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Low prevalence of infection by *Sarcocystis neurona* in horses from the State of Alagoas, Brazil

Baixa prevalência da infecção por *Sarcocystis neurona* em cavalos do estado de Alagoas, Brasil

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Abstract

The aim of this study was to determine the prevalence of infection by *Sarcocystis neurona* in horses and identify potential risk factors. Were analyzed 427 samples from 36 farms in 21 municipalities in the Alagoas State, Brazil. Presence of anti-*S. neurona* antibodies was diagnosed by indirect immunofluorescence antibody test (IFAT) and was confirmed using the immunoblot test. Risk factors were assessed through investigative questionnaires on animal management on the farms. The prevalence of anti-*S.neurona* antibodies was 2.8% (confidence interval, CI: 1.5-4.9%) from IFAT and 1.6% (CI:0.8-3.34%) from immunoblot, and there were positive horses on 16.6% of the studied farms. None of the variables studied presented associations with serological status for *S. neurona*. This is the first report on infection by *S. neurona* in horses reared in Alagoas, Brazil showing a low exposure to *S. neurona* in this region, but with significant numbers of foci.

Keywords: Equine protozoan myeloencephalitis, epidemiology, serology, indirect immunofluorescence, immunoblot.

Resumo

Objetivou-se neste estudo determinar a prevalência e os fatores de risco associados à infecção por *Sarcocystis neurona* em equinos. Foram analisadas 427 amostras de 36 propriedades localizadas em 21 municípios do estado de Alagoas. O diagnóstico de anticorpos anti-*S. neurona* foi realizado pela técnica de Imunofluorescência Indireta (IFI) e confirmada por *immunoblot*. O estudo dos fatores de risco foi realizado a partir de questionários investigativos sobre o manejo dos animais nas propriedades. A prevalência de anticorpos anti-*S. neurona* foi de 2,8% (I.C. 1,5-4,9%) na IFI e de 1,6% (I.C. 0,8-3,34%) no *immunoblot* com equinos positivos em 16,6% das propriedades estudadas. Nenhuma variável estudada apresentou associação com o status sorológico para *S. neurona*. Este é o primeiro relato da infecção por *S. neurona* em equinos criados no Estado de Alagoas, Brasil, confirmando que os animais desta região têm baixa exposição a *S. neurona*, mas com significativo número de focos.

Palavras-chave: Mieloencefalite protozoária equina, epidemiologia, sorologia, imunofluorescência indireta, *immunoblot*.

Introduction

Sarcocystis neurona is a coccidian parasite from Sarcocystidae family of the phylum Apicomplexa. It definitive hosts are opossums of the species *Didelphis virginiana* in North America and *Didelphis albiventris* in South America, and a variety of other mammals could act as intermediate hosts (FENGER et al., 1995; DUBEY et al., 2001, 2015). This coccidian is the main causative agent of equine protozoan myeloencephalitis (EPM), is widely distributed across the Americas and has a significant economic impact on the horse-rearing industry (MACKAY et al., 2000; DUBEY et al., 2015). The risk factors associated with EPM are related with geographical proximity to areas of definitive host occurrence, age, stress, exercise intensity and seasonal factors (SAVILLE et al., 2000).

Despite the importance of this disease, few studies on infection by *S. neurona* in horses in Brazil have been conducted. Hoane et al. (2006) found 69.6% of seropositive animals to *S. neurona* in

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different regions of Brazil, which shows the great exposure of the animals to the protozoan. In particular, little is known about whether animals in the northeastern region of Brazil are exposed to this coccidian.

The state of Alagoas, northeastern Brazil, has 64,126 horses, representing the 22nd equine herd among the States of Brazil (ALAGOAS, 2015). However, there are no studies demonstrating *S. neurona* infection. Therefore, the objectives of the present study were to determine the prevalence of anti-*S. neurona* antibodies in horses reared in the State of Alagoas, and to identify potential risk factors associated with such infection.

Materials and Methods

Samples

A cross-sectional study was conducted on 427 blood serum samples from healthy horses (no MEP signs) of both sexes, aged over 36 months that were kept on 36 farms in rural and urban areas of 21 municipalities in the State of Alagoas, Brazil (Figure 1). Horses of different breeds were sampled from farms that employed semi-intensive or intensive animal farming. Each animal's diet was primarily based on green forage and/or hay as well as balanced ration. The animals were used for work, sport and recreation/leisure activities.

To determine the sample size, the expected prevalence was estimated to be 50%, with a 95% confidence level and statistical error of 5%. The farms were selected according to the convenience of access and agreement of the farmers with the study.

Indirect Fluorescent Antibody Test (IFAT)

Initially, the serum samples were screened by IFAT to detect anti-*S. neurona* IgG antibodies using merozoites of the strain SN37R that were fixed in 12-well plates (12,000-15,000 merozoites/well). Serum dilutions started at 1:80 (DUARTE et al., 2003) and positive samples underwent serial two-fold dilutions until the endpoint titer was reached. Fluorescein isothiocyanate-labeled goat anti-horse IgG (Sigma-Aldrich®, St. Louis, USA) was used as a secondary antibody. Negative and positive control serum samples were included on each slide.

Immunoblot test

The immunoblot test was performed as previously described by Moré et al. (2014) to confirm the IFAT-positive samples. A concentration of 2×10^7 purified merozoites of the *S. neurona* isolate (strain SN-3) was used as the antigen. Positive serum samples were diluted 1:10 and control reference serum samples (positive and negative) were used on each routine. Anti-horse immunoglobulin G peroxidase conjugate (1:500) was used as the secondary antibody. Reactivity to antigens with relative mobility of 7-10 and 16 kDa and additionally to the 30 kDa was considered specific for antibodies against *S. neurona.*

Risk factor assessment and statistical analysis

Risk factors were assessed through application of investigative questionnaires consisting of objective questions about animals' productive and sanitary management, like: history of neurological problems; health status; presence of marsupials (*Didelphis* spp.) in the farms (frequency seen and access to facilities); origin, types and stocks of feed; horses' water sources; structure and management of the farms. This information served as independent variables for subsequent analyses that evaluated the associations with seropositivity to the protozoa. Univariate analysis was conducted on the variables (chi-square test or Fisher's exact test), followed by multivariate analysis (logistic regression). To perform the frequency and statistical calculations, the EpiInfo software (CDC, version 7) was used.

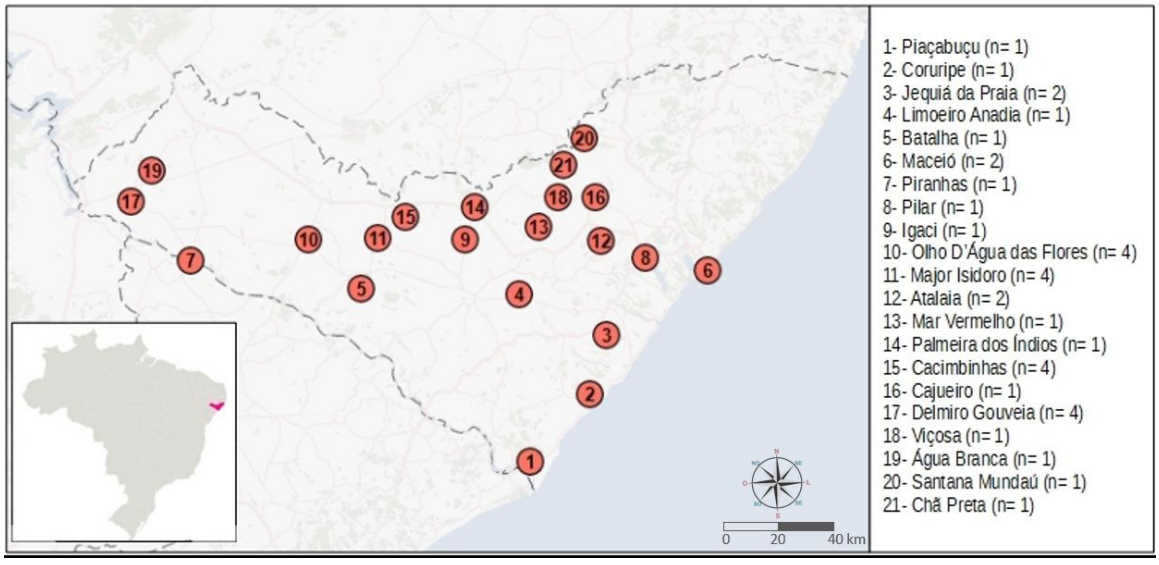

Figure 1. Distribution of the municipalities and the numbers of farms sampled (n) in each of them, in Alagoas State, Brazil.

Results, Discussion and Conclusion

The prevalence of antibodies against *S. neurona* was 2.8% by IFAT (confidence interval, CI: 1.5-4.9%; 12/427), with titers of 80 (66.7%; 8/12), 160 (25%; 3/12) and 320 (8.3%; 1/12). IFAT-positive serum samples were then subjected to the immunoblot test and this confirmed that the prevalence of positive samples with specific antigens against *S. neurona* was 1.6% (CI: 0.8-3.34%; 7/427). It was observed that 16.6% (6/36) of the farms presented at least one positive animal. Table 1 shows the frequency of positive animals per property obtained by IFAT and Western blot. Out of the 12 samples that were positive in IFAT, seven were confirmed as immunoreactive to specific antigens against *S. neurona* (Table 2).

The frequency of infection by *S. neurona* in horses in the State of Alagoas was considered low in comparison with previous investigations conducted in Brazil. These included 26.0% by IFAT in the State of Minas Gerais (RIBEIRO et al., 2016); three studies in Rio Grande do Sul: 33.7% by ELISA (PIVOTO et al., 2014), 33.8% by IFAT (ANTONELLO et al., 2015) and 37.7% by Immunoblot (LINS et al., 2012); and 36% by Immunoblot in Thoroughbreds horses from Rio de Janeiro, São Paulo and Rio Grande do Sul (DUBEY et al., 1999). In a study on horses from ten different States from Brazil, Hoane et al. (2006) found that 69.6% of the samples were positive by ELISA. Worldwide, the

Table 1. Frequency of antibodies anti-*Sarcocystis neurona* in horses per farms from Alagoas State, Brazil.

Farms	Municipies	IFAT positive	Western blot positive[a]	Negative[b]	Total[b]
1	**Água Branca**	1	-	12	12
2	**Atalaia**	-	-	34	34
3	**Atalaia**	2	1	11	12
4	**Batalha**	-	-	22	22
5	**Cacimbinhas**	-	-	8	8
6	**Cacimbinhas**	1	-	11	11
7	**Cacimbinhas**	1	-	11	11
8	**Cacimbinhas**	-	-	11	11
9	**Cajueiro**	-	-	22	22
10	**Chá Preta**	-	-	9	9
11	**Coruripe**	-	-	6	6
12	**Delmiro Goveia**	1	1	45	46
13	**Delmiro Goveia**	-	-	6	6
14	**Delmiro Goveia**	-	-	3	3
15	**Delmiro Goveia**	-	-	4	4
16	**Igaci**	-	-	7	7
17	**Jequiá da Praia**	-	-	4	4
18	**Jequiá da Praia**	-	-	4	4
19	**Limoeiro Anadia**	-	-	3	3
20	**Maceió**	3	2	8	10
21	**Maceió**	-	-	11	11
22	**Major Isidoro**	-	-	11	11
23	**Major Isidoro**	1	1	10	11
24	**Major Isidoro**	-	-	13	13
25	**Major Isidoro**	-	-	16	16
26	**Mar vermelho**	-	-	11	11
27	**Olho D'águas das Flores**	-	-	7	7
28	**Olho D'águas das Flores**	-	-	6	6
29	**Olho D'águas das Flores**	-	-	8	8
30	**Olho D'águas das Flores**	-	-	16	16
31	**Palmeira dos ìndios**	-	-	16	16
32	**Piaçabuçu**	1	1	9	10
33	**Pilar**	-	-	25	25
34	**Piranhas**	-	-	5	5
35	**Santana Mundaú**	-	-	11	11
36	**Viçosa**	1	1	4	5
TOTAL		12 (2.8%)	7 (1.6%)	420 (98.4%)	427 (100%)

[a]Confirmation of IFAT-positive samples; [b]Considering the results of the confirmation for immunoblot.

Table 2. Titers of the positive samples in IFAT and relationship with the *S. neurona* immunoblot results from horses from Alagoas State, Brazil.

Sample ID	IFAT titer	Immunoblot
1	80	Positive
2	80	Positive
3	80	Positive
4	80	Positive
5	80	Negative
6	80	Negative
7	80	Negative
8	80	Negative
9	160	Positive
10	160	Positive
11	160	Negative
12	320	Positive

prevalence of seropositivity for *S. neurona* in horses ranged from 0% to 89.2%, according to data reviewed by Dubey et al. (2015). Discrepancies in the frequencies have been explained in terms of use of animals of different ages in different states of health, the distribution of definitive hosts and the choice of serological test (HOANE et al., 2006; DUBEY et al., 2015), along with variations in management practices (SAVILLE et al., 2000). In our study, the use of young adult animals without apparent clinical disease that had been reared on farms that followed good management practices must have contributed towards the low seroprevalence detected.

Gennari et al. (2016) investigated the exposure of donkeys from different States of northeastern Brazil (including Alagoas State) to *S. neurona*, demonstrating frequency of infection of 3% (10/333), using IFAT. Donkeys and horses belong to the genus *Equus*, differ physiologically and socioeconomically but have similarities in feeding habits. Considering that the ingestion of oocysts is the main route of infection both animals, reinforces the findings of this work that shows the low exposure of the agent in the studied region.

Investigation of antibodies using serological techniques is the main tool for making *ante mortem* diagnosis of EPM, and this only indicates whether horses have been exposed to *S. neurona*. Serological techniques cannot provide certainty that infection by this protozoan is present, or even whether clinical signs are caused by *S. neurona* (JOHNSON et al., 2013). The combination of factors, such as presence of specific antibodies, typical clinical signs and the complete differential diagnosis, defines EPM diagnosis and the therapeutic actions (DUBEY et al., 2015).

In our study, the choice of cutoff titer in IFAT (1:80) was based on the findings of Duarte et al. (2003), in which choosing this cutoff point resulted in sensitivity of 88.9% (CI: 51.8-99.7%) and specificity of 100% (CI: 91-100%). Elevation of the cutoff avoids or diminishes the possibility of detecting cross-reactions (false positives), given that because of phylogenetic proximity, species of the genus *Sarcocystis* may present the same or similar surface proteins (SAVILLE et al., 2004). A serological study developed by Antonello et al. (2015) on 189 samples from mares demonstrated that 64 (33.6%) of the animals were positive for antigens against *S. neurona* in IFAT. When these same samples were subjected to IFAT with *Sarcocystis cruzi* antigen (cattle-canids life cycle), 57 samples were positive, i.e. there were only seven samples that were positive solely for antigens against *S. neurona*. Because of this possibility of cross-reactions, combinations of diagnostic serological tests are recommended (DAFT et al., 2002).

We also used the Immunoblot test, which has been considered as "gold standard" for identification of specific antibodies against *S. neurona* in horses (DUBEY et al., 2015). This confirmed that seven of the twelve samples that were positive in IFAT were also positive for reactivity to specific antigens against *S. neurona*. Even though we chose a cutoff that maximized the specificity of IFAT, five samples were considered as false positives based in 2 tests results.

The univariate analysis did not detect any variable to productive or sanitary management with infection by *S. neurona*, probably due to the low prevalence of positive animals in the farms. However, *Didelphis* spp. were frequently observed in the farms, leading to likely contamination with *S. neurona*. According to Fenger et al. (1995), horses are infected through accidental ingestion of sporocysts that were eliminated in the feces of opossums. According to Dubey et al. (2015), the species responsible for this in South America is *Didelphis albiventris,* which is a definitive competent host. Saville et al. (2000) conducted a seroepidemiological study in the United States and found that poor storage of hay (OR=3.1), the season of the year (spring, OR=3.1; summer, OR=3.2; or autumn, OR=6.0) and previous stressful events such as racing, reproduction or concomitant diseases (OR=10.0) were factors that could favor infection and emergence of EPM. In Brazil, Ribeiro et al. (2016) found that the risk factors relating to the farm were the presence of forest reserves either within or adjacent to the farm (OR=10.5) and use of animal feed that the farm itself produced (OR=6.73).

This is the first report on the presence of antibodies against *S. neurona* in horses reared in the State of Alagoas, Brazil. Despite low prevalence, seropositive horses were found to be present with a wide distribution.

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