MÔNICA RAQUEL SBEGHEN

AVALIAÇÃO DA INFECÇÃO POR *Paracoccidioides brasiliensis* E *Leishmania* sp. EM ANIMAIS SILVESTRES DE PEQUENO PORTE

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, para obtenção do título de Doutor em Patologia Experimental.

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"A compaixão pelos animais está intimamente ligada à bondade de caráter, sendo que se pode afirmar que quem é cruel com os animais não pode ser um bom homem"

(Arthur Schopenhaeur)

SBEGHEN, Mônica Raquel. **Avaliação da infecção por *Paracoccidioides brasiliensis* e *Leishmania* sp. em animais silvestres de pequeno porte.** 2015. 53 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina. 2015

RESUMO

A paracoccidioidomicose (PCM) é a principal micose sistêmica prevalente na América Latina, causada pelos fungos termo-dimórficos *Paracoccidioides brasiliensis* e *P. lutzii*. Considerando que o habitat destes fungos na natureza permanece indefinido, animais silvestres podem ser úteis como indicadores da presença de *Paracoccidioides* sp. no ambiente. Outra doença endêmica no estado do Paraná é a leishmaniose tegumentar americana (LTA). A LTA é uma doença parasitária causada por protozoários do gênero *Leishmania* e os animais silvestres podem ser hospedeiros e reservatórios biológicos destes patógenos. O objetivo deste estudo foi avaliar a infecção de mamíferos silvestres de pequeno porte por *P. brasiliensis* e *Leishmania* sp. em uma área endêmica para PCM e LTA. Os mamíferos silvestres (n=38) foram capturados por meio de armadilhas, em uma Reserva Nacional do Patrimônio Natural (RPPN) no município de Mauá da Serra**-**PR e a infecção por *P. brasiliensis* e *Leishmania* sp. foi avaliada por meio de métodos sorológicos (ELISA e imunodifusão), reação em cadeia da polimerase (*Nested*-PCR), cultura e exame histopatológico. Os animais utilizados para investigação da infecção por *P. brasiliensis* e *Leishmania* sp. foram: *Akodon* sp (n=12), *Thaptomys nigrita* (n=8), *Euryoryzomys russatus* (n=7), *Oligorizomys nigripes* (n=3), *Monodelphis* sp (n=3), *Sooretamys angouya* (n=2), *Abrawayaomys angouya* (n=1), *Abrawayaomys ruschii* (n=1) e *Akodontinae* sp (n=1). Além destes animais, um tatu atropelado *Dasypus novemcinctus* foi avaliado quanto à infecção por *Leishmania* sp. A análise das amostras de soro por ELISA demonstrou uma positividade de 23.7% (n=9) para gp43 de *P. brasiliensis* e 36,8% (n=14) para antígeno bruto de *Leishmania* sp. Amostras de coração e fígado de um *O. nigripes* apresentaram positividade para *P. brasiliensis* na PCR e o animal também apresentou positividade para o fungo no ELISA. O exame histopatológico, a imunodifusão e a cultura foram negativos *P. brasiliensis*. Amostras de tecidos de *Dasypus novemcinctus* (baço), *Akodon* sp. (fígado) e de *Thaptomys nigrita* (baço) apresentaram positividade para *Leishmania* sp. na PCR e estes mesmos animais também apresentaram positividade para *Leishmania* sp. no teste de ELISA. Estes dados sugerem que mamíferos silvestres de pequeno porte são reservatórios de *P. brasiliensis* e *Leishmania* sp..

Palavras-chave: Paracoccidioidomicose. Leishmaniose. PCR. Epidemiologia.

SBEGHEN, Mônica Raquel. **Evaluation of infection by *Paracoccidioides brasiliensis* and *Leishmania* sp. in small wild animals.** 2015. 53 p. Thesis (Doctoral Degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America, caused by the termo dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*. Taking into account that the habitat of these fungi is undefined, wild animals may be useful as indicators of the presence of *Paracoccidioides* sp. in the environment. Another endemic disease in the state of Paraná is the American Cutaneous Leishmaniasis (ACL). The ACL is a parasitic disease caused by protozoans of the genus *Leishmania*. Wild animals can be host and biological reservoirs of these pathogens. The aim of this study was to evaluate the infection of small wild mammals by *P. brasiliensis* and *Leishmania* sp. in an endemic area for PCM and ACL. The wild mammals (n=38) were captured by traps, in a National Natural Heritage Reserve (RPPN) in Mauá da Serra-PR and the infection by *P. brasiliensis* and *Leishmania* sp. was evaluated by serological methods (ELISA and immunodiffusion), polymerase chain reaction (Nested-PCR), culture and histopathological exam. The animals used for evaluation of infection by *P. brasiliensis* and *Leishmania* sp. were *Akodon* sp (n = 12), *Thaptomys nigrita* (n = 8), *Euryoryzomys russatus* (n = 7), *Oligorizomys nigripes* (n = 3), *Monodelphis* sp (n = 3), *Sooretamys angouya* (n = 2) *Abrawayaomys angouya* (n = 1), *Abrawayaomys ruschii* (n = 1) and *Akodontinae* sp (n = 1). Additionally, a road killed armadillo *Dasypus novemcintus* was evaluated for infection by *Leishmania* sp.. The analysis of serum samples by ELISA showed a positivity of 23.7% (n=9) for *P. brasiliensis* gp43 and 36.8% (n=14) for crude antigen of *Leishmania* sp. Tissue samples from one *O. nigripes* (heart and liver) were positive for *P. brasiliensis* in PCR and the animal was also seropositive for gp43 in ELISA. Histopathology, immunodiffusion and culture were negative for *P. brasiliensis*. Tissues samples from *Dasypus novemcintus* (spleen), *Akodon* sp. (liver) and *Thaptomys nigrita* (spleen) were PCR positive for *Leishmania* sp., and these animals were also seropositive for *Leishmania* sp. by ELISA. These data suggest that small wild mammals are reservoirs of *Paracoccidioides* and *Leishmania* sp.

Keywords: Paracoccidioidomycosis. Leishmaniasis. PCR. Epidemiology.

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1 INTRODUÇÃO

Os fungos *P. brasiliensis* e *Paracoccidioides lutzii* são os agentes causadores da paracoccidioidomicose (PCM) (Theodoro et al; 2012). Estes microrganismos apresentam-se na forma de micélio em temperaturas abaixo de 28°C, e na forma de levedura em temperaturas em torno de 37°C (San-Blas, 1993; Lacaz et al., 1991). Esta mudança na morfologia ocorre devido às alterações bioquímicas e fisiológicas estimuladas pela variação na temperatura (Bastos et al., 2007). O habitat de *Paracoccidioides* sp. na natureza ainda não está estabelecido, embora se saiba que estes fungos se adaptam facilmente a solos úmidos, com pH ácido, e rico em material orgânico (Restrepo et al., 1985; Theodoro et al; 2012).

A PCM apresenta alta prevalência na América Latina (Franco et al., 1987) e no Brasil, onde se encontram a maioria dos casos da doença (80%) (Lacaz et al., 1991; Brummer et al., 1993). A maioria dos indivíduos infectados pelo *P. brasiliensis* permanecem assintomáticos (Franco et al., 1987).

A infecção em seres humanos provavelmente ocorre devido à inalação de propágulos que ao chegarem ao alvéolo pulmonar convertem para a forma de levedura, e desenvolvem o foco inflamatório (Brummer et al., 1993; Camargo et al., 2000), no entanto o desenvolvimento da PCM doença irá depender da resposta imune do hospedeiro (Franco et al., 1987).

Esta micose pode se apresentar na forma aguda, com o desenvolvimento mais rápido; sendo a forma mais grave da doença e com pior prognóstico para o

paciente, pois pode levar o indivíduo a óbito. A forma crônica é responsável por 90% dos casos da PCM, com desenvolvimento mais lento. Em geral o órgão mais atingido nesta forma são os pulmões, podendo, também, acometer diversos órgãos como baço, fígado, mucosas nasal e oral (Franco et al., 1987).

Os animais domésticos e silvestres podem ser utilizados como marcadores epidemiológicos da PCM e contribuir em elucidar do habitat de *Paracoccidioides* sp.. A infecção pelo *P*. *brasiliensis* tem sido demonstrado em animais selvagens e domésticos, como cães (Ono et al, 2001; Ricci, et al 2004; Farias et al.2005), cavalos (Conti-Diaz et al, 1972, Corte et al, 2009), vacas (Gutierrez et al, 1974; Silveira et al, 2008), ovinos e caprinos (Oliveira et al, 2011; Costa et al. 1978), galinhas (Oliveira et al de 2011), macacos, (Costa et al, 1995; Johnson, et al 1977, Corte et al, 2007.) e tatus (Naiff, 1986; Bagagli, 1998; Silva - Vergara, 2000) e morcegos (Grose, 1965).

Outra doença endêmica no Brasil e no estado do Paraná é a leishmaniose tegumentar americana (LTA) (Gontijo et al, 2003). A LTA é uma zoonose que ocorre nas Américas desde o sul dos Estados Unidos até o norte da Argentina (WHO, 2010). No Brasil, esta doença encontra-se em todos os estados brasileiros, possivelmente devido à invasão dos vetores no ambiente peridoméstico (Laison et al., 1987). Na região sul do Brasil, em que se encontram 3% dos casos da doença no país, há destaque para o estado do Paraná onde há o maior registro de casos, sendo mais de 95% daqueles da região sul do país (Ministério da Saúde, 2010).

A importância clínica da LTA decorre da alta incidência e das graves manifestações clínicas (lesões destrutivas, desfigurantes e incapacitantes) conseqüentes da doença (Gontijo e Carvalho, 2003). A LTA pode apresentar-se na forma cutânea (mais freqüente e com lesões restritas a pele), cutâneo-mucosa

(envolvimento das mucosas e nasofaringe) (Gontijo e Carvalho, 2003; WHO, 2010), disseminada (presença abundante de úlceras cutâneas) e difusa (lesões nodulares não ulcerativas).

Esta parasitose é transmitida aos seres humanos (e aos demais hospedeiros vertebrados) pela picada de insetos do gênero *Phlebotomus* sp, e *Lutzomyia* sp, infectados com promastigotas de *Leishmania* sp . O ciclo biológico do parasita envolve duas formas distintas, a promastigota (vida extracelular), presente no sistema digestivo do vetor e amastigotas (intracelular) do sistema imune do hospedeiro vertebrado, que inclui o ser humano, animais domésticos e silvestres (Gontijo & Carvalho, 2003).

A leishmaniose, assim como a PCM é endêmica no Brasil e em regiões demográficas semelhantes, como o estado do Paraná, além de possivelmente possuir vários reservatórios ambientais em animais silvestres e domésticos. Uma vez que os animais silvestres estão constantemente expostos a organismos patogênicos eles podem ser considerados os melhores indicadores ambientais de risco humano e de animais domésticos. Esta abordagem, que já foi aplicada em outros estudos, se torna útil para auxiliar a elucidar a eco-epidemiologia da PCM, bem como melhorar a compreensão dos reservatórios ambientais da leishmaniose (Richini-Perreira, 2008; Richini- Perreira, 2014).

2 OBJETIVOS

2.1 Geral

Detectar a presença de *P.brasiliensis* e *Leishmania* sp. em mamíferos de pequeno porte por *P. brasiliensis* e *Leishmania* sp, por meio de métodos imunológicos, de biologia molecular e histopatológicos.

2.2 Específicos

Analisar e diagnosticar a infecção de mamíferos silvestres de pequeno porte por *P. brasiliensis* e *Leishmania* sp. por ELISA e com o uso de imunodifusão radial dupla para *P.brasiliensis*.

Detectar a infecção de mamíferos silvestres de pequeno porte por *P. brasiliensis* e *Leishmania* sp. por meio da Reação em Cadeia da Polimerase. e por meio de exame histopatológico.

Isolar *Paracoccidioides* sp. por meio de cultura, a partir de tecido de mamíferos silvestres de pequeno porte.

Paracoccidioides brasiliensis infection in small wild mammals

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Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis prevalent in Brazil and other Latin American countries. The etiological agents of PCM are the thermo-dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*. Taking into account that the natural habitat of *Paracoccidioides* spp. is still undefined, domestic and wild animals could be useful as indicators of *Paracoccidioides* spp. presence in endemic areas. The objective of this study was to evaluate the infection of small wild mammals by *P. brasiliensis* in an endemic area for human paracoccidioidomycosis. Samples from 38 wild mammals from different species such as *Akodon* sp, *Thaptomys nigrita*, *Euryoryzomys russatus*, *Oligoryzomys nigripes*, *Monodelphis* sp., *Sooretamys angouya*, *Abrawayaomys angouya*, *Abrawayaomys ruschii* and *Akodontinae* sp. were evaluated by ELISA, immunodiffusion, histopathology, Nested-PCR and culture. The overall positivity to gp43 observed in the ELISA was 23.7 %. Samples from heart and liver of one *O. nigripes* were PCR positive and the animal was also seropositive to gp43 in ELISA. This study showed that wild animals living in endemic areas for PCM are infected with *P. brasiliensis* and can be valuable epidemiological markers of the fungus presence in the environment. This is the first evidence of paracoccidioidomycosis infection in *Akodon* sp., *E. russatus*, *T. nigrita* and *O. nigripes*.

Key Words: Paracoccidioidomycosis, ecoepidemiology, wild animals.

INTRODUCTION

Paracoccidioidomycosis is a prevalent systemic mycosis in Brazil and other Latin Americas countries [1]. The etiological agents of paracoccidioidomycosis are the thermo-dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii* [2].

The infection is probably acquired by inhalating infective propagules present in the environment [3]. Paracoccidioidomycosis can be classified as paracoccidioidomycosis infection (infected individuals living in paracoccidioidomycosis endemic areas without symptoms of disease) and paracoccidioidomycosis disease (patients with clinical symptoms) [4].

Taking into account that the habitat of *Paracoccidioides* spp. in nature is still undefined, domestic and wild animals could be useful as indicators of *Paracoccidioides* spp. presence in endemic areas. Infection by *P. brasiliensis* has been reported in domestic and wild animals such as dogs [5,6,7], horses in Uruguay and in Brazil [8,9], cows in Colombia and Brazil [10,11], sheep, goats and chickens in Brazil [12,13,14] monkeys [15] and armadillos in Brazil [16]. *P. brasiliensis* isolates were obtained from soil samples in Argentina [17], Venezuela [18] and Brazil [19]. The fungus was also isolated several times from armadillos *Dasypus novemcinctus* in Brazil and Colombia [20,22] and two cases of natural disease in dogs were described in Brazil [23,24].

Terrestrial wild animals live in close contact with the soil, the probable habitat of *Paracoccidioides* spp. and may be good epidemiological markers of paracoccidioidomycosis. Epidemiological studies of paracoccidioidomycosis in animals are based mainly on detecting antibodies against the fungus. Serological methods cannot confirm the presence of the pathogen in the host, but only previous contact. On the other hand, molecular methods, such as PCR, can detect the pathogen in biological samples. PCR reactions with primers from the rDNA genomic

region are considered very sensitive because they usually target a multicopy gene [25]. The use of specific primers with Nested-PCR may increase the specificity of *P. brasiliensis* detection [26].

The objective of this study was to evaluate the infection of small wild mammals by *P. brasiliensis* in an endemic area for paracoccidioidomycosis using immunological and molecular methods.

MATERIALS AND METHODS

Study area and animals

The wild animals were captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná State, Brazil, an endemic area for human paracoccidioidomycosis. The altitude is 1089.8 m and the climate is humid subtropical mesothermal, with relative humidity 72 %, not very warm summers, and cold winters with frequent frosts with average annual temperature of 17.4 ^{o}C and average annual rainfall of 1560 mm. Sherman traps, Tomahawk and pitfall type were used to capture small non-flying mammals. The animals were captured monthly, from March to December 2012 and at each sample phase traps were arranged in transects over three consecutive nights with daily reviews. The traps were placed in the soil (70%) and undergrowth (30%) 10 m apart. The animals were euthanized by exsanguination after anesthesia with ketamine (60 mg/kg) and xylazine (16 mg/kg) and samples from liver, spleen, lungs, kidneys and heart were collected and processed for DNA extraction at necropsy or preserved at -80 °C. This study was developed after receiving authorization from the Brazilian Ministry of Environment

(No. 30025-1), Paraná State Protection Agency (No. 370.12) and Animal Ethics Committee of the State University of Londrina (No. 2597.2012.97).

ELISA for detection of antibodies against gp43

Polystyrene flat-bottom microtiter plates were coated with purified gp43 [6] in 0.1 M carbonate buffer, pH 9.6 (250 ng well^{-1}). The plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-T) and blocked with PBS-T 5% skim milk (PBS-T-M). After washing with PBS-T, the serum samples were diluted 1:100 in PBS 1% skim milk (PBS-T-M) and incubated at 37 °C for 1 h. The plates were washed and incubated at 37 °C for 1 h with protein A-peroxidase conjugate. After washing with PBS-T a solution of substrate/chromogen (H_2O_2/tetramethylbenzidine) was added to each well, and the reaction was stopped with 4 N H_2SO_4. Absorbance was measured with an ELISA reader at 450 nm and the serum samples were considered positive with two and half times the absorbance of the well without serum.

Agar gel immunodiffusion

The serum samples were analyzed by immunodiffusion test as described previously using *P. brasiliensis* exoantigen as reagent [27]. The serum samples were added at peripheral orifices and the exoantigen at the central orifice. A serum sample from a human patient with paracoccidioidomycosis was used as a positive control.

Histopathology

Fragments of liver, spleen, lungs, kidneys and heart were fixed in 10% neutral formalin for 24 hours and embedded in paraffin. Serial sections (5 μm thick) were

prepared and stained by the hematoxylin-eosin (HE) and Gomori-Grocott methods. The analyses were performed in duplicate.

PCR Analysis

The DNA was extracted from tissue samples (liver, spleen, lungs, kidneys and heart) according to Corredor [22]. The primers and PCR conditions are listed in table 1. DNA from *P. brasiliensis* B-339 and ultrapure water were used as positive and negative control, respectively. The Nested-PCR amplicons were purified with ammonium acetate and the sequencing reactions were carried out by Big Dye Terminator Sequencing Kits. The sequences were compared to the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Attempt to Isolate Paracoccidioides sp. from tissue samples

Fragments of tissues (liver, spleen and lung) were incubated at 35 °C for six weeks in Sabouraud and Mycobiotic Agar medium.

RESULTS

Animal capture

Thirty-eight small mammals were captured and identified as *Akodon* sp (n=12), *Thaptomys nigrita* (n=8), *Euryoryzomys russatus* (n=7), *Oligoryzomys nigripes* (n=3), *Monodelphis* sp (n=3), *Sooretamys angouya* (n=2), *Abrawayaomys angouya* (n=1), *Abrawayaomys ruschii* (n=1) and *Akodontinae* sp. (n=1) (Table 2).

Detection of antibodies against P. brasiliensis in serum samples of wild mammals by ELISA

A positivity of 23.7 % (n=9) was observed in ELISA with *P. brasiliensis* gp43 (Table 3).

Immunodiffusion

No serum sample from wild animals showed positivity by the immunodiffusion test.

Attempt to isolate* Paracoccidioides spp. *from tissue samples

No fungus cells with morphological characteristics of *Paracoccidioides spp.* were detected in liver, spleen, and lung tissue fragments cultured in Sabouraud and Mycobiotic Agar.

Histopathology

No cells similar to *Paracoccidioides* spp. were detected in tissue samples (spleen, lung, liver, kidney and heart) from the animals submitted to histopathological examination.

Detection of* Paracoccidioides *spp. by Nested PCR in tissue samples

Amplicons of *Paracoccidioides* sp. were detected by Nested-PCR in tissue fragments from the heart and liver from one *O. nigripes* (Figure 1).

The molecular identities of amplicons were confirmed by direct double strand sequencing which showed 99% similarity with *P. brasiliensis* DNA sequences deposited in the Gen Bank (Figure 2).

DISCUSSION

Epidemiological studies of paracoccidioidomycosis in domestic and wild animals have been based mainly on immunological reactions using crude paracoccidioidin and purified gp43 as antigens [5,16, 32].

Previously our research group showed high rates of gp43 ELISA positivity in dogs [6], horses [9], sheep [12], goats [13], pigs [29], rabbits [30] and wild monkeys [15] from Paraná State, Brazil. The present study reinforces that ELISA is a useful method for detection of *P. brasiliensis* infection in animals.

Wild armadillos that live in burrows underground are considered natural reservoirs of *P. brasiliensis* [16] and natural disease was reported in armadillos (*Dasypus novemcinctus*) captured in an area where the fungus was isolated from soil [31]. An epidemiological study with several species of wild mammals from a Brazilian Zoo showed higher positivity to paracoccidioidin skin test in terrestrial animals (82.98%) than arboreal animals (22.45%), reinforcing that soil is the habitat of *P. brasiliensis* [32]. A seroepidemiological study evaluated *P. brasiliensis* infection in several wild animal species from the State of Rio Grande do Sul, Brazil, and showed positivity in animals from the orders Artiodactyla, Rodentia, Xenarthra, Carnivora, Marsupialia and Primata [33].

Other wild animal species living in endemic areas for paracoccidioidomycosis are potential hosts and consequently valuable epidemiological markers.

PCR methods may be valuable tools to detect *Paracoccidioides* spp. infection in epidemiological studies. Infection by *P. brasiliensis* was detected in road-killed animals by Nested-PCR in tissue samples from armadillos, guinea pigs, porcupines, raccoons and grisons in Botucatu, an endemic area for paracoccidioidomycosis in State of São Paulo, Brazil [26].

Although infection by *P. brasiliensis* has been demonstrated in several wild animal species [15,16,26,31-33], this is the first evidence of paracoccidioidomycosis in *Akodon sp.*, *E. russatus*, *T. nigrita* and *O. nigripes*. The negativity observed in histophatological examination, imunodiffusion and culture probably is due to the lower sensitivity of these methods. The *O. nigripes* positive by ELISA with gp43 also showed PCR positive results in the liver and heart, suggesting a disseminated infection.

There are several reports in the literature indicating that small wild rodents can be reservoirs of human pathogens. *Akodon* sp, *Euryoryzomys russatus* and *O. nigripes* were reported with *Leishmania* (Viania) *braziliensis* [34] and other parasites such as *Hymenolepis* sp., *Longistriata* sp., *Strongyloides* sp., *Trychomonas* sp., *Ancilostomatidae*, *Trichuridae*, *Oxyuridae* [35].

This study showed that wild mammals living in endemic areas for paracoccidioidomycosis are infected with *P. brasiliensis* and consequently, are potential new hosts and good sentinels for paracoccidioidomycosis.

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Figure captions

Figure 1. Analysis of PCR products by polyacrylamide gel electrophoresis with silver nitrate staining. Ladder (1), Positive Control (2), *O. nigripes* liver (3), *O. nigripes* heart (4), and negative control (5).

Figure 2. Direct sequencing of the PCR product. The specificity of the *P. brasiliensis* detection was confirmed by sequencing by Sanger methods using Big Dye. The resulting sequences were analyzed by comparison with the GenBank database.

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Figure 1

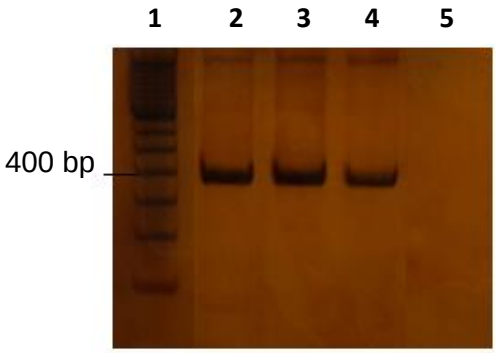

Figure 2

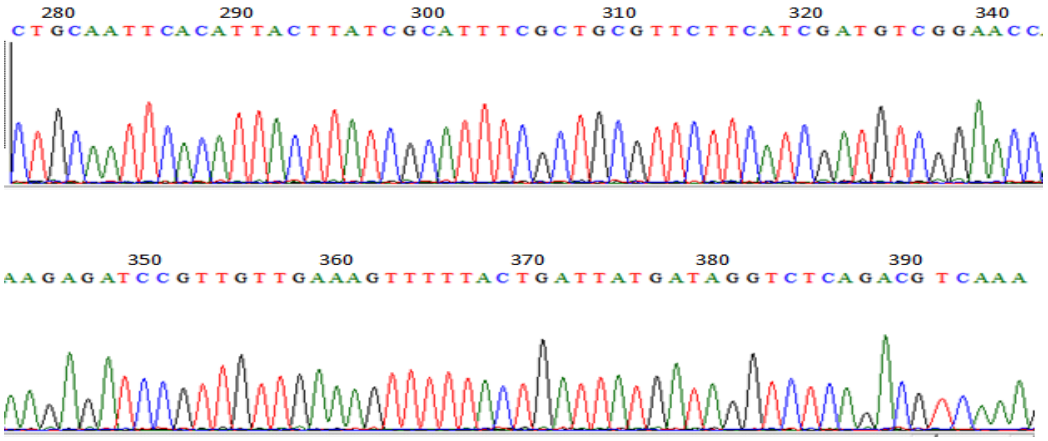

Table 1. Primers, genetic sequence, amplicons size and annealing temperature.

Primer	Genetic Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature
ITS-4	TCCTCCGCTTATTGATATGC	634	60°C
ITS-5	GGAAGTAAAAGTCGTAACAACG	634	60°C
PB-ITS-E	GAGCTTTGACGTCTGAGACC	387	62°C
PB-ITS-R	AAGGGTGTCGATCGAGAGAG	387	62°C

Table 2. Wild animals captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná, Brazil.

Animal Species	n (%)
Akodon sp.	12 (31.6)
Thaptomys nigrita	8 (21.1)
Euryoryzomys russatus	7 (18.4)
Oligoryzomys nigripes	3 (7.9)
Monodelphis sp.	3 (7.9)
Sooretamys angouya	2 (5.3)
Abrawayaomys angouya	1 (2.6)
Abrawayaomys ruschii	1 (2.6)
Akodontinae sp.	1 (2.6)
Total	**38 (100)**

Table 3. Detection of antibodies against gp43 protein by ELISA in sera from wild animals captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná, Brazil.

Animal Species	**Positive n(%)**	**Negative n(%)**
Akodon sp.	6 (50.0)	6 (50.00)
Euryoryzomys russatus	1 (14.3)	6 (85.7)
Thaptomys nigrita	1 (12.5)	7 (87.5)
Oligoryzomys nigripes	1 (33.3)	2 (66.7)
Monodelphis sp.	0 (0)	3 (100)
Abrawayaomys angouya	0 (0)	1 (100)
Abrawayaomys ruschii	0 (0)	1(100)
Sooretamys angouya	0 (0)	2 (100)
Akodontinae sp.	0 (0)	1 (100)
Total	**9 (23.7)**	**29 (76.3)**

Infection of small wild mammals by Leishmania sp.

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Abstract

American cutaneous leishmaniasis (ACL) is a zoonotic disease that occurs from the southern United States to northern Argentina. The disease is transmitted to humans by sandflies *Phlebotomus* sp. and *Lutzomyia* sp. infected with promastigotes of *Leishmania* sp. Wild animals can be valuable epidemiological markers for the presence of *Leishmania* sp. in endemic areas. The objective of this study was to evaluate the infection of small wild mammals by *Leishmania* sp. in an endemic area for ACL. *Leishmania* sp. infection was evaluated by ELISA, histopatology and PCR in samples collected from 39 wild mammals such as *Akodon* sp. (n=12), *Thaptomys nigrita* (n=8), *Euryoryzomys russatus* (n=7), *Oligorizomys nigripes* (n=3), *Monodelphis* sp (n=3), *Sooretamys angouya* (n=2), *Abrawayaomys angouya* (n=1), *Abrawayaomys ruschii* (n=1), *Akodontinae* sp. (n=1) and *Dasypus novemcinctus* (n=1). The overall positivity observed in ELISA was 36.8% (n=14) and positive PCR results were observed in tissue samples from one armadillo *D. novemcinctus* (spleen), one *T. nigrita* (spleen) and one *Akodon* sp. (liver). The positive PCR animals also showed positive ELISA results. These data suggest that these animal species are reservoirs of *Leishmania* sp..

Key Words: Leishmaniasis, epidemiology, wild animals.

INTRODUCTION

American cutaneous leishmaniasis (ACL) is a zoonotic disease that occurs from the southern United States to northern Argentina. ACL has clinical importance due to the high incidence and severe clinical manifestations [1].

The ACL can present two forms, the skin (most frequent form of disease with skin lesion) and mucocutaneous (partial or total destruction of the mucous membranes surrounding the region of nasopharynx) [1, 2]. The ACL may be disseminated with skin ulcers and diffuse characterized by nodular lesions without the presence of ulceration [1].

ACL is transmitted to humans by sandflies *Phlebotomus* sp. and *Lutzomyia* sp. infected with promastigotes of *Leishmania* sp [3]. In Brazil, 634,914 cases of ACL were recorded during 1980 to 2006, and 13,762 occurred in Paraná State [4].

Taking into account that wild animals can be valuable epidemiological markers for the presence of *Leishmania* sp. in endemic areas, the objective of this study was to evaluate the infection of small wild mammals by *Leishmania* sp. in an endemic area for ACL.

MATERIALS AND METHODS

Study area and animals

The wild animals were captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná State, an endemic area for ACL. The altitude is 1089.8 m and the climate is humid subtropical mesothermal, with relative humidity 72 %, little hot summers, and cold winters with frequent frosts with average annual temperature of 17.4 ^{o}C and average annual rainfall of 1560 mm.

Sherman traps, Tomahawk and pitfall type were used for the capture of small mammals. Animals were captured monthly, from March to December 2012 and each sample phase traps were arranged in transects over three consecutive nights with daily reviews. The traps were placed in the soil (70%) and undergrowth (30%) with a distance of 10 m between them. To euthanasia were used 80mg/kg de cetamina + 30mg/kg de xilazina and matherial were preserved at -80^{o}C. The animal organs (liver, spleen, lungs, kidneys and heart) were collected and processed for DNA extraction at necropsy or preserved at 80ºC. This study was developed after receiving authorization from the Instituto Ambiental do Paraná (IAP) n. 370.12 and Ministério do Meio Ambiente (MMA) n.29957-2.

ELISA for *Leishmania* sp crude antigen

Polystyrene flat-bottom microtiter plates were coated with crude antigen of *Leishmania* sp. in 0.1 M carbonate buffer, pH 9.6. The crude antigen supernatant fraction was prepared by sonication and freezing-thawing of *L. amazonensis* (1.0 x

10^7 promastigotes). The plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-T) and blocked with PBS-T 5% skim milk (PBS-T-M). After washing with PBS-T, the serum samples were diluted 1:100 in PBS 1% skim milk (PBS-T-M) and incubated at 37°C for 1 h. The plates were washed and incubated at 37 °C for 1 h with protein A-peroxidase conjugate. After washing with PBS-T the solution of substrate/chromogen (H_2O_2/tetramethylbenzidine) was added to each well, and the reaction was stopped with 4 N H_2SO_4. The negative control were water. Absorbance was measured with an ELISA reader at 450 nm and serum samples with two and half times the absorbance of the well without serum were considered positive.

Histopathology

Fragments of liver, spleen, lungs, kidneys and heart liver, kidney, heart, were fixed in 10% neutral formalin during 24 hours and embedded in paraffin. Serial sections (5 µm thick) were prepared and stained by hematoxylin-eosin method.

PCR Analysis

The DNA was extracted from tissue samples (liver, spleen, lungs, kidneys and heart) according to Corredor [5]. The molecular detection was performed by PCR reactions, using the primers MP3-H (5-GAACGGGGTTTCTGTATGC-3) e MP1-L (5-TACTCCCCGACATGCCTCTG-3), annealing temperature of 56ºC, and these primers produce a fragment of 90bp [6]. DNA from *Leishmania amazonensis* and ultrapure water were used as positive and negative control, respectively.

Silver Nitrate Revelation

For amplicons visualization were used the Silver Nitrate methodology. After electrophoresis, the gel was fixed for 20 '(10ml ethanol, 5ml acetic acid, q.s.p. 100ml), stained by a 15' (10% silver nitrate) and revealed (13g of sodium hydroxide, 10 ml formaldehyde PA , 100ml q.s.p.) to visualize the bands. The reaction was stopped to begin displaying the bands with 5% acetic acid.

RESULTS

Animal capture

The captured animals were identified as *Akodon* sp. (n=12), *Thaptomys nigrita* (n=8), *Euryoryzomys russatus* (n=7), *Oligorizomys nigripes* (n=3), *Monodelphis* sp (n=3), *Sooretamys angouya* (n=2), *Abrawayaomys angouya* (n=1), *Abrawayaomys ruschii* (n=1) and *Akodontinae* sp. (n=1) and *Dasypus novemcinctus* (n=1) (Table 1).

Detection of antibodies against Leishmania sp in serum samples of wild mammals by ELISA

The overall serum positivity observed in the ELISA with *Leishmania amazonensis* crude antigen was 36.8% (n=14) (Table 2).

Histopathology

No *Leishmania* amastigote was observed in the tissue samples (spleen, lung, liver, kidney and heart) from captured animals.

DNA amplification

The specificity of the MP3-H e MP1-L primers were successfully tested against a panel of DNA samples from *Leishmania* sp. The PCR products with these primers resulted in amplified 90bp. With respected to animal samples, predictive specific amplicons of *Leishmania* sp were detected by PCR reactions in tissue fragments from spleen in *Dasypus novemcinctus* (AM 1), liver in *Akodon* sp (AM2) and spleen in *Thaptomys nigrita* (AM3) To control positive were used DNA by *Leishmania amazonensis* and to negative control were used Ultra Pure Water. Figure 1.

DISCUSSION

Reports about leishmaniasis in domestic and wild animals had been based mainly on immunological methods. Animals living in areas of hot and humid climates next to sandflies show high rates of infection [7]. Paraná state is an endemic area for ACL [8] and high rates of infection by *Leishmania* sp were reported in sandflies living near the shelter of small wild animals [9, 10, 11].

Other research [12] observed that several wild rodents and marsupials showed positive results in PCR for leishmaniasis (*Leishmania (Viania)*) (*Nectomys*

squamipes, *Rattus rattus*, *Bolomys lasiurus*, *Holochilus scieurus*, *Akodon arviculoides*, *Marmosa* sp., *Didelphis albiventris*) and Santiago (2007) [13] showed antibodies against *Leishmania* sp in 71% *Didelphis* spp..

Infection by *Leishmania* sp. in domestic and wild animals has been detected by immunological and molecular methods [14,15,16,17,18]. In this study, 36.8% of 39 animals showed antibodies against *Leishmania*, however only three animals (*D. novemcinctus*, *Akodon* sp., *T. nigrita*) also showed positive PCR results [13,19].

Wild animals such as armadillos, that live in areas with a lot of organic matter near the ground and frequent contact with shadflies, can be natural reservoirs of *Leishmania* sp. [20,21].

Richini-Pereira (2014) [18]detected DNA by *Leishmania* ssp in liver, heart, mesenteric lymph node, Kidney, spleen and lung in wild animals in São Paulo Stade (Brazil), as 41.67% in *Cerdocyon thous*, 33,3% in *Procyon cancrivorus*, 25% in *Didelphis* sp, indicating that wild animals, even at climate humid and subtropical may have had infected with *Leishmania* sp. The same authors found DNA of *Leishmania* in 22.9% of the animals. However, our study presents the first evidence of natural infection by *Leishmania* sp. in *T. nigrita*.

Wild animals are difficult to capture, hindering the diagnosis of leishmaniasis in these animals that are possibly reservoirs of *Leishmania* sp.. The use of molecular methods for detection of *Leishmania* sp in tissue samples from animals is important for the study of ecology and epidemiology of leishmaniasis. In this study the species *Dasypus novemcinctus*, *Akodon* sp. and *Thaptomys nigrita* showed positive PCR for *Leishmania* sp., in liver, liver and spleen, respectively,

confirming the infection. The failure in detection of *Leishmania* sp. in the histopathological exam probably is due to its lower sensitivity.

This study showed the importance of use of different methods for diagnosis of infection by *Leishmania* sp*,* because the PCR positive animals also were positive in ELISA, but not all seropositive animals showed positive PCR results. PCR is a high sensitivity method and rarely is false positive, although cross-reactions with other tripanossomatides may occur in immunological methods.

There are several reports in the literature indicating that wild rodents can be reservoirs for human pathogens as *Leishmania* sp [7, 12,18,22,23].

These data demonstrate that the wild animals living in the forest in endemic areas of leishmaniasis are infected with *Leishmania* sp and acting as reservoirs of this pathogen [20,21].

Our study is the first evidence of natural infection by *Leishmania* sp. in *T. nigrita* and verificated the species *Dasypus novemcinctus*, *Akodon* sp. and *Thaptomys nigrita* were positive to PCR for *Leishmania* sp., in tissues of wild animals. Several wild animal species may be acting as reservoirs of *Leishmania* sp in endemic area for leishmaniasis and immunological and molecular methods are valuable tools for investigating the infection in new animal species.

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Figure captions

Figure 1. Map showing the municipality of Mauá da Serra, in Paraná State, Brazil.

Figure 2. Analysis of PCR products for *Leishmania* sp. Ladder (Sigma, Canadá), Positive control (C+), spleen of *Dasypus novemcinctus* (AM1), liver of *Akodon* sp (AM2), spleen of *Thaptomys nigrita* (AM3), Negative Control (C-) with Ultra Pure Water.

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Figure 1.

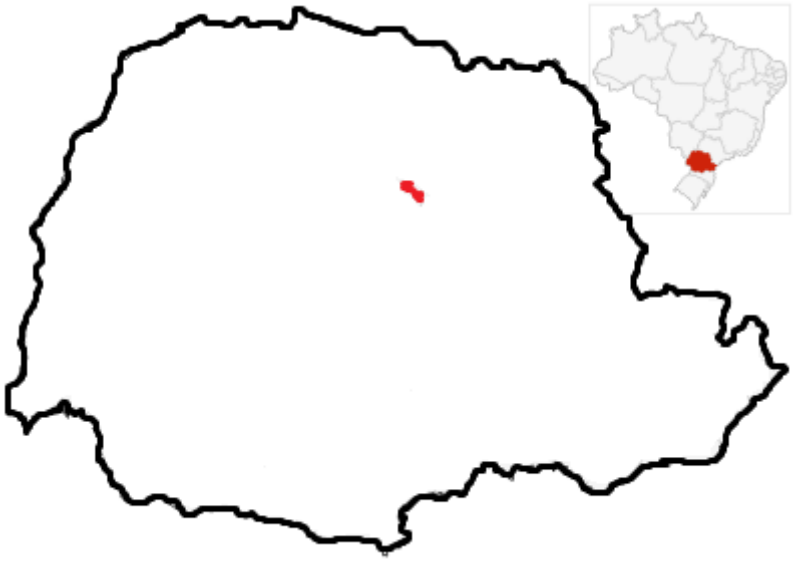

Figure 2.

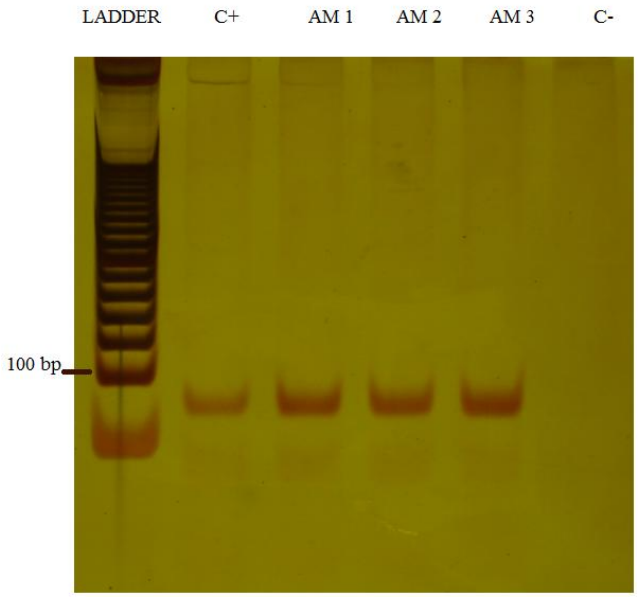

Table 1. Wild animals captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná.

Animal Species	**Number**	**Percentage (%)**
Akodon sp.	12	30.8
Thaptomys nigrita	8	20.5
Euryoryzomys russatus	7	17.9
Oligoryzomys nigripes	3	7.7
Monodelphis sp.	3	7.7
Sooretamys angouya	2	5.1
Abrawayaomys angouya	1	2.6
Abrawayaomys ruschii	1	2.6
Akodontinae sp.	1	2.6
Dasypus novemcinctus	1	2.6
Total	39	100

Table 2. Detection of antibodies against *Leishmania amazonensis* crude antigen by ELISA in sera from wild animals captured in a Private Nature Reserve located in the municipality of Mauá da Serra, Paraná.

Animal Species	**Positive n(%)**	**Negative n(%)**
Akodon sp.	5 (41.7)	7 (58.3)
Euryoryzomys russatus	3 (42.8)	4 (57.2)
Thaptomys nigrita	2 (25.0)	6 (75.0)
Oligoryzomys nigripes	2 (66.6)	1 (33.3)
Monodelphis sp.	0 (0)	3 (100)
Abrawayaomys angouya	0 (0)	1 (100)
Abrawayaomys ruschii	0 (0)	1 (100)
Dasypus novemcinctus	-	-
Sooretamys angouya	1 (50)	1 (50)
Akodontinae sp.	1 (100)	0 (0)
Total	**14 (36.8)**	**24 (63.2)**

(-): Not done.

CONCLUSÃO FINAL

Conclui-se neste trabalho que os mamíferos silvestres *Akodon sp., E. russatus, T. nigrita* and *O.nigripes*. podem se infectar por *P.brasiliensis* e serem considerados indicadores epidemiológicos deste fungo no ambiente; assim como as espécies *Akodon* sp, *E. russatus, T. nigrita, O.nigripes, Sooretamys angouya, Akodontinae* sp. foram positivos para *Leishmania* sp. no teste de ELISA, podendo ser infectados e atuar como marcadores epidemiológicos deste parasito.

Este trabalho apresentou os primeiros relatos da literatura incluindo:

- A primeira PCR positiva para *Leishmania* sp em *T. nigrita*.
- A primeira PCR positiva em *O.nigripis* para *P. brasiliensis*
- O primeiro relato de *P.brasiliensis* no coração de animais naturalmente infectados.
- Primeira sorologia positiva para *P.brasiliensis* em *Akodon* sp. , *E.russatus* e *T. nigrita*;
- Primeira evidência de infecção natural de *Thaptomys nigrita* por *Leishmania* sp.

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