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Molecular detection of *Leishmania* spp. in road-killed wild mammals in the Central Western area of the State of São Paulo, Brazil

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Abstract

Background: Road-killed wild animals have been classified as sentinels for detecting such zoonotic pathogens as *Leishmania* spp., offering new opportunities for epidemiological studies of this infection.

Methods: This study aimed to evaluate the presence of *Leishmania* spp. and *Leishmania chagasi* DNA by PCR in tissue samples (lung, liver, spleen, kidney, heart, mesenteric lymph node and adrenal gland) from 70 road-killed wild animals.

Results: DNA was detected in tissues of one *Cavia aperea* (Brazilian guinea pig), five *Cerdocyon thous* (crab-eating fox), one *Dasypus septemcinctus* (seven-banded armadillo), two *Didelphis albiventris* (white-eared opossum), one *Hydrochoerus hydrochoeris* (capybara), two *Myrmecophaga tridactyla* (giant anteater), one *Procyon cancrivorus* (crab-eating raccoon), two *Sphiggurus spinosus* (porcupine) and one *Tamandua tetradactyla* (lesser anteater) from different locations in the Central Western part of São Paulo state. The *Leishmania chagasi* DNA were confirmed in mesenteric lymph node of one *Cerdocyon thous*. Results indicated common infection in wild animals.

Conclusions: The approach employed herein proved useful for detecting the environmental occurrence of *Leishmania* spp. and *L. chagasi*, as well as determining natural wild reservoirs and contributing to understand the host-parasite interaction.

Keywords: Road-killed animal, *Leishmania* spp, *Leishmania chagasi*, PCR, Zoonosis

Background

Leishmaniosis is a zoonotic, parasitic disease caused by kinetoplastid flagellate protozoan parasites of the genus *Leishmania* that infects several mammal species, including humans, and is transmitted by the phlebotomine sandfly. *Leishmania* species include visceral, cutaneous and mucocutaneous forms of the disease in both the Old and New Worlds [1,2].

Great concern has been sparked by the contribution that global warming might be making to the recent increase in the number of reported cases and geographical areas [3]. Environmental, demographic and human behavioral factors contribute to the changing landscape of leishmaniasis, which includes increased risk factors for zoonotic cutaneous leishmaniasis and new scenarios associated with the zoonotic visceral leishmaniasis [4].

Studies on *Leishmania* spp. in wild animals have become more numerous in Brazil due to the importance of these species in the life cycle of leishmaniasis [5]. Studies involving road-killed instead of laboratory research animals have become more frequent in helminthological, epidemiological, morphological and genetic areas [6-12]. However, the use of molecular techniques for detection of microorganisms in these samples is recent [13-17].

Despite the difficulty of culturing and histopathologically analyzing tissue samples from road-killed animals, molecular techniques can be used in the identification and typing of pathogens by polymerase chain reaction (PCR), which presents high specificity and sensitivity to a certain fragment of the pathogen's specific DNA [18].

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Leishmania kinetoplastic DNA (kDNA)-specific probes have been used for the detection and identification of this protozoan, and demonstrated to be useful for epidemiological field studies because a large number of samples can be handled simultaneously [19]. A minicircle of kDNA (0.8 to 1 kb in length) is an ideal target, since it is present in 10,000 copies per cell and because its sequences are known for most *Leishmania* species [20].

The present work aimed to describe possible new hosts for leishmaniasis by using molecular tools to detect *Leishmania* spp. in tissues of road-killed wild mammals. Thus, research into new hosts by molecular techniques is distinctive in epidemiological studies of pathogens and represents suitable indicators of environmental contamination by *Leishmania* spp. [21].

Methods
Animals and studied area
Seventy road-killed wild animals were studied: one *Callithrix penicillata* (black-tufted marmoset), four *Cavia aperea* (Brazilian guinea pig), one *Cebus apella* (capuchin monkey), 13 *Cerdocyon thous* (crab-eating fox), three *Dasypus novemcinctus* (nine-banded armadillo), one *Dasypus septemcinctus* (seven-banded armadillo), nine *Didelphis albiventris* (white-eared opossum), one *Eira barbara* (tayra), one *Euphractus sexcinctus* (six-banded armadillo), two *Gallictis vittata* (grison), two *Hydrochoerus hydrochaeris* (capybara), one *Leopardus tigrinus* (leopard cat), five *Lepus europaeus* (brown hare), three *Lutreolina crassicaudata* (latrine opossum), two *Mazama gouazoubira* (brown brocket deer), one *Myocastor coypus* (coypu), six *Myrmecophaga tridactyla* (giant anteater), three *Procyon cancrivorus* (crab-eating raccoon), two *Puma concolor* (cougar), two *Rattus rattus* (black rat), five *Sphiggurus spinosus* (porcupine) and two *Tamandua tetradactyla* (lesser anteater). Only recently killed animals (1–7 hours) and those with no exposed viscera were collected. This study is in accordance with the Brazilian Institute of Environment and Renewable Natural Resources' (IBAMA) normative statement n. 119 of October 11, 2006, chapter VI, art .26, which authorizes the collection and transport of animals that were found dead for scientific or didactic purposes. This work was also approved by the Ethics Committee for Animal Experimentation at our Institution (CEEA/FMVZ n.211/2008).

The geographic positions of the road-killed animals, established through global positioning system (GPS), were plotted on a digital map using a geographic database by the TerraView 3.6.0 [22].

Molecular detection
DNA extraction from the animals' tissue samples (lung, spleen, liver, kidney, heart, mesenteric lymph node and adrenal gland) was carried out by using the kit Illustra™ Tissue & Cells Genomic Prep Mini Spin (GE Healthcare, USA). PCR reactions were performed by employing the primers LinR4 (5'-GGGTTGGTGTAAAATAGGG-3') and Lin19 (5'-CAGAACGCCCCTACCCG-3'), described by Aransay *et al.* [20], to amplify a 720 bp fragment. Samples positive for *Leishmania* spp. PCR were also assayed for *Leishmania braziliensis* complex and *Leishmania mexicana* complex [23,24]. Genus-specific primers for *Leishmania* spp. were used in order to identify the DNA of all possible *Leishmania* species that cause visceral or cutaneous leishmaniasis. The cycling profile consisted of an initial denaturation at 95°C for three minutes, followed by 30 cycles at 95°C for 30 seconds, 63°C for 30 seconds and 72°C for one minute, and a final extension at 72°C for seven minutes. Positive controls were included in each assay and consisted of 10 ng of DNA extracted from *Leishmania major* (MHOM/SU/1973/5-ASKH) and *L. chagasi* (MHOM/BR/2002/LPC-RPV). Negative controls were: ultrapure water and DNA from *T. cruzi* (ColTryp 0032/MCAN/BR/2008/CAO) that were added to the mix-PCR. The PCR mixture was composed of 10 mM Tris HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 ρmol of each primer, 0.2 units of *Taq* DNA polymerase, and 10 ng DNA template.

The amplification of *Leishmania chagasi* DNA was performed utilizing primers Lc14 (5'-CGCACGTTATATCTACAGGTTGAG-3') and Lc15 (5'- TGTTTGGGATTGAGGTAATAGTGA-3') on a 190 bp fragment, by using the following cycling profile: initial denaturation at 94°C for four minutes, 40 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and 70°C for ten minutes. Positive controls were included in each assay and consisted of 10 ng of DNA extracted from *L. chagasi* (MHOM/BR/2002/LPC-RPV). Negative controls were: ultrapure water and DNA from *T. cruzi* (ColTryp 0032/MCAN/BR/2008/CAO) that were added to the mix-PCR. The PCR mixture was composed of 10 mM Tris HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 ρmol of each primer, 0.5 units of *Taq* DNA polymerase, and 10 ng DNA template.

Amplification was performed in a MasterCycler EP gradient (Eppendorf, USA). The sequence was analyzed by electrophoresis in 1.5% agarose with SYBR® safe DNA gel stained (Invitrogen, USA), and visualized in an image analyzer (GelDoc-It™ Imaging System – UVP, USA) by using VisonWorks®LS Software. Amplicons were purified by using ExoSap (USB, USA) and the sequencing reactions were carried out on both strands in a 3500 Genetic Analyzer (Applied Biosystems). The obtained sense and antisense sequences were visualized (Chromas 2.3 software, Technelysium Pty Ltd, Australia), aligned by the software MEGA 4 and compared with the NCBI database using BLASTn (Basic Local Alignment Tool for Nucleotide) [25].

Results and discussion

The present results draw attention to a very important source of research and emphasize the importance of using this biological resource in an epidemiological study of zoonotic infection.

One of the main problems in elucidating leishmaniasis epidemiology is to identify and confirm that a vertebrate host is a natural reservoir. The natural reservoirs are widely unknown because of the difficulties in capturing a sufficient number of wild animals and due to the techniques used in isolating and identifying the parasite.

This approach of using road-killed wild animals for the molecular detection of *Leishmania* spp. may represent a useful alternative to the utilization of captured ones in research studies, as indicated by animal research ethics committees. In the present study, a great diversity of road-killed wild mammal species was found. Culture analysis and histopathology are difficult and laborious. Sensitive and specific molecular tools allow pathogens to be identified without the need of culturing.

In this paper, molecular detection of *Leishmania* spp. and *Leishmania chagasi* was attempted from several wildlife species using PCR. Several studies have reported the presence of this parasite in mammalian species, including rodents, carnivores, primates and marsupials [5,26-29].

Table 1 contains the results of the PCR and identifies percentages of amplicon obtained in road-killed wild animals positive for *Leishmania* spp. and *Leishmania chagasi* from deposited homologue DNA sequences, as determined by BLASTn analysis. Figure 1 illustrates the human cutaneous leishmaniasis data corresponding to the cases seen in the central western area of the state of São Paulo, Brazil, from 1998 to 2010 [30]. Figure 2 displays the human visceral leishmaniasis data corresponding to the cases seen in the central western area of the state of São Paulo, Brazil, from 1998 to 2010 and geographic location of the positive road-killed animals evaluated [31].

Leishmania DNA was detected in 5/12 (41.67%; CI95% 19.22-68.42%) samples from *Cerdocyon thous* (crab-eating fox). A previous report indicates seropositivity in wild non-captive *Cerdocyon thous* [5,28]. The importance of these animals as reservoirs depends on their ability to transmit the infection to sandflies rather than on their infection rate; it is also a function of their capability to (re)introduce the pathogen into *Leishmania*-free dog populations [32].

In the current study, *Leishmania* spp. DNA was detected in 1/3 *Procyon cancrivorus* (crab-eating raccoon). Voltarelli *et al.* [33] reported the presence of *Leishmania* antibodies in *Procyon cancrivorus* in Northwestern Paraná. These findings suggest that these species can act as a reservoir for *Leishmania* spp.

The members of Didelphidae, represented by *Didelphis albiventris* specimens (white-eared opossums), are habitat generalists and currently occur in areas near dwellings, including farms, yards and urban centers [34]. This species is already proven to be a leishmaniasis reservoir and, for its synanthropic habits, it plays an important role in the peridomestic-forest traffic of degraded areas [35-37]. The present study confirms that molecular detection of

Table 1 Data on road-killed wild animals, including the sex, tissue, PCR and sequencing results for molecular detection on *Leishmania* spp. and *Leishmania chagasi*

| Species	Animal	Sex	Tissue (PCR positive)	% identity/GenBank access
Procyon cancrivorus	A1	Male	kidney	99%/AJ270142.1 *Leishmania* spp.
Cerdocyon thous	A4	Male	heart, mesenteric lymph node	100%/AJ270142.1 *Leishmania* spp.
Cerdocyon thous	A6	Male	spleen, heart	100%/AJ270142.1 *Leishmania* spp.
Cerdocyon thous	A7	Male	heart	100%/AJ270141.1 *Leishmania* spp.
Cavia aperea	A17	Male	heart	99%/AJ270141.1 *Leishmania* spp.
Dasypus septemcinctus	A18	Male	liver	100%/AJ270141.1 *Leishmania* spp.
Sphiggurus spinosus	A22	#	liver, spleen	100%/AJ270142.1 *Leishmania* spp.
Tamandua tetradactyla	A23	Male	lung, liver, mesenteric lymph node	100%/AJ270142.1 *Leishmania* spp.
Shiggurus spinosus	A24	Female	spleen, kidney, heart	100%/AJ270142.1 *Leishmania* spp.
Cerdocyon thous	A41	Male	liver, mesenteric lymph node	100%/AF308682.1 *Leishmania chagasi*
Myrmecophaga. tridactyla	A42	Male	lung, kidney, heart, mesenteric lymph node	100%/AJ270142.1 *Leishmania* spp.
Didelphis albiventris	A46	Male	liver, spleen, kidney	100%/AJ270142.1 *Leishmania* spp.
Didelphis albiventris	A47	Male	lung	100%/AJ270142.1 *Leishmania* spp.
Hydrochoerus hydrochaeris	A50	Female	lung	100%/AJ270142.1 *Leishmania* spp.
Cerdocyon thous	A54	Male	lung, spleen	100%/AJ270142.1 *Leishmania* spp.
Myrmecophaga tridactyla	A60	Male	lung	100%/AJ270142.1 *Leishmania* spp.

#not available.

Figure 1 Geographic location of road-killed animals employed for *Leishmania* spp. molecular detection, correlating to the occurrence of cases of human cutaneous leishmaniasis.

Figure 2 Geographic location of the road-killed animals employed for *Leishmania* spp. molecular detection, correlating to the occurrence of cases of human visceral leishmaniasis.
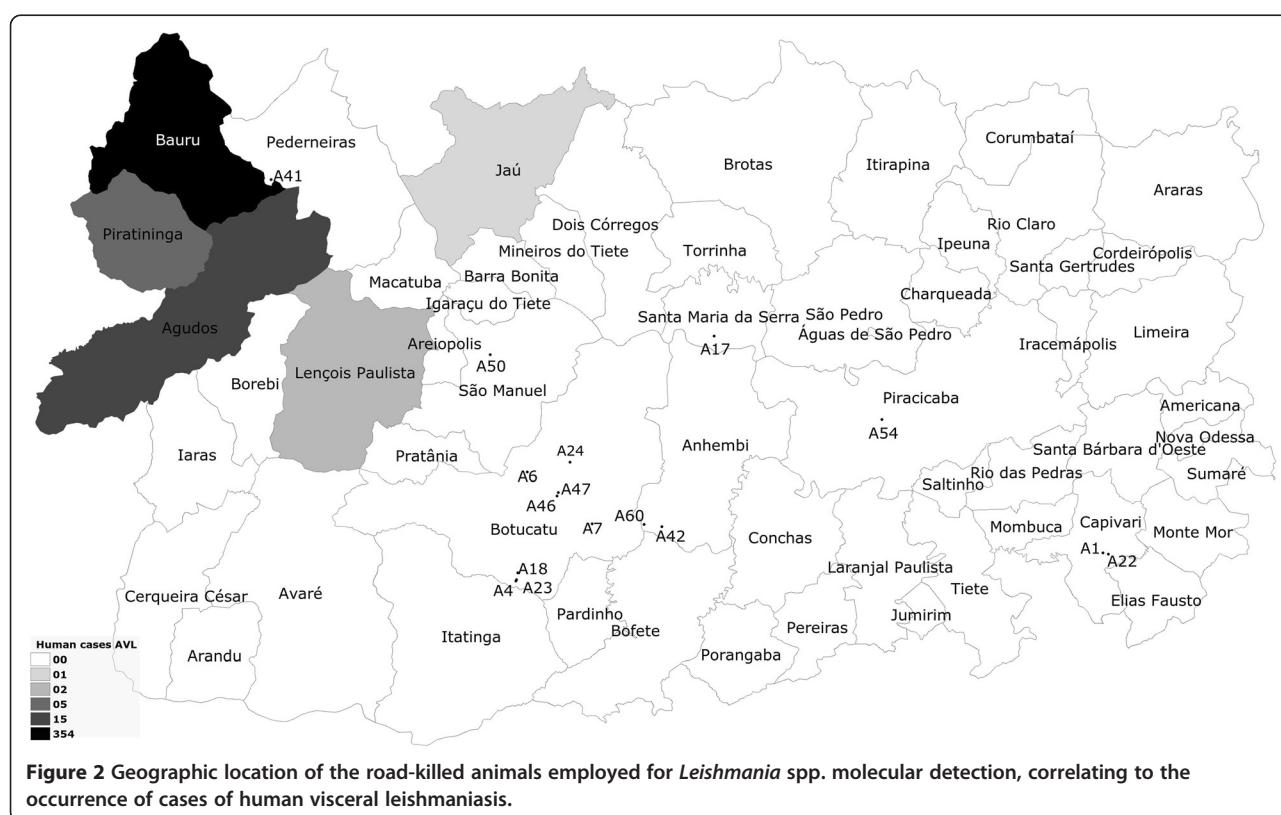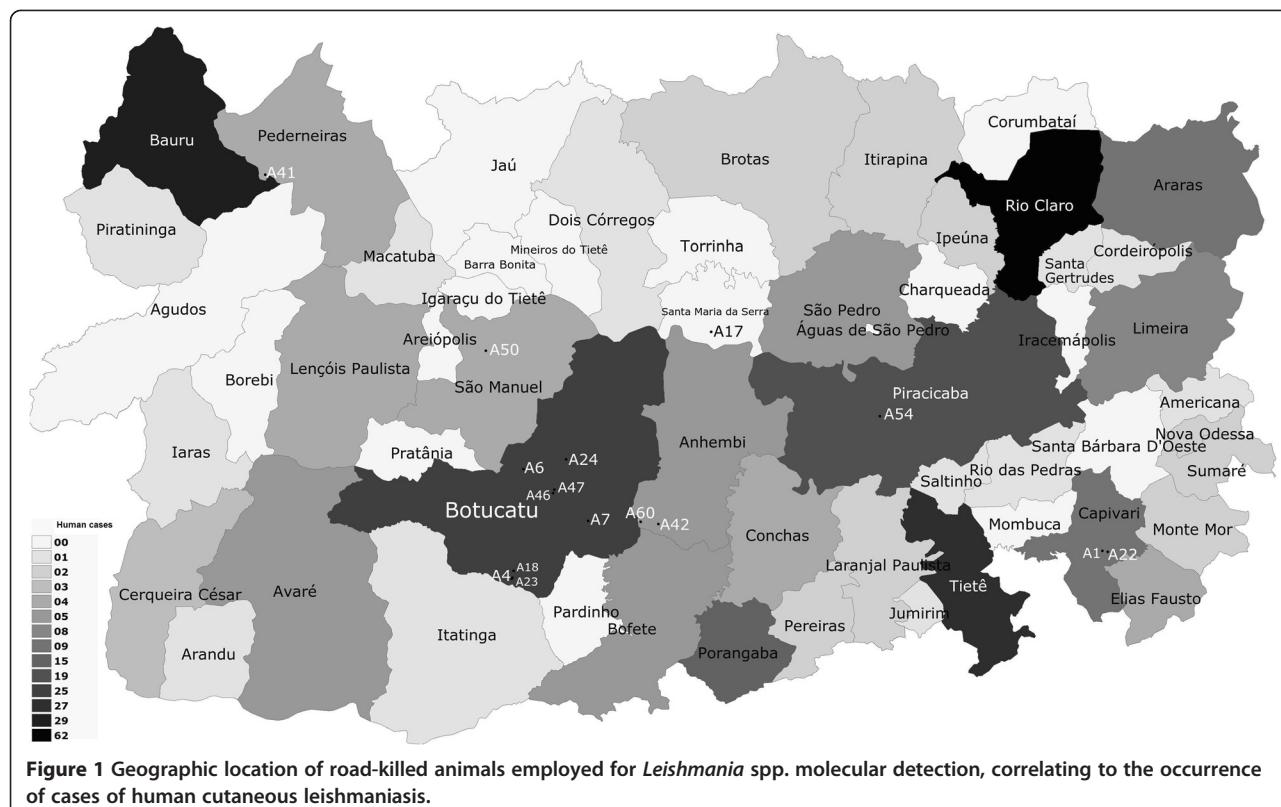
Leishmania spp. in 2/8 group members may be common, as was already described in several regions of Brazil: Manaus, Amazonas state; in Barra de Guarituba, Rio de Janeiro state; in Amaraji, Pernambuco; and Bauru, São Paulo state [38-41].

Rodents were represented by five *S. spinosus* (porcupine), four *C. aperea* (Brazilian guinea pig), two *Rattus rattus* (black rat), two *Hydrochoerus hydrochaeris* (capybara) and one *M. coypus* (coypu). Current data show that 8/14 (57.14%; CI95% 35.14-82.34%) specimens of wild rodents were positive, a finding that corroborates the literature that considered some rodent as reservoirs of *Leishmania* spp.

The superorder Xenarthra was represented by 13 specimens: three *D. novemcinctus* (nine-banded armadillo), one *E. sexcinctus* (six-banded armadillo), one *D. septemcinctus* (seven-banded armadillo), two *T. tetradactyla* (lesser anteater) and six *M. tridactyla* (giant anteater). These animals present some peculiar physiological and ecological characteristics including a weak immune system and low body temperature, besides the fact that they live literally immersed in soil and organic matter, mainly in tropical and subtropical regions, under biotic and abiotic conditions that promote multiple encounters with a diverse group of pathogens and vectors.

The present study confirms the occurrence of *Leishmania* spp. DNA in armadillos (one *D. septemcintus*) and anteaters (one *T. tetradactyla* and two *M. tridactyla*). Casadeval and Pirofski [42] clarified many points on virulence and pathogenicity regarding host immune response and pathogen activity. According to the authors, there are classes of pathogenic microorganisms varying from those that provoke damage in hosts that present an extremely weak immune response to others that cause disease only in a situation of very strong immune response. Therefore, it seems reasonable to consider *Leishmania* spp. to be a pathogen whose ability to provoke disease also depends on host immune response. Since the cellular immune response is weak in armadillos and anteaters, it is possible to detect yeast cells in many of their organs; however, this is not sufficient to cause disease as observed in human hosts. Taken together, these factors make xenarthrans suitable models for studying host-pathogen interaction [43].

These animals are assumed to be sources of infection since the agent's DNA was found in internal organs; in addition, parasitism may occur in internal and cutaneous organs, facilitating transmission from the blood meal by the vector that inoculates promastigote forms of the agent into the man while sucking.

The identities of the amplicon were confirmed by direct double-strand sequencing which showed 100% similarity with *L. chagasi* sequence deposited at GenBank (access number AF308682.1) (Table 1).

Even without the DNA detection of the cutaneous leishmaniasis agents, the positive results for *Leishmania* spp. are interesting. Of the 20 species described in the New World, five have never been reported to have caused visceral human leishmaniasis: *Leishmania enriettii*, *Leishmania hertigi*, *Leishmania deanei*, *Leishmania aristidesi* and *Leishmania forattinii* [44-48]. The *L. forattinii* was isolated from pooled liver and spleen of opossum *Didelphis marsupialis* captured in Conchas, SP, Brazil [48,49].

It is suggested that the species is *Leishmania forattinii* and that the evaluated site is close to that where the parasite was first isolated, since the species nucleotide sequence deposited at the GenBank was not found. Considering the occurrence of both the cutaneous and visceral form, in the studied municipalities, it must be emphasized that the sandfly vector may be present and serve as transmitter of *Leishmania* to these animals and humans.

These findings corroborate the worldwide distribution of *Leishmania* spp., considering the wide variety of intermediate hosts that contribute to the epidemiological transmission chain of this infection.

It is important to emphasize that Bauru, SP, is endemic for leishmaniasis; therefore, our results indicate the need for epidemiological molecular biology research on environmental contamination by *L. chagasi.*

It was possible to evaluate 22 different wild species, without the necessity of exerting a laborious sampling effort. In fact, the numbers and diversity of road-killed animals are considerably higher and, in general, they are killed after their own natural habitats had been invaded by roads [50]. In this manner, the geographic coordinates of the locations of the infected animals are well-integrated in databases that use the geographical information systems (GIS), thus contributing to a better understanding of pathogen distribution.

These results show risk factors such as free movement of the circulating parasite and vectors, as well as the importance of road-killed animals as possible reservoirs for the transmission of *Leishmania* spp. in addition to the significance of the environment and ecology of these positive mammals in the interaction of Leishmania with different *Leishmania* species that may be pathogenic to humans.

Conclusions
The presented results focus that road-killed animals may serve as an important reservoir for transmission of *Leishmania* spp. and *L. chagasi*, as well as contributing to understand the host-parasite interaction.

Ethics committee approval
This study is in accordance with the Brazilian Institute of Environment and Renewable Natural Resources' (IBAMA) normative statement n. 119 of October 11, 2006, chapter VI, art .26, which authorizes the collection and transport of animals that were found dead for scientific or didactic purposes. This work was also approved by the Ethics Committee for Animal Experimentation of our Institution (CEEA/FMVZ n.211/2008).

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