

# Antibodies against Apicomplexa protozoa and absence sarcocysts in heart tissues from horses in southern Brazil

Anticorpos contra protozoários do filo Apicomplexa e ausência de sarcocistos no miocárdio de equinos no sul do Brasil

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## Abstract

*Sarcocystis* spp., *Neospora* spp., and *Toxoplasma gondii* are Apicomplexa protozoa that can infect horses. This study aimed to investigate the occurrence of antibodies against *Sarcocystis* spp., *Neospora* spp., and *T. gondii* in horses slaughtered in southern Brazil. The presence of histological lesions, tissue cysts, and *Sarcocystis* spp. DNA in the hearts of these horses was also investigated. A total of 197 paired serum and heart samples were evaluated by serology and direct microscopic examination; 50 of these samples were subjected to histopathological and PCR analyses. Antibodies against at least one of the protozoa were detected in 146 (74.1%) of the serum samples. The frequencies of positive serology were: 36% (71/197) against *Sarcocystis* spp., 39.1% (77/197) against *Neospora* spp., and 47.2% (93/197) against *T. gondii*. No cysts, *Sarcocystis* spp. DNA, or histopathological lesions were observed in myocardial tissue samples. The frequencies of antibody seropositivity against *Sarcocystis* spp., *Neospora* spp., and *T. gondii* showed that horses are frequently infected by these parasites in southern Brazil. The absence of sarcocysts in horse tissues is compatible with their role as aberrant/accidental hosts in the life cycle of *Sarcocystis* spp..

**Keywords:** Accidental hosts, epidemiology, *Sarcocystis* spp., *Neospora* spp., *Toxoplasma gondii*.

## Resumo

*Sarcocystis* spp., *Neospora* spp. e *Toxoplasma gondii* são protozoários que pertencem ao filo Apicomplexa e que podem afetar equinos. O objetivo deste estudo foi investigar a ocorrência de anticorpos contra *Sarcocystis* spp., *Neospora* spp. e *T. gondii*. A presença de lesões histológicas, cistos teciduais e DNA de *Sarcocystis* spp. no miocárdio de equinos abatidos no sul do Brasil também foi investigado. Um total de 197 amostras de soro juntamente com as respectivas amostras de coração, foram avaliadas por sorologia e exame microscópico direto. Destas amostras, 50 foram selecionadas e submetidas a análise histopatológica e PCR. Anticorpos contra pelo menos um dos protozoários foi detectado em 146 (74,1%) das amostras de soro. As frequências de sorologia positiva foram: 36% (71/197) para *Sarcocystis* spp., 39,1% (77/197) para *Neospora* spp. e 47,2% (93/197) para *T. gondii*. Não foram encontradas lesões histopatológicas, cistos e DNA de *Sarcocystis* spp. nas amostras de miocárdio dos equinos. As frequências de soropositividade para *Sarcocystis* spp., *Neospora* spp. e *T. gondii* mostra que os equinos podem ser frequentemente infectados por estes parasitas no sul do Brasil. A ausência de sarcocistose no coração dos equinos é compatível com seu papel como hospedeiro errático/acidental no ciclo de vida deste protozoário.

**Palavras-chave:** Hospedeiro acidental, epidemiologia, *Sarcocystis* spp., *Neospora* spp., *Toxoplasma gondii*.

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## Introduction

*Sarcocystis neurona*, *Neospora* spp., and *Toxoplasma gondii* are Apicomplexa protozoan parasites that can cause encephalomyelitis in horses. Equine protozoal myeloencephalitis (EPM) is a severe and debilitating neurological disease that is most often caused by *S. neurona* (DUBEY et al., 2001a) and less frequently by *Neospora hughesi* (REED et al., 2016). Commonly, a diagnosis of EPM is based on neurological signs and serology indicating prior exposure to *Sarcocystis* spp. and/or *Neospora* spp. (RENIER et al., 2016). Clinical signs depend on the distribution of the parasite in the central nervous system (DUBEY et al., 2001a). Equines become infected with *S. neurona* by ingesting sporocysts excreted in the feces of opossums (*Didelphis* spp.), which are definitive hosts for the pathogen (FENGER et al., 1995; DUBEY et al., 2001a). Horses are generally considered accidental hosts of this protozoan (DUBEY et al., 2006). However, Mullaney et al. (2005) suggested that horses can act as natural intermediate hosts of *S. neurona*, since cysts have been found within their muscle tissues.

*T. gondii* and *Neospora* spp. are biologically similar protozoa (DUBEY et al., 2009). Neosporosis is a widely recognized reproductive disease in cattle caused by *Neospora* spp.; this agent can also cause EPM (HAMIR et al., 1998). Horses can be infected by both *N. caninum* and *N. hughesi*; however, EPM seems to be caused specifically by *N. hughesi* (REED et al., 2016). Toxoplasmosis is a zoonotic disease caused by *T. gondii* and the infection with this protozoan in horses is usually subclinical (AL-KHALIDI & DUBEY, 1979). However, clinical signs of infection, including hyperirritability, motor incoordination, ocular disorders, and abortions, have been reported (DUBEY & PORTERFIELD, 1986). Therefore, considering the importance of Apicomplexa protozoa as etiologies of neurological diseases in horses, the objectives of this study were to investigate (a) the presence of antibodies against *Sarcocystis* spp., *Neospora* spp., and *T. gondii* in horses slaughtered in Rio Grande do Sul, Brazil, and (b) the presence of histological lesions, tissue cysts, and *Sarcocystis* spp. DNA in the hearts of these horses.

## Materials and Methods

### Animals and samples

Heart tissues and serum samples of 197 horses were collected from a slaughterhouse located in São Gabriel, Rio Grande do Sul, Brazil. The samples were collected between September and November 2014. The animals were clinically healthy horses, ranging from 6 months to 18 years of age and originating from two states of southern Brazil: Rio Grande do Sul and Paraná. Collected blood samples were centrifuged at  $250 \times g$  for 10 minutes to obtain serum and then stored at  $-20^\circ\text{C}$  until testing. Harvested heart tissue samples (50 g) were stored individually in plastic bags and kept refrigerated until processing.

### Direct examination and histopathology

Each heart tissue sample was directly examined. Approximately 25 g of tissue from each sample was macerated and homogenized with phosphate-buffered saline (PBS; 0.1 M phosphate,

0.33 M NaCl, pH 7.2) and filtered with gauze. This homogenate was examined for sarcocysts using an inverted microscope at  $400\times$ . For the histopathological examination, 50 of the 197 heart tissue samples were randomly selected, sectioned transversely, and fixed in 10% formaldehyde. Samples were steam autoclaved after 7 days of formaldehyde fixation, and then processed for histology and stained with hematoxylin and eosin. The resulting material was analyzed by light microscopy.

### DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from approximately 50 mg of each heart tissue sample using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer's instructions, with modifications in the lysis step, as outlined by Moré et al. (2011).

Total DNA was subjected to PCR, using primers specific for the 18S rDNA region of *Sarcocystis* spp. (forward: 5'-CGCAAATTACCCAATCCTGA-3' and reverse: 5'-ATTTCTCATAAGGTGCAGGAG-3'). The PCR reaction was performed in a total volume of 25  $\mu\text{L}$ , containing 5  $\mu\text{L}$  of buffer, 100 ng of each primer; 10 mM deoxynucleotide triphosphate (dNTPs); 1 U of DNA polymerase GoTaq<sup>®</sup> (Hot Start Polymerase, Promega, Madison, WI, USA), and 100 ng of total DNA used as a template. Amplification was performed with an initial denaturation for 4 min at  $95^\circ\text{C}$ ; followed by 40 cycles of denaturation for 40 sec at  $94^\circ\text{C}$ , annealing for 30 sec at  $59^\circ\text{C}$ , and extension for 60 sec at  $72^\circ\text{C}$ ; with a final extension for 6 min at  $72^\circ\text{C}$ . The PCR products were visualized by UV illumination after electrophoresis on a 1% agarose gel stained with GelRed<sup>®</sup> (Biotium Inc., CA, USA).

### Antibody survey

Anti-*Sarcocystis* spp., anti-*Neospora* spp., and anti-*T. gondii* immunoglobulin G (IgG) antibodies were detected by indirect fluorescence antibody test (IFAT) on separate slides. Antigens used to detect antibodies were merozoites of *S. neurona* (SN-37R strain) cultivated in CV-1 cells, tachyzoites of *N. caninum* (NC-1 strain), and tachyzoites of *T. gondii* (RH strain), all maintained in Vero cells. The cell were cultured in RPMI 1640 culture medium (Invitrogen, Brazil), supplemented with 10% fetal bovine serum (Nutricell, Brazil) at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ .

Serum samples were diluted 1:50 in PBS, and equine sera that were known to be positive or negative for the tested protozoa were used as controls. Commercial fluorescein-labeled anti-equine IgG (Goat Anti-Equine IgG FITC<sup>®</sup>, 160A, Southern Biotech, Birmingham, USA) was used as the secondary antibody. Slides were observed at  $400\times$  magnification using a fluorescent microscope (Leica CTR 4000/EBQ 100, Leica Microsystems, Germany). Samples with titers  $\geq 50$  were considered positive.

## Results

Serologic results for the 197 horses evaluated by IFAT showed that 146 (74.1%) were positive for antigens of at least one of the protozoa. The frequency of detection of antibodies

against each of the protozoa were the following: 36% (71/197) anti-*Sarcocystis* spp., 39.1% (77/197) anti-*Neospora* spp., and 47.2% (93/197) anti-*T. gondii*. Mixed infection by *Sarcocystis* spp. and *Neospora* spp. occurred in 5.6% (11/197) of the horses, while 11.7% (23/197) were positive for *Sarcocystis* spp. and *T. gondii*, and equines infected by *Neospora* spp. and *T. gondii* was 12.7% (25/197). In 19 (9.6%) serum samples, antibodies were detected against all three protozoa. The antibody seropositivity of horses for *Sarcocystis* spp., *Neospora* spp., or *T. gondii* alone were 9.1% (18/197), 12.2% (24/197), and 13.2% (26/197), respectively.

No sarcocysts were found in the 197 cardiac muscle tissue by direct examination. Of the 50 cardiac muscle samples subjected to histopathological examination and PCR, 25 (50%) were from animals seropositive for *Sarcocystis* spp. antibodies. No *Sarcocystis* spp. DNA was amplified by PCR of the 50 samples tested. Furthermore, no morphological alterations, lesions, or tissue cysts consistent with *Sarcocystis* spp. infection were observed in the histopathological analysis.

## Discussion

Serological analysis indicated a frequency of 36% (71/197) seropositivity to *Sarcocystis* spp. in clinically healthy horses slaughtered in southern Brazil. This result was similar to the 35.6% seropositivity found in 101 thoroughbreds in Brazil (DUBEY et al., 1999a). In addition, Hoane et al. (2006) detected seropositivity to *S. neurona* in 69.6% of horses tested in Brazil. These results indicate consistent, widespread distribution of this parasite in Brazil. This is probably a result of the free access of definitive hosts to horse pastures and feed, resulting in broad environmental contamination by sporocysts and oocysts from this protozoan. *Didelphis albiventris* is a broadly distributed opossum that was confirmed to be definitive host of *S. neurona* in South America (DUBEY et al., 2001b) and due to its synanthropy, this species is a potential disseminator of pathogens between domestic animals and man (MULLER et al., 2005).

An antibody seropositivity of 39.1% (77/197) against *Neospora* spp. is relatively high when compared with another study performed in southern Brazil, in which 15.4% seropositivity was found in draft horses (SANGIONI et al., 2011) and 23.9% detected in horses from state of Minas Gerais (RIBEIRO et al., 2016). Moreover, Hoane et al. (2006) detected a very low prevalence (2.5%) of antibodies against *Neospora* spp. in Brazilian horses. Neosporosis in horses has been described in several countries with different infection rates, which may be a result of environmental conditions, sampling methods, or the type of tests used for diagnosis (DUBEY et al., 1999a, b). Regardless of the variation, these results indicate that *Neospora* spp. infection is significant in the horses tested. Environmental contamination by oocysts excreted by dogs is a potential route for infection of horses by *N. caninum* (GONDIM et al., 2004); however, the transplacental route also allows *Neospora* spp. to be maintained in horse populations (DUBEY & PORTERFIELD, 1990). Based in these results, the neosporosis should be included in the differential diagnosis as a potential etiologic agent of EPM in southern Brazil.

Toxoplasmosis is a zoonosis of great interest due to its impact on public health and that can be transmitted through consumption

of raw or undercooked meat (DUBEY & SU, 2009). *T. gondii* infections in equines have been reported in Brazil, with variable antibody detection rates (DUBEY et al., 1999a; RIBEIRO et al., 2016). In the present study, seropositivity to *T. gondii* was 47.2% (93/197), which is high when compared to similar studies (DUBEY et al., 1999a, b), but similar results were found in Egypt (GHAZY et al., 2007). In state of Minas Gerais, Brazil, Ribeiro et al. (2016) showed a wide dispersion of *T. gondii* among horse farms, revealing that in 71.6% of the assessed farms, at least one presented one seropositive equine. The great differences in seroprevalence may be associated with various factors, including the type of feed given and the source of the water provided to animals; the intrinsic resistance to infection of horses tested; the population density of domestic or wild cats in the environment; environmental conditions; and the type of diagnostic test used (KOUAM et al., 2010). The high frequencies of antibodies detected against each of the parasites tested might be related to the origin of the blood samples. Although only clinically normal horses were sampled, it is important to highlight that most (85%) of these animals were over 10 years of age, which increases the probability of horizontal infection.

Despite the importance of *S. neurona* as an etiologic agent of EPM, the epidemiology of this protozoan disease in horses is not completely known. There are three other *Sarcocystis* species that infect equines and form cysts in their muscle tissue, *S. bertrami*, *S. equicanis*, and *S. fayeri* (LEVINE, 1986). Therefore, equine cardiac muscle was examined for sarcocysts to investigate whether these horses could serve as intermediate hosts in the parasite's life cycle. For most authors, in spite of the clinical importance of EPM, equines are considered as accidental hosts of *S. neurona*, where the infection does not determine the tissue cysts formation (DUBEY et al., 2006). However, Mullaney et al. (2005) found sarcocysts in the tongue muscle tissue of a horse with clinical signs of EPM, and they confirmed the presence of *S. neurona* DNA by restriction fragment length polymorphism and PCR. Thus, more studies are needed to clarify the epidemiological role of horses in the life cycle of this protozoan. In this study, no cysts or *Sarcocystis* spp. DNA were detected in the myocardium tissue samples evaluated, reinforcing the idea that infected horses act as accidental or final hosts of the parasite. *S. neurona* infection in Brazil is associated with cases of EPM (MASRI et al., 1992) and the presence of the definitive host *D. albiventris* (DUBEY et al., 2001b). This opossum is frequently encountered in rural areas, and usually lives in forests. However, with an increase in deforestation, the opossums are in closer proximity to farms and urban areas, increasing the possibility of environmental contamination and potential for infection of horses (DUBEY et al., 2001a).

## Conclusion

The high frequencies of antibody seropositivity against *Sarcocystis* spp., *Neospora* spp., and *T. gondii* in clinically healthy horses showed that these animals are at risk of infection by Apicomplexa parasites in southern Brazil. The absence of sarcocysts in the heart tissues of anti-*Sarcocystis* spp. seropositive horses is compatible with their role as aberrant/accidental hosts in the life cycle of *S. neurona*.



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