Diagnosis of *Leishmania infantum* infection by Polymerase Chain Reaction in wild mammals[1]

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ABSTRACT.- Lombardi M.C., Turchetti A.P., Tinoco H.P., Pessanha A.T., Soave S.A., Malta M.C.C., Paixão T.A. & Santos R.L. 2014. **Diagnosis of *Leishmania infantum* infection by Polymerase Chain Reaction in wild mammals.** *Pesquisa Veterinária Brasileira 34(12):1243-1246.* Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: rsantos@vet.ufmg.br

Visceral leishmaniasis is a chronic infectious disease caused by *Leishmania infantum* (synonym: *Leishmania chagasi*) and transmitted by the sandfly *Lutzomyia longipalpis* in Brazil. It is an endemic zoonosis in several regions of the country, including Belo Horizonte (State of Minas Gerais). In urban areas, the domestic dog is susceptible and considered the most important animal reservoir. However, *L. infantum* has been previously diagnosed in other species, including captive primates and canids. This study aimed to evaluate the presence of the agent DNA in captive animals as well as some free ranging animals from the Zoo-Botanical Foundation of Belo Horizonte by Polymerase Chain Reaction. Eighty one blood samples from primates, carnivores, ruminants, edentates, marsupial, and a monogastric herbivore were analyzed. Three primates *Alouatta guariba* (brown howler monkey), and two canids *Speothos venaticus* (bush dog) were positive, demonstrating the importance of leishmaniasis control in endemic areas for preservation of wildlife species in captivity.

INDEX TERMS: Leishmaniasis, *Leishmania infantum*, zoo, visceral leishmaniasis, PCR.

RESUMO.- [Diagnóstico de infecção por *Leishmania infantum* pela reação em cadeia da polimerase em mamíferos silvestres.] A leishmaniose visceral é uma doença infecciosa crônica de mamíferos causada, no Brasil, pelo protozoário *Leishmania infantum* (sinonímia: *Leishmania chagasi*) e transmitida pelo flebótomo *Lutzomyia longipalpis*. Trata-se de uma zoonose endêmica em muitas regiões do Brasil, inclusive em Belo Horizonte, Minas Gerais. Em centros urbanos, leishmaniose visceral acomete principalmente o cão doméstico. Entretanto, *L. infantum* já foi diagnosticada em outras espécies, incluindo canídeos e primatas de cativeiro em zoológicos. Este estudo buscou avaliar a presença do DNA deste agente em animais de cativeiro e de vida livre da Fundação Zoobotânica de Belo Horizonte através da reação em cadeia da polimerase. Foram analisadas oitenta e uma amostras de sangue oriundas de primatas, carnívoros, ruminantes, edentatos, marsupial e herbívoro de estômago simples. Três primatas *Alouatta guariba* (bugio marrom) e dois canídeos *Speothos venaticus* (cachorro-do-mato-vinagre), foram positivos, demonstrando a importância do controle da leishmaniose em áreas endêmicas com a finalidade de conservar a fauna silvestre mantida em cativeiro.

TERMOS DE INDEXAÇÃO: Leishmaniose, *Leishmania infantum*, zoológico, leishmaniose visceral, PCR.

INTRODUCTION

Visceral leishmaniasis (VL) is an important zoonosis characterized by a chronic systemic disease. In Brazil, VL is caused by *Leishmania infantum* (synonym: *Leishmania chagasi*). Its definitive hosts are mammals, mainly domestic

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dogs (*Canis familiaris*) that play a major epidemiological role in urban areas, where it is considered the most important reservoir for human infections (Diniz et al. 2008). Dogs are in close contact with humans and they may be asymptomatic, which increases the risk of transmission. VL is endemic in Belo Horizonte, where it has become a major public health issue (Diniz et al. 2008). Although transmission may occur in the absence of the biological vector (Pangrazio et al. 2009, Silva et al. 2009), usually *Leishmania* spp. is transmitted by an invertebrate vector, which in the case of *L. infantum* in Brazil is the sand fly *Lutzomyia longipalpis* (Rosypal et al. 2003), which is abundant in the metropolitan area of Belo Horizonte (Resende et al. 2006).

L. infantum infection has also been detected in wildlife species in Brazil, including canids (Figueiredo et al. 2008, Luppi et al. 2008, Souza et al. 2010), primates (Malta et al. 2010), and felids (Dahroug et al. 2010). The role of these species as reservoirs is unclear, but some species are susceptible to clinical development of the disease (Luppi et al. 2008, Malta et al. 2010). Furthermore, *L. infantum* has been detected in other groups of wild mammals that are present in urban areas in Brazil, including rodents (Oliveira et al. 2005) and marsupials (Schallig et al. 2007, Santiago et al., 2007) that may also function as potential reservoirs. Leishmaniasis affecting zoo and wildlife has been recently reviewed (Souza et al., 2014).

This study aims to investigate the presence of the *L. infantum* DNA by PCR (Polymerase Chain Reaction) in blood samples from several captive and free-ranging mammal species potentially susceptible to infection at the Zoo-Botanical Foundation of Belo Horizonte (ZBF-BH).

MATERIALS AND METHODS

From April 2011 to August 2012, 81 blood samples were collected from captive or free-ranging wild mammals that underwent medical procedures at the veterinary hospital from the ZBF-BH. A total of 81 blood samples were analyzed: 26 samples from primates, 26 from carnivores (including 14 felids, 10 canids, and 2 mustelids), 10 from edentates, 9 from ruminants, 8 from rodents, 1 from a marsupial, and 1 from a monogastric herbivore. Blood samples were collected into EDTA sterile tubes and kept refrigerated at 4ºC. Blood samples were centrifuged at 1500 g for 10 minutes to separate the buffy coat and extract its DNA by the guanidine method (Pitcher, Saunders and Owen 1989). DNA concentrations were measured and adjusted to 100-500ng/µL. PCR was performed according to Lachaud et al. (2002) to amplify minicircles of kinetoplast DNA from *Leishmania* spp. belonging to the donovani complex using 23µL of PCR Supermix (Invitrogen, Brazil), 0.5µL of 10µM sense primer (5'- CTT TTC TGG TCC CGC GGG TAGG -3'), 0.5µL of 10µM antisense primer (5'- CCA CCT GGC CTA TTT TAC ACCA -3'), 0.7µL of $MgCl_2$, 0.2µL of Taq Polymerase (Invitrogen, Brazil) and 1µL of extracted DNA (100-500ng/µL). PCR products were visualized under UV light after electrophoresis in a 2% agarose gel stained with ethidium bromide. Samples that yielded a 145 bp product were considered positive. Positive and negative controls were included in all reactions.

RESULTS

PCR results and detailed species identification and their respective numbers are shown in Table 1. Five samples, three from *Alouatta guariba* (brown howler monkey) and two from *Speothos venaticus* (bush dog), were positive for leishmanial DNA (Table 1). Therefore, only one primate and one carnivore species were PCR-positive for *Leishmania infantum. The* remaining 10 primate species, seven carnivore species, two edentate species, five ruminant species, three rodent species, one marsupial species, and one monogastric herbivore species were all negative by PCR (Table 1).

Table 1. Detection of *Leishmania infantum* DNA by Polymerase Chain Reaction (PCR) in blood samples from wild mammals from the Zoo-Botanical Foundation of Belo Horizonte. Samples were collected from April 2011 to August 2012

Group	Species	Number of sampled animals	PCR positive
Primata (primates)	*Aotus infulatus*	1	0
	Alouatta guariba	8	3
	Brachyteles hypoxanthus	1	0
	Callicebus personatus	2	0
	Cebus apella xanthosternos	4	0
	Chiropotes satanas	2	0
	Leontopithecus rosalia	1	0
	Mandrillus sphinx	1	0
	Pan troglodythes	2	0
	Pithecia irrorata	2	0
	Saguinus imperator	2	0
Carnivora (carnivores)	*Chrysocyon brachyurus*	4	0
	Leopardus pardalis	3	0
	Mustela putorius furo	2	0
	Panthera leo	4	0
	Panthera onca	1	0
	Panthera tigris altaica	2	0
	Puma concolor	4	0
	Speothos venaticus	6	2
Pilosa (edentates)	*Myrmecophaga tridactyla*	6	0
	Tamandua tetradactyla	4	0
Artiodactyla (ruminants)	*Camellus bactrianus*	1	0
	Cervus elaphus	2	0
	Dama dama	1	0
	Lama glama	2	0
	Oryx gazella	3	0
Rodentia (rodents)	*Dasyprocta agouti*	6	0
	Hydrochaeris hydrochaeris	1	0
	Rattus norvegicus	1	0
Didelphimorphia (marsupial)	*Didelphis albiventris*	1	0
Perissodactyla (monogastric herbivore)	*Tapirus terrestris*	1	0

DISCUSSION

Among eleven different primate species that were sampled, all three positive animals belonged to the same species, namely *Alouatta guariba* (brown howler monkey). *Leishmania* sp. has been previously detected by PCR in the peripheral blood of this species (Malta et al. 2010). Anti-*L. mexicana* IgG antibodies have been detected in two species of the *Alouatta* genus, i.e. *A. pigra* and *A. palliate*, in Mexico (Rovirosa-Hernandez et al. 2013). Leishmaniasis affecting zoo and wildlife species have been recently reviewed (Souza et al., 2014).

Among the carnivore species sampled in this study, only two bush dogs (*Speothos venaticus)* were positive. The bush dog is known to be susceptible to *Leishmania infan-*

tum since previous studies have reported direct detection the agent (Figueiredo et al. 2008, Luppi et al. 2008, Lima et al. 2009, Souza et al. 2010) or detection of anti-*Leishmania* antibodies (Lima et al. 2009, Jusi et al. 2011) in various biological samples. Considering that this is an endangered species according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES - www.cites.org), prevention and control of *L. infantum* infection in captive specimens is extremely important for conservation purposes. Therefore, epidemiological studies, clinical monitoring, and *L. longipalpis* control are extremely important measures in zoos located in endemic areas as is the case of ZBF-BH. The significance of wild canids in the pathobiology of leishmaniasis is reflected by the large number of recent publications on this topic, which includes the report of three wolves (*Canis lupus*) out of 33 sampled in Southwestern Europe that were positive to *L. infantum* by PCR (Sastre et al. 2008). In addition, Sobrino et al. (2008) detected *L. infantum* by PCR in eight of 39 wolves, 23 of 162 foxes (*Vulpes vulpes)* in Spain, whereas Dipineto et al (2007) detected the parasite from 20 of 50 fox carcasses in Italy. Antibodies against *Leishmania* spp. were found in one gray fox (*Urocyon cinereoargenteus*) from the United States, out of 49 sampled (Rosypal et al. 2010). In Brazil, *Leishmania* sp. has been reported in both free-ranging and captive canids. In the State of Minas Gerais, antibodies against *Leishmania* sp. have been detected in hoary zorros (*Lycalopex vetulus*) (Luppi et al. 2008), crab-eating foxes (*Cerdocyon thous*) (Curi et al. 2006, Luppi et al. 2008) and maned wolves (*Chrysocyon brachyurus*) (Curi et al. 2006, Luppi et al. 2008, Curi et al. 2012). In São Paulo State, in addition to the bush dog and maned wolf, *Leishmania* spp. was detected in the crab-eating fox through different techniques such as ELISA, PCR and indirect fluorescent antibody test (IFAT) (Jusi et al. 2011). *L. infantum* was also detected in skin, bone marrow and lymph node samples by PCR from six crab-eating foxes and one bush dog in state of Mato Grosso (Souza et al. 2010). Furthermore, *L. infantum* has been detected in wild felids such as two captive barbary lions (*Panthera leo leo*) in France (Libert et al. 2012), one genet (*Geneta geneta*) and one Iberian lynxes (*Lynx pardinus*) in Spain (Sobrino et al. 2008). In Brazil, it was detected in five captive cougars (*Puma concolor)* and in one captive jaguar *(Panthera onca)* in state of Mato Grosso (Dahroug et al. 2010).

Importantly, none of the edentates, ruminants, rodents, marsupial, and monogastric herbivore sampled in this study were positive. However, some species belonging to these groups had been previously diagnosed with *Leishmania* sp. and, therefore, they may be considered potential reservoirs. Indeed, *Leishmania* sp. infection in opossums has been detected by PCR in 91.6% of 112 sampled *Didelphis* spp. from São Paulo State, and, among these, 71% had anti-*Leishmania* antibodies (Santiago et al. 2007). In a similar study performed in Minas Gerais, five of 20 *Didelphis marsupialis* were PCR positive for *Leishmania* sp., and 24 out of 111 were positive by IFAT (Schallig et al. 2007). *L. infantum* DNA was detected by PCR in 15 out of 54 *Didelphis albiventris* opossums in the State of Mato Grosso do Sul, Brazil (Humberg et al. 2012). *Leishmania* sp. was also detected by PCR in three rodent species, *Rattus rattus*, *Thrichomys apereoides*, and *Oryzomys subflavus* in Brazil (Oliveira et al. 2005), and *Rattus norvegicus* in Greece (Papadogiannakis et al. 2010). In Spain, *Lepus granatensis* were reported to be infected with *L. infantum,* and they were capable of infecting sandflies as confirmed by xenodiagnosis (Molina et al. 2012).

Not much is known about the *Leishmania* sp. cycle in wild animals. Theoretically, exposed animals might be resistant and overcome the parasite; alternatively some host species might allow the protozoa to multiply but not develop disease, being able to transmit amastigotes to the sandfly and thus contribute to the transmission of the disease; whereas other host species might be susceptible, develop disease and also contribute to the dissemination of leishmaniasis. PCR results, which detect only the presence of the parasite DNA, should be analyzed carefully, as it is an extremely sensible test able to detect low concentrations of *Leishmania* DNA, what does not necessarily indicate persistent infection or disease. However, these results are relevant since it clearly indicates exposition of captive wild animals to the parasite. In addition, Luppi et al. (2008) reported previously a case of a captive brush dog from the Zoo-Botanical Foundation of Belo Horizonte (ZBF-BH) that died due to clinical VL. The animal presented cachexia, anemia, generalized hemorrhage and chronic renal failure. *L. infantum* was detected by immunohistochemistry and PCR. A crab-eating fox from São Paulo State, Brazil, also died due to VL. The diagnosis was confirmed by IFAT, PCR and direct observation of amastigotes in macrophages from liver, spleen, lymph nodes and skin (Tenório et al. 2011). Malta et al. (2010) reported a case of confirmed clinical leishmaniasis in a black-fronted titi monkey (*Callicebus nigrifrons*) also from ZBF-BH.

Although blood may not be considered the biological sample of choice for the diagnosis of *Leishmania* sp. infection, the method used in this study is highly sensitive since it amplifies a sequence of the kDNA minicircle that has multiple copies, which increases the sensitivity of the method (Lachaud et al., 2002). Importantly, even with blood samples, PCR is a suitable method for diagnosis of Leishmania sp. infection when compared to other methods (Assis et al., 2010). Finally, in this study, serological methods could not be applied due to the variability of species included in this survey. Furthermore, the use of additional biological samples, such as bone marrow or lymph node aspirates is not feasible due to limitations to manipulate these animals.

CONCLUSION

Leishmania infantum DNA was detected in five out of the 81 blood samples from wild mammals of the ZBF-BH, three positive samples were from *Alouatta guariba* and two from *Speothos venaticus*.

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