JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Serologic evidence of the exposure of small mammals to spotted-fever *Rickettsia* and *Rickettsia bellii* in Minas Gerais, Brazil

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Abstract

Introduction: Sources of pathogenic *Rickettsia* in wildlife are largely unknown in Brazil. In this work, potential tick vectors and seroreactivity of small mammals against four spotted-fever group *Rickettsia* (*R. rickettsii*, *R. parkeri*, *R. amblyommii* and *R. rhipicephali*) and *Rickettsia bellii* from peri-urban areas of Uberlândia, a major town in Brazil, are described for the first time.

Methodology: Small mammals were captured and blood samples collected. Ticks were collected from the surface of the host and the environment and posteriorly identified. Reactivity of small mammal sera to *Rickettsia* was tested by indirect immunofluorescence assay (IFA) using crude antigens from five Brazilian *Rickettsia* isolates.

Results: Information was obtained from 416 small mammals (48 Marsupialia and 368 Rodentia). Forty-eight animals were parasitized and two tick species, *Ixodes loricatus* and *Amblyomma dubitatum*, were found on several host species, with a few tick-host relationships described for the first time. From the 416 tested sera, 70 reacted to at least one *Rickettsia* antigen (prevalence of 16.8%) and from these, 19 (27.1%) reacted to two or more antigens. Seroprevalence was higher for marsupials (39.6%) than for rodents (13.8%). Marsupial and *Rhipidomys* spp. sera reacted mainly (highest seroprevalence and titers) to *R. bellii*, and that of *Necromys lasiurus* mainly to *R. rickettsii*.

Conclusions: Although the serologic assays poorly discriminate between closely related spotted-fever group *Rickettsia*, the observed small mammal seroreactivity suggests the circulation of *Rickettsia* in the peri-urban area of Uberlândia, albeit at low levels.

Key words: *Rickettsia;* Ixodidae; Rodentia, Marsupialia; seroepidemiologic studies; Brazil.

J Infect Dev Ctries 2016; 10(3):275-282. doi:10.3855/jidc.7084

(Received 5 May 2015 – Accepted 2 October 2015)

Introduction

Rickettsia spp. are intracellular Gram-negative bacteria. The genus comprises human and animal pathogens, with a few causing very severe, sometimes lethal disease. Spotted-fever *Rickettsia* is a group strongly associated with ticks as vectors and includes both pathogenic species such as *Rickettsia rickettsii*, *R. conorii*, *R. parkeri*, *R. africae*, as well as *Rickettsia* with undetermined pathogenicity. The world has witnessed the discovery of several *Rickettsia* species in the last two decades and description of new species can be expected [1]. At the same time, the knowledge about the relationship between human disease and specific *Rickettsia* has also increased with the use of molecular diagnosis. Similarly, in Brazil, where only one spotted-fever human rickettsial disease was known, a new disease caused by an *R. parkeri*-like agent was recently discovered [2,3]. The range of this new agent is rather extensive along the Atlantic coast, and human infection may be hidden by other febrile diseases [4-6]. These findings underscore the possible existence of other tick-borne rickettsial agents in the country.

Brazil is a huge country with continental dimensions, and research of rickettsial disease is non-existent in several locations, as is the case for Uberlândia, the second-most populous county of Minas Gerais state. Initial information of *Rickettsia* circulation on a given area may be provided by serology of some key hosts [7]. Small mammals such as rodents are hosts for immature stages of several tick species and may amplify *Rickettsia* infection in tick populations; thus, they are an important study target for the epidemiology

of rickettsiosis [8,9]. We herein present tick species and a serosurvey of small mammals from peri-urban areas of Uberlândia to four spotted-fever *Rickettsia* found elsewhere in Brazil as well as *Rickettsia bellii*, a common *Rickettsia* in the country but with unknown pathogenicity [10].

Methodology

Sampling location

Sampling was conducted in Uberlândia municipality (18°54′S; 48°15'W), Minas Gerais state, southeast Brazil. The city is the second-most populous of the state, with approximately 646,000 inhabitants. The region belongs to the Cerrado biome, a savannah, and has a tropical climate with two distinct seasons: a hot and rainy summer and a cool and dry winter. Small mammals were captured at nine locations surrounding the urban area. Locations were characterized by either native Cerrado, its fragments, or anthropogenically altered landscapes (Table 1).

Small mammal capture

Small mammals were captured from July 2011 to August 2012 along two to nine campaigns set by convenience at each of the nine sites distributed around the city. Overall sampling effort at each location is shown in Table 1. At locations 1 and 2, small Sherman-type live traps (25 x 8 x 9 cm) one to two meters above ground on trees, and bigger ones (30 x 8 x 9 cm) on the ground, were distributed in grids for four consecutive nights at each campaign. In all other locations, Sherman (30 x 8 x 9 cm) and Tomahawk (45 x 16 x16 cm) live traps at a 3:1 proportion were placed in linear transects set at 10- to 20-meter intervals on the ground for five consecutive nights. Traps were baited daily with a mix of banana, peanut, and oat. Animals recaptured within the same campaign were immediately released and no sample was collected from these.

Captured animals were anesthetized with ketamine cloridrate (Syntec, Santana de Parnaíba, Brazil) and acepromazine (Vetnil, Louveira, Brazil) and blood samples were collected. Animals were identified according to morphological characters, and in a sample, taxonomic identification was determined by karyotype analysis [11]. Animals were marked with an ear code and released at the capture site after full recovery from anesthesia. Voucher rodent species were euthanized by deepening anesthesia, and carcasses were deposited at the National Museum of the Federal University of Rio de Janeiro (UFRJ).

All procedures were submitted to and approved by the ethics committee of the Federal University of Uberlândia (permit 071/11) and Brazilian Environment Institute (IBAMA) (permits 22629-1, 13373-1, and 10762-1).

Tick collection and identification

Hosts were thoroughly examined for ticks by gently rubbing fine forceps against the fur on the whole body surface. Located ticks were collected with the aid of forceps. Flat ticks were placed in alcohol, and engorged larvae and nymphs were kept alive for molting to the next stage at 27°C, 80% relative humidity, and 12:12

Table 1. Location, vegetation type, and trapping effort of small mammal capture from July of 2011 to August 2012 in the peri-urban areas of Uberlândia, State of Minas Gerais, southeastern Brazil

Location	Coordinates	Vegetation	Trapping effort (trap/night)
Glória farm	18°57'S; 48°12'W	Inside dry semideciduous forest and its border with pasture	3,280
Panga reserve	19°09'S; 48°23'W	Cerrado *sensu stricto*[a]	1,400
Veadinho farm	18°57'S; 48°03'W	Pasture and sugar cane	700
Fernanda farm	18°57'S; 48°04'W	Dry semideciduous forest	300
Federal Institute	18°46'S; 48°17'W	Dry semideciduous forest/sugar cane plantation border	400
El Dourado farmstead	18°59'S; 48°26'W	Pasture by a dam	400
Bálsamo farmstead	19°01'S; 48°11'W	Vereda[b] and pasture	600
Morada do Sol residential park	18°53'S; 48°21'W	Dry semideciduous forest	680
Campo Florido roadway	19°00'S; 48°19'W	Cerrado *sensu stricto*	1,080
Total			8,840

[a] Vegetation dominated by trees and shrubs often 3–8 meters tall and giving more than 30% crown cover but with still a fair amount of herbaceous vegetation between them; [b] Valley-side marshes where the water table reaches or almost reaches the surface during the rainy season

photoperiod. Samples of host-seeking ticks were collected from the vegetation by dragging at each location a white 1 x 2-meter cloth over 100 meters on animal trails as described before [12]. Dragging was performed always in the morning (between 8:00 and 10:00 a.m.) and repeated in at least three different months in each location to sample during both dry (May, June, and August) and rainy (December, January, February, and March) seasons. The dragging time schedule was intended to avoid both morning dew that would soak the cloth and periods of the day with direct exposure of vegetation to sun (when ticks descend to lower levels, making them unavailable to drag sampling). Both rainy and dry seasons were sampled because *Amblyomma* ticks in Brazil have a one-year life cycle, with adults prevailing in the rainy season and immatures in the dry one [13]. Collected ticks were stored in alcohol and identified based on dichotomous keys for adults and *Amblyomma* nymphs [14-16]. Lack of keys for Neotropical *Amblyomma* larvae and immatures of *Ixodes* as well as damaged samples precluded identification of several ticks retained as either *Amblyomma* spp. or *Ixodes* spp. Several engorged *Ixodes* immatures were identified after molting to the next stage, and others by comparison to descriptions by Marques *et al*. and/or reference specimens kept in alcohol [17].

Serology for Rickettsia

Reactivity of small mammal sera to *Rickettsia* was tested by indirect immunofluorescence assay (IFA) using crude antigens from five Brazilian *Rickettsia* isolates (*R. bellii* strain Mogi, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ5, *R. rickettsii* strain Taiaçu, and *R. parkeri* strain At24) available at the Faculty of Veterinary Medicine of the University of São Paulo, as previously described [7,18,19]. Briefly, starting from the 1:64 dilutions, sera were diluted in twofold increments with phosphate-buffered saline (PBS), pH 7.4, until the last reactive dilution. Slides were incubated with fluorescein isothiocyanate-labelled goat anti-mouse IgG (Sigma, St. Louis, USA), goat anti-rat IgG (Sigma), or sheep anti-opossum IgG (CCZ, São Paulo, Brazil). For each sample, the endpoint IgG titer reacting with each of the five *Rickettsia* antigens was determined. Reaction was considered homologous when an endpoint titer to a *Rickettsia* species was at least fourfold higher than those observed for all other *Rickettsia* species. In this case, the *Rickettsia* species or a closely related species was considered the possible antigen involved in a homologous reaction. In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested at the 1:64 dilutions, as previously reported [6].

Results

Small mammal capture

Overall, 416 small mammals were captured, 48 Marsupialia from two species (*Didelphis albiventris* and *Gracilinanus agilis*) and 368 Rodentia with one species from Murinae subfamily (*Mus musculus*) and several species from nine genera from Sigmodontinae subfamily (*Calomys* spp., *Cerradomys* spp., *Hylaeamys* spp., *Necromys* spp., *Oecomys* spp., *Oligoryzomys* spp., *Oxymycterus* spp., *Pseudoryzomys* spp., and

Table 2. Prevalence and mean intensity of tick infestation of small mammals captured from July 2011 to August 2012 in the peri-urban areas of Uberlândia, State of Minas Gerais, southeastern Brazil

Order/species	No. infested / No. captured (%)	No. ticks	Mean intensity[a]
Marsupialia			
Didelphis albiventris	3/8 (37.5)	19	6.3
Gracilinanus agilis	7/40 (17.5)	16	2.3
Rodentia			
Calomys spp.	0/1 (0.0)	0	-
Calomys expulsus	0/23 (0.0)	0	-
Calomys tener	0/9 (0.0)	0	-
Cerradomys maracajuensis	1/7 (14.3)	27	27
Cerradomys scotti	0/2 (0.0)	0	-
Cerradomys subflavus	0/3 (0.0)	0	-
Hylaeamys megacephalus	21/35 (60.0)	47	2.2
Mus musculus	0/2 (0.0)	0	-
Necromys lasiurus	4/165 (2.4)	4	1
Oecomys spp.	0/12 (0.0)	0	-
Oligoryzomys spp.	5/13 (38.5)	14	2.8
Oligoryzomys mattogrossae	0/3 (0.0)	0	-
Oligoryzomys nigripes	0/1 (0.0)	0	-
Oxymycterus delator	0/5 (0.0)	0	-
Pseudoryzomys simplex	0/1 (0.0)	0	-
Rhipidomys spp.	7/86 (8.1)	8	1.1
	48/416 (11.5)	135	2.8

[a] Mean number of ticks per infested animal

Rhipidomys spp.). The number of individuals captured from each species of Rodentia is shown in Table 2.

The capture of 416 small mammals included one *D. albiventris* captured three times and a *G. agilis* and two *Hylaeamys megacephalus* captured twice, all in different campaigns.

Ticks collected

Information on tick infestation of small mammals is presented in Tables 2 and 3. From the 416 captured small mammals, 48 were infested by 135 ticks (prevalence: 11.5%; mean intensity [mean number of ticks per infested host]: 2.8 ticks). Only two tick species (*Amblyomma dubitatum* and *Ixodes loricatus*) were found on animals, but other species among unidentified ticks (*Amblyomma* spp. or *Ixodes* spp.) cannot be ruled out. Ticks found were overwhelmingly in immature stages (larvae and nymphs). In fact, only two adults of *I. loricatus* were found and solely on *D. albiventris*.

Dragging on vegetation yielded ticks from five locations (locations 1, 2, 3, 8, and 9) and included 29 *Amblyomma* spp. larvae as well as 9 nymphs and 10 adults of *Ambyomma cajennense* sensu lato [20]. All larvae and nymphs were collected in the dry season and most adults in the rainy season.

Seroreactivity to Rickettsia

From the 416 tested sera, 70 reacted to at least one *Rickettsia* antigen (16.8% overall prevalence) (Table 4). Seroprevalence was higher for marsupials (39.6%, 19 reactive animals from 48) than for rodents (13.8%, 51 reactive animals from 368). With the exception of location 8, all other locations had animals with seroreactivity to *Rickettsia* spp. However, only three animals were captured in this residential park (location 8).

Forty-six (65.7%), eight (11.4%), five (7.1%), and six (8.6%) serum samples were reactive to, respectively, one, two, three or five, and four species of *Rickettsia*. Homologous reaction (endpoint titer to a *Rickettsia* species at least fourfold higher than those observed for the other *Rickettsia* species) to *R. rickettsii*, *R. parkeri*, *R. rhipicephali*, and *R. bellii* was detected in, respectively, nine, four, four, and twenty animals (Table 4). Marsupials and *Rhipidomys* spp. reacted overwhelmingly to *R. bellii*, whereas *N. lasiurus* reacted to *R. rickettsii*. Endpoint titers of small mammals ranged from 64 to 1,024 against *R. amblyommii* and *R. bellii*, and 64 to 2,048 against *R. rickettsii*, *R. parkeri*, and *R. rhipicephali*. Two *Rhipidomys* spp. were the hosts that exhibited the highest titers to all five antigens; all animals from these genera were captured at location 1 (Glória farm).

Discussion

With the exception of two adult ticks on *D. albiventris*, ticks of solely immature stages were found on small mammals. Previous reports on ticks of small mammals from various regions of Brazil showed a similar tendency [6,19,21,22]. Two tick species, *A. dubitatum* and *I. loricatus*, were recovered from small mammals, whereas only *A. cajennense* sensu lato, both nymphs and adults, were found in the environment. This apparent disconnection of environmental and host infestation may be explained by limitations of the sampling method of the environment, differing micro-environmental preferences of the host and parasite, and/or the lack of attraction of these small mammal species to specific ticks. In fact, *A. cajennense* sensu lato in Brazil are known to feed mainly on large mammals such as horses and capybaras in the adult stage [23]. Further, samplings by cloth dragging, which

Table 3. Tick species found on small mammals captured from July 2011 to August 2012 in the peri-urban areas of Uberlândia, State of Minas Gerais, southeastern Brazil

Host species	*Amblyomma dubitatum*	*Amblyomma* spp.	*Ixodes loricatus*			*Ixodes* spp.	
	Nymph	Larva	Adult	Nymph	Larva	Nymph	Larva
Didelphis albiventris	3	14	2	-	-	-	-
Gracilinanus agilis	-	-	-	3	4	4	5
Cerradomys maracajuensis	4	23	-	-	-	-	-
Hylaeamys megacephalus	2	-	-	18	9	15	3
Necromys lasiurus	1	3	-	-	-	-	-
Oligoryzomys spp.	2	1	-	6	-	5	-
Rhipidomys spp.	1	-	-	3	3	-	1

were restricted to mornings, as well as temperature variations may have caused sampling bias with reflections on tick species and numbers collected. Irrespective of this disconnection between ticks from the environment and on small mammals, rodents are a common source of microorganisms for arthropods worldwide and thus may contribute to the transmission of several infectious agents to other hosts during the tick's feeding and in the subsequent developmental stages of the tick [24]. For this reason, rodent-tick associations herein described are evaluated with care.

Finding *A. dubitatum* nymphs on all tick-infested mammals with the exception of *G. agilis* was a noteworthy observation. In fact, *A. dubitatum* on *C. marinhus*, *H. megacephalus*, *N. lasiurus*, and *Rhipidomys* spp. is, to our knowledge, the first report of these host-tick relationships. This tick species (*A. dubitatum = A. cooperi*) [25] has been shown to harbor *R. bellii*, a species with unknown pathogenicity, and at least two other undefined *Rickettsia* species [26-28]. At the same time, it is a tick species with strong association in all parasitic stages with capybaras (*Hydrochoerus hydrochaeris*) [29]. Capybara, the biggest rodent in the world, also withstands high *A. cajennense* infestations, and this host and this tick together provide the major epidemiological background for Brazilian spotted fever (BSF) in southeast Brazil [30]. Nonetheless, the initial *Rickettsia* source for ticks and capybaras is still undetermined [23]. In fact, even though BSF has not been diagnosed in Uberlândia region, capybaras are common and were seen in several of the studied locations. Thus, infestations of *A. dubitatum* on small mammals and later on capybaras together with *A. cajennense* should be investigated further in endemic areas as a possible bridge for *R. rickettsii*.

Ixodes loricatus is a tick species common on New World Marsupials but found on Sigmodontinae rodents as well [25,31]. This tick species frequently harbors *R. bellii*, and both ticks and opossums (*Didelphis* spp.) are common in BSF-endemic areas [6,32]. There are two reports of *R. rickettsii* isolation from opossums from the states of São Paulo and Minas Gerais, Brazil [33,34]. Thus, *I. loricatus* is another tick species that deserves attention as a potential pathogenic *Rickettsia* vector among small mammals.

Table 4. Homologous and non-homologous seroreactivity to *Rickettsia* species of small mammals captured from July 2011 to August 2012 in the peri-urban areas of Uberlândia, State of Minas Gerais, southeastern Brazil

Species	No. seroreactive/ No. captured (%)	PAIHR[a]				NHR[b]
		R. rickettsii	*R. parkeri*	*R. rhipicephali*	*R. bellii*	
Didelphis albiventris	3/8 (37.5)	1	-	-	2	-
Gracilinanus agilis	16/40 (40.0)	-	1	1	9	5
Calomys spp.	0/1 (0.0)	-	-	-	-	-
Calomys expulsus	1/23 (4.3)	-	-	-	-	1
Calomys tener	0/9 (0.0)	-	-	-	-	-
Cerradomys maracajuensis	4/7 (57.1)	-	-	-	1	3
Cerradomys scotti	0/2 (0.0)	-	-	-	-	-
Cerradomys subflavus	1/3 (33.3)	-	-	-	-	1
Hylaeamys megacephalus	2/35 (5.7)	-	-	1	-	1
Mus musculus	0/2 (0.0)	-	-	-	-	-
Necromys lasiurus	18/165 (11.0)	4	1	1	1	11
Oecomys spp.	0/12 (0.0)	-		-	-	-
Oligoryzomys spp.	1/13 (7.7)	-	1	-	-	-
Oligoryzomys mattogrossae	1/3 (33.3)	1	-	-	-	-
Oligoryzomys nigripes	0/1 (0.0)	-	-	-	-	-
Oxymycterus delator	3/5 (60.0)	1	-	1	-	1
Pseudoryzomys simplex	0/1 (0.0)	-	-	-	-	-
Rhipidomys spp.	20/86 (23.0)	2	1	-	7	10
Total	**70/416 (16.8)**	**9**	**4**	**4**	**20**	**33**

[a]A homologous reaction was determined when an endpoint titer to a *Rickettsia* species was at least fourfold higher than to those observed for the other *Rickettsia* species. In this case, the *Rickettsia* species involved in the highest endpoint titer was considered the possible antigen involved in a homologous reaction (PAIHR); [b] NHR: non-homologous reaction

Finding *I. loricatus* and *A. dubitatum* on small rodents and marsupials, as seen in our work, indicates the possibility of ticks feeding on these different hosts throughout their life cycles, with disease agent transmission from one to the other. In this regard, it is worthwhile to mention that it was previously observed that opossums from both endemic and non-endemic areas displayed titers to *R. rickettsii* at least fourfold higher than to any of the other rickettsial antigens [6,32]. Moreover, it was shown that, under laboratory conditions, *R. rickettsii* was capable of infecting opossums without causing illness [34]. In these experiments, animals developed a rickettsemia capable of causing infection in guinea pigs and ticks, although the infection rate in ticks was low.

Overall, serological prevalence of small mammals to the five *Rickettsia* antigens from our work (16.8%) was low, but within the wide range described in the country. However, due to the lack of antigens until a few years ago, there are only few reports on small mammal sera reactivity to several *Rickettsia* species in Brazil [6,19,21,32,36]. Unfortunately, these reports are not enough to establish a standard serological profile for circulation of *Rickettsia* species in small mammals. At the same time, low prevalence of seroreactivity of small rodents, as seen in our work, must be evaluated with caution. Szabó *et al*., for example, observed an almost absolute seroconversion of dogs to *Rickettsia* spp. Atlantic rainforest strain, agent of spotted-fever human rickettsioses in Brazil, at forest sites that harbor infected *Amblyomma ovale* ticks, whereas seroprevalence was half in the case of *Euryorysomys russatus*, the small rodent that feeds *A. ovale* juveniles [6]. Although other factors may be involved, it is possible that dogs, by crossing longer distances, enhance the chance of encounter with infected ticks. Thus, at endemic sites, seroprevalence of small mammal population with restricted home range might be decreased in comparison to other animals even if such hosts have an important role in disease epidemiology.

In our case, a probable homologous reaction of small mammals was found against four of the five *Rickettsia* antigens, overwhelmingly against *R. bellii* but also against *R. rickettsii*, *R. parkeri*, and *R. rhipicephali*. *Rickettsia bellii* has been found in several tick species in the country, attaining high prevalence in both *I. loricatus* and *A. dubitatum* ticks, which may explain our serological results [6,10,27,32,37]. Reasons for the other probable homologous reactions, however, are less straightforward and deserve further investigation, particularly in the case of *R. rickettsii.* The aforementioned *Rickettsia* species may be circulating but in lower levels, or serologic results may reflect cross-reactivity with closely related *Rickettsia* from the spotted-fever group and to which small mammals were exposed to.

Non-homologous reactions to antigens from several *Rickettsia* species from our work may be a result of infection with unknown *Rickettsia* species and/or mixed infections. In fact, antigenic cross-reactivity among *Rickettsia* of different species occurs [38]. In this regard, it is important to note that several *Rickettsia* species may be present at one location and even in the same tick, and thus animals may be exposed to more than one species simultaneously or over time with an unknown effect on seroreactivity [6].

In the few existing reports from Brazil, small mammal seroreactivity to *Rickettsia* and BSF relationship is not very clear. Reactivity was observed in BSF-endemic areas, in areas of silent focus, and also in apparently non-endemic areas [19,21,36]. In this regard, it was observed in the state of São Paulo, Brazil, in areas with or without a history of recent confirmed cases of human BSF, that rather than prevalence of opossum serum reactivity, the height of titers was related to endemicity [32]. In our case, overall titers of small mammals were low, with only two *Rhipidomys* displaying higher titers, but none of them exhibiting a homologous reaction, suggesting a lack of BSF endemicity or its restriction to specific, non-sampled spots.

Conclusions

The relatively low prevalence of small mammal seroreactivity and the observed homologous reactions suggest that in the peri-urban area of Uberlândia, there is a circulation of *Rickettsia*, albeit at low levels. Among these, *R. bellii* and probably one or more spotted-fever group *Rickettsia* species may be present. In this context, apart from opossums and *I. loricatus*, both *Rhipidomys* spp. and *A. dubitatum* deserve further investigation as well as molecular identification of *Rickettsia* in ticks. Last but not least, a follow-up is mandatory as endemicity may vary over time for reasons yet unknown.

Acknowledgements

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Academic Career Research Fellowship to M.B. Labruna and M.P.J. Szabó), Fundação de Amparo á Pesquisa do Estado de Minas Gerais - FAPEMIG (Rede Mineira), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES

(scholarship to M.G. Coelho). The authors acknowledge Zoonosis Control Center of Uberlândia and Laboratório de Ecologia de Mamíferos (LEMA), Universidade Federal de Uberlândia for logistic collaboration. The authors are indebted to Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres do Instituto Oswaldo Cruz (FIOCRUZ) for the identification of several small mammals.

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Conflict of interests: No conflict of interests is declared.