

Molecular detection of *Leishmania infantum* in donkeys and mules under semiarid conditions in Brazil

Detecção molecular de *Leishmania infantum* em asininos e muare sob condições semiáridas

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Abstract

Visceral leishmaniasis is a parasitic zoonosis that mainly affects poorest and most vulnerable populations, and domestic dogs are considered to be the main source of infection to the vector and therefore humans. However, several studies have investigated the role of other vertebrate hosts in the disease cycle. In this context, the aim of the present study was to conduct a survey of *Leishmania infantum* infection in donkeys and mules living in a semiarid region of Brazil. Whole blood sampled from 72 equids (65 donkeys and 7 mules) was used to perform molecular diagnosis using the real-time polymerase chain reaction (qPCR) technique. A total of 25% of the samples (18/72) were positive through qPCR, but there were no significant differences between the species (donkeys or mules), sex (male or female) and abandonment situation of the animals (yes or no). Donkeys and mules living under semiarid conditions have high frequency of *L. infantum* infection. It is therefore worth assigning importance to these species in the epidemiological cycle of visceral leishmaniasis, either as potential reservoirs or just as an abundant food source for vectors.

Keywords: Epidemiology, equids, leishmaniasis, qPCR, zoonosis.

Resumo

A leishmaniose visceral é uma zoonose parasitária que afeta principalmente populações mais pobres e vulneráveis, e os cães domésticos são considerados as principais fontes de infecção para o vetor e, portanto, para os humanos. Porém diversos estudos têm pesquisado o papel de outros hospedeiros vertebrados no ciclo da doença. Neste contexto, objetivou-se realizar um levantamento da infecção por *Leishmania infantum* em asininos e muare, vivendo em região semiárida do Brasil. Foi utilizado sangue total de 72 equídeos (65 asininos e 7 muare) para a realização de diagnóstico molecular por meio da técnica de Reação em Cadeia de Polimerase em Tempo Real (qPCR). Um total de 25% das amostras (18/72) resultaram positivas na qPCR, porém não houve diferença significativa entre as espécies (asininos e muare), sexo (macho e fêmea) e situação de abandono dos animais (sim ou não). Asininos e muare, vivendo em condições semiáridas, apresentam alta frequência de infecção por *L. infantum*, sendo válido atribuir importância a essas espécies no ciclo epidemiológico da leishmaniose visceral, seja como um reservatório em potencial, seja apenas como uma fonte alimentar abundante para os vetores.

Palavras-chave: Epidemiologia, equídeos, leishmaniose, qPCR, zoonose.

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Introduction

Leishmaniasis is a parasitic zoonosis caused by several species of the genus *Leishmania*, endemic in many regions of the world, especially in underdeveloped and developing countries. They are considered to be a neglected disease that affects the poorest and most vulnerable populations that do not have adequate access to healthcare services (WHO, 2017).

In areas that are endemic for visceral leishmaniasis, domestic dogs have been reported as the main source of infection to the vector and, therefore, humans (Pace, 2014). However, several studies have demonstrated the possibility that other vertebrate hosts might harbor parasites and participate in the disease cycle (Quaresma et al., 2011; Gao et al., 2015; Kenubih et al., 2015; Rohousova et al., 2015). In this regard, Limeira et al. (2019) conducted a systematic analysis on several studies that indicated the presence of different *Leishmania* species, such as *L. braziliensis*, *L. infantum* and *L. siamensis* parasitizing donkeys, horses and mules. This may suggest that the parasites are adapting to these new hosts (Soares et al., 2013). In equids, the clinical manifestations described so far have comprised a benign cutaneous form of the disease, with lesions observed at the inoculation site, of self-limiting nature (Limeira et al., 2019).

Semi-arid regions of emerging countries generally have low rates of socioeconomic development, which leads the population of these areas to indiscriminately exploit the natural resources of native vegetation (Silva et al., 2016b). This gives rise to environmental imbalance that can influence the epidemiological aspects of vector-borne diseases such as dengue, leishmaniasis and Chagas disease. In addition, the functions previously performed by equids in these regions have been replaced by forms of mechanical traction, which has resulted in abandonment of these animals and uncontrolled population growth, especially of donkeys (Carneiro et al., 2018). In periods of drought, these abandoned animals can move close to homes in search of food, water and/or shelter, which increases the food sources for phlebotomines (sandflies) in peridomestic environments. This can generate a risk of infection by *Leishmania* spp. in the population of these areas.

Thus, considering the specific characteristics of semiarid regions, the increasing numbers of abandoned equids and the proximity of the population to these animals, the objective of this study was to conduct a survey of *L. infantum* infection among donkeys and mules living under semiarid conditions.

Material and Methods

The procedures developed in this study were previously submitted to and analyzed by the Ethics Committee for Animal Use of the Health and Rural Technology Center, Federal University of Campina Grande (CEUA/CSTR/UFPG), in accordance with the current rules and regulations, and the protocol approved was registered under the number 011/2019.

Study area

This study was conducted in rural areas of the municipality of Salgueiro (8°4'20" S; 39°7'36" W), in the semiarid region of the state of Pernambuco, northeastern Brazil. This municipality has a human development index (HDI) of 0.669 (IBGE, 2020) and is considered endemic for human visceral leishmaniasis (Brasil, 2021).

Animals, clinical evaluation and sampling

Through non-probabilistic sampling, for convenience, a total of 72 equids (65 donkeys and 7 mules) living in rural areas of the municipality of Salgueiro were included in the study. Both animals living under the care of owners, with shelter and food provided, and animals in situations of abandonment, without fixed shelter and living freely off the local vegetation (Caatinga), were evaluated. The latter were captured using lassoes, by a trained and experienced person. This study was developed during the months of October 2018 and March 2019.

From each animal, 4 mL of whole blood were collected directly from the jugular vein using a vacuum tube containing EDTA. The samples were then packed in Styrofoam boxes with ice and sent to the laboratory, where they were frozen and kept at a temperature of -20 °C until the time of use. At the time when the animals were being restrained for blood collection, they were evaluated by means of visual inspection and palpation, seeking to observe any possible skin lesions.

Molecular diagnosis

Genetic material, i.e. deoxyribonucleic acid (DNA), was extracted from the samples by means of the DNeasy blood and tissue kit (Qiagen®, Hilden, Germany), using an aliquot of 180 µL of the whole blood, following the manufacturer's recommendations. A qualitative real-time polymerase chain reaction (qPCR) was performed as previously described by Silva et al. (2016a), using the primers Linf kDNA-F 5'-GGCGTTCTGCAAAATCGGAAA-3', Linf kDNA-R 5'-CCGATTTTTGGCATTGTTGGTCGAT-3' and Linf kDNA_FAM-5'-TTTTGAACGGGATTCTG-3' in order to amplify the kinetoplast minicircle gene (kDNA) of *L. infantum*. A culture of *L. infantum* was used as a positive control, and ultrapure water was used as a negative control.

Samples that were found to be positive through qPCR were then sequenced by means of conventional PCR for *L. infantum*, using the following specific 447-base primers, with a larger amplification fragment of the kinetoplast minicircle gene (kDNA): MC1 (5'-GTTAGCCGA TGGTGGTTG-3') and MC2 (5'-CACCCATTTTCCGATTG-3') (Cortes et al., 2004; Benassi et al., 2018), following the methodology of Benassi et al. (2018).

The sequencing reaction was performed with the MC1 and MC2 primers described above, using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Capillary electrophoresis was performed using the Genetic Analyzer 3130 and POP-7 polymer (Applied Biosystems), as described by Platt et al. (2007).

Nucleotide sequences were elaborated using the Sequencing Analysis software v5.3.1 (Applied Biosystems), constructed and aligned using the BioEdit software (Hall, 1999). These sequences were then compared with *L. infantum* strains obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

A phylogenetic tree was generated using the software Seaview4 (Gouy et al., 2010), in which its tree was built using the Bio Neighbor-Joining method and Kimura 2p model bootstrap with 1,000 repetitions. This was visualized through FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/>). Phylogenetic reconstruction included sequences of *Leishmania* for comparison.

Statistical analysis

Prevalence ratios were used to measure effects (Coutinho et al., 2008) and Fisher's exact test (with significance level of 5%) was used to investigate the existence of associations between the dependent variable and the independent variables (species, sex and abandonment situation), using the "epiR" package (Stevenson et al., 2020) in the statistical program R, version 3.5.1 (R Core Team, 2020).

Results

Out of the total number of samples analyzed, 25% (18/72) presented genetic material of *L. infantum* in peripheral blood according to the qPCR technique. However, none of the animals demonstrated any visible clinical alterations at the time of the sampling.

In analyses on the data according to categories, 23.08% (15/65) of the donkeys and 42.86% (3/7) of the mules were positive; regarding sex, 22.62% (12/53) of the males and 31.58% (6/19) of the females were positive; and regarding the abandonment situation, 35.29% (6/17) of the animals in this condition and 21.82% (12/55) of those who were not in this condition tested positive in qPCR, but without any statistical differences between the categories (Table 1).

Because of the low quantity of amplicons, it was only possible to sequence genetic material from one of the positive samples. The phylogenetic tree thus constructed demonstrated that the *L. infantum* found in this sample had evolutionary relationships with others available in GenBank (Figure 1).

Discussion

Leishmania spp. infection has been described in equids in various regions of the world, with a combined global prevalence of 25% (CI 15-35%), according to a meta-analysis conducted by Limeira et al. (2019). That prevalence was equal to what was found in this study for *L. infantum* infection. Other studies that used molecular tests (PCR) have also indicated that *L. infantum* is able to infect significant percentages of equids: Gao et al. (2015) described a prevalence of 21.63% in the Jiashi desert, China; Benassi et al. (2018) found a prevalence of 15% in healthy horses

Table 1. Frequency by category (species, sex and abandonment status) of *L. infantum* detected through qPCR in donkeys and mules living under semiarid conditions in Brazil.

Variables	Categories	Number of animals	Positives (%)	Prevalence Ratio (CI 95%)	p-value*
Species	Mules	65	15 (23,08)	1,86 (0,71 - 4,87)	0,356
	Donkeys	7	3 (42,86)		
Sex	Males	53	12 (22,64)	1,39 (0,61 - 3,19)	0,539
	Females	19	6 (31,58)		
Abandonment situation	Yes	55	12 (21,82)	1,62 (0,72 - 3,66)	0,338
	No	17	6 (35,29)		
Overall		72	18 (25)	-	-

*Fisher's exact test ($\alpha=5\%$).

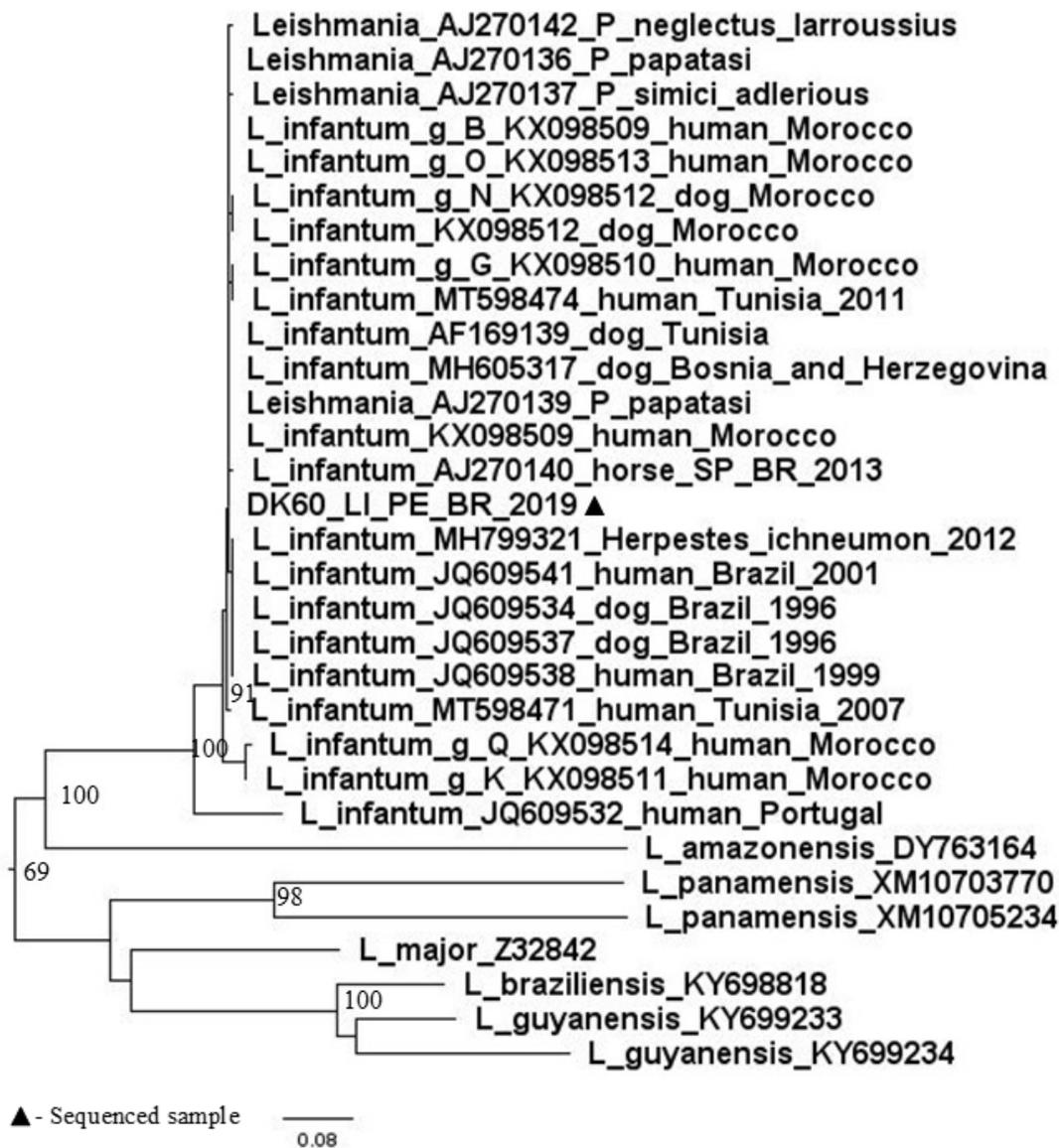


Figure 1. Phylogenetic analysis on *L. infantum* detected in peripheral blood samples from donkeys, constructed through the Bio Neighbor-Joining method and Kimura 2p model bootstrap with 1,000 repetitions.

and Escobar et al. (2019) reported a prevalence of 14.3% in southeastern and southern Brazil. These findings suggest that these domestic animals participate in the visceral leishmaniasis cycle. It is interesting to note that in these studies cited, most of the infected equids did not present apparent clinical manifestations (Gao et al., 2015; Benassi et al., 2018); while in others, clinical signs such as cutaneous lesions, nodules and lymphadenopathy could be seen. This was similar to the findings among dogs living in areas of occurrence of visceral leishmaniasis in southern Brazil (Escobar et al., 2019).

Several techniques with different methodologies have been described, both for the purposes of cross-sectional prevalence studies (Truppel et al., 2014; Aharonson-Raz et al., 2015; Evers et al., 2017; Nardoni et al., 2019) and for making clinical diagnoses of skin diseases involving *Leishmania* spp. infection. (Müller et al., 2009; Soares et al., 2013; Menezes et al., 2019). The results have been heterogeneous, especially those from serological tests, due to the methodological variations used (Limeira et al., 2019). Molecular techniques have become increasingly popular within research, however, despite being very effective for making the diagnosis of leishmaniasis (Galluzzi et al., 2018), these techniques still have a high cost, which makes it unfeasible to use them on a larger scale, such as in prevalence studies.

Over the five-year period from 2015 to 2019, 41 cases of human visceral leishmaniasis were recorded in the municipality where the present study was conducted. This represented 4.77% of the total number of cases reported throughout the state of Pernambuco during this period (Brasil, 2021), thus demonstrating that the disease in humans is endemic in this region. Therefore, the detection of genetic material of *L. infantum* in 25% of the samples analyzed is a worrying finding, as it indicates that donkeys and mules living in rural areas of the semiarid region of Brazil are exposed to the vector *Lutzomyia longipalpis* and are frequently infected. This suggests that parasites of the genus *Leishmania* are becoming adapted to these hosts (Soares et al., 2013).

From analysis on the data according to categories (Table 1), the crude frequency was higher among mules, females and animals in abandonment situations, although no statistical differences between the categories was detected when using the Fisher test ($p > 0.05$). However, these statistical analyses should be interpreted with caution, considering the limitations imposed by the small quantity of samples collected, due to the low number of animals found during visits to farms and the difficulty in capturing the abandoned animals.

Our finding that these equids did not present clinical signs suggestive of leishmaniasis at the time of blood collection was in line with data from several other studies (Sgorbini et al., 2014; Oliveira et al., 2017; Benassi et al., 2018; Nardoni et al., 2019). This demonstrating that even when infected, these animals remain asymptomatic, which contributing to the idea that in these hosts, the immune response that is mounted is effective against parasites (Fernández-Bellon et al., 2006). On the other hand, in some reports on cutaneous leishmaniasis in horses, lesions arise or progress when females are pregnant and regress immediately after delivery or abortion. Likewise, use of immunotherapy may aggravate the clinical picture of the disease, thus indicating that stressful factors reduce the immune response to infection (Barbosa-Santos et al., 1994; Müller et al., 2009; Reuss et al., 2012). Therefore, further research is needed in order to better analyze this immune response in equids and to try to understand at what point in the life of these hosts their protection levels would decrease to a level that would allow emergence of clinical signs and the possibility of transmission of the protozoa to other hosts. If such situations can develop, these equids would then fit within the concept of "reservoir hosts", as described by Ashford (1996).

According to Ashford (1996), merely identifying hosts that are infected by *Leishmania* spp. is not enough to define them as reservoirs. Certain ecological factors relating to the hosts are particularly important for enabling them to become reservoirs, such as their geographical distribution and habitat, and the local climate, as well as host population age structure, density, dispersion, movements and social structure. The donkeys and mules used in this study present many of these characteristics, given that they can be found both in urban and in rural areas; their population density has increased significantly, due to abandonment and uncontrolled reproduction (Carneiro et al., 2018); and they move over long distances in their search for food and water. They are therefore exposed to the vectors that are present in a considerably large region.

However, to be characterized as a reservoir host, an infected individual must possess the ability to infect at least one other individual, on average (Ashford, 1997), and this requirement has not yet been reported for equids. Experimentally, Cerqueira et al. (2003) infected four females of *Equus asinus* aged 3 to 4 months with an inoculum of 10^8 promastigote forms of *L. chagasi* (synonym *L. infantum*) (strain IOCLC2455) per kg of body weight, intravenously. However, in xenodiagnoses at follow-ups conducted 2, 4, 6, 8, 10 and 12 months later, the researchers did not recover any flagellated forms of the parasite in females of *Lu. longipalpis*. This suggested that donkeys are devoid of importance as a reservoir in the transmission chain for visceral leishmaniasis (Cerqueira et al., 2003).

However, the above-mentioned experiment used promastigote forms, which have low infectivity in relation to amastigote forms, and was based on data (dose and route of administration of the inoculum) from previous studies on dogs, due to the lack of research on the species *E. asinus*. Thus, further research should be done taking into account important ecological factors such as population density and the proximity of these animals to dogs in peridomestic environments. Studies on invertebrate vectors in semiarid regions and on the immune response levels of donkeys and mules to *L. infantum* infection should also be conducted.

Conclusion

Donkeys and mules living under semiarid conditions present high frequency of *L. infantum* infection, and their habits of life allow them to present some characteristics necessary to become a reservoir for the disease. However, investigations on specific characteristics of these species in semiarid environments are needed. Although transmission of infection from parasitized equids has not yet been proven, it is worth ascribing some importance to donkeys and mules in the epidemiological cycle of visceral leishmaniasis in the semiarid region of northeastern Brazil, either as potential reservoirs or just as an abundant food source for vectors, which in the end converge for introduction, reintroduction or increase of cases of the disease in a given region.

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