Acta Tropica 166 (2017) 121–125

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Molecular characterization of *Leishmania infantum* in domestic cats in a region of Brazil endemic for human and canine visceral leishmaniasis

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ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form
26 September 2016
Accepted 11 November 2016
Available online 13 November 2016

Keywords:
Protozoa
Zoonosis
Reservoir host
Feline leishmaniasis
PCR

ABSTRACT

Leishmaniasis is a "neglected tropical disease" and serious public health issue in Brazil. While dogs are recognized as particularly important reservoirs, recent reports of domestic cats infected with *Leishmania* sp. in urban areas suggest their participation in the epidemiological chain of the parasite in endemic areas. The aim of this study was to screen domestic cats for *Leishmania* sp. infection in an area where human and canine visceral leishmaniasis are endemic, followed by the identification of the species circulating in cats. We collected peripheral blood, lymph-node aspirates and bone marrow from 100 adult animals, both male and female, and analyzed the samples using cytological and molecular (PCR) detection techniques. We detected *Leishmania* in 6% of animals, which were then analyzed by RFLP-PCR to identify the species. *Leishmania infantum* (synonym: *L. chagasi*), a species responsible for visceral leishmaniasis in humans and other animals, was identified from all six samples. Amastigotes were observed in the peripheral blood, bone marrow and lymph-node aspirates in 4 of the 6 PCR-positive animals. The presence of infected cats in endemic areas should not be neglected, because it demonstrates the potential role of these animals in the biological cycle of the pathogen.

1. Introduction

Leishmaniasis is one of the world's "neglected tropical diseases" and a major public health problem in Brazil, due to its high incidence and wide geographical distribution (Bern et al., 2008). It is a zoonotic disease caused by protozoans of the genus *Leishmania*, which are transmitted by sandflies (Diptera: Psychodidae: Phlebotominae). While there have been significant advances in treatment, particularly in Brazil, controlling the disease remains a significant challenge and epidemics occur frequently (Harhay et al., 2011; Dantas-Torres et al., 2012).

The city of Campo Grande in mid-western Brazil is an endemic area of epidemiological relevance for human and canine visceral leishmaniasis (VL). Like other urban centers in Brazil, it has seen an increasing number of cases over the past decades (Harhay et al., 2011). The city has the fourth highest number of cases of leishmaniasis in humans in Brazil, with an incidence rate in 2012 of 24.21 cases per 100 000 inhabitants (Brasil, 2016).

Both domestic and wild animals may serve as host reservoirs of *Leishmania* sp. (Brandão-Filho et al., 2003; Quinnel and Courtenay 2009). In particular, the role of dogs in the zoonotic cycle of VL, especially in urban and peri-urban areas, has long been understood

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http://dx.doi.org/10.1016/j.actatropica.2016.11.013
0001-706X/

(Costa, 2008). They are known to be an important reservoir of *Leishmania infantum* (Dantas-Torres, 2007; WHO 2012), the causative agent of human and canine VL.

Recent studies have detected *Leishmania* infections in domestic cats as well (e.g. Savani et al., 2004; Souza et al., 2005, 2009; Bresciani et al., 2010; Vides et al., 2011). Worldwide, five species of *Leishmania* have been reported in cats, although most cases involve *L. infantum* (Pennisi et al., 2013). Prevalence rates may be over 40% in some endemic areas (Chatzis et al., 2014). Although these factors indicate that domestic cats likely play an important role in leishmaniasis epidemiology, their role has been largely neglected and remains controversial; it is unknown whether domestic cats serve as primary, secondary, or accidental hosts (Maia and Campino, 2011; Pennisi et al., 2015).

Feline leishmaniasis is highly prevalent in Brazil, with the highest number of reported cases worldwide (Dantas-Torres et al., 2006). Three different species of *Leishmania* have been described from cats in Brazil: *Leishmania amazonensis* (Souza et al., 2005), *Leishmania braziliensis* (Schubach et al., 2004) and *L. infantum* (Savani et al., 2004; Silva et al., 2008). Incidents of the infection have been observed throughout the country, including the states of Pará (Mello, 1940), Minas Gerais (Passos et al., 1996), São Paulo (Savani et al., 2004), Rio de Janeiro (Schubach et al., 2004; Silva et al., 2008), Mato Grosso do Sul (Souza et al., 2005, 2009) and Pernambuco (Silva et al., 2014).

The records of natural infection in domestic cats and the high population density of cats in urban areas highlight the need to analyze the ability of these animals to sustain and spread the infection in natural and urban environments (Maia and Campino, 2011). The prevalence of infection in endemic areas and the identification of the species of *Leishmania* present in domestic cats are among the key issues requiring attention. Therefore, we surveyed domestic cats for *Leishmania* infection in Campo Grande, Mato Grosso do Sul, evaluated clinical symptoms, and molecularly characterized *Leishmania* species detected, which is the first study to do so in Campo Grande.

2. Materials and methods

2.1. Study population

Samples were collected from 100 randomly chosen, mixed-breed adult domestic cats (43 males and 57 females), one to ten years old, from either veterinary practices (n = 3) or the Zoonosis Control Center of Campo Grande-MS, Brazil (n = 97) from November 2013 to March 2014. Physical examinations of the animals consisted of inspections of general appearance, body condition, mucous-membrane color, nasal and oral cavities, eyes and skin.

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Mato Grosso do Sul under protocol 546/2013.

2.2. Biological samples

Peripheral blood, bone marrow and popliteal lymph-node aspirates were collected following the intravenous anesthesia of the animals using a barbiturate (thiopental) at a dose of 12.5 mg/kg. The samples were examined using parasitological and molecular diagnostic techniques. Three milliliters of bone marrow tissue were obtained from the tibial tuberosity after careful disinfection of the region. Three milliliters of peripheral blood were collected by jugular venipuncture. Aspirates of the popliteal lymph nodes were collected by fine-needle aspiration cytology using a 25 × 7 hypodermic needle (22G × 25 mm) and 5-ml syringes containing 0.5 mL of sterile saline solution. Samples for PCR analysis were placed in tubes containing EDTA and stored at −20 °C until analysis.

2.3. Sample processing for parasitological examination

Thin smears of peripheral blood, bone marrow tissue and lymph-node aspirates were prepared for the direct parasitological diagnosis of parasites. Air-dried smears were prepared in duplicate and subsequently stained with Diff-Quick (Panótico Rápido®, LABORCLIN, São Paulo, Brazil) and were then examined microscopically (1000×) for the presence of amastigotes.

2.4. Molecular diagnosis

2.4.1. DNA extraction

Genomic DNA was extracted from the samples of peripheral blood and bone marrow using a PureLink® Genomic DNA kit (Invitrogen Life Technologies, São Paulo, Brazil) following the manufacturer's instructions. DNA was extracted from the lymph-node samples following a procedure described by Sambrook et al. (1989), with minor modifications. Five hundred microliters of lymph-node aspirate were added to a sterile physiological solution and centrifuged at 13 000 rpm for 5 min, and the supernatant was discarded by inversion. Three hundred microliters of lysis buffer (1 M NaCl, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0) and 200 ul of 10% SDS were added to the pellet, which was subsequently homogenized by vortexing for 3 min; Twenty microliters of proteinase K (20 mg/mL, Invitrogen Life Technologies, São Paulo, Brazil) were added. The mixture was incubated for 1 hour at 65 °C. Afterwards, 400 μL of chloroform was added and the mixture was vortexed until completely homogenized. This product was then centrifuged at 10,000 rpm for 10 min. The supernatant was then transferred to a new tube. One milliliter of 100% ethanol was added and the mixture was homogenized by inversion and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded. One milliliter of 70% ethanol was added to the sediment, which was then centrifuged again for 3 min at 10,000 rpm. This previous step was repeated twice and then the tube was inverted to dry the sediment. Fifty microliters of ultrapure water were added to the tube, which was incubated overnight at 20 °C to dissolve the pellet. The extracted samples were labelled and stored at −20 °C for later use in the PCR reactions.

2.4.2. PCR

All samples were subjected to PCR for the detection of *Leishmania* DNA using two pairs of primers. The first pair was described by Rodgers et al. (1990): 13A (GTG GAG GGG GGG CGT TCT) and 13 B (ATT TTA CAC CAA GTT CCC CCA). The second pair was designed by Degrave et al. (1994): A: (C/G)(C/G)(G/C) CC(C/A) CTA T(T/A)T TAC ACC AAC CCC and B: GGG GAG GGG CGT TCT GCG AA. Both pairs amplified DNA fragments of 120 base pairs (bp) from conserved regions of kinetoplast DNA minicircles.

The PCR solutions for primers 13A and 13 B contained 2× Phoneutria buffer, 0.2 mM dNTPs, 0.3 mM $MgCl_2$, 1 μL of formamide (100%), 0.4 pmol of each primer, 4U of Taq polymerase (Phoneutria, Belo Horizonte, MG, Brazil) and 1 μL of DNA (30–200 ng/μL) in a final volume of 25 μL. The solutions were cycled on a (BIO RAD T100 Thermal cycler) with an initial denaturation step at 95 °C for 7 min, followed by 35 cycles of 95 °C for 30 s, 55.2 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR solutions for primers A and B contained 2× Phoneutria buffer, 0.2 mM dNTPs, 0.3 mM $MgCl_2$, 1 μL of formamide (100%), 10 pmol primers, 4U of Phoneutria Taq polymerase and 1 μL of DNA (30–200 ng/μL) in a final volume of 25 μL. The solutions were cycled on a Biocycler thermal cycler with an initial denaturation step at 94 °C for 4 min,

Table 1
Cytological and PCR results for the tissues collected from the six positive cats (from 100 analyzed) in an area in Brazil where visceral leishmaniasis is endemic.

Animal	Cytological analysis			PCR		
	Peripheral blood	Bone marrow	Lymph-node aspirate	Peripheral blood	Bone marrow	Lymph-node aspirate
40	+	+	+	+	+	–
73	–	–	–	–	+	–
88	–	–	–	–	+	–
96	+	+	+	+	+	+
97	+	+	+	+	+	+
98	+	+	+	+	+	+

followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

The PCR products were electrophoresed in a 2% agarose gel (Amersham Biosciences AB, Uppsala, Sweden) with 1× Tris-borate-EDTA buffer (TBE) pH 8.0 at 100 V and 400 mA for 90 min. The gels were stained using ethidium bromide (0.5 mg/ml) and were visualized in ultraviolet light.

WHO reference strains were provided by the Leishmaniasis Laboratory of the Research Center René Rachou/Fiocruz (Belo Horizonte, Brazil) and were used as positive controls: L. (*Leishmania*) *infantum* (MHOM/BR/74/PP/75), L. (*Viannia*) *braziliensis* (MHOM/BR/75/M2903) and L. (*Leishmania*) *amazonensis* (IPLA/BR/67/PH8).

2.4.3. PCR–RFLP

Positive PCR samples were further analyzed using the primers LITSR (5′-CTGGATCATTTTCCGAT-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′) (El Tai et al., 2000). These primers amplify internal transcribed spacer 1 (ITS1) to produce amplicons between 300 and 350 bp. The PCR solutions contained 1× Invitrogen buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μL of formamide (100%), 0,4 pmol/uL primers, 0,2 u/uL of Phoneutria Taq polymerase and 1 μL of DNA (30–200 ng/μL) in a final volume of 25 μL. The solutions were cycled on a BIO-RAD T100™ thermal cycler with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

The PCR amplicons were analyzed for restriction fragment length polymorphisms (RFLPs) using HaeIII GG▼CC/CC▲GG (Schonian et al., 2003). The RFLP digestion reactions contained 1 μL (10 u/μL) of HaeIII (Promega, Madison, WI, USA) – 10000u, 2 μL of 10× buffer (10 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol), 5 μL of sterile water and 17 μL of the amplicon in a final volume of 25 μL. The reactions were incubated at 37 °C for 3 h. The PCR-RFLP digests were electrophoresed in a 3% high-resolution agarose gel (Sigma-Aldrich, St. Louis, USA) with 1× TBE pH 8.0 at 400 mA and 100 V for 3 h. The gels were stained with ethidium bromide (0.5 mg/ml) and were visualized in ultraviolet light.

3. Results

The cytological examination identified amastigotes of *Leishmania* spp. in four of the 100 animals (4%) in all three tissues (peripheral blood, bone marrow and lymph-node aspirate). The PCR analysis detected two additional positive animals (6% total) (Table 1). The PCR-RFLP analysis identified the species as *L. infantum* (Fig. 1) in the six naturally infected cats.

The physical examination identified clinical signs in 58% of the animals, some of which had more than one clinical sign. The main findings were: weight loss (30/58, 51.7%), skin diseases (24/58, 41.3%), gingivitis/stomatitis complex (10/58, 17.2%), ectoparasites (7/58, 12.0%), eye discharge (6/58, 10.3%), jaundice (5/58, 8.6%), pale mucous membranes (5/58, 8.6%), lymphadenopathy (3/58, 5.1%) nasal discharge (3/58, 5.1%), uveitis (3/58, 5.1%), corneal ulcers (2/58, 3.4%) and diarrhea (2/58, 3.4%).

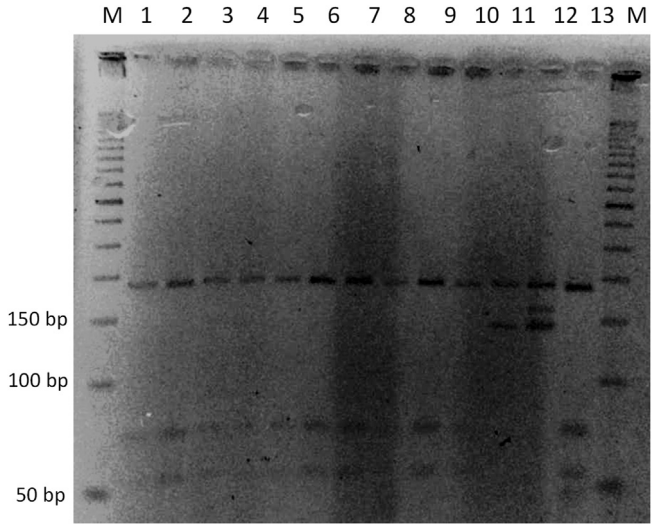

Fig. 1. Agarose gel electrophoresis of the PCR-RFLP analysis of the ITS1 region. Lanes: (M) 50-bp ladder, (1) DNA from peripheral blood of animal 40, (2) DNA from bone marrow of animal 40, (3) DNA from bone marrow of animal 73, (4) DNA from bone marrow of animal 88, (5) DNA from peripheral blood of animal 96, (6) DNA from bone marrow of animal 96, (7) DNA from bone marrow tissue of animal 97, (8) DNA from peripheral blood of animal 98, (9) DNA from bone marrow of animal 98, (10) DNA from lymph node of animal 98, (11) *L.* (*Leishmania*) *amazonensis* control, (12) *L.* (*V.*) *braziliensis* control, (13) *L. infantum* control, (M) 50-bp ladder.

The clinical symptoms in the six animals diagnosed with *L. infantum* were: gingivitis/stomatitis complex (4/6), weight loss (3/6), nasal and eye mucopurulence (1/6), oral ulcers (1/6), flea-allergy dermatitis (1/6), lice infestation (1/6) and alopecia (1/6). Only one of the infected cats had no clinical symptoms.

4. Discussion

The cytological examination had a positivity rate of 4%, while the PCR analysis found an additional two animals which were positive, for a total prevalence rate of 6%. Rates of *Leishmania* prevalence in cats vary widely. The rate of prevalence that we found was higher than that found in other regions of Brazil (Coelho et al., 2011; Vilhena et al., 2013) but lower than some highly endemic regions of southern Europe (Martin-Sanchez et al., 2007; Maia et al., 2008; Chatzis et al., 2014). All cats were infected with *L. infantum* and this is the first record of this species from cats in Mato Grosso do Sul, although other studies have detected *L. amazonensis* in cats in this state (Souza et al., 2005, 2009). *L. infantum* has been identified in Mato Grosso do Sul in humans (Oliveira et al., 2006a; Lima Junior et al., 2009) and dogs (Savani et al., 2005) and causes frequent epidemics throughout Mato Grosso do Sul (Oliveira et al., 2006a).

Leishmania prevalence in dogs in Campo Grande has reached up to 20% in some years (Oliveira et al., 2006b). The lower, but significant prevalence of 6% in cats suggests they may be a secondary reservoir in this area. Further, the presence of amastigotes in multiple tissues supports the idea that cats could be a host reservoir (Maia and Campino, 2011). However, additional studies to compare rates of infection in cats and dogs over the same time periods may further clarify the role of cats in the epidemiological cycle of leishmaniasis in Campo Grande and determine whether they should be considered primary, secondary, or accidental hosts.

The clinical findings observed in our study were similar to those previously reported for cats infected with *L. infantum* (Poli et al., 2002; Vides et al., 2011; Chatzis et al., 2014). However, because the presence of co-infections was not investigated, we cannot confirm that the clinical signs were caused exclusively by the *Leishmania* parasite. In other cases, cats infected with *Leishmania* were also infected with *Toxoplasma gondii*, FIV, and feline leukemia (Miró et al., 2014).

In addition, some symptoms, such as gingivitis/stomatitis complex and weight loss occurred both in infected and uninfected cats. Other symptoms, such as nasal and eye mucopurulence, oral ulcers, flea-allergy dermatitis, lice infestation and alopecia were found exclusively in infected cats, but only 1/6 (11%) of animals testing positive exhibited those symptoms. Finally, one cat positive for *L. infantum* in this study showed no clinical signs, consistent with a previous study in cats experimentally infected with a Brazilian strain of *L. infantum* (Kirkpatrick et al., 1984). Such cases of asymptomatic infection and survival of infected animals could further point to their ability to play a role as a potential reservoir and maintain the parasite in the region (Maia and Campino, 2011).

Although leishmaniasis is usually not suspected in cats, our results show that it is necessary for veterinary professionals working in endemic areas to thoroughly examine their feline subjects, be aware of the possibility of *Leishmania* infection in cats, and screen animals that exhibit potential symptoms. In many cases, diagnosis occurs accidentally when performing laboratory tests for other diseases. Monitoring for leishmaniasis in all host animals is necessary to effectively control the spread and transmission of the disease. Cytological examinations may thus be adopted as an initial screen for leishmaniasis due to the high specificity, speed, low cost and ease. Supplementary tests using molecular techniques should be performed in cases of low sensitivity under certain conditions, especially in negative but symptomatic animals (Maia and Campino, 2011).

Many control efforts, such as culling of infected dogs and insecticide campaigns have had limited effects on preventing leishmaniasis outbreaks in Brazil (Dantas-Torres et al., 2012). This may point to the importance of other important host reservoirs, such as cats, that require further investigation. Since culling infected animals has had limited success, strategies for controlling *Leishmania* infection in both cats and dogs should employ a wider range of interventions, such as sensitive screenings of domestic animals, insecticide spraying, and insecticide animal collars (Dantas-Torres et al., 2012). This study is an important step in determining what role domestic cats may play in the epidemiology of leishmaniasis in Campo Grande and lead to more effective monitoring and control efforts for all hosts of *L. infantum*.

Acknowledgements

We thank Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior (Capes) for the MSc scholarship to I.P. Metzdorf and the National Science Foundation Graduate Research Fellowship for support of J.T. Shapiro (DGE-1315138). We also thank the Leishmaniasis Laboratory of the Research Center René Rachou/Fiocruz (Belo Horizonte, Brazil) for kindly providing WHO reference strains.

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