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Urban Horses As Environmental Bioindicators for Leishmaniasis

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Abstract

The presence of DNA and anti-*Leishmania* spp. antibodies in the serum of 112 healthy horses was investigated by evaluating the physical examination, from a rural society located in the north central region of Paraná. The antigens of *Leishmania amazonensis*, *Leishmania braziliensis*, and *Leishmania infantum* were used to perform the indirect enzyme-linked immunosorbent assay, where it was possible to detect the reaction in 27.67% of the samples. These were also subjected to the real-time quantitative PCR, which confirmed the presence of *Leishmania* spp. DNA in 67.34% of the tested samples. The results show that the tested animals were previously exposed to the protozoan. Thus, these animals can be considered environmental bioindicators of the presence of *Leishmania* spp. at the study site. The material used in this study (serum), although not ideal, proved to be effective and less invasive. Taking into account the importance of the disease, the absence of more in-depth information on the species, the high zootechnical value of these animals, and their strictly close contact with the urban area and the human species, it is essential that further studies are carried out to elucidate the epidemiological profile of them in the face of the disease, as well as the possibility of them acting not only as hosts but also as reservoirs.

Keywords: urban area, Phlebotomines, *Leishmania* spp., One Health, zoonosis

Introduction

Horses are medium-to-large animals widely known and distributed in the world since the dawn of humanity. Initially, they were used as a food source and for transportation purposes. Today, they are part of an agribusiness complex that directly influences the Brazilian economy, with a turnover of more than R$ 16 billion annually (Cintra 2010, Ministério da Agricultura, Pecuária e Abastecimento 2017).

The Brazilian national troop is >5 million horses, counted among animals for working, breeding, competition, and leisure. Of all these categories mentioned, we can highlight the animals used for sport and leisure activities, whose number increased from 800,000 to 1,100,000 heads between the years 2006 and 2016 (Lima et al. 2006, Ministério da Agricultura, Pecuária e Abastecimento 2017).

These animals are distributed in different establishments such as stud farms, equestrian centers, ranches, training centers, or rural societies, with different objectives: commercial (breeding to sell products), private (breeding for own use), and professional (providing services to third parties, such as, *e.g.*, sports and eco therapies) (Lima et al. 2006).

In order for them to perform as expected when performing sports, it is necessary that these animals are healthy, that is,

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showing complete physical, mental, and social well-being, which is associated with the absence of injuries and diseases (Broom and Molento 2004).

As they are animals in stables, factors such as stress and intense and frequent training can be considered aggravating the incidence of infections, since these animals can present the most vulnerable immune system, making them more susceptible to different diseases, such as, for example, leishmaniasis (Murray et al. 1989, Bird 2004, Cintra 2010, Ministério da Agricultura, Pecuária e Abastecimento 2017, Benassi et al. 2018).

Leishmaniasis is an anthropozoonosis, caused by protozoa of the genus *Leishmania*, considered of great importance for public health, representing a complex of diseases with high incidence, wide geographical distribution, epidemiological diversity, and an important clinical spectrum, which include the forms: cutaneous, mucocutaneous, and visceral (World Health Organization 2010, Silva 2018, Pan-americana de Saúde Organização 2019).

The disease can be caused by >20 species of *Leishmania*, digenetic protozoa that develop with the promastigote form in the intestine of insects belonging to the Lutzomya genus, and with the intracellular amastigote form in the mononuclear phagocytic system of vertebrate hosts, such as dogs, humans, and, even equines (Furusawa and Borges 2014, Chagas 2017, Silva 2018, Morais 2019).

Infected horses may have different clinical manifestations, the cutaneous symptoms being the most common in the species, observed through large, granulomatous, and exudative ulcerated lesions, located predominantly on the head, limbs, and regions with absence or little presence of hair, which facilitates the access of the sandfly vector (Soares 2012, Escobar et al. 2019, Limeira et al. 2019). However, there are reports of animals that, even when infected, did not manifest evident clinical signs of the infection (Soares 2012, Escobar et al. 2019, Limeira et al. 2019).

The identification of host species that act as possible reservoirs of infectious agents is the first step in predicting the risks of zoonotic diseases, offering clarity about the specificity of the pathogen, the geographical distribution, as well as the risks of transmission to humans and also other species of animals (Cooper and Numm 2013).

Working together with multidisciplinary teams is essential to identify, characterize, and carry out zoonoses surveillance, using strategies based on acting and intervening, directly or indirectly, on the target animal populations, thus reflecting a direct benefit to One Health (Brazil 2016). Considering the proximity to humans and the presence of these animals in the urban context, the objective of this study was to investigate the presence of *Leishmania* spp. antibodies and DNA in horses, healthy by physical examination evaluation, from a rural society located in the north central region of the state of Paraná, an endemic region for Leishmaniasis.

Materials and Methods

Ethical aspects

This study was approved by the Research Ethics Committee Involving Animal Experimentation (CEPEEA) of Universidade Paranaense (UNIPAR), under protocol No. 30442/2016.

Animals and place of study

The horses used in this study came from a rural society in the urban area of a city located in the north central region of Paraná.

The animals were in stalls of 9 meters^2, 3 meters long and 3 meters wide, being mixed stalls, that is, some of masonry and others with wooden partitions. Food and water were provided separately. The feeder is at the front of the stall with a separation to supply mineral salt at will. Water drinkers have a float system, specific for horses, and are cleaned daily.

The environment is constantly crowded with people (horse keepers, veterinarians, sportsmen, and people undergoing treatment) and is close to a closed forest environment.

For this study, 112 horses were used, 28 females and 84 males. Of these horses, 98 were American Quarter Horse, 9 Grade Horses, 3 American Paint Horses and 2 Criollo Horses; body score, size, and behavior were varied and did not show any clinical signs at the time of physical examination before blood collection.

Regarding the activities practiced by the animals, it was found that 2 animals were used for dressage, 5 for ranch sorting, 7 for equine-assisted therapy, 20 for team roping, 76 for barrel racing, and 2 were colts and had not yet been tamed.

Collection of biological samples

Blood collection was performed by a veterinarian from July to August 2017 through the puncture of the jugular vein, where $\sim$10 mL of blood was collected and immediately forwarded to the Laboratory of Preventive Veterinary Medicine and Public Health at UNIPAR. In the laboratory, the samples were centrifuged to obtain the serum, where they were divided into two aliquots with equal volumes, packed in sterile flasks, and kept at −20°C until the moment of the laboratory examinations.

Serological diagnosis: indirect enzyme-linked immunosorbent assay

The indirect enzyme-linked immunosorbent assay (ELISA) was carried out according to Szargiki et al. (2009) with adaptations. The standardization of concentrations for the detection of antigens against *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum*, equine serum and protein A conjugate with peroxidase (Sigma Aldrich) (Fernandez-Bellon et al. 2006) were, respectively, 2.5 lg/mL, 1:25 and 1:500. All samples were tested in duplicate.

The optimal conditions for the dilutions of antigen, serum, and conjugate were established by the highest proportion between the average absorbances of the samples known to be positive and negative used as controls. The cutoff point for each plate was obtained through the mean absorbance of the negative sera plus 3 standard deviations. After calculating by plate, the cutoff point was obtained using the receiver operating characteristic (ROC) curve constructed by the MedCalc Statistical Software program (version 13.2.0) (Schoonjans et al. 1995).

Molecular diagnosis: real-time quantitative PCR

Good laboratory practices were used to avoid cross-contamination of DNA and negative controls were included during all DNA extraction procedures and in the performance of the molecular technique. DNA was extracted only from samples that were considered to be reactive in the ELISA test. The total genomic DNA was extracted from $\sim$100 μL of

serum wherein the DNA purification kit QIAamp® DNA Mini Kit (Qiagen, Germany) was used according to the manufacturer's instructions. The concentration and quality of the DNA obtained from the tissues were determined using a spectrophotometer (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers).

DNA samples were tested in real-time PCR for the presence of *Leishmania* spp. using 150 forward (kDNA) 5 - GGG (G/T) A GGG GCG TTC T (G/C) CGA A-3 and 50 reverse (kDNA) 5 - (G/C) (G/C) (G/C) (A/T) CT AT (A/T) TTA CAC CAA CCC C-3 according to Reis et al. (2013).

The PCR was performed in a final volume of 20 μL containing 0.5 pmol of forward and reverse primers, Master Mix of QuantiNova® SYBR® Green PCR (Qiagen, Germany), and 10 μL of DNA template.

The reactions were performed in a Real-Time PCR System Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System, following the next parameters for the reaction: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60 cycles for 1 min.

Reactions were considered positive when the cycle threshold was ≤ 37 for the pathogen analyzed and ≤ 34 for the positive control. As a positive control of the real-time PCR, a positive sample of *Leishmaniose* spp. and *L. infantum* was used, amplified in the mentioned primers, and confirmed by sequencing.

Results

Of the 112 serum samples tested in the ELISA, 31 (27.67%) were reactive, 25 (22.32%) of which were reactive samples for *L. amazonensis*, 17 (15.18%) for *L. infantum*, and 17 (15, 18%) for *L. braziliensis*, with some positive for more than one species (Table 1).

Of the 31 reactive samples in the ELISA test, for any of the 3 *Leishmania* spp. antigens tested, 21 (67.34%) were also reactive by the quantitative PCR (qPCR) test, confirming the presence of *Leishmania* spp. DNA (Table 1).

Discussion

The central north mesoregion of Paraná covers an area of 2,453,216 ha, corresponding to 12% of the state territory, located in its largest portion in the *Terceiro Planalto Paranaense*, bordering the State of São Paulo by the Paranapanema River and having as main borders the Tibagi River to the east and Rio Ivaí to the west. In most of the territory, the humid subtropical mesothermal climate occurs, with annual average temperatures ranging from 22°C in warmer months to 18°C in the coldest and relative humidity of 75% (Instituto Paranaense de Desenvolvimento Econômico e Social 2004).

It is an endemic region for the detection of Tegumentary Leishmaniasis, with high rates recorded in recent years in humans, with 1156 confirmed cases between the years 2008 and 2018, rates higher than those recorded in the metropolitan region during the same period, which is 426 cases, this region being three times larger than that of this study, which highlights the urgency in understanding which species may be contributing to the maintenance of the protozoan, acting as hosts or reservoirs (Sistema de Informação de Agravos de Notificação 2020).

The humid climate, characteristic of the north central region of Paraná, associated with environments with a lot of organic matter and low light, favors the development of larvae, one of the phases of the metamorphosis of the Phlebotomines that can contribute to the population increase of the vector (Chagas 2017).

Table 1. Indirect Immunoenzymatic Assay (ELISA) Using *Leishmania amazonensis*, *Leishmania braziliensis*, and *Leishmania infantum* Antigen and Real-Time Quantitative PCR from Serum Samples from Equine Athletes from the North Central Region of the State of Paraná, Brazil, July to August 2017

	ELISA			
Samples	*Leishmania amazonensis*	*Leishmania braziliensis*	*Leishmania infantum*	*qPCR*
1		+	+	
8	+	+	+	+
9	+	+	+	+
13	+			
15	+	+	+	+
16	+	+		+
20	+		+	+
22		+	+	
24	+	+	+	+
33	+	+	+	
45	+	+	+	+
47	+			
48	+			
50	+	+	+	+
51	+			
54	+		+	+
56	+			+
61	+			+
64	+			+
65	+			
72		+		+
76	+		+	+
78	+		+	+
81	+	+	+	+
86	+		+	+
89	+	+	+	
91	+			
94	+	+		+
105		+	+	+
115		+		+
124		+		+
Total	25	17	17	21
(%)	(22.32)	(15.18)	(15.18)	(67.34)

"+" denotes reactive samples for ELISA and qPCR.
ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative PCR.

Owing to the fact that they are invertebrate insects of the order Diptera, capable of covering short distances in each flight, they are predominantly close to the hosts, as in animal breeding environments, which may explain the detection of leishmaniasis in different species such as horses, species investigated in this study (Chagas 2017, Escobar et al. 2019). Other important factors that may favor transmission to horses are the high blood volume they present and the fact that they travel long distances in endemic regions due to the numerous activities to which they can be subjected (Chagas 2017).

The results of this study were superior to those found by Chagas (2017) and Lopes et al. (2013), who showed 17.6% and 4% positivity regarding the detection of anti-*L. infantum* antibodies, being the first compared with the ELISA test and the last one by direct agglutination (DAT). The variation in prevalence between the results of this study and those of

Lopes et al. (2013) can be explained by the specificity of the ELISA technique when compared with DAT.

The presence of anti-*Leishmania* antibodies in the serum of the horses analyzed in this study allows us to infer, as was done by Chagas (2017), Escobar et al. (2019), and Benassi et al. (2018), that the equine species is capable of becoming infected and that the animals in this study had previous exposure to the protozoan, evidencing its presence in the study area, thus associating the species with a role of environmental bioindicators for the disease (Chagas 2017, Vasconcelos et al. 2018).

Regarding molecular diagnosis, this study presented results superior to those presented by Escobar et al. (2019), who detected DNA from *Leishmania* spp. in 13.7% of the horses analyzed and inferior to those from Benassi et al. (2018), which detected DNA in 100% of the analyzed samples. This variation in prevalence can be attributed to different factors such as the variation in the transmission cycle in the respective study sites, the reservoirs, the Phlebotomine vectors, the diagnostic technique performed, and the biological sample used to perform the diagnostic test.

The use of serum for the diagnosis of leishmaniasis by molecular techniques is still contradictory. It is known that this biological material for antibody research is the material of choice. However, for DNA research, it is not the most satisfactory material. In contrast, when tested in this study, the results proved to be efficient (67.34%) in view of the objective of this study, which was to investigate *Leishmania* spp. DNA, being even less invasive for the animal when compared, for example, with puncture of lymph node and confirming what had already been observed in dogs, that qPCR using blood can be highly sensitive, with a high rate of positivity, especially in asymptomatic cases (Assis et al. 2010).

The importance of the species of *Leishmania* in the pathogenesis of the disease is indisputable, as well as the complex interaction of the immune response of the vertebrate host with the parasitic species, which can generate diverse pathogenic profiles (Pires et al. 2012). In this study, the animals presented no evident clinical signs, which can be attributed to the *Leishmania* species present in the animal and the type of cellular immune response triggered.

Corroborating what has already been observed in dogs and humans, if the response is of the Th1 type, cytokines such as IL-2, IFNγ, TNF-α, and IL-12 will be produced, inducing the activation of macrophages and the production of antimicrobial compounds, such as nitric oxide, blocking the action of the protozoan in the animal (absence of symptoms) or leading to the destruction of parasites. However, if the response is of the Th2 type, IL-4 and IL-10 will be produced, which inhibit macrophage activation, thus triggering the clinical symptoms of the disease (Alvar et al. 2004, Fernández-Bellon et al. 2006, Feitosa et al. 2012, Pires et al. 2012, Chagas 2017).

This is the first study to detect antibodies anti-*L. amazonensis*, anti-*L. infantum*, and anti-*L. braziliensis*, as well as to confirm the presence of *Leishmania* DNA in horses without clinical signs from that region. The lack of knowledge about the clinical aspects of the disease in this animal species underscores the importance of further studies through interdisciplinary teams, reinforcing the importance of addressing this issue from the concept of One Health.

Conclusions

The horses in this study, coming from the north central region of the state of Paraná (PR), were previously exposed to protozoa, since the antibodies anti-*L. amazonensis*, anti-*L. infantum*, and anti-*L. braziliensis* were detected. Although the biological material (serum) used in this study is not ideal for DNA research, it proved to be satisfactory when related to the objective of this study, which was to investigate the presence of *Leishmania* spp. DNA in animals without evident clinical signs for this disease, being even less invasive for the animal.

The presence of humans (horse keepers, veterinarians, sportsmen, or even people undergoing treatments) attending the environment in which these animals are housed and the possible exposure to an infected vector can be an aggravating factor for the incidence of the disease in the study region.

Taking into account the importance of the disease, the absence of more specific information about the species, the high zootechnical value of these animals, and their close contact with the urban area and the human species, it is essential that further studies are conducted to elucidate their epidemiological profile in the face of this disease, as well as the possibility of them acting not only as hosts but also as reservoirs.

In addition, intensifying health education measures and devising strategies that aim to contain the vector's reproduction are fundamental in the place of this study, considering the relevance of the disease and its high incidence in the region.

Author Disclosure Statement

No conflicting financial interests exist.

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