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. No cisto, Sarcocystis spp. DNA, ou histopatológicas lesões foram observadas em amostras de coração. As frequências de antibióticos soropositividade contra Sarcocystis spp., Neospora spp., e T. gondii mostrou que cavalos são frequentemente infectados por esses parasitas no sul do Brasil. A ausência de sarcocistose no coração dos equinos é compatível com seu papel como hospedeiro errático/accidental no ciclo de vida deste protozoário.


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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 x g for 10 minutes to obtain serum and then stored at −20 °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 400x. 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′-CGCAAAATTACCCAATCTTGTA-3′ and reverse: 5′-ATTTCCTCATAAGGGTCAGGAGAG-3′). The PCR reaction was performed in a total volume of 25 µL, containing 5 µL of buffer, 100 ng of each primer; 10 mM deoxynucleotide triphosphate (dNTPs); 1 U of DNA polymerase GoTaq® (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 °C; followed by 40 cycles of denaturation for 40 sec at 94 °C, annealing for 30 sec at 59 °C, and extension for 60 sec at 72 °C; with a final extension for 6 min at 72°C. The PCR products were visualized by UV illumination after electrophoresis on a 1% agarose gel stained with GelRed® (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 °C under 5% CO₂. 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®, 160A, Southern Biotech, Birmingham, USA) was used as the secondary antibody. Slides were observed at 400× magnification using a fluorescent microscope (Leica CTR 4000/EBQ 100, Leica Microsystems, Germany). Samples with titers ≥ 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.
References


