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Original Article

Serological study of feline leishmaniasis and molecular detection of *Leishmania infantum* and *Leishmania braziliensis* in cats (*Felis catus*)

Pesquisa sorológica da leishmaniose felina e detecção molecular de *Leishmania infantum* e *Leishmania braziliensis* em gatos (*Felis catus*)

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Abstract

Blood samples and swabs from ocular conjunctiva and mouth were obtained from 64 cats. Of 64 serum samples, 19 were positive for *Leishmania* antibodies by ELISA (29.80%). Eight cats were positive by PCR (12.5%) in swab samples from mouth and/or ocular mucosa. Poor kappa agreement between serological and molecular results (k = 0.16) was obtained. From five positive PCR samples one was *L. braziliensis* and four were *L. infantum.* Phylogenetic analysis performed with the five isolates of *Leishmania*, showed that samples of *L. infantum* isolated from the cats were phylogenetically close to those isolated from domestic dogs in Brazil, while the *L. braziliensis* is very similar to the one described in humans in Venezuela. The study demonstrated that, despite high seropositivity for *Leishmania* in cats living in the study region, poor agreement between serological and molecular results indicate that positive serology is not indicative of *Leishmania* infection in cats. Parasite DNA can be detected in ocular conjunctiva and oral swabs from cats, indicating that such samples could be used for diagnosis. Results of phylogenetic analyzes show that *L. infantum* circulating in Brazil is capable of infecting different hosts, demonstrating the parasite's ability to overcome the interspecies barrier.

Keywords: ELISA, *Leishmania infantum*, *Leishmania braziliensis*, oral and conjunctival swabs, PCR swab.

Resumo

Amostras de sangue e swabs da conjuntiva ocular e oral foram obtidas de 64 gatos. Das 64 amostras de soro, 19 foram positivas para anticorpos contra *Leishmania* por ELISA (29,80%). Oito gatos foram positivos por PCR (12,5%) em amostras de swab da boca e / ou mucosa ocular. Demonstrou-se baixa concordância kappa entre os resultados sorológicos e moleculares (k = 0,16). Das cinco amostras positivas para PCR, uma era *L. braziliensis* e quatro eram *L infantum*. A análise filogenética realizada com os cinco isolados de *Leishmania*, mostrou que amostras de *L. infantum,* isoladas dos gatos, eram filogeneticamente próximas às isoladas de cães domésticos do Brasil enquanto *L. braziliensis* era muito semelhante ao descrito em humanos na Venezuela. O estudo demonstrou que, apesar da alta soropositividade para *Leishmania,* em gatos que vivem na região do estudo, pouca concordância entre os resultados sorológicos e moleculares indica que a sorologia positiva não é indicativa de infecção por *Leishmania* em gatos. O DNA do parasita pode ser detectado na conjuntiva ocular e nas zaragatoas orais de gatos, indicando que essas amostras podem ser usadas para o diagnóstico. Resultados de análises filogenéticas mostram que *L. infantum,* circulando no Brasil, é capaz de infectar diferentes hospedeiros, demonstrando a capacidade do parasita de superar a barreira interespécies.

Palavras-chave: ELISA, *Leishmania infantum*, *Leishmania braziliensis*, swabs oral e conjuntival, PCR de swab.

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Introduction

Visceral Leishmaniasis (VL) is a zoonosis caused by *Leishmania infantum* (Baneth et al., 2008). Dogs are considered the main reservoir of the parasite in domestic and peridomestic areas; however, recent studies have reported cats infected with the protozoan and capable of acting as infectious reservoirs (Maroli et al., 2007; Maia & Campino 2011).

Leishmania infantum is transmitted by the bite of phlebotomine sandflies, primarily by *Lutzomyia longipalpis* and secondarily by *Lutzomyia cruzi* in Brazil (Andrade-Filho et al., 2017). The most frequent clinical signs of leishmaniasis in cats include skin changes, such as papules, nodules, ulcers, and alopecia (Silveira et al., 2015); ocular signs have also been described (Pimenta et al. 2016). *Leishmania* species identified in cats include *L. mexicana, L. venezuelensis, L. braziliensis, L. amazonensis,* and *L. infantum* (Pennisi et al., 2015). *Leishmania* infection shows different clinical presentations in the affected hosts as a result of the *Leishmania* species and the host immune response. *L. braziliensis* is associated with cutaneous and mucosal leishmaniasis and *L. infantum* with visceral and cutaneous (unusual presentation) (Pace, 2014). Notably, it was demonstrated that cats showing dermatological lesions were highly associated with visceral leishmaniasis (Vides et al., 2011).

Studies have examined the seroprevalence of *Leishmania* (Baldini-Peruca et al., 2017; Benassi et al., 2017; Braga et al., 2014; Cardia et al., 2013; Coura et al., 2018; Noe et al., 2015; Oliveira et al., 2015a; Silva et al., 2014; Sobrinho et al., 2012) or the occurrence of natural cases of leishmaniasis (Passos et al., 1996; Savani et al., 2004; Schubach et al., 2004; Souza et al., 2005; Coelho et al., 2010; Silva et al., 2010) in cats in Brazil.

The use of noninvasive sampling techniques is of interest, especially in feline medicine (Athanasiou et al., 2018). Recently *Leishmania* DNA was detected from conjunctival (Benassi et al., 2017) and oral swabs of cats (Persichetti et al., 2018).

The *Leishmania* genus is a slowly evolving monophyletic group and includes important human pathogens (Marcili et al., 2014). Despite the widespread distribution of leishmaniasis among humans and animals in Brazil, little is known about the genetic characteristics of the causative agents. Moreover, phylogenetic studies have focused on human or canine strains (Cortes et al., 2014), while very few of them involve genetic analysis of *Leishmania* obtained from cats (Ceccarelli et al., 2018; Pereira et al., 2020).

Since cats are potential reservoirs of *Leishmania* and no study has identified the presence of *Leishmania* DNA in oral swabs from cats in Brazil, the objectives of the present study were to investigate the occurrence of *Leishmania* antibodies in cats from a region endemic for canine and human leishmaniasis by ELISA and the presence of *Leishmania* DNA using PCR analysis of ocular conjunctiva and oral swab samples. From the genomic sequences of the *Leishmania* isolates, a characterization and evaluation of the phylogenetic relationships between *Leishmania* strains based on the *Leishmania* Internal transcribed spacer 1 (ITS1) gene was made.

Materials and Methods

Animals

The study included 64 cats (*Felis catus*) living in Belo Horizonte and Confins in Minas Gerais State, Brazil. These municipalities are endemic for canine leishmaniasis (CanL) and have also registered human cases of leishmaniasis (Silva, 2015; Belo Horizonte, 2019). The collection of the biological samples was carried out with the permission of the owners and the experimental procedures were approved by the Ethics Committee on Animal Experimentation, UFMG (CETEA 242/2014). All procedures performed were in accordance with the ethical standards of the institution or practice at which the studies were conducted

Cats were clinically evaluated before sample collection and were clinically healthy upon examination. Cats of either sex and of any breed and age were included in this study.

Sample collection

Using disposable syringes and needles, 5 mL blood samples were collected from the jugular or cephalic veins of the cats and transferred into tubes without anticoagulant. Blood samples were centrifuged at 1500 x *g* for 6 min, and 1 mL of the separated serum was transferred into plastic microtubes and stored at -20°C for serological analysis.

Sterile swabs manufactured for use in bacteriological isolation were used for the collection of specimens for *Leishmania* DNA analysis. Swabs were rubbed on the lower portion of both ocular conjunctiva and on the right of the oral mucosal of the lips of each cat. The ends of the swabs were separated and stored in 1.5 mL microtubes at -20°C until processing for PCR analyses. DNA extraction was performed using Gentra® Puregene® Tissue Kits (Qiagen), according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were tested for *Leishmania* antibodies by ELISA (Voller et al., 1979). Soluble *Leishmania* antigen was produced from promastigote forms of *Leishmania* (*Leishmania*) *infantum* strain MHOM/BR/1967/BH46. Previously known positive (optical density [OD]: 0.340) and negative (OD: 0.060) sera were used as controls. The cutoff point was calculated as the median of positive and negative sera, plus four times standard value (Solano-Gallego et al., 2007). Values above OD: 0.955 were considered positive. All samples were tested in duplicate.

PCR assays

Leishmania kinetoplast DNA

All ocular conjunctiva, and mouth mucosa cells were analyzed by PCR to detect *Leishmania* minicircle kinetoplast DNA (kDNA), generating a 120bp fragment as described by Degrave et al. (1994) and Passos et al. (1996). Briefly, PCR assays were performed in a total volume of 25 µL containing 5 µL DNA template, 0.2 mM DNTPs, 1.5 mM $MgCl_2$, 1.5 µL DMSO 5%, 0.4 µM each primer, buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.0) and 1.25 U of Taq DNA polymerase (Invitrogen™, Carlsbad, CA, USA). PCR conditions were as follows: initial denaturation at 94ºC for 5 min, followed by 35 cycles at 94ºC for 30 sec, 61ºC for 1 min, and 72ºC for 3 min, with a final extension at 72ºC for 10 min. Negative (no DNA) and positive (0.01 and 1.0 pg of *Leishmania* DNA) PCR controls were included in each set of reactions. Positive control for the PCR reactions included DNA extracted from promastigote forms of the *L. infantum* (MHOM/BR/74/PP75) strain.

Leishmania internal transcribed spacer 1 (ITS1)

Further analyses from PCR positive samples were performed to identify the *Leishmania* species. DNA extracted from cat samples was used as a template for detection of *Leishmania* via amplification of a 300-350 bp fragment of an intergenic region in the *Leishmania* genome (ITS1), using the primers LITSR: 5'-CTGGATCATTTTCCGATG-3' and L5.8S: 5'-TGATACCACTTATCGCACTT-3' (El Tai et al., 2000; Schönian et al., 2003). Positive controls for the PCR reactions included DNA extracted from promastigote forms of the following *Leishmania* strains: *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), *L. infantum* (MHOM/BR/74/PP75), and *L. guyanensis* (MHOM/BR/75/M4147).

Gel electrophoresis of PCR products

Amplification products were subjected to electrophoresis in 2% agarose gels, with a 100 bp DNA Step Ladder as a molecular weight size standard and stained with ethidium bromide (10 mg/mL). To identify *Leishmania* species ITS1 PCR products were digested with HaeIII (10 U/µL) using the conditions recommended by the supplier (Promega). Restriction digest profiles were analyzed by electrophoresis in 3% agarose gels, stained with ethidium bromide (10 mg/mL), and compared with *Leishmania* reference strains.

Sanger sequencing

Amplicons from positive samples by ITS1-PCR were purified using QIAquick PCR Purification kit (QIAGEN, Germantown, MD, USA), following the manufacturer's instructions. Purified fragments were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 8 µL containing 10 ng of purified PCR product and 5.0 pmol of the forward and reverse primers. Sequences were generated using an ABI 3730xl DNA Analyzer, and the software Finch TV (Geospiza, Inc., USA) was used to analyze electropherograms and align sequences with others obtained from GenBank.

Phylogenetic analysis

We retrieved from GenBank database fifty-nine published sequence of internal transcribed spacer 1 and 5.8S ribosomal gene of *Leishmania infantum* and eighteen complete sequence of *Leishmania braziliensis*, sampled of cats, dogs and human in worldwide. The *L. infantum* sequences were collected from the Middle east (Turkey, Iran, Israel, Tunisia), Southeast Europe (Italy, Spain, Romania and Greece) and South America (Argentina and Brazil) while the *L. braziliensis* sequences were collected from the South America (Brazil and Venezuela) and North America (United States of America).

The sequences were aligned for ClustalW method using the BioEdit v7.0.5 software (Sanchez-Villeda et al., 2008). A maximum likelihood (ML) phylogenetic analysis was performed through the IQ-TREE software (Nguyen et al., 2015). Prior the analysis, the jModelTest 2.1.7 software (Darriba et al., 2012), was used to select the best-fitted nucleotide substitution model for the dataset. The ML estimate tree was subsequently visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Support was evaluated using the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010) and the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013) with 1000 replicates each.

Statistical analysis

The kappa agreement between the results of PCR from oral and ocular conjunctiva swabs was determined using Cohen's kappa test. The results are expressed as follows: no agreement ($k < 0$), poor agreement ($0 < k < 0.2$), fair agreement ($0.2 < k < 0.4$), moderate agreement ($0.4 < k < 0.6$), substantial agreement ($0.6 < k < 0.8$), and almost perfect agreement ($k > 0.8$) (Altman, 1999). Analyses were conducted using R (R version 3.1.1, R Development Core Team, New Zealand).

Results

Of 64 feline serum samples collected, 19 were positive for *Leishmania* antibodies based on ELISA results, representing 29.80% seropositivity.

PCR analysis revealed that oral swabs from four animals (6.25%) were positive for *Leishmania*, only one of which was also positive by ELISA. PCR analysis of the ocular conjunctiva revealed that two cats were positive in the right eye, two in the left eye, and one animal positive in both eyes, totaling five positive cats (7.81%), one of which was also positive by PCR from an oral swab sample. In total, eight cats were positive by PCR (12.5%). There was poor agreement between the serological and molecular test results ($k = 0.16$). Results are summarized in Table 1.

Table 1 . Results of the diagnostic tests for *Leishmania* applied to 64 cats from Belo Horizonte and Confins, Minas Gerais, Brazil.

Diagnostic Testing Technique				Nº of observations
ELISA	OM PCR	LOM swab PCR	ROM swab PCR	
-	-	-	-	41
-	-	+	-	1
-	+	-	-	2
+	-	-	-	16
-	-	+	+	1
+	-	-	+	2
-	+	+	-	1
+	+	-	-	1

Abbreviations: OM: oral mucosa; LOM: left ocular mucosa, ROM: right ocular mucosa, PCR polimerase chain reaction.

PCR RFLP ITS1 detected *Leishmania* DNA in five of eight kDNA positive samples. It was not possible to perform PCR analysis on DNA of three cats. At the species level, four samples were identified as positive for *L. infantum* and one for *L. braziliensis,* the latter detected in an oral swab. The five positive samples were subjected to sequencing

analysis, which confirmed four as *L. infantum* and one as *L. braziliensis*. *L. infantum* was found in both oral and ocular conjunctival swabs. Results and GenBank accession numbers for the nucleotide sequences are summarized in Table 2.

Table 2. Results of *Leishmania* species isolation of 5 cats from Belo Horizonte and Confins, Minas Gerais, Brazil.

Cat	Source of biological material	Species isolated	GenBank accession number
C6	OM	*L. infantum*	MN245031
C7	OM	*L. infantum*	MN245032
C10	LOM/ROM	*L. infantum*	MN245033
C21	OM	*L. braziliensis*	MN245034
C46	OM	*L. infantum*	MN245035

Abbreviations: OM: oral mucosa; LOM: left ocular mucosa, ROM: right ocular mucosa.

Phylogenetic analysis (Figure 1) showed that *Leishmania* spp. found in this study form two monophyletic groups. The first group is composed of *L. infantum* isolates, and the subdivision of isolates has no relation to origin or with the species in which the parasite was found. However, in the first group, composed basically of *L. infantum*, a clade of *L. braziliensis* isolates from Brazilian dogs and a human from the USA was formed.

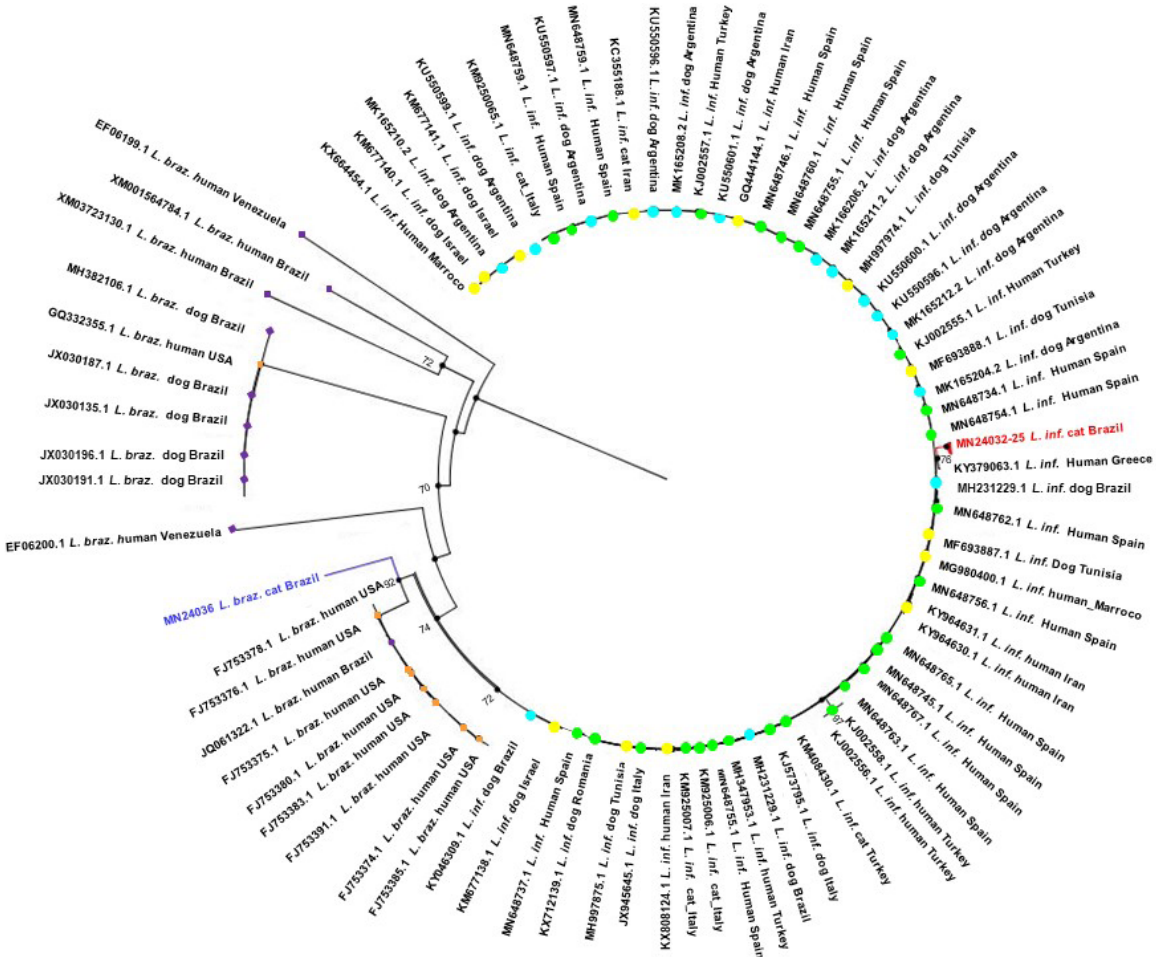

Figure 1. The Phylogeny of *Leishmania* species according to the maximum-likelihood (ML), tree was conducted based on the multiple sequence alignment of ITS-rDNA by IQTREE 1.6.1. Only bootstrap values of higher than 80% are indicated on each branch. Distance represents the number of base substitutions per site. The tree was constructed by using the Kimura 2-parameter model. Besides the haplotypes reported in this study, a number of accession numbers from other countries were applied in phylogenetic analysis.

In addition, the second group was formed exclusively by *L. braziliensis* isolates from humans from Brazil, Venezuela and the USA.

Although, the *L. infantum* isolates from cats detected in this study form a single cluster, similar to the human isolate from Greece and the isolates found in dogs in Brazil, they are phylogenetically distant from the *L. infantum* strains found in domestic cats from other countries as Italy, Turkey and Iran (Supplementary Material Figure S1 A). However, the *L. braziliensis* isolate found in a Brazilian domestic cat is very similar to the strains circulating in Venezuela (Supplementary Material Figure S1 B).

Discussion

Studies in Brazil and other countries have investigated the occurrence of feline leishmaniasis; however, many questions remain to be answered (Maia & Campino, 2011; Pennisi & Persichetti, 2018). The present study was conducted in Belo Horizonte and Confins, Minas Gerais, which are both regions where cases of leishmaniasis have been registered in humans and dogs. Although dogs are the main hosts and reservoir for *Leishmania infantum* (Maia & Campino, 2011), our study demonstrated the presence of DNA from this parasite in conjunctiva swabs from cats and, for the first time in Brazil, in oral mucosa swabs, calling attention to the presence of parasite DNA in these sites and suggesting that these tissues could be sampled for diagnosis. Moreover, sequence analysis identified *L. braziliensis* in one oral swab and *L. infantum* in an eye conjunctiva sample.

Leishmania infection results in different clinical manifestations depending on the immune response of the host and the protozoan species detected, being *L. infantum* associated with visceral and cutaneous *Leishmaniasis* (unusual) and *L. braziliensis* with cutaneous manifestation of the disease (tegumentary leishmaniasis) (Loeuillet et al., 2016; Pace, 2014). Cats with dermatological lesions might present visceral leishmaniasis (Vides et al., 2011) and both cited species have been identified in cats (Pennisi & Persichetti, 2018). Positive cats subjected to xenodiagnosis assays can infect the *Leishmania* vector (Maroli et al., 2007) and *Leishmania braziliensis* can be detected in skin samples from experimentally chronically infected cats (Simões-Mattos et al., 2005). Recently, in the same region of the present study, *L. braziliensis* DNA was detected in conjunctival swab of dogs (Souza et al., 2019), highlighting the importance of detecting *Leishmania* species not only in dogs but also in cats, to better understand the role of these hosts in the epidemiology of tegumentary leishmaniasis.

In our study, 40.63% of evaluated cats were seropositive for antibodies against *Leishmania* by ELISA. The frequency of feline seropositivity varies between regions, and caution is advised when comparing this result with those of other studies, since variables, such as serological technique, the level of endemicity, and cross-reaction with other *Leishmania* species or parasites, can interfere with the results. The seropositivity rate for *Leishmania* antibodies has been reported as 16.1% in Spain (Martín-Sánchez et al., 2007), 0% in Angola (Lopes et al., 2017), 6.7% in Jerusalem (Nasereddin et al., 2008), 24.27% in Iran (Mohebali et al., 2017), 3.87% to 21.6% in Greece (Diakou et al., 2009; Chatzis et al., 2014), and 25.3% in Italy (Spada et al., 2013). In Brazil, the reported frequency of seropositivity for feline *Leishmania* antibodies determined by ELISA varies from 3.9% in Pernambuco (Silva et al., 2014) to 11.50% and 15.23% in São Paulo (Costa et al., 2010; Sobrinho et al., 2012). The discrepancies are due to different levels of endemicity, type of feline populations included in the study and, mainly, different methodologies, especially validation of the serological test (Pennisi, 2015). A previous study conducted in Minas Gerais, showed that 54% of the cats were seropositive for *Leishmania* antibodies by immunofluorescence antibody test (Coura et al., 2018). The high frequency of feline *Leishmania* seropositivity observed in the present study can be explained by possible occurrence of co-infections with other pathogens (Sobrinho et al., 2012), or the assay performed may have exhibited cross-reaction with antibodies against other parasites (Andrade et al., 2009; Luciano et al., 2009; Soares et al., 2016), although cats do not mount a humoral response like dogs (Solano-Gallego et al., 2007).

Studies of feline leishmaniasis using PCR have used DNA from blood (Martín-Sánchez et al., 2007; Maia et al., 2010; Sherry et al., 2011; Spada et al., 2013; Akhtardanesh et al., 2017), ocular conjunctiva (Chatzis et al., 2014; Oliveira et al., 2015b), urine (Persichetti et al., 2018) and lymphoid organs (Coelho et al., 2011; Noe et al., 2015; Mohebali et al., 2017; Otranto et al., 2017); however, this is the first report of *Leishmania* DNA identification in oral mucosa swabs from cats in Brazil, supporting recently found results (Persichetti et al., 2018). This finding is important, since there is no consensus on the ideal biological sample for use in cytological or molecular diagnosis of feline leishmaniasis (Braga et al., 2014; Chatzis et al., 2014; Noe et al., 2015; Akhtardanesh et al., 2017). Low production of antibodies by cats (Solano-Gallego et al., 2007) and cross-reactions with other microorganisms that may occur highlights the need for different diagnostic tools for feline leishmaniasis (Noe et al., 2015). Furthermore, we performed sequencing

analysis of five DNA samples, which confirmed the presence of *L. infantum* and *L. braziliensis* DNA in swab samples evaluated, indicating that infections with these two species might occur in the region of the study.

Phylogenetic analyzes for *L. infantum* showed low divergence between parasite isolates in cats from Minas Gerais, Brazil. However, there was no direct correlation between the isolates of this study and the other *L.infantum* isolates from domestic cats described in countries such as Italy, Iran or Turkey (Can et al., 2016; Spada et al., 2016; Mohebali et al., 2017). These results show the genetic complexity of *Leishmania* spp. populations which circulate in a different geographical area but in the same species (Cupolillo et al., 1998; Ortuño et al., 2019). On the other hand, isolated samples from Brazilian domestic cats were phylogenetically close to isolated samples from domestic dogs, which indicates that possibly *L. infantum* circulating in Brazil is present in both species. Mohammadiha et al.(2013) identified similar results for dogs and humans, demonstrating the parasite's ability to overcome the interspecies barrier.

Regarding *L. braziliensis*, the feline sample found in the country is very similar to the isolate described in Venezuela. Aguilar et al. (1989) have already observed that the *L. braziliensis* strain responsible for an outbreak of cutaneous leishmaniasis that affected humans, dogs and horses in Venezuela was the same strain that circulated in Brazil. The circulation of *L. braziliensis* between the countries occurs through the presence of the hematophagous vectors *Lutzomyia panamensis* in Venezuela and *Lutzomyia intermedia* in Brazil, which perform the blood support in horses and eventually dogs. These hosts transit between Brazil and Venezuela border and have peridomestic habits, allowing the maintenance of the parasite in the environment. Because of this, the strains of *Leishmania* spp. circulating between the hosts of the two countries have high genetic similarity.

Our study found poor agreement between the results generated using ocular conjunctiva and those from oral mucosa swabs. Other studies have reported inconsistent results when using different types of tissue samples for PCR-based diagnosis compared with parasitological and serological data (Akhtardanesh et al., 2017; Baldini-Peruca et al., 2017; Braga et al., 2014; Chatzis et al., 2014; Diakou et al., 2009; Maia et al., 2010; Noe et al., 2015; Oliveira et al., 2015b; Sherry et al., 2011), indicating that sampling from different tissues together with serological analysis is required when screening for feline leishmaniasis. The study was conducted with 64 cats and use ELISA for serological tests, and these factors should be considered as limitations for further conclusions of cat role in the epidemiology of leishmaniases.

Conclusion

Our findings revealed a high seroprevalence for leishmaniasis in cats and parasite DNA was detected in feline oral and conjunctival swabs. Moreover, sequence analysis detected *L. infantum* and *L. braziliensis* in these sites. These results highlight that oral mucosa swabs can also be used for diagnosis, alongside another diagnostic tool. Poor agreement between serological and molecular results indicates that positive serology cannot be used as indicative of *Leishmania* infection in cats. The results of phylogenetic analyzes show that *L. infantum* circulating in Brazil is capable of infecting different hosts, demonstrating the parasite's ability to overcome the interspecies barrier. In addition, the *L. braziliensis* transits between hosts with peridomestic habitats, present on the Brazil and Venezuela border, allowing the maintenance of the parasite in the environment. There is a need to continue the standardization of diagnostic procedures for feline leishmaniasis and for further investigation of the role of cats as reservoirs of *Leishmania.*

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1: Phylogenetic trees of Brazilian field sequences and foreign sequences.

This material is available as part of the online article from http://www.scielo.br/rbpv