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Serological evidence of *Leishmania* infection by employing ELISA and rapid tests in captive felids and canids in Brazil

Natalia Tolentino[a], Guilherme Rafael Gomide Pinheiro[b], Jennifer Ottino[c], Ayisa Rodrigues de Oliveira[e], Carlyle Mendes Coelho[d], Herlandes Penha Tinoco[d], Ricardo Toshio Fujiwara[c], Renato Lima Santos[e], Vitor Márcio Ribeiro[a,f,*]

[a] *Departamento de Medicina Veterinária, Pontifícia Universidade Católica de Minas Gerais, Rua do Rosário, 1081, Bairro Angola, CEP 32630-000 Betim, Minas Gerais, Brazil*
[b] *Departamento de Medicina Preventiva Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Bairro Pampulha, CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[c] *Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Bairro Pampulha, CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[d] *Fundação de Parques Municipais e Zoobotânica de Belo Horizonte, 31365-450 Belo Horizonte, Minas Gerais, Brazil*
[e] *Departamento de Clínica e Cirurgias Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Bairro Pampulha, CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[f] *Santo Agostinho Hospital Veterinário, Avenida Amazonas, 2218, Santo Agostinho, Belo Horizonte, Minas Gerais 30180-003, Brazil*

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ABSTRACT

Visceral leishmaniasis (VL) is a zoonosis and the dog is considered the most important urban reservoir. Cases in cats have been reported, but little is known about *Leishmania* infection and disease in wild felids and canids kept in captivity in endemic areas. Thus, the serological pattern of wild felids and canids kept in captivity at the Belo Horizonte Zoological Garden was investigated using two primary antigens for conventional ELISA: k39 and rKDDR, as well as three serological rapid kits: Dual Path Platform (DPP®) immunochromatographic test, rKDDR Immunochromatographic assay and ELISA SNAP *Leishmania* IDEXX®. A total of 21 serum samples, 13 of wild felids and 8 wild canids of varying age and sex were evaluated. The results obtained in the tests were analyzed by agreement using Kappa coefficient, and between ELISA antigens all the analysis performed had showed significant agreement among both of them, as well between the three immunochromatographic tests. The results demonstrated that there is serological evidence of wild animals seropositive for *Leishmania* antibodies at the Belo Horizonte Zoological Garden, and that all the antigens and rapid tests used can be employed in serological screening for VL in wild felids and canids.

1. Introduction

Leishmaniasis is caused by protozoans of the genus *Leishmania*, which are considered obligatory intracellular parasites, transmitted during the blood meal of female phlebotomine sand flies. The main clinical manifestations are cutaneous and visceral forms that affect animals and men, constituting a zoonosis and a neglected tropical disease (Carvalho et al., 2015; Desjeux, 2004).

The urban cycle of visceral leishmaniasis (VL) is mostly associated with transmission of *Leishmania infantum* from domestic animals, mainly the dog, in which is it associated with canine visceral leishmaniasis (CVL), and man. Therefore, this is a disease of great importance for public health, with estimated 200,000 to 400,000 new human cases annually, and > 50,000 deaths, being one of the most important vector-borne diseases worldwide (World Health Organization, 2017). Importantly, these numbers are underestimated since the disease does not require mandatory reporting in all countries, particularly those with low socioeconomic status, poor surveillance, and lack of proper epidemiological investigation.

Currently, VL is a reality in the urban context in most of the Brazilian territory, affecting man and susceptible animals, but also it is relevant among wild animals kept in captivity which must be considered in surveillance programs, prevention and treatment of the disease (Bresciani et al., 2010; Palatnik-De-Sousa et al., 2001; Tinoco et al., 2018). In this context, it is well known that wild animal species including canids: *Dusicyon vetulus*, *Cerdocyon thous*, *Chrysocyon*

* Corresponding author at: Amazonas Avenue, 2218, Santo Agostinho, Belo Horizonte, Minas Gerais 30180-003, Brazil.
E-mail address: ribeirovm@yahoo.com.br (V.M. Ribeiro).

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brachyurus, *Speothos venaticus*; marsupials: *Didelphis albiventris*; and non-human primates: *Alouatta guariba*, *Callicebus nigrifrons*, *Leontopithecus crysomelas*; play an important role in the transmission cycle of leishmaniasis in the wild (Diniz et al., 2008; Figueiredo et al., 2008; Lombardi et al., 2014; Malta et al., 2010; Mol et al., 2015). Felidae family also had shown to be susceptible to *Leishmania* parasites infection in endemic areas where they are kept in captivity (Souza et al., 2014). Natural infection has been reported in ocelot (*Leopardus pardalis*), puma (*Puma concolor*), jaguar (*Panthera onca*) and African lion (*Panthera leo*) (Dahroug et al., 2011; Dahroug et al., 2010; Libert et al., 2012).

The implementation of health program of captive wild animals including diagnostic and screening tests for VL in enzootic areas should be highly desirable since captive animals in urban centers are considered a public patrimony and possibly a source of infection to the insect vector. Hence, less invasive and more practical diagnostic techniques may be employed to avoid subjecting these animals to stressful situations such as prolonged anesthesia and unnecessary restraint. In this sense, serological approaches as ELISA (enzyme-linked immunosorbent assay) and immunochromatography techniques, molecular diagnostic that employs PCR techniques (polymerase chain reaction) and, when necessary parasitological approaches, histopathology and immunohistochemistry have been performed in wild animals samples (Felix Lima et al., 2009; Lombardi et al., 2014; Luppi et al., 2008; Mol et al., 2015; Quaresma et al., 2011; Rosypal et al., 2010; Sobrino et al., 2008; Souza et al., 2014)

Thus, the scope of this work was to assess the serological status for L. *infantum* infection in felids and wild canids kept at the Belo Horizonte Zoological Garden, employing five different serological methods based on ELISA and immunochromatography assays for the screening of anti-*Leishmania* antibodies in wildlife animals living in an urban environment.

2. Material and methods

2.1. Animals

A retrospective study of serum samples collected between 2015 and 2017 was performed. Twenty-one samples were analyzed, being 13 from felids and 8 from canids, including three pumas (*Puma concolor*), two Siberian tigers (*Panthera tigris altaica*), two jaguars (*Panthera onca*), two ocelots (*Leopardus pardalis*), two African lions (*Panthera leo*), two pampas cat (*Leopardus pajeros*), four bush dogs (*Speothos venaticus*), and four maned wolves (*Chrysocyon brachyurus*). All the animals were adults and kept in captivity at the Belo Horizonte Zoological Garden. Data regarding gender are plotted in Table 1.

2.2. Ethical statement

This experimental protocol has been approved by Institutional Ethics Committee for Animal Experimentation of the Pontifícia Universidade Católica de Minas Gerais (CEUA/PUC MINAS, protocol number 011/2017).

2.3. Samples processing

Blood samples were obtained during handling for general or special examinations. Chemical containment was performed with ketamine (10–12 mg/kg), xylazine (1–2 mg/kg) and butorphanol (0.15–0.2 mg/kg) in the same syringe for felids. For wild canids chemical containment was performed with xylazine and ketamine (6–8 mg/kg + 0.5–1 mg/kg, respectively), also associated. Sera were separated by centrifugation and aliquoted in 1.5 mL microtubes, and stored at -20 °C until serological assays were performed.

2.4. Enzyme linked immunosorbent assay (ELISA)

Sera samples were evaluated for *Leishmania* antibodies detection by using a commercial kit (Safetest® Diagnostics, Brazil) and an ELISA *in house* protocol employing the rK39. The last was performed using 96-well plates coated with rK39 in carbonate buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). After coating, plates were washed four times with PBS containing Tween-20 at 0.05% concentration. Unspecific reactions were inhibited by incubating with 2% bovine serum albumin (BSA) in PBS. Serum samples (1:100 dilution) were added to the well and incubated at 4 °C for 12 h. Plates were then washed and incubated with the secondary antibody (anti-canine IgG - 1:2500 dilution or anti-cat IgG – 1:2500 dilution) for 60 min, followed by another wash, and incubation for 10 min in citrate buffer (0.1 M citric acid, 0.2 M bibasic sodium phosphate, pH 5.0) containing 0.02 g of *o*-phenylenediamine (OPD) and 16 µL de H2O2. Optical density (OD) was measured at 492 nm and cut off was established at three standard deviations above the average OD of the negative controls (see Suplementary Material). The commercial ELISA kit (Safetest® Diagnostics) was used according to the manufacturer's instructions.

2.5. Serological assays employing rapid kits

Three commercial rapid kits for *Leishmania* antibodies detection were used for each sample. DPP® (Dual Path Platform) and rKDDr kits are classified as immunochromatographic tests while ELISA SNAP *Leishmania* IDEXX® is a rapid test that employed a dry ELISA technology. They were employed according to manufacturer's instructions.

2.5.1. Statistical analysis

Statistical comparisons to determine agreement between the tests employed in the two animal family groups were performed using Kappa coefficient (Cohen, 1960). Kappa values were interpreted according to Landis and Koch (1977) with a confidence interval of 95%.

3. Results

3.1. Canids data analysis

Table 1 summarizes results for canids included in the study (five males and three females), in which 5/8 were positive in ELISA for both antigens employed. In rapid tests, 2/8 tested positive for KDDR® Immunochromatographyc assay and SNAP *Leishmania* IDEXX®, and 3/8 were positive in DPP® Test. Two canids presented clinical signs suggestive of Leishmaniasis.

Kappa analysis comparing the agreement between the primary antigens employed in ELISA showed an almost perfect correlation between rK39 and ELISA Safetest® Diagnósticos ($p = 0{,}005$), and a substantial degree of agreement between all the three rapid tests employed ($p < 0{,}001$) (Table 2).

3.2. Felids data analysis

The felids included in the study (seven males and five females), showed 33,33% of positivity in both ELISA primary antigens employed in the study. In rapid tests, 1/8 tested positive for KDDR® Immunochromatographyc assay and SNAP *Leishmania* IDEXX®, and 2/8 were positive in DPP® Test. Only one felid presented clinical signs suggestive of Leishmaniasis (Table 1).

Kappa analysis comparing the agreement between the primary antigens employed in ELISA showed a substantial correlation between rK39 and ELISA Safetest® Diagnósticos ($p = 0{,}03$), and a substantial degree of agreement between all the three rapid tests employed ($p < 0{,}001$) (Table 2).

Table 1
Results obtained for canids and felids in ELISA and rapid tests.

Animal specie	Animal ID	Gender	Common animal name	ELISA Safetest® Diagnósticos	ELISA rK39	KDDR® Immunochromatographyc assay	SNAP *Leishmania* IDEXX®	DPP® Test	Suggestive Leishmaniasis clinical signs?
Canids									
Speothos venaticus	C1	M	Bush dog	+	+	−	−	+	No
Speothos venaticus	C4	M	Bush dog	+	+	+	+	+	No
Speothos venaticus	C8	M	Bush dog	+	+	+	+	+	No
Speothos venaticus	C10	M	Bush dog	+	+	−	−	−	No
Chrysocyon brachyurus	C5	F	Maned wolf	−	−	−	−	−	No
Chrysocyon brachyurus	C6	F	Maned wolf	−	−	−	−	−	Yes
Chrysocyon brachyurus	C7	M	Maned wolf	−	−	−	−	−	No
Chrysocyon brachyurus	C9	F	Maned wolf	+	+	−	−	−	Yes
Felids									
Panthera tigris altaica	F01	F	Siberian tiger	+	+	+	+	+	Yes
Panthera tigris altaica	F17	M	Siberian tiger	−	−	−	−	−	No
Panthera onca	F05	M	Jaguar	−	−	−	−	−	No
Panthera onca	F20	F	Jaguar	−	+	−	−	+	No
Panthera leo	F12	M	Lion	−	−	−	−	−	No
Panthera leo	F19	F	Lion	+	+	−	−	−	No
Puma concolor	F06	M	Cougar/Puma	−	−	−	−	−	No
Puma concolor	F11	M	Cougar/Puma	−	−	−	−	−	No
Puma concolor	F14	F	Cougar/Puma	−	−	−	−	−	No
Leopardus pardalis	F13	M	Ocelot	+	+	−	−	−	No
Leopardus pardalis	F15	F	Ocelot	+	−	−	−	−	No
Leopardus pajeros	F16	M	Ocelot	−	−	−	−	−	No

C = canids; F = felids; F = female; M = male.

Table 2
Kappa values obtained for canids and felids between two different primary antigens in ELISA and three rapid tests.

Technique	Total of animals	ELISA rK39	DPP® Test	ELISA Safetest® Diagnostics	SNAP *Leishmania* IDEXX®	KDDR® Immunochromatographyc Assay
Canids						
ELISA rK39	8	–	NA	5 Agreement 1000 (95%CI)	NA	NA
DPP® Test	8	NA	–	NA	2 Agreement 0,798 (95%CI)	2 Agreement 0,798 (95%CI)
ELISA Safetest® Diagnostics	8	5 Agreement 1000 (95%CI)	NA	–	NA	NA
SNAP *Leishmania* IDEXX®	8	NA	2 Agreement 0,798 (95%CI)	NA	–	2 Agreement 0,798 (95%CI)
KDDR® Immunochromatographyc Assay	8	NA	2 Agreement 0,798 (95%CI)	NA	2 Agreement 0,798 (95%CI)	–
Felids						
ELISA rK39	12	–	NA	3 Agreements 0,625 (95%CI)	NA	NA
DPP® Test	12	NA	–	NA	1 Agreement 0,719 (95%CI)	1 Agreement 0,719 (95%CI)
ELISA Safetest® Diagnostics	12	3 Agreements 0,625 (95%CI)	NA	–	NA	NA
SNAP *Leishmania* IDEXX®	12	NA	1 Agreement 0,719 (95%CI)	NA	–	1 Agreement 0,719 (95%CI)
KDDR® Immunochromatographyc Assay	12	NA	1 Agreement 0,719 (95%CI)	NA	1 Agreement 0,719 (95%CI)	–

CI = confidence interval; NA = not applicable.

4. Discussion

This study evaluated serologic evidences of *Leishmania* sp. infection of wild felids and canids kept in captivity in an urban area endemic for VL. Furthermore, this study evaluated the suitability of different serologic methods for screening of these animals, which is a highly relevant contribution under the perspectives of conservational medicine and public health. Visceral leishmaniasis in dogs is a well-studied disease and the diagnosis relies on well standardized tests (Coura-Vital et al., 2014; Paz et al., 2018). However, for wild animals it is important to evaluate other important criteria before establishing a reliable diagnostic protocol including animal species, management, chemical and physical containment protocols, and stressing conditions, which can turn the screening of these animals laborious (Lombardi et al., 2014; Muñoz-Madrid et al., 2013; Souza et al., 2014).

In endemic areas some clinical signs can suggest the possibility of wildlife infection with *Leishmania* sp.. The Siberian tiger, which had positive results in all serological tests also presented cutaneous lesions, weight loss, reduced appetite, dehydration and weight loss, similar to the clinical signs described for domestic felines (Maroli et al., 2007; Poli et al., 2002). However, once the clinical manifestations might not be uniquely associated with leishmaniasis a confirmatory laboratory test becomes essential for a conclusive diagnosis, which is extremely important considering the animal maintenance in an area with continuous public access. The jaguar and the African lion had no reports of symptomatic disease, which was also verified by other authors that found felines infected by L. *infantum* without clinical manifestations (Dahroug et al., 2011; Dahroug et al., 2010). In fact, reports of VL are rare in wild animals, mainly among felids, with a large proportion of clinically healthy seropositive animals (Braga et al., 2014).

Among the seropositive canids one bush dog (C9) was described with chronic kidney and skin disease, and since this animal did not show any comorbidity these clinical signs might be associated with VL, as previously described, in L. *infantum* infection in other species, including man (Ciaramella et al., 1997; Dahroug et al., 2011; Dahroug et al., 2010; Miró et al., 2014; do Prado et al., 2011). In a distinct manner, one maned wolf (C6) was negative in all performed tests, although presented chronic kidney disease. This data raises the importance of performing complementary tests to confirm VL diagnose (Dhom-Lemos et al., 2019).

Also, two other bush dogs (C4 and C8) that were positive by all five tests performed had no physical or laboratory signs of disease. For this reason, the role of these wild animals as reservoirs of the parasite is considered uncertain (Diniz et al., 2008), although vectors may readily become infected after feeding on infected asymptomatic animals (Mol et al., 2015; Molina et al., 2012).

For *Leishmania* infection serological, molecular and parasitological tests are the most common diagnostic assays (Dantas-Torres et al., 2018; Dantas-Torres et al., 2010; Lombardi et al., 2014; Otranto et al., 2017). Among captive wildlife animals there is not a standardized protocol for investigating the presence of *Leishmania* infected animals, although different methods are currently applied (Souza et al., 2014). In this context there was considerable agreement between k39 or KDDR primary antigens for canids or felids from FZB-BH. Mol et al. (2015) also performed ELISA in maned wolves and bush dogs from the same zoo using a commercial kit (Biomanguinhos®) and rk39, and found 6/9 seropositive animals. In a study with dogs Dantas-Torres et al. (2018) had observed agreement by using DPP® and ELISA SNAP *Leishmania* IDEXX® testing 95 dog serum sample from Pernambuco state, Brazil.

In the present study for both animal families, was observed a substantial correlation between the three rapid tests, DPP®, rKDDR or ELISA SNAP *Leishmania* IDEXX®. In this way the rapid tests used in the study had show potential application for use as screening test for VL in the wild animals from Belo Horizonte Zoological Garden.

The contribution of serological tests in the epidemiological surveys of diseases is of great relevance, particularly in leishmaniasis. The results obtained in this study are consistent in identifying anti-*Leishmania* antibodies in both wild felids and canids, and the inclusion of other serological tests as well as parasitological and molecular diagnostics methods will allow a grater screening to locate the actual scenario of wild animals kept in the Belo Horizonte Zoological Garden.

5. Conclusion

In conclusion, this study demonstrated serological evidence of infection by *Leishmania* sp. in wild canids and felids kept in captivity at the Belo Horizonte Zoological Garden. In addition, according to the agreement between the assays employed, the two primary antigens used in ELISA and the three commercial rapid kits used in this study had show to have potential to be used in leishmaniasis screening in wild

animals.

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Ethical statement

This experimental protocol has been approved by Institutional Ethics Committee for Animal Experimentation of the Pontifícia Universidade Católica de Minas Gerais (CEUA/PUC MINAS, protocol number 011/2017).

Conflict of interest

The authors declare have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2019.100308.

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