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MEDICINA VETERINÁRIA

"BAIXADEIROS" HORSES: PREVALENCE OF ANTI-*Trypanosoma* spp. AND ANTI-*Leishmania* spp. ANTIBODIES

CAVALOS "BAIXADEIROS": PREVALÊNCIA DE ANTICORPOS ANTI-Trypanosoma spp. *E ANTI-Leishmania* spp.

Fernanda Pinto Ferreira[1*]
Eloiza Teles Caldart[1]
Danilo Rodrigues Barros Brito[2]
Daniel Praseres Chaves[3]
João Luis Garcia[1]
Italmar Teodorico Navarro[1]

[1]Universidade Estadual de Londrina, Londrina, Paraná, Brasil
[2]Instituto Federal do Maranhão, São Luis, Maranhão, Brasil
[3]Universidade Estadual do Maranhão, São Luis, Maranhão, Brasil
[*]Corresponding author - nandaferreiravet@gmail.com

Abstract
A total of 138 horse blood samples were collected from 2012 to 2013 for the subsequent separation of serum collection. For detection of anti-*Leishmania* spp. and anti-*Trypanosoma* spp. antibodies, indirect immunofluorescence reactions and immunoenzymatic assays were performed. Samples with titers ≥40 were considered positive in the indirect immunofluorescence (IFAT), and the cut off for the enzyme immunoassay (ELISA) was calculated with three standard deviations. When tested for *Trypanosoma* spp., 50.37% (68/135) of the samples were ELISA reagents, 18.84% (8/136) were positive for IFAT and 2.89% (4/138) for both tests. A significant statistical association was observed for the municipality (p = 0.013) and collection period (p = 0.042) in the ELISA. When tested for *Leishmania* spp., 25.4% (35/138) of the samples were ELISA reagents, 13.00% (18/138) were positive for the IFAT and 4.34% (6/138) were positive for two tests, there were no variables associated with seropositivity. A statistical association (p = 0.0034) was observed between the presence of anti-*Trypanosoma* spp. and anti-*Leishmania* spp. antibodies when the ELISA was used. The results suggest that this horse breed is in contact with leishmaniasis and trypanosomiasis, which demonstrates the need for a more accurate investigation into the real role of horses in these diseases to assist in disease control measures.
Keywords: leishmaniasis, trypanosomiasis, equines, serology

Resumo
Um total de 138 amostras de sangue de equinos foram coletadas nos anos de 2012 a 2013 para posterior obtenção de soro. Para detecção de anticorpos anti-*Leishmania* spp. e anti-*Trypanossoma* spp. realizou-se a reação de imunofluorescência indireta e o ensaio imunoenzimático. Consideraram-se positivas, na RIFI, amostras com títulos iguais ou superiores a 40; o ponto de corte do ELISA foi

calculado com três desvios padrão. Quando testadas para *Trypanosoma* spp., 50,37% (68/135) das amostras foram reagentes ao ELISA, 18,84% (8/136) à RIFI e 2,89% (4/138) para os dois testes. Observou-se associação estatística significativa quanto ao município (p= 0.013) e período de coleta (p=0.042) no ELISA. Quando testadas para *Leishmania* spp., 25,4% (35/138) das amostras foram positivas ao ELISA, 13,00% (18/138) à RIFI e 4,34% (6/138) nos dois testes, não havendo variáveis associadas estatisticamente à soropositividade. Foi observada associação estatística (p=0,0034) entre presença de anticorpos anti-*Trypanosoma* spp. e anti-*Leishmania* spp., quando utilizada a técnica de ELISA. Os resultados obtidos sugerem que esta raça de cavalo está em contato com os agentes das leishmanioses e tripanossomíases, demonstrando a necessidade de uma investigação mais precisa sobre o real papel de cavalos nessas enfermidades, a fim de auxiliar nas medidas de controle da doença.

Palavras-chave: leishmanioses, tripanossomíases, equinos, sorologia

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Introduction

Baixada Maranhense is located in the northern mesoregion of the state of Maranhão, which is characterized by vast low plains that flood in the rainy season and create huge lakes between January and July. It is also called Pantanal Maranhense. This region stands out due to a breed of native horses called "baixadeiros", which are characterized by their small size, robustness, strength and resistance to work[(1)].

The genus *Trypanosoma* comprises parasitic species of fish, amphibians, reptiles, birds and mammals, and these parasites are transmitted mainly by hematophagous invertebrate vectors. The main species responsible for trypanosomiasis in horses are *Trypanosoma equiperdum* and *T. evansi*, which are endemic in the Pantanal of Mato Grosso due to the animal, climate and environmental conditions that favor the development of the vectors[(2)].

Cutaneous leishmaniasis (CL) in horses has been reported in several Brazilian states since the 1950s; however, there are no characteristic clinical patterns. Usually, papules and nodules occur with single and multiple ulcerated lesions as well as proliferative lesions or crusts[(3)]. With regard to visceral leishmaniasis (VL), seropositive animals were found in endemic areas[(4)]. The role of horses as reservoirs has not been definitively confirmed[(5)]; however, studies have shown that Equidae can be infected by species of *Leishmania* and are preferred food sources for sandflies[(6)]. Experimentally, they do not cause infection to the vector, which suggests that they are not important reservoirs in the transmission chain[(7)].

The objective of this study was to determine prevalence of anti-*Leishmania* spp. and anti-*Trypanosoma* spp. antibodies in "Baixadeiro" horse in the region of Baixada Maranhense, state of Maranhão, Brazil.

Material and Methods

The Baixada Maranhense (01°59'-04°00'S, 44°00'-45°33'W), is located in Maranhão northern mesoregion and has a total area of approximately 18 thousand km^2 and an estimated population of 518,241 inhabitants. It is comprised of 21 municipalities and its climate is classified by Köppen as Aw (Tropical Climate with dry season in winter)[8]. A total of 138 horse blood samples from the municipalities of Pinheiro, Arari and Anajatuba (Figure 1), which belong to the protected area of Baixada Maranhense, were collected between April 2012 and March 2013.

The determination of anti-*Trypanosoma* spp. and anti-*Leishmania* spp. antibody titers was performed using an indirect immunofluorescence reaction according to Marzochi et al.[9] with some adaptations as well as an antigen for the epimastigote forms of *Trypanosoma cruzi* and promastigotes of *L. amazonensis*. During the analysis, the equine anti-IgG conjugate (FITC - Sigma Chemicals®) was used, and positive and negative patterns were included in each slide. Samples with titers ≥40[5] were considered positive and diluted in ratio two until a negative titer was obtained.

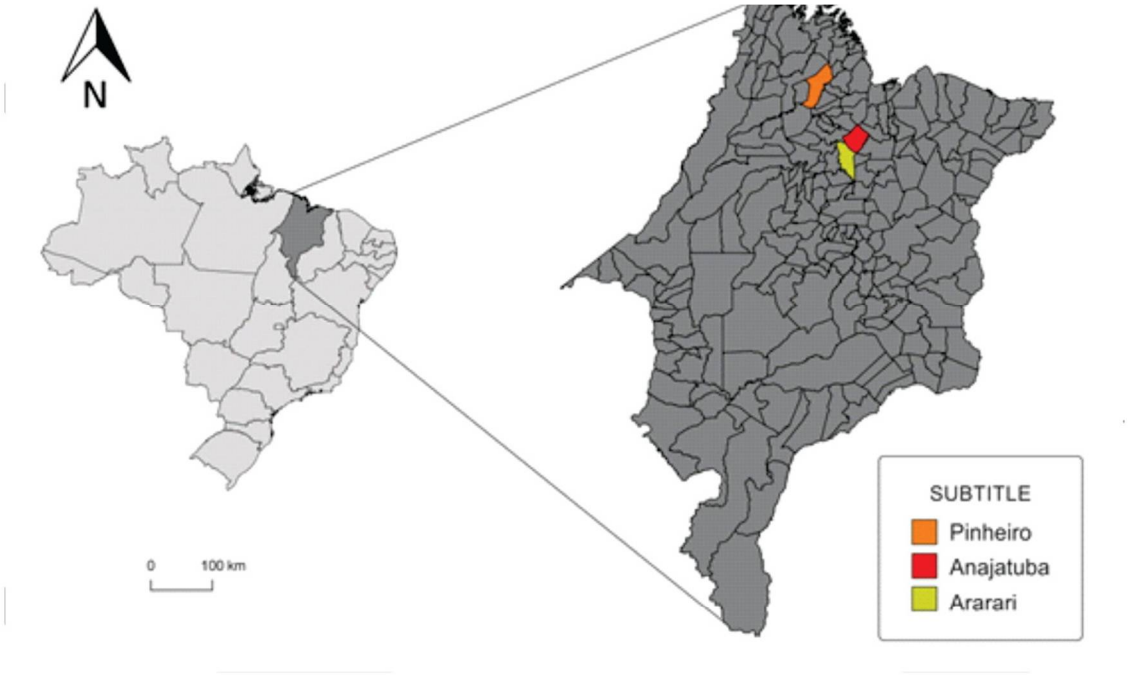

Figure 1. Geographic location of the cities of Pinheiro, Arari and Anajatuba, Baixada Maranhense, Maranhão, Brazil.

The indirect enzyme-linked immunosorbent assay (ELISAi) was performed as described by Szargiki et al.[10] with adaptations. The standard concentrations of *T. cruzi* and *L. amazonensis* antigen, serum and peroxidase-conjugated protein A conjugate (Sigma Aldrich, USA)[11] were 2.5 µg/mL, 1:25 and 1: 500, respectively. All samples were tested in duplicate. The optimum conditions concerning dilutions of antigen, serum and conjugate were established by the highest ratio between the mean absorbances of the known positive and negative samples used as controls. The cutoff of each plate

was obtained by the mean absorbance of the negative sera added by three standard deviations. After the number of calculations per plate, the corrected cutoff was estimated using the ROC curve constructed by the MedCalc Statistical Software program (version 13.2.0)[12].

The statistical association between the results in the above tests and the characteristics of the horses, such as gender, age, municipality and date of collection were analyzed using the Epi Info program 7.1.5.2[13]. Chi-square or Fisher exact tests were conducted with a level of significance of 5%. For tables 2 x 2, the odds ratio (OR) calculation with a 95% confidence interval (CI) was used as a measure of association. For larger tables, standardized residue analysis (ARP)[14] was used with the objective of verifying which categories contributed most to the statistics.

Results

Among the 138 horses studied, their age were between seven and 120 months old (mean of 49 months), 61.6% (85/138) were female, 4.3% (3/138) were from the municipality of Anajatuba, 6.5% (9/138) were from Arari and 89.1% (123/138) were from Pinheiro.

For *Trypanosoma cruzi*., 50.4% (68/135) were positive for ELISA, 18.84% (8/136) for IFAT and 2.96% (4/135) for both tests. The RIFI titers ranged between 40 and 160, 75.00% (6/8) 40; 12.5% (1/8) 80; 12.5% (1/8) 160 (Figure 2). The Fisher exact test showed a statistically significant association with municipality in which there was a higher seropositivity in the ELISA for *T. cruzi* (P = 0.013) Arari. The differences in the total number of samples analyzed between the tests are due to the insufficient quantity of samples.

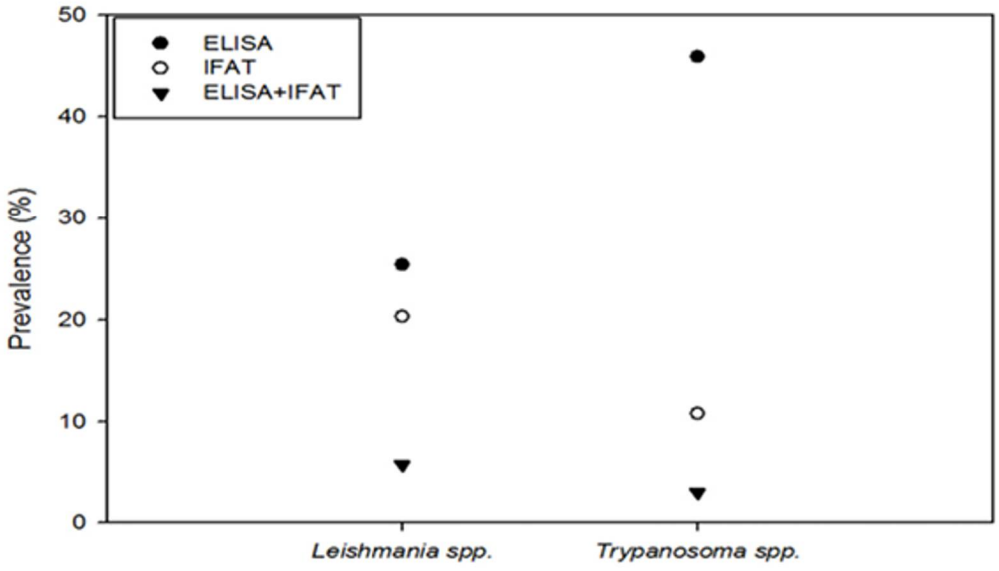

Figure 2. Prevalence of anti-*Trypanosoma* spp. and anti-*Leishmania* spp. antibodies by IFAT, ELISA and both tests in "baixadeiro" horses from Baixada Maranhense, Maranhão, Brazil.

When tested for *L. amazonensis,* 25.4% (35/138) of the samples were ELISA positive, 13.00% (18/138) were IFAT positive and 4.34% (6/138) were positive for both tests. Titers varied between

40 and 320 with 66.67% (12/18) 40; 16.67% (3/18) 80; 11.11% (2/18) 160 and 5.55% (1/18) 320.
A statistical association (p = 0.0034) was observed between the presence of anti-*T. cruzi* and anti- *L. amazonensis a*ntibodies in 18.38% (25/136) of the samples when the ELISA technique was used. Of these animals, 64.00% (16/25) presented serum with a higher optical density for *T. cruzi*, while 36.00% (9/25) had a higher optical density for *L. amazonensis.*
The samples were collected in four periods: April/2012, August/2012, September/2012 and March/2013, with 81 (60.0%), 6 (4.4%), 39 (28.9%) and 9 (6.7%) samples. A significant association (p = 0.042) was observed between the collection period and the seropositivity for *T. cruzi* in the ELISA (Figure 3).

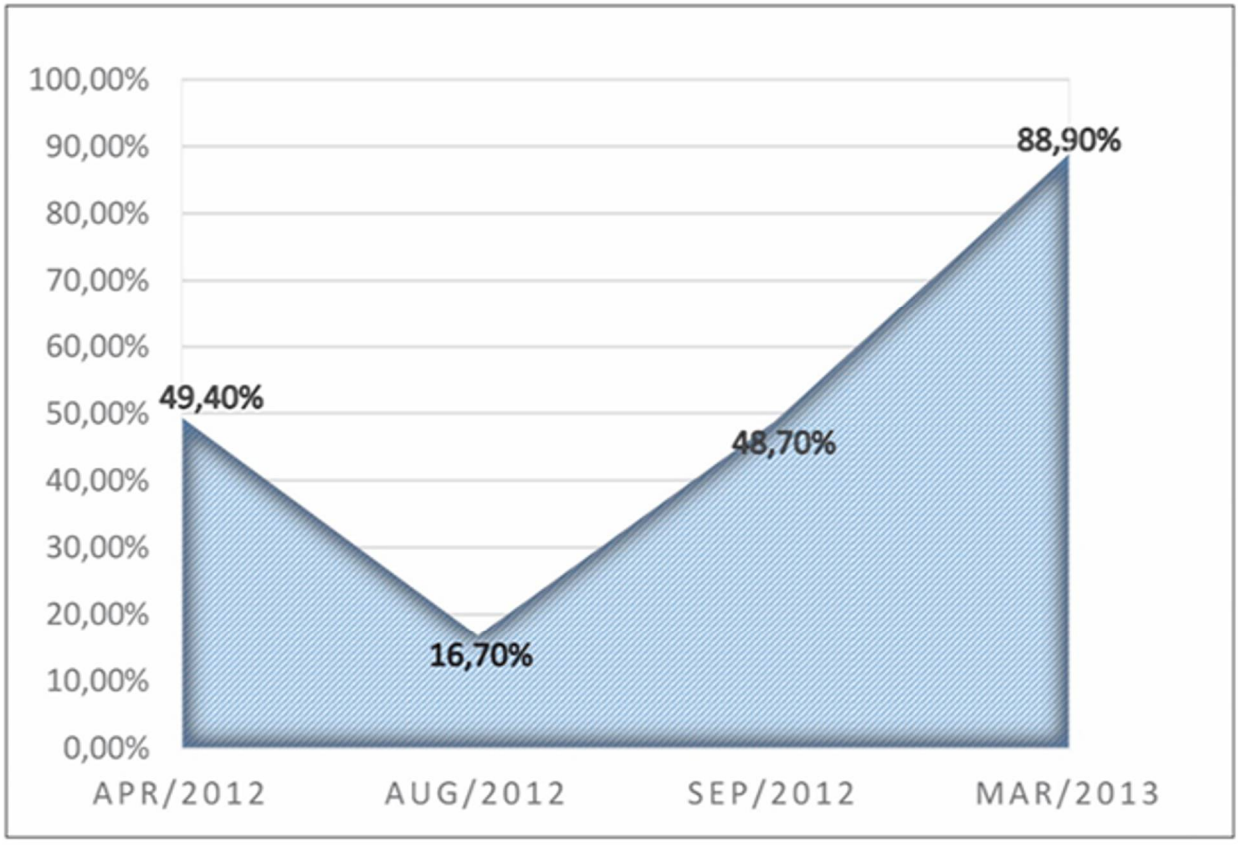

Figure 3. Equine seropositivity for *Trypanosoma* spp., according to the collection month, Baixada Maranhense, Brazil, 2012 - 2013.

Discussion

This is the first seroepidemiological investigation of anti-*Trypanosoma* spp. and anti-*Leishmania* spp. antibodies in horses in the Baixada Maranhense region. There was no statistical association between seropositivity and sex and age.

Regarding *Trypanosoma* spp., the ELISA technique had an antibody prevalence of 50.4%, which was lower than the prevalence observed by Dávila et al.[15] in a study in Pantanal, Mato Grosso do Sul, in which a prevalence of 79.2% was observed. When analyzed by RIFI, the samples had a prevalence of 18.84%, which was also lower than the prevalence found by Herrera et al.[16], who observed

73.50% seropositivity in horses from Nhecolândia, Mato Grosso do Sul. Studies were carried out in Pantanal regions with similar characteristics as those of the cities of this study; yet, the high prevalence results may be due to the collection season being the rainy season, unlike our study in which a large part of the samples were collected during the dry season and may have led to a low number of vectors as well as low seropositivity.

Among the three cities studied, a higher prevalence of antibodies against *Trypanosoma* spp. were observed in the city of Arari ($p = 0.013$) in which the horse permanence was close to the floodplain and forest areas as well as farther from the urban area, this proximity to the wild environment may facilitate greater contact with Triatominae, which are vector transmitters of *Trypanosoma* spp. and naturally occupy wild environments as their habitat[17]. In addition, the collections in this municipality were all carried out in the month of March, which was the rainiest season. Mendes and Lima[18] evaluated the influence of climate on the occurrence of triatomines over a period of three years in Uberlândia, state of Minas Gerais. They observed a higher catch rate in the rainy season. Some studies indicate that the dispersion and formation of colonies occurs in the rainy season, while mating and oviposition are more concentrated during the dry season[18]

The role of equine in the transmission chain of leishmaniasis has not yet been fully clarified, and it is known that there is seroconversion[4] and disease development[3]. In addition, a study conducted in the state of Maranhão showed that the horse is the animal of choice as a food preference for sand flies[6]. Using ELISA for screening and IFAT as confirmation for *Leishmania* spp., we obtained 4.34% of reactive animals, which was a result lower than the result found by Soares et al.[5] in a study with horses from Belo Horizonte, Minas Gerais, where they observed a seroprevalence of 30.48% using both techniques. When the ELISA was used, 25.40% of reagent samples were found by Duarte et al.[19] in a serological survey in the municipality of Rio de Janeiro, which is an endemic region for the two clinical forms of leishmaniasis. Researchers observed 11.6% of positive horses, which is a result lower than the outcome of this study, and the difference possibly occurred due to the use of heterologous antigens of *Leishmania* major-like parasites[20].

The statistical association ($p = 0.003$) observed between the presence of anti-*Trypanosoma* spp. and anti-*Leishmania* spp. antibodies in ELISA can indicate cross-reaction between the tests, or even a possible co-infection. The cross-reaction in serological tests for parasites of species belonging to the Trypanosomatidae family is quite common because they are phylogenetically close species[21]. Of these animals, 64.00% (16/25) presented sera with higher optical density for *Trypanosoma* spp., while 36.00% (9/25) presented higher optical density for *Leishmania* spp., and it is possible that the most likely agent is that with higher optical density, although more studies are needed to confirm that.

As for antibody titers, for the two diseases, most of the tested animals showed titer 40. Studies with other species of mammals show that exposure to CL agents may not trigger a production of antibodies with high titers. This trend likely occurred because humoral immunity in cutaneous leishmaniasis is not as expressive as in VL and trypanosomiasis, in which immune responses are more exacerbated and low titers suggest that it is an early exposure[22–24].

Conclusions

The results obtained in this study suggest that this horse breed is in contact with the protozoa that cause leishmaniasis and trypanosomiasis, which demonstrates the need for a more accurate investigation of the real role of horses in these diseases to enhance control measures for the disease. The protozoa of the family Trypanosomatidae cross-react with each other in indirect diagnostic methods.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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