UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

CARACTERIZAÇÃO PATOLÓGICA E PARASITOLÓGICA DE CASOS DE
MIELOENCEFALITE PROTOZOÁRIA EQUINA NA REGIÃO SUL DO BRASIL

LUAN CLEBER HENKER

PORTO ALEGRE

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RESUMO

A mieloencefalite protozoária equina (MEP) é uma doença neurológica que acomete equinos no continente americano. MEP é frequentemente causada por *Sarcocystis neurona* e ocasionalmente por *Neospora hughesi*. A maior parte do conhecimento atual sobre MEP é proveniente de estudos conduzidos na América do Norte, enquanto dados referentes à MEP na América do Sul são escassos. O objetivo deste estudo é descrever os aspectos epidemiológicos, patológicos, imuno-histoquímicos e moleculares de casos de MEP em equinos submetidos à necropsia entre os anos de 2010 e 2017 na região sul do Brasil. No período estudado, 13 casos histologicamente compatíveis com MEP foram diagnosticados, representando 2,64% do total de diagnósticos em equinos e 34,21% das doenças inflamatórias afetando o sistema nervoso central (SNC) nesta espécie. MEP correspondeu à segunda condição inflamatória do SNC mais comum em equinos, apenas atrás da infecção pelo vírus da raiva. A idade mediana dos equinos afetados foi de oito anos, a maioria dos animais acometidos eram machos (10/13), e a doença foi observada ao longo de todo o ano. Lesões macroscópicas, caracterizadas por áreas multifocais de coloração avermelhada foram detectadas na medula espinhal em cinco equinos. Lesões microscópicas apresentaram-se de forma multifocal em todos os casos, e foram mais frequentemente observadas em segmentos da medula espinhal e no rombencéfalo. As lesões encontravam-se restritas a medula espinhal e ao encéfalo em três e dois casos, respectivamente. Protozoários intralesionais consistentes com *S. neurona* foram observados em secções histológicas em cinco equinos, e imunomarcação positiva para *S. neurona* foi observada em onze casos (11/13). Através de técnicas moleculares, cinco casos positivos para o ITS-1 e para o 18S rRNA foram detectados. Após o sequenciamento obtiveram-se fragmentos de qualidade de duas amostras referentes ao gene 18S rRNA e quatro referentes ao ITS-1. No presente estudo, o agente associado com casos de MEP detectado por imuno-histoquímica e técnicas moleculares foi *Sarcocystis* spp. Portanto, MEP deve ser incluída entre os diagnósticos diferenciais de doenças neurológicas em equinos na região sul do Brasil, mesmo em casos em que parasitas não são prontamente observados na avaliação histológica.

Palavras-chave: *Sarcocystis neurona*; doença neurológica; equino; parasita; diagnóstico.

ABSTRACT

Equine protozoal myeloencephalitis (EPM) is a neurologic disease affecting horses in the American continent. EPM it is often caused by *Sarcocystis neurona*, and occasionally by *Neospora hughesi* infection. Most current knowledge regarding EPM comes from studies conducted in North America, while in South America these data are scant. The aim of this study is to describe the epidemiological, pathological, immunohistochemical and molecular findings of EPM cases in horses necropsied in Southern Brazil from 2010 to 2017. In the studied period, 13 cases compatible with EPM were diagnosed, representing 2.64% of the overall postmortem diagnosis in horses, and 34.21% of the central nervous system (CNS) inflammatory conditions in the species. EPM corresponded to the second most common cause of CNS inflammatory disease in horses, only after rabies virus infection. Median age of affected horses was of eight years, males were overrepresented (10/13), and the disease was observed all year round. Gross lesions were detected in the spinal cord in five horses (5/13), and these were characterized by multifocal red areas. Microscopic lesions were multifocal in all cases, and these were more frequently observed in the spinal cord segments and in the rhombencephalon. Lesions were restricted to the spinal cord and to the brain in three and two cases, respectively. Intralesional protozoans consistent with *S. neurona* were seen in histopathological sections in five horses, and positive immunostaining for *S. neurona* was observed in eleven cases (11/13). Through molecular techniques, five positive cases for the ITS-1 and 18S rRNA genes were detected. After sequencing, two quality samples of 18S rRNA gene and four of the ITS-1 were obtained. In the present study, the only agent associated with EPM cases detected by immunohistochemistry, and molecular analysis was *Sarcocystis* spp. Therefore, EPM should be included among the differential diagnosis of neurological disease in horses in Southern Brazil, even in the cases where parasites are not readily seen in the histological examination.

Keywords: *Sarcocystis neurona*; neurologic disease; equine; parasite; diagnosis.

SUMÁRIO

1. INTRODUÇÃO

A mieloencefalite protozoária equina ou mieloencefalite equina por protozoário (MEP) é uma das principais doenças neurológicas desta espécie no continente americano (DUBEY *et al*., 2001a; DUBEY *et al*., 2015) com relatos nos Estados Unidos da América (EUA) (FAYER *et al*.,1990), Canadá (CLARK; TOWNSEND; MCKENZIE, 1981), Panamá (GRANSTROM *et al*., 1992) e Brasil (BARROS et al., 1986; MASRI; ALDA; DUBEY, 1992; PAIXÃO; RÊGO; SANTOS, 2007). A enfermidade geralmente é progressiva e cursa com sinais neurológicos diversos que variam de acordo com a localização do parasita no sistema nervoso central (SNC) do hospedeiro (REED *et al*., 2016).

A MEP foi descrita pela primeira vez nos EUA na década de 60, sendo designada como mielite segmentar (ROONEY *et al*., 1970). Estudos iniciais suspeitavam do envolvimento de *Toxoplasma gondii* (CUSICK *et al*., 1974), entretanto pesquisas subsequentes caracterizaram o parasita envolvido nesses casos como pertencente ao gênero *Sarcocystis* (SIMPSONS; MAYHEW, 1980), e posteriormente como *Sarcocystis neurona* (DUBEY *et al*., 1991).

Atualmente tem-se estabelecido como agentes etiológicos da MEP os protozoários *S. neurona* (DUBEY *et al*., 1991) e *Neospora hughesi* (MARSH *et al*., 1998), entretanto acredita-se que a maioria dos casos de MEP sejam ocasionados por *S. neurona* (REED *et al*., 2016). MEP associada à infecção por *Neospora* sp. foi descrita pela primeira vez na década de 90 nos EUA (MARSH *et al*., 1996). Posteriormente, com base em estudos moleculares, o agente do gênero *Neospora* associado com a condição foi classificado como uma nova espécie, sendo esta denominada *N. hughesi* (MARSH *et al*., 1998). Neosporose associada à MEP tem sido apenas ocasionalmente reportada, e casos confirmados por avaliação *post mortem* encontram-se restritos a América do Norte (WOBESER *et al*., 2009; DUBEY; SCHARES, 2011). O ciclo de *Neospora hughesi* não é totalmente elucidado. Não se conhece o hospedeiro definitivo deste agente, e, por conseguinte as vias de transmissão são pobremente compreendidas (REED *et al*., 2016).

S. neurona é um protozoário pertencente ao filo Apicomplexa que apresenta ciclo parasitário heteroxeno estabelecido entre o hospedeiro definitivo, e inúmeros mamíferos que atuam como hospedeiros intermediários (REED *et al*., 2016). O hospedeiro definitivo de *S. neurona* é o gambá, gambá-da-virgínia (*Didelphis*

virginiana) na América do Norte (FENGER *et al*., 1997) e gambá-de-orelha-branca (*Didelphis albiventris*) na América do Sul (DUBEY *et al*., 2001b). Desta forma, a distribuição geográfica de casos de MEP tem sido consistentemente associada à presença deste mamífero marsupial, que habita as Américas do Sul, Central e do Norte (DUBEY *et al*., 2015). Assim, casos confirmados de MEP têm sido relatados exclusivamente em equinos no continente americano, ou em animais importados dessas áreas (KATAYAMA *et al*., 2003).

A reprodução sexuada de *S. neurona* ocorre no epitélio intestinal do hospedeiro definitivo, este então elimina esporocistos contendo esporozoítos através das fezes (REED *et al*., 2016). Os esporozoítos são infectantes para hospedeiros intermediários, e a infecção ocorre através da ingestão desta forma parasitária a partir de alimentos e água contaminados com material fecal do hospedeiro definitivo (REED *et al*., 2016). Hospedeiros intermediários de *S. neurona* incluem inúmeros mamíferos como o tatu-galinha (*Dasypus novemcinctus*) (CHEADLE *et al*., 2001a), o cangambá (*Mephitis mephitis*) (CHEADLE *et al*., 2001b), o guaxinim (*Procyon lotor*) (DUBEY *et al*., 2001c), a lontra-marinha (*Enhydra lutris*) (DUBEY *et al*., 2001d) e o gato doméstico (*Felis catus*) (DUBEY *et al*., 2000a). Nestas espécies, *S. neurona* forma cistos latentes, principalmente no tecido muscular (REED *et al*., 2016). Gambás tornam-se infectados a partir da ingestão de tecidos contendo tais cistos, o que dá continuidade ao ciclo parasitário (DUBEY *et al*., 2000a).

Equinos por sua vez atuam como hospedeiros intermediários aberrantes, erráticos ou terminais (DUBEY *et al*., 1991), uma vez que cistos teciduais de *S. neurona* não são usualmente detectados nesta espécie, desta forma equinos não contribuem para o ciclo biológico do parasita (REED *et al*., 2016). Em hospedeiros aberrantes, apenas esquizontes e merozoítos de *S. neurona* são detectados nos tecidos, e no caso da espécie equina, estas formas parasitárias tem sido detectadas exclusivamente no SNC (DUBEY *et al*., 2015). Além de equinos, doença neurológica associada à *S. neurona* tem sido ocasionalmente descrita em uma série de mamíferos terrestres (DUBEY; HAMIR, 2000b). Ainda, doença neurológica causada por *S. neurona* tem sido descrita como uma importante causa de mortalidade em mamíferos marinhos nas últimas décadas na América do Norte (BARBOSA *et al*., 2015).

O mecanismo pelo qual o parasita adentra o SNC do equino não é totalmente conhecido, mas acredita-se que envolva a infecção de células endoteliais e leucócitos

(DUBEY *et al*., 2001a). Fatores de risco para o desenvolvimento da doença incluem o acesso do hospedeiro definitivo a piquetes e instalações destinadas aos equinos, com consequente contaminação ambiental de fontes de alimentação e água (MORLEY *et al*., 2008). Além disso, fatores imunossupressores como estresse e transporte parecem estar relacionados com o desenvolvimento da enfermidade (SAVILLE *et al*., 2000).

A soroprevalência para *S. neurona* é elevada dependendo da região geográfica pesquisada (VARDELEON *et al*., 2001). Entretanto, apenas uma pequena proporção de animais infectados desenvolve a doença clínica, o que sugere a existência de fatores individuais relativos ao agente, como virulência da cepa envolvida, ou ainda, características relativas à suscetibilidade do hospedeiro intermediário (REED *et al*., 2016).

O curso clínico da doença é variável e tende a ser progressivo (FURR; HOWE, 2008). Os sinais clínicos observados dependem da região do SNC afetado e da porção comprometida (substância branca ou substância cinzenta) (REED *et al*., 2016). Lesões em medula espinhal estão associadas com sinais clínicos de incoordenação motora, dificuldade em manter-se em estação, fraqueza e atrofia muscular, principalmente, dos membros pélvicos (FURR; HOWE, 2008). O acometimento do tronco encefálico e cérebro pode cursar com diminuição do nível de consciência, paralisia de nervos faciais, dificuldade mastigatória e respiratória (DUBEY *et al*; 2001a; CANTILE; YOUSSEF, 2016).

O diagnóstico clínico presuntivo é obtido através da associação dos sinais clínicos sugestivos, juntamente com a exclusão de demais possíveis causas (FURR; HOWE, 2008). A detecção de anticorpos no soro sanguíneo e líquido cefalorraquidiano dos animais afetados podem ser realizadas, entretanto, estas técnicas apresentam limitações e devem ser interpretadas com cautela (REED *et al*., 2016). Testes sorológicos apresentam valor diagnóstico reduzido (FURR; HOWE, 2008), uma vez que parte significativa dos equinos apresenta anticorpos contra os agentes causadores de MEP, porém apenas um pequeno número desenvolve a doença clínica (VARDELEON *et al*., 2001). A avaliação de anticorpos no líquido cefalorraquidiano constitui um recurso diagnóstico mais específico, entretanto deve-se levar em consideração a passagem de anticorpos da corrente sanguínea para o líquor, bem como a possível contaminação sanguínea decorrente da coleta (FURR; HOWE, 2008). O tratamento da MEP é realizado através da administração de drogas antiprotozoário, incluindo

ponazuril, diclazuril e a associação de sulfadiazina e pirimetamina (DUBEY *et al*., 2015). O tratamento é prolongado, e o tempo mínimo para a administração de drogas como ponazuril é de 28 dias, entretanto vários meses de tratamento são por vezes necessários (DUBEY *et al*., 2015).

O diagnóstico definitivo, na maioria das vezes, somente é possível através do exame *post mortem*, a partir da associação dos achados macroscópicos e microscópicos característicos, além da comprovação do agente nos tecidos através de técnicas como a imuno-histoquímica e a PCR (REED *et al*., 2016). As lesões macroscópicas geralmente encontram-se presentes apenas em casos severos e variam de focos aleatórios de hemorragia a áreas multifocais discretas acinzentadas ou amareladas, observadas a partir da secção transversal do tecido nervoso (FURR; HOWE, 2008). Os achados histopatológicos consistem de meningoencefalomielite com infiltrado inflamatório misto de linfócitos, neutrófilos e eosinófilos, além de eventuais células gigantes, áreas de necrose e hemorragia (DUBEY *et al.,* 2001a). Esquizontes e merozoítos do parasita podem ser ocasionalmente visualizados em neurônios, células da glia e macrófagos, entretanto na maioria das vezes estes não são observados, o que dificulta o diagnóstico (CANTILE; YOUSSEF, 2016; DUBEY *et al*., 2015).

Embora um grande número de estudos envolvendo MEP encontre-se disponível, a maioria das pesquisas no tópico tem sido conduzida na América do Norte, principalmente nos EUA (REED *et al*., 2016). Assim, informações referentes a esta enfermidade são escassas no continente sul-americano, e são frequentemente representadas por relatos de casos isolados (PAIXÃO *et al*., 2007) e estudos de soroprevalência (HOANE *et al*., 2006; RIBEIRO *et al*., 2016). Adicionalmente, informações referentes a achados imuno-histoquímicos e moleculares em casos clínicos de MEP na América do Sul são limitadas. Portanto, o objetivo deste trabalho é descrever os aspectos epidemiológicos, patológicos, imuno-histoquímicos e moleculares de 13 casos fatais que apresentaram o diagnóstico de MEP, diagnosticados entre 2010 e 2017 na região sul do Brasil.

Neste item é apresentado o artigo "Pathological, immunohistochemical and molecular findings in cases of Equine Protozoal Myeloencephalitis in horses in Southern Brazil", redigido conforme as normas do período *Veterinary Parasitology*, a ser submetido após as contribuições da banca examinadora.

2. ARTIGO

Pathological, immunohistochemical and molecular findings of equine protozoal myeloencephalitis in horses in Southern Brazil

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Abstract

Equine protozoal myeloencephalitis (EPM) is an important neurologic disease affecting horses in the American continent. EPM it is often caused by *Sarcocystis neurona*, and occasionally by *Neospora hughesi* infection. Most current knowledge regarding EPM comes from studies conducted in North America, while in South America these data are scant. The aim of this study is to describe the epidemiological, pathological, immunohistochemical and molecular findings of EPM cases in horses necropsied in Southern Brazil from 2010 to 2017. In the studied period, 13 cases compatible with EPM were diagnosed, representing 2.64% of the overall postmortem diagnosis in horses, and 34.21% of the CNS inflammatory conditions in the species. EPM corresponded to the second most common cause of CNS inflammatory disease in horses, only after rabies virus infection. Median age of affected horses was of eight years, males were overrepresented (10/13), and the disease was observed all year round. Gross lesions were characterized by multifocal red areas detected at the spinal cord level in five horses. Microscopic lesions were multifocal in all cases, and more

frequently observed in the spinal cord segments and the rhombencephalon. Lesions were restricted to the spinal cord and to the brain in three and two cases, respectively. Intralesional protozoans were seen in histopathological sections in five horses, and positive immunostaining for *S. neurona* was observed in eleven cases (11/13). Through molecular techniques, five positive cases for the ITS-1 and 18S rRNA genes were detected. After sequencing, two quality samples of 18S rRNA gene and four of the ITS-1 gene were obtained. In the present study, the only agent associated with EPM cases detected by immunohistochemistry, and molecular analysis was *Sarcocystis* spp. Therefore, EPM should be included among the differential diagnosis of neurological disease in horses in Southern Brazil, even in the cases where parasites are not readily seen in the histological examination.

1. Introduction

Equine protozoal myeloencephalitis (EPM) is an important neurologic disease of horses in the American continent (Dubey et al., 2015a), which was first described in the 1960s (Rooney et al., 1970), and, since then, it has been reported in the USA, Canada (Fayer et al., 1990), Panama (Granstrom et al., 1992), and Brazil (Barros et al., 1986; Masri et al., 1992; Paixão et al., 2007). The condition may be caused either by *Sarcocystis neurona* (Dubey et al., 2015a) or *Neospora hughesi* infection (Marsh et al., 1998); nonetheless the majority of the cases are associated with the first (Reed et al., 2016). EPM is usually a debilitating progressive disease characterized by a wide range of neurological signs according to the parasite distribution in the central nervous system (CNS), frequently representing a challenge for the clinical diagnosis (Dubey et al., 2001a).

Opossums, the Virginia opossum (*Didelphis virginiana*) in North America (Fenger et al., 1995), and the white-eared opossum (*Didelphis albiventris*) in South America (Dubey et al., 2001b), act as the definitive hosts of *S. neurona*. Thus, the occurrence of EPM has been consistently related to the presence of opossums (Dubey et al., 2015a), with the condition being only described in horses born and raised in the Americas (Dubey et al., 2015a), or imported from these areas (Katayama et al., 2003). *S. neurona* infection occurs through the fecal-oral route, and known intermediate hosts include the nine-banded armadillos (*Dasypus novemcinctus*) (Cheadle et al., 2001a), striped skunks (*Mephitis mephitis*) (Cheadle et al., 2001b), raccoons (*Procyon lotor*)

(Dubey et al., 2001c), sea otters (*Enhydra lutris*) (Dubey et al., 2001d), and domestic cats (Dubey et al., 2000a). Horses are believed to act as aberrant or dead-end hosts, since *S. neurona* cysts are not usually observed in muscle samples from this species (Reed et al., 2016). Still, EPM-like illness induced by *S. neurona* infection has been occasionally reported in several other terrestrial (Dubey et al., 2000b), and marine mammals in North-America (Barbosa et al., 2015).

Neosporosis as a cause of EPM has been first identified in the USA (Marsh et al., 1996; Marsh et al., 1998), and to this day, EPM due to *N. hughesi* infection has been only described in the referred country (Dubey et al., 2001e), and in Canada (Wobeser et al., 2009). Since *N. hughesi* definitive hosts are unknown, information regarding parasite life cycle and pathogenesis are lacking (Reed et al., 2016). Even though a representative amount of research regarding EPM is currently available, most information arises from studies conducted in North America, mainly in the USA (Reed et al., 2016). Yet, these data are scarce in South America, and mainly represented by isolated case reports (Paixão et al., 2007), and seroprevalence surveys (Hoane et al., 2006; Ribeiro et al., 2016). Furthermore, little information regarding molecular and immunohistochemical findings in cases of EPM is available in Brazil. Therefore, the aim of this work is to describe the pathological, immunohistochemical, molecular, and some epidemiological findings of 13 fatal cases compatible with EPM diagnosed in horses between 2010 and 2017 in Southern Brazil.

2. **Material and methods**

2.1. Case selection

A retrospective study was conducted in the Veterinary Pathology Laboratory database of the Federal University of Rio Grande do Sul. All cases of CNS inflammatory conditions, including myelitis, encephalitis and meningitis diagnosed in horses from 2010 to 2017 were searched. Epidemiological information regarding age, sex, breed, clinical signs, disease progression, treatments, comorbidities and seasonality were obtained from the necropsy reports. Cases were reviewed and classified according to the etiology, and those presenting CNS histopathological features consistent with EPM, including occasional intralesional protozoan parasitic structures, and all the following criteria were compiled: i) CNS mononuclear perivascular cuffing; ii) inflammatory infiltrate of eosinophils; iii) multinucleated giant cells.

2.2. Pathological examination

Data regarding gross lesions observed at necropsy in the CNS, as well as in other organs were obtained from the necropsy reports. All histological slides were revised, and the microscopic lesions were characterized according to the CNS distribution as affecting the spinal cord (cervical, thoracic, lumbar, sacral and cauda equina segments), the encephalon (prosencephalon, mesencephalon, and rhombencephalon), as well as paravertebral ganglia and the trigeminal ganglion. Histological lesions were classified as absent, and when present these were graded as mild, moderate and marked. Microscopic features evaluated included mononuclear perivascular cuffing, inflammatory infiltrate of eosinophils, multinucleated giant cells, meningitis, gliosis, vacuolation of the white matter, axonal spheroid formation, liquefactive necrosis, hemorrhage, endothelial cell swelling, perivascular edema, and the presence of visible protozoan parasitic structures.

2.3. Immunohistochemistry

After histological grading, two slides of each case were selected from the regions which presented visible protozoan parasitic structures, or from regions with the most severe inflammatory lesions in cases where protozoan parasitic structures were not detected. These slides were submitted for immunohistochemistry (IHC) anti-*S. neurona*, employing a primary polyclonal antibody produced in rabbits (non-commercial), as previously described (Hamir et al., 1993). In addition, one slide from each case, selected based on the same criteria, was submitted for IHC anti-*Neospora caninum* and anti-*T. gondii*.

To produce the serum anti-*S. neurona*, merozoites (SN138 strain) (Lindsay et al., 2004) were cultivated in cell culture flasks (Vero cell line) as previously described (Lindsay et al., 2004). Inoculum was produced through partial scraping of the cell culture, which was subsequently passed three times through a 24-gauge needle to disrupt cells, and filtered in a 5µm filter. Three rounds of centrifugation (1500G, 10 min) and washing with PBS were conducted in order to remove cell debris. The resultant pellet was suspended in 1mL PBS solution, which was used as the final inoculum. Parasites were observed in a Neubauer chamber; and average number of merozoites ranged from 12,000 to 18,000 per inoculum. The inoculum was

intramuscularly administered to a rabbit twice, with a 20 days interval between administrations (Hamir et al., 1993). Eight days after the second boost, the rabbit was euthanized, blood was collected, and serum was sampled and stored (-20 °C) until used. Rabbit serum was tested with known positive tissue samples of *T. gondii* and *Neospora* sp. infection, and no cross immunolabeling was detected.

Polyclonal antibodies anti-*S. neurona* at a dilution of 1:200 in phosphate buffered saline (PBS), anti-*T. gondii* (VRMD, Pullman, WA, USA) at a dilution of 1:1000, and anti-*N. caninum* (VRMD, Pullman, WA, USA) at a dilution of 1:1000 were used. Antigen retrieval was performed with proteinase K for 1 min for *S. neurona*, and 0.1% trypsin for 10 min for *N. caninum* and *T. gondii*. LSAB-HRP Universal kit (Dakocytomation, Carpinteria, CA, USA) was used for *N. caninum* and *T. gondii*, and MACH 4 Universal HRP-Polymer (Biocare, Pacheco, CA, USA) for *S. neurona*. The reactions were revealed with 3-Amino-9-Ethylcarbazole chromogen (AEC; Sigma, St. Louis, Missouri, USA). Slides from known cases of CNS infection caused by *S. neurona*, *T. gondii* and *N. caninum* were used as positive controls. Primary antibodies were replaced by PBS in the negative control sections.

2.4. Molecular analysis

Molecular analysis was performed in all cases from fresh frozen CNS samples (five horses) and from formalin-fixed paraffin-embedded tissues (eight remaining horses). Total DNA was extracted from fresh samples using QIAmp DNA Mini Kit according to the manufacturer's instructions. For DNA extraction from Paraffin-embedded tissues, 20µm thick sections of CNS were used. Samples were pretreated for paraffin removal with a commercial deparaffinization solution, and subsequently DNA was extracted according to the manufacturer's instructions (QiAamp; Qiagen®). DNA was eluted in a final 80uL elution buffer.

Two PCRs were used for the identification of the parasites; the first detected a portion of the 18S rRNA gene, and used Primer 1L as forward primer (CCA TGC ATG TCT AAG TAT AAG C) and Primer 3H as reverse primer (GGC AAA TGC TTT CGC AGT AG) (Yang et al., 2001). The second protocol was a nested PCR targeting a gene portion from the internal transcribed spacer 1 (ITS-1 gene), based on primers directed to 18S and 5.8S rRNA coding genes. The forward primers anneal to 3'end of 18S locus whereas the reverse primers are directed to the 5'end of 5.8S locus. The primers flank

the ITS-1, and sequences used as outer primers were JS4 (CGA AAT GGG AAG TTT TGT GAA C) and CT2c (CTG CAA TTC ACA TTG CGT TTC GC), and as inner primers were JS4b (AGT CGT AAC AAG GTT TCC GTA GG) and CT2b (TTGCGC GAG CCA AGA CAT C) (Soares et al., 2011). Protocols for the 18S rRNA PCR and ITS-1 nested PCR reactions used the following parameters: 200 nM of dNTPs, 15 pmol of each primer, 1 mM of MgCl2, 1x Taq DNA polymerase buffer 10, 1 unit of Taq DNA polymerase (Invitrogen) and 1 µL of total DNA. PCR cycling conditions for primary (18S rRNA), and primary and secondary amplifications (ITS-1) were set as follows: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30s, at 58°C for 30s and at 72°C for 30s, and a final extension at 72°C for 5 min. PCR products were separated by agarose gel electrophoresis (2%) and DNA was viewed under ultraviolet (UV) light.

Amplicons of the expected size were purified with a PureLink PCR purification Kit (Invitrogen®) and quantified with a Qubit DNA quantification method (Invitrogen), following manufacturer instructions. Amplicons were sequenced using Sanger sequencing with the same forward and reverse primers employed in the PCR reactions. Generated sequences were submitted to BLAST analysis (Altschul et al., 1990) to determine the closest similarities present in GenBank.

Partial sequences of the 18S rRNA were aligned with twenty-six 18S rRNA gene sequences of protozoa retrieved from Genbank, using Clustal/W v.1.8.1 (Thompson et al., 1994). A maximum likelihood phylogenetic tree using Tamura 3 parameter+G+I substitution was generated using Mega 7 software (Kumar et al., 2016) with 100 bootstrap replicates. The substitution model was selected using Mega 7 software (Kumar et al., 2016) according to the lowest Bayesian Information Criterion (BIC) score. Sequences of the subfamily Toxoplasmatinae were used as outgroup.

Partial sequences of the ITS-1 gene were aligned with thirteen ITS-1 sequences of protozoa retrieved from Genbank, using Clustal/W v.1.8.1 (Thompson et al., 1994). The evolutionary distance (Neighbor-Joining method) was calculated by p-distance using Mega 7 software (Kumar et al., 2016) with 100 bootstrap replicates. Sequences of the subfamily Toxoplasmatinae were used as outgroup. Using the same sequences above mentioned for the Neighbor-Joining method, Maximum-Parsimony method was performed, also using Mega 7 software (Kumar et al., 2016) with 100 bootstrap replicates.

Identity matrices were calculated with the BioEdit software using the 18S rRNA and ITS-1 sequences of *Sarcocystis* spp. deposited in GenBank and the ones found in this study to evaluate their similarity.

3. **Results**

3.1. Epidemiological findings

A total of 492 horses were submitted for necropsy examination from 2010 to 2017 at the Veterinary Pathology Laboratory of the Federal University of Rio Grande do Sul, southern Brazil. Of these, 38 horses were diagnosed with myelitis, encephalitis, meningitis or the association of such conditions, which represented 7.72% of the causes of death in the species. From this group, 13 cases had a diagnosis of EPM or had histological features consistent with EPM, totalizing 34.21% (13/38) of the CNS inflammatory lesions and 2.64% of the overall postmortem diagnosis in the species in the comprehended period (13/492). EPM represented the second most common cause of inflammatory CNS disease in horses after rabies virus infection (21/492).

Data regarding breed, sex, age, season of diagnosis, clinical progression length, treatments, and clinical outcome (euthanasia or spontaneous death) of affected horses are shown in table 1. All animals were referred from cities in the State of Rio Grande do Sul, predominantly in the metropolitan area of Porto Alegre, Brazil. Information regarding age was available for 10 horses. In these cases, age range varied from 2.5 to 22 years, with mean and median age of affected animals of 10.2 and 8 years, respectively. Cases were diagnosed randomly all year round, with a relatively higher occurrence in the fall, and lower occurrence in the spring. Three cases were diagnosed in the summer, six in the fall, three in the winter, and one in the spring.

Data regarding clinical disease length was available for eight horses. In these animals, clinical progression from the onset of clinical signs until the time of death or euthanasia ranged from five to 50 days, with median and mean of 20 and 23 days, respectively. In the majority of cases (8/13), euthanasia was elected due to the animal's poor general health condition, secondary traumatic lesions due to neurologic signs, and unfavorable prognosis. In five cases, nonsteroidal or steroidal anti-inflammatory drugs were administered. In two cases (horses 6 and 8), antiprotozoal drugs were administered; however, no detailed information regarding the utilized compound, and prescribed treatment were available.

Clinical signs cited in the necropsy reports ranged from acute to chronic and comprised a wide variety of neurological clinical signs, including locomotor deficits, head signs and muscle atrophy, and these are shown in table 2. Gait abnormalities, such as mild asymmetric hind limb incoordination or stumbling, progressing to ataxia were reported in 12 horses, and forelimb lameness culminating in ataxia was described in five cases. Twelve animals presented locomotion and standing difficulties, and showed frequent falls. Clinical course culminated in persistent lateral recumbency in ten horses. Other clinical signs presenting lower frequency such as circling, paddling movements, compulsive walking, muscle atrophy and poor body condition are depicted in table 2.

In addition to that, clinical signs related to cranial nerve deficits were noted in four affected animals (horses 2, 7, 8 and 12). Of these, horses 7, 8 and 12 showed difficulty apprehending and swallowing feed. In addition, horse 7 displayed horizontal nystagmus, horse 2 had unilateral moderate masseter muscle atrophy, and horse 12 presented blindness. Horse 8 had upper airway dysfunction due to laryngeal hemiplegia as well as severe right masseter muscle atrophy.

3.2. Pathological findings

3.2.1. Gross lesions

CNS gross lesions were detected in 5/13 cases (horses 4, 5, 6, 10 and 12), and were noted at the spinal cord level at cross sectioning. Such gross changes were characterized by multifocal mild to moderate dark-red to brown areas, which usually extended from the grey matter to the white matter (Fig 1). No gross lesions were noted in the brain.

Regarding secondary muscular lesions, horses 2 and 8 had head muscle changes characterized by moderate to severe unilateral masseter atrophy. Horse 8, which clinically displayed laryngeal hemiplegia, showed moderate left cricoarytenoid dorsalis muscle atrophy. Such muscle presented pale appearance intermixed by moderate amount of gelatinous material (edema). Likewise, cases presenting hind limb atrophy (horses 11 and 12) were grossly characterized by different degrees of unilateral muscle size reduction and pale discoloration affecting the quadriceps and gluteal muscles.

Besides direct CNS damage induced by protozoan infection, and muscular secondary changes, gross findings, likely secondary to neurological deficits (standing difficulties and frequent falls), included cervical vertebral fracture (horse 9), cervical

extradural hematoma (horses 4 and 10) and multiple rib fractures associated to hemothorax (horse 1). In addition to that, five horses showed cutaneous lesions related to prolonged lateral recumbency (horses 5, 6, 7, 10, and 13). Comorbidities detected at necropsy included fibrinonecrotic enterocolitis consistent with salmonellosis infection (horse 12), granulomatous typhlocolitis related to cyathostomins (horse 7), metastatic melanoma (horse 10) and metastatic preputial squamous cell carcinoma (horse 11).

3.2.2. Histological lesions

Histologically, CNS lesions were restricted to the spinal cord in 3/13, and limited to the brain in 2/13 of the horses. In the remaining horses (8/13), associated lesions were noted at both anatomic sites. Anatomic distribution of histological changes in the CNS is depicted along with the level of lesion severity in table 3. Studied horses presented all spinal cord segments and brain regions for evaluation, expect for the cauda equina, which was only available in eight out of 13 cases. Spinal cord segments were more frequently affected (cervical, thoracic and lumbar), and tended to display more severe changes when compared with the brain. On the other hand, lesions affecting the cauda equina were noted only in horse 4. Regarding encephalic lesion distribution, the rhombencephalon was more frequently affected than the mesencephalon and prosencephalon. No histological changes were observed in paravertebral ganglia and trigeminal ganglion.

Histological lesions had a multifocal or segmental distribution in all cases, and both white and grey matter were affected. The main histological findings are shown in table 4. Microscopic changes varied in severity among cases, but perivascular inflammatory infiltrate of lymphocytes, plasma cells, macrophages, fewer eosinophils, multinucleated giant cells, and rare neutrophils was a constant finding in all cases. Perivascular cuffing frequently coalesced and involved adjacent neuropil areas in all horses. Such areas were associated with liquefactive necrosis (8/13), white matter vacuolation (10/13), axonal spheroids (9/13), endothelial cell swelling and tumefaction (10/13), hemorrhage (3/13) and perivascular edema (5/13). In addition, gliosis was noted in all but one case, and leptomeningitis was seen in 10 horses. Intralesional protozoans were rarely or occasionally observed in histological sections of 5/13 horses (38.46%). These parasitic structures were noted predominantly in areas of inflammation and liquefactive necrosis, and were consistent with schizonts in different stages of

maturation and merozoites of *S. neurona.* Mature schizonts were round to oval, measured from 8-15μm in diameter, and contained 1x3μm slender merozoites frequently arranged haphazardly, and rarely organized resembling a rosette-like structure. Immature schizonts presented multilobed nuclei. The main histological changes are depicted in figure 2. A-E.

3.3. . Immunohistochemistry

At the IHC, positive immunostaining for *S. neurona* was observed in 11/13 (84.6%) of the horses. These varied from moderate to marked labeling for schizonts and merozoites in three (horses 3, 4 and 9) and two cases (horses 12 and 13), respectively (Fig 2. F). Six additional cases had a mild to moderate immunostaining for merozoites only (horses 2, 5, 6, 7, 10 and 11). The evidenced parasitic structures were often intermixed by areas of inflammation, liquefactive necrosis and gliosis, and sometimes were within the cytoplasm of astrocytes, neurons and macrophages. No immunostaining was noted for *Neospora* sp. and *T. gondii* in any of the evaluated samples.

3.4. Molecular findings

Five out of thirteen cases presented positive PCR results for the 18S rRNA gene and ITS-1 gene. All of which corresponded to fresh frozen samples (horses 6, 9, 10, 12 and 13). While none of the formalin-fixed paraffin-embedded tissue samples yielded positive PCR results. Results regarding the type of tissue sample available for molecular analysis (fresh or formalin-fixed) and PCR results are shown in table 4.

Sequencing of the 18S rRNA gene yielded two quality samples (horses 9 and 13). The remaining cases (6, 10 and 12) did not have a satisfactory sequencing, and these will be retested. Horse 9 yielded a sequence of 341bp with 100% identity with *S. halieti* (MH898962.1), *S. speeri* (KP681854.1) and *S. neurona* (KJ957839.1). Horse 13 yielded a 694bp sequence with 100% identity with *Sarcocystis* sp. (KX470746.1), and *S. speeri* (KT207459.1), as well as 99% identity with *S. neurona* (KX610767.1). Horse 13 sequence was used to construct the 18S rRNA maximum likelihood phylogenetic tree, shown in figure 3.

ITS-1 gene sequencing yielded four quality samples (horses 6, 9, 10 and 12). The remaining case, horse 13, did not have a satisfactory sequencing, and it will be retested. Horse 6 yielded a sequence of 333bp with 98% identity with *S. neurona* (AH009986.2)

and *S. speeri* (KT207458.1). Horse 9 had a 404bp sequence with 99% identity with *S. neurona* (AH009986.2) and *S. speeri* (KT207458.1). Horse 10 yielded a 513bp sequence with 98% identity with *S. dasypi* (AY082633.1), *S. neurona* (AH009986.2) and *S. speeri* (KT207458.1). Horse 12 had a 334bp sequence with 98% identity with *S. neurona* (AH009986.2), and *S. speeri* (KT207458.1). The referred samples were used to construct the ITS-1 Neighbor-Joining and Maximum-Parsimony trees, which are shown in figures 4 and 5, respectively.

In the present work, phylogenetic analysis based on the 18S rRNA gene were not sufficient to make a clear distinction between the obtained sequence (horse 13) and other *Sarcocystis* species, including *S. neurona* and *S. speeri*. The referred sequences were all part of a clade, along with *S. falcatula* and a sequence of *Sarcocystis* spp. identified in *D. aurita*, from Brazil. It is worth to highlight that all sequences in this clade corresponded to *Sarcocystis* species shed by opossums (Fig. 3).

Similarly, ITS-1 gene distance analysis (Neighbor-Joining tree) inserted the *Sarcocystis* spp. sequences obtained in the present study (horses 6, 9, 10 and 12) in a polytomous clade (bootstrap of 91), along with *S. neurona*, *S. dasypi* and *S. speeri*. This clade was phylogenetically related to a sequence of *Sarcocystis* spp. obtained from a Brazilian opossum, as well as *S. falcatula* (Fig. 4).

Maximum parsimony analysis of the ITS-1 gene sequences demonstrated a clear phylogenetic relationship between the *Sarcocystis* spp. sequence obtained from horse 10 and *Sarcocystis dasypi*. Nonetheless, all *Sarcocystis* spp. sequences from the present study were inserted in a macro-clade (bootstrap of 80), along with *S. neurona*, *S. speeri* and a sequence of *Sarcocystis* spp. obtained from a Brazilian opossum (Fig. 5).

Regarding the identity matrices, the evaluated portion of the 18S rRNA gene was not capable of demonstrating any difference among the obtained sequences (horses 9 and 13) and other *Sarcocystis* sequences, as shown in table 5. The differences demonstrated in the ITS-1 identity matrix highlight the similarity among the sequences obtained in the present study (6, 9, 10 and 12) with other species in the genus *Sarcocystis*, mainly *S. neurona*, *S. dasypi* and *S. speeri* (Fig. 6).

4. **Discussion**

EPM accounted for 2.64% of the diagnosis in the horses of the present study, and the only agent detected by immunohistochemistry and molecular analysis was

Sarcocystis spp. Even though the condition presented a relatively low overall frequency, EPM represented the second most common cause of inflammatory CNS disease in horses in the analyzed period (34.21%). EPM frequency in this case series was somehow higher when compared with other retrospective studies evaluating general causes of death in horses in Brazil, which have described the disease as a rare illness (Pierezan et al., 2009; Marcolongo-Pereira et al., 2014). Conversely, serological studies conducted in Brazil have revealed a high prevalence of *S. neurona* infection in horses, which often varies according to the geographical area studied, with values ranging from 26% (Ribeiro et al., 2016) to 69.6% (Hoane et al., 2006). On the other hand, *Neospora* spp. seroprevalence in Brazilian horses seems to be considerably lower, with reported values ranging from 2.5% (Hoane et al., 2006) to 23.9% (Ribeiro et al., 2016); corroborating with the present study, which found no evidence of EPM associated with *Neospora* spp. or other protozoan infections in the evaluated cases. Even though *T. gondii* is not associated with EPM, IHC for this agent was included in this study since apicomplexan parasites share numerous morphological similarities, which could be a cause for misinterpretation (Dubey et al., 2001a).

Mean and median age of affected horses, 10.2 and 8 years, respectively, were higher than those previously described. Young horses tend to be more commonly affected, with over 50% of clinical cases diagnosed in animals with age ranging from one to four years (Saville et al., 2000). Likewise, a retrospective study based on histologically confirmed cases indicated that approximately 60% of cases were diagnosed in horses younger than four years of age (Fayer et al., 1990). Nonetheless, EPM was diagnosed in horses of virtually all ages in the referred studies (Fayer et al., 1990; Saville et al., 2000), similarly to the present study, where the age range varied from 2.5 to 22 years. Still, it is possible that the present result may have been skewed due to the small number of analyzed cases.

The majority of horses included in the study were male (10/13), and mixed breed (7/13); nonetheless, no correlation could be established linking these parameters to the occurrence of EPM. Previous studies have shown that sex is likely not an important predisposing factor for the disease (Fayer et al., 1990; Cohen et al., 2007), even though one retrospective case series indicated a disproportionate representation of males (Boy et al., 1990). Previous EPM studies have demonstrated an overrepresentation of certain breeds, such as American Quarter, Thoroughbreds, and Standardbreds (Boy et al., 1990;

Saville et al., 2000). However, most horses in the present study were mixed breed, and this result is probably a consequence of the horse population present in the analyzed region, as corroborated by a previous report (Cohen et al., 2007), and, therefore, does not indicate any specific breed susceptibility.

Regarding seasonality, EPM cases were diagnosed all year round, with a relatively higher number of cases diagnosed in the fall-winter (9/13) than in the spring-summer period (4/13). Epidemiological studies conducted in North America have shown that EPM was significantly less frequent in the winter, when compared with other seasons, mainly the fall (Saville et al., 2000). Reduced exposure to opossums has been proposed as one of the factors related to the lower occurrence of the disease during the winter months in the USA (Saville et al., 2000). Nevertheless, this epidemiological factor may be more relevant in countries such as Canada and most of the USA states, which present a temperate climate, and not as important in tropical and subtropical areas, such as in the Brazilian territory.

EPM commonly presents as a debilitating disease with acute to chronic clinical signs, and frequently with a long clinical course (Reed et al., 2016), which in our cases ranged from five to 50 days, with a mean of 23 days. As it could be observed in the present case series, clinical progression length may be influenced by several factors, including disease severity, comorbidities, secondary traumatic lesions, as well as the development of permanent lateral recumbency, which frequently is associated with poor prognosis, and ultimately euthanasia. In addition, the establishment of an early and appropriate therapy may lead to prolonged clinical course or cure (Furr et al., 2008). In the present study, therapy with antiprotozoal drugs was conducted in two horses, which may have influenced the length of clinical progression, as well as decreased parasite detection rate. However, no further information regarding administered protocols was available in such cases.

Histological characterization described in the present study is consistent with prior descriptions of EPM (Furr et al., 2008; Zoll et al., 2018). A multifocal random distribution of microscopic lesions was observed, affecting unpredictably both the white and grey matter. This has been directly associated with the wide range of clinical signs observed in EPM cases (Furr et al., 2008; Dubey et al., 2015a). When lesions affect more severely the grey matter, muscle weakness and atrophy are commonly associated, as observed in the head and hind limb musculature of two horses each in the present

case series. Conversely, white matter lesions are frequently associated with ataxia (Reed et al., 2016), which was observed in different degrees in all reported cases.

Histological lesions presented a segmental random distribution affecting the brain and the spinal cord in 8/13 cases (61.54%); however, lesions were restricted to the brain or the spinal cord in two and three cases, respectively. This finding reiterates the importance of evaluating the entire CNS in cases of such neurologic disease in horses (Furr et al., 2008). Even though microscopic changes were detected in all CNS parts in the analyzed case series, lesions tended to be more frequent and more severe in the rhomboencephalon and in the cervical, thoracic, and lumbar spinal cord segments, similarly to previous descriptions for the condition (Dubey et al., 2001a).

The observation of parasitic structures in EPM histological sections occurs in a small percentage of suspected cases, and detection rates may vary from 20% (Hamir et al., 1993) to 36% (Boy et al., 1990). Similar findings were observed in the present study; in which parasites were detected in 5/13 cases (38.46%). Since histological identification of protozoan parasites is often difficult, immunohistochemistry is considered of great value to improve diagnostic accuracy in EPM suspected cases (Hamir et al., 1993). A previous study compared the histological and immunohistochemical intralesional parasite detection rates, with 20% of suspected cases presenting intralesional parasites by histology, while positive immunolabeling ranged from 51 to 67% (Hamir et al., 1993). Likewise, immunohistochemical evaluation in the present case series revealed 11/13 (84.61%) positive samples. This demonstrates an increase of confirmed cases by IHC, when compared to the histopathological detection of the protozoa (38.46%). It is also important to highlight that 1/2 negative cases on immunohistochemistry corresponded to a horse which had been previously treated (horse 8), which may have negatively influenced immunostaining. While such information was not available for horse 1. Even though confirmatory diagnosis of *S. neurona*-associated EPM (histological visualization of parasites, IHC or molecular findings) could not be established for horses 1 and 8, these cases were maintained in the present study, since histopathological features observed were consistent with EPM.

All frozen samples (horses 6, 9, 10, 12 and 13) presented positive results for the 18S rRNA gene and ITS-1 gene PCR's. Regarding formalin-fixed paraffin-embedded tissue samples (8/13), none presented positive results. All these eight cases showed histological features consistent with EPM; in addition, of these eight cases presenting

negative PCR's, six had *S. neurona* positive immunostaining, which strengths the diagnosis of EPM in such cases. The lack of positive samples in the PCR'S found in these cases is likely related to the degradation of nucleic acids, a process commonly reported in fixed tissues, which may be intensified by the type of fixative, and time of fixation, among numerous other factors (Miething et al., 2006).

After sequencing, both 18S rRNA and ITS-1 obtained sequences presented high identity with *Sarcocystis* spp., mainly with species shed by opossums. 18S rRNA gene analysis has been frequently used in phylogenetic studies of eukaryotes; however, since this gene is more conserved than other markers, its usage may be more valuable when trying to differentiate sequences at a higher taxonomic level (Tang et al., 2012). This was found to be accurate in the present study, in which the obtained 18S rRNA gene sequences presented 99-100% identity with several species in the *Sarcocystis* genus; thus preventing a classification at the species level. In the 18S rRNA phylogenetic tree, the evaluated sequence clustered with several *Sarcocystis* species excreted by opossums, and in the identity matrix all evaluated sequences presented 100% identity.

The assessment of the ITS-1 gene has been commonly performed to infer phylogenetic relationships at lower taxonomic levels in eukaryotes, including *Sarcocystis* species (Valadas et. al, 2016), since this marker presents a relatively high evolutionary rate (Prasad et al., 2007). However, in the present study, the ITS-1 obtained sequences presented high identity, as well as a significantly close phylogenetic relationship with several *Sarcocystis* species, especially the ones excreted by *Didelphis* spp., such as *S. neurona*. It is plausible to think that this may have been caused due to the great genetic similarity shared among the *Sarcocystis* species which present opossums as definitive host. Such inference is corroborated by a previous study which aimed to characterize *S. speeri* using several genetic markers (Dubey et al., 2015b). In the referred study, ITS-1 gene assessment indicated a significantly close phylogenetic relationship among *S. speeri*, *S. neurona*, *S. falcatula*, *S. lindsayi* and *S. dasypi*, all of which clustered in the same clade (Dubey et al., 2015b). In addition, in the mentioned study, few SNPs (single nucleotide polymorphisms) could be detected when comparing *S. speeri* and *S. neurona*, and the evaluated sequence of *S. speeri* presented 99.4% identity with *S. neurona* (AF252407) (Dubey et al., 2015b).

Sarcocystis species shed in the feces of opossums include avian infective species, such as *S. falcatula* and *S. lindsayi*, and mammalian infective species, including

S. neurona and *S. speeri* (Dubey et al., 1998; Dubey et al., 1999). These species share innumerous morphological and genetic similarities; nonetheless, bioassay in immunodeficient mice and budgerigars has been used to distinguish among these, mainly when evaluating fecal samples from the definitive host or isolates from clinical cases (Dubey et al., 1998; Dubey et al., 1999). Taking into account the fact that *S. neurona* is known to be the main agent associated with EPM (Reed et al., 2016), and to this day it represents the only protozoan in the genus *Sarcocystis* associated with the referred disease (Dubey et al., 2015a), it is highly likely that *S. neurona* is the agent associated with the reported cases. However, further studies focusing on more specific genetic markers are necessary in order to rule out the participation of other *Sarcocystis* species in the present EPM reported cases.

5. **Conclusion**

In conclusion, EPM presents a relatively low frequency in Southern Brazil; yet, it represents the second most common inflammatory CNS disease of horses after rabies. Lesions affected concomitantly the brain and spinal cord in most cases. Still, two and three horses had lesions that were restricted to the brain and to the spinal cord, respectively. IHC provided a more accurate detection rate of parasites than histopathological evaluation alone. The assessment of the ITS-1 and 18S rRNA genes indicated that the agent involved in the cases was *Sarcocystis* spp., however, due to the great genetic similarity shared among *Sarcocystis* species shed by opossums, it was not possible to determine with certainty the involved species. In the present case series, the only protozoan associated with EPM cases detected through immunohistochemistry and molecular analysis was *Sarcocystis* spp.

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Table 1: Epidemiological data in cases of equine protozoal myeloencephalitis in southern Brazil.

Horse	Breed	Age (years)	Sex	Clinical progression length (days)	Season of diagnosis	Euthanasia	Anti-Inflammatory Drugs	Antiprotozoal drugs
				Epidemiological data			Treatment	
1	AQH	22	M	-	Winter	No	-	-
2	TB	9	F	50	Fall	No	Yes	-
3	MB	2.5	M	-	Summer	Yes	-	-
4	MB	-	M	-	Fall	Yes	-	-
5	MB	6	M	30	Fall	Yes	-	-
6	MB	7	M	15	Spring	Yes	-	Yes
7	TB	-	F	9	Fall	No	-	-
8	MB	-	M	-	Winter	No	-	Yes
9	MB	6	M	20	Fall	Yes	Yes	-
10	Criollo	5	F	-	Winter	No	Yes	-
11	Criollo	16	M	20	Fall	Yes	-	-
12	MB	13	M	5	Summer	Yes	Yes	-
13	Criollo	15	M	30	Summer	Yes	Yes	-

Breed: AQH (American Quarter Horse), TB (Thoroughbred) and MB (Mixed breed). Sex: M (male) and F (female). Information not reported or not available is represented for evaluation (-).

Table 2: Clinical signs reported in cases of equine protozoal myeloencephalitis.

Horse	Hind limb sings	Forelimb signs	Difficulty standing and falls	Persistent recumbency	Paddling	Compulsive walking	Circling	Poor body condition	Cranial nerve deficits	Muscle atrophy Hind limbs	Head
				Clinical signs						Muscle atrophy	
1	+	-	+	-	-	-	-	-	-	-	-
2	+	+	+	+	+	-	-	-	+	-	+
3	+	-	+	+	-	-	-	-	-	-	-
4	+	-	+	+	+	-	-	-	-	-	-
5	+	-	+	+	-	-	-	-	-	-	-
6	+	-	+	+	+	-	-	-	-	-	-
7	+	+	+	+	+	+	+	-	+	-	-
8	+	-	-	-	-	-	-	-	+	-	+
9	+	+	+	+	-	-	-	-	-	-	-
10	+	+	+	+	+	-	-	-	-	-	-
11	+	-	+	+	+	-	-	+	-	-	-
12	-	+	+	+	+	-	-	-	+	+	-
13	+	-	+	+	+	-	-	-	-	+	-

Clinical signs are classified as reported (+) or not reported (-).

Table 3: Anatomic distribution and severity of central nervous system lesions in horses with equine protozoal myeloencephalitis.

Horse	Gross lesion	Anatomic Distribution	Cervical	Thoracic	Lumbar	Sacral	Rhomb[a]	Mes[b]	Pros[c]
						Histological lesions			
1	-	Brain	-	-	-	-	++	-	+
2	-	Both	+	+	+++	+	+	-	+
3	-	SC[d]	-	++	++	-	-	-	-
4	Present	Both	+++	+++	+++	++	+	-	+
5	Present	Both	+	+	-	-	+	-	-
6	Present	Both	++	++	+	+	+	+	+
7	-	Both	+	-	-	-	+	+	+
8	-	Both	-	-	+	-	+	-	-
9	-	SC[d]	++	+	-	-	-	-	-
10	Present	SC[d]	++	++	-	-	-	-	-
11	-	Brain	-	-	-	-	++	-	+
12	Present	Both	+++	-	++	+	+	+	-
13	-	Both	+	+++	-	-	+	+	++

Lesion severity was classified as absent (-) or present, and when the latter, as mild (+), moderate (++) and marked (+++). [a] Rhombencephalon, [b] Mesencephalon, [c] Prosencephalon. [d] Spinal cord.

Table 4: Histopathological, immunohistochemical, and molecular findings in cases of EPM.

Horse	Histological findings						IHC	PCR sample	PCR results
	Perivascular Cuffing	Giant Cells	Eosinophils	Liquefactive Necrosis	Meningitis	Parasites*			
1	+	+	+	-	-	-	-	FFPET	-
2	++	+	+	+	+	-	+	FFPET	-
3	+++	+	+	+	+	-	++	FFPET	-
4	+++	+	++	++	+	Rare	++	FFPET	-
5	+	+	+	+	+	-	+	FFPET	-
6	++	+	+	++	+	-	++	FFS	Positive
7	+	+	+	-	-	Rare	+	FFPET	-
8	+	+	+	-	+	-	-	FFPET	-
9	+++	+	++	-	+	-	++	FFS	Positive
10	++	+	+	+	-	Rare	+	FFS	Positive
11	++	++	+	-	+	-	+	FFPET	-
12	+++	+	+	+++	++	Rare	+++	FFS	Positive
13	++	+	+++	++	++	Occasional	+++	FFS	Positive

Lesion severity and immunostaining intensity are classified as absent (-) or present, and when the latter, as mild (+), moderate (++) and marked (+++). *Parasites: protozoa observed in histological sections. FFPET: formalin-fixed paraffin embedded tissues. FFS: fresh frozen samples.

Table 5: Nucleotide identity matrix between the 18S rRNA gene sequences of the two *Sarcocystis* spp. sequences identified in the present study (horses 9 and 13), and three of the most similar *Sarcocystis* species retrieved from GenBank.

Species and sequence	*Sarcocystis* spp. (Horse 13)	*Sarcocystis* spp. (Horse 9)	*S. neurona* (KJ957839)	*S. speeri* (KT207459)	*Sarcocystis* sp. *D. aurita* from Brazil (KX470744)
Sarcocystis spp. (Horse 13)	ID				
Sarcocystis spp. (Horse 9)	1	ID			
S. neurona (KJ957839)	1	1	ID		
S. speeri (KT207459)	1	1	1	ID	
Sarcocystis sp. *D. aurita* from Brazil (KX470744)	1	1	1	1	ID

Sequences showing values close to 1 indicate greater identity. The smaller sequence obtained, horse 9 (341bp), was used to adjust the length of the remaining sequences.

Table 6: Nucleotide identity matrix between the ITS-1 gene sequences of the four *Sarcocystis* spp. sequences identified in the present study (horses 6 , 9, 10 and 12), and five of the most similar *Sarcocystis* species retrieved from GenBank.

Species and Sequences	*Sarcocystis* spp. (Horse 12)	*Sarcocystis* spp. (Horse 9)	*Sarcocystis* spp. (Horse 10)	*Sarcocystis* spp. (Horse 6)	*S. neurona* (AF252407)	*S. dasypi* (AY082631)	*S. falcatula* (AF098244)	*S. speeri* (KT207458)	*Sarcocystis* sp. Isolate – Opossum from Brazil (KP871743)
Sarcocystis spp. (Horse 12)	ID								
Sarcocystis spp. (Horse 9)	1	ID							
Sarcocystis spp. (Horse 10)	1	1	ID						
Sarcocystis spp. (Horse 6)	1	1	1	ID					
S. neurona (AF252407)	0.981	0.981	0.981	0.981	ID				
S. dasypi (AY082631)	0.981	0.981	0.981	0.981	1	ID			
S. falcatula (AF098244)	0.943	0.943	0.943	0.943	0.952	0.952	ID		
S. speeri (KT207458)	0.981	0.981	0.981	0.981	1	1	0.952	ID	
Sarcocystis sp. Isolate – Opossum from Brazil (KP871743)	0.943	0.943	0.943	0.943	0.952	0.952	0.961	0.952	ID

Sequences showing values close to 1 indicate greater identity. The length of the sequences was adjusted based on 241bp.

Figure 1. Spinal cord gross appereance at cross section (thoracic segment). Multifocal moderate areas presenting dark red coloration are observed extending from the grey matter to the white matter (horse 6).
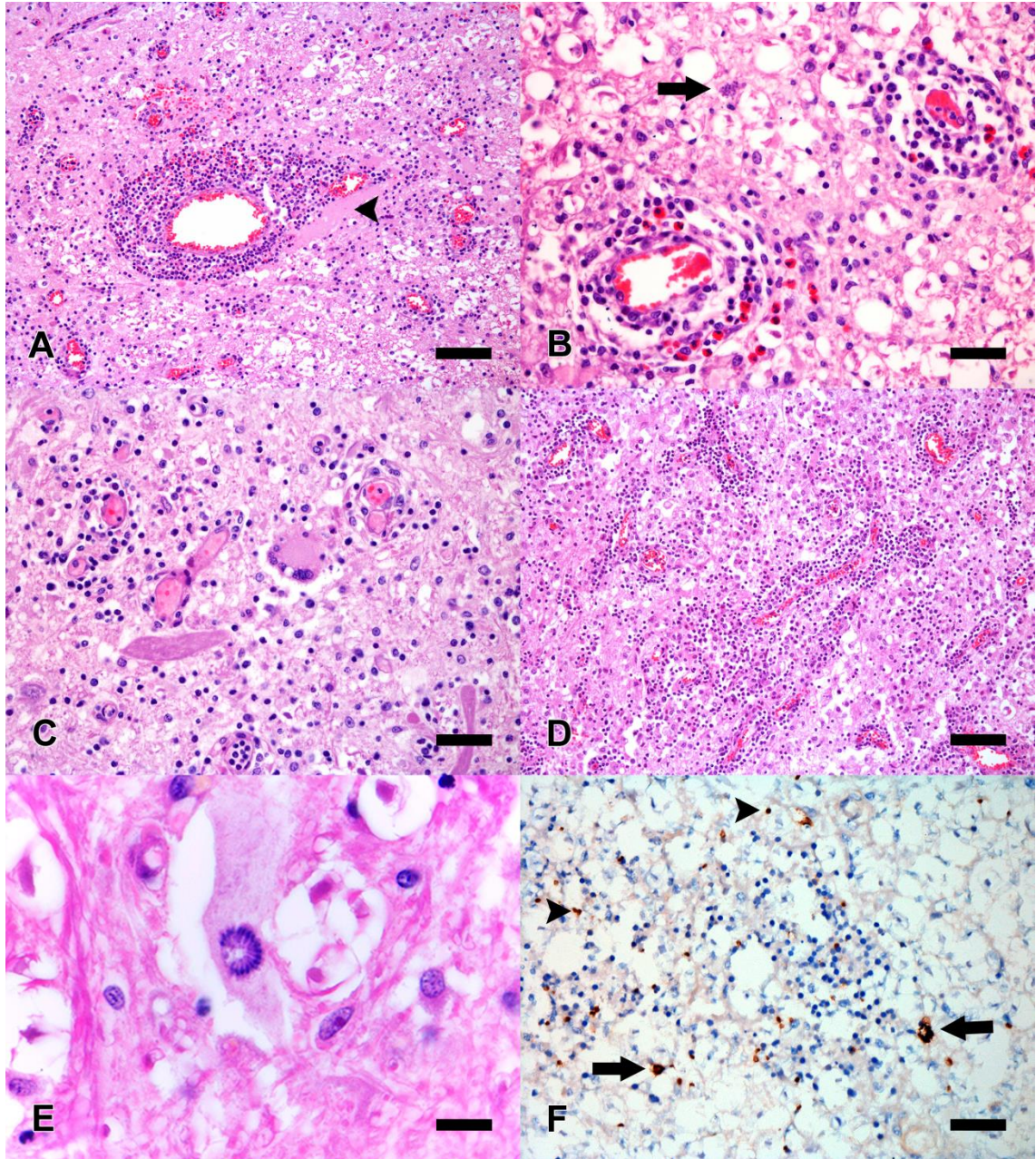
Figure 2. Sections of spinal cord of horses with EPM. Hematoxylin and eosin stain.
(A) Multifocal marked perivascular cuffing composed predominantly of lymphocytes, fewer macrophages and plasma cells is noted. Mild hemorrhage and moderate perivascular edema are also observed (arrow head). Bar 120μm. (B) Multifocal moderate perivascular cuffing of eosinophils and lymphocytes is present in the white matter. In addition, a schizont of *S. neurona* containing merozoites is observed (arrow). Bar 70μm. (C) A multinucleated giant cell is observed in the neuropil, as well as mild perivascular infiltrate of lymphocytes. Bar 70 μm. (D) A focally extensive, marked area of liquefactive necrosis associated with large number of gitter cells is observed. Additionally, multifocal moderate inflammatory infiltrate of lymphocytes, fewer plasma cells and eosinophils is seen, frequently extending to the adjacent neuropil. 120μm bar. (E) A mature schizont containing peripherally arranged merozoites is observed. 14μm bar. **IHC** (F) Strong positive immunostaining for *S. neurona* is noted. Schizonts (arrow) and merozoites (arrow head) are observed intermixed with inflammatory cells in the neuropil. 70μm bar.
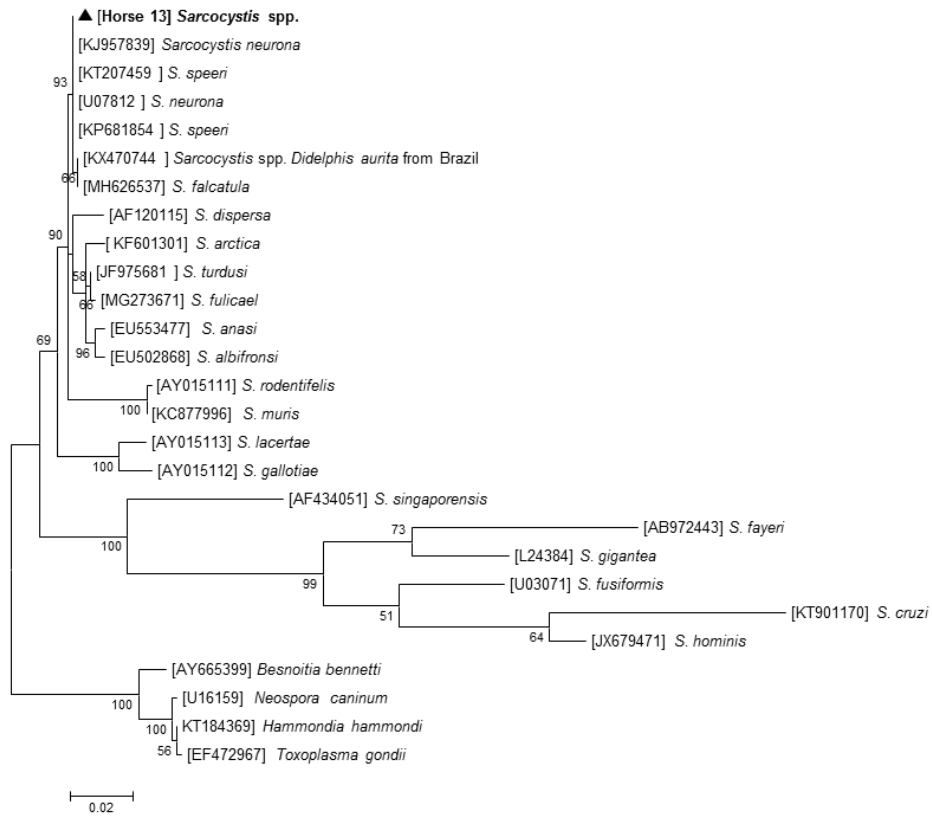
Figure 3. Maximum likelihood phylogenetic tree based on the 18S rRNA sequence obtained and other protozoans. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (only bootstraps >50 are depicted). The accession number of the sequences is given in brackets, followed by the species name. The *Sarcocystis* spp. sequence generated in the present work is in bold, and indicated by a black triangle. Sequences of the subfamily Toxoplasmatinae were used as outgroup.
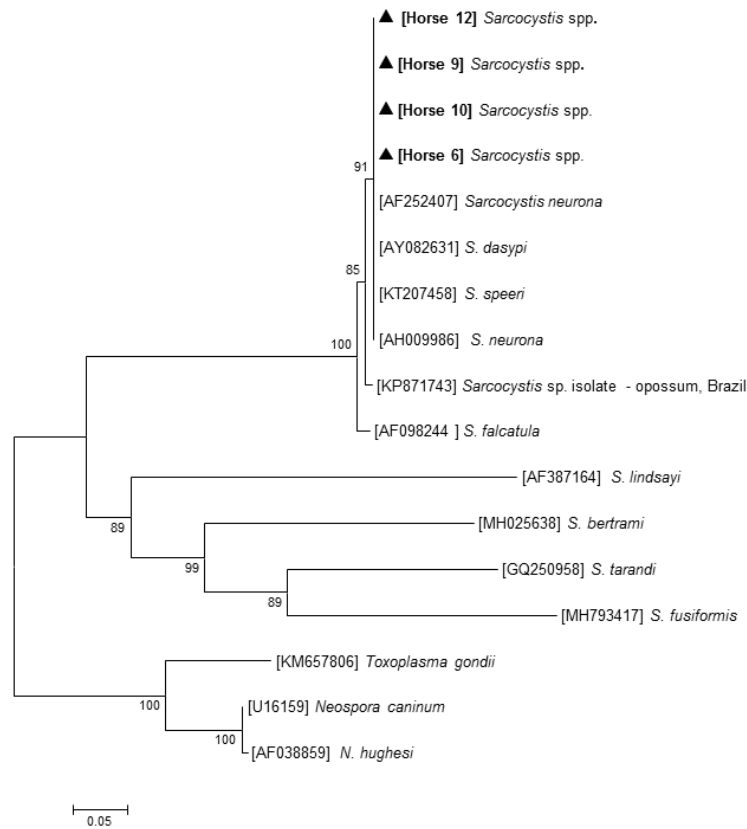
Figure 4. Neighbor-Joining tree based on ITS-1 sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (only bootstraps >50 are depicted). The accession number of the sequences is given in brackets, followed by the species name. Generated *Sarcocystis* spp. sequences in the present work are in bold, and indicated by black triangles (horses 6, 9, 10 and 12). Sequences of the subfamily Toxoplasmatinae were used as outgroup.
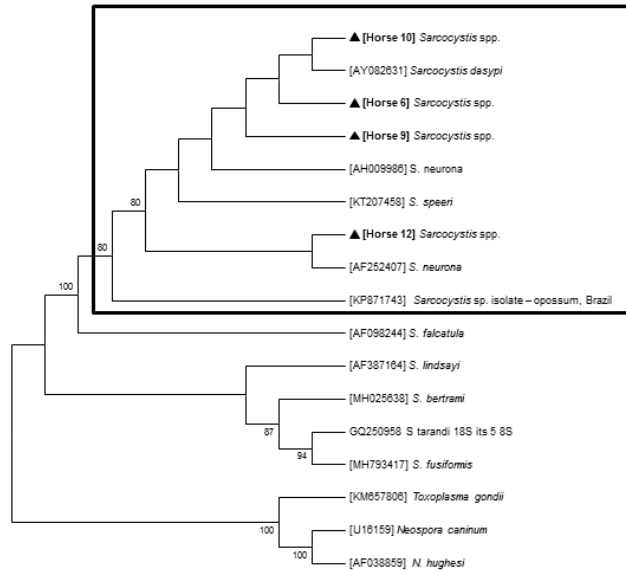
Figure 5. Maximum Parsimony tree based on ITS-1 sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (only bootstraps >50 are depicted). The accession number of the sequences is given in brackets, followed by the species name. Generated *Sarcocystis* spp. sequences in the present work are in bold, and indicated by black triangles (horses 6, 9, 10 and 12). Generated sequences are arranged in a clade with 80 bootstraps with other *Sarcocystis* species (black rectangle). Sequences of the subfamily Toxoplasmatinae were used as outgroup.

3. CONCLUSÕES

No período estudado, 13 casos compatíveis com MEP foram diagnosticados, representando 2,64% do total de diagnósticos em equinos e 34,21% das doenças inflamatórias afetando o sistema nervoso central (SNC) nesta espécie. MEP correspondeu à segunda condição inflamatória do SNC mais comum em equinos, apenas atrás da infecção pelo vírus da raiva. Desta maneira, MEP representa uma das principais doenças neurológicas diagnosticadas na espécie equina no sul do Brasil.

A idade mediana dos equinos afetados foi de oito anos, a maioria dos animais acometidos era macho, e a doença foi observada ao longo do ano. Lesões macroscópicas foram detectadas em 38,46% dos casos, e estas foram observadas apenas na medula espinhal. Lesões microscópicas apresentaram-se de forma multifocal em todos os casos, e foram mais frequentemente observadas em segmentos da medula espinhal e no rombencéfalo. As lesões encontravam-se restritas a medula espinhal e ao encéfalo em três e dois casos, respectivamente. Protozoários intralesionais consistentes com *S. neurona* foram observados em secções histológicas em cinco equinos.

A técnica imuno-histoquímica aumentou significativamente a taxa de detecção de parasitas, e dessa maneira representou uma importante ferramenta para o estabelecimento de um diagnóstico final de MEP associada à *Sarcocystis* sp. Através de técnicas moleculares, cinco casos positivos para o ITS-1 e para o 18S rRNA foram detectados. Após o sequenciamento obtiveram-se fragmentos de qualidade de duas amostras referentes ao gene 18S rRNA e quatro referentes ao ITS-1. Através de avalições filogenéticas foi possível inferir que todas as sequências obtidas pertencem ao gênero *Sarcocystis*, entretanto, devido a grande similaridade genética compartilhada entre as espécies de *Sarcocystis* excretadas por gambás, não foi possível determinar de maneira definitiva a espécie envolvida.

No presente estudo, o único agente associado com casos de MEP detectado por imuno-histoquímica e técnicas moleculares foi *Sarcocystis* spp.

Portanto, MEP deve ser incluída entre os diagnósticos diferenciais de doenças neurológicas em equinos na região sul do Brasil, mesmo em casos em que parasitas não são prontamente observados na avaliação histológica.

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