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PROTOZOOLOGY - REVIEW

The role of sloths and anteaters as *Leishmania* spp. reservoirs: a review and a newly described natural infection of *Leishmania mexicana* in the northern anteater

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Abstract

For years, mammals of the order Pilosa have been considered *Leishmania* reservoirs. But while most studies have focused on sloth species, anteaters have been overlooked, and in many *Leishmania* endemic countries like Mexico, no studies have been carried out. The aims of this work were to identify the presence of *Leishmania* spp. in tissue samples from road-killed northern tamanduas (*Tamandua mexicana*), using PCR amplification and sequencing of ITS1 DNA, and to discuss the role of Pilosa mammals as reservoirs of *Leishmania* based on available scientific records. This is the first study that identifies *Leishmania* in *T. mexicana*, from 1 of 16 individuals analyzed, so the estimated prevalence (CI 95%) of infection was 6.3% (0.3–27.2). Amplified sequence exhibited a 98.9% (727/735) similarity with *L. mexicana*, and phylogenetic analysis grouped the species in the *L. mexicana-amazonensis* cluster. The literature review revealed 241 cases of *Leishmania* spp. infection among 1219 Pilosa mammals evaluated, with prevalence between studies ranging from 3.5% in the brown-throated three-toed sloth (*Bradypus variegatus*) to 78% in the Hoffman's two-toed sloth (*Choloepus hoffmanni*). Current scientific information indicates that *C. hoffmanni* sloths are reservoirs of *Leishmania*, and further studies are needed in order to clarify if other Pilosa species play a role in *Leishmania* transmission.

Keywords Edentata · Leishmaniasis · *Tamandua mexicana* · Mexico · *Myrmecophaga* · Xenarthra

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Introduction

Leishmaniasis is one of the top three protozoan neglected tropical diseases (Gradoni 2018). Every year, 1.3 million new human cases occur, and 350 million people are considered at risk of transmission (Gradoni 2018). In the New World, zoonotic dermotropic *Leishmania* species are endemic causing tegumentary clinical manifestations (Gradoni 2018). Until now, seven orders of American mammals have been found positive for *Leishmania*, including the order Pilosa (Roque and Jansen 2014). This order is composed of four anteater species of the families Cyclopedidae and Myrmecophagidae and six sloth species of the families Bradypodidae and Megalonychidae (Moraes-Barros and Arteaga 2015). Pilosa order is exclusively distributed across the American continent and is one of the oldest placental lineages that irradiated from South America to North America, as early as 9 million years ago (Woodburne 2010).

Because of the high prevalence of *Leishmania* and *Trypanosoma* infections in some species of the order Pilosa, some authors have proposed these animals as trypanosomatid reservoirs (Lainson et al. 1981a). This hypothesis is based on the high frequency of *Leishmania* infection reports in sloths, which is the largest group of Pilosa mammals that have been evaluated (Roque and Jansen 2014; Maia et al. 2018); however, infection in anteaters has been rarely studied. Until now, *Leishmania* has been recorded in two of the four anteater species in the southern tamandua (*Tamandua tetradactyla*) Linnaeus, 1758 and in the giant anteater (*Myrmecophaga tridactyla*) Linnaeus, 1758, in South American countries where both anteater species are sympatric with sloths (Lainson et al. 1981a; Mimori et al. 1989; Richini-Pereira et al. 2014). In contrast, no studies are available from *Leishmania* endemic countries, such as Mexico where sloths do not exist and anteater species are other than those aforementioned.

In Mexico, there are two anteater species, the silky anteater (*Cyclopes didactylus*) Linnaeus, 1758 and the northern tamandua (*Tamandua mexicana*) Saussure, 1860 (Moraes-Barros and Arteaga 2015), the latter being distributed from the north of Colombia to the Mexican neotropics (Navarrete and Ortega 2011). The northern tamandua is categorized by Mexican legislation as "under extinction risk" (Semarnat 2010), because it faces many conservation threats like habitat loss and road traffic accidents (Nuñez-Perez et al. 2011). However, other threats, such as infectious diseases, have been scarcely investigated or there is no information on the role of *T. mexicana* as reservoir of endemic pathogens, such as *Leishmania* spp. Interestingly, studies from Colombia indicate that the northern tamandua is an attractive host for the *Leishmania* blood-feeding sand fly vector species *Lutzomyia shannoni* and *Lu. trinidadensis* (Paternina et al. 2016). Furthermore, this anteater has also been theoretically proposed, using a mathematical model, as *Leishmania* wild reservoir in Mexico (Stephens et al. 2009).

The aims of this work were (i) to identify the presence of *Leishmania* spp. in road-killed individuals of *T. mexicana* from Mexico and (ii) to review *Leishmania* spp. records in the order Pilosa from scientific bibliographic databases, in order to discuss the role of these wild hosts as potential *Leishmania* reservoirs.

Material and methods

Between 2009 and 2015, in the course of the "Parasites in road-killed wildlife" project, carried out by the universities "Universidad Autónoma Metropolitana" and "Universidad Juárez Autónoma de Tabasco," the main highways of Chiapas, Guerrero, and Tabasco states in Mexico were surveyed by car, and road-killed northern anteaters were found and collected. The collection of road-killed animals was carried out under authorization of the Secretaria de Medio Ambiente y Recursos Naturales (Semarnat), permit references: SGPA/DGVS/03663/11, SGPA/DGVS/04726/13, and SGPA/DGVS/07303/14. Animals had been run over less than 12 h before collection, based on the absence of blowfly eggs or maggots typically observed when the post-mortem interval is greater (Reibe and Madea 2010). The animal's gender was recorded, and the age category (juvenile or adult) was determined based on morphometric criteria (Navarrete and Ortega 2011). Necropsies were performed under laboratory conditions, and tissues—namely samples of spleen, lymph node, liver, lung, and kidney—were stored frozen at − 20 °C until analyzed for *Leishmania* DNA.

Tissues were thawed, and in order to avoid cross contamination between animals, the surgical material was disinfected with 5% chlorine between each of the specimens. Defrosted tissues were placed in a 1.5-mL Eppendorf tube and macerated using disposable polypropylene pellet pestles (Sigma Cat. No. Z359947). For DNA extraction, we performed the Chelex-100 Chelating Resin (Bio-Rad, USA) protocol, previously reported by Ballados-González et al. (2018). For initial screening of *Leishmania* DNA, we amplified a 589-bp fragment of the alanine aminotransferase gene (*alat*) using the ALAT.F/ALAT.R primers and conditions reported by Marco et al. (2015). The reaction mixture consisted of 12.5 μL of GoTaq® Green Master Mix, 2× of Promega Corporation (Madison, WI, USA), the pair of primers (100 ng each), 6.5-μL nuclease-free water, and 200-ng DNA in a final volume of 25 μL (Espinosa-Martínez et al. 2015).

For *Leishmania* species identification, the positive sample was tested for the amplification of ~ 800-bp fragment of the Internal Transcriber Subunit I (ITS1) (Cupolillo et al. 1995). Ten microliters of each PCR product was resolved in 2% agarose gels using TAE buffer at 85 V during 45 min and visualized using an ODYSSEY CLx Imaging System (LICOR Biosciences). PCR products were submitted for sequencing at Laboratorio de Biología Molecular y de la Salud, Universidad Nacional Autónoma de México. Sequences were edited using Bioedit and deposited in GenBank™ under accession number: MH885526.

Global alignments were done using Clustal W, and the best substitution model was selected based on the lowest BIC (Bayesian information criterion) score. Additionally, a phylogenetic reconstruction was done using maximum likelihood, with 10,000 replicates of bootstrap in Mega 6.0 software (Tamura et al. 2013).

The literature search was conducted using the scientific electronic databases PubMed, Web of Science, Google Scholar, Research Gate, Redalyc, and Scielo. The combinations of key words included in this literature search were "anteater" and "Leishmania," "Tamandua" and "Leishmania," "Pilosa" and "Leishmania," "Bradypus" and "Leishmania," "Choloepus" and "Leishmania," "Myrmecophaga" and "Leishmania," "Cyclopes" and "Leishmania," and "Xenarthra" and "Leishmania." Literature search was limited to research articles and scientific reviews, without time restrictions, and information related with Pilosa host species, *Leishmania* species, country, tissues evaluated, diagnostic techniques, and number of individuals studied was extracted.

Results

Sixteen road-killed adults *T. mexicana* were collected: one female and two males from Chiapas, one male from Guerrero, and seven females and five males from Tabasco. Of the 16 samples analyzed (11 spleens, one liver, one lymph node, one lung, and two kidneys), *Leishmania* DNA was detected in a single spleen sample, obtained from a female collected in Tabasco; so, the estimated prevalence (CI 95%) of *Leishmania* infection was 6.3% (0.3–27.2). The recovered sequence exhibited a similarity of 98.9% (727/735) with an isolate of *L. mexicana* from a South American human patient, of unknown origin, deposited in Genbank (FJ948433). The phylogenetic analysis grouped the sequence in the cluster of *Leishmania mexicana-amazonensis* with a bootstrap value of 99 (Fig. 1).

The literature search for *Leishmania* spp. records in Pilosa mammals spawned 17 research articles; the studies that identified the parasite are shown in Table 1. From 1969 until now, the number of Pilosa mammals evaluated for *Leishmania* spp. was 1219 individuals, belonging to seven species. Studies differed greatly with regard to the number of individuals evaluated (range 1–498, median 18), the total number of individuals of each species examined, and the country where the study was conducted (see Table 2, which also includes the 16 northern anteaters of our study). The estimated prevalence of *Leishmania* infection in Pilosa mammals ranged from 3.5% in the brown-throated three-toed sloth (*Bradypus variegatus*) Schinz, 1825 to 78% in the Hoffman's two-toed sloth (*C. hoffmanni*) Peters, 1858 (Table 2). Records came from six countries: the most numerous from Brazil that had five studies, followed by Panama with three, and Colombia, Costa Rica, Ecuador, and French Guiana with two studies each. Table 2 also shows the 13 continental American countries where Pilosa species exist, but no *Leishmania* investigations have been carried out.

The literature search shows that *Leishmania* has been detected in nine different tissues (Table 1). The most commonly used were liver and spleen (in 15 and 14 studies, respectively), skin and blood (in nine and seven studies, respectively), and bone marrow, lung, mesenteric lymph node, kidney, and heart (in four or fewer studies). Eight diagnostic techniques were used for *Leishmania* diagnosis in Pilosa mammals. The most commonly used was the experimental infection of hamsters in 11 studies published between 1969 and 1991, in eight of which biochemical characterization with enzymatic profiles were also carried out. From the late 1980s to the early 1990s, three serological surveys of *Leishmania* antibodies were done using the techniques radioimmune binding assay and monoclonal antibodies specific to *L. braziliensis*; two of these studies were also complemented with biochemical characterization and a single study that used only the radioimmune binding assay. Xenodiagnosis with *Lutzomyia* sand flies was used in two studies in 1979 and 1991; both studies examined samples only from the species *C. hoffmanni*. Finally, molecular techniques were used for the first time in a study published in 1992, then used again since 2012 (for details see Table 1).

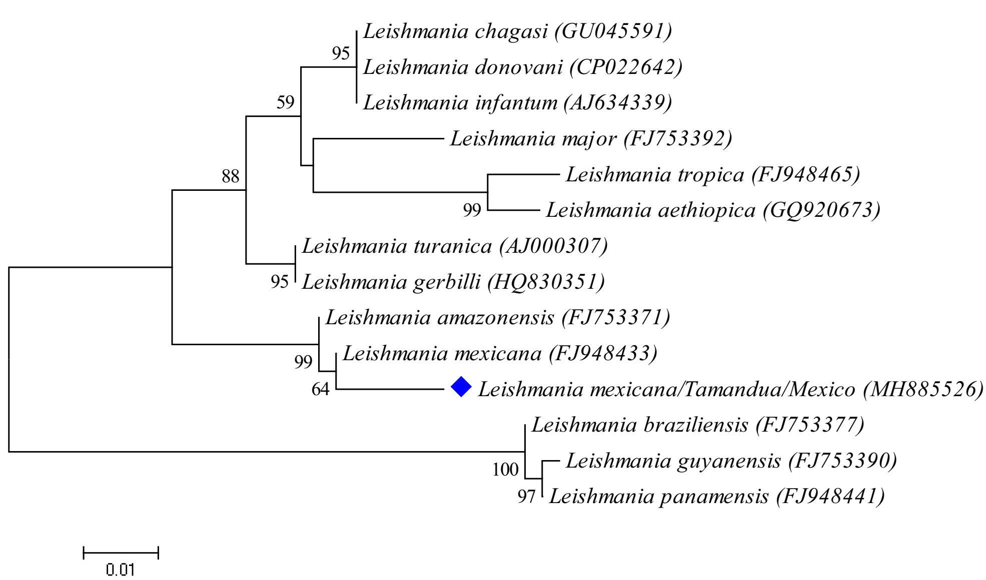

Fig. 1 Maximum likelihood (ML) phylogenetic tree generated using a fragment of 735 bp of the rRNA internal transcribed spacer 1 based on the Tamura 3-parameter model with a discrete gamma distribution (+ *G*)

Table 1 Frequency of *Leishmania* infection in Pilosa mammals according to the parasite and host species and the diagnosis sample and technique used

Leishmania species	Pilosa host	Common name	Evaluated individuals (% positivity)	Country	Tissues isolation	Diagnostic technique	Reference
L. infantum	*Tamandua tetradactyla*	Southern tamandua	1 (100)	Brazil	BM	HInc, Mol	De Aráujo et al. 2013
L. amazonensis	*Tamandua tetradactyla*	Southern tamandua	1 (100)	Ecuador	L, S	IRBA	Mimori et al. 1989
L. braziliensis	*Bradypus variegatus*	Brown-throated sloth	3/77 (4)	Panama	B, BM, L, S, SK	HInc, Mor	Herrer and Telford 1969
	Bradypus variegatus	Brown-throated three-toed sloth	8/47 (17)	Costa Rica	B, L, S, SK	HInc, Bch	Zeledón et al. 1975
	Bradypus variegatus	Brown-throated three-toed sloth	3/86 (3)	Costa Rica	B, L, S, SK	HInc, Bch	Zeledón et al. 1979
	Choloepus hoffmanni	Hoffman's two-toed sloth	24/85 (28)	Panama	B, BM, L, S, SK	HInc, Bch	Herrer and Telford 1969
	Choloepus hoffmanni	Hoffman's two-toed sloth	14/18 (78)	Costa Rica	B, L, S, SK	HInc, Bch	Zeledón et al. 1975
	Choloepus hoffmanni	Hoffman's two-toed sloth	20/72 (28)	Panama	B, BM, L, LU, S, SK	HInc, X, Mor	Christensen and Herrer 1979
	Choloepus hoffmanni	Hoffman's two-toed sloth	96/498 (19)	Panama	B, BM, L, LU, S, SK	HInc	Herrer and Christensen 1980
L. colombiensis	*Choloepus hoffmanni*	Hoffman's two-toed sloth	1 (100)	Colombia/Panama	NR	HInc, X, Bch	Kreutzer et al. 1991
L. equatoriensis	*Choloepus hoffmanni*	Hoffman's two-toed sloth	1 (100)	Ecuador	L, S	Bch, IRBA, Mol	Grimaldi Júnior et al. 1992
L. braziliensis guyanensis	*Choloepus didactylus*	Linnaeus's two-toed sloth	7/15 (47)	French Guiana	L, S, SK	Bch	Gentile et al. 1981
	Choloepus didactylus	Linnaeus's two-toed sloth	27/59 (46)	Brazil	L, S, SK	HInc, Bch	Lainson et al. 1981a
	Choloepus didactylus	Linnaeus's two-toed sloth	11/31 (35)	French Guiana	L, S, SK	HInc, Bch	Dedet et al. 1989
	Tamandua tetradactyla	Southern tamandua	6/27 (22)	Brazil	L, S	HInc, Bch	Lainson et al. 1981a
	Tamandua tetradactyla	Southern tamandua	1/2 (50)	Brazil	L, S	HInc, Bch	Lainson et al. 1981b
L. braziliensis panamensis	*Choloepus hoffmanni*	Hoffman's two-toed sloth	2/2 (100)	Colombia	B, L, S	HInc, Bch	Loyola et al. 1988
L. shawi	*Bradypus tridactylus*	Pale-throated three-toed sloth	1 (100)	Brazil	L, S	Bch, MA	Lainson et al. 1989
	Choloepus didactylus	Linnaeus's two-toed sloth	1 (100)	Brazil	L, S	Bch, MA	Lainson et al. 1989
L. herreri	*Bradypus variegatus*	Brown-throated three-toed sloth	3/86 (3)	Costa Rica	B, S	HInc, Bch	Zeledón et al. 1979
	Choloepus hoffmanni	Hoffman's two-toed sloth	7/63 (11)	Costa Rica	B, L, S, SK	HInc, Bch	Zeledón et al. 1979
Leishmania sp.	*Myrmecophaga tridactyla*	Giant anteater	2/6 (33)	Brazil	H, K, L, LNM, LU	PCR and sequencing	Richini-Pereira et al. 2014
	Tamandua tetradactyla	Southern tamandua	1/2 (50)	Brazil	L, LNM, LU	PCR and sequencing	Richini-Pereira et al. 2014

B: blood, *BM*: bone marrow, *H*: heart, *L*: liver, *LNM*: mesenteric lymph node, *LU*: lung, *S*: spleen, *SK*: skin, *NR*: no reported, *Bch*: biochemical characterization with enzymatic profiles, *HInc*: detection of lesions in hamsters experimentally infected, *X*: xenodiagnostic, *MA*: monoclonal antibodies, *Mol*: molecular identification, *Mor*: morphological identification, *IRBA*: Indirect radioimmune binding assay, *PCR*: polymerase chain reaction

Discussion

This is the first report of *L. mexicana* in a Pilosa mammal and in the northern tamandua. Also, this is the first record of *Leishmania* in a member of the order Pilosa in Mexico. Until now, *Leishmania* has been identified in Mexico only in small wild mammals of the orders Rodentia (two heteromid and five cricetid species), Marsupialia (only in the Mexican mouse-opossum [*Marmosa mexicana*] Merriam, 1897), and Chiroptera (13 microchiroptera bats) (Van Wynsberghe et al. 2009; Berzunza-Cruz et al. 2015). Sequence recovered in our study exhibited a high similarity with those deposited in Genbank from several *L. mexicana* isolates from South America and Mexico (accession numbers AF466381.1 and FJ948433.1).

Table 2 Country-specific frequency of *Leishmania* infection according to the Pilosa species

	Bradypus tridactylus		*Bradypus variegatus*		*Choloepus didactylus*		*Choloepus hoffmanni*		*Tamandua tetradactyla*		*Tamandua mexicana*		*Myrmecophaga tridactyla*		Total Ev.	Total Leish +	%
	Ev	Leish +	Ev	Leish +	Ev	Leish +	Ev	Leish +	Ev	Leish +	Ev	Leish +	Ev	Leish +			
Brazil	1	1			60	28			32	9			6	2	99	40	40.0
Colombia			3	0			3	3			1	0			7	3	42.9
Costa Rica			219	14			81	21							300	35	11.7
Ecuador							1	1	1	1					2	2	100
French Guiana	26	0			46	18			6	0			1	0	79	18	22.8
Panama			77	3			498	140							732	143	19.5
Mexico											16	1			16	1	6.25
Total	27	1	299	17	106	46	583	165	39	10	17	1	7	2	1235	242	
Leish +%	3.7		5.7		43.4		28.3		25.6		5.9		28.6		19.6		
Countries with this Pilosa species but no research	V, Gy, S		A, Bl, H, N, Pe, V		Gy, Pe, S, V		Bl, H, N, Pe, V		A, Bl, Gy, Pa, Pe, S, U, V		Be, ES, Gu, H, N, Pe, V		A, Bl, Gy, ES, H, N, Pa, S, U, V				

Ev: number of animals evaluated, *Leish +*: number of *Leishmania*-positive animals, *Leish +%*: percentage of *Leishmania*-positive animals

Black squares are countries where the Pilosa species does not exist

Gray squares are countries where the mentioned Pilosa species exist, but no research has been done on it

A: Argentina, *Bl*: Bolivia, *Be*: Belize, *ES*: El Salvador, *Gt*: Guatemala, *Gy*: Guyana, *H*: Honduras, *N*: Nicaragua, *Pa*: Paraguay, *Pe*: Peru, *S*: Suriname, *U*: Uruguay, *V*: Venezuela

In relation to *Leishmania* reports in Pilosa mammals, nine *Leishmania* species have been recognized so far, though not *L. mexicana*. The nine *Leishmania* species were found in four of the six existing sloths species and in one of the four anteater species (*T. tetradactyla*) (Table 1). Noteworthy, in some studies, *Leishmania* has been identified only at the genus level, like in Richini-Pereira et al. (2014) that detected the parasite for the first time in the anteater *M. tridactyla*, but were unable to identify the *Leishmania* species. Table 2 shows the estimated prevalence of *Leishmania* infection in Pilosa mammals, which ranged from 3.5% in the sloth *B. variegatus* to 78% in the sloth *C. hoffmanni* (Zeledón et al., 1975; Zeledón et al., 1979), and considerable high prevalence was also detected in two populations of the Linnaeus's two-toed sloth (*C. didactylus*) Illiger, 1811 from Brazil (46%) and French Guiana (47%) (Gentile et al. 1981; Lainson et al. 1981a). The wide range in prevalence may be partly explained by the limited number of animals studied, since half of the studies referred to less than 10 individuals (Loyola et al. 1988; Lainson et al. 1989; Kreutzer et al. 1991; Grimaldi Júnior et al. 1992), particularly in the case of anteaters (Lainson et al. 1981a; Mimori et al. 1989; De Aráujo et al. 2013; Richini-Pereira et al. 2014). Variable prevalence estimates may also reflect differences in *Leishmania* susceptibility between host species or could be related to ecological factors, such as the extent to which *Lutzomyia* sand flies are attracted and share a common habitat with putative hosts (Maia et al. 2018).

Controversy about the *Leishmania* reservoirs status of Pilosa mammals is ongoing. The most recent studies of *Leishmania* in these mammals recognize them as susceptible to infection (Roque and Jansen 2014). Instead, the oldest records incriminate them as reservoir hosts, based on the high *Leishmania* infection prevalence observed in sloths populations of the Megalonychidae family, which only includes two species of *Choloepus* genus (Lainson et al. 1981a). The most comprehensive of all *Leishmania* studies in Pilosa specimens is that of Herrer and Christensen (1980) on Panamanian Hoffman's two-toed sloths, in which 498 animals were analyzed over a 10-year period and 19% were infected with *L. braziliensis*. These sloths varied greatly with respect to the length of time they remained infected with naturally acquired *L. braziliensis*, ranging from 5 to 23 months, and prevalence was highest among the youngest animals. Besides, the authors reported the absence of transplacental or perinatal (acquired through contact with the birth canal) *Leishmania* transmission. This study plus the ones performed by Christensen and Herrer (1979) and Kreutzer et al. (1991) show that the Hoffman's two-toed sloth can transmit infection to the sand fly species *Lu. trapidoi*, *Lu. gomezi*, and *Lu. sanguinaria*. However, transmission efficiency varies between sand fly species, and Christensen and Herrer (1979) reported a limited 7.7% *L. braziliensis* transmission success to *Lu. sanguinaria* sand flies.

Judging by their ability to infect sand flies, this sloth species can be considered a *Leishmania* reservoir (Quinnell and Courtenay 2009). However, with current knowledge, it is not possible to state if it is a primary reservoir host which is able to maintain infection in the absence of other hosts, or a secondary reservoir which in spite of being able to transmit infection to the vector, it cannot maintain the parasite indefinitely by itself (Quinnell and Courtenay 2009). Besides the studies in the Hoffman's two-toed sloth, no other studies have been carried out with the objective of revealing the anteaters' or sloths' reservoir capacity. In this sense, it is noticeable that there are studies that were unsuccessful in detecting *Leishmania* in these species, like the ones by Dedet et al. (1989) in 26 *B. tridactylus*, six *T. tetradactyla* and one *M. tridactyla* from French Guiana, and Loyola et al. (1988) in three *B. variegatus* and one *T. mexicana* from Colombia.

Unfortunately, except for the study of Richini-Pereira et al. (2014) and ours, all *Leishmania* infection studies in Pilosa mammals were "lethal studies," i.e., animals were captured in the wild and euthanized thereafter in order to examine infection in tissues. Lethal studies should be avoided since many Pilosa species face conservation problems (Superina and Loughry 2015), especially those species of restricted distribution and in which *Leishmania* has never been studied, such as the pygmy three-toed sloth (*B. pygmaeus*) Anderson and Handley, 2001 and the maned three-toed sloth (*B. torquatus*) Illiger, 1811. Instead of lethal studies, other effective approaches should be undertaken, such as the use of live trapping methods for taking blood samples and skin biopsies, or the collection of dead animals from wildlife rescue centers, zoos, and road-killed (Muñoz-García et al. 2018).

Conclusion

Based on available information, the sloth *C. hoffmanni* is the only representative of Pilosa mammals in which a role as reservoir of *Leishmania* infection has been unambiguously demonstrated. In order to clarify if a similar role is played by other Pilosa species, it will be necessary to increase the number of studies on those species, with particular emphasis on the transmission efficiency to vectors, while avoiding the use of lethal techniques.

Compliance with ethical standards

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

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