Leishmania infection in bats from a non-endemic region of Leishmaniasis in Brazil

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SUMMARY

Leishmaniasis is a complex of zoonotic diseases caused by parasites of the genus *Leishmania*, which can develop in domestic as well as wild animals and humans throughout the world. Currently, this disease is spreading in rural and urban areas of non-endemic regions in Brazil. Recently, bats have gained epidemiological significance in leishmaniasis due to its close relationship with human settlements. In this study, we investigated the presence of *Leishmania* spp. DNA in blood samples from 448 bats belonging to four families representing 20 species that were captured in the Triangulo Mineiro and Alto Paranaiba areas of Minas Gerais State (non-endemic areas for leishmaniasis), Brazil. *Leishmania* spp. DNA was detected in 8·0% of the blood samples, 41·6% of which were *Leishmania infantum*, 38·9% *Leishmania amazonensis* and 19·4% *Leishmania braziliensis*. No positive correlation was found between *Leishmania* spp. and bat food source. The species with more infection rates were the insectivorous bats *Eumops perotis*; 22·2% (4/18) of which tested positive for *Leishmania* DNA. The presence of *Leishmania* in the bat blood samples, as observed in this study, represents epidemiological importance due to the absence of Leishmaniasis cases in the region.

Key words: Bats, *Leishmania infantum*, *Leishmania amazonensis*, *Leishmania braziliensis*, CytB, HSP70, Brazil.

INTRODUCTION

The leishmaniases are considered zoonoses where humans are accidental hosts. Natural vertebrate hosts include mammals of the Cingulata, Pilosa, Carnivora, Rodentia, Primate, Marsupialia and Perissodactyla orders. The primary hosts are wild mammals, such as rodents and canids (Ashford, 1996). Globally, more than 30 species of trypanosomes have been recorded in over a hundred of species of bats (Molyneux, 1991). In the Americas, families of the Phyllostomidae, Vespertilionidae and Molossidae are the most commonly infected bats (Brener and Andrade, 1979).

Despite its close relationship with trypanosomatids, the role of bats in the biological cycle of *Leishmania* has receiving increasing attention. However, some species of sand flies can live in the same locations inhabited by these mammals, such as caves, crevices, houses or abandoned buildings. In these environments, bats are a food source for insects (Lampo *et al*. 2000) and vice versa. Some studies have reported direct (Mutinga, 1975) and indirect evidence (Morsy *et al*. 1987) that bats have been infected by *Leishmania* spp. The only report of parasites from the genus *Leishmania* isolated from bats was observed with the growth of *Leishmania infantum* (syn. *L. chagasi*) in a blood culture from the bat species *Carollia perspicillata* (De Lima *et al*. 2008). The presence of *L. infantum*, *L. amazonensis* and *L. braziliensis* DNA in bats was already demonstrated in studies conducted in Brazil (Savani *et al*. 2010).

Diverse natural *Leishmania* infection in bats has been reported around the World (Mutinga, 1975; Rajendran *et al*. 1985; Morsy *et al*. 1987; Rotureau *et al*. 2006; De Lima *et al*. 2008; Savani *et al*. 2010; Shapiro *et al*. 2013; Millan *et al*. 2014*a*; Berzunza-Cruz *et al*. 2015). These findings suggest their possible epidemiological incrimination in the transmission cycle of *Leishmania*. The aim of this study was to investigate the presence of *Leishmania* DNA

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in blood samples of bats captured in a region considered non-endemic for Leishmaniasis in Brazil (Triângulo Mineiro and Alto Paranaiba regions of Minas Gerais).

METHODS

Study area and capture of bats

Bats were captured in a study conducted previously in different rural regions of the Triangulo Mineiro (Agua Comprida S21°54′0″, W45°40′0″; Conceição das Alagoas S19°54′51″, W48°23′11″; Conquista S19°56′20″, W47°32′38″; Uberaba S19°45′27, W47°55′36″; Uberlandia S18°54′41″, W48°15′44″) and Alto Paranaiba (Araxá S19°35′36″, W46°56′ 27″; Nova Ponte S19°9′46″, W47°40′42″ and Perdizes S19°21′19″, W47°16′58″) (Bento, 2006; Bento, 2013). These regions are areas of 'cerrado' (Brazilian savanna) (Ab'sáber, 1971; Alho, 1993; Rizzini, 1997). Climate in most of the study area is classified as Aw (Tropical savannah climate, with summer rains) with two defined seasons (dry and rainy). The rains occur most often in the hottest season from October to March, when the average minimum temperature is higher than 18 °C. The dry season is between April and September. The annual precipitation varies between 1400 and 17 000 mm, and is concentrated between December and February (Motta, 1993). These regions were classified as non-endemic areas by the time bat were captured. The captures occurred during the night, between approximately 18 and 24 h, using mist nets, dip nets or manually. The taxonomic classification of the captured bats was conducted using keys for the family (Gregorin, 2002), genus and species (Vizotto and Taddei , 1973; Reis *et al*. 2007).

Blood collection

From each of the 448 bats captured, 0·5–1·0 mL of blood was collected by cardiac puncture and blood samples were preserved in guanidine-EDTA solution V/V (6 M Guanidine–HCl and 0·2 M disodium EDTA, pH 8·0) and stored at 4 °C.

DNA extraction and polymerase chain reaction (PCR)

The DNA extraction from blood was performed using the phenol–chloroform method (Gomes *et al*. 1998). In order to amplify *Leishmania* DNA, we employed primers Jw11 (5′-CCTATTTTACACC AACCCCCAGT-3′) and Jw12 (5′-GGGTAGGG GCGTTCTGCGAAA-3′) targeting the *Leishmania* kDNA region (Nicolas *et al*. 2002). The electrophoresis was performed in agarose gel 1% stained with GelRed™ 1× (Biotium, USA) and visualized in UV transiluminator.

The positives sample for kDNA was performed PCR of the partial region of the cytochrome b gene (marker encoded on maxicircles), it was amplified in a total volume of 50 µL containing 1× of buffer, 100 mM of dNTPs, 50 pM of each primer, 1·25 U of GoTaq Polymerase (Promega) and 10 ng of DNA. The PCR products were precipitated with 70% and sequenced by the dideoxy-terminal method in an automated capillary sequencer (AB3730, Applied Biosystems) by both strands in ACTGene Analises Moleculares (Brazil). The sequences were submitted to BLASTn for similarity search with *Leishmania* sequences deposited on the databases. Since Cytb marker is not able to discriminate *L*. *braziliensis* from *L*. *peruviana* species, the positive samples were subsequently typed by the direct sequencing of HSP70 as recommended elsewhere (Hernandez *et al*. 2014; Ramirez and Galetti, 2015).

Identification of Leishmania species

The resulting sequences were edited in MEGA 5·0 and aligned using ClustalW 1·8 with sequences from *L*. *donovani donovani* (AB095957), *L*. *donovani infantum* (AB095958), *L*. *donovani chagasi* (AB095959), *L*. *tropica* (AB095960), *L*. *major* (AB095961), *L*. *aethiopica* (AB095962), *L*. *mexicana mexicana* (AB095963), *L*. *amazonensis* (AB095964), *L*. *garnhami* (AB095965), *L*. *braziliensis* (AB095966), *L*. *panamensis* (AB095968), *L*. *guyanensis* (AB095969), *L*. *equatoriensis* (AB43 4687), *L*. *pifanoi* (EF579907), *L*. *lainsoni* (AB433280), *L*. *colombiensis* (KF302738) and *L*. *peruviana* (AB4 33282) retrieved from GeneBank as suggested by different studies (Luyo-Acero *et al*. 2004). A maximum composite likelihood (MCL) analysis using a Tamura-3 parameter was run in MEGA 7·0. To evaluate the robustness of the nodes in the resulting phylogenetic tree, 1000 bootstrap replicates were performed. All sequences obtained in this work were deposit in the Genbank (MF344824–MF344895).

Statistical analysis

The Chi-square (χ^2) test was used to determine possible associations between variables (feeding habits and county capture) and positive PCR results for *Leishmania* sp. using Statistica software 10·0 (Statsoft, Tulsa, OK, 2011), with a significance level of 5% ($P < 0{\cdot}05$).

Ethical considerations

This study was approved by the Ethics Committee for Use of Animals (CEUA) of the Federal University of Triângulo Mineiro, protocol n. 51 and authorized by the Brazilian Environmental Institute (IBAMA) under licenses: 076/2003, 121/2004, 143/06-NUFAS/MG and 13206-1.

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Table 1. Bat species captured in Minas Gerais, Brazil by family, feeding habits and county origin

Family	Species	Feeding habit	Mubicipality	Bats captured by municipality	Total (%)
Molossidae	*Eumops perotis*	Insectivorous	Agua Comprida	18	18 (4·02%)
	Molossops neglectus	Insectivorous	Conquista	2	2 (0·45%)
	Molossops temminckii	Insectivorous	Uberlândia	1	1 (0·22%)
	Molossus molossus	Insectivorous	Uberaba	61	61 (13·62%)
	Nyctinomops laticaudatus	Insectivorous	Araxá	1	2 (0·45%)
			Uberaba	1	
	Tadarida brasiliensis	Insectivorous	Uberaba	1	1 (0·22%)
Noctilionidae	*Noctilio albiventris*	Insectivorous	Conceição das Alagoas	1	1 (0·22%)
Phyllostomidae	*Phyllostomus discolor*	Omnivorous	Uberlândia	2	2 (0·45%)
	Phyllostomus hastatus	Omnivorous	Perdizes	5	34 (7·59%)
			Uberaba	29	
	Anoura caudifera	Nectarivorous	Agua Comprida	2	16 (3·57%)
			Conquista	1	
			Perdizes	13	
	Glossophaga soricina	Nectarivorous	Agua Comprida	19	213 (47·5%)
			Araxá	10	
			Conquista	76	
			Nova Ponte	16	
			Perdizes	25	
			Uberaba	67	
	Carollia perspicillata	Frugivorous	Agua Comprida	8	35 (7·8%)
			Conquista	8	
			Perdizes	19	
	Artibeus obscurus	Frugivorous	Perdizes	3	3 (0·67%)
	Artibeus planirostris	Frugivorous	Perdizes	5	5 (1·12%)
	Artibeus cinereus	Frugivorous	Uberlândia	2	2 (0·45%)
	Artibeus planirostris	Frugivorous	Agua Comprida	1	1 (0·22%)
	Platyrrhinus lineatus	Frugivorous	Agua Comprida	2	11 (2·45%)
			Conquista	4	
			Perdizes	4	
			Uberlândia	1	
	Sturnira lilium	Frugivorous	Agua Comprida	4	8 (1·78%)
			Perdizes	4	
	Desmodus rotundus	Hematofagous	Agua Comprida	15	31 (6·91%)
			Perdizes	6	
			Uberaba	1	
			Uberlândia	9	
Vespertilionidae	*Eptesicus furinalis*	Frugivorous	Agua Comprida	1	1 (0·22%)
Total				448	

RESULTS

Leishmania infection

Blood samples of 448 bats representing 20 different species captured in eight districts of Triângulo Mineiro and Alto Paranaiba, MG, Brazil (Table 1) were analysed. In five out of the eight studied municipalities, 36 bats were positive for *Leishmania* spp. The highest frequency of bats infected with *Leishmania* was in Água Comprida (11·4%), followed by Conquista (9·9%), Uberaba (9·4%), Araxá (9·1%) and Perdizes (3·6%). Regarding the bat's alimentary habit, the highest percentage bats that tested positive of *Leishmania* spp. DNA were insectivores (12·6%), followed by omnivores (8·3%), nectarivores (7·5%), frugivorous (6·1%) and haematophagous (3·2%). However, there was no significant association between feeding habits and presence of *Leishmania* sp. ($P = 0{\cdot}416$) or the municipality of origin ($P = 0{\cdot}431$).

Identification of Leishmania *species*

Eight out of the 20 species of bats captured were positive for *Leishmania* spp. (40·0%). The species with multiple representative samples that had the highest rate of *Leishmaina* presence was *Eumops perotis* (22·2%), followed by *Platyrrhinus lineatus* (18·2%), *Artibeus planirostris* (16·7%), *Molossus molossus* (11·5%), *Phyllostomus hastatus* (8·8%), *Glossophagas soricina* (8·0%) and *Desmodus rotundus* (3·2%) (Table 2). Additionally, only one species of *Eptesicus furinalis* was captured, and it was found to positive for *Leishmania* spp. (Table 2).

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Table 2. Positivity for *Leishmania* species in bats captured in Minas Gerais, Brazil

Bat species	Positive for *Leishmania* sp.	*Leishmania* species		
		L. infantum	*L. amazonensis*	*L. braziliensis*
Artibeus planirostris	1/6 (16·7%)	1/36 (2·8%)		
Desmodus rotundus	1/31 (3·2%)			1/36 (2·8%)
Eptesicus furinalis	1/1 (100·0%)	1/36 (2·8%)		
Eumops perotis	4/18 (22·2%)	2/36 (5·5%)	1/36 (2·8%)	1/36 (2·8%)
Glossophaga soricina	17/212 (8·0%)	6/36 (16·6)	9/36 (25·0%)	2/36 (5·5%)
Molossus molossus	7/61 (11·5%)	3/36 (8·3%)	1/36 (2·8%)	3/36 (8·3%)
Phyllostomus hastatus	3/34 (8·8%)	2/36 (5·5%)	1/36 (2·8%)	
Platyrrhinus lineatus	2/11 (18·2%)		2/36 (5·5%)	
Total	36/448 (8·0%)	15/36 (41·6%)	14/36 (38·9%)	7/36 (19·4%)

Leishmania species identified in the infected bats were: *L. infatum* in 41·6%, *L. amazonensis* in 38·9% *L. braziliensis* in 19·4%, respectively (Table 2). These results were supported by the maximum-likelihood (ML) phylogenetic tree constructed based on Cytb marker (Fig. 1).

DISCUSSION

Leishmaniases comprise a set of pathologies caused by different species of the genus *Leishmania*. This infection is considered an anthropozoonosis where more than 100 mammals have been naturally infected with these parasites. Among those mammal hosts, bats arise as some of the most important putative reservoirs of some *Leishmania* species. Herein, we investigated the presence of *Leishmania* DNA in bat's blood samples collected in areas of Triângulo Mineiro and Alto Paranaíba from Brazil that are considered non-endemic for leishmaniasis. We observed 8·0% of positive samples for *Leishmania* infection. Diverse studies have reported natural infection of *Leishmania* in bats suggesting a relevant epidemiological role in the transmission cycle of leishmaniasis (Rotureau *et al*. 2006; Savani *et al*. 2010; Shapiro *et al*. 2013; Millan *et al*. 2014*b*; Berzunza-Cruz *et al*. 2015; de Rezende *et al*. 2017). Also, the fact of finding *Leishmania* infection in a geographical region that is not considered endemic for leishmaniasis shows the relevant role that bats play for the dispersal of distinct pathogens as *Leishmania*. The ecology of these mammals highlights their putative importance as reservoirs of infectious diseases and zoonotic pathogens due to their high ability of mobility, broad distribution and social behaviour (communal roosting and fission–fusion social structure). Among these pathogens emerge rabies, severe acute respiratory syndrome (SARS), henipavirus, possibly *Ebola* and distinct *Trypanosoma* species (Halpin *et al*. 2000; Guyatt *et al*. 2003; Li *et al*. 2005; Hamilton *et al*. 2012*a*,*b*).

Frugivores, insectivorous, omnivores and vampire bats were positive for the presence of *Leishmania* DNA, but no significant association was found between the bat-feeding preference and parasite infection rates (Brener and Andrade, 1979; Marinkellei, 1982) as observed in our study. The identification of *Leishmania* hosts is crucial to determine the natural transmission cycle of this parasite and to understand the epidemiology of the disease (Brandao-Filho *et al*. 2003). Some authors consider animals as potential reservoirs when the infection rate is above 20%, while lower percentages indicate that the animal is an accidental host (Berzunza-Cruz *et al*. 2015). On the other hand, studies define a reservoir as one or more epidemiologically connected populations or environments in which the parasite can be permanently maintained and from which infection is transmitted to the defined target population (Haydon *et al*. 2002; Thompson and Etter, 2015). In this pragmatic scenario, bats from our study would not be considered strict reservoirs. This is clearly depicted to the fact that the area where bats were captured is not considered endemic for leishmaniasis. However, the presence of *Leishmania* DNA implies that the circulation of parasites could provoke future likely emergent outbreaks. In the light of our data, these findings represent relevant information in order to consider prevention and control strategies in the region.

The biggest limitation in the understanding of the ecology of leishmaniasis is the lack of sensitive markers for the identification and discrimination of *Leishmania* species. Traditionally, microscopic examination is considered the diagnostic routine method for identification of *Leishmania*. However, more sensitive methods such as the PCR have been developed as an alternative for the diagnosis and identification of *Leishmania* species. PCR platforms show sensitivity values between 92 and 100% and specificity of 100% (Shahbazi *et al*. 2008; Mohammadiha *et al*. 2013; Adams *et al*. 2014). For the amplification of DNA fragments of *Leishmania* species, the use of genetic targets such as kinetoplast DNA (kDNA), which has a sensitivity of 97% and a specificity of 87%, has been reported in several studies (Marques *et al*. 2001; Rodriguez *et al*. 2002). The internal heat shock protein 70 kDa (HSP70) also reflects a sensitivity of 95% and a specificity of 100% (Garcia *et al*.

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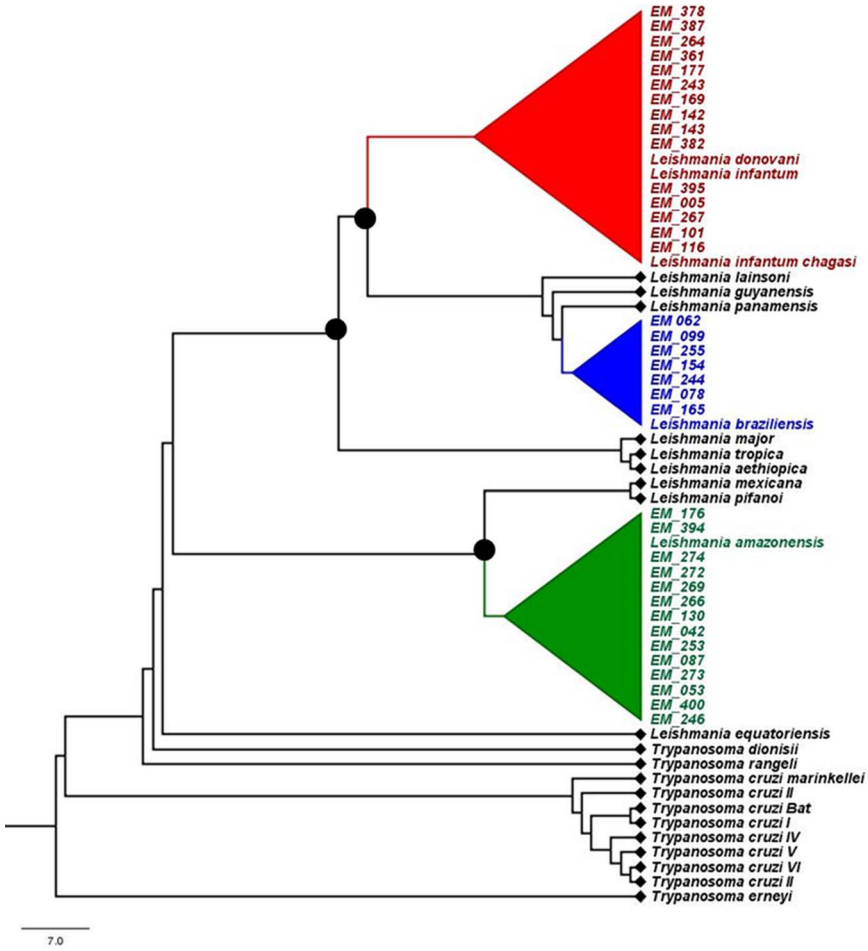

Fig. 1. Molecular phylogenetic analysis by the maximum-likelihood (ML) method. The evolutionary history was inferred by using the ML method based on the Tamura–Nei model. The tree with the highest log likelihood (−2763,0697) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 61 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 405 positions in the final dataset. Black dots indicate bootstrap values over 80%.

2007; Montalvo *et al*. 2014) and the transcribed internal spacer (ITS-1) with 40 and 96%, respectively (Marfurt *et al*. 2003; Kumar *et al*. 2007; Ovalle Bracho *et al*. 2007) and finally the small 18S ribosomal subunit with a similar behaviour as kDNA in terms of sensitivity and specificity (Adams *et al*. 2014). These are the most commonly used markers for the identification of *Leishmania* DNA by PCR and real-time PCR. Regarding the use of molecular markers used to discriminate in mammals, Cytb and 18S have been widely used in mammals (De Lima *et al*. 2008; Pinto *et al*. 2015). Herein, we employed kDNA to detect *Leishmania* infection and subsequently amplified and sequenced Cytb. All samples positive by kDNA amplified by Cytb. kDNA represents around the 10% of *Leishmania* genome and Cytb marker is located in the maxicircle molecule with dozens of copies (Liu *et al*. 2005). The number of copies suggests, Cytb is a very sensitive marker to conduct *Leishmania* species discrimination in mammals. This marker has been used in other trypanosomes with relevant results in terms of sensitivity (De Lima *et al*. 2008; Marcili *et al*. 2014; Ramirez and Llewellyn, 2014). Also, several authors have shown the high specificity of Cytb to discriminate *Leishmania* species (Foulet *et al*. 2007; Ramirez *et al*. 2016) Therefore, future studies should consider Cytb as a suitable marker for *Leishmania* species discrimination in mammals.

A vast number of *Leishmania* species have been described in bats from Brazil such as *L. infantum*, *L. amazonensis* and *L. braziliensis* (De Lima *et al*. 2008; Savani *et al*. 2010; de Rezende *et al*. 2017). Herein, we detected single infections of *L. infantum*, *L. amazonensis* and *L. braziliensis*, which highlights the epidemiological threat in the transmission to humans causing visceral, cutaneous and/or mucocutaneous leishmaniasis (Lemos and Lima 2005; Paula *et al*. 2013). This work opens new perspectives to study the importance of bats in the epidemiology of leishmaniases, though the presence of amastigotes in the skin or blood of the animal has not been

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directly demonstrated, which would provide the greatest opportunity for phlebotomine infection.

In conclusion, our results showed that bats from the Triângulo Mineiro and Alto Paranaiba regions of Brazil are involved in the transmission cycles of *L*. *infantum*, *L*. *amazonensis* and *L*. *braziliensis*. The epidemiological importance of the role bats play in leishmaniasis has not yet been defined.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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