (World Health Organization 2005), and human health problems of morbidity and mortality from leishmaniasis occur in 98 countries (World Health Organization 2014). Interestingly, Michalsky et al. (2009) reported that some areas in Brazil do not have sand flies, but visceral leishmaniasis occurs in both humans and dogs in these areas. Thus, other ectoparasites, such as fleas and ticks, have been suspected of playing major roles in transmission of leishmaniasis in the sand fly-free regions of Brazil.

The sand fly, *Lutzomyia longipalpis*, is the main vector of *Leishmania infantum* and visceral leishmaniasis in Brazil

(Deane and Deane 1962). However, *Leishmania* spp. has been detected in fleas (Coutinho and Linardi 2007; Ferreira et al. 2009; Colombo et al. 2011) and ticks (Coutinho et al. 2005; Dantas-Torres et al. 2010a; Colombo et al. 2011; Trotta et al. 2012; Solano-Gallego et al. 2012; Morais et al. 2013) removed from dogs infected by leishmaniasis. Generally in these studies, PCR was used to indicate the presence of *Leishmania* spp. in the flea or tick samples. However, the role of these ectoparasites in the transmission or epidemiology of leishmaniasis cannot be definitively assigned solely by whole organism polymerase chain reaction (PCR). The gut of the ectoparasite would harbor remnants of blood directly ingested from feeding upon a leishmania-infected dog and this blood could serve as the direct reason for the PCR-positive result (Coutinho and Linardi 2007; Dantas-Torres et al. 2010a; Paz et al. 2010; Dantas-Torres 2011).

Rhipicephalus sanguineus, the brown dog tick, is a globally distributed ectoparasite which can ingest several types of pathogens while taking its blood meal, including *Ehrlichia*, *Anaplasma*, *Babesia*, *Leishmania*, and *Hepatozoon* (Dantas-Torres 2008). The role of this tick in the transmission of *Leishmania* has not been established despite several reports describing the detection of *Leishmania* DNA in *R. sanguineus* ticks removed from dogs naturally infected with *L. infantum* (Dantas-Torres et al. 2010a; Trotta et al. 2012; Solano-Gallego et al. 2012; Morais et al. 2013). Conversely, the study by Paz et al. (2010) concluded that it was unlikely that *Leishmania* maintenance and multiplication occurs within *R. sanguineus*. Our research group has focused upon the study of the epidemiology and diagnosis of canine visceral leishmaniasis, with an interest in the role nontraditional vectors might have in the biological cycle of this disease (Coelho and Bresciani 2013). We utilized immunohistochemistry (IHC) and PCR to test for the presence and prevalence of *Leishmania* spp. promastigotes in *R. sanguineus* ticks removed from tick-infested dogs in two urban areas of Brazil. Both sand flies and canine visceral leishmaniasis occur in Araçatuba, São Paulo State (SP), and Campo Grande, Mato Grosso do Sul State (MS), and those two cities were the source of infected dogs. Dogs were tested for their infection status regarding *Leishmania* spp. Engorged female ticks were removed from each dog and dissected, such that tick intestines, ovaries, and salivary glands could be individually tested by IHC and RT-PCR. Both the IHC and RT-PCR assays found that a large percentage of tick tissues contained *Leishmania* spp. DNA and/or promastigotes.

Material and methods

Approval by the ethics committee

This study was approved by the Ethics Committee on Animal Use (CEUA) of the School of Agricultural and Veterinary Sciences of UNESP, Universidade Estadual Paulista, Jaboticabal Campus, Process No. 011800/11.

Canine selection and testing

A total of 99 adult dogs, 65 male and 34 female, most of undefined breed and infested with ticks, were used in the study. The aim was to include canine visceral leishmaniasis-positive dogs from two endemic urban areas where each dog was infested by at least 10 ticks. We also included canine visceral leishmaniasis-negative dogs from one urban area not endemic for the disease. Cytological testing (described below) verified the presence or absence of the amastigote stage in the lymph nodes and/or bone marrow of each dog. The data from this testing can be found in Online Resource 1. Of 66 positive animals, 33 came from the Zoonosis Control Centers in the city of Campo Grande, MS, and 33 from the city of Araçatuba, SP, two areas endemic for canine visceral leishmaniasis. All 33 dogs negative for this infection were from the Center for Animal Health Research, CPPAR, UNESP, Jaboticabal, SP, which is generally considered a nonendemic area for canine visceral leishmaniasis (Sakamoto et al. 2007). The popliteal or pre-scapular lymph nodes were sampled using 5-ml syringes and 25 × 7-mm needles such that multiple aspirations were obtained from the same puncture. Bone marrow material was collected from the iliac crest, using 20-ml syringes and size 40 × 12-mm needles. Cytological prints on microscope slides were stained with Fast Panoptic (Laborclin, Pinhais, PR, Brazil) and the direct parasitological examinations for the *Leishmania* amastigote forms performed at ×100 magnification (Troncarelli et al. 2009).

Collections and dissections of ticks

Ten engorged female *R. sanguineus* ticks were collected from each dog, and immediately rinsed in buffered saline solution pH 7.4 for 5 min, in alcohol for 5 min, then air-dried at room temperature on paper towels. All 10 ticks from each dog were pooled together for collection of dissected organ material. Ticks were dissected within 1–2 h after removal from the dog, mounting the ticks on Petri plates with paraffin. The intestines, ovaries, and salivary glands were removed from all 10 ticks and placed into microtubes containing buffered saline solution, one tube for each tissue type. Dissections were carefully performed to prevent cross contamination or rupture of the organs and any ruptured organs were discarded (Edwards et al. 2009; Dantas-Torres et al. 2010a). After dissection, approximately half of the material in each tube was transferred to another microtube containing 1.5 ml of 10 % buffered formalin, pH 7.4 for IHC.

IHC to detect LPG

Tick ovaries, salivary glands, and intestines were fixed in formaldehyde, embedded in paraffin, and subjected to microtomy for the preparation of 3-μm thickness histological sections on silanized slides. The sections were deparaffinized, rehydrated, subjected to antigen recovery under humid heat, and immersed in 10 mM citric acid solution pH 6.0 in a pressure cooker for 3 min similar to the method described in Norton et al. (1994). After cooling, slides were rinsed in H_2O and subjected to endogenous peroxidase blockage by incubation in 20 volumes of 6 % hydrogen peroxide solution, at 37 °C, for 30 min. Final washes in H_2O and phosphate-buffered saline solution (PBS) preceded storage at room temperature prior to IHC.

The sectioned tissues were incubated with mouse monoclonal primary anti-*Leishmania* lipophosphoglycan (LPG) antibody (clone CA7AE raised against *Leishmania donovani* promastigotes, Thermo Fisher Pierce Biotechnology, Rockford, IL, USA) diluted 1:10,000 in 1 % bovine serum albumin in PBS at 4 °C, for 18 h in a humid chamber without shaking. Sections were washed in PBS for 3–5 min at room temperature and the antigen-antibody complex amplified by the addition of a third generation polymer (Shi et al. 1999) tagged with anti-mouse and anti-rabbit immunoglobulins and peroxidase enzyme (Reveal Polyvalent HRP, Spring Biosciences, Pleasanton, CA, USA), using an incubation at 37 °C for 30 min in a humid chamber according to the manufacturer's protocol. Excess unbound polymer was removed by washing for 3–5 min at 37 °C with PBS. Reaction products were visualized by addition of chromogenic substrate 0.1 % diaminobenzidine solution (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 % hydrogen peroxide in PBS and incubating for 3–5 min at 37 °C. Positive signals were evidenced by a golden brown color. Subsequently, slides were extensively washed in H_2O and subjected to light counterstaining with Harris hematoxylin followed by mounting with cover slip and Entellan permanent medium (Merck, Darmstadt, Germany) for microscopy. Negative controls were obtained by omission of the primary antibody.

DNA extraction from tick organs

DNA samples were extracted by protocols similar as reported by Sangioni et al. (2005) and stored at −80 °C. Briefly, the organs were suspended in 150 μL of buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and homogenized with 450 μL of 5 M guanidine thiocyanate. Following extraction with phenol and chloroform, the aqueous phase was recovered and nucleic acids precipitated with 3 M sodium acetate and isopropanol at −80 °C for 18 h. The nucleic acids were recovered by centrifugation, rinsed in 70 % ethanol, and the washed pellet dried at 56 °C in a thermoblock for 15 min with the tube cap opened. The pellet was resuspended in 50 μl of H_2O, assisted by mixing and heating at 65 °C for 15 min in a thermoblock. The DNA was quantified and stored at −80 °C.

Diagnostic RT-PCR

The primers ITS1-F (5′-CCTATTTTACACCAACCCCCAGT-3′) and ITS1-R (5′-GGGTAGGGGCGTTCTGCGAAA-3′) were used with protocols similar as reported in Perosso et al. (2014). The specificity of the primer was tested using the Primer-Blast program of the National Center for Biotechnology Information (Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers amplify the intergenic internal transcribed spacer region of the rRNA gene. RT-PCR was carried out in a Bio-Rad thermocycler (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μl, which consisted of 1.0 μL of each primer (10 pmol), 1.0 μl sample DNA, 7.0 μL H_2O, and 10 μL of the Quantifast Syber Green mix (Qiagen, Valencia, CA, USA). Samples were run in duplicate on a program consisting of 2-min incubation at 50 °C followed by 95 °C for 2 min and then 40 amplification cycles that each consisted of 95 °C for 15 s followed by 60 °C for 30 s. The melt-curve protocol was 95 °C for 15 s, 60 °C for 15 s, followed by a 20-min gradient step from 60 to 95 °C which was maintained for 15 s.

Statistical analysis

For statistical analysis, McNemar's test (Zar 2010) was adopted to compare the proportion of positive results for *Leishmania* among the tick organ samples.

Results and discussion

Prior to designing and conducting the diagnostic RT-PCR assay, analysis of the prospective PCR primers by the Primer-Blast program verified that significant sequence similarity did not exist between the primers and DNA from *Trypanosoma cruzi*, *Babesia* spp., and *Ehrlichia* spp. All tick tissue pools obtained from each of the 33 dogs testing negative for *Leishmania* spp. by cytological testing also tested negative in both the RT-PCR and IHC assays of the dissected tissues (Online Resource 1).

The tick tissue pools of intestines, ovaries, and salivary glands obtained from the 66 dogs that tested positive for *Leishmania* spp. by cytological tests showed varying results in the IHC and RT-PCR assays (Online Resource 1). In this group consisting of all infected canines, positive IHC immunolabeling for *Leishmania* spp. was detected in 98.5, 13.6, and 7.6 % of the 66 pools of intestines, ovaries, and salivary glands, respectively. Figure 1 shows representative photographs of *Leishmania* spp. promastigotes in dissected

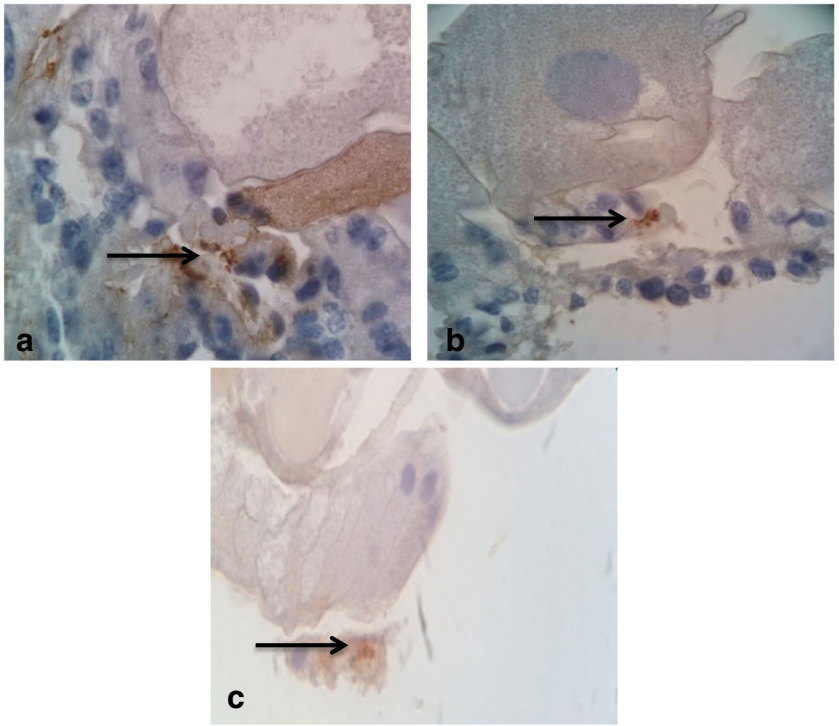

Fig. 1 Photomicrographs representing positive immunolabeling of *Leishmania* spp. promastigotes in organs of *R. sanguineus*-engorged females. **a** Intestine. **b** Ovary. **c** Salivary gland (magnification ×100)

tick tissues. Diagnostic RT-PCR assay results from these pooled tick tissue samples showed that *Leishmania* spp. DNA was detected in 89.4, 40.9, and 33.3 % of the intestines, ovaries, and salivary glands, respectively. Figure 2 graphically compares overall results of the IHC and RT-PCR tissue assays. The RT-PCR gave an approximately fourfold higher percentage of positives than the IHC for both ovary and salivary gland (Fig. 2). In the intestine samples, the IHC detected a slightly higher incidence of positives than the RT-PCR (98.5 vs. 89.4 %). This might be due to carryover of RT-PCR inhibitory substances that occur in blood-containing samples, such as the intestinal materials of our samples. A caveat in our using RT-PCR as a diagnostic assay with dissected tick materials is that cross-contaminating intestinal materials might have affected the ovary and salivary gland sample results. Engorged female ticks contain large amounts of ingested blood and extended intestinal organ that can be damaged and burst during dissections. Although care was taken to prevent tissue damage during dissections, damaged leaking intestinal material could have been the cause of the higher RT-PCR-positive percentages in the ovary and salivary gland samples compared to the IHC.

Table 1 shows the IHC and RT-PCR results according to location. Interestingly, the ovary and salivary gland tissue pools from Campo Grande, MS, showed a greater level of positive results compared to the tissue pools from

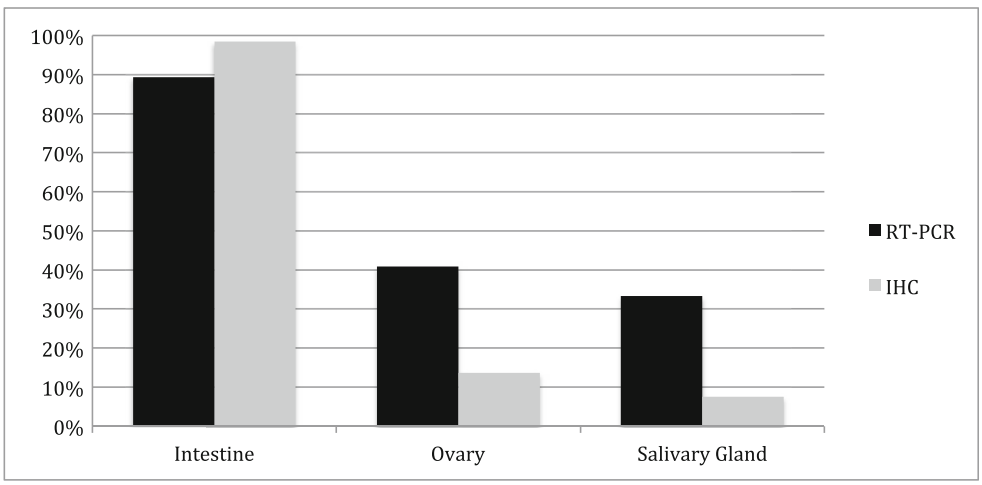

Fig. 2 Comparison of results for the IHC (*gray*) and RT-PCR (*black*) assays of tick intestine, ovary, and salivary gland

Table 1 IHC and RT-PCR assay results according to canine city of origin

City	No. of dogs	RT-PCR			IHC		
		Intestine	Ovary	Salivary gland	Intestine	Ovary	Salivary gland
Campo Grande	33	87.9	51.5	48.5	100	24.2	15.2
Araçatuba	33	90.9	30.3	18.2	97.0	3.0	0
Jaboticabal	33	0	0	0	0	0	0

Araçatuba, SP. This occurred despite both cities showing similar results for the intestine samples. The reason for the higher positive results in the Campo Grande ovary and salivary gland samples is not known. Perhaps an environmental or biological factor differentially influences the developmental process of the *Leishmania* spp. within the canines of the two cities.

Our IHC technique used a primary LPG monoclonal antibody raised against *L. donovani* promastigotes as the molecule to interact with putative *Leishmania* spp. promastigotes in our samples. We selected this diagnostic assay because LPG is known to be present on the surface of the promastigote glycocalyx (Naderer et al. 2004; McConville and Blackwell 1991; Moody et al. 1993; Assis et al. 2012; Forestier et al. 2015). The promastigote form of all members of the genus *Leishmania* is believed to synthesize LPG, and its main glycoconjugate covers the entire parasite surface, including the flagellum (Turco and Descoteaux 1992). There is less known about LPG synthesis in the amastigote stage, although McConville and Blackwell (1991) reported that the amastigotes of *L. donovani* do not appear to synthesize LPG. This uncertainty in the synthesis of LPG by amastigotes limits our findings somewhat. However, Forestier et al. (2015) discuss the observation that the progressive development of promastigotes into amastigotes within a mammalian host is accompanied by a large downregulation of LPG expression. The specificity of our LPG antibody must be investigated further to allow us to unequivocally state that our IHC findings in the tick tissues relate specifically to the promastigote stage. The ability to distinguish between amastigotes and promastigotes is important because promastigotes develop within an arthropod host, whereas amastigotes develop within a mammalian host. Ticks feeding upon infected canines would ingest amastigotes present in the host blood. Assuming that the antibody is specific for the promastigote stage, our detection of promastigotes in tick ovaries and salivary glands provides evidence that *R. sanguineus* ticks can propagate *Leishmania* infections as alternate arthropod hosts feeding upon infected canines.

Detection of the *Leishmania* DNA by diagnostic PCR has been previously described in other Brazil-based studies. Although these studies tested smaller numbers of dogs and ticks compared to our study, Coutinho et al. (2005), Colombo et al. (2011), Morais et al. (2013), and Campos and Costa (2014) detected *Leishmania* DNA in 33, 50, 44, and 23 % of sampled ticks, respectively. None of these studies used dissected ticks nor did they use an independent technique such as IHC to verify the presence of promastigotes in their *Leishmania* spp. RT-PCR-positive samples. Thus, these studies could not assess if the positive results were due to remnants of ingested *Leishmania* DNA in the intestines of the sampled ticks or if the positive results truly represented development of infective promastigotes within the tick. Our study is the first report using biologically relevant tick organ samples dissected from individual ticks to assay for *Leishmania* spp. with both molecular and immunohistological tools. It should be noted that a study of Ixodid ticks sampled from serologically positive dogs from Italy detected *Leishmania* in some tick salivary gland samples (Dantas-Torres et al. 2010a). Also, transovarian dissemination of *Leishmania* was detected by PCR assays performed on eggs from experimentally infected female ticks (Dantas-Torres et al. 2010b).

We expected to detect *Leishmania* spp. on ticks feeding upon infected dogs. The sensitivity of RT-PCR meant extra care was needed in the tick collection and template preparation process. Also, the feeding ticks are taking up infected blood into their intestines. We desired to detect if ticks could play biologically relevant roles in Leishmania transmission in regions where the sand fly vector is absent. By dissecting tick tissues, we found that a significant percentage of tick salivary glands had *Leishmania* spp. DNA and promastigotes (Figs. 1 and 2). Tick susceptibility to infection by *Leishmania chagasi* was proven by using inoculation via peritoneal and oral route in hamsters (Coutinho et al. 2005). This finding has particular importance when we consider that canines can eventually ingest ectoparasites, but this question still needs to be elucidated. To extend our knowledge and confirm the role of *R. sanguineus* as a vector of canine visceral leishmaniasis, it remains necessary to establish the tick's ability to support the growth, multiplication, and transstadial transmission of this protozoon. This could be accomplished using in vivo transmission studies in uninfected canines, using ticks harboring *Leishmania* promastigotes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical standards All experiments were conducted in a manner that complies with the current laws and regulations of Brazil.

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