**UNIVERSIDADE ESTADUAL PAULISTA - UNESP**
**CÂMPUS DE JABOTICABAL**

# MOLECULAR CHARACTERIZATION OF *Sarcocystis* spp. FROM OPOSSUMS (*Didelphis* spp.) AND SEROLOGICAL DETECTION OF ANTI-*Sarcocystis* spp. ANTIBODIES IN HORSES FROM SOUTHEASTERN AND MIDWESTERN BRAZIL

**Mariele De Santi**
**Médica Veterinária**

**2023**

**UNIVERSIDADE ESTADUAL PAULISTA - UNESP**
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**Orientadora: Profa. Dra. Rosangela Zacarias Machado**
**Coorientadora: Profa. Dra. Karin Werther**

Tese de apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária.

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## IMPACTO ESPERADO DA PESQUISA

De acordo com a INSTRUÇÃO AT/PROPG Nº 02, DE 22 DE DEZEMBRO DE 2022, abaixo estão descritos os impactos esperados a partir desta pesquisa.

Mieloencefalite Protozoária Equina é uma doença grave que acomete os equinos. A doença é causada pelo protozoário *Sarcocystis neurona*, e é caracterizada pelo aparecimento de lesões em encéfalo e/ou medula espinhal. O quadro clínico cursa com sintomatologia neurológica, com sinais tais como dificuldade de ficar em pé, andar ou engolir podendo rapidamente progredir para a morte do animal. Trata-se, portanto, de uma enfermidade de elevada importância à qual se deve dar especial atenção. A presente pesquisa, realizada com base em biologia molecular e testes imunológicos, gerou dados importantes, que auxiliam no melhor entendimento da dinâmica da enfermidade. O conhecimento sobre a distribuição do protozoário nos seus hospedeiros definitivos (gambás) é importante pois pode auxiliar a identificar áreas com maior probabilidade de ocorrência da doença. O conhecimento das características genéticas do protozoário pode auxiliar no entendimento da patogênese da doença, ou seja, quais são os mecanismos utilizados pelo protozoário para causar a doença nos equinos. Com base nesse entendimento, metodologias mais efetivas e confiáveis para o diagnóstico precoce da doença podem ser desenvolvidas. Além disso, os dados gerados neste estudo podem auxiliar na criação de novas drogas para tratamento da enfermidade. A prevenção da doença também é um ponto importante, visto que que seu prognóstico é desfavorável. Sendo assim, os dados aqui gerados podem servir de base para o desenvolvimento de formas efetivas de prevenção, tais como as vacinas.

## IMPACT EXPECTED FROM THE STUDY

In accordance with the INSTRUÇÃO AT/PROPG Nº 02, DE 22 DE DEZEMBRO DE 2022, here are the impacts expected from the study.

Equine Protozoal Myeloencephalitis is a disease that affects horses. The disease is caused by the protozoa *Sarcocystis neurona*, and it is characterized by the occurrence of lesions in brain and/or in spinal cord. The clinical picture presents with neurological symptomatology, with signs such as difficulty on standing, walking, or swallowing, that could quickly progress to the death of the animal. It is, therefore, a very important disease, to which special attention should be given. The present study, based on molecular biology and immunological tests, lead to important knowledge that can help the better understanding of the dynamics of the disease. The information about the protozoa distribution in their definitive hosts (opossums) is important, as it could help to identify areas with higher probability of occurrence of the disease. Information about the protozoa's genetic characteristics may help the better understanding of the pathogenesis of the disease, i.e., which are the mechanisms used by the protozoa to cause the disease in the horses. Based on that, more effective and reliable methodologies may be developed for the early diagnostic of the disease. Besides, the information obtained in the present study may help in the development of new drugs for the treatment of the disease. The prevention is also important, as the prognostic of the disease is not favorable. Therefore, the knowledge shared here may serve as base to the development of effective forms of prevention, such as the vaccines.

**UNIVERSIDADE ESTADUAL PAULISTA**

**Câmpus de Jaboticabal**

## CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: MOLECULAR CHARACTERIZATION OF *Sarcocystis* spp. FROM OPOSSUMS (*Didelphis* spp.) AND SEROLOGICAL DETECTION OF ANTI-*Sarcocystis* spp. ANTIBODIES IN HORSES FROM SOUTHEASTERN AND MIDWESTERN BRAZIL

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Jaboticabal, 24 de fevereiro de 2023

## DADOS CURRICULARES DO AUTOR

**MARIELE DE SANTI** – Nascida em 13 de abril de 1988, em Concórdia, Santa Catarina. Filha de Ivanir Antonio De Santi e Geni Maria De Santi. Formada em Medicina Veterinária pelo Instituto Federal Catarinense – IFC Campus Concórdia em 2014. Em fevereiro de 2017 concluiu o Curso de Mestrado em Medicina Veterinária, na área de concentração - Patologia Animal, na Universidade Estadual Paulista "Júlio de Mesquita Filho" – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Jaboticabal, São Paulo, com bolsa concedida pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq. Em março deste mesmo ano, ingressou no Programa de Aprimoramento Profissional do Hospital Veterinário "Governador Laudo Natel" da FCAV/UNESP, o qual foi finalizado em fevereiro de 2019. Em março de 2019, ingressou no Curso de doutorado em Medicina Veterinária, na área de concentração - Patologia Animal na mesma instituição sob a orientação da Profa. Dra. Rosangela Zacarias Machado e coorientação da Profa. Dra. Karin Werther. Durante o curso de doutorado usufruiu de bolsa da Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP. Entre dezembro de 2021 e junho de 2022, realizou Estágio de Doutorado Sanduíche na University of Kentucky, sob orientação do Prof. Dr. Daniel K. Howe, com bolsa concedida pela FAPESP.

**EPÍGRAFE**

Calma,
Se você está no ponto de estourar mentalmente, silencie alguns instantes para pensar.
Se o motivo é moléstia no próprio corpo, a intranquilidade traz o pior.
Se a razão é enfermidade em pessoa querida, o seu desajuste é fator agravante.
Se você sofreu prejuízos materiais, a reclamação é bomba atrasada, lançando caso novo.
Se perdeu alguma afeição, a queixa tornará você uma pessoa menos simpática, junto de outros amigos.
Se deixou alguma oportunidade valiosa para trás, a inquietação é desperdício de tempo.
Se contrariedades aparecem, o ato de esbravejar afastará de você o concurso espontâneo.
Se você praticou um erro, o desespero é porta aberta a faltas maiores.
Se você não atingiu o que desejava, a impaciência fará mais larga a distância entre você e o objetivo a alcançar.
Seja qual for a dificuldade, conserve a calma, trabalhando, porque, em todo problema, a serenidade é o teto da alma, pedindo o serviço por solução.

Autor: André Luiz
Psicografia de Francisco Cândido Xavier

**DEDICO...**

Aos meus pais, pelo esforço e apoio incondicionais.

## AGRADECIMENTOS

A Deus, por seu infinito amor, bondade e justiça.

À minha família, pelo suporte e pelos ensinamentos que moldaram meu caráter.

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À professora Cláudia Momo pelo auxílio na realização dos trabalhos de campo.

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À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela bolsa de estudos (Processo 2019/08594-0) e pela bolsa de estágio de pesquisa no exterior (Processo 2021/06779-6), concedidos para a realização deste trabalho.

À banca examinadora do exame geral de qualificação: Profa. Dra. Darci Moraes Barros-Battesti, Prof. Rodrigo Martins Soares e Profa. Rosangela Zacarias Machado e à banca examinadora da defesa, pelas valiosas críticas e contribuições.

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O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

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com competência, sabedoria e paciência em todos os momentos
desta jornada. Sempre a terei como um exemplo a ser seguido.

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Câmpus de Jaboticabal

## CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

# CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Detecção e caracterização molecular de *Sarcocystis* spp. em marsupiais (*Didelphis* spp.) amostrados na cidade de Campo Grande, Mato Grosso do Sul"**, protocolo nº 003444/19, sob a responsabilidade da Profª. Drª. Rosangela Zacarias Machado, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de maio de 2019.

| | |
|---|---|
| Vigência do Projeto | 01/05/2019 a 11/12/2022 |
| Espécie / Linhagem | Gambá *(Didelphis* spp.) |
| Nº de animais | - |
| Peso / Idade | 0,4 – 1,5 kg/ 0,5 – 4 anos |
| Sexo | Macho e fêmea |
| Origem | Animais serão capturados na cidade de Campo Grande, MS, região centro-oeste do Brasil. |

Jaboticabal, 16 de maio de 2019.

Fabiana Pilarski

**Prof.ª Dr.ª Fabiana Pilarski**
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal/ SP - Brasil
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## CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

## DECLARAÇÃO

Declaramos que o trabalho de pesquisa intitulado "Detecção e caracterização molecular de *Sarcocystis spp*. em marsupiais (*Didelphis spp.*) amostrados na cidade de Campo Grande, Mato Grosso do Sul", sob a responsabilidade da Profa. Dra. Rosangela Zacarias Machado e Certificado CEUA protocolo nº 3444/2019, aprovado em reunião ordinária em 16 de maio de 2019, teve o local de captura dos animais alterado de "Campo Grande, Mato Grosso do Sul" para "São Paulo / SP".

Solicitação aprovada em reunião ordinária de 10 de dezembro de 2020.

Jaboticabal, 10 de dezembro de 2020.

Fabiana Pilarski

**Profa. Dra. Fabiana Pilarski**
Coordenadora – CEUA

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Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

São Paulo, 3rd February 2022

**CERTIFIED**

We certify that the Research "Detection and molecular characterization of Sarcocystis spp. in opossums (Didelphis spp.)", protocol number CEUAx 1558030821 (ID 002048), under the responsibility Cláudia Momo, agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of School of Veterinary Medicine and Animal Science (University of São Paulo), and was approved in the meeting of day February 03, 2022.

Certificamos que o protocolo do Projeto de Pesquisa intitulado "Detecção e caracterização molecular de Sarcocystis spp. em gambás (Didelphis spp.)", protocolado sob o CEUAx nº 1558030821, sob a responsabilidade de Cláudia Momo, está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, e foi aprovado na reunião de 03 de fevereiro de 2022.

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
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Ministério do Meio Ambiente - MMA
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### Cronograma de atividades

| # | Descrição da atividade | Início (mês/ano) | Fim (mês/ano) |
|---|---|---|---|
| 1 | Padrão alimentar dos triatomíneos | 10/2018 | 10/2022 |
| 2 | Coleta Mensal de hospedeiros vertebrados e invertebrados | 10/2018 | 10/2022 |
| 3 | Modelagem de Nicho Ecológico | 10/2018 | 10/2022 |
| 4 | Exames Parasitologicos | 10/2018 | 10/2022 |

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## Dados do titular



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| Nome da Instituição: UNESP JABOTICABAL | CNPJ: 48.031.918/0012-87 |

## Cronograma de atividades

| # | Descrição da atividade | Início (mês/ano) | Fim (mês/ano) |
|---|---|---|---|
| 1 | processamento das amostras | 10/2020 | 12/2020 |
| 2 | captura dos gambás | 10/2020 | 12/2022 |

## Equipe

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**CARACTERIZAÇÃO MOLECULAR DE *Sarcocystis* spp. EM GAMBÁS (*Didelphis* spp.) E DETECÇÃO DE ANTICORPOS ANTI-*Sarcocystis* spp. EM EQUINOS DAS REGIÕES SUDESTE E CENTRO-OESTE DO BRASIL**

**RESUMO -** Gambás (*Didelphis* spp.) são hospedeiros definitivos de *Sarcocystis neurona*, *Sarcocystis speeri, Sarcocystis lindsayi* e *Sarcocystis falcatula.* No Brasil, diversos estudos vêm demonstrando a alta frequência de *Sarcocystis falcatula*-like em esporocistos recuperados dos intestinos de gambás, com grande diversidade nos genes codificadores de antígenos de superfície (*SAGs*). Neste estudo, a diversidade genética de *Sarcocystis* spp. oriundos de *Didelphis albiventris* e *Didelphis aurita* capturados nos municípios de Campo Grande e São Paulo, foi acessada por meio do sequenciamento de *SAG2, SAG3* e *SAG4*, da primeira região espaçadora interna transcrita (ITS-1) e da subunidade I da citocromo c oxidase (*cox1*). Adicionalmente, reação de imunofluorescência indireta (RIFI) foi empregada para detecção de anticorpos (IgG) contra *S. falcatula*-like (Dal-CG23) e *S. neurona* (SN138) no soro de 342 equinos amostrados nos mesmos municípios. Identificação molecular foi realizada em 16 amostras de DNA obtidas de esporocistos recuperados do intestino de gambás ou merozoítos derivados de cultivo *in vitro* de *Sarcocystis* spp. Os fragmentos de ITS-1, *cox1*, e *SAG3* foram clonados, enquanto *SAG2* e *SAG4* foram sequenciados diretamente dos produtos de PCR. Vinte e sete alelos foram observados em ITS-1, todos filogeneticamente relacionados à *S. falcatula*-like previamente detectado em aves e/ou em gambás no Brasil. *Sarcocystis* sp. filogeneticamente relacionado à *Sarcocystis rileyi* foi evidenciado por *cox1* em três gambás. Vinte e um novos alelos *SAGs* foram descritos, sendo quatro em *SAG2* (n=4), 13 em *SAG3* (n=13) e quatro em *SAG4* (n=7). Nos Imunoensaios utilizando RIFI com ponto de corte de 1:25, anticorpos IgG anti-*S. falcatula*-like foram detectados em 177/342 amostras de soro equino (51.75%), e anticorpos IgG anti-*S. neurona* foram detectados em 239/342 amostras (69.88%). Anticorpos IgG contra ambos os isolados foram observados no soro de 132 equinos (38,59%). Ausência de reatividade foi verificada em 16.95% (58/342) animais. A elevada soroprevalência observada neste estudo pode estar relacionada ao baixo ponte de corte utilizado e à presença de gambás infectados com *Sarcocystis falcatula*-like e *Sarcocystis* spp. nas áreas onde os equinos foram

amostrados. Devido à similaridade entre antígenos de superfície detectados nos imunoensaios, relatos de soropositividade contra *S. neurona* no Brasil podem resultar da exposição dos equinos à outras espécies de *Sarcocystis*. O papel de outras espécies de *Sarcocystis* como causa de doença neurológica nos equinos em Brasil permanece incerto.

**Palavras-chave:** *Didelphis* spp., *Sarcocystis* spp., identificação molecular, Imunoensaios.

**MOLECULAR CHARACTERIZATION OF *Sarcocystis* spp. FROM OPOSSUMS (*Didelphis* spp.) AND SEROLOGICAL DETECTION OF ANTI-*Sarcocystis* spp. ANTIBODIES IN HORSES FROM SOUTHEASTERN AND MIDWESTERN BRAZIL**

**ABSTRACT** – Opossums (*Didelphis* spp.) are definitive hosts of *Sarcocystis neurona*, *Sarcocystis speeri, Sarcocystis lindsayi* and *Sarcocystis falcatula.* In Brazil, several studies have demonstrated a high frequency of *Sarcocystis falcatula*-like in sporocysts derived from opossums, and high genetic diversity has been observed in surface antigen-encoding genes (*SAGs*). In this study, genetic diversity of *Sarcocystis* spp. derived from *Didelphis albiventris* and *Didelphis aurita* from the cities of Campo Grande and São Paulo, was accessed by sequencing *SAG2*, *SAG3*, *SAG4,* the first internal transcribed spacer (ITS-1) and cytochrome c oxidase subunit I (*cox1*). Additionally, indirect fluorescent antibody test (IFAT) was used to detect IgG antibodies against *Sarcocystis falcatula*-like (Dal-CG23) and *S. neurona* (SN138) in equine sera from 342 horses sampled in the same regions. Molecular identification was performed for 16 DNA samples obtained from sporocyst or culture-derived merozoites. The ITS-1, *cox1*, and *SAG3* fragments were cloned, whereas *SAG2* and *SAG4* were sequenced directly from PCR products. Twenty-seven allele variants were found for ITS-1, all phylogenetically related to *S. falcatula*-like previously described in birds and/or opossums in Brazil. *Sarcocystis* sp. phylogenetically related to *Sarcocystis rileyi* was evidenced by *cox1* in three opossums. Twenty-one new *SAG* alelles were described, four in *SAG2* (n=4), 13 in *SAG3* (n=13) and four in *SAG4* (n=7). On IFAT using 1:25 cutoff, IgG antibodies against *S. falcatula*-like were detected in 177/342 samples (51.75%), whereas IgG antibodies against *S. neurona* were detected in 239/342 samples (69.88%). IgG antibodies against both isolates were detected in 132 samples (38,59%). No reactivity was observed in 16.95% (58/342) horses. The high seroprevalence observed here may be related to the lower cutoff, and the presence of opossums infected with *Sarcocystis falcatula*-like and *Sarcocystis* spp. in the regions where the horses were sampled. Owing to the similarity among antigens targeted in immunoassays, reports on *S. neurona*-seropositive horses in Brazil might also derived from exposure of horses to other *Sarcocystis* species. The role of other Sarcocystis species in causing neurological diseases in horses in Brazil remains unclear.

**Keywords:** *Didelphis* spp., *Sarcocystis* spp., molecular identification, immunoassays.

## LIST OF ABBREVIATIONS

*cox1*: Cytochrome c oxidase 1
cPCR: Conventional Polymerase chain reaction
DNA: Deoxyribonucleic acid
ELISA: Enzyme-Linked Immunosorbent Assay
EPM: Equine protozoal myeloencephalitis
IFAT: Immunofluorescence antibody test
IMDM: Iscove's Modified Dulbecco's Medium
ITS-1: First internal transcribed spacer
q.s.p: 'Quantidade Suficiente Para'
*SAGs*: surface antigen-encoding genes
SNC: Central Nervous System

# CHAPTER 1 – General consideration

## 1 INTRODUCTION

Tissue cysts-forming coccidia are a vast group of organisms within the Phylum Apicomplexa Levine, 1979. Apicomplexans from the family Sarcocystidae Poche, 1913, such as those in the genus *Toxoplasma* Nicolle and Manceaux, 1908, *Neospora* Dubey, Carpenter, Speer, Topper and Uggla, 1988 and *Sarcocystis* Lankester, 1882, have the ability of infecting a broad range of hosts. They multiply into tissue cysts inside muscle cells of intermediate hosts, and the ingestion of the cysts by definitive hosts propagates their life cycle (BLAZEJEWSKI et al., 2015). *Sarcocystis* is considered the most diverse genera withing the family Sarcocystidae, infecting birds, reptiles, amphibians, fish, and mammals, including humans (BLAZEJEWSKI et al., 2015).

Currently, the genus *Sarcocystis* encompasses over 200 species, that vary considerably in their biology. Therefore, caution should be used when making generalized statements. Not all *Sarcocystis* spp. are pathogenic for the hosts. Notwithstanding, infections may have economic importance in farm animals, such as horses, cattle, sheep, goats, and pigs (DUBEY et al., 2016).

In the last several years, substantial number of studies have been conducted on a global level in order to investigate the role of *Sarcocystis* species causing disease in vast number of animal species. Several genetic markers have been used to molecularly characterize *Sarcocystis* strains isolated from various host species, and considerable progress has been made regarding genetic and immunogenic aspects of these parasites. In the Americas, studies based on natural and experimental infections have contributed to the understanding of the biology of *Sarcocystis* spp., especially those using opossums (*Didelphis* Linnaeus, 1758) as definitive hosts. The genome annotation of North American *S. neurona* SO SN1 (BLAZEJEWSKI et al., 2015) and *S. neurona* SN3 provided important insights into the genetics of the parasite. Immunological techniques, such as Immunofluorescent antibody test (IFATs), Western blotting (WB) and various enzyme-linked immunosorbent assays (ELISAs) have been used in diagnosis and in seroepidemiological studies (DUBEY et al., 2016).

Despite the countless number of studies, and the undeniable advances achieved, several aspects of *Sarcocystis* species infecting opossums in the Americas remain poorly understood. The elucidation of the distribution, genetic diversity, and evolutionary relationships of the parasite is of great importance. Therefore, the present study sought: 1- to assess the genetic diversity of *Sarcocystis* spp. in *Didelphis albiventris* and *Didelphis aurita* sampled in the cities of Campo Grande (Midwestern) and São Paulo (Southeastern), Brazil; 2- to evaluate sera reactivity against *S. falcatula*-like (strain Dal23-CG) and a North American strain of *S. neurona* (strain SN138), among horses sampled in the above cited cities.

## 2 LITERATURE REVIEW

Vast literature related to *Sarcocystis* spp. has been produced in the last several years. This review does not intend to exhaust the subject, simply representing an overview that aims to provide background, therefore contributing to the better understanding of the present work.

### 2.1 The genus *Sarcocystis*

*Sarcocystis* spp. are two-host single-celled parasites of medical and veterinary importance. These protozoans can be found in muscle and central nervous system (CNS) of a broad range of poikilothermic and homeothermic animals (DUBEY et al., 2016). *Sarcocystis* was first reported by Miescher, in 1843, as “milky white threads” in skeletal muscle of a deer mouse in Switzerland. The genus was named as *Sarcocystis* by Lankester in 1882 (DUBEY et al., 2016).

*Sarcocystis* species can be found in mammals, birds, reptiles, amphibians, and fishes (Figure 1) (PRAKAS; BUTKAUSKAS, 2012). In the last taxonomical review of the genus, 196 valid species were proposed (DUBEY et al., 2016). Since them, several new *Sarcocystis* spp. have been named and *Sarcocystis*-like parasites have been observed in muscles or nervous tissue of various animal species. At present, their number is estimated to be over 200. The majority of *Sarcocystis* species are described in intermediate host, and complete life cycle is ascertained to the majority of them.

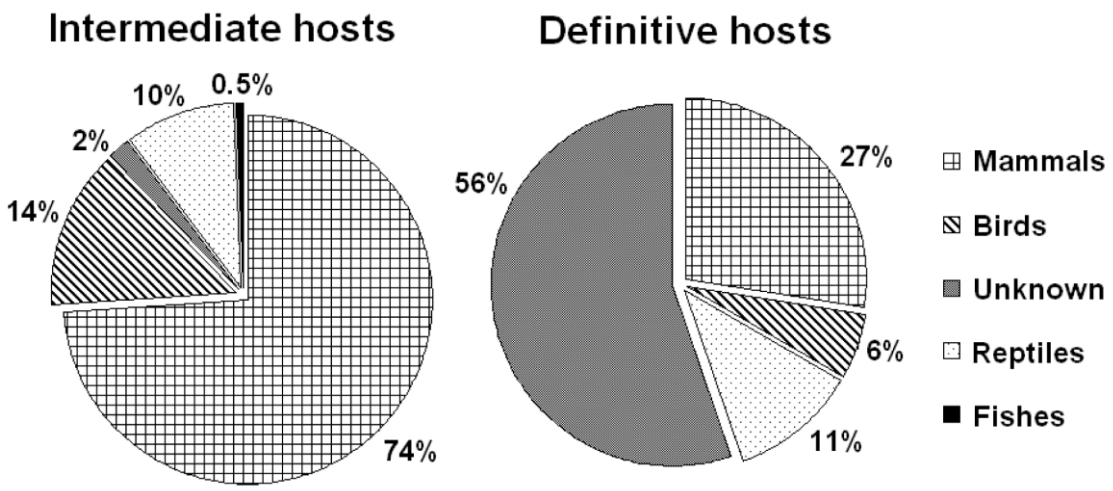


Figure 1. Intermediate and definitive hosts of *Sarcocystis* species (PRAKAS; BUTKAUSKAS, 2012).

*Sarcocystis* has an obligatory prey–predator two-host life cycle (Figure 2). Asexual stages develop in intermediate hosts, while sexual stages develop in definitive hosts. Definitive hosts become infected by ingesting mature cysts in muscle (sarcocysts) of intermediate hosts. The sarcocysts vary in size (from micro to macroscopic) and shape, according to the time of development, and the species of *Sarcocystis*. Also, more than one sarcocyst may be found within a host cell. Mature sarcocysts harbor banana-shaped zoites (bradyzoites), representing the infective form of the parasite. After sarcocyst ingestion by the definitive host, bradyzoites are released in the stomach and small intestines. Bradyzoites penetrate the enterocytes, converting into micro (male) and macro (female) gamonts. The fertilization originates an oocyst, which sporulate (sporocyst) in the lamina propria, is released into the intestinal lumen and excreted in feces. Each sporocyst contain four infective sporozoites (DUBEY et al., 2016).

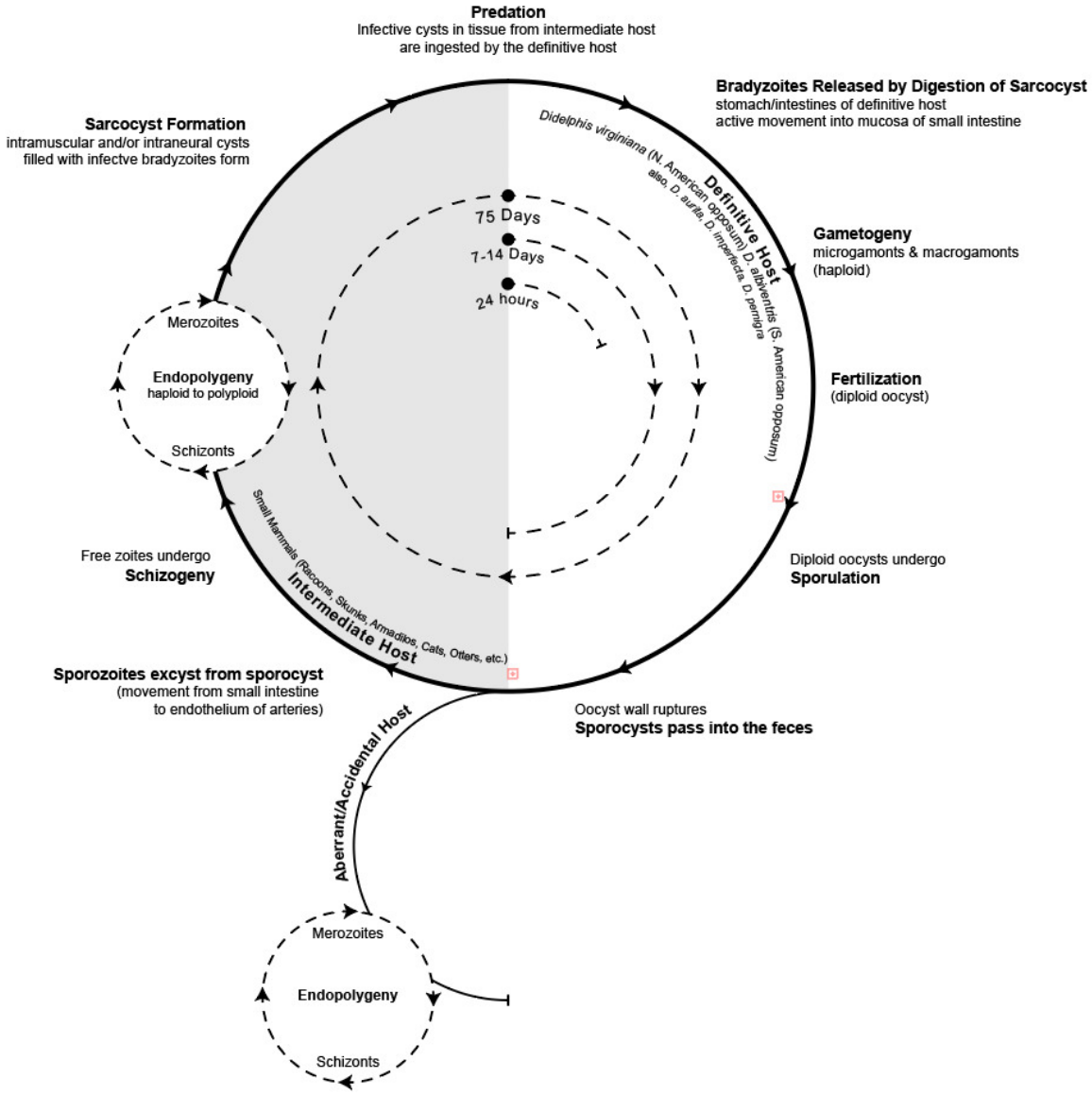


Figure 2. Two-host life cycle of *Sarcocystis* spp. (Courtesy of Jamie K. Norris).

Intermediate hosts become infected by ingesting sporocysts present in the environment. In the small intestines, sporozoites penetrate endothelial cells and initiate the first generation of schizogony. *Sarcocystis* schizonts divide by endopolygeny. The schizogonic cycle is asynchronous, therefore, schizonts of different maturity stages may be found in a single host cell. The number of generations of schizogony and the type of host cell invaded vary depending on the *Sarcocystis* species. Merozoites penetrate striated and cardiac muscle cells, and initiate cyst formation. Muscle cysts harbor asexual stages. After repeated cycles of endodyogeny, the sarcocyst is filled

with bradyzoites. Sarcocysts' maturation time is considerable variate depending on the *Sarcocystis* species. Immature sarcocysts and schizonts are not infectious for the definitive host and tissue stages are not infectious between intermediate hosts. Merozoites may also achieve and penetrate cells from CNS, where they develop as schizonts (DUBEY et al., 2016).

Intermediate and definitive hosts vary for each *Sarcocystis* species. Biological characteristics, such as sarcocysts morphology, and host specificity may be used to differentiate *Sarcocystis* species. Some *Sarcocystis* can infect numerous hosts, and some hosts may be infected by more than one *Sarcocystis* species. The parasite is generally more host-specific for intermediate hosts than for definitive hosts. This trait does not apply to *Sarcocystis* species shed by opossums (*Didelphis* spp.), as they have a wide range of intermediate hosts, while opossums are the only known definitive hosts (DUBEY et al., 2016). Not all species of *Sarcocystis* are pathogenic for intermediate hosts, and the severity of clinical sarcocystosis is dose dependent. Definitive hosts usually don't present clinical disease (DUBEY et al., 2016).

*Sarcocystis* infections have economic importance in farm animals. It can cause signs such as fever, anemia, muscle pain, and anorexia, reducing feed efficiency, weight gain, and milk yield. Abortion, neurologic impairment, and even death may also occur (DUBEY et al., 2016). Humans are definitive hosts for two *Sarcocystis* species, *Sarcocystis hominis* (Railliet and Lucet 1891) Dubey 1976 and *Sarcocystis suihominis* (Tadros and Laarman 1976) Heydorn 1977 (PENA; OGASSAWARA; SINHORINI, 2001) and serve as accidental intermediate or aberrant host for several other species that may cause muscular and intestinal disease (DUBEY et al., 2016).

Several conditions account for the high prevalence of *Sarcocystis* spp. worldwide: a single host may harbor several species of *Sarcocystis,* and a wide number of species may serve as definitive hosts; oocysts and sporocysts are eliminated in the infective form, may be excreted in large number and over a long period; sporocysts remain viable in the environment for several months, being resistant to disinfectants (MCKENNA; CHARLESTON, 1992, 1994), freezing (FAYER; JOHNSON, 1975) and low humidity (SAVINI; ROBERTSON; DUNSMORE, 1996).

## 2.2 *Sarcocystis* spp. and opossums

Opossums (*Didelphis* spp.) are marsupials from the order Didelphimorphia Gill, 1872. The species within the *Didelphis* genus are extremely adaptable to different habitats, inhabiting either forests or anthropized areas. They have lonely and nomad habits, traversing large areas, which enhances their role as pathogens spreaders. They are essentially terrestrial, however the presence of five digits in both hands and feet, including an opposable thumb, gives the opossums the ability to grab and escalate. Opossums are essentially crepuscular and nocturnal (CERQUEIRA, 1985; ROSSI; BIANCONI; PEDRO, 2006).

*Didelphis* spp. inhabit exclusively the American continent. In the entire North America, a single species of opossum, named *Didelphis virginiana*, is observed, whereas in South America five species of opossum are described. South American opossums have been grossly divided into white-eared opossums (*Didelphis albiventris* Lund, 1840, *Didelphis imperfecta* Mondolfi and Pérez-Hernández, 1984 and *Didelphis pernigra* Allen, 1900) and black-eared opossums (*Didelphis aurita* Wied-Neuwied, 1826 and *Didelphis marsupialis* Linnaeus, 1758) (CERQUEIRA, 1985; LEMOS; CERQUEIRA, 2002). The most common and most widely distributed South American opossum is *D. albiventris*, especially in Argentina and Brazil (CERQUEIRA, 1985).

Opossums act as definitive hosts for *Sarcocystis neurona* Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir, Cummings, and Suter 1991, *Sarcocystis speeri* Dubey and Lindsay 1999, *Sarcocystis falcatula* (Stiles 1893) Box, Meier, and Smith 1984, and *Sarcocystis lindsayi* Dubey, Rosenthal, and Speer 2001 (BOX; MEIER; SMITH, 1984; DUBEY et al., 1991a; DUBEY; ROSENTHAL; SPEER, 2001; DUBEY and LINDSAY, 1999). *Sarcocystis neurona* is the causative agent of equine protozoal myeloencephalitis (EPM), a neurological disorder affecting horses and some marine mammals (DUBEY et al., 1991a; WENDTE et al., 2010; ACOSTA et al., 2018). *Sarcocystis speeri* is biologically similar to *S. neurona*, being experimentally infective in immunodeficient mice (DUBEY; LINDSAY, 1999), but it was named a new species because of its morphological and antigenic differences. *Sarcocystis speeri* forms cysts in the tissues of infected mice, which do not occur with *S. neurona* (DUBEY; LINDSAY, 1999). *Sarcocystis falcatula* and *S. lindsayi* cause acute

pulmonary sarcocystosis in birds (DUBEY; ROSENTHAL; SPEER, 2001; HILLIER et al., 1991; ACOSTA et al., 2018). *Sarcocystis lindsayi* is biologically similar to *S. falcatula*, however, it was named a new species predominantly because of the differences in ITS-1 locus of the rDNA (DUBEY; ROSENTHAL; SPEER, 2001).

Extensive literature about the occurrence of *Sarcocystis* spp. in intestinal scrapings of *D. virginiana* in North America is currently available. Dubey (2000) reported 54.5% (24/44) of prevalence of *Sarcocystis* sporocysts in wild-caught opossums from diverse areas in the United States. In that work mixed *Sarcocystis* infections (*S. falcatula, S. neurona* and *S. speeri*) were present in 21 opossums. Subsequently, Dubey et al. (2001a) reported *S. neurona* sporocysts in intestinal scrapings of 24/72 (33%) *D. virginiana* from rural Mississippi. After bioassay in KO mice and immunostaining, the authors confirmed the presence of *S. neurona* in 19 opossums. Elsheikha, Murphy and Mansfield (2004a) reported *S. neurona* sporocysts in intestinal scrapings of 31/206 (15%) *D. virginiana* from Southern Michigan. Rejmanek et al. (2009) reported *S. neurona* sporocysts in intestinal scrapings of 53/288 (18.4%) *D. virginiana* from central California.

In Brazil, several studies have reported the prevalence of *Sarcocystis* spp. sporocysts in intestinal scrapings of opossums. Casagrande et al. (2009) observed sporocysts from *Sarcocystis* spp. in intestinal scrapings of 6/66 (9.1%) *D. aurita* in São Paulo. Lins et al. (2011) observed sporocysts from *Sarcocystis* spp. in intestinal scrapings of 19/19 (100%) *D. albiventris* in Rio Grande do Sul. Valadas (2015) observed sporocysts from *Sarcocystis* spp. in intestinal scrapings of 33/50 (66%) *Didelphis* spp. in São Paulo and Rio Grande do Norte.

Infected opossums may shed large numbers of *Sarcocystis* spp. sporocysts over a long period of time. Once infected, these animals may serve as a reservoir throughout their entire life span (2–3 years) (PORTER et al., 2001). Opossums fed muscles from brown-headed cowbird (*Molothrus ater*) infected with *S. falcatula* initiated to shed sporocysts 7-16 days after infection and continued to shed sporocysts until they were euthanized (46-200 days after infection) (PORTER et al., 2001). Dubey et al. (2001a) reported sporocysts in intestinal scrapings of opossums ranging from less than 100.000 to 187 million. In places with high population density, significant environmental loading rates of the parasite may occur. *Sarcocystis* sporocysts may be

found even in apparently healthy opossums (ELSHEIKHA; MURPHY; MANSFIELD, 2004b). This could evidence a nonharmful co-existence between parasite and opossums, indicating a long evolutionary history of host–parasite interaction (ELSHEIKHA; MURPHY; MANSFIELD, 2004b).

The most frequently method applied to determine the prevalence of *Sarcocystis* in opossums is the microscopic observation of oocysts/sporocysts on feces. However, as sporocysts are trapped in the lamina propria, they are excreted irregularly and sometimes they are not detectable in feces although millions are present in the intestines (DUBEY et al., 2016). Casagrande et al. (2009) observed sporocysts in intestinal scrapings of 6/66 (9.1%) *D. aurita,* however, only in four of them sporocysts were observed in the feces. Structure of the oocyst and sporocysts has little or no taxonomic value because morphological characteristics are shared by several closely related species (DUBEY et al., 2016). Therefore, biologic and/or molecular methods are necessary to correct identification. Bioassay in immunodeficient mice and budgerigars can distinguish *S. falcatula* from *S. neurona* and *S. speeri*. *Sarcocystis neurona* and *S. speeri* are not infective to budgerigars, and *S. falcatula* is not infective to mice (DUBEY et al., 2016).

Most of *Sarcocystis* derived from opossums in Brazil were identified as *S. falcatula*-like, due to their genetic characteristics and/or infectivity to birds (VALADAS et al., 2016; GONDIM et al., 2017, 2019; ACOSTA et al., 2018; CESAR et al., 2018; GALLO et al., 2018). It has been suggested that the diversity of *Sarcocystis* species in the intestine of opossums may enable sexual recombination, contributing to their allelic variability (MONTEIRO et al., 2013).

### *2.2.1 Sarcocystis neurona*

*Sarcocystis neurona*, first identified in the United States in 1991, is the main etiological agent of equine protozoal myeloencephalitis (EPM) (DUBEY et al., 1991a), a debilitating and potentially fatal neurologic syndrome, first described in equine. After some years from description in equids, an EPM-like fatal disease was also recognized in many other hosts, especially marine mammals (LINDSAY; THOMAS; DUBEY,

2000). This progressive disease is characterized by a wide range of neurological signs according to the parasite distribution in the CNS (DUBEY et al., 2015a).

In Brazil, the disease was first observed several decades ago. One of the very first reports identified protozoal organisms in the spinal cord of a 10-year-old horse (LOMBARDO DE BARROS; BARROS; SANTOS, 1986). However, at that time, the causative agent was not elucidated. Few years later, mature schizonts and merozoites of a *Sarcocystis* sp. were observed in the CNS of two horses presenting neurological signs (MASRI; LOPEZ DE ALDA; DUBEY, 1992). Horses affected by EPM usually present progressive symmetrical ataxia that may be accompanied by focal muscular atrophy. Detailed information about clinical presentations and pathogenesis of EPM can be found on published reviews (DUBEY et al., 2001b; DUBEY et al. 2015a).

*Sarcocystis neurona* presents a wide host range. White-nosed coati (*Nasua narica molaris* Merriam, 1902) skunks (*Mephitis mephitis* Schreber, 1776), raccoons (*Procyon lotor* Linnaeus, 1758), armadillos (*Dasypus novencinctus* Linnaeus, 1758), sea otter (*Enhydra lutris* Linnaeus, 1758), and cats (*Felis catus* Linnaeus, 1758), have already been described as intermediate hosts (DUBEY; HAMIR, 2000; CHEADLE et al., 2001a; CHEADLE 2001b; TANHAUSER et al., 2001; DUBEY et al., 2001c; DUBEY et al., 2001d; DUBEY et al., 2017; HAMMERSCHMITT et al., 2020). In addition, *S. neurona*-like sarcocysts have been reported in the muscles of horses (MULLANEY et al., 2005), dogs (VASHISHT et al., 2005; DUBEY et al., 2014), minks (*Mustela vison* Schreber, 1777) (RAMOS-VARA et al., 1997), and bobcats (*Lynx rufus* Schreber, 1777) (VERMA et al., 2015). Equines are considered aberrant hosts for *S. neurona*, with the parasite multiplying as schizonts in neural and inflammatory cells in the CNS, but failing to encyst (DUBEY et al., 2016). In North America, the parasite has arisen as a significant cause of mortality in marine mammals (WENDTE et al., 2010). A common described victim of the disease is the threatened southern sea otter (*Enhydra lutris nereis* Linnaeus, 1758). Between 1998 and 2001, *S. neurona* accounted for nearly 7% of all southern sea otter mortality (KREUDER et al., 2003).

The North and the South American opossums, *D. virginiana* and *D. albiventris*, respectively, are the known definitive hosts for *S. neurona* (DUBEY et al., 2016). Multiple studies in the United States have reported varying levels of *S. neurona*

infection among opossum based on the detection of *S. neurona* sporocysts in their intestinal scrapings. A study performed by Dubey (2000) found 8/44 (18.1%) opossums infected with *S. neurona* sporocysts, compared to 19/72 (26%) opossums from rural Mississippi (DUBEY et al., 2001a), 31/206 (15%) opossums from Michigan (ELSHEIKHA; MURPHY; MANSFIELD, 2004b) and 17/288 (5.9%) opossums from central California (REJMANEK et al., 2009). Only on one occasion *S. neurona* was isolated from *D. albiventris* in Brazil (DUBEY et al., 2001e). South American opossums appears to present a lower incidence of *S. neurona*, comparing to its North American relatives.

*Sarcocystis neurona* is highly prevalent in horses in the Americas. Antibodies to *S. neurona* were found in 1056 (53.6%) horses from Ohio (SAVILLE et al., 1997), 117(45.3%) horses from Pennsylvania (BENTZ; GRANSTROM; STAMPER, 1997), 334 (45%) horses from Oregon (BLYTHE et al., 1997), and in 5250 (78%) horses sampled across the United States (JAMES et al., 2017). In Brazil, seroepidemiological studies have been carried out in several states from North, Northeast, Center-West, Southeast and South regions, and seroprevalence as high as 84% were observed (RIBEIRO et al., 2016; BORGES et al., 2017; VALENÇA et al., 2019; KOCH et al., 2019; BORGES-SILVA et al., 2020). Seroprevalence may be associated to the age of the horse, the presence of opossums, and the serological test used (DUBEY et al., 2015a; BORGES et al., 2017). Immunofluorescent antibody test (IFAT), Western blotting (WB) and various enzyme-linked immunosorbent assays (ELISAs) have been widely used for EPM diagnosis and seroepidemiological studies. However, due to the presence of antibodies against other *Sarcocystis* spp. in equine sera, these tests may generate false-positive results. Studies using combinations of tests or immunoblotting as confirmatory after a positive IFAT or ELISA result, presented a lower number of “true positive” samples (BORGES et al., 2017; VALENÇA et al., 2019), denoting that, reports on *S. neurona*–seropositive horses in Brazil may also derived from exposure of horses to other *Sarcocystis* species, such as *S. falcatula, S. lindsayi*, *S. speeri*, *S. fayeri* and *S. bertrami* (BORGES-SILVA et al., 2020).

### *2.2.2 Sarcocystis falcatula*

*Sarcocystis falcatula* is one of the several species of *Sarcocystis* that affect birds. The parasite was first described by Stiles in muscles of the rose-breasted grosbeck (*Pheucticus ludovicianus*), and subsequently re-described by Box, Meier and Smith (1984). Similar to what occurs with other intermediate hosts, birds become infected with *S. falcatula* through ingestion of food or water contaminated with sporocysts. The extensive multiplication of the parasite in endothelial cells may cause physical damage and vasculitis (DUBEY et al., 2016). Respiratory alterations and death of heavily infected birds have been described in exotic species (VILLAR et al., 2008). Cases of encephalitis in birds have also been assumed to be caused by *S. falcatula* (JACOBSON et al., 1984; SIEGAL-WILLOTT et al., 2005; WÜNSCHMANN et al., 2009; KONRADT et al., 2017). Conversely, muscle cysts have been incidentally found in Brazilian native species, not associated with clinical disease (LLANO et al., 2022).

*Sarcocystis falcatula*, just as its relative *S. neurona*, present an unusual wide host range. Numerous cases of acute sarcocystosis presumed to be caused by *S. falcatula* or related species (named *S. falcatula*-like) have been reported in captive passerine (DUBEY et al., 2001f), psittacine (SMITH et al., 1990; HILLIER et al., 1991; VILLAR et al., 2008; GODOY et al., 2009; ECCO et al., 2008), and pigeons (ECCO et al., 2008; SUEDMEYER et al., 2001) in the Americas. The parasite was also reported in a free-ranging great horned owl (*Bubo virginianus*) (WÜNSCHMANN et al., 2009), and in free-ranging eagles (*Haliaeetus leucocephalus* and *Aquila chrysaetos*) (DUBEY et al., 1991b; WÜNSCHMANN et al., 2010).

The first description of *S. falcatula* outside the United States was in 1999, from the intestines of *D. albiventris* from Argentina (DUBEY et al., 1999). That isolate was later re-examined using enzyme restriction of a 1100-pb PCR product obtained from RAPD marker 33/54. The authors observed that fragment digestion with Dra I resulted in products similar to that produced by *S. neurona* digestion, and digestion with Hinf I resulted in products similar to that produced by *S. falcatula* digestion. The isolated was then named *S. falcatula*-like (DUBEY et al., 2000a).

Most *Sarcocystis* derived from opossums in Brazil were described as *S. falcatula*-like, as they were phylogenetically related to *S. falcatula* and infective for birds, but presented molecular differences compared with *S. falcatula* described in North America (GONDIM et al., 2017, 2019; ACOSTA et al., 2018; CESAR et al., 2018). Previous studies have shown that *S. falcatula* constitutes a heterogeneous population (MARSH et al., 1999; VALADAS et al., 2016; GONDIM et al., 2017, 2019; CESAR et al., 2018; LLANO et al., 2022). *In vitro* culture, single-cell cloning and molecular assays will be needed to resolve this speciation issue (DUBEY et al., 2016).

### 2.2.3 Other *Sarcocystis* that use opossums as definitive hosts

*Sarcocystis speeri* and *Sarcocystis lindsayi* are the other species that use opossums as definitive hosts. *Sarcocystis speeri* was first described in 1999, and it was the third species recognized in the North American opossum (*D. virginiana*) (DUBEY; LINDSAY, 1999). Later *D. albiventris* and *D. marsupialis* were also described as definitive hosts for *S. speeri* (DUBEY et al., 2000b; 2000c). However, probably *D. aurita* was mistakenly identified as *D. marsupialis*, as the latter species does not occurs in the geographical area where it was described (Gondim et al., 2021). The natural intermediate hosts for *S. speeri* are not known.

*Sarcocystis speeri* is infective for mammals and present high genetic similarity with *S. neurona* (DUBEY et al., 2015b). However, some characteristics differentiate the two parasites. *Sarcocystis neurona* does not produce sarcocysts in KO mice, whereas *S. speeri* does. Schizonts may be found in many organs, including liver, brain, and uterus (DUBEY; LINDSAY, 1999; DUBEY; SPEER; LINDSAY, 1999; DUBEY et al., 2015b). While few *S. neurona* sporocysts were lethal for KO mice (DUBEY et al., 2013), a thousand *S. speeri* sporocysts were necessary to be pathogenic for KO mice (DUBEY; LINDSAY, 1999). Also, *S. speeri* schizonts are antigenically and morphologically distinct from *S. neurona* schizonts (DUBEY; LINDSAY, 1999). Based on bioassays in KO mice and budgerigars, prevalence of *S. speeri* was 18.8% (44 opossums examined) (DUBEY, 2000).

*Sarcocystis lindsayi* was described for the first time in *D. albiventris* from Brazil (DUBEY; ROSENTHAL; SPEER, 2001). Posteriorly, it was also described in *D. aurita*

(STABENOW et al., 2012). *Sarcocystis lindsayi* is biologically similar to *S. falcatula*, but it was proposed as a new species mostly due to differences in the first internal transcribed spacer 1 (ITS-1) locus of the rDNA. At ITS-1, *S. lindsayi* has 93.3% identity with *S. falcatula,* although at 28S rDNA these two species are almost identical (DUBEY; ROSENTHAL; SPEER, 2001). Budgerigars fed sporocysts of *S. lindsayi* from opossum feces died from acute sarcocystosis, similar to what occur in infection with *S. falcatula.* Natural intermediate hosts of *Sarcocystis lindsayi* are still unknown.

### 2.2.4 Molecular identification of *Sarcocystis* spp. from opossums

Each *Sarcocystis* species has a particular spectrum of hosts and full knowledge of the host specificity is often difficult to obtain (GONDIM et al., 2021). Therefore, molecular data have become mandatory for identification of the species in the genus (GJERDE; VIKOREN; HAMNES, 2018). To compare and accurately describe closely related pathogens, the use of multiple genetic markers is essential. The annotation of the genomes of *S. neurona* SO SN1 (BLAZEJEWSKI et. al, 2015), and *S. neurona* SN3, considerably increased the repertoire of known genetic markers. The Sanger sequencing method directed to complete or partial gene segments, have been used to access the diversity of *Sarcocystis* spp. from opossums in the Americas (DUBEY et al., 2016; GONDIM et al., 2021).

The gene encoding the small unit of ribosomal DNA (18S rDNA) is considered a universal marker for molecular identification of *Sarcocystis* spp., especially when first characterizing an isolate (MORRISON et al., 2004). This gene was already sequenced for several *Sarcocystis* species (DUBEY et al., 2016). Ribosomal RNA is ubiquitous and present highly conserved and highly variable domains. Conserved domains allow easy application of universal primers, whereas variable domains allow distinction among related biological species and lineages (MORRISON et al., 2004). However, closely related species, such as *S. falcatula* and *S. neurona*, and certain *Sarcocystis* species that use birds as intermediate hosts are almost identical at 18S rDNA (DAME et al., 1995; MARSH et al., 1999; OLIAS et al., 2010; PRAKAS et al., 2013). Therefore, other markers with better phylogenetic resolution, such as the first

internal transcribed spacer (ITS-1) and cytochrome c oxidase subunit 1 (*cox1*), have been associated with 18S rDNA (TANHAUSER et al., 1999; MARSH et al., 1999).

The *cox1* has shown good phylogenetic resolution for differentiation among *Sarcocystis* that use ruminants as intermediate hosts (GJERDE, 2013). However, it may not be variable enough to discriminate between *Sarcocystis* species that use birds or carnivorous mammals as intermediate hosts (GJERDE; VIKOREN; HAMNES, 2018, LLANO et al., 2022). The ITS-1 locus, located between 18S rDNA and 5.8S rDNA, enhances sensitivity of molecular assays, as it is present in several copies within the eukaryote genome. Besides, it present high evolutionary rates (HILLIS; DIXON, 1991; ALVAREZ; WENDEL, 2003). Sequencing of ITS-1 have allowed differentiation between *S. falcatula* and *S. neurona*, species that had been previously considered synonymous (MARSH et al., 1999).

Isolates of a *Sarcocystis* sp. divergent from *S. falcatula* and *S. neurona* at ITS-1 were detected in opossums in Brazil. Due to infectivity to budgerigars, the isolate was named *Sarcocystis falcatula*-like (DUBEY et al., 2001g). Over the years, this genotype has been molecularly detected in opossums (VALADAS et al., 2016; GONDIM et al., 2019), in budgerigars experimentally infected with opossums sporocysts (CESAR et al., 2018; GONDIM et al., 2017), and in naturally infected birds (ACOSTA et al., 2018, KONRADT, et al., 2017, LLANO et al., 2022) in the country.

Surface antigen-encoding genes (*SAGs*) express immunodominant antigens present on the surface of merozoites in both extracellular and intracellular stages of development. These genes, first observed in *S. neurona* and therefore named SnSAG1-6, present differently depending on the strain. Some strains lack SAG1, expressing instead one or two alternative major surface antigens, SAG5 and SAG6. The SAGs have been assessed to evaluate diversity in *Sarcocystis* excreted by opossums (WENDTE et al., 2010; REJMANEK et al., 2010; GONDIM et al., 2017, 2019). The allelic combinations of SAG2, SAG3 and SAG4 observed among *S. falcatula* related isolates suggest that they may suffer sexual recombination processes with exchange of high divergent alleles (MONTEIRO et al., 2013).

## 2.3 Serological studies on *Sarcocystis neurona* in South American horses

The first assay developed for detecting antibodies against *S. neurona* was a Western blot (WB) (GRANSTROM et al., 1993). *S. neurona* WB test was modified over the years to improve the diagnostic accuracy of EPM. However, WB is a fairly laborious technique that requires significant expertise to accurately interpretation of results. Therefore, other serologic assays, such as immunofluorescent antibody tests (IFATs), agglutination tests and enzyme-linked immunosorbent assays (ELISAs), more informative and with greater throughput, have been developed (DUBEY et al., 2015). IFATs and various ELISAs have been widely used for EPM diagnosis and seroepidemiological studies in South America. Most of these studies have been conducted in Brazil, where serum samples from horses tested using different methods have revealed variable results.

Horse serum tested by IFAT in Brazil have shown a range of seropositivity varying from 2.8 to 84.1% (summarized by Gondim et al., 2021). It is important to stress that on IFAT the use of whole merozoites allow the detection of antibodies against surface antigens. Some of these antigens are suspect to be shared among different *Sarcocystis* spp., which could lead to the false assumption that a horse is seropositive for *S. neurona* due to seroconversion after infection with other *Sarcocystis* species. A recent study performed with 409 horses sampled in Bahia and Rio Grande do Sul states, used *S. falcatula–like* (Sarco-BA1) and *S. neurona* (SN138) as antigens for IFAT and WB. The authors observed that 43 (10.5%) and 70 (17.1%) samples tested by IFAT, were reactive to *S. falcatula*-like and *S. neurona,* respectively. A total of 25 samples (6.1%) were reactive for both parasites. The fair agreement observed between the two IFATs indicated that horses were exposed to more than one *Sarcocystis* species. Results from WB in the same work suggested that antigens in the range of 16 and 30 KDa are probably homologous in the two parasites (BORGES-SILVA et al., 2020).

In a study performed with sera from 427 horses from the state of Alagoas, antibodies against *S. neurona* (strain SN37R) were observed in 2.8% (12/427) of the samples evaluated by IFAT (the authors used a more conservative cutoff of 1:80), and in 1.6% (7/427) of the samples evaluated by immunoblot. In the immunoblot, the

authors observed reactivity to antigens of 7-10KDa, 16 and 30 KDa. In that study confirmation of IFAT using immunoblot revealed a low number of “true positive” samples (VALENÇA et al., 2019). Cross-reactivity between *S. neurona* (strain SN138) and *S. falcatula-like* (strain Sarco-BA1) was also reported in experimentally infected Mongolian gerbils (*Meriones unguiculatus*) tested by WB (DE JESUS et al., 2019). The authors reported absence of cross-reactivity in samples tested using IFAT, while similar reactivity to proteins of 30 and 16 KDa was observed in both parasites.

ELISAs using *S. neurona* merozoite surface antigens (SnSAGs), mainly expressed as recombinant proteins have been developed and applied in the EPM diagnosis and seroepidemiological studies (DUBEY et al., 2016). A study performed in Brazil evaluated sera from 961 horses from ten different states using an ELISA based on the SnSAG4. In that study the authors observed a positivity of 69.6% (669/991) (HOANE et al., 2006). To prevent from false-positive results derived from the use of a single antigen protein, improved SnSAG ELISAs were developed in North America. Chimeras combining two (YEARGAN; HOWE, 2011) and three (YEARGAN et al., 2015) of the major SnSAGs have been used in EPM diagnosis.

In Brazil, seroepidemiological studies on *S. neurona* have been conducted using antigens and recombinant proteins based in North American strains (DUBEY et al., 2002; ONUMA et al., 2014; GENNARI et al., 2016; GOMES et al, 2019; VALENÇA et al., 2019), as no isolation of *S. neurona* has been obtained from affected horses in South America. Notably, despite the occurrence of a disease resembling EPM (MASRI et al., 1992) and the high seroprevalence of *S. neurona* observed in equids in Brazil, only on one occasion *S. neurona* was isolated from opossums in the country. Sporocysts of *Sarcocystis* spp. obtained from opossums (*D. albiventris*) were shipped to the United States and bioassayed in interferon gamma gene knock out (KO) mice. The mice developed neurologic sarcocystosis, and *S. neurona* was demonstrated in their brains by immunohistochemical staining (DUBEY et al., 2001e). No subsequent studies were published on that isolate.

Several authors have discussed the hypothesis that horses in Brazil are being exposed and seroconverting to other *Sarcocystis* species shed by opossums (*S. falcatula-like*, *S. neurona*, *S. lindsayi* and *S. speeri*) as well as to *S. bertrami* (syn. *S. fayeri*) from which horses are intermediate hosts. However, the potential of other

*Sarcocystis* spp. present in south America in causing neurological disorders and EPM in horses remains uncertain.

## 3 GENERAL OBJECTIVES

The current study aimed: 1- to assess the genetic diversity of *Sarcocystis* spp. in opossums (*D. albiventris* and *D. aurita*) sampled in the cities of Campo Grande (Midwestern) and São Paulo (Southeastern), Brazil; 2- to evaluate the horse sera reactivity against *S. falcatula*-like (strain Dal23-CG) and *S. neurona* (North American strain SN138), among horses sampled in the same cities.

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## CHAPTER 2 – Molecular diversity of *Sarcocystis* spp. in opossums (*Didelphis* spp.) from Southeastern and Midwestern Brazil

**Abstract**

South American opossums (*Didelphis* spp.) are definitive hosts of *Sarcocystis neurona, Sarcocystis speeri, Sarcocystis lindsayi* and *Sarcocystis falcatula*. In Brazil, diverse studies have demonstrated a high frequency of *Sarcocystis falcatula*-like in sporocysts derived from opossums, and high genetic diversity has been observed in surface antigen-encoding genes (*SAGs*). In this study, genetic diversity of *Sarcocystis* spp. derived from *Didelphis albiventris* and *Didelphis aurita* from the cities of Campo Grande and São Paulo, was accessed by sequencing *SAG2*, *SAG3*, *SAG4,* the first internal transcribed spacer (ITS-1) and cytochrome c oxidase subunit I (*cox1*). Molecular identification was performed for 16 DNA samples obtained from sporocyst or culture-derived merozoites. The ITS-1, *cox1*, and *SAG3* fragments were cloned, whereas *SAG2* and *SAG4* were sequenced directly from PCR products. Four alleles variants were found for *SAG2*, 13 for *SAG3* and seven for *SAG4,* from which 04, 13 and 04, respectively, were novel. Twenty-seven allele variants were found for ITS-1, all phylogenetically related to *S. falcatula*-like previously described in Brazil. *Sarcocystis* sp. phylogenetically related to *Sarcocystis rileyi* was evidenced by *cox1* in three opossums. Further studies are needed to clarify the role of *Didelphis* spp. as definitive hosts of *Sarcocystis* spp. other than those previous described.

**Keywords:** *Sarcocystis* spp., opossums, molecular characterization, ITS-1, *cox1, SAGs*

**Resumo**

Gambás sul-americanos (*Didelphis* spp.) são hospedeiros definitivos de *Sarcocystis neurona, Sarcocystis speeri, Sarcocystis lindsayi* e *Sarcocystis falcatula*. No Brasil, diversos estudos têm demonstrado alta frequência de *Sarcocystis falcatula*-like em esporocistos derivados de gambás, com grande diversidade nos genes que codificam antígenos de superfície (*SAGs*). Neste estudo, a diversidade genética de *Sarcocystis* spp. oriundos de *Didelphis albiventris* e *Didelphis aurita* dos municípios de Campo Grande e São Paulo, foi acessada por meio do sequenciamento de *SAG2*,

*SAG3* e *SAG4,* da primeira região espaçadora interna transcrita (ITS-1) e citocromo c oxidase subunidade I (*cox1*). Identificação molecular foi realizada em 16 amostras de DNA obtidas de esporocistos ou merozoítos derivados de cultivo. Os fragmentos de ITS-1, *cox1*, e *SAG3* foram clonados, enquanto *SAG2* e *SAG4* foram sequenciados diretamente dos produtos de PCR. Quatro alelos foram observados em *SAG2*, 13 em *SAG3* e sete em *SAG4,* sendo novos quatro, 13 e quatro, respectivamente. Em ITS-1, 27 alelos foram observados, todos filogeneticamente relacionados à *S. falcatula*-like previamente detectados no Brasil. *Sarcocystis* sp. filogeneticamente relacionado à *Sarcocystis rileyi* foi evidenciado por *cox1* em três gambás. Mais estudos são necessários para entender o papel de *Didelphis* spp. como hospedeiro definitivo de *Sarcocystis* spp. diferentes daqueles previamente descritos.

**Palavras-chave:** *Sarcocystis* spp., gambás, caracterização molecular, ITS-1, *cox1, SAGs*

## Introduction

*Sarcocystis* Lankester 1882, are obligate intracellular protozoa belonging to the phylum Apicomplexa Levine 1979. Species of the genus *Sarcocystis* have an obligatory prey-predator two-host life cycle. Opossums (*Didelphis* Linnaeus, 1758), which exclusively inhabit the American continents, act as definitive hosts for *Sarcocystis neurona* Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir, Cummings, and Suter 1991, *Sarcocystis speeri* Dubey and Lindsay 1999, *Sarcocystis falcatula* (Stiles 1893) Box, Meier, and Smith 1984, and *Sarcocystis lindsayi* Dubey, Rosenthal, and Speer 2001 (Box et al., 1984; Dubey et al., 1991, 2001a; Dubey & Lindsay, 1999).

*Sarcocystis neurona* is the chief etiological agent of equine protozoal myeloencephalitis (EPM) (Dubey et al., 2001b). *Sarcocystis falcatula* has been associated with numerous cases of pulmonary sarcocystosis in free-living and captive birds (Smith et al., 1990; Hillyer et al., 1991; Clubb & Frenkel, 1992; Page et al., 1992; Dubey et al., 2001c; Suedmeyer et al., 2001; Villar et al., 2008; Wünschmann et al., 2009, 2010; Verma et al., 2018). *Sarcocystis speeri* and *S. lindsayi* are respectively, experimentally infective to mice and budgerigars (*Melopsittacus undulatus*), but their natural intermediate hosts are unknown (Dubey & Lindsay, 1999; Dubey et al., 2001a).

Several genetic markers have been used to molecularly characterize *Sarcocystis* spp. shed by opossums in the Americas. Genome annotation of the North American *S. neurona* SO SN1 (Blazejewski et al., 2015) and *S. neurona* SN3 provided important insights into the molecular biology of the parasite. In Brazil, various studies have demonstrated the presence of *Sarcocystis* spp. in sporocysts derived from opossums (Casagrande et al., 2009; Monteiro et al., 2013; Gallo et al., 2018; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018). The majority of the *Sarcocystis* identified in the country have been classified as *Sarcocystis falcatula*-like, due to genetic characteristics and/or experimental infectivity to budgerigars (Gondim et al., 2017, 2019; Acosta et al., 2018; Cesar et al., 2018). Extensive variability has been observed in surface antigen-encoding genes (*SAGs*) of *Sarcocystis* spp. derived from opossums in Brazil (Monteiro et al., 2013; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018). It has been suggested that the diversity of *Sarcocystis* species in the intestine of opossums could enable allele exchange through sexual recombination, contributing to their allelic variability (Monteiro et al., 2013).

Considering the widespread occurrence of *Sarcocystis* spp. in opossums in Brazil and the wide genetic variation observed in previous studies, this study sought to assess the genetic diversity of *Sarcocystis* spp. in *D. albiventris* and *D. aurita* sampled in the cities of Campo Grande (midwestern) and São Paulo (southeastern), Brazil. The detection and molecular characterization of these agents in the opossums of these regions contribute to increasing the knowledge related to the genetic diversity of *Sarcocystis* spp. in Brazil.

## Material and Methods

### Sampling

Between July 2019 and April 2021, five expeditions were performed for capturing free-ranging opossums. Four expeditions were performed in the city of Campo Grande, Mato Grosso do Sul state (Midwestern) and one expedition was performed in the city São Paulo, São Paulo state (Southeastern), Brazil. The animals were caught in six locations in the urban region of Campo Grande (1–20°41’37.51” S, 54°61’54.65” O; 2– 20°44’88.11” S, 54°57’95.99” O; 3–20°43’95.15” S, 54°57’43.24”

O; 4–20°49’96.79” S, 54°61’35.94” O; 5–20°47’17.08” S, 54°65’60.08” O; 6–20°49’32.15” S, 54°58’09.15” O) and in an equestrian club in the city of São Paulo (23°38'31.3" S, 46°42'35.0" O), using Tomahawk and Sherman live traps baited with a mix of bananas, peanut butter, tinned sardines, and bacon. Together, these five expeditions resulted in the capture of 37 opossums: 26 *Didelphis albiventris* from Campo Grande and 11 *Didelphis aurita* from São Paulo (*D. aurita* was differentiated from the phenotypical similar species *Didelphis marsupialis* based on their distribution in the country). Trapped opossums were transported to the laboratory, where they were chemically restrained with a combination of cetamina and xilazina (30 mg/Kg and 5 mg/Kg, respectively, intramuscular), followed by euthanasia with T-61 (MSD) (0.3 mL/Kg, intravenously). Necropsy was performed. The small intestine was separated, longitudinally sectioned, and the internal surface was scraped and processed as previously described (Dubey et al., 2016; Gondim et al., 2019). Briefly, intestinal scraping was homogenized with a mixture of 30 mL of sodium hypochlorite (2.5% active chlorine) and 70 ml of distilled water, to disrupt cell clumps and release *Sarcocystis* spp. sporocysts. The solution was filtered through gauze and centrifuged at 2000 x *g* at 4 °C for 10 min. A small drop of the sediment was examined using light microscopy (Olympus BX-51, 400x magnification) for the presence of *Sarcocystis* spp. sporocysts. For positive samples, sodium hypochlorite was removed by two additional washes with PBS (pH 7.2). Sporocysts were purified by sucrose gradient flotation, washed in distilled water, and stored in a commercial 100x concentrated antibiotic/antimycotic solution (10.000 units/mL of penicillin, 10.000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B- Gibco) at 4 °C.

### *In vitro* growth of *Sarcocystis* spp.

Sporocysts of *Sarcocystis* spp. were processed as previously described (Gondim et al., 2019). The antibiotic/antimycotic solution containing sporocysts was adjusted to a minimum of $1 \times 10^4$ sporocysts/mL and 1 mL of the resulting solution was treated with 2.5% sodium hypochlorite for 30 min. Simultaneously, ~0.5 mL glass beads (400–600 µm in diameter, Sigma-Aldrich) were treated with 2.5% sodium hypochlorite. Sporocysts and glass beads were washed thrice with Iscove's modified Dulbecco medium (IMDM) (Invitrogen/Gibco, Carlsbad, USA) to remove hypochlorite.

The sporocysts were resuspended in 700 µL of IMDM and added to a microtube containing glass beads. The mixture was vortexed at maximum speed for 3 min. A drop of the solution was observed under a light microscope (Olympus BX-51, 400x magnification) to examine the released sporozoites. The solution was filtered using a sterile 5 µm-pore size filter and subsequently inoculated into T25 flasks containing confluent monolayers of Vero cells (BCRJ: 0245). Cultures were maintained in IMDM supplemented with 1% penicillin/streptomycin/amphotericin B and 5% inactivated bovine calf serum, in a 37 °C incubator with 5% $CO_2$ (Nuare, NU-4750E, Plymouth, MN, USA). Cultures without parasite propagation were discarded 60 d post-infection.

**DNA extraction**

DNA was extracted from culture-derived merozoites using the DNeasy blood and tissue kit (Qiagen, Valencia, California, USA), and from sporocysts using the QIAamp DNA stool mini kit (Qiagen, Valencia, California, USA), according to the manufacturer's instructions. The DNA concentration and quality were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, San Jose, CA, USA).

**Molecular detection of *Sarcocystis* spp. based on ITS-1, *cox1*, *SAG2*, *SAG3* and *SAG4* gene fragments**

Conventional PCR assays were performed to amplify *Sarcocystis* spp. DNA of the *loci* encoding *cox1*, *SAG2*, and ITS-1. Nested and hemi-nested PCR assays were used to amplify *loci* encoding *SAG3* and *SAG4*, respectively. Primers that amplify most of the open reading frames from *SAG2* and *SAG3* were used to maximize the likelihood of detecting polymorphisms at these *loci*. The primer sequences and cycling conditions are shown in Supplementary Table S1.

The first round of PCR was conducted in a 25-µL total reaction volume containing ~200 ng of target DNA, 0.2 mM mixed deoxynucleotide triphosphates, 3.0 mM $MgCl_2$, 1.25 U Taq Platinum DNA Polymerase (Life Technologies, Carlsbad, CA, USA), 0.4 µM of each primer, 2.5 µL of 10X reaction buffer, and sterile ultra-pure water. For *SAG2, SAG3* and *SAG4,* 2 µL of the product derived from the first amplification was used as a template in an additional round of conventional PCR, nested PCR, and hemi-nested PCR, respectively. A conventional thermocycler device (T100 Thermal

Cycler, Bio-Rad, Hercules, CA, USA) was used to conduct the PCR. Ultrapure water and DNA from *S. neurona* merozoites obtained from the intestines of an opossum (*Didelphis virginiana*) (Lindsay et al., 2004) were used as negative and positive controls, respectively. PCR products were separated by electrophoresis on 1% agarose gels containing 0.5 µg/mL ethidium bromide. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System, Bio-Rad, Hercules, CA, USA) using the Image Lab Software v4.1 (Bio-Rad, Hercules, CA, USA).

**Cloning and sequencing**

The ITS-1, *cox1*, and *SAG3* amplicons were cloned using the pGEM-T vector (Promega, Madison, WI, USA). Ligation reaction products were used to transform One Shot Mach1 T1 *Escherichia coli* cells (Invitrogen Cat # C8620-03) (109–1010 CFU/ng DNA) by thermal shocking. Plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). All the procedures were performed following the manufacturer's recommendations.

Up to three clones from each sample were selected for sequencing in an automatic sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystem/Perkin Elmer) (Sanger et al., 1977), using primers M13 F and M13 R (Lau et al., 2010) that flank the cloning site of the pGEM-T vector. Additionally, primers ITS-720R19 (Valadas et al., 2016) and *cox1*-275F22 (Gondim et al., 2019) were used to sequence an internal region of ITS-1 and *cox1* fragments to increase the Phred quality of the consensus sequence. The amplicons obtained from the *SAG2*- and *SAG4*-based PCR assays were purified using EXOSAP-IT (Applied Biosystems) and sequenced with the same primer pairs used in the PCR assays.

**Sequence editing**

Consensus sequences were obtained through analysis of electropherograms with Phred-base calling and the Phrap-assembly tool available in the suite Codoncode aligner v.4.2.1. (Codoncode Corp. Dedham, MA, USA) with a Phred quality score of ≥ 20 (99% accuracy of the base call). Consensus sequences were submitted to BLASTn (http://www.ncbi.nlm.nih.gov) to determine sequence identity by comparison with the sequences available in the GenBank database. The significance of the alignments was

determined based on the E-value analysis. A BLAST search, using the newly generated sequences as a query, allowed the retrieval of homologous fragments from *Sarcocystis* spp. with similar lengths.

**Identification of genetic relationship of identified *Sarcocystis***

Genetic diversity was assessed with sequences from all five molecular markers used in this study. The sequences were aligned and used to calculate nucleotide diversity ($\pi$), polymorphism level (allele diversity [ad]), number of alleles (a), average number of nucleotide differences (K), and number of variable sites (v), using DnaSP v5.10 (Librado & Rozas, 2009). The alleles were then aligned with sequences available in the GenBank database using ClustalW (Thompson et al., 1994), and adjusted using Bioedit v. 7.0.5.3. (Hall, 1999). Phylogenetic reconstructions using the maximum likelihood (ML) method were performed on MEGA-X software (Tamura et al., 2011). The model for evolutionary distances was also calculated using the MEGA-X software and was applied to identify the most appropriate model for nucleotide substitution. The robustness of the ML tree was statistically evaluated using bootstrap analysis with 1.000 replicates. Representative genomic DNA sequences for each *Sarcocystis* spp. allele observed in this study were deposited in GenBank under the following accession numbers: OL830303–OL830329 (ITS-1), OL780806–OL780816 and OL780818–OL780820 (*cox1*), OL809965–OL809968 (*SAG2*), OP321544–OP321555 and OP811212 (*SAG3*), and OL862280–OL862286 (*SAG4*).

**Results**

*Sarcocystis* spp. sporocysts were observed in the intestinal scrapings of 18 out of 37 opossums: 13 *D. albiventris* from Campo Grande, and five *D. aurita* from São Paulo. From the 18 sporocysts suspensions, 12 presented more than $1 \times 10^4$ sporocysts/mL and were subjected to *in vitro* culture. Replication of *Sarcocystis* spp. was observed in six cultures: four inoculated with *D. albiventris*-derived sporozoites and two inoculated with *D. aurita*-derived sporozoites. Therefore, from six samples, DNA was obtained from culture-derived merozoites, and from 12 samples, DNA was obtained from sporocysts.

Sequencing could not be performed for all samples due to unsuccessful amplification or low DNA concentration (faint bands) in some PCR amplicons. At least one molecular marker was sequenced from 16 samples, and sequences from all five molecular markers were obtained from 8 samples. From two sporocyst samples none of the molecular markers were successfully sequenced. After cloning, 29 sequences were obtained from ITS-1, 39 from *cox1,* and 13 from *SAG3*. Additionally, nine sequences were obtained from *SAG2* and 15 from *SAG4*. After genetic diversity assessment using DNAsp v5.10, 27 allele variants were obtained for ITS-1, 14 for *cox1*, four for *SAG2*, 13 for *SAG3*, and seven for *SAG4. SAG3* was the molecular marker with the highest allele diversity (1.00 ± 0.03), nucleotide diversity per site (0.055 ± 0.00), and number of nucleotide differences between all sequences (55.4) (Table 1). Samples were named with alphanumeric code (Dal00-CG or Dau00-SP), and allele variants from each locus were designated with Arabic numerals (Table 2).

**Table 1.** Polymorphism and genetic diversity of *Sarcocystis* spp. detected in wild-caught opossums (*Didelphis albiventris* and *Didelphis aurita*) captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Molecular marker | Size of the fragment analyzed* | Number of sequences analyzed | Number of variable sites (VS) | Number of Alleles (a) | GC content (%) | Allele Diversity (mean ± SD) | Nucleotide Diversity per site = $\pi$ (mean ± SD) | Average Number of Nucleotide Differences between All Sequences (K) |
|---|---|---|---|---|---|---|---|---|
| ITS-1 | 1075 | 29 | 54 | 27 | 46.3 | 0.99 ± 0.01 | 0.006 ± 0.00 | 7.32 |
| *cox1* | 972 | 39 | 53 | 14 | 37.2 | 0.81 ± 0.04 | 0.013 ± 0.00 | 13.01 |
| SAG2 | 676 | 9 | 5 | 4 | 54.9 | 0.80 ± 0.08 | 0.003 ± 0.00 | 2.27 |
| SAG3 | 1106 | 13 | 159 | 13 | 51.8 | 1.00 ± 0.03 | 0.055 ± 0.00 | 55.4 |
| SAG4 | 280 | 15 | 22 | 7 | 58.9 | 0.87 ± 0.05 | 0.034 ± 0.00 | 9.71 |

* The number of nucleotides in all the sequences was standardized based on the length of the shorter sequence.

**Table 2.** *Sarcocystis* spp. detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively. Numbers represent the alleles identified at ITS-1 (27), *cox1* (14), *SAG2* (4), *SAG3* (13), and *SAG4* (7) at genetic diversity assessment. Superscribed letters refer to each of the clones sequenced.

| Samples | | | Molecular Markers | | | | |
|---|---|---|---|---|---|---|---|
| **ID** | **Origin of DNA** | **Species of opossum** | **ITS-1** | ***cox1*** | **SAG2** | **SAG3** | **SAG4** |
| Dal1-CG | Sporocyst | *Didelphis albiventris* | N | N | N | NS | NS |
| Dal3-CG | Sporocyst | *Didelphis albiventris* | NS | N | NS | 1 | 1 |
| Dal5-CG | Sporocyst | *Didelphis albiventris* | N | N | NS | NS | 2 |
| Dal6-CG | Sporocyst | *Didelphis albiventris* | $1^{a}$, $2^{b}$ | $1^{a}$, $2^{b}$, $3^{c}$ | 2 | 2 | 3 |
| Dal9-CG | Sporocyst | *Didelphis albiventris* | $3^{a}$, $4^{b}$, $5^{c}$ | $2^{a,b}$ | 1 | NS | 4 |
| Dal11-CG | Sporocyst | *Didelphis albiventris* | $6^{a}$, $7^{b}$, $8^{c}$ | $2^{a,c}$, $4^{b}$ | 2 | 3 | 1 |
| Dal12-CG | Merozoite | *Didelphis albiventris* | $9^{a}$, $10^{b}$, $11^{c}$ | $5^{a,b,c}$ | 2 | 4 | 2 |
| Dal13-CG | Sporocyst | *Didelphis albiventris* | NS | NS | NS | N | N |
| Dal16-CG | Sporocyst | *Didelphis albiventris* | NS | $2^{a}$, $6^{b}$, $7^{c}$ | N | N | N |
| Dal18-CG | Merozoite | *Didelphis albiventris* | $12^{a}$, $13^{b}$ | $5^{a,b,c}$ | 2 | 5 | 2 |
| Dal23-CG | Merozoite | *Didelphis albiventris* | $12^{a}$ | $2^{a,b,c}$ | 3 | 6 | 1 |
| Dal26-CG | Sporocyst | *Didelphis albiventris* | N | $8^{a}$ $9^{b,c}$ | N | 7 | 5 |
| Dal27-CG | Merozoite | *Didelphis albiventris* | $14^{a}$, $15^{b}$, $16^{c}$ | $2^{a,b,c}$ | 3 | 8 | 1 |
| Dau28-SP | Sporocyst | *Didelphis aurita* | $17^{a}$, $18^{b}$ | $10^{b}$, $2^{c}$, $5^{a}$ | N | 9 | 4 |
| Dau29-SP | Sporocyst | *Didelphis aurita* | $19^{a}$, $20^{b}$ | $2^{b}$, $11^{a}$, | 1 | 10 | 4 |
| Dau30-SP | Sporocyst | *Didelphis aurita* | $17^{b}$, $21^{a}$ | $12^{a}$, $13^{b}$ | NS | 11 | 6 |
| Dau36-SP | Merozoite | *Didelphis aurita* | $22^{a}$, $23^{b}$, $24^{c}$ | $9^{a,b,c}$ | NS | 12 | 7 |
| Dau37-SP | Merozoite | *Didelphis aurita* | $25^{a}$, $26^{b}$, $27^{c}$ | $14^{a}$, $5^{b,c}$ | 4 | 13 | 6 |

N = Negative at PCR. NS = Positive at PCR, but not sequenced due to low DNA concentration (faint band).

After BLASTn search for ITS-1, all 27 alleles disclosed identity ranging from 98.98–100% with a sequence of *S. falcatula* from a rainbow lorikeet (*Trichoglossus moluccanus*) from the United States (MH626538), with a query cover value of 100% (Supplementary Table S2). The phylogenetic reconstruction of ITS-1 exhibited three clades (Figure 1). The first clade was formed by the 27 alleles from this study and sequences from *S. falcatula* detected in rainbow lorikeet (*Trichoglossus moluccanus*, MH626538), brown boobies (*Sula leucogaster*, MW822665, MW822670), and ducks (*Anas* sp., OL323100), and sequences of *Sarcocystis* sp. detected in Magellanic penguins (*Spheniscus magellanicus*, MG493471) and opossums (*D. virginiana*, AY082647, and *D. aurita/D. marsupialis*, MK803362). The second clade was formed by sequences from *S. neurona* (MN172273, AF252407, AF081944, and AF204230) and *S. speeri* (KT207458). A third clade was formed exclusively with sequences from *S. falcatula* from the United States (AF098244, AF098242, AY082639, and AY082638).

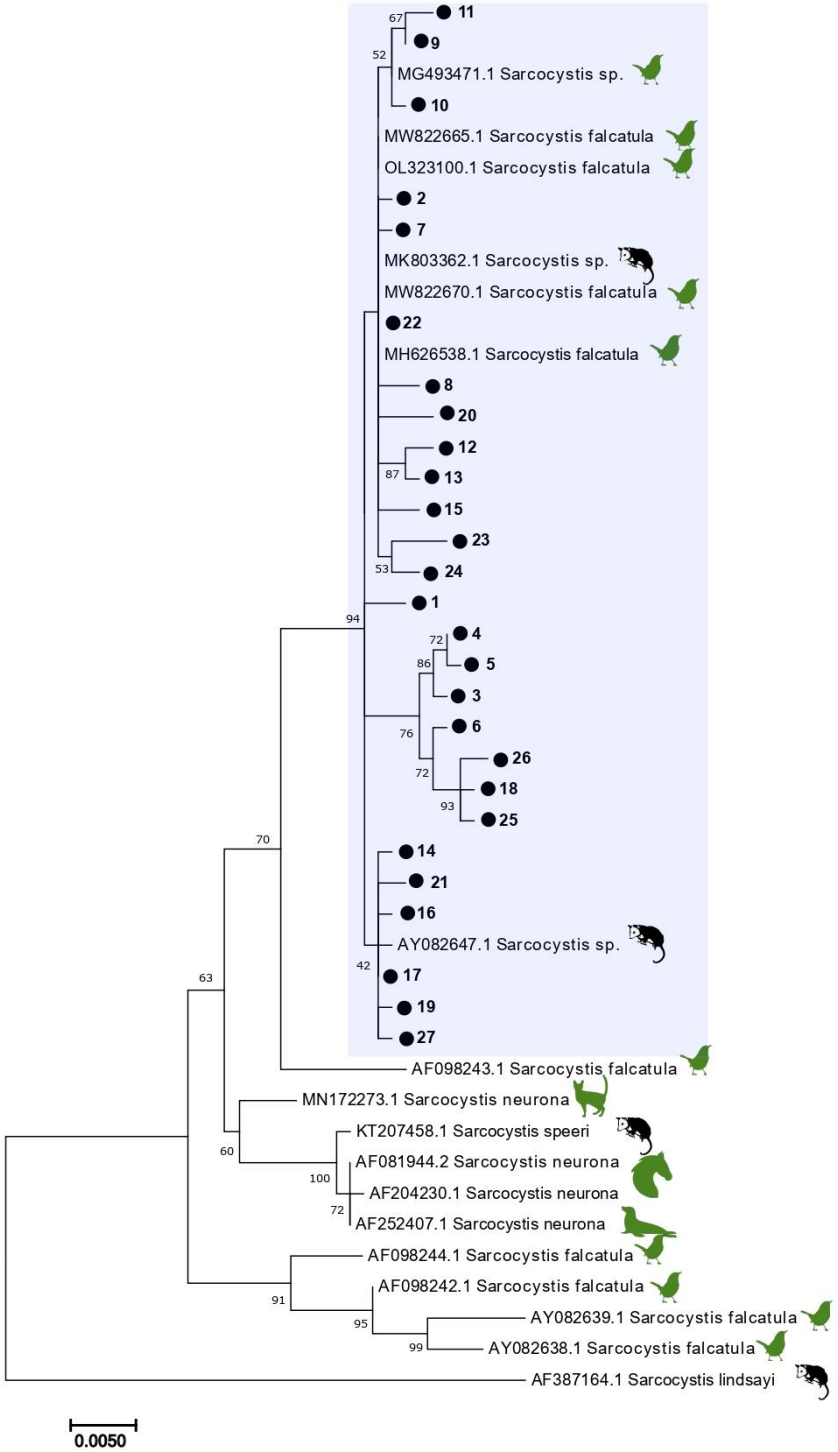


**Figure 1.** Phylogenetic relationships within the *Sarcocystis* genus based on 965 bp fragment of ITS-1. The tree was inferred by using the maximum likelihood (ML) method with the evolutionary model Kimura 2-parameter with gamma distributed rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with

1000 replicates. A sequence from *Sarcocystis lindsayi* was used as an outgroup. The black dots identify the sequences obtained in the present study.

In the BLASTn analysis of *cox1*, alleles 8, 9, and 11 disclosed identities ranging from 96.40–96.81% with *S. rileyi* from a common eider (*Somateria mollissima*) from Norway (KJ396582), with a query cover value of 100% (Supplementary Table S3). The remaining alleles showed identities ranging from 99.69–100% with sequences from *S. falcatula* (MH665257) and *S. speeri* (KT207461), with a query cover value of 100%. The phylogeny for *cox1* positioned the alleles from this study into two well-separated clades (Figure 2). The first clade (clade A) was formed by alleles 8, 9, and 11 and a sequence from *Sarcocystis rileyi* Stiles, 1893 (*Somateria mollissima*) (KJ396582) found in avian muscle tissues. Another clade was formed with sequences of *Sarcocystis* spp. from birds, *S. arctica* from red fox (*Vulpes vulpes*) (MF596306), *S. caninum* from domestic dogs (MH469240), *S. canis* from polar bear (*Ursus maritimus*) (KX721495), and *S. lutrae* from otters (*Lutra lutra*) (KM657808). The second clade (clade B) was formed by the remaining alleles, and sequences from *S. falcatula* detected in the muscle tissues of psittacine birds (*Trichoglossus moluccanus*, MH665257; *Polytelis alexandrae*, MZ962977; *Psittacula krameri*, MZ962975) and a sequence from *S. speeri* detected in opossums intestines (*D. albiventris*, KT207461).

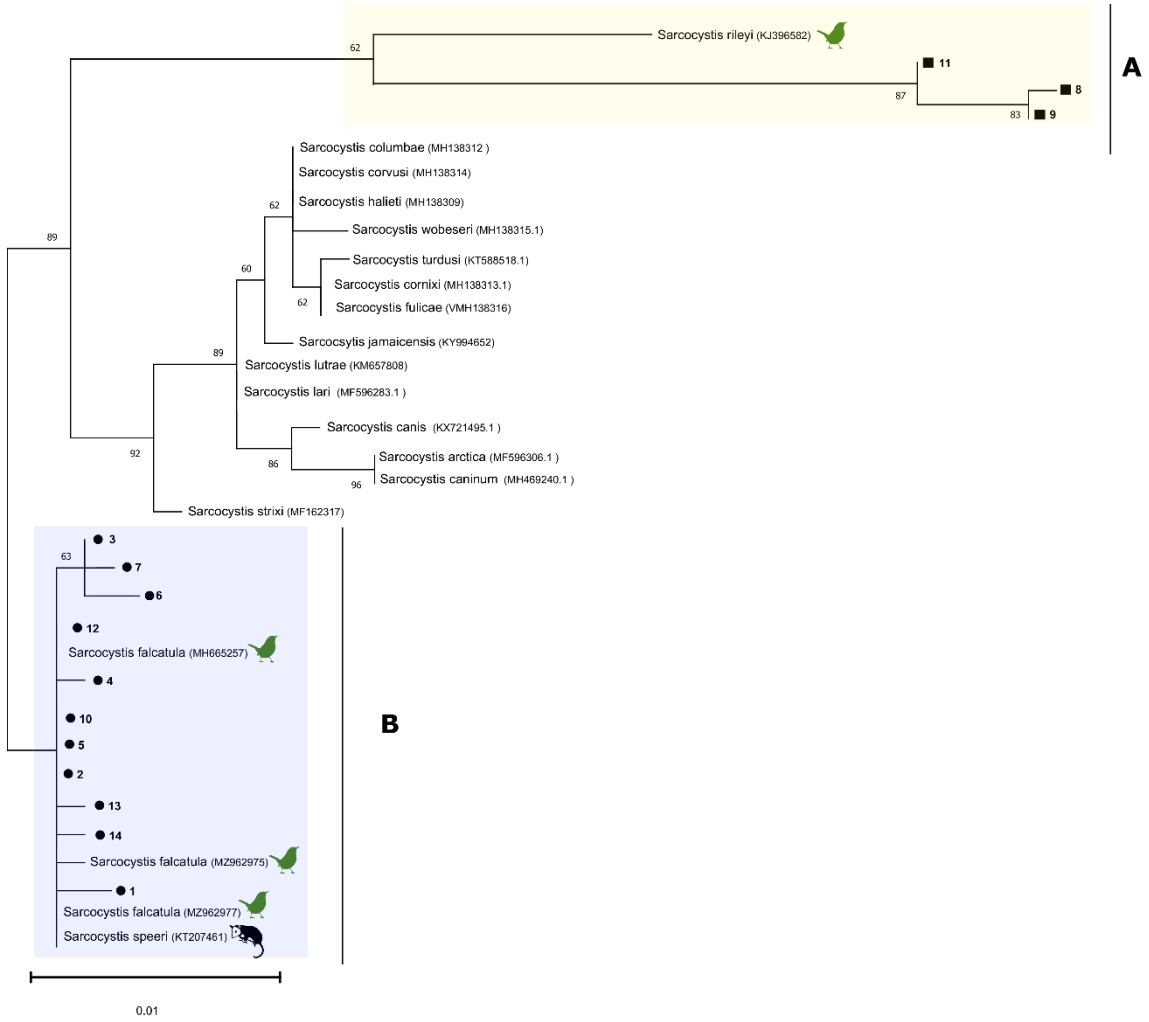


**Figure 2.** Phylogenetic relationships within the *Sarcocystis* genus based on 916 pb fragment of *cox1*. The tree was inferred by using the maximum likelihood (ML) method with the evolutionary model Tamura 3-parameter with gamma distributed rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. Sequences from *Toxoplasma gondii, Neospora caninum* and *Hammondia heydorni* were used as an outgroup (hidden). The black dots and black squares identify the sequences obtained in the present study.

Although the *SAG2* and *SAG3* nucleotide sequences from this study contained most of the open reading frame of the genes, the majority of the sequences available in GenBank were substantially shorter. Therefore, two phylogenetic reconstructions were performed for these *loci*: one with short fragments and other composed exclusively of longer sequences. The *SAG2* phylogeny based on 211 bp fragment

showed four clades with high statistical support (Figure 3). All alleles obtained in this study were positioned in clades along with sequences of *Sarcocystis* sp. detected in sporocysts shed by opossums (*Didelphis* spp.) and *S. falcatula* detected in experimentally and naturally infected birds. The *SAG2* phylogenetic tree based on 645 bp (Supplementary Figure S1) fragment positioned the alleles from this study in the same clade as *S. falcatula* (GQ851953). The other clade was formed exclusively with sequences from *S. neurona* (BLASTn information on Supplementary Table S4).

For *SAG3,* five alleles (1, 2, 4, 5, and 10) were related to *S. falcatula* (GQ851956), with nucleotide identities ranging from 90.4–99.9% (Supplementary Table S5). The remaining eight alleles (3, 6, 7, 8, 9, 11, 12, and 13) were monophyletic and closely related to a sister clade exclusively formed by the *S. neurona* genetic sequences (Supplementary Figure S1). The alleles related to *S. neurona* were typically larger than the rest and showed the presence of “AT” repetitions (approximately 90 nucleotides long), which were absent in the *S. falcatula SAG3*-related alleles. In the phylogenetic reconstruction based on 317 bp fragment, the alleles split into six highly supported clades (Figure 3). All *SAG3* sequences in this study were phylogenetically related to *Sarcocystis* sp. and *S. falcatula*, either shed by opossums and/or detected in birds. One of the six *SAG3* clades was formed exclusively the *S. neurona* genetic sequences.

Phylogenetic reconstruction of *SAG4* failed to fully resolve branching in some samples. As with other SAG phylogenies, *S. neurona*-derived sequences were grouped into a highly statistically supported branch. The majority of *SAG4* alleles were closely related to sequences annotated as *Sarcocystis* sp. or *S. falcatula*, either detected in *Didelphis* spp. or birds. Allele 3 was the most divergent taxon and could not be associated with any known *Sarcocystis*-derived sequences (Figure 3) (BLASTn information on Supplementary Table S6).

None of the molecular markers used in this study demonstrated host specificity for different *Sarcocystis* lineages or alleles, as the distribution of the alleles among *D. aurita* and *D. albiventris* was random. Alleles derived from both species clustered together on several occasions.

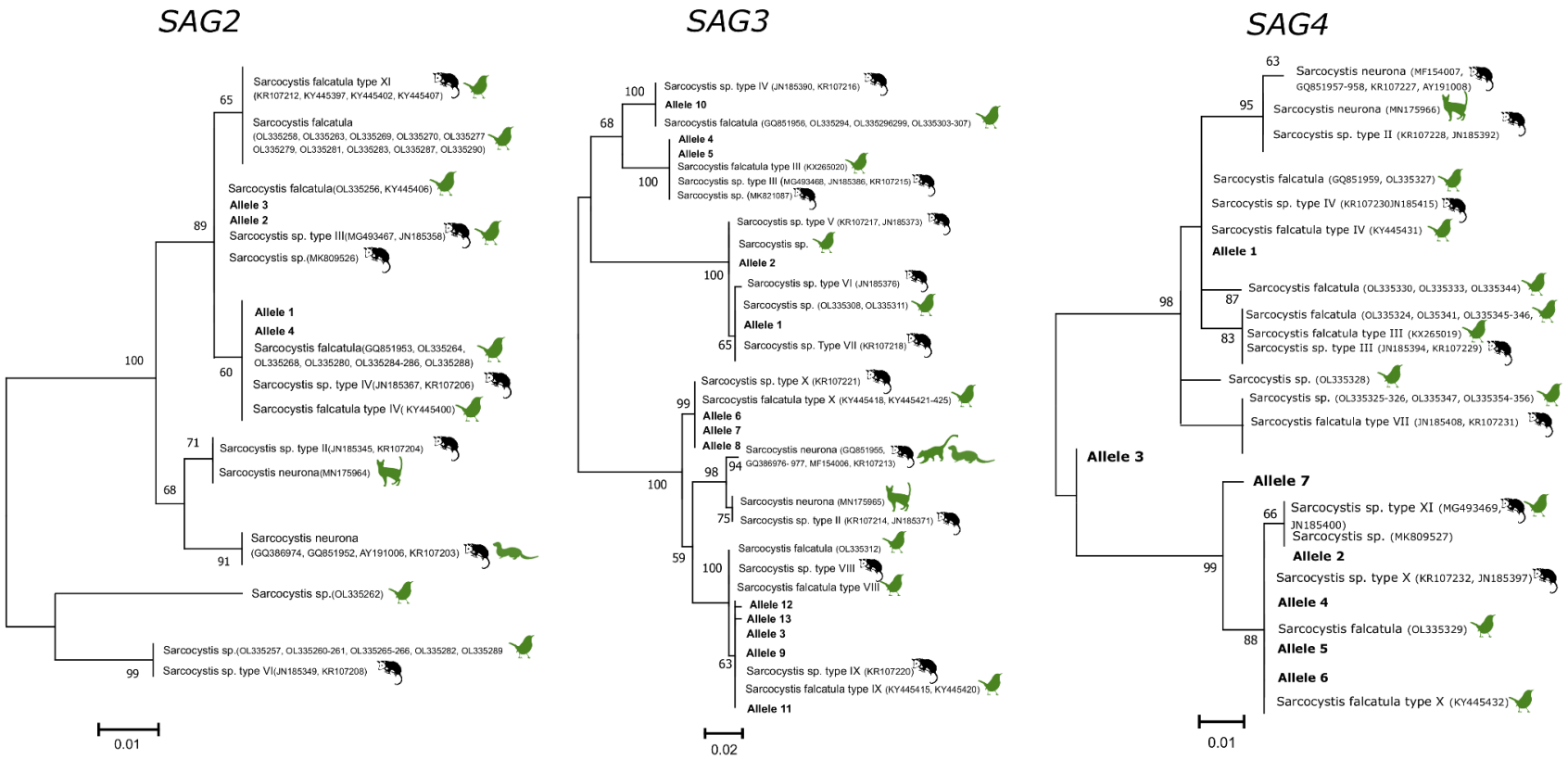


**Figure 3.** Phylogenetic relationships within the *Sarcocystis* genus based on *SAG2* (211 bp), *SAG3* (317 bp) and *SAG4* (220 bp). The trees were inferred by using the maximum likelihood (ML) method with the evolutionary models Jukes-Cantor (*SAG2* and *SAG4*) and Kimura 2-parameter (*SAG3*) with uniform rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. The sequences from the present study are marked in bold.

**Discussion**

Previous studies have shown that *S. falcatula* constitutes a heterogeneous population (Marsh et al., 1999; Dubey et al., 2001d; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018; Llano et al., 2022). Thus far, two lineages of *S. falcatula* have been described in the Americas, based on ITS-1. One was described only in North America, while the other was described in both North and South America. In this study, the ITS-1-based phylogeny separated the two *S. falcatula* lineages into two well-supported clades. All 27 alleles obtained in this study belonged to the same lineage and positioned in a clade along with sequences from *S. falcatula*-like detected in naturally infected birds from Brazil (MG493471, MK803362, MW822665, MW822670, OL323100, MH626538,) and North America (AY082647). The second clade comprised *S. falcatula* sequences detected only in birds from North America. Most *Sarcocystis* found in Brazil were identified as *S. falcatula*-like, as they were phylogenetically related to *S. falcatula* and infective for birds, but had molecular differences compared with *S. falcatula* described in North America. Several *Sarcocystis,* primarily found in opossums and described as *Sarcocystis* spp., were after observed in natural and experimental infections in birds (Valadas et al., 2016; Gondim et al., 2017, 2019; Konradt et al., 2017; Acosta et al., 2018; Cesar et al., 2018; Gallo et al., 2018; Llano et al., 2022).

All the *cox1* alleles from this study showed BLASTn identity (> 96%) with a wide variety of *Sarcocystis* species. Phylogenetic reconstruction positioned the alleles in two well-separated clades with high statistical support. The fragment from *cox1* was too conserved to allow differentiation between the alleles from this study and *S. falcatula* sequences present in clade B from *S. speeri*. Llano et al. (2022) also reported the failure of *cox1* to differentiate *Sarcocystis* species obtained from the muscle tissue of wild birds, even though the phylogeny based on ITS-1 demonstrated the presence of different species of *Sarcocystis*. Herein, while only *Sarcocystis falcatula*-like was detected in ITS-1, phylogeny based on *cox1* demonstrated the presence of two species of *Sarcocystis*: *Sarcocystis falcatula*-like sequences from clade B and *Sarcocystis* sp. phylogenetically related to *S. rileyi*, positioned in clade A. The evolutionary divergence revealed between clades B and A suggests that alleles present in the later branch belong to some species not yet described in opossums.

Multiple *Sarcocystis* infections may occur in the intestines of opossums; therefore, more than one *Sarcocystis* species may be detected in DNA obtained from sporocysts (Dubey et al., 2000). Allele 11 from *cox1* was identical to allele 2 from bases 1–193 and 768–972, and to alleles 8 and 9 from bases 194–767. These findings suggest that allele 11 might be a chimera, that is, an artifact sequence formed by the incorrect junction of two biological sequences. Chimeras may occur during PCR using mixed templates and do not represent sequences that exist in nature (Kalle et al., 2014). The presence of chimeras could lead to the misinterpretation of the results. Therefore, the scientific community should be aware of the need to avoid polluting public databases with artifact sequences.

Twenty-one new *SAG* alleles were found in comparison with those previously described: four at *SAG2*, 13 at *SAG3* and four at *SAG4*. This result reinforces that *Sarcocystis* spp. from opossums in Brazil exhibit a diverse portfolio of surface antigen-encoding genes (Monteiro et al., 2013, Valadas et al., 2016; Cesar et al., 2018). The amplification of the entire open reading frame of *SAG2* and *SAG3* demonstrated that the use of primers that amplify short fragments of *SAGs* may be underestimating the variation in *SAG* alleles from *Sarcocystis* shed by opossums in Brazil. Most of the *SAG3* alleles from this study presented insertions of “AT” repetitions of ~90 nucleotides in the region of the intron, resembling a micro-satellite pattern. Alleles presenting “AT” repetitions disclosed identity with *S. neurona* (96.3–96.7%), but phylogeny positioned them in a separate clade. “AT” repeats have been previously described in sequences from *S. neurona* obtained from sea otters (*Enhydra lutris nereis* - GQ851954 and GQ851955) and white-nosed coati (*Nasua narica molaris*- MF154006), and in *Sarcocystis* sp. types VIII, IX, and X obtained from opossums in Brazil (Valadas et al., 2016). Sequences identical to those observed in *Sarcocystis* sp. types VIII, IX, and X have also been found in *S. falcatula* infecting budgerigars (*M. undulattus*) (Gondim et al., 2017; Cesar et al., 2018). Interestingly, *S. falcatula* that present “AT” repeats were more similar to *S. neurona* than to the other *S. falcatula*. Among the three *SAGs* evaluated in this study, *SAG3* had the highest number of alleles, similar to that previously observed by Valadas et al. (2016). The phylogeny based on *SAG4* failed to fully resolve branching in some samples. However, it was possible to notice that none

of the *SAG4* alleles from this study was phylogenetically related to *S. neurona*. All the *SAG4* alleles in this study were closely related to *S. falcatula.*

The diversity of South American fauna acting as definitive hosts for *Sarcocystis* spp. is higher than that of North America. A single species of opossum *D. virginiana* is found in North America, while, in South America, five species, grossly divided into white-eared opossums (*D. albiventris, D. pernigra*, and *D. imperfecta*) and black-eared opossums (*D. aurita* and *D. marsupialis*), have been described (Cerqueira, 1985; Lemos & Cerqueira, 2002). In this study, host specificity for different *Sarcocystis* species was not observed, as the distribution of the alleles among *D. aurita* and *D. albiventris* was random. This suggests that both species of opossum might present the same susceptibility as definitive hosts for different *Sarcocystis* species.

Although *D. albiventris* is considered the definitive host for *S. neurona* in South America, only on one occasion *S. neurona* was isolated from *D. albiventris* in Brazil (Dubey et al., 2001e). Hammerschmitt et al. (2020) reported *S. neurona* causing meningoencephalitis in domestic cats. The authors observed significant molecular differences related to *S. neurona* detected elsewhere in the Americas. At three *SAG* loci (2, 3, and 4), the parasite found in the cat was identical to *Sarcocystis* sp. genotype II derived from opossums (*D. albiventris* and *D. aurita*) in the state of Rio Grande do Sul (Monteiro et al., 2013). None of the molecular markers used in this study demonstrated the presence of *S. neurona* in the sampled opossums. The results observed here, along with numerous other studies performed in Brazil, imply that *Sarcocystis* spp. that use opossums as definitive hosts in Brazil are different from those found in North America. Furthermore, the frequency of infection caused by *S. falcatula*-related organisms in opossums from Brazil is much higher than that of *S. neurona* infection (Monteiro et al., 2013; Valadas et al., 2016; Acosta et al., 2018; Cesar et al., 2018; Gondim et al., 2017, 2019).

## Conclusions

This study reported the occurrence, genetic diversity, and phylogenetic relationships of *Sarcocystis* spp. derived from the wild-caught opossums *D. albiventris* and *D. aurita* sampled in midwestern and southeastern Brazil. *S. falcatula*-like was detected by ITS-1, whereas *S. falcatula*-like and *Sarcocystis* spp. were detected by

*cox1.* The assessment of phylogenetic inferences based on *SAG2, SAG3* and *SAG4* revealed the genetic richness of *Sarcocystis* spp. occurring among opossums and highlighted the presence of several genotypes infecting these animals in the country.

**Conflicts of interest:** None

**Ethics statement**

The present study was approved by the Ethics Committees in the Use of Animals of the School of Agricultural and Veterinarian Sciences, São Paulo State University (protocol number 013444/2019) and Faculty of Veterinary Medicine and Animal Science, University of São Paulo (1558030821), and by Institute Chico Mendes for Conservation of Biodiversity (ICMBio numbers 49662-8 and 76295-1).

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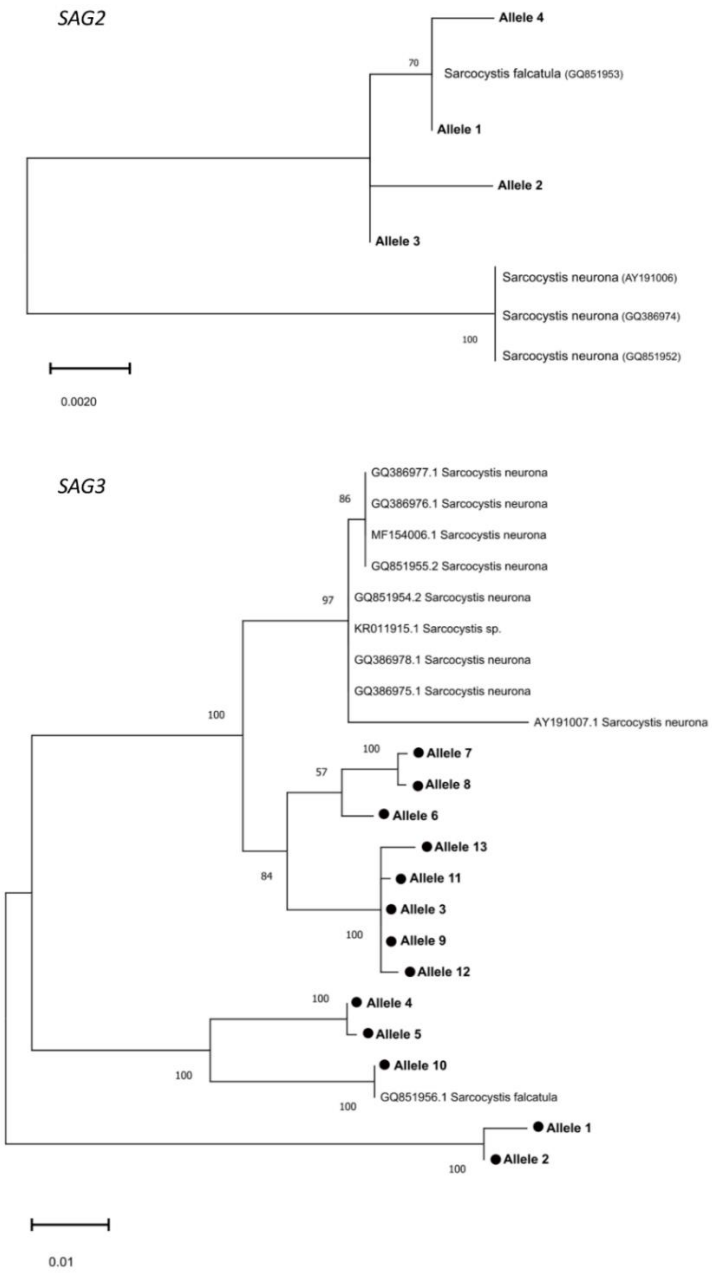
## Supplementary material




**Supplementary Figure S1.** Phylogenetic relationships within the *Sarcocystis* genus based on *SAG2* (645 bp) and *SAG3* (889 bp). The trees were inferred by using the maximum likelihood (ML) method with the evolutionary model Kimura 2-parameter with uniform rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. The sequences from the present study are marked in bold.

**Supplementary Table S1.** Oligonucleotide primers utilized for DNA amplification of ITS-1, *cox1*, SAG2, SAG3, and SAG4 from *Sarcocystis* spp. detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Target gene | Primer sequence | Fragment size | Thermal conditions | References |
|---|---|---|---|---|
| ITS-1 | JS4- CGA AAT GGG AAG TTT TGT GAA C<br>CT2c- CTG CAA TTC ACA TTG CGT TTC GC | ~1000 | 94°C/3 min., 35 cycles of 94°C/30 s., 56°C/30 s., 72°C/90 s., and 1 cycle of 72°C/5 min. | Slapeta et al., 2002; Acosta et al., 2011 |
| cox1 | SF1- ATG GCG TAC AAC AAT CAT AAA GAA<br>SR5- TAG GTA TCA TGT AAC GCA ATA TCC AT | ~1000 | 95°C/3 min., 35 cycles of 94°C/45 s., 52°C/45 s., 72°C/90 s., and 1 cycle of 72°C/10 min. | Gjerde, 2015 |
| SAG-2 | 1st and 2nd rounds:<br>SnSAG2F- AGC GGC GTT TTC AGA TTG TA<br>SnSAG2R- AAA ACG AAG GCA AGT GTG CT | ~900 | 94°C/4 min., 35 cycles of 94°C/40 s., 58°C/40 s., 72°C/90 s., and 1 cycle of 72°C/5 min. | Wendte et al., 2010 |
| SAG-3 | 1st round:<br>SnSAG3EXT-F- TCA AGG ACG TTT TTC CCT GT<br>SnSAG3EXT-R- CTC TGC ATG CTG CAA TGA AT<br>2nd round:<br>SnSAG3INT-F CCC TGC CTT TCT GGT CTC TT<br>SnSAG3INT-R TTC TCC CCA AAG ACC ATC TG | ~1100 | 94°C/4 min., 40 cycles of 94°C/40 s., 58°C/40 s., 72°C/90 s., and 1 cycle of 72°C/5 min. | Wendte et al., 2010 |
| SAG-4 | 1st round:<br>SAG4-F2- CCG AGG TAC AGT TCA AGG CG<br>SAG4-R- CGA CGA CGA TAC CCA ATG CC<br>2nd round:<br>SAG4-541-F21- GGC AAC GCC GCA GCM CTG CAA<br>SAG4-R- CGA CGA CGA TAC CCA ATG CC | ~295 | 94°C/3 min., 35 cycles of 94°C/30 s., 56°C/30 s., 72°C/30 s., and 1 cycle of 72°C/5 min. | Monteiro et al., 2013; Valadas et al., 2016 |

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**Supplementary Table S2.** BLASTn analysis of each *Sarcocystis* ITS-1 detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Allele | Clone Id | Species of opossum | GenBank Accession Number | BLASTn Best Hit | Host | Country | Query Cover (%) | Identity (%) | Best Hit GenBank Accession Number |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6.5 | *Didelphis albiventris* | OL830303 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.53 | MH626538 |
| 2 | 6.6 | *Didelphis albiventris* | OL830304 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.91 | MH626538 |
| 3 | 9.2 | *Didelphis albiventris* | OL830305 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.35 | MH626538 |
| 4 | 9.3 | *Didelphis albiventris* | OL830306 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.26 | MH626538 |
| 5 | 9.4 | *Didelphis albiventris* | OL830307 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.26 | MH626538 |
| 6 | 11.1 | *Didelphis albiventris* | OL830308 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.26 | MH626538 |
| 7 | 11.2 | *Didelphis albiventris* | OL830309 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.91 | MH626538 |
| 8 | 11.3 | *Didelphis albiventris* | OL830310 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 9 | 12.1 | *Didelphis albiventris* | OL830311 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.81 | MH626538 |
| 10 | 12.2 | *Didelphis albiventris* | OL830312 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.91 | MH626538 |
| 11 | 12.4 | *Didelphis albiventris* | OL830313 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.63 | MH626538 |
| 12 | 18.5; 23.5 | *Didelphis albiventris* | OL830314 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.63 | MH626538 |
| 13 | 18.7 | *Didelphis albiventris* | OL830315 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 14 | 27.1 | *Didelphis albiventris* | OL830316 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 15 | 27.2 | *Didelphis albiventris* | OL830317 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 16 | 27.3 | *Didelphis albiventris* | OL830318 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 17 | 28.3; 30,3 | *Didelphis aurita* | OL830319 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.81 | MH626538 |

**Supplementary Table S2**. Continued…

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 18 | 28.4 | *Didelphis aurita* | OL830320 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.07 | MH626538 |
| 19 | 29.1 | *Didelphis aurita* | OL830321 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.72 | MH626538 |
| 20 | 29.2 | *Didelphis aurita* | OL830322 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.63 | MH626538 |
| 21 | 30.1 | *Didelphis aurita* | OL830323 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.63 | MH626538 |
| 22 | 36.1 | *Didelphis aurita* | OL830324 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 100 | MH626538 |
| 23 | 36.2 | *Didelphis aurita* | OL830325 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.53 | MH626538 |
| 24 | 36.4 | *Didelphis aurita* | OL830326 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.63 | MH626538 |
| 25 | 37.1 | *Didelphis aurita* | OL830327 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.07 | MH626538 |
| 26 | 37.3 | *Didelphis aurita* | OL830328 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 98.98 | MH626538 |
| 27 | 37.4 | *Didelphis aurita* | OL830329 | *Sarcocystis falcatula* | *Trichoglossus moluccanus* | United States | 100 | 99.7 | MH626538 |

**Supplementary Table S3.** BLASTn analysis of each *Sarcocystis cox1* sequence detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Allele | Clone Id | Species of opossum | GenBank Accession Number | BLASTn Best Hit | Host | Country | Query Coverage (%) | Identity (%) | Best Hit GenBank Accession Number |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6.1 | *Didelphis albiventris* | OL780806 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 99.79 | KT207461<br>MH665257 |
| 2 | 6.2; 9.1; 9.4; 11.1; 11.3; 16.1; 23.1; 23.2; 23.3; 27.1; 27.2; 27.3; 28.4; 29.2 | *Didelphis albiventris* and *Didelphis aurita* | OL780807 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 100 | KT207461<br>MH665257 |
| 3 | 6.3 | *Didelphis albiventris* | OL780808 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 99.90 | KT207461<br>MH665257 |
| 4 | 11.2 | *Didelphis albiventris* | OL780809 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 99.90 | KT207461<br>MH665257 |
| 5 | 12.1; 12.2; 12.3; 18.1; 18.2; 18.3; 28.1; 37.3; 37.4 | *Didelphis albiventris* and *Didelphis aurita* | OL780810 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 99 | 100 | KT207461<br>MH665257 |
| 6 | 16.2 | *Didelphis albiventris* | OL780811 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 99.69 | KT207461<br>MH665257 |
| 7 | 16.3 | *Didelphis albiventris* | OL780812 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 100 | 99.79 | KT207461<br>MH665257 |
| 8 | 26.1 | *Didelphis albiventris* | OL780813 | *S. rileyi* | *Somateria mollissima* | Norway | 100 | 96.50 | KJ396582 |
| 9 | 26.2; 26.3; 36.1; 36.2; 36.4 | *Didelphis albiventris* and *Didelphis aurita* | OL780814 | *S. rileyi* | *Somateria mollissima* | Norway | 100 | 96.40 | KJ396582 |
| 10 | 28.2 | *Didelphis aurita* | OL780815 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 99 | 99.79 | KT207461<br>MH665257 |

**Supplementary Table S3**. Continued…

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 11 | 29.1 | *Didelphis aurita* | OL780816 | *S. rileyi* | *Somateria mollissima* | Norway | 100 | 96.81 | KJ396582 |
| 12 | 30.2 | *Didelphis aurita* | OL780818 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 99 | 99.90 | KT207461<br>MH665257 |
| 13 | 30.4 | *Didelphis aurita* | OL780819 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 99 | 99.90 | KT207461<br>MH665257 |
| 14 | 37.2 | *Didelphis aurita* | OL780820 | *Sarcocystis speeri*<br>*Sarcocystis falcatula* | *Didelphis albiventris*<br>*Trichoglossus moluccanus* | Argentina<br>United States | 99 | 99.90 | KT207461<br>MH665257 |

**Supplementary Table S4**. BLASTn analysis of each *Sarcocystis* SAG2 sequence detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Allele | Sample | Species of opossum | GenBank Accession Number | BLASTn Best Hit | Host | Country | Query Coverage (%) | Identity (%) | Best Hit GenBank Accession Number |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 9; 11; 29 | *Didelphis albiventris* and *Didelphis aurita* | OL809965 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 99,8 | GQ851953 |
| 2 | 6; 12;18 | *Didelphis albiventris* | OL809966 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 99.2 | GQ851953 |
| 3 | 23; 27 | *Didelphis albiventris* | OL809967 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 99,8 | GQ851953 |
| 4 | 37 | *Didelphis aurita* | OL809968 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 99,7 | GQ851953 |

**Supplementary Table S5.** BLASTn analysis of each *Sarcocystis* SAG3 sequence detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Allele | Clone Id | Species of opossum | GenBank Accession Number | BLASTn Best Hit | Host | Country | Query Coverage (%) | Identity (%) | Best Hit GenBank Accession Number |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.1 | *Didelphis albiventris* | OP321544 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 90,4 | GQ851956 |
| 2 | 6.1 | *Didelphis albiventris* | OP321545 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 90.6 | GQ851956 |
| 3 | 11.1 | *Didelphis albiventris* | OP321546 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,5 | GQ851954 |
| 4 | 12.1 | *Didelphis albiventris* | OP321547 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 96,0 | GQ851956 |
| 5 | 18.1 | *Didelphis albiventris* | OP321548 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 95,9 | GQ851956 |
| 6 | 23.1 | *Didelphis albiventris* | OP321549 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,3 | GQ851954 |
| 7 | 26.1 | *Didelphis albiventris* | OP321550 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,4 | GQ851955 |
| 8 | 27.1 | *Didelphis albiventris* | - | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,5 | GQ851955 |
| 9 | 28.1 | *Didelphis aurita* | OP321551 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,3 | GQ851954 |
| 10 | 29.1 | *Didelphis aurita* | OP321552 | *Sarcocystis falcatula* | Not mentioned | United States | 100 | 99,9 | GQ851956 |
| 11 | 30.1 | *Didelphis aurita* | OP321553 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,7 | GQ851954 |
| 12 | 36.1 | *Didelphis aurita* | OP321554 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,3 | GQ851954 |
| 13 | 37.1 | *Didelphis aurita* | OP321555 | *Sarcocystis neurona* | *Enhydra lutris nereis* | United States | 100 | 96,3 | GQ851954 |

**Supplementary Table S6.** BLASTn analysis of each *Sarcocystis* SAG4 sequence detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

| Allele | Sample Id | Species of opossum | GenBank Accession Number | BLASTn Best Hit | Host | Country | Query Coverage (%) | E-value | Identity (%) | Best Hit GenBank Accession Number |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3, 11, 23, 27 | *Didelphis albiventris* | OL862280 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 1,00E-148 | 100 | JN185415 |
| 2 | 5, 12, 18 | *Didelphis albiventris* | OL862281 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 1,00E-142 | 100 | JN185400 |
| 3 | 6 | *Didelphis albiventris* | OL862282 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 1,00E-127 | 96,7 | JN185397 |
| 4 | 9, 28, 29 | *Didelphis albiventris* and *Didelphis aurita* | OL862283 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 1,00E-142 | 100 | JN185397 |
| 5 | 26 | *Didelphis albiventris* | OL862284 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 7,00E-142 | 99,6 | JN185397 |
| 6 | 30, 37 | *Didelphis aurita* | OL862285 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 7,00E-141 | 99,6 | JN185397 |
| 7 | 36 | *Didelphis aurita* | OL862286 | *Sarcocystis sp.* | *Didelphis sp.* | Brazil | 100 | 3,00E-134 | 98,2 | JN185397 |

## CHAPTER 3 – Reactivity against *Sarcocystis neurona* and *Sarcocystis falcatula*-like in horse sera from Southeastern and Midwestern Brazil

**Abstract**

Equine protozoal myeloencephalitis (EPM) is a neurological disease caused by *Sarcocystis neurona*. Immunofluorescence antibody tests (IFATs) have been widely used to identify exposure of horses to *S. neurona* in Brazil. Here we used IFAT to search for IgG antibodies against *Sarcocystis falcatula*-like (Dal-CG23) and *S. neurona* (SN138) in sera from 342 horses sampled in Campo Grande, Mato Grosso do Sul state (Midwestern), and São Paulo, São Paulo state (Southeastern), Brazil. The 1:25 cutoff value was chosen to maximize sensitivity. IgG antibodies against *S. falcatula*-like were detected in 177 horses (51.75%), whereas IgG antibodies against *S. neurona* were detected in 239 horses (69.88%). Sera from 132 horses (38.59%) reacted against both isolates. No reactivity was observed in 16.95% (58/342) horses. The high seroprevalence observed here may be related to the lower cutoff, and the presence of opossums infected with *Sarcocystis falcatula*-like and *Sarcocystis* spp. in the regions where the horses were sampled. Owing to the similarity among antigens targeted in immunoassays, reports on *S. neurona*-seropositive horses in Brazil may also be derived from the exposure of horses to other *Sarcocystis* species. The role of other *Sarcocystis* species in causing neurological diseases in horses in Brazil remains unclear.

**Keywords:** *Sarcocystis* spp., IFAT, EPM, horses, cross-reactivity

**Resumo**

Mieloencefalite protozoária equina (MPE) é uma doença neurológica causada por *Sarcocystis neurona*. Reação de imunofluorescência indireta (RIFI) tem sido utilizada para identificar a exposição de equinos à *S. neurona* no Brasil. Neste estudo, RIFI foi utilizada para avaliar a presença de anticorpos IgG anti-*Sarcocystis falcatula*-like (Dal-CG23) e anti-*S. neurona* (SN138) no soro de 342 equinos de Campo Grande, Mato Grosso do Sul (Centro-oeste) e São Paulo, São Paulo (Sudeste), Brasil. O ponto de corte de 1:25 foi escolhido para maximizar a sensibilidade. Anticorpos IgG anti-*S. falcatula*-like foram detectados em 177 cavalos (51,75%), enquanto anticorpos IgG

anti-*S. neurona* foram detectados em 239 cavalos (69.88%). Soro de 132 animais (38.59%) reagiu contra ambos os isolados. Nenhuma reatividade foi observada em 58/342 animais (16.95%). A alta soroprevalência observada aqui pode estar relacionada ao baixo ponto de corte e à presença de gambás infectados com *Sarcocystis falcatula*-like e *Sarcocystis* spp. nas regiões onde os equinos foram amostrados. Devido à similaridade entre antígenos de superfície detectados nos imunoensaios, relatos de soropositividade contra *S. neurona* no Brasil podem resultar da exposição dos equinos à outras espécies de *Sarcocystis*. O papel de outras espécies de *Sarcocystis* como causa de doença neurológica nos equinos em Brasil permanece incerto.

**Palavras-chave:** *Sarcocystis* spp., RIFI, MPE, equinos, reatividade-cruzada

## Introduction

Equine protozoal myeloencephalitis (EPM) is a neurological disease caused by *Sarcocystis neurona* (Dubey et al., 1991). Opossums (*Didelphis* spp.) are the definitive hosts and sources of *S. neurona* infections in horses. *Didelphis virginiana* is the definitive host of *S. neurona* in North America (Fenger et al., 1995), and *Didelphis albiventris* is the definitive host of *S. neurona* in Brazil (Dubey et al., 2001). Opossums are definitive hosts also for *Sarcocystis speeri, Sarcocystis lindsayi*, and *Sarcocystis falcatula.* Coinfection with different *Sarcocystis* species have already been described in opossums (Dubey et al., 2016).

In Brazil, the majority of *Sarcocystis* shed by opossums has been identified as *Sarcocystis falcatula*-like due to genetic characteristics and/or experimental infectivity to budgerigars (*Melopsittacus undulatus*) (Gondim et al., 2019; Acosta et al., 2018; Cesar et al., 2018). *Sarcocystis falcatula*-like in Brazil present high allelic variation in genes coding for immunodominant surface antigens (*SAGs*), which may represent genetic recombination between *S. neurona*, *S. falcatula*, or other *Sarcocystis* species shed by opossums (Monteiro et al., 2013; Cesar; Gondim et al., 2019).

Serological techniques such as immunofluorescence antibody tests (IFAT), Western blot (WB), and enzyme-linked immunosorbent assays (ELISAs) based on *SAGs* have been used to identify exposure of horses to *S. neurona* in Brazil (summarized by Gondim et al., 2021). So been evidenced in central nervous system

of Brazilian horses by means of immunohistochemistry (Masri et al., 1992; Paixão et al., 2007; Henker et al., 2020) and molecular methods (Henker et al., 2020). However, the protozoal has never been isolated from horses in the country.

Due to the high similarity among *SAGs* in *Sarcocystis* shed by opossums, serological cross-reactivity between *S. neurona* and *S. falcatula*-like was hypothesized and experimentally demonstrated in infected Mongolian gerbils (*Meriones unguiculatus*) (de Jesus et al., 2019). The aim of the present study was to evaluate horse sera reactivity against *S falcatula*-like (strain Dal23-CG isolated from an opossum in Campo Grand) and *S. neurona* (North American strain SN138) among horses sampled in the cities of Campo Grande, Mato Grosso do Sul state (Midwestern), and São Paulo, São Paulo state (Southeastern), Brazil.

## Material and methods

### *Samples*

To detect anti-*S. neurona* and anti-*S. falcatula*-like antibodies, IFAT was performed using serum samples obtained by convenience from 342 horses. Samples from 262 horses were collected in 12 properties located in the peri-urban region of Campo Grande for screening of *Rickettsia*, *Borrelia*, and Anaplasmataceae agents, and were kindly supplied by Dr. Heitor Miraglia Herrera from the Universidade Católica Dom Bosco (UCDB). Samples from 30 horses were collected for routine exams and were kindly supplied by the veterinary staff of the military police cavalry from Campo Grande. Samples from 50 horses were collected for routine exams and were kindly supplied by the veterinary staff of an equestrian club in São Paulo. The samples included males and females of diverse ages and breeds (**Supplementary Table S1**).

### *Merozoites and Antigen Production*

Merozoites of *S. neurona* (strain SN138) isolated from *Didelphis virginiana* (Lindsay et al., 2004) and *S. falcatula*-like (strain Dal23-CG) isolated from *Didelphis albiventris* (De Santi et al., 2023), were used as antigens. The parasites were propagated in T25 flasks with confluent monolayers of Vero cells (BCRJ:0245), supplemented with Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen/Gibco®,

Carlsbad, USA), 1% antibiotic–antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B; Gibco®, Carlsbad, USA), and 5% inactivated fetal bovine serum (Invitrogen/Gibco®, Auckland, NZ). Flasks were maintained at 37 °C in a humidified incubator with 5% $CO_2$ (Nuare NU-4750E, Plymouth, MN, USA).

*Indirect Fluorescent Antibody Test (IFAT)*

Extracellular merozoites were harvested from the flasks, purified using a sterile 5 µm pore size filter, washed in phosphate-buffered saline (PBS) by centrifugation (1500 × g for 5 min), fixed in 10% formalin for 10 min, washed twice in PBS, and used to cover 12-well slides in 10 µL aliquots per well. The slides were allowed to air dry and stored at -20 °C. Prior to use, the slides were removed from storage and allowed to thaw at room temperature (approximately 22 °C) for 30 min. IFAT was performed as previously described (André et al., 2010). Briefly, serum samples were diluted at 1:25 in PBS and pipetted into each well. The slides were incubated in a humid chamber at 37 °C for 45 min, washed for 15 min with PBS, and dried at 37 °C. The delimited areas were covered with 10 µL of commercial fluorescein-labeled anti-horse immunoglobulin G (IgG) (Sigma–Aldrich®, St. Louis, USA) at a 1:64 dilution in PBS containing 10% Evans blue. The slides were then incubated for 45 min in a dark and humid chamber, washed, and dried. For microscopic evaluation, slides were overlaid with buffered glycerin (pH 8.7), covered with glass coverslips, and examined at 400 x magnification using an epifluorescence microscope (Olympus, Japan). Only samples presenting fluorescence in the entire periphery of merozoites were considered positive (**Figure 1**). Positive controls consisted of sera from horses that reacted at 1:100 solely for each parasite (*S. neurona* or *S. falcatula*–like). Negative controls consisted of horse sera that tested negative for both parasites at 1:25 dilution. Positive samples at 1:25 were further diluted in two-fold increments to obtain the final titer, with the endpoint titer being the last serum dilution showing distinct whole parasite fluorescence.

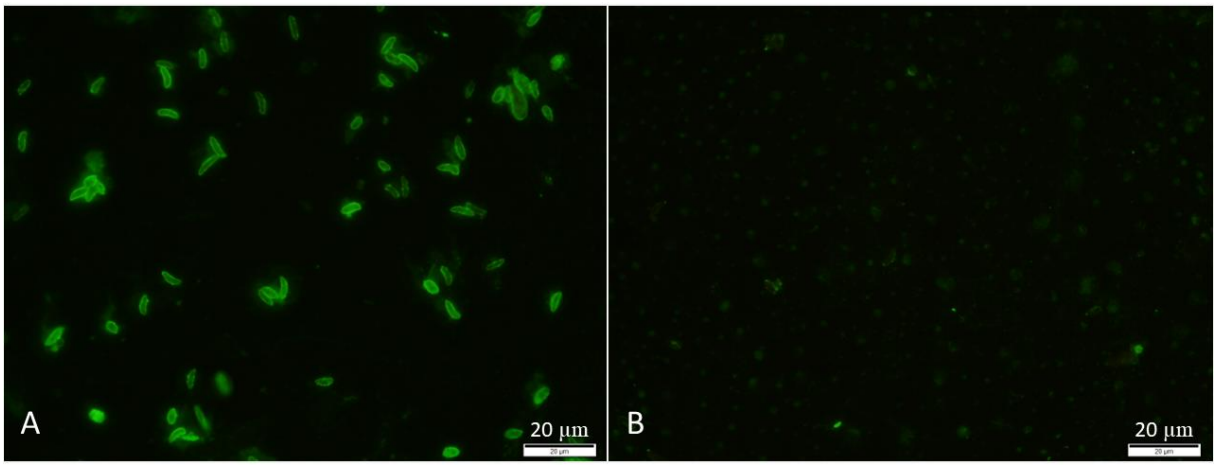


**Figure 1.** Indirect fluorescence antibody test (IFAT) in horses. *Sarcocystis falcatula*-like (isolate Dal-23CG) and *Sarcocystis neurona* (isolate SN138) were used as antigens. A- Positive titer with merozoites of *Sarcocystis neurona*. 400x. B- Negative control with absence of fluorescence. 400x.

**Results and discussion**

From the 342 horse serums analyzed using IFAT in the present study, 239 (69.88%) reacted against *S. neurona* (209 from Campo Grande and 30 from São Paulo), whereas 177 samples (51.75%) reacted against *S. falcatula*-like (163 from Campo Grande and 14 from São Paulo). IgG antibodies against both *S. neurona* and *S. falcatula*-like were detected in 132/342 (38.59%) samples (122 from Campo Grande and 10 from São Paulo). No reactivity was observed in 58/342 (16.95%) samples (42 from Campo Grande and 16 from São Paulo) (**Table 1**). A total of 107 horses (31.28%) presented IgG antibodies solely against *S. neurona* (87 from Campo Grande and 20 from São Paulo), while 45 (13.15%) presented IgG antibodies solely against *S. falcatula*-like (41 from Campo Grande and 04 from São Paulo). From the 132 samples that reacted against both isolates, a 4-fold increase in the titer of *S. neurona* was observed in 18 samples (12.87%), while a 4-fold increase in titers of *S. falcatula*-like was observed in 13 samples (10.6%) (**Supplementary Table S1**). Titers for *S. falcatula*-like and for *S. neurona* are described in **Table 2**.

Table 1. Seroprevalence for *Sarcocystis falcatula*-like (isolate Dal-23CG) and *Sarcocystis neurona* (isolate SN138) in 342 horses from Campo Grande Mato Grosso do Sul state (Midwestern), and São Paulo, São Paulo state (Southeastern) tested by immunofluorescence antibody test (IFAT).

| Isolate | *Sarcocystis falcatula*-like | | *Sarcocystis neurona* | | *Sarcocystis neurona* and *Sarcocystis falcatula*-like | |
|---|---|---|---|---|---|---|
| Seropositive | 51.75% (177/342) | Campo Grande = 163<br>São Paulo = 14 | 69.89% (239/342) | Campo Grande = 209<br>São Paulo = 30 | 38.59% (132/342) | Campo Grande = 122<br>São Paulo = 10 |
| Seronegative | 48.25% (165/342) | Campo Grande = 129<br>São Paulo = 36 | 30.11% (103/342) | Campo Grande = 83<br>São Paulo = 20 | 16.95% (58/342) | Campo Grande = 42<br>São Paulo = 16 |

Table 2. Titration of *Sarcocystis falcatula*-like (isolate Dal-23CG) and *Sarcocystis neurona* (isolate SN138)-seropositive horses from Campo Grande Mato Grosso do Sul state (Midwestern), and São Paulo, São Paulo state (Southeastern) tested by immunofluorescence antibody tests (IFAT).

| Titers | *Sarcocystis falcatula*-like | | *Sarcocystis neurona* | |
|---|---|---|---|---|
| | Campo Grande | São Paulo | Campo Grande | São Paulo |
| 1:25 | 69 | 1 | 49 | 20 |
| 1:50 | 38 | 2 | 84 | 3 |
| 1:100 | 39 | 6 | 47 | 1 |
| 1:200 | 13 | 5 | 20 | 3 |
| 1:400 | 4 | 0 | 9 | 2 |
| 1:800 | 0 | 0 | 0 | 1 |
| Total | 163 | 14 | 209 | 30 |

Results obtained by IFAT indicated that horse sera tested in our study reacted to more than one *Sarcocystis* species. Notably, 69.88% (239/342) of the samples reacted against *S. neurona*, and 51.75% (177/342) reacted against *S. falcatula*. This is a high seroprevalence compared to previous investigations conducted in Brazil. These include horse serum analyzed by IFAT in the states of Alagoas (Northeast, 2.8%) (Valença et al., 2019), Mato Grosso (Midwestern, 20.8%) (Borges et al., 2017), Minas Gerais (Southeastern, 23.09%) (Ribeiro et al., 2016), Rio Grande do Sul (South, 33.86%) (Antonello et al., 2015), São Paulo (Southeastern, 23.8%) (Oliveira et al., 2017) and Roraima (North, 40.4%) (Gomes et al., 2019). In the present study, the highest titer observed for *S. neurona* was 1:800, whereas 1:400 was the highest titer observed for *S. falcatula*-like. Titers up to 1:5120 were reported in 8.13% (7/86) of the *S. neurona*-seropositive horses in the state of Roraima (Gomes et al., 2019).

Discrepancies of our results in comparison with previous studies may be associated with the lower cutoff titer used here, and with the presence of opossums infected with *Sarcocystis falcatula*-like and *Sarcocystis* spp. in the same regions where the horses were sampled (De Santi et al., 2023). A starting serum dilution of 1:25 was selected based on previous study from Duarte et al. (2003), who observed that a gold standard panel of horses infected with *S. neurona* showed seropositivity starting at

1:20 at IFAT. The authors recommended a 1:80 cutoff in IFAT for horses suspected to have EPM. As none of the horses sample in the present study presented neurological signs at the moment of blood collection, a less conservative cutoff was used. Exposure to different *Sarcocystis* species may also generate different immunological responses (Borges-Silva et al., 2020), which could explain why horses in the present study reacted differently to *S. neurona* and *S. falcatula*-like antigens.

Serological assays are the main tools for *ante mortem* diagnosis of EPM. However, they only indicate whether horses have been exposed to *S. neurona*, not providing certainty of current infection (Dubey et al., 2015). Surface antigens, targeted in serological tests, are highly similar among *Sarcocystis* spp. shed by opossums (Gondim et al., 2021). Therefore, immunological assays that use whole merozoites as antigens, such as IFAT, and SAG-based ELISAs may result in the false assumption that horses are seropositive for *S. neurona* due to seroconversion after infection with other *Sarcocystis* species. Confirmation of IFAT using immunoblot assays revealed a low number of “true positive” samples in horse sera tested against *S. neurona* (SN37R) in Brazil (Valença et al., 2019). In an IFAT survey performed with serum samples from mares, it was observed that 64/189 (33.6%) samples tested were positive against *S. neurona,* and 57/64 samples were also positive to *Sarcocystis cruzi*, i.e., only seven samples were positive solely for *S. neurona* (Antonello et al., 2015). The presence of specific antibodies, associated with typical clinical signs, and the complete differential diagnosis, is currently used to define EPM diagnosis (Dubey et al., 2015).

The hypothesis of cross reactivity between different *Sarcocystis* species shed by opossums in Brazil could justify the high seropositivity observed for *S. neurona* in horses despite the low number of reports on EPM in the country (Gondim et al., 2021). It is worth to note that not all horses infected with *S. neurona* develop clinical disease. To date, the exact mechanisms that favor an asymptomatic infection to progress to a severe neurological disease remain unclear. Although the clinical history of the horses in this study was not available, none of the animals presented neurological signs at the time of blood collection.

Based on the results presented here along with that from previous studies, the high seroprevalence of *S. neurona* reported in horses in Brazil should be interpreted with caution. Further studies are necessary to elucidate the role of *Sarcocystis* species

other than *S. neurona* in infecting and causing clinical diseases in horses in the country.

**Conflicts of interest:** None

**Ethics statement**

The present study was approved by the Ethics Committee on the Use of Animals of the Dom Bosco Catholic University, number 020/2017.

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## Supplementary material

**Supplementary Table S1:** Titration for *Sarcocystis falcatula*-like (isolate Dal-23CG) and *Sarcocystis neurona* (isolate SN138) in 342 horses from Campo Grande, Mato Grosso do Sul state (Midwestern), and São Paulo, São Paulo state (Southeastern), Brazil, tested by immunofluorescence antibody test (IFAT).

| **Sample Id** | **Location** | **Sex** | **Age** | **Breed** | ***Sarcocystis neurona* titers** | ***Sarcocystis falcatula*-like titers** |
|---|---|---|---|---|---|---|
| Baião | Campo Grande | M | NI | NI | 1:100 | 1:200 |
| Bela Vista | Campo Grande | F | NI | NI | N | 1:25 |
| Tordilha | Campo Grande | F | NI | NI | N | 1:50 |
| Foguete | Campo Grande | M | NI | NI | 1:200 | 1:100 |
| Absoluto | Campo Grande | M | NI | NI | N | 1:100 |
| Elus | Campo Grande | M | NI | NI | 1:25 | 1:25 |
| Alabama | Campo Grande | M | NI | NI | 1:400 | 1:100 |
| Pingo | Campo Grande | M | NI | NI | 1:400 | 1:50 |
| Pelanca | Campo Grande | F | NI | NI | 1:50 | 1:25 |
| Coragem | Campo Grande | NI | NI | NI | 1:200 | 1:100 |
| Eclipse | Campo Grande | F | NI | NI | 1:100 | 1:100 |
| Diamante | Campo Grande | NI | NI | NI | 1:100 | 1:100 |
| Canário | Campo Grande | M | NI | NI | 1:200 | 1:200 |
| Ferrugem | Campo Grande | M | NI | NI | N | 1:50 |
| 496 | Campo Grande | NI | NI | NI | 1:25 | 1:25 |
| 499 | Campo Grande | NI | NI | NI | 1:25 | 1:25 |
| 505 | Campo Grande | F | NI | NI | N | 1:200 |
| 513 | Campo Grande | F | NI | NI | N | 1:25 |
| 514 | Campo Grande | NI | NI | NI | 1:25 | 1:200 |
| 518 | Campo Grande | NI | NI | NI | 1:25 | 1:200 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 530 | Campo Grande | NI | NI | NI | N | 1:25 |
| 538 | Campo Grande | NI | NI | NI | N | 1:50 |
| 540 | Campo Grande | NI | NI | NI | 1:100 | 1:100 |
| 543 | Campo Grande | M | NI | NI | 1:50 | 1:25 |
| 544 | Campo Grande | NI | NI | NI | 1:100 | 1:25 |
| 545 | Campo Grande | NI | NI | NI | 1:200 | 1:100 |
| 546 | Campo Grande | M | NI | NI | N | 1:25 |
| 557 | Campo Grande | NI | NI | NI | 1:200 | 1:200 |
| 576 | Campo Grande | M | NI | NI | 1:400 | 1:400 |
| 579 | Campo Grande | NI | NI | NI | 1:100 | 1:50 |
| 1 | Campo Grande | F | 08 Y | AQH | 1:50 | 1:50 |
| 2 | Campo Grande | F | 10 Y | NI | 1:50 | N |
| 3 | Campo Grande | F | 03 Y | AQH | 1:25 | 1:50 |
| 4 | Campo Grande | F | 05 Y | AQH | N | N |
| 5 | Campo Grande | F | 07 Y | AH | 1:25 | N |
| 6 | Campo Grande | M | 03 Y | AQH | N | N |
| 7 | Campo Grande | M | 03 Y | PH | N | N |
| 8 | Campo Grande | M | 08 Y | AQH | N | N |
| 9 | Campo Grande | M | 01 Y | AQH | N | N |
| 10 | Campo Grande | M | 08 Y | Appaaloosa | N | N |
| 11 | Campo Grande | F | 11 Y | AQH | 1:100 | 1:100 |
| 12 | Campo Grande | M | 08 Y | AQH | N | N |
| 13 | Campo Grande | F | 05 Y | AH | N | N |
| 14 | Campo Grande | F | 07 Y | AQH | N | N |
| 15 | Campo Grande | F | 06 Y | AQH | N | N |
| 16 | Campo Grande | F | 01 Y | AQH | N | N |
| 17 | Campo Grande | F | 05 Y | Pantaneira | 1:50 | N |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 18 | Campo Grande | M | 08 Y | AQH | 1:25 | 1:200 |
| 19 | Campo Grande | F | 08 Y | PH | 1:50 | N |
| 20 | Campo Grande | F | 03 Y | AQH | 1:100 | 1:200 |
| 21 | Campo Grande | M | 06 Y | AQH | N | N |
| 22 | Campo Grande | F | 14 Y | AQH | 1:50 | N |
| 23 | Campo Grande | F | 09 Y | Mangalarga | N | N |
| 24 | Campo Grande | F | 13 Y | Crioulo | 1:100 | 1:200 |
| 25 | Campo Grande | M | 11 Mo | AQH | N | N |
| 26 | Campo Grande | F | 04 Y | AQH | 1:25 | N |
| 27 | Campo Grande | M | 10 Y | Mixed breed | 1:100 | 1:200 |
| 28 | Campo Grande | F | 03 Y | AQH | 1:50 | N |
| 29 | Campo Grande | M | 15 Y | AQH | 1:200 | 1:100 |
| 30 | Campo Grande | M | 12 Y | Mixed breed | 1:25 | 1:50 |
| 31 | Campo Grande | F | 06 Y | AQH | N | N |
| 32 | Campo Grande | F | 12 Y | AQH | 1:25 | 1:100 |
| 33 | Campo Grande | F | 06 Y | AQH | N | N |
| 34 | Campo Grande | F | 15 Y | AQH | 1:50 | N |
| 35 | Campo Grande | F | 10 Y | AQH | 1:50 | N |
| 36 | Campo Grande | M | 05 Y | PH | 1:50 | N |
| 37 | Campo Grande | M | 08 Y | AQH | 1:25 | N |
| 38 | Campo Grande | M | 09 Y | AQH | 1:50 | N |
| 39 | Campo Grande | F | 04 Y | AQH | 1:50 | N |
| 40 | Campo Grande | F | 17 Y | AQH | 1:50 | N |
| 41 | Campo Grande | F | 09 Y | AQH | 1:50 | N |
| 42 | Campo Grande | F | 08 Y | AQH | 1:25 | 1:25 |
| 43 | Campo Grande | F | 11 Mo | AQH | 1:50 | N |
| 44 | Campo Grande | F | 06 Y | AQH | 1:50 | N |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 45 | Campo Grande | F | 10 Y | AQH | 1:100 | 1:25 |
| 46 | Campo Grande | M | 08 Y | AQH | 1:100 | 1:25 |
| 47 | Campo Grande | M | 06 Y | AQH | 1:50 | N |
| 48 | Campo Grande | M | 04 Y | AQH | N | 1:25 |
| 49 | Campo Grande | M | 10 Y | AQH | N | 1:50 |
| 50 | Campo Grande | M | 12 Y | AQH | 1:100 | 1:25 |
| 51 | Campo Grande | F | 08 Y | AQH | 1:50 | N |
| 52 | Campo Grande | F | 06 Y | AQH | N | N |
| 53 | Campo Grande | M | 04 Y | AQH | 1:25 | N |
| 54 | Campo Grande | M | 04 Y | AQH | 1:50 | N |
| 55 | Campo Grande | M | 10 Y | AQH | 1:25 | 1:100 |
| 56 | Campo Grande | M | 08 Y | AQH | 1:50 | N |
| 57 | Campo Grande | M | 02 Y | AQH | N | N |
| 58 | Campo Grande | M | 08 Y | AQH | 1:50 | 1:25 |
| 59 | Campo Grande | F | 08 Y | AQH | 1:50 | N |
| 60 | Campo Grande | M | 10 Y | AQH | 1:200 | 1:25 |
| 61 | Campo Grande | F | 12 Y | AQH | N | N |
| 62 | Campo Grande | F | 09 Y | AQH | 1:25 | N |
| 63 | Campo Grande | M | 08 Y | Crioulo | 1:50 | N |
| 64 | Campo Grande | M | 10 Y | AQH | 1:50 | 1:25 |
| 65 | Campo Grande | M | 12 Y | AQH | N | N |
| 66 | Campo Grande | F | 07 Y | AQH | N | N |
| 67 | Campo Grande | F | 06 Y | PH | 1:50 | N |
| 68 | Campo Grande | F | 02 Y | AQH | 1:100 | N |
| 69 | Campo Grande | M | 07 Y | AQH | 1:25 | N |
| 70 | Campo Grande | F | 18 Y | AQH | 1:100 | N |
| 71 | Campo Grande | F | 08 Y | AQH | 1:25 | 1:100 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 72 | Campo Grande | F | 10 Y | AQH | 1:200 | 1:100 |
| 73 | Campo Grande | F | 03 Y | AQH | 1:25 | N |
| 74 | Campo Grande | F | 13 Y | AQH | 1:50 | N |
| 75 | Campo Grande | M | 10 Y | AQH | 1:50 | 1:100 |
| 76 | Campo Grande | F | 06 Y | AQH | 1:25 | N |
| 77 | Campo Grande | F | 08 Y | AQH | 1:25 | 1:25 |
| 78 | Campo Grande | M | 09 Mo | AQH | N | N |
| 79 | Campo Grande | F | 07 Y | AQH | 1:25 | 1:50 |
| 80 | Campo Grande | F | 12 Y | AQH | N | N |
| 81 | Campo Grande | M | 03 D | AQH | N | N |
| 82 | Campo Grande | F | 10 Y | AQH | 1:25 | 1:25 |
| 83 | Campo Grande | M | 06 Mo | AQH | 1:25 | N |
| 84 | Campo Grande | F | 03 Y | AQH | N | N |
| 85 | Campo Grande | F | 02 Y | AQH | 1:50 | N |
| 86 | Campo Grande | M | 03 Y | AQH | N | N |
| 87 | Campo Grande | F | 01 Y | AQH | 1:50 | N |
| 88 | Campo Grande | F | 08 Y | AQH | N | N |
| 89 | Campo Grande | F | 03 Mo | AQH | N | N |
| 90 | Campo Grande | M | 01 Y | AQH | 1:200 | 1:25 |
| 91 | Campo Grande | F | 01 Y | AQH | 1:50 | N |
| 92 | Campo Grande | M | 07 Y | AQH | 1:50 | N |
| 93 | Campo Grande | M | 15 Y | AQH | 1:50 | N |
| 94 | Campo Grande | F | 10 Y | AQH | 1:100 | 1:50 |
| 95 | Campo Grande | M | 08 Y | AQH | 1:100 | N |
| 96 | Campo Grande | M | 04 Y | AQH | 1:50 | N |
| 97 | Campo Grande | M | 13 Y | AQH | 1:50 | N |
| 98 | Campo Grande | M | 31 Y | AQH | 1:400 | N |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 99 | Campo Grande | F | 17 Y | AQH | 1:50 | N |
| 100 | Campo Grande | F | 10 Y | AQH | 1:200 | N |
| 101 | Campo Grande | M | 10 Y | AQH | 1:100 | 1:100 |
| 102 | Campo Grande | M | 10 Y | AQH | 1:50 | N |
| 103 | Campo Grande | F | 10 Y | AQH | 1:50 | N |
| 104 | Campo Grande | F | 05 Y | AQH | 1:200 | N |
| 105 | Campo Grande | M | 04 Y | AQH | 1:50 | N |
| 106 | Campo Grande | M | 30 Y | AQH | 1:200 | N |
| 107 | Campo Grande | F | 09 Y | AQH | N | N |
| 108 | Campo Grande | F | 08 Y | AQH | 1:50 | N |
| 109 | Campo Grande | M | 04 Y | AQH | N | N |
| 110 | Campo Grande | M | 04 Y | AQH | N | N |
| 111 | Campo Grande | M | 06 Y | AQH | 1:100 | N |
| 112 | Campo Grande | F | 09 Y | AQH | 1:50 | N |
| 113 | Campo Grande | M | 03 Y | AQH | 1:50 | 1:25 |
| 114 | Campo Grande | M | 04 Y | AQH | 1:25 | 1:25 |
| 115 | Campo Grande | M | 04 Y | AQH | 1:50 | 1:25 |
| 116 | Campo Grande | F | 04 Y | AQH | 1:25 | 1:25 |
| 117 | Campo Grande | M | 13 Y | DW | N | 1:25 |
| 118 | Campo Grande | F | 15 Y | BSH | 1:100 | 1:25 |
| 119 | Campo Grande | M | 06 Y | AQH | N | 1:25 |
| 120 | Campo Grande | M | 06 Y | BSH | 1:25 | 1:25 |
| 121 | Campo Grande | F | 08 Y | BSH | 1:200 | 1:50 |
| 122 | Campo Grande | M | 10 Y | BSH | N | 1:25 |
| 123 | Campo Grande | M | 16 Y | BSH | N | 1:25 |
| 124 | Campo Grande | F | 16 Y | BSH | N | 1:50 |
| 125 | Campo Grande | F | 06 Y | Thoroughbred | N | 1:25 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 126 | Campo Grande | F | 10 Y | BSH | 1:400 | 1:25 |
| 127 | Campo Grande | M | 09 Y | BSH | 1:50 | 1:25 |
| 128 | Campo Grande | M | 05 Y | BSH | 1:100 | 1:50 |
| 129 | Campo Grande | F | 09 Y | Thoroughbred | N | N |
| 130 | Campo Grande | M | 20 Y | BSH | 1:100 | 1:25 |
| 131 | Campo Grande | M | 10 Y | BSH | N | 1:25 |
| 132 | Campo Grande | M | 05 Y | BSH | N | 1:100 |
| 133 | Campo Grande | F | 08 Y | BSH | 1:200 | N |
| 134 | Campo Grande | M | 22 Y | BSH | 1:50 | N |
| 135 | Campo Grande | F | 10 Y | BSH | 1:200 | 1:100 |
| 136 | Campo Grande | M | 08 Y | BSH | 1:100 | 1:100 |
| 137 | Campo Grande | M | 09 Y | BSH | 1:50 | N |
| 138 | Campo Grande | M | 08 Y | BSH | 1:100 | 1:100 |
| 139 | Campo Grande | F | 10 Y | BSH | 1:50 | N |
| 140 | Campo Grande | M | 14 Y | AQH | 1:200 | 1:100 |
| 141 | Campo Grande | M | 14 Y | BSH | 1:25 | 1:200 |
| 142 | Campo Grande | F | 05 Y | Thoroughbred | 1:100 | 1:100 |
| 143 | Campo Grande | M | 09 Y | Thoroughbred | 1:25 | 1:25 |
| 144 | Campo Grande | M | 04 Y | BSH | 1:50 | N |
| 145 | Campo Grande | M | 14 Y | Thoroughbred | N | 1:25 |
| 146 | Campo Grande | F | 20 Y | BSH | 1:50 | 1:50 |
| 147 | Campo Grande | M | 04 Y | BSH | 1:100 | 1:100 |
| 148 | Campo Grande | F | 06 Y | AQH | N | N |
| 149 | Campo Grande | M | 12 Y | AQH | 1:25 | 1:50 |
| 150 | Campo Grande | M | 12 Y | BSH | 1:50 | 1:100 |
| 151 | Campo Grande | M | 10 Y | AAH | 1:100 | 1:100 |
| 152 | Campo Grande | M | 14 Y | AQH | 1:100 | 1:50 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 153 | Campo Grande | F | 07 Y | BSH | 1:50 | N |
| 154 | Campo Grande | F | 21 Y | BSH | 1:50 | N |
| 155 | Campo Grande | F | 09 Y | BSH | 1:25 | 1:50 |
| 156 | Campo Grande | M | 17 Y | Thoroughbred | 1:100 | N |
| 157 | Campo Grande | F | 10 Y | BSH | N | N |
| 158 | Campo Grande | M | 15 Y | BH | N | N |
| 159 | Campo Grande | M | 15 Y | Thoroughbred | 1:50 | N |
| 160 | Campo Grande | M | 08 Y | BSH | 1:50 | N |
| 161 | Campo Grande | M | 13 Y | BSH | 1:25 | 1:50 |
| 162 | Campo Grande | M | 15 Y | AAH | N | N |
| 163 | Campo Grande | M | 18 Y | AQH | 1:50 | 1:100 |
| 164 | Campo Grande | M | 15 Y | AQH | 1:25 | 1:100 |
| 165 | Campo Grande | M | 33 Y | AAH | 1:50 | 1:50 |
| 166 | Campo Grande | M | 13 Y | BSH | 1:50 | N |
| 167 | Campo Grande | M | 14 Y | Lusitano | 1:50 | N |
| 168 | Campo Grande | M | 05 Y | BSH | 1:50 | N |
| 169 | Campo Grande | M | 08 Y | Mixed breed | N | 1:25 |
| 170 | Campo Grande | M | 05 Y | Mixed breed | 1:100 | 1:100 |
| 171 | Campo Grande | F | 15 Y | Mixed breed | 1:25 | 1:100 |
| 172 | Campo Grande | F | 03 Y | Thoroughbred | 1:25 | 1:25 |
| 173 | Campo Grande | F | 12 Y | Mixed breed | 1:200 | 1:25 |
| 174 | Campo Grande | M | 08 Y | Mixed breed | N | 1:400 |
| 175 | Campo Grande | M | 03 Y | Mixed breed | 1:400 | 1:400 |
| 176 | Campo Grande | M | 10 Y | Mixed breed | 1:50 | N |
| 177 | Campo Grande | M | 12 Y | Mixed breed | 1:25 | 1:50 |
| 178 | Campo Grande | F | 13 Y | Mixed breed | 1:100 | 1:50 |
| 179 | Campo Grande | F | 12 Y | Mixed breed | N | 1:50 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 180 | Campo Grande | F | 07 Y | Thoroughbred | 1:50 | 1:100 |
| 181 | Campo Grande | M | 03 Y | BSH | 1:200 | 1:200 |
| 182 | Campo Grande | F | 32 Y | Mixed breed | 1:50 | 1:100 |
| 183 | Campo Grande | F | 03 Y | Mixed breed | 1:50 | N |
| 184 | Campo Grande | M | 12 Y | Mixed breed | 1:25 | 1:25 |
| 185 | Campo Grande | M | 10 Y | Mixed breed | 1:400 | 1:25 |
| 186 | Campo Grande | M | 08 Y | Mixed breed | 1:100 | 1:100 |
| 187 | Campo Grande | M | 06 Y | Mixed breed | 1:100 | 1:100 |
| 188 | Campo Grande | M | 05 Y | AQH | 1:50 | 1:100 |
| 189 | Campo Grande | F | 04 Y | AQH | N | N |
| 190 | Campo Grande | M | 12 Y | Mixed breed | 1:25 | 1:25 |
| 191 | Campo Grande | M | 10 Y | Mixed breed | 1:25 | 1:50 |
| 192 | Campo Grande | F | 07 Y | Mixed breed | N | 1:50 |
| 193 | Campo Grande | M | 10 Y | Mixed breed | 1:50 | N |
| 194 | Campo Grande | F | 12 Y | Mixed breed | N | 1:25 |
| 195 | Campo Grande | F | 12 Y | Mixed breed | 1:100 | 1:50 |
| 196 | Campo Grande | F | 08 Y | Mixed breed | 1:50 | 1:100 |
| 197 | Campo Grande | F | 12 Y | Mixed breed | N | 1:50 |
| 198 | Campo Grande | M | 05 Y | Mixed breed | 1:50 | 1:100 |
| 199 | Campo Grande | M | 06 Y | Mixed breed | 1:50 | N |
| 200 | Campo Grande | M | 07 Y | Mixed breed | 1:50 | N |
| 201 | Campo Grande | F | 15 Y | Mixed breed | 1:100 | 1:100 |
| 202 | Campo Grande | M | 17 Y | Mixed breed | 1:400 | 1:400 |
| 203 | Campo Grande | M | 17 Y | Mixed breed | N | 1:50 |
| 204 | Campo Grande | F | 20 Y | Thoroughbred | 1:400 | 1:200 |
| 205 | Campo Grande | M | 15 Y | Mixed breed | 1:25 | 1:25 |
| 206 | Campo Grande | M | 04 Y | Mixed breed | 1:100 | 1:50 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 207 | Campo Grande | F | 16 Y | Mixed breed | 1:25 | 1:25 |
| 208 | Campo Grande | M | 12 Y | Mixed breed | 1:50 | 1:25 |
| 209 | Campo Grande | F | 10 Y | Mixed breed | 1:50 | N |
| 210 | Campo Grande | F | 13 Y | Mixed breed | 1:100 | N |
| 211 | Campo Grande | F | 10 Y | Mixed breed | N | 1:50 |
| 212 | Campo Grande | F | 09 Y | Mixed breed | 1:100 | N |
| 213 | Campo Grande | M | 12 Y | Mixed breed | N | N |
| 214 | Campo Grande | F | 16 Y | Mixed breed | 1:25 | N |
| 215 | Campo Grande | M | 15 Y | Mixed breed | N | 1:50 |
| 216 | Campo Grande | M | 08 Y | Mixed breed | 1:50 | N |
| 217 | Campo Grande | F | 14 Y | Mixed breed | N | N |
| 218 | Campo Grande | M | 06 Y | Mixed breed | 1:100 | N |
| 219 | Campo Grande | F | 13 Y | Mixed breed | 1:100 | 1:50 |
| 220 | Campo Grande | F | 04 Y | Mixed breed | 1:50 | N |
| 221 | Campo Grande | F | 06 Y | Crioulo | 1:100 | 1:50 |
| 222 | Campo Grande | F | 04 Y | Mixed breed | 1:25 | 1:25 |
| 223 | Campo Grande | F | 18 Y | Mixed breed | N | 1:25 |
| 224 | Campo Grande | F | 08 Y | Mixed breed | N | 1:25 |
| 225 | Campo Grande | F | 02 Y | Mixed breed | N | 1:25 |
| 226 | Campo Grande | F | 07 Y | AQH | N | 1:25 |
| 227 | Campo Grande | F | 10 Mo | AQH | 1:50 | N |
| 228 | Campo Grande | M | 07 Y | Crioulo | N | 1:25 |
| 229 | Campo Grande | F | 08 Y | Crioulo | 1:100 | 1:50 |
| 230 | Campo Grande | F | 06 Y | Crioulo | 1:100 | 1:25 |
| 231 | Campo Grande | F | 03 Y | AQH | N | N |
| 232 | Campo Grande | F | 04 Y | AQH | 1:25 | N |
| 233 | Campo Grande | F | 04 Y | AQH | 1:25 | 1:25 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 234 | Campo Grande | M | 15 Y | Mixed breed | N | 1:25 |
| 235 | Campo Grande | M | 15 Mo | Crioulo | N | 1:25 |
| 236 | Campo Grande | F | 03 Y | Mixed breed | N | 1:25 |
| 237 | Campo Grande | F | 04 Y | Crioulo | 1:200 | 1:50 |
| 238 | Campo Grande | F | 04 Y | AQH | 1:50 | 1:25 |
| 239 | Campo Grande | M | 20 Y | AQH | 1:100 | 1:25 |
| 240 | Campo Grande | M | 04 Y | AQH | N | 1:25 |
| 241 | Campo Grande | M | 06 Y | Mixed breed | 1:50 | 1:25 |
| 242 | Campo Grande | M | 04 Y | AQH | 1:200 | 1:100 |
| 243 | Campo Grande | M | 05 Y | AQH | N | 1:25 |
| 244 | Campo Grande | M | 05 Y | AQH | 1:100 | 1:50 |
| 245 | Campo Grande | M | 05 Y | AQH | 1:50 | N |
| 246 | Campo Grande | F | 06 Y | AQH | 1:100 | 1:50 |
| 247 | Campo Grande | F | 02 Y | AQH | 1:50 | N |
| 248 | Campo Grande | M | 04 Y | AQH | 1:50 | 1:50 |
| 249 | Campo Grande | F | 10 Y | Mixed breed | 1:50 | N |
| 250 | Campo Grande | M | 14 Y | Mixed breed | 1:100 | N |
| 251 | Campo Grande | M | 02 Y | Mangalarga | 1:50 | N |
| 252 | Campo Grande | F | 04 Y | Mangalarga | N | N |
| 253 | Campo Grande | M | 10 Y | Mangalarga | N | 1:25 |
| 254 | Campo Grande | M | 08 Y | Mangalarga | N | 1:25 |
| 255 | Campo Grande | M | 10 Y | Mangalarga | 1:25 | 1:100 |
| 256 | Campo Grande | F | 08 Y | Mangalarga | 1:50 | N |
| 257 | Campo Grande | M | 04 Y | Mangalarga | N | N |
| 258 | Campo Grande | M | 10 Y | Mangalarga | N | N |
| 259 | Campo Grande | F | 10 Y | Mangalarga | 1:50 | N |
| 260 | Campo Grande | F | 08 Y | Mangalarga | 1:25 | 1:25 |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 261 | Campo Grande | F | 10 Y | Mangalarga | 1:25 | 1:25 |
| 262 | Campo Grande | F | 05 Y | Mangalarga | 1:25 | 1:25 |
| 1 | São Paulo | M | 12 Y | DW | 1:25 | N |
| 2 | São Paulo | M | 15 Y | BSH | 1:25 | N |
| 3 | São Paulo | M | 08 Y | BSH | N | N |
| 4 | São Paulo | M | 07 Y | BSH | N | 1:50 |
| 5 | São Paulo | NI | 09 Y | BSH | 1:25 | N |
| 6 | São Paulo | NI | 11 Y | DW | 1:25 | N |
| 7 | São Paulo | M | NI | NI | 1:25 | N |
| 8 | São Paulo | F | NI | NI | 1:25 | N |
| 9 | São Paulo | M | 08 Y | Lusitano | N | 1:50 |
| 10 | São Paulo | M | 01 Y | BSH | 1:25 | N |
| 11 | São Paulo | F | 09 Y | BSH | 1:50 | 1:100 |
| 12 | São Paulo | M | 10 Y | Lusitano | N | N |
| 13 | São Paulo | M | 10 Y | Lusitano | 1:25 | 1:100 |
| 14 | São Paulo | M | NI | NI | N | N |
| 15 | São Paulo | M | 14 Y | BSH | 1:200 | 1:200 |
| 16 | São Paulo | M | 09 Y | BSH | 1:25 | N |
| 17 | São Paulo | F | 07 Y | BSH | 1:25 | N |
| 18 | São Paulo | M | 17 Y | BSH | 1:50 | 1:200 |
| 19 | São Paulo | NI | 05 Y | BSH | 1:25 | N |
| 20 | São Paulo | F | 11 Y | BSH | 1:25 | N |
| 21 | São Paulo | F | 12 Y | BSH | 1:25 | N |
| 22 | São Paulo | M | 21 Y | DW | N | N |
| 23 | São Paulo | M | 08 Y | BSH | N | 1:200 |
| 24 | São Paulo | NI | 09 Y | BSH | 1:25 | N |
| 25 | São Paulo | M | 17 Y | BSH | 1:25 | N |

**Supplementary Table S1.** Continued…

| | | | | | | |
|---|---|---|---|---|---|---|
| 26 | São Paulo | M | 12 Y | Lusitano | N | 1:100 |
| 27 | São Paulo | M | 05 Y | BSH | 1:100 | 1:100 |
| 28 | São Paulo | M | 13 Y | BSH | 1:200 | 1:25 |
| 29 | São Paulo | NI | NI | NI | 1:25 | N |
| 30 | São Paulo | F | 15 Y | BSH | 1:25 | N |
| 31 | São Paulo | M | 06 Y | BSH | 1:400 | 1:200 |
| 32 | São Paulo | M | 10 Y | BSH | N | N |
| 33 | São Paulo | NI | 12 Y | Lusitano | 1:25 | N |
| 34 | São Paulo | NI | 07 Y | BSH | N | N |
| 35 | São Paulo | M | 08 Y | BSH | 1:200 | 1:100 |
| 36 | São Paulo | NI | NI | BSH | N | N |
| 37 | São Paulo | NI | NI | BSH | N | N |
| 38 | São Paulo | NI | 06 Y | BSH | 1:25 | N |
| 39 | São Paulo | NI | 13 Y | BW | 1:25 | N |
| 40 | São Paulo | M | 10 Y | BSH | 1:800 | N |
| 41 | São Paulo | M | 11 Y | DW | N | N |
| 42 | São Paulo | F | 10 Y | BSH | N | N |
| 43 | São Paulo | F | 10 Y | BSH | 1:400 | 1:100 |
| 44 | São Paulo | M | 08 Y | BSH | 1:50 | N |
| 45 | São Paulo | NI | 08 Y | BSH | N | N |
| 46 | São Paulo | F | 14 Y | BSH | N | N |
| 47 | São Paulo | NI | 05 Y | BSH | N | N |
| 48 | São Paulo | M | 05 Y | BSH | 1:50 | 1:200 |
| 49 | São Paulo | M | 09 Y | BSH | N | N |
| 50 | São Paulo | F | 11 Y | BSH | N | N |

Legend: NI- Not informed, N- Negative, Y- Years, Mo- Months, D- Days, M- Male, F- Female, BW- Belgian Warmblood, DW- Deutsch Warmblood, AAH- Anglo Arabian Horse, AQH- American Quarter Horse, BSH- Brazilian Sport Horse, PH- Paint Horse, AH- Arabian Horse.

## CHAPTER 4 – Final considerations

*Sarcocystis* spp. are an interesting protozoal group. The evolutionary success of the group is irrefutable and may be verified by its wide geographic distribution associated with the richness of intermediate and definitive hosts. Over the years, several studies performed in the Americas have endorsed the improvement of the knowledge about the group. Despite the undeniable advances achieved, biological issues – e.g., intermediate, and definitive hosts, and genetic diversity – remain poorly understood for *Sarcocystis* species that use opossums as definitive hosts.

Using serological, molecular, and bioinformatic tools, the current study increased the knowledge about the occurrence, genetic diversity, and phylogenetic relationships of *Sarcocystis* spp. shed by opossums, as well as identified the exposure of horses to *Sarcocystis* spp. The results obtained demonstrated that *Sarcocystis* spp. shed by opossums in the studied areas are close related to *Sarcocystis falcatula*-like previous described in Brazil. *Sarcocystis* spp. phylogenetically related to *S. rileyi* was revealed by new *cox1* alleles. Expressive number of horses in that areas presented antibodies against *S. neurona*, *S. falcatula*-like, or against both species.

The findings presented here have contributed adding new elements in the complex network involving *Sarcocystis* spp. shed by opossums in the Americas. In Brazil, horses have been serologically tested for *S. neurona* using North American strains of the parasite, as no viable isolate of *S. neurona* is available in Brazil. Besides, Brazilian horses have been demonstrated to be reactive to *S. falcatula*-like antigens used in serological tests. Whether horses have been naturally exposed and seroconverting to *S. falcatula*-like or whether the seropositivity resulted from cross-reactivity with other *Sarcocystis* species, remains unclear. Therefore, the isolation of the parasite from the CNS of EPM confirmed horses, as well as the development a serum panel of truly *S. neurona*-infected horses is very important. The development of recombinant proteins based on *S. falcatula*-like antigens to be used in serological tests, could also help to better identify the exposure of horses to different *Sarcocystis* species. It is also relevant to investigate whether horses in Brazil can be infected and develop disease with other *Sarcocystis* spp. shed by opossums in the country.