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Clécio Henrique Limeira

Detecção molecular de *Leishmania infantum* e *Trypanosoma cruzi* em equídeos em condições semiáridas

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutor em Ciência e Saúde Animal.

Prof. Titular Dr. Clebert José Alves
Orientador

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CLÉCIO HENRIQUE LIMEIRA

DETECÇÃO MOLECULAR DE LEISHMANIA INFANTUM E TRYPANOSOMA CRUZI EM EQUÍDEOS EM CONDIÇÕES SEMIÁRIDAS

Tese apresentada ao Programa de Pós-Graduação em Ciência e Saúde Animal como pré-requisito para obtenção do título de Doutor em Ciência e Saúde Animal.

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BANCA EXAMINADORA:

Prof. Dr. Clebert José Alves (Orientador - PPGCSA/UFCG)

Prof. Dr. Sergio Santos de Azevedo (Examinador Interno - PPGCSA/UFCG)

Prof. Dr. Inácio José Clementino (Examinador Interno - UFPB)

Profa. Dra. Raizza Barros Sousa Silva (Examinadora Externa - FACENE)

Dra. Maria Luana Cristiny Rodrigues Silva (Examinadora Externa - UFCG)

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André Henrique Limeira*

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RESUMO

A leishmaniose e a doença de Chagas são infecções causadas por protozoários pertencentes aos gêneros *Leishmania* e *Trypanosoma*, respectivamente, considerados os mais importantes dentro da família Trypanosomatidae, devido seu caráter zoonótico. Classificadas como doenças tropicais negligenciadas, ocorrem com maior prevalência em países pobres, causando problemas de ordem econômicos, social e político. A presente tese foi dividida em três capítulos, que objetivaram descrever aspectos epidemiológicos e do diagnóstico da infecção por *Leishmania infantum* e *Trypanosoma cruzi* em equídeos. No capítulo I, foi desenvolvida uma revisão sistemática com metanálise sobre as características clínicas e a prevalência da leishmaniose em equídeos. Os principais sintomas da leishmaniose em equídeos são cutâneos (pápulas, nódulos, úlceras, crostas), autolimitantes e localizadas principalmente na cabeça e membros. A metanálise evidenciou uma prevalência combinada de 25%. Como conclusão, evidenciou-se que a leishmaniose em equídeos é uma doença benigna, e deve ser incluída como diagnóstico diferencial para doenças cutâneas nessas espécies. No capítulo II realizou-se um levantamento da infecção por *Leishmania infantum* em asininos e muares em condições semiáridas do Brasil. Sangue total de 72 equídeos (65 asininos e 7 muares) foi usado para 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, não havendo diferença significativa entre as espécies, sexo e situação de abandono dos animais (sim ou não). Asininos e muares vivendo em área rural do semiárido do Brasil apresentam alta frequência de infecção por *L. infantum*, sendo válido atribuir importância a essas espécies no ciclo epidemiológico da leishmaniose. No capítulo III objetivou-se detectar a infecção por *Trypanosoma cruzi* em asininos e muares de áreas rurais do semiárido brasileiro. Sangue total de 72 equídeos (65 asininos e 7 muares) foram analisadas por Nested Polymerase Chain Reaction (nested-PCR). Um total de 51,39% das amostras (37/72) resultaram positivas. A análise filogenética identificou os tipos TcI e TcII de *T. cruzi*. Esse é o primeiro relato de infecção por *T. cruzi* em asininos e muares no Brasil, e o modo de vida deles, a adaptabilidade dos vetores na Caatinga e as características socioeconômicas da população do semiárido criam interações que podem favorecer a transmissão e a sobreposição dos ciclos silvestre, peridoméstico e doméstico de infecção de *T. cruzi*.

PALAVRAS-CHAVE: Diagnóstico molecular; Epidemiologia; Equídeos; Leishmaniose; Doença de Chagas; Zoonoses.

ABSTRACT

Leishmaniasis and Chagas disease are infections caused by protozoa belonging to the genera *Leishmania* and *Trypanosoma*, respectively, considered the most important within the Trypanosomatidae family, due to their zoonotic character. Classified as neglected tropical diseases, they occur with greater prevalence in poor countries, causing economic, social and political problems. This thesis was divided into three chapters, which aimed to describe epidemiological and diagnostic aspects of infection by *Leishmania infantum* and *Trypanosoma cruzi* in equids. In chapter I, a systematic review with meta-analysis about the clinical characteristics and prevalence of leishmaniasis in equids was developed. The main symptoms of leishmaniasis in equids are cutaneous (papules, nodules, ulcers, crusts), self-limiting and mainly located on the head and limbs. The meta-analysis showed a combined prevalence of 25%. In conclusion, it was shown that leishmaniasis in equids is a benign disease and should be included as a differential diagnosis for skin diseases in these species. In chapter II, a survey of *Leishmania infantum* infection in donkeys and mules in semi-arid conditions in Brazil was carried out. Whole blood from 72 equidae (65 donkeys and 7 mules) was used to perform molecular diagnosis through the Real Time Polymerase Chain Reaction (qPCR) technique. A total of 25% samples (18/72) were positive in the qPCR, with no significant difference between species, sex and abandonment status of the animals (yes or no). Donkeys and mules living in rural area of the semiarid region of Brazil have a high frequency of infection by *L. infantum*, and it is valid to attribute importance to these species in the epidemiological cycle of leishmaniasis. Chapter III aimed to detect *Trypanosoma cruzi* infection in donkeys and mules from rural areas of the Brazilian semiarid region. Whole blood from 72 equidae (65 donkeys and 7 mules) were analyzed by Nested Polymerase Chain Reaction (nested-PCR). A total of 51.39% samples (37/72) were positive. Phylogenetic analysis identified the TcI and TcII types of *T. cruzi*. This is the first report of *T. cruzi* infection in donkeys and mules in Brazil, and their way of life, the adaptability of vectors in the Caatinga and the socioeconomic characteristics of the semi-arid population create interactions that can favor the transmission and overlap of wild, peridomestic and domestic cycles of *T. cruzi* infection.

KEY-WORDS: Molecular diagnosis; Epidemiology; Equidae; Leishmaniasis; Chagas disease; Zoonoses.

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INTRODUÇÃO GERAL

A leishmaniose e a doença de Chagas são infecções causadas por protozoários pertencentes à ordem Kinetoplastida, família Trypanosomatidae, dos gêneros *Leishmania* e *Trypanosoma*, respectivamente. Ambos possuem um ciclo heteroxênico, no qual ocorre a participação de hospedeiros invertebrados e vertebrados. São considerados os gêneros mais importantes dentro da família Tryponosomatidae, por incluírem espécies patogênicas para humanos e animais domésticos (VOTÝPKA *et al.*, 2015). As duas enfermidades constam no rol das doenças tropicais negligenciadas (DTN's), que ocorrem com maior prevalência em países pobres localizados entre as latitudes de 35ºN e 35ºS, como consequência de fatores econômicos, sociais e políticos (LINDOSO; LINDOSO, 2009).

Mais de 20 espécies do gênero *Leishmania* já foram incriminadas como agente etiológico das leishmanioses, e a via de transmissão mais comum requer a participação de vetores flebotomíneos, sendo as principais formas da doença a leishmaniose visceral (ou calazar) e a leishmaniose cutânea ou tegumentar. Em humanos, a manifestação clínica mais comum é a forma cutânea, enquanto a forma visceral é a mais grave e quase sempre fatal se não tratada (WORLD HEALTH ORGANIZATION - WHO, 2017). Além dos humanos, a leishmaniose acomete diversos outros mamíferos, e embora o cão doméstico seja considerado o principal reservatório, várias pesquisas têm buscado identificar outros hospedeiros vertebrados que possam albergar os parasitas, incluindo os equídeos (QUARESMA *et al.*, 2011; LIMEIRA *et al.*, 2019).

Já a doença de Chagas é causada pelo *Trypanosoma cruzi* e é considerada uma zoonose parasitária endêmica em 21 países da América, transmitida por insetos hemípteros da subfamília *Triatominae,* principalmente dos gêneros *Triatoma, Panstrongylus* e *Rhodnius.* Além da via vetorial, outras vias incluem transmissão oral por alimentos contaminados (principal via de transmissão no Brasil), transfusão sanguínea, transmissão congênita, transplante de órgãos e acidentes laboratoriais (WHO, 2015). A doença de Chagas pode evoluir em três ciclos: o silvestre, no qual diversas espécies de mamíferos selvagens albergam o parasita, o peridoméstico e o doméstico, sendo que nesses últimos os animais domésticos (cães e gatos, por exemplo) e os humanos são os principais hospedeiros (ORGANIZAÇÃO PAN-AMERICANA DE SAÚDE – OPAS, 2019).

O estudo foi desenvolvido no semiárido do Nordeste brasileiro, visto que é uma região que detém características que favorecem a ocorrência de DTN's, como baixo Índice de Desenvolvimento Humano (IDH), acesso inadequado aos serviços de saúde, saneamento básico, água potável, telefonia, internet, transporte, entre outros, e uso indiscriminado de recursos naturais (SILVA *et al*., 2016; OPAS, 2019; BRASIL, 2020). Outro fator a ser considerado é o crescente nível de abandono de equídeos, principalmente asininos em áreas da caatinga, acarretando crescimento populacional descontrolado (CARNEIRO *et al*., 2018) e, consequentemente, mais fonte de alimentação para invertebrados vetores de doenças. Portanto, é necessário realizar pesquisas que forneçam informações a respeito da participação desses animais domésticos na cadeia epidemiológica da leishmaniose e doença de Chagas, uma vez que a relação entre os hospedeiros, vetores e ambiente podem colocar em risco a saúde de populações mais vulneráveis.

Dessa maneira, o objetivo deste trabalho foi descrever aspectos relacionados ao diagnóstico da infecção por *Leishmania infantum* e *Trypanosoma cruzi* em equídeos de áreas rurais do semiárido brasileiro. Para isso, a tese foi dividida em três capítulos: o primeiro corresponde a uma revisão sistemática com metanálise dos aspectos clínicos e epidemiológicos da leishmaniose em equídeos, escrito em forma de artigo científico e publicado no periódico "Brazilian Journal of Veterinary Parasitology". O segundo capítulo consiste em um artigo científico que teve como objetivo realizar o diagnóstico molecular da infecção por *Leishmania infantum* em asininos e muares em localidades rurais do semiárido brasileiro, e foi submetido ao periódico "Veterinary Parasitology". E o terceiro capítulo é composto por um artigo científico que objetivou determinar a infecção por *Trypanosoma cruzi* em asininos e muares em localidades rurais do semiárido brasileiro, e foi submetido ao periódico "Zoonoses and Public Healh".

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CAPÍTULO I

Clinical aspects and diagnosis of leishmaniasis in equids: a systematic review and meta-analysis

Clinical aspects and diagnosis of leishmaniasis in equids:
a systematic review and meta-analysis

Aspectos clínicos e diagnóstico da leishmaniose em equídeos: uma revisão sistemática e metanálise

Clécio Henrique Limeira[1,2*]; Clebert José Alves[1]; Sérgio Santos de Azevedo[1]; Carolina de Souza Américo Batista Santos[1]; Marcia Almeida de Melo[1]; Rafael Rodrigues Soares[1]; Nathanael Natércio da Costa Barnabé[1]; Gabriel de Queiroz Rodrigues[1]

[1] Programa de Pós-graduação em Ciência e Saúde Animal, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande – UFCG, Patos, PB, Brasil

[2] Instituto Federal de Educação, Ciência e Tecnologia do Pará – IFPA, Santarém, PA, Brasil

Abstract

Leishmaniases are a group of diseases of zoonotic importance caused by over 20 species of protozoa of the genus *Leishmania*, in which domestic dogs are considered to be the main reservoir for the disease. However, the involvement of other vertebrates as reservoirs for these parasites has also been investigated. Therefore, the objective of the present study was to carry out a systematic review with meta-analysis on occurrences of leishmaniasis in equids. The case reports described animals with cutaneous symptoms of leishmaniasis (papules, nodules, ulcers or crusts) that regressed spontaneously, located mainly on the head and limbs, from which three species of protozoa were identified in the lesions: *Leishmania braziliensis, Leishmania infantum* and *Leishmania siamensis*. In turn, the meta-analysis showed a combined prevalence of 25%, although with high heterogeneity among the studies, which was attributed to the use of different methods for diagnosing the disease. Leishmaniasis in equids is a benign disease but it should be included in the differential diagnosis of cutaneous diseases among these species. Seroepidemiological studies are important in investigating and monitoring suspected exposure of these hosts to the parasite, especially in endemic areas. However, there is also a need to standardize diagnostic methods.

Keywords: Horse, diagnosis, *Leishmania*, meta-analysis, zoonosis.

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* Autor para correspondência: Clécio Henrique Limeira. Programa de Pós-graduação em Ciência e Saúde Animal, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande – UFCG, Av. Universitária, Santa Cecília, CP 61, CEP 58708-110, Patos, PB, Brasil.
E-mail: cleciolimeira@hotmail.com

Resumo

As leishmanioses são um grupo de doenças de importância zoonótica causadas por mais de 20 espécies de protozoários do gênero *Leishmania*, sendo o cão doméstico considerado o principal reservatório da doença. No entanto, diversas pesquisas têm investigado o envolvimento de outros vertebrados como reservatórios do parasita. Portanto, o objetivo do presente estudo foi realizar uma revisão sistemática com meta-análise da ocorrência de leishmaniose em equídeos. Os relatos de caso descreviam animais com sintomas cutâneos de leishmaniose (pápulas, nódulos, úlceras, crostas) que regrediam espontaneamente, localizadas principalmente na cabeça e membros, sendo identificadas três espécies do protozoário nas lesões: *Leishmania braziliensis, Leishmania infantum* e *Leishmania siamensis*. Por sua vez, a meta-análise evidenciou uma prevalência combinada de 25%, porém com alta heterogeneidade entre os estudos, atribuída às diferenças nos métodos utilizados no diagnóstico da doença. A leishmaniose em equídeos é uma doença benigna, porém deve ser incluída no diagnóstico diferencial de doenças cutâneas nessas espécies. Os estudos soroepidemiológicos são importantes para investigar e monitorar a suspeita de exposição desses hospedeiros ao parasita, principalmente em áreas endêmicas, porém há necessidade de padronização dos métodos de diagnóstico.

Palavras-chave: Equino, diagnóstico, *Leishmania*, meta-análise, zoonose.

Introduction

According to the World Health Organization (WHO, 2017), leishmaniases are a group of parasitic diseases caused by over 20 different species of Leishmania spp., protozoa that are transmitted mainly through the bite of sandflies. Four forms of the disease are known: visceral leishmaniasis (or kala-azar), post-kala-azar dermal leishmaniasis, cutaneous leishmaniasis and mucocutaneous leishmaniasis.

In humans, the most common clinical manifestation is the cutaneous form, which is considered endemic in 44% of the countries that notified cases of the disease in 2015 (WHO, 2017); while the visceral form is the most severe, nearly always fatal if untreated, and is endemic in 38% of the countries that reported occurrence of the disease in that same year (WHO, 2017). In addition to affecting humans, leishmaniasis also affects several domestic mammals. Infected dogs are the most important reservoirs for the parasite in urban areas, which makes them the main source of infection for people living in endemic areas (PACE, 2014).

However, several studies have sought to identify other vertebrates that can host and participate in the cycle of these protozoa (QUARESMA et al., 2011; GAO et al., 2015; KENUBIH et al., 2015; ROHOUSOVA et al., 2015). Among the investigations that have sought new possible reservoirs, there have been reports on equids showing clinical manifestations of cutaneous leishmaniasis (KOEHLER et al., 2002; SOLANO-GALLEGO et al., 2003; MÜLLER et al., 2009; SOARES et al., 2013). Furthermore, prevalence studies conducted in Europe (FERNÁNDEZ-BELLON et al., 2006; LOPES et al., 2013; SGORBINI et al., 2014), Asia (GAO et al., 2015; AHARONSON-RAZ et al., 2015), Africa (MUKHTAR et al., 2000; KENUBIH et al., 2015; ROHOUSOVA et al., 2015) and South America (AGUILAR et al., 1989; FEITOSA et al., 2012; TRUPPEL et al., 2014; OLIVEIRA et al., 2017; BENASSI et al., 2018) have demonstrated that horses (Equus caballus), donkeys (Equus asinus), mules (Equus asinus caballus) and ponies (E. caballus) are parasitized by different species of Leishmania, such as Leishmania braziliensis, Leishmania infantum and Leishmania siamensis, including cases of mixed infections.

The importance of research on leishmaniasis in equids lies in the fact that these species of domestic animal, just like dogs and cats, are in close contact with humans, which may be through use as a means of transportation or for work or leisure activities. Moreover, the low socioeconomic level of the population living in endemic areas for the disease could generate risks of infection across the zoonotic cycle, such that equids would play the role of either potential reservoirs or sources of food for sandflies in peridomestic areas.

Therefore, the objective of the present study was to carry out a qualitative and quantitative synthesis (with meta-analysis) based on a systematic review of the literature. Greater clarification regarding the main clinical aspects of leishmaniasis in equids and methods for diagnosing it was sought, with a view to enabling support for future studies on this subject.

Materials and Methods

Study design

The present study consisted of a systematic review of the literature, with synthesis and analysis of clinical findings and meta-analysis on the quantitative data available in articles from indexed journals, both from Brazil and from other countries. The study was conducted based on the methodological recommendations of PRISMA: the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (MOHER et al., 2009).

Article eligibility

Articles published in indexed journals were considered eligible if they consisted of case reports and cross-sectional studies describing the clinical and epidemiological characteristics (prevalence, species, sex and age) and diagnostic methods that are used to identify equids naturally infected with Leishmania spp. There were no restrictions regarding the year in which the study was developed or published, the language or the country where the study was conducted.

The types of publications included were complete articles, short communications and case reports that addressed issues within the following criteria: (I) information on the clinical presentation of leishmaniasis in equids; (II) prevalence of the disease in equid populations; or (III) diagnosis of leishmaniasis in equids. Reviews of the literature, research notes, editorials, experimental assays and other types of publications not within the inclusion criteria were excluded.

Information sources and search strategies

Considering the pre-established inclusion criteria, the process of identifying articles was developed using the PubMed, SciELO, ScienceDirect, Scopus and Web of Science databases. The following combination of search terms in English was used: {leishmania OR leishmaniosis} AND {equids OR equine OR horses OR donkeys OR mules}. The citations of studies thus identified, containing title and abstract, were saved in BibTex format and were exported to a bibliographic manager for subsequent selection. The searches were conducted between October 19 and 26, 2018.

Selection of studies and data extraction

A bibliographic manager tool was used to exclude duplicate records. After this stage, two researchers selected studies independently based on an analysis of titles and abstracts, followed by a full reading of the text. Through this full evaluation of the texts, other studies were excluded because they did not meet the eligibility criteria. Occurrences of divergences between the two researchers were resolved by reaching a consensus.

To make it easier to extract and analyze the data, the articles selected were divided into two groups: the first included all case reports of leishmaniasis in equids, for qualitative synthesis; while the second included cross-sectional studies, for quantitative synthesis and meta-analysis.

Data extraction was conducted independently by two researchers and the information was added to a previously developed electronic spreadsheet. The qualitative data extracted from the first group of articles comprised the authors, year of publication, country, clinical characteristics (type and location of lesions), etiological agent and identification of animals (species, sex and age). In turn, the quantitative data extracted from the second group of articles comprised the references (authors and year of publication), country where the study was conducted, sample size, number of positive animals, prevalence (%) and diagnostic methods.

Data analysis

The qualitative data were analyzed using descriptive statistics, by means of absolute and percentage distributions, to characterize the clinical aspects of leishmaniasis in equids and methods for diagnosing it. The primary outcome for the quantitative data was the crude prevalence of leishmaniasis in equids, with a 95% confidence interval (95% CI).

Heterogeneity was assessed using Cochran's Q test and was quantified through the I2 test of Higgins and Thompson. The combined estimates and 95% confidence interval were calculated based on the random-effects model through the inverse of variance, using the DerSimonian-Laird method. Funnel plots were also analyzed visually and Egger's test was applied, as alternatives for identifying possible biases. All analyses were conducted using the R statistical software (version 3.5.1), through the RStudio software interface (version 1.1.463).

Results

The initial search of databases and study selection are presented in Figure 1. Out of the total number of studies searched (n = 531), 29 met the eligibility criteria and were divided as follows: 11 studies described the clinical characteristics of leishmaniasis in equids, and thus were included in the qualitative synthesis; and another 18 were considered to be cross-sectional studies (prevalence studies), with sufficient data for quantitative synthesis and meta-analysis.

Qualitative synthesis of the clinical aspects of leishmaniasis in equids

The 11 studies included in the qualitative synthesis were conducted in seven different countries (Germany, Brazil, Spain, United States, Puerto Rico, Portugal and Switzerland) and reported occurrences of cutaneous leishmaniasis in a total of 22 equids
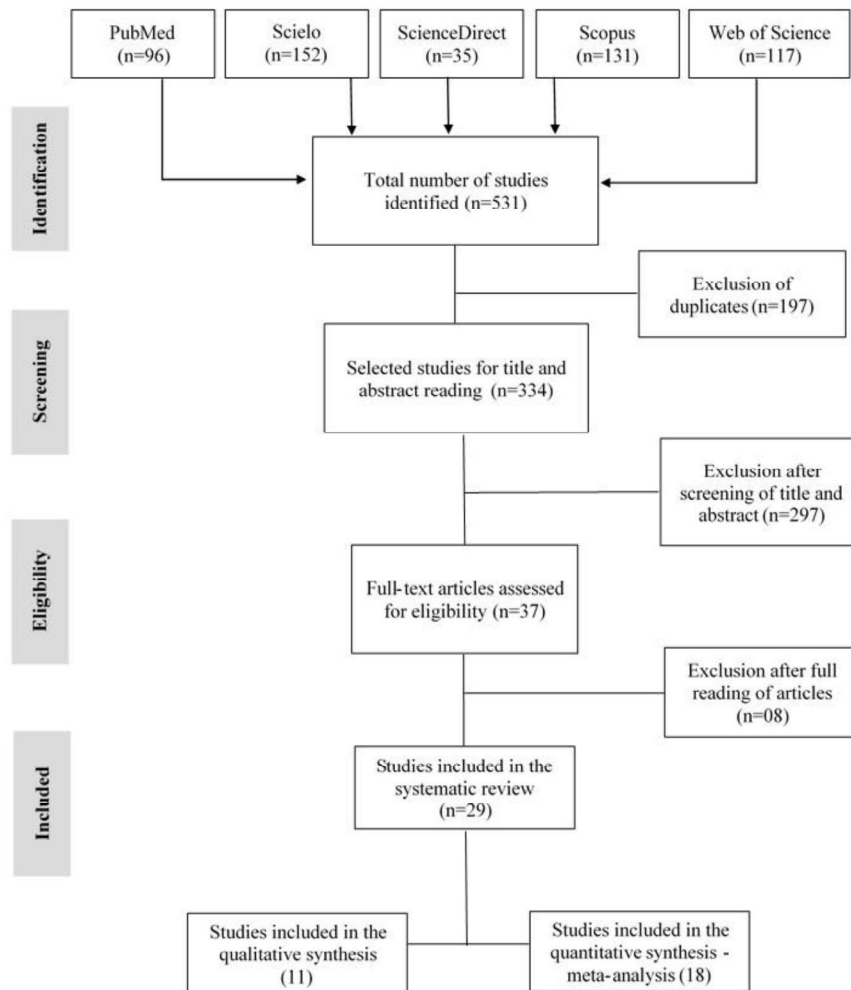
Figure 1. Flow chart of the search, selection and inclusion process for studies in the systematic review.

(*E. caballus*), of which 15 were male (68.18%) and 7 were female (31.82%), with ages between 0 and 2 (n = 2), 2 and 5 (n = 8), 5 and 10 (n = 9) and over 10 years (n = 3).

The major clinical manifestations described in the studies were limited to the skin, and included ulcers (n = 10), nodules (n = 7), crusts (n = 2), papules (n = 1), areas of alopecia (n = 1), presence of exudate (n = 1) and pruritus (n = 1). Most lesions were described as multiple, although single lesions were also observed in some studies, distributed over different parts of the body. The protozoa identified in the studies were *Leishmania braziliensis, Leishmania infantum, Leishmania siamensis* and also mixed infection by *L. braziliensis* and *L. infantum*. Further details of the clinical factors (characteristics and locations of lesions), methods used for diagnosis and etiological agents are described in Table 1.

Table 1. Characteristics of the studies included in the qualitative synthesis regarding leishmaniasis in equids.

References		Clinical aspects		Diagnostic	
Authors (year)	Country	Clinical manifestation	Location	Method	Etiological agent
Yoshida et al. (1988)	Brazil	Ulcers	Prepuce	Parasitological	Not identified
Yoshida et al. (1990)	Brazil	Ulcers	Unknown	Parasitological	Leishmania braziliensis
Barbosa-Santos et al. (1994)	Brazil	Nodules and ulcers	Genital organs, limbs, nasal cavity, neck and jaw	Parasitological + IFAT	Leishmania braziliensis
Ramos-Vara et al. (1996)	Puerto Rico	Ulcers, crusts and nodules	Pinna, neck, maxilla and shoulder	Parasitological	Not identified
Koehler et al. (2002)	Germany	Nodules and ulcers	Eyelid	Parasitological	Leishmania infantum
Solano-Gallego et al. (2003)	Spain	Papules, nodules, alopecia, ulcers and crusts	Face, axillary and inguinal regions	Parasitological + ELISA	Leishmania infantum
Rolão et al. (2005)	Portugal	Ulcers	Pelvic limb	Parasitological + PCR	Leishmania infantum
Müller et al. (2009)	Germany Switzerland	Nodules	Head, flank, axilla, ear and thorax	Parasitological + PCR	Leishmania siamensis
Reuss et al. (2012)	United States	Nodules and ulcers	Pinna, neck, shoulders and withers	Parasitological + PCR	Leishmania siamensis
Soares et al. (2013)	Brazil	Ulcer, exudation and pruritus	Pelvic limb and vulvar region	Parasitological + PCR + ELISA + IFAT	L. braziliensis and L. infantum
Gama et al. (2014)	Portugal	Nodules and ulcers	Face	Parasitological + DAT + PCR	Leishmania infantum

Quantitative synthesis and meta-analysis on leishmaniasis prevalence in equids

The studies included in this stage were conducted in Brazil (n = 10), China (n = 1), Spain (n = 1), Ethiopia (n = 2), Greece (n = 1), Israel (n = 1), Italy (n = 1), Portugal (n = 1), Sudan (n = 1) and Venezuela (n = 2). In one of these studies (AGUILAR et al., 1989), data collected in both Brazil and Venezuela were published. In turn, in two other studies (TRUPPEL et al., 2014; BENASSI et al., 2018), two different diagnostic techniques were used. We therefore considered the use of these different techniques to constitute different studies and, for this reason, the initial total of 18 studies identified was then counted as 21 in the quantitative analysis phase.

Table 2 summarizes the main characteristics of the studies included in the meta-analysis. In evaluating the prevalence results through Cochran's Q test (in which p = 0) and the I2 statistic of Higgins and Thompson (in which I2 = 99.5%), high heterogeneity was observed among the studies.

Table 2. Quantitative synthesis regarding the main characteristics of the studies included in the meta-analysis.

References	Country	Sample	Positive	Prevalence (%)	Diagnosis
Aguilar et al. (1984)	Venezuela	28	6	21,43	Parasitological
Aguilar et al. (1989)	Brazil	26	8	30,77	Parasitological
Aguilar et al. (1989)	Venezuela	32	9	28,13	Parasitological
Mukhtar et al. (2000)	Sudan	96	66	68,75	DAT
Fernández-Bellon et al. (2006)	Spain	112	16	14,29	ELISA
Vedovello et al. (2008)	Brazil	55	42	76,36	DAT
Kouam et al. (2010)	Greece	773	2	0,26	ELISA
Feitosa et al. (2012)	Brazil	466	68	14,59	ELISA
Lopes et al. (2013)	Portugal	173	7	4,05	DAT
Sgorbini et al. (2014)	Italy	277	18	6,50	IFAT
Truppel et al. (2014)	Brazil	227	25	11,01	ELISA
Truppel et al. (2014)	Brazil	227	37	16,30	PCR
Acosta et al. (2014)	Brazil	20	0	0,00	IFAT

Aharonson-Raz et al. (2015)	Israel	319	6	1,88	DAT
Gao et al. (2015)	China	37	8	21,62	PCR
Kenubih et al. (2015)	Ethiopia	15	5	33,33	DAT
Rohousova et al. (2015)	Ethiopia	20	2	10,00	PCR
Oliveira et al. (2017)	Brazil	257	62	24,12	IFAT
Evers et al. (2017)	Brazil	398	183	45,98	IFAT
Benassi et al. (2018)	Brazil	40	40	100,00	PCR
Benassi et al. (2018)	Brazil	40	1	2,50	IFAT

Therefore, a random-effects model was used, according to subgroup, to conduct the meta-analysis. This model yielded a combined prevalence of leishmaniasis in equids of 25% (CI: 15-35%), among the studies included (Figure 2). To assess the possible causes of heterogeneity, the studies were divided into subgroups according to the technique that had been used for the diagnosis: parasitological; enzyme-linked immunosorbent assay (ELISA); direct agglutination test (DAT); indirect fluorescence antibody test (IFAT); and polymerase chain reaction (PCR). These results are shown in Figure 2.

The visual analysis on the funnel plot (Figure 3) showed that there was asymmetrical distribution among the 21 studies, thus demonstrating the possibility of publication bias, which was confirmed through applying Egger's test ($p = 0.01$).

Discussion

The qualitative analysis on the case reports showed that all the equids were affected by the cutaneous form of leishmaniasis (Table 1), presenting lesions that began with papules and nodules and progressed to ulcers, with presence of crusts, alopecia, exudate and pruritus as a result of the evolving inflammatory process that was underway. There is a consensus among researchers that the common infection site of *L. braziliensis* is the skin, while *L. infantum* is responsible for visceral infection (KOEHLER et al., 2002). However, the present review shows that this has not been observed among equids, given that all the reports cited in this review

showed predominance of the cutaneous form of the disease, regardless of which species of *Leishmania* was identified.
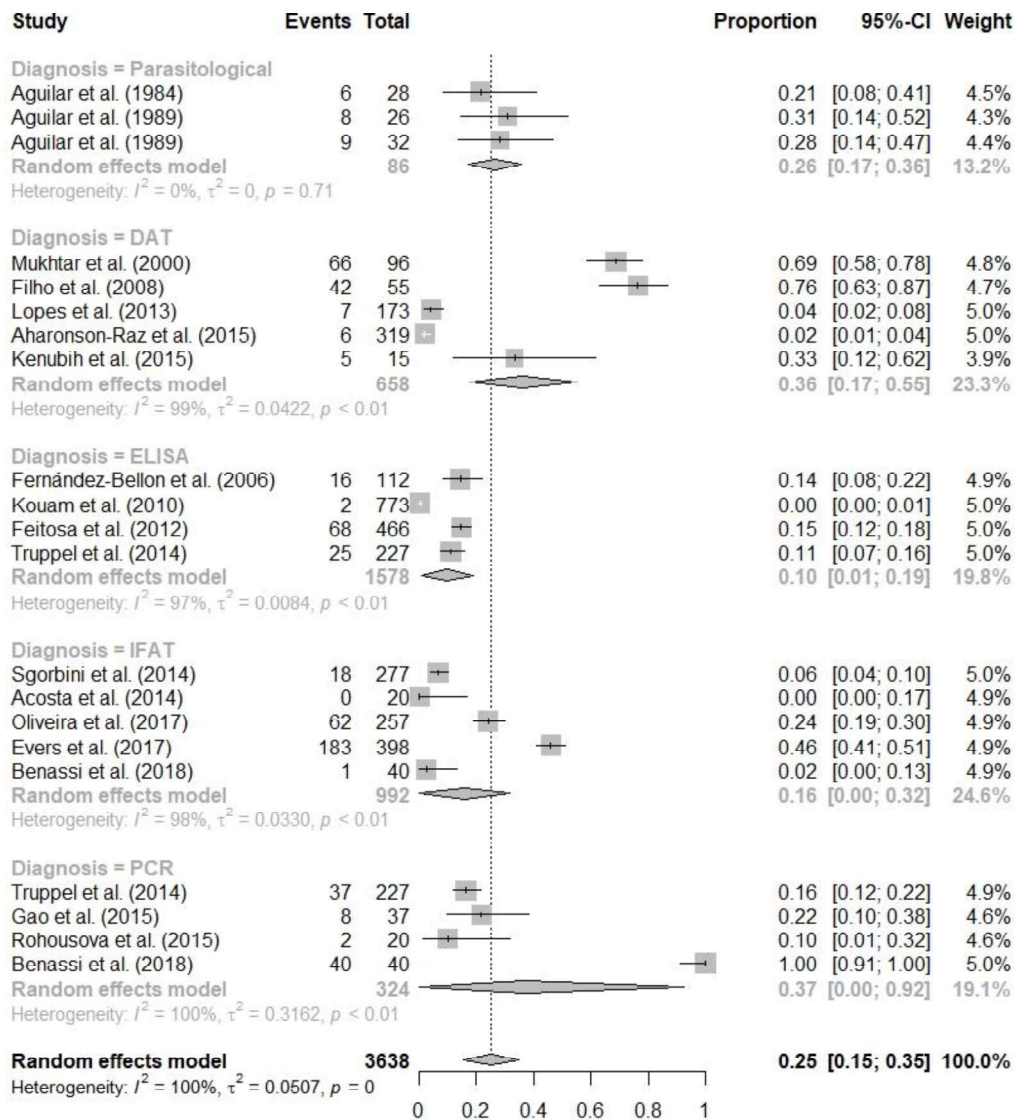
Figure 2. Combination of 21 prevalence studies on leishmaniasis in equids, according to the diagnosis method used.

When an etiological diagnosis of the disease was possible, the most frequent species found was *L. infantum* (n = 8), followed by *L. siamensis* (n = 5), *L. braziliensis* (n = 2) and mixed infection of *L. infantum* and *L. braziliensis* (n = 1). In the remaining cases (n = 6), the agent was not identified. Occurrences of species with higher pathogenicity towards humans (*L. infantum* and *L. siamensis*) infecting equids is a reason for greater concern, given that vectors use this mammal species as a source of food and could consequently ingest and become

contaminated by the protozoa, although this type of transmission has not yet been demonstrated in equids in a general manner.
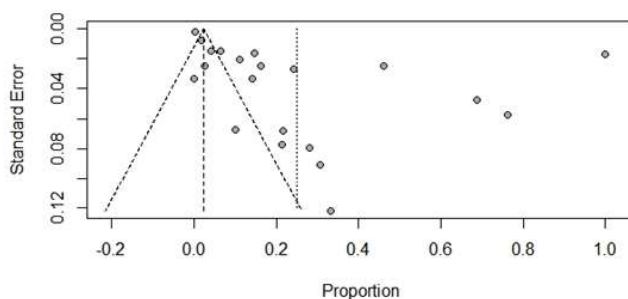
Figure 3. Funnel plot presenting the asymmetrical distribution of studies on the revalence of leishmaniasis in equids.

The lesions that were described in the studies surveyed here were located mainly on the head and limbs, although they were also observed on the neck, genital organs, abdomen and thorax, which are regions where there are few or even no hairs, which facilitates access by the vector mosquito to begin feeding and, consequently, to inoculate the parasite into the skin. Moreover, research on humans has shown that attractive volatile substances are exhaled from specific parts of the body, such as the ears, which attract greater numbers of insects to that region (REBOLLAR-TELLEZ et al., 1999). Although no such reports relating to equids have been conducted, presence of volatile attractants forms a plausible argument for explaining the presence of lesions on certain body parts rather than on others, thus suggesting that this occurs not just because of absence of hair in these places.

Skin diseases are common among equids, and some present characteristics similar to those described for leishmaniasis, such as equine sarcoid, squamous cell carcinoma, pythiosis, habronemiasis (KOEHLER et al., 2002; SOLANO-GALLEGO et al., 2003) or any other cutaneous disorder that causes either papular or nodular lesions and/or ulcers in equids (SOARES et al., 2013). Thus, leishmaniasis should be considered in making differential diagnoses of dermatopathies in equids, especially when the animals inhabit regions that are endemic for leishmaniasis and lesions occur in areas such as the genital organs, head, neck, ears and inguinal and axillary regions, and when there is no satisfactory response from administration of antimicrobial or antifungal therapies (RAMOS-VARA et al., 1996).

Most lesions that were described in previous studies either regressed spontaneously without the need for treatment or did not recur after surgical removal (RAMOS-VARA et al., 1996; KOEHLER et al., 2002; SOLANO-GALLEGO et al., 2003; ROLÃO et al., 2005;

MÜLLER et al., 2009; GAMA et al., 2014). This emphasizes the idea that the immune response of equids against the parasite is effective (FERNÁNDEZ-BELLON et al., 2006).

Use of medication to treat the disease was reported in three studies. In the first, Barbosa-Santos et al. (1994) initially used immunotherapy and observed an increase in antibody levels and worsening of lesions, which then led them to use conventional chemotherapy (pentavalent antimony), through which regression of lesions was achieved after a second application. In the second report, Ramos-Vara et al. (1996) used sodium stibogluconate and reached complete cure for the lesions. Lastly, Solano-Gallego et al. (2003) used anti-inflammatory therapy with dexamethasone, but without any satisfactory response.

Regarding the quantitative synthesis, the meta-analysis indicated that the prevalence of leishmaniasis in equine populations was 25% (CI:15-35%), with distribution over four different continents (Africa, South America, Asia and Europe). However, the high heterogeneity of the studies analyzed gives rise to some reflection regarding the degree of reliability of this combined prevalence among the studies.

In seeking to elucidate this high heterogeneity, we were particularly interested in the number of different diagnostic techniques that have been used to investigate leishmaniasis in equids. Thus, one meta-analysis per subgroup was conducted, with separation according to the method used for the diagnosis: parasitological examinations, IFAT, ELISA, PCR and DAT.

The forest plot (Figure 2) brings the 21 studies included in the meta-analysis, where the points inside the boxes in the center of the graph represent the prevalence of each study individually and the horizontal line the confidence interval, and the diamonds demonstrate the combined prevalence among studies. Prevalence results and confidence intervals are shown in the fourth and fifth columns, respectively. The last column represents the weight with which each study participated in the aggregate result.

The heterogeneity was high both in the aggregate outcome of the 21 studies ($I2 = 100\%$) and in the DAT ($I2 = 99\%$), ELISA ($I2 = 97\%$), IFAT ($I2 = 98\%$) and PCR ($I2 = 100\%$) subgroups. As shown in Figure 2, heterogeneity is clearly present between the studies that used different diagnostic techniques, and it is also present among studies that used the same technique. One exception were the three studies that used parasitological techniques, which did not show heterogeneity between each other ($I2=0\%$). Parasitological examinations are considered to be the gold standard for diagnosis, and thus their results are more reliable.

Serological tests were the diagnostic techniques that presented greatest methodological differences when used to diagnose leishmaniasis in equids. Regarding DAT, for example, five studies used different antigen concentrations, serum dilutions and cutoff points (LOPES et al., 2013; AHARONSON-RAZ et al., 2015; KENUBIH et al., 2015), along with positive control serums from either humans or dogs (MUKHTAR et al., 2000; VEDOVELLO et al., 2008). In ELISA, there were also methodological differences, especially regarding the types of antigens and conjugates used in serum dilutions (FERNÁNDEZ-BELLON et al., 2006; KOUAM et al., 2010; FEITOSA et al., 2012; TRUPPEL et al., 2014). Other serological studies used IFAT and also presented differences regarding the cutoff points and conjugates used (SGORBINI et al., 2014; ACOSTA et al., 2014; OLIVEIRA et al., 2017; EVERS et al., 2017; BENASSI et al., 2018).

Regarding molecular tests through PCR, the meta-analysis results also demonstrated high heterogeneity (I2 = 100%) among the four studies that used this technique (TRUPPEL et al., 2014; GAO et al., 2015; ROHOUSOVA et al., 2015; BENASSI et al., 2018). Although the authors of these studies used different primers to identify parasite DNA, we do not believe that this was the source of the heterogeneity that was observed in the meta-analysis of this subgroup. In this case, we believe it is coherent to attribute this heterogeneity to the variation in real prevalence of the disease in the studies indicated, given the high sensitivity and specificity of the PCR technique as a diagnostic method and the combined prevalence and confidence interval observed among the studies in which it was applied (37%; 0 − 92%).

Publication bias is often responsible for the heterogeneity between studies that is found through meta-analysis. In Figure 3 each point on the plot represents a study, with the prevalence arranged on the X axis and the standard error on the Y axis. In the absence of bias, the points are expected to have a symmetrical distribution under the dashed area of the triangle (inverted funnel), with the most accurate studies occupying the vertex (lowest standard error) and the least accurate distributed at the base of the triangle. It was possible to verify by observing the funnel plot (Figure 3) a marked asymmetry of the points (studies), indicating possible publication bias in the present meta-analysis, a fact also confirmed by the Egger's test (p = 0.01). In relation to cross-sectional studies, it is especially common to find that researchers are not interested in publishing their studies when they do not find significant prevalence in their investigations. Moreover, the editors of journals even appear reluctant to publish these negative findings. This lack of negative findings generates bias that can interfere in the results from a meta-analysis (Pereira & Galvão, 2014). However, we believe that the difference in the

methodologies that were used for diagnosing the disease in equids was the factor that most contributed towards the high heterogeneity observed.

Conclusion

The clinical disease caused by different species of *Leishmania* spp. is benign to equids, thus not requiring treatment in most cases, with the exception of basic care to avoid secondary contaminations, or cases in which the lesions reach larger proportions. However, leishmaniasis is clinically important, given that it can be mistaken for other common skin diseases in equids. Thus, leishmaniasis should be considered in the differential diagnosis for these diseases. Moreover, more detailed investigations are needed regarding the pathology of the disease in these hosts, particularly if development of visceral lesions occurs, and regarding the role of the immune response in determining whether clinical signs will appear.

Hence, seroepidemiological studies are important for investigating and monitoring suspected exposure of these hosts to the parasite, especially in endemic areas, given that natural equid-vector-human transmission has not yet been either demonstrated or refuted and could, therefore, represent a risk to vulnerable populations. However, the diagnostic techniques used for this purpose have not yet been standardized for equids and this may generate conflicting results and erroneous conclusions regarding prevalence of the disease in this host population, as observed in the present study.

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CAPÍTULO II

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

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

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

Clécio Henrique Limeira[1, *]; Murilo Duarte de Oliveira[2]; João Pessoa Araújo Júnior[3]; Camila Dantas Malossi[3]; Leila Sabrina Ullmann[3]; Maria Luana Cristiny Rodrigues Silva; Sergio Santos de Azevedo[1]; Clebert José Alves[1]

[1]Programa de Pós-graduação em Ciência e Saúde Animal, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande – UFCG, Patos, PB, Brasil

[2] Instituto Federal do Sertão Pernambucano, Salgueiro, PE, Brasil

[3] Universidade Estadual Paulista (Unesp), Av. Prof. Mário Rubens Guimarães Montenegro, Botucatu, SP 18618-687, Brasil

Abstract

Leishmaniasis is a parasitic zoonosis that mainly affects poorer and more vulnerable populations, and domestic dogs are considered to be the main source of infection for 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 leishmaniasis, either as potential reservoirs or just as an abundant food source for vectors.

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

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*Corresponding author: Clécio Henrique Limeira. Programa de Pós-graduação em Ciência e Saúde Animal, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande – UFCG, Av. Universitária, Santa Cecília, CP 61, CEP 58708-110, Patos, PB, Brasil. Fone (83) 3423-9523. e-mail: cleciolimeira@hotmail.com. https://orcid.org/0000-0002-3151-3680

Resumo

A leishmaniose é uma zoonose parasitária que acomete principalmente populações mais pobres e vulneráveis, sendo o cão doméstico considerado a principal fonte de infecção para humanos. Porém diversos estudos têm pesquisado o papel de outros hospedeiros vertebrados no ciclo da doença. Nesse contexto objetivou-se realizar um levantamento da infecção por *Leishmania infantum* em asininos e muares vivendo em região semiárida do Brasil. Foi utilizado sangue total de 72 equídeos (65 asininos e 7 muares) para 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 muares), sexo (macho e fêmea) e situação de abandono dos animais (sim ou não). Asininos e muares vivendo em condições semiáridas apresentam alta frequência de infecção por *L. infantum*, sendo válido atribuir importância a essas espécies no ciclo epidemiológico da leishmaniose, seja como um reservatório em potencial ou apenas como uma fonte alimentar abundante para os vetores.

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

Introduction

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

In areas that are endemic for leishmaniasis, domestic dogs have been reported to be the main source of infection for 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 *Leishmania braziliensis*, *Leishmania infantum* and *Leishmania 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., 2016). 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 *Leishmania infantum* infection among donkeys and mules living under semiarid conditions, with a view to contributing information about the epidemiology and role of these animals in the parasite cycle.

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/UFCG), 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 (Brazilian Institute for Geography and Statistics - 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 animals 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. (2016), using the primers Linf kDNA-F 5′-GGCGTTCTGCAAAATCGGAAA-3′, Linf kDNA-R 5′-CCGATTTTTGGCATTTTTGGTCGAT-3′ and Linf kDNA_FAM-5′-TTTTGAACGGGATTTCTG-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 TGGTGGTTTG-3') and MC2 (5'-CACCCATTTTTCCGATTTG-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 et al., 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 constructed using the Seaview4 software (Gouy et al., 2010), by means of the maximum parsimony method (Nei and Kumar, 2000) with 1,000 bootstraps. The tree was viewed through FigTree v1.4.3. The phylogenetic reconstruction included *L. infantum* sequences obtained from GenBank 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 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 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, such as the qPCR used in this study, have become increasingly popular within research, and can be used both for detection and quantification of the agent and for identification of *Leishmania* species. 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 (Brazil, 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.

Corroborating the results from the present study, Truppel et al. (2014) did not find any significant differences between the categories of species (horses, donkeys or mules) or sex (male or female) in a study on *Leishmania braziliensis* infection in equids in the state of Paraná, Brazil. This differed from the results of Oliveira et al. (2017), who found a significant association with equid species, in a rural area of the municipality of Uberlândia, southeastern Brazil. In their study, mules presented higher prevalence of infection (80%), such that their risk of being seropositive for *Leishmania* spp. was 3.22 times higher than that of horses.

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).

In this manner, the protection levels against infection among the donkeys and mules sampled in this study may become reduced at some phases of their development. It needs to be borne in mind that they live in remote rural areas and are exposed to vectors and to stressful conditions specific to this region, such as malnutrition, low water intake and extreme cold and heat. These factors reduce immunity and can facilitate reproduction and dissemination of the parasite. However, 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 *Leishmania 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 *L. longipalpis*. This suggested that donkeys are devoid of importance as a reservoir in the transmission chain for 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 *Equus 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 leishmaniasis in the semi-arid 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.

Acknowledgements

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World Health Organization – WHO. Integrating neglected tropical diseases into global health and development: fourth WHO report on neglected tropical diseases [online]. 2017 [cited 2020 oct 26]. Available from: https://apps.who.int/iris/bitstream/handle/10665/255011/9789241565448-eng.pdf;jsessi onid=FF58D6943C1C407719FAF54A2A29FA05?sequence=1.

Tables

Table 1. Frequency 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)	-	-

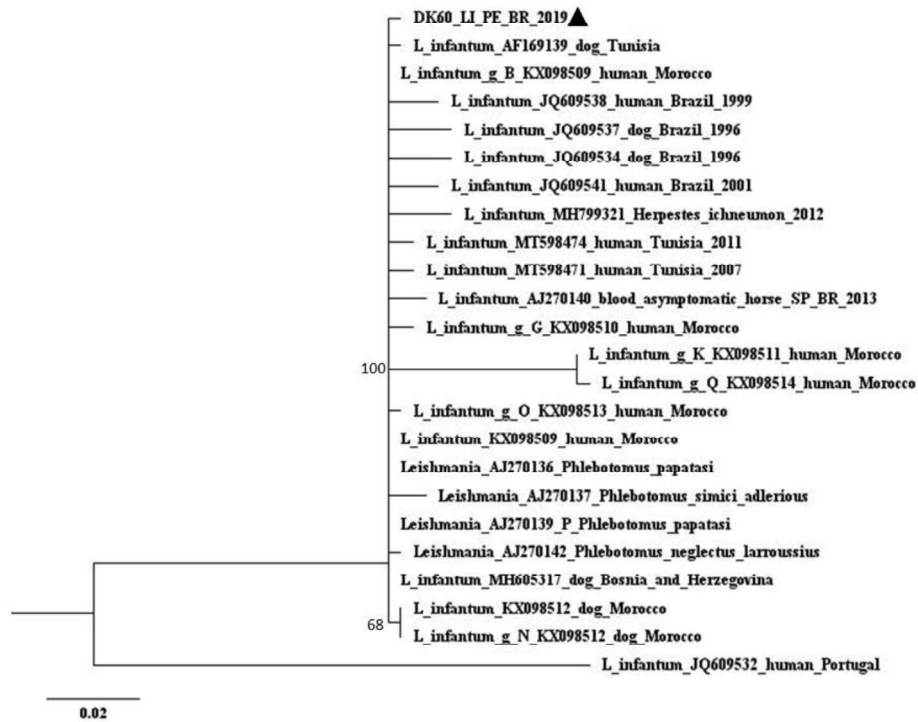*Fisher's exact test (α=5%)

Figure 1

Figure 2. Phylogenetic analysis on *L. infantum* detected in peripheral blood samples from donkeys, constructed through the maximum parsimony method.

▲ - Sequenced sample

CAPÍTULO III

Molecular detection of *Trypanosoma cruzi* in donkeys and mules under semiarid conditions in Brazil

Manuscrito submetido ao periódico "Zoonoses and Public Health"

Molecular detection of *Trypanosoma cruzi* in donkeys and mules under semiarid conditions in Brazil

Clécio Henrique Limeira[1] | Murilo Duarte de Oliveira[2] | João Pessoa Araújo Júnior[3] | Camila Dantas Malossi[3] | Leila Sabrina Ullmann[3] | Maria Luana Cristiny Rodrigues Silva[1] | Sergio Santos de Azevedo[1] | Clebert José Alves[1]

[1] Post-Graduate Program in Animal Science and Health, Federal University of Campina Grande, Av. Universitária S/N, Santa Cecília, Patos, PB, Brazil

[2] Federal Institute of Sertão Pernambucano, Salgueiro, PE, Brazil

[3] Paulista State University (Unesp), Av. Prof. Mário Rubens Guimarães Montenegro, s / n, Botucatu, SP 18618-687, Brazil

Abstract

Trypanosoma cruzi is the etiological agent of Chagas disease, a neglected and frequently occurring zoonosis in South American countries. Wild mammals and domestic dogs are the main reservoirs of the parasite in the wild and domestic cycles, respectively. The vectors have a wide variety of food sources that can influence transmission cycles. The aim of this study was to detect *T. cruzi* infection in donkeys *(Equus asininos)* and mules *(Equus mulus)* living in rural areas of the Brazilian semi-arid region. Whole-blood samples from 72 equids (65 donkeys and 7 mules) were analyzed by means of the nested polymerase chain reaction (nested PCR). A total of 51.39% of the samples (37/72) were positive. Phylogenetic analysis identified discrete typing units TcI and TcII, which suggested the possibility that donkeys and mules might be participating in domestic/peridomestic and wild transmission cycles. This was the first report of *T. cruzi* infection in donkeys and mules in Brazil. It demonstrated that the particular features of these hosts, the presence of vectors and the socioeconomic characteristics of the population under semiarid conditions create interactions that can favor transmission and overlapping of *T. cruzi* infection cycles.

KEYWORDS

Chagas disease, epidemiology, equids, nested PCR, zoonosis

Impacts

- This was the first report of *T. cruzi* infection in donkeys and mules in Brazil, with high frequency of positive animals (51.39%) diagnosed through nested PCR
- Equids infected with *T. cruzi* in a given region can interfere in serological tests for other trypanosomiases such as *T. equiperdum, T. vivax* and *T. evansi*

- Phylogenetic analysis identified discrete typing units I and II of *T. cruzi* (TcI and TcII), thus suggesting that donkeys and mules under semiarid conditions present a link between the domestic and wild cycles of *T. cruzi* transmission

1 | INTRODUCTION

The protozoon *Trypanosoma cruzi* is the etiological agent of Chagas disease, a neglected endemic parasitic zoonosis in 21 countries of the Americas that is transmitted by hemipteran insects of the subfamily *Triatominae,* especially of the genera *Triatoma, Panstrongylus* and *Rhodnius.* Depending on how reservoirs and vectors are established or circulate, Chagas disease can evolve in three cycles: the wild cycle, in which several species of wild mammals harbor the parasite, the peridomestic cycle and the domestic cycle. In the latter two, domestic animals (dogs and cats, for example) and humans are the main hosts (Organización Panamericana de la Salud, 2019).

Because of genetic diversity, *T. cruzi* has been divided into six groups according to different molecular markers, called discrete typing units (DTUs): *T. cruzi* I (TcI) to *T. cruzi* VI (TcVI) (Zingales et al., 2009). In Brazil, the main DTUs found are TcI and TcII. TcI is associated more with the wild cycle of the disease, while TcII relates mainly to the domestic transmission cycle and is the form most frequently diagnosed in chronic infections in humans (Zingales et al., 1998). Among domestic animals, *T. cruzi* infection has been shown to be pathogenic and is well characterized in dogs (Andrade & Andrade, 1980; Souza et al., 2008; Mendes et al., 2013). On the other hand, in production animals, although *T. cruzi* infection is only moderately pathogenic or nonpathogenic, its presence may interfere in diagnostic tests for other clinically important trypanosomes of equids, such as those caused by *Trypanosoma equiperdum, T Trypanosoma vivax* and *Trypanosoma evansi* (World Organization for Animal Health, OIE, 2004).

Research on *T. cruzi* infection and diagnosis in equids is scarce, and most studies available in the literature have mainly used serological tests as diagnostic tools, which can generate cross-reactions with other species of *Trypanosoma* (Fujita et al., 1994; Desquesnes et al., 2007; Ferreira et al., 2018). On the other hand, using molecular techniques, Viettri et al. (2018) found high prevalence of *T. cruzi* in donkeys *(Equus asininos)*, mules *(E. mulus)* and horses (*E. caballus*) in Venezuela. In a study in the semiarid region of the state of Ceará, northeastern Brazil, Bezerra et al. (2018) detected genetic material (DNA) of horses in the digestive tract contents of *Triatoma brasiliensis*, caught in a domestic environment. This

demonstrated that horses are feeding sources for these vectors and that they can participate as a bridge between the peridomestic and domestic cycles *of T. cruzi*. Thus, considering the zoonotic importance of this parasite and the scarcity of studies on its diagnosis in equids in Brazil, the aim of this study was to detect, through the nested-PCR (nPCR) technique, *T. cruzi* infection in donkeys and mules that were kept under semiarid conditions.

2 | MATERIALS AND METHODS

This research project was previously submitted to and analyzed by the Ethics Committee for Animal Use of the Center for Rural Health and Technology, Federal University of Campina Grande (CEUA/CSTR/UFCG). It was approved in accordance with the rules and regulations in force, with a research protocol registered under the number 011/2019.

2.1 | Study area

This study was carried out in the rural area of the municipality of Salgueiro (8°4′20″ S; 39°7′36″ W), state of Pernambuco, northeastern Brazil. This location is within a region of semiarid tropical climate, with an average annual rainfall of 431.8 mm, concentrated between November and April. The vegetation is basically composed of hyperxerophilic Caatinga (Brazil, 2005).

2.2 | Animals and sample collection

The experimental design consisted of non-probabilistic sampling, for convenience, and included both animals that had owners and animals in situations of abandonment that were found living within the local vegetation. The abandoned animals were caught by a trained and experienced person, using lassoes.

In total, the sample included 72 equids (65 donkeys and 7 mules). Peripheral blood (4 mL) was collected from each animal, directly from the jugular vein, using vacuum tubes containing EDTA. These tubes containing the samples were numbered and identified with animal data (species and sex) and were kept frozen at a temperature of -20ºC until the time of use. The sampling was done in October 2018 and March 2019.

2.3 | Molecular diagnosis

Genetic material, i.e. deoxyribonucleic acid (DNA), was extracted from the blood samples using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following the

manufacturer's recommendations. The molecular test consisted of nested PCR (nPCR), using the primers described by Moser et al. (1989) and Ochrs et al. (1996). In the first reaction, the primers used were TcZ1 (5'-CGAGCTCTTGCCCACACGGGTGCT-3') and TcZ2 (5'-CCTCCAAGCAGCGGATAGT TCAGG-3'). The second nPCR was amplified using the primers TcZ3 (5'-TGCTGCASTCGGCTGATCGTTTTCGA-3') and TcZ4 (5'-CARTGSTGtTTGGTGT CCAGTGTTGTGA-3'). A culture of *T. cruzi* was used as the positive control, and ultrapure water was used as the negative control.

2.4 | Nucleotide sequencing

The sequencing reaction was performed using the primers TcZ3 (sense) and TcZ4 (antisense), as described above, in the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Capillary electrophoresis was performed using the Genetic Analyzer 3130 with POP-7 polymer (Applied Biosystems), as described by Platt et al. (2007).

The analysis was elaborated using the Sequencing Analysis Software v5.3.1 (Applied Biosystems) and sequences constructed through SeqMan v7.0 (DNASTAR, Madison, WI, USA). These sequences were then aligned using the BioEdit software (Hall et al., 1999), and *T. cruzi* strains obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) were used as references (http://www.ncbi.nlm.nih.gov/BLAST/).

A phylogenetic tree was elaborated using the Seaview4 software (Gouy et al., 2010), through the generalized time-reversible (GTR) method with 1,000 bootstraps, using PhyML 3. The tree was viewed by means of FigTree v1.4.3. The phylogenetic reconstruction included *T. cruzi* sequences obtained from GenBank, for comparison.

2.5 | Statistical analysis

The prevalence ratio (PR) was used to measure performance (Coutinho et al., 2008) and Fisher's exact test (with significance level of 5%) was used to investigate the existence of associations between the result from the diagnostic test and the variables of species, sex and abandonment situation, using the "epiR" package (Stevenson et al., 2020) in the R statistical software, version 3.5.1 (R Core Team, 2020).

3 | RESULTS

Out of the total number of samples analyzed, 51.39% (95% CI 39.51-63.22%) were positive in the molecular test, thus demonstrating the presence of genetic material from *T. cruzi* in peripheral blood from these donkeys and mules living under semiarid conditions. All the samples that tested positive were collected during the dry period of the region studied.

In analyzing the data according to categories, it was observed that 85.7% (6/7) of the mules and 47.69% (31/65) of the donkeys were positive. Regarding sex, 56.6% (30/53) of the males and 36.84% (7/19) of the females were positive; and 47.06% (8/17) of the animals in abandonment situations and 52.73% (29/55) of the animals that had owners tested positive in the molecular test. Thus, the categories that presented the highest frequencies of positivity were mules, males and animals that had owners, but there was no significant difference in the statistical test used (Table 1).

Genetic material from five samples that were positive in the molecular test was sequenced. This made it possible to identify two DTUs of *T. cruzi*: TcI (four samples from male donkeys, among which three belonged to residents of the region and one was in a situation of abandonment) and TcII (one sample, from a female donkey, in a situation of abandonment). The evolutionary relationships of the sequencing are shown in Figure 1.

4| DISCUSSION

When we started our visits to farms to collect information and collect blood from animals to continue with the research, many residents of the region reported that they did not have any equids, because there was no longer a need to use these animals for working in their fields. They had therefore left them to live freely within the local vegetation. This imposed a limitation on the total number of samples collected, due to the low number of animals found during visits and the difficulty in catching animals that were in situations of abandonment.

From our analysis on several bibliographic sources, it was observed that this was the first study to report on *T. cruzi* infection in donkeys and mules in Brazil. There was high frequency of positive animals (51.39%) in molecular diagnoses that were made using nested PCR. Among horses in the state of Maranhão, northeastern Brazil, Ferreira et al (2018) found that 50.4% of the animals were seropositive for *T. cruzi*, using the enzyme-linked immunosorbent assay (ELISA). However, ELISA has low specificity for differentiating protozoa of the family Trypanosomatidae, which gives rise to the possibility of cross-reactions between *T. evansi, T. equiperdum* and *T. cruzi* in horses (OIE, 2004). Using molecular diagnoses, Viettri et al (2018) found *T. cruzi* DNA in blood samples from donkeys, mules and horses, with prevalences of 23.7% (32/135), 20.8% (5/24) and 12.5% (1/8), respectively, in rural and urban areas of different states in Venezuela. These data corroborate the findings of the present study in that they show that the prevalence of *T. cruzi* infection among equids was high.

According to the OIE (2004), *T. cruzi* infection in production animals presents moderate or no pathogenicity. However, further studies are needed to evaluate the pathogenicity of *T. cruzi* in equids, given that Bryan et al. (2016) described a case of a neurological condition in a 10-year-old horse with a history of ataxia and claudication in the hind limbs six months previously, in which *T. cruzi* was then detected in histological sections from the spinal cord, with subsequent confirmation of this diagnosis through conventional PCR and real-time PCR (qPCR). Thus, *T. cruzi* infection can be considered to be a differential diagnosis for neurological diseases of equids.

The climate of the study area is characteristic of semiarid regions, with high temperatures and average rainfall of 431.8 mm annually, concentrated between November and April (Brazil, 2005). It should be noted that other similar studies were developed in regions with different climatic characteristics (Ferreira et al., 2018; Viettri et al., 2018), which demonstrates that the vectors can adapt to distinct ecological niches. In a study on the ecology of *Triatoma brasiliensis* in northeastern Brazil, Sarquis et al. (2010) observed a higher proportion of triatomines infected with *T. cruzi* in the rainy season (April) and at the beginning of the drought (July), thus indicating higher availability of blood sources in this period. However, these same authors observed greater dispersion of *T. brasiliensis* in the dry period (December), which would reflect more active movement in search of food. In addition, the present study was developed during a dry period, when the number of wild mammals (such as armadillos and some rodent species, for example) that serve as a food source for vectors is reduced. Even if equids are not the preferred sources (Sarquis et al., 2010; Bezerra et al. 2018), they may become the only food option for triatomes during long periods of drought.

There was no statistical difference in the frequency of positivity between animals in different categories (Table 1). However, the proportion of positive samples was higher among mules than among donkeys. In the present study, all the positive mules were living in peridomestic environments, where there are corrals, chicken coops and other installations that serve as artificial shelters for vectors, especially during the dry period (Sarquis et al, 2010; Bezerra et al., 2018). The animals in the present study were also classified according to whether they had owners and were living in a peridomestic environment, or whether they had been abandoned and were living freely in the Caatinga. These two categories presented similar proportions of positive animals (52.73% and 47.06%, respectively), thus demonstrating that the donkeys and mules in the area studied are exposed to vectors both in the peridomestic and in the wild environment.

In addition, during the dry season, abandoned animals approached human homes more frequently, in search of water and food, thus forming a link between the wild and peridomestic environments that can cause overlapping of the *T. cruzi* infection cycles, as also occurs with other domestic animals (Bezerra et al., 2018). This overlapping of cycles was demonstrated through the *T. cruzi* strains found in the samples analyzed. From our phylogenetic analysis (Figure 1), two DTUs were identified: TcI and TcII. In Brazil, TcI relates mainly to the wild cycle, while TcII relates to the domestic transmission cycle (Zingales et al., 1998). We therefore demonstrated that these donkeys and mules were in contact with vectors belonging both to the domestic/peridomestic and to the wild environment.

5 | CONCLUSION

The present study provides the first report of *T. cruzi* infection in donkeys and mules in Brazil, with high frequency of positive animals identified through the nested PCR technique. This places these animals as potential reservoirs for the parasite. The particular features of these hosts, the presence of vectors and the socioeconomic characteristics of the population under semiarid conditions create interactions that may favor transmission and overlapping of *T. cruzi* infection cycles.

Conflict of interest

The authors declare that they did not have any conflict of interest.

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Table 1. Frequency of occurrence of donkeys and mules positive for *T. cruzi* through the nested PCR technique in the rural area of the semiarid region of northeastern Brazil.

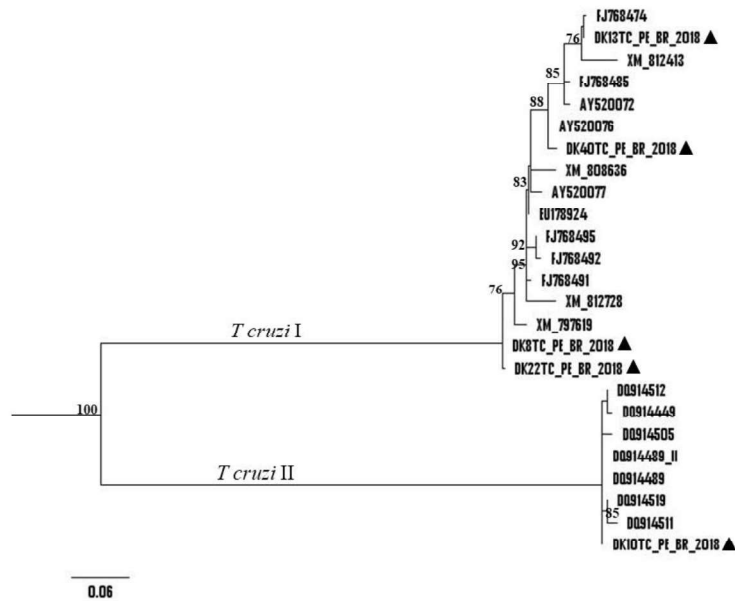| Variables	Categories	Number of animals	Positives	% positives	Prevalence Ratio (CI 95%)	p-value*
Species	Mules	7	6	85,7	1,80 (1,21-2,67)	0.062
	Donkeys	65	31	47,69		
Sex	Males	53	30	56,6	1,54 (0,81-2,90)	0.112
	Females	19	7	36,84		
Abandonment situation	Yes	55	29	52,73	1,12 (0,64-1,97)	0.447
	No	17	8	47,06		
Overall		**72**	**37**	**51,39%**	**-**	**-**

*Fisher's exact test (α=5%)

Figure 1. Phylogenetic tree of *T. cruzi* (TcI and TcII) detected in whole-blood samples from donkeys and mules living in the semiarid region of northeastern Brazil, elaborated by means of the generalized time-reversible (GTR) method, with 1,000 bootstraps.

CONCLUSÃO GERAL

Os resultados obtidos no presente trabalho de tese possibilitam concluir que:

A leishmaniose em equídeos é predominantemente cutânea, devendo ser considerada no diagnóstico diferencial de enfermidades da pele nessas espécies. Há necessidade de mais investigações sobre a patologia da doença nesses hospedeiros, sobretudo se há desenvolvimento de lesões viscerais e a respeito do papel da resposta imune no surgimento ou não de sinais clínicos.

Asininos e muares vivendo em área rural do semiárido nordestino apresentam alta frequência de infecção por *L. infantum*. Embora a transmissão da infecção a partir de equídeos parasitados ainda não tenha sido comprovada, é válido creditarmos uma importância aos asininos e muares no ciclo epidemiológico da leishmaniose no semiárido nordestino, seja como um reservatório em potencial ou apenas como uma fonte alimentar abundante para os vetores.

Com relação ao *T. cruzi*, foi descrito o primeiro relato de infecção em asininos e muares no Brasil, com alta frequência de animais positivos identificados pela técnica de nested-PCR, o que os colocam como potenciais reservatórios do parasita. O modo de vida desses animais, a adaptabilidade dos vetores ao bioma Caatinga e as características socioeconômicas da população criam interações que podem favorecer a transmissão e a sobreposição dos ciclos de infecção de *T. cruzi* em regiões semiáridas.