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PROTOZOOLOGY - ORIGINAL PAPER

# First molecular characterization of *Sarcocystis neurona* causing meningoencephalitis in a domestic cat in Brazil

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## Abstract

*Sarcocystis neurona* is the main agent associated with equine protozoal myeloencephalitis (EPM). Apart from horses, *S. neurona* has been occasionally described causing neurologic disease in several other terrestrial animals as well as mortality in marine mammals. Herein, we describe the clinical, pathological, and molecular findings of a fatal case of *S. neurona*-associated meningoencephalitis in a domestic cat. The causing agent was analyzed by multilocus genotyping, confirming the presence of *S. neurona* DNA in the tissue samples of the affected animal. Significant molecular differences were found in relation to *S. neurona* isolates detected in other regions of the Americas. In addition, the parasite was identical to *Sarcocystis* sp. identified in opossum sporocysts in Brazil at molecular level, which suggests that transmission of. *S. neurona* in Brazil might involve variants of the parasite different from those found elsewhere in the Americas. Studies including more samples of *S. neurona* would be required to test this hypothesis, as well as to assess the impact of this diversity.

**Keywords** Protozoal meningoencephalitis · Feline · Genotyping · Polymerase chain reaction

## Introduction

*Sarcocystis neurona*, *Sarcocystis speeri*, and *Sarcocystis falcatula* are very similar species, transmitted exclusively in the Americas by opossums of the genus *Didelphis* spp. (Dubey et al. 2015). *S. neurona* and *S. falcatula* have already been described in Brazil. Birds of various species are susceptible to *S. falcatula*, while mammals, predominantly, are susceptible to *S. neurona*/*S. speeri* (Dubey et al. 2015). It is worth mentioning that *S. neurona* and *S. speeri* are almost identical species according to criteria based on molecular identification (Cesar et al. 2018; Acosta et al. 2018).

*Sarcocystis neurona* is the main agent associated with equine protozoal myeloencephalitis (EPM), an important neurologic disease of horses in the American continent (Dubey et al. 2015). In addition, neurologic disease caused by *S. neurona* has been occasionally described in several other terrestrial animals (Dubey et al., 2000), and this parasite has arisen as a significant cause of mortality in marine mammals (Barbosa et al. 2015). Opossums, *Didelphis virginiana* in North America (Fenger et al. 1995) and *Didelphis albiventris* in South America (Dubey et al., 2001a), are known definitive

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hosts of *S. neurona*, while intermediate hosts include domestic cats, sea otters, armadillos, raccoons, and skunks (Dubey et al., 2001b). Central nervous system (CNS) disease caused by *S. neurona* has been rarely described in domestic cats (Dubey et al., 1994, 2003).

Therefore, the objective of this study is to describe the clinical, pathological, immunohistochemical, and molecular findings of a fatal case of *S. neurona*-associated meningoencephalitis in a domestic cat in Southern Brazil.

## Material and methods

### Animal

A 1.7-year-old domestic cat, with free access to the street, presented a clinical history of anorexia and dyspnea, which lasted 7 days. At clinical examination, hyperthermia and pleural effusion were detected. Serological tests yielded positive results for feline leukemia virus (FeLV) and negative results for feline immunodeficiency virus (FIV). Pleural effusion was submitted for cytological evaluation, and pyogranulomatous inflammation was observed. Thoracic effusion was subjected for coronavirus immunocytochemistry, yielding negative results. Thoracic radiography was performed, and a radiopaque area in the cranial mediastinal region was noted. Due to the clinical suspicion of mediastinal lymphoma, chemotherapeutic treatment was prescribed. Treatment with prednisolone (2 mg/kg, S.I.D., P.O.) was established, associated with vincristine administrations with a 7-day interval (0.75 mg/m2, I.V.). In addition, a single administration of cyclophosphamide (250 mg/kg, P.O.), at the third week after treatment onset, was prescribed. Twenty-one days after the first clinical examination, the cat returned to the hospital presenting neurological signs, such as compulsive walking, head pressing, and vocalization. The cat died and was referred for necropsy. At the necropsy, the animal showed severe dehydration, emaciation, as well as mild cerebellar herniation through the foramen magnum. No mass consistent with lymphoma was evidenced at the mediastinal region or any other organ. In addition, no cavitary effusions or inflammatory lesions compatible com FIP were observed. At necropsy, multiple tissue samples were collected and fixed in a 10% neutral buffered formalin solution. Samples were routinely processed for histopathological examination and stained with hematoxylin and eosin (HE). Fresh frozen samples of brain and cerebellum were collected and posteriorly submitted for DNA extraction and molecular analysis. At the histological examination, lesions were restricted to the encephalon (spinal cord was not available for evaluation). In the gray and white matter and sometimes extending to the leptomeninges (Fig. 1a), there was a marked inflammatory infiltrate consisting of degenerate neutrophils, macrophages, and lymphocytes (Fig. 1b). These areas were often associated with parasitic structures measuring 10–15 μm in diameter (schizonts), which contained numerous elongated structures measuring around 1.5 × 4 μm (merozoites), morphologically consistent with *S. neurona* (Fig. 1c). Schizonts were observed freely in the midst of inflammation areas, as well as in the soma of neurons and in the cytoplasm of astrocytes. In addition, multifocal moderate to severe perivascular inflammatory infiltrate of lymphocytes, plasma cells, and macrophages was seen in the brain and in the cerebellum. Furthermore, multifocal areas of gliosis associated with gemistocytic astrocytes, few gitter cells, and endothelial cell hypertrophy were seen. Lesions were marked in the telencephalon, diencephalon, cerebellum, and corpus striatum, and moderate in the mesencephalon and in the brainstem.

### Immunohistochemistry

CNS sections were submitted for immunohistochemistry anti-*S. neurona* (polyclonal antibody non-commercial, 1:200), anti-*Toxoplasma gondii* (VRMD, Pullman, WA, USA, dilution of 1:1000), and anti-*Neospora caninum* (VRMD, Pullman, WA, USA, dilution of 1:1000). Antigen retrieval was performed with proteinase K for 1 min for *S. neurona* and 0.1% trypsin for 10 min for *N. caninum* and *T. gondii*. Blocking of nonspecific reactions was performed with 5% skim milk for 15 min. The amplification signal was achieved by using MACH 4 Universal HRP-Polymer (Biocare, Pacheco, CA, USA) for *S. neurona* and LSAB-HRP Universal kit (Dakocytomation, Carpinteria, CA, USA) for *N. caninum* and *T. gondii*. The reactions were visualized with 3-amino-9-ethylcarbazole chromogen (AEC; Sigma, St. Louis, Missouri, USA), and subsequently, all slides were counterstained with Harris' hematoxylin. Positive control samples consisted of known cases of CNS disease caused by *S. neurona*, *T. gondii*, and *N. caninum*. In the negative control slides, primary antibodies were replaced by PBS.

### DNA extraction, PCR amplification, and sequencing

DNA was extracted from frozen samples of the brain of the infected cat with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer's, except for elution of the final product in 50 μL of AE buffer. The DNA samples were tested for the presence of *S. neurona* by means of multilocus genotyping as described below.

*Sarcocystis* spp. sequences of 18S small subunit rRNA region, gene coding for cytochrome c oxidase subunit I, and internal transcribed spacer 1, SAG2, SAG3, and SAG4 were nested PCR amplified. Nested PCR directed to 18S (nPCR-18S) was performed using primers 18S9L and 18S1H (Li et al. 2002). DNA amplification of *Sarcocystis* spp. cytochrome c oxidase subunit I (nPCR-COX1) was performed using primers designed by Gondim et al. (2019). Complete internal

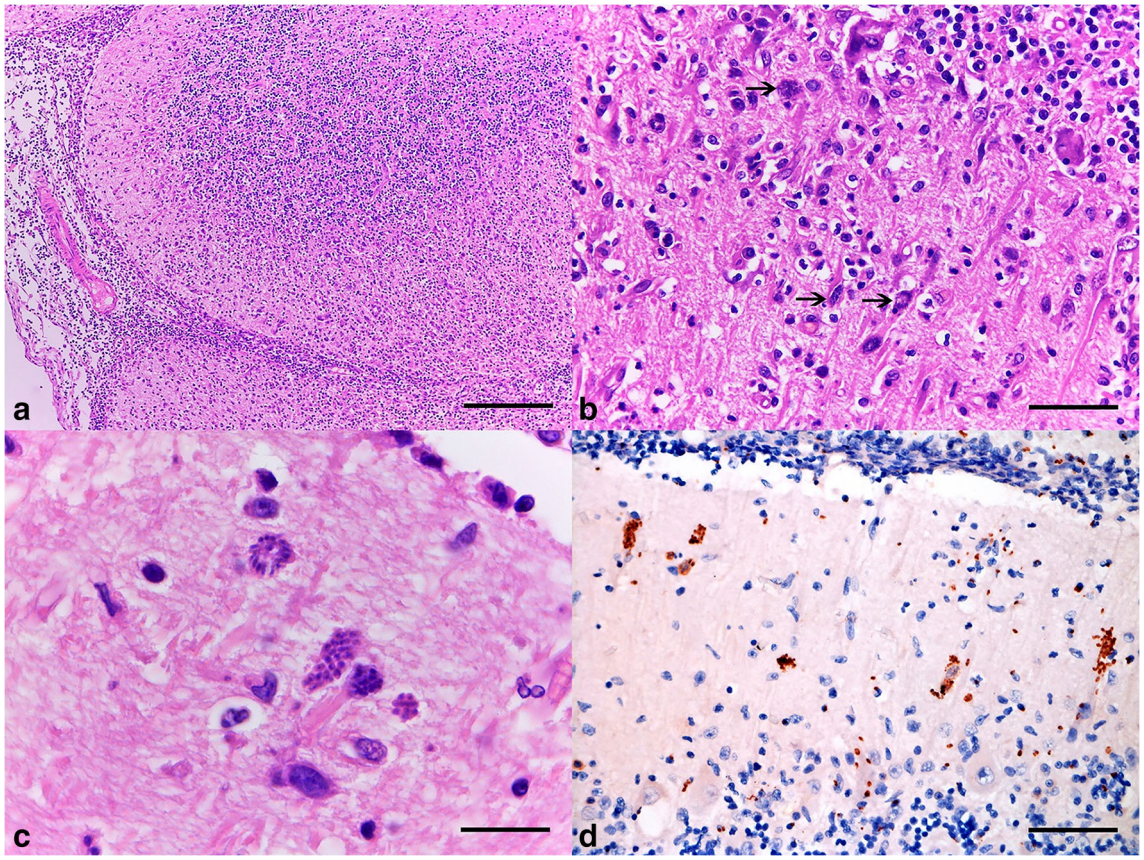


**Fig. 1** *S. neurona*-associated meningoencephalitis in a domestic cat. **a** Cerebellum, marked diffuse mixed inflammatory infiltrate is observed distending the leptomeninges as well as partially effacing the neuropile or the trilaminar aspect of the cerebellum. HE, bar 300 μm. **b** Cerebellum, there is moderate inflammatory infiltrate of lymphocytes, plasmocytes and macrophages associated with parasitic schizonts (arrows). HE, bar 100 μm. **c** Cerebellum, multiple schizonts of *S. neurona* filled by numerous elongated structures (merozoites) are seen in the neuropile. HE, bar 30 μm. **d** Cerebellum, marked multifocal anti-*S. neurona* immunostaing is noted in the neuropile. Numerous schizonts and merozoites are evidenced freely in the neuropile, as well as in the cytoplasm of CNS cells. IHC, chromogen AEC, bar 45 μm

transcribed spacer 1 was nested PCR amplified using the primer set described by Soares et al. (2011). Finally, genetic sequences of SAG2, SAG3, and SAG4 were nested PCR amplified using the primers as designed in Monteiro et al. (2013), Valadas et al. (2016), and Gondim et al. (2019). Primers used to amplify genetic sequences of *Sarcocystis* spp. using nested PCR are depicted in Table 1.

DNA amplification was done in two rounds, in all cases. The first round of amplifications (external primers) was conducted by adding 4 μL of extracted DNA, 2.5 μL of 10x PCR Buffer (KCL 50 mM; Tris HCl 10 mM; pH 9.0), 1 μL of MgCL2 (50 mM), 0.5 μL of mixed dNTPs (10 mM), 0.35 μL of each primer (10 μM), 0.2 μL of Taq DNA Polymerase Platinum-Invitrogen (5 U/μL), and 16.1 μL of ultrapure autoclaved water to a volume of 25 μL per reaction. The PCR thermal cycling consisted of an initial incubation at 94 °C for 3 min, followed by 40 cycles (94 °C for 30 s, 56 °C for 30 s, 72 °C for 50 s) and a final extension at 72 °C for 5 min. For the second rounds of amplifications (internal primers), 2 μL of template derived from the first reactions was added to 2.5 μL of 10x PCR Buffer (KCL 50 mM; Tris HCl 10 mM; pH 9.0), 1 μL of MgCL2 (50 mM), 0.5 μL of mixed dNTPs (1.25 mM), 1 μL of each primer (10 μM), 0.2 μL of Taq DNA Polymerase Platinum-Invitrogen (5 U/μI), and 16.8 μL of ultrapure autoclaved water to a volume of 25 μL per reaction. The thermal cycling was the same used in the first round.

The nested PCR products were analyzed under ultraviolet transilluminator after electrophoresis in 2% agarose gels and ethidium bromide (0.5 μg/mL) staining for 60 min. The targeted bands were excised from the gel and purified using a commercial purification kit (Illustra GFX PCR DNA and Gel Band Purification, Amersham Biosciencies) according to the manufacturer's instructions. The purified DNA was bidirectionally sequenced using forward and reverse primers with the Kit ABI PRISM Big Dye Terminator (Applied Biosystem) following the manufacturer's protocol.

## Sequence edition and sequence analysis

The quality of nucleotide sequences, the contig assembly, and sequence edition were assessed using the program Phred-Phrad

**Table 1** Primers for the detection of *Sarcocystis* spp. genetic sequences in brain tissue sample of an infected cat

| Locus | Primers | Sequences | PCR step[a] | Size (bp)[b] | Reference |
|---|---|---|---|---|---|
| 18S | 18S9L | GGATAACCTGGTAATTCTATG | 1 + 2 | 825 | Li et al. 2002 |
| | 18S1H | GGCAAATGCTTTCGCAGTAG | 1 + 2 | | Li et al. 2002 |
| COX1 | COX1-227F25 | GTTTTGGTAACTACTTTGTACCGAT | 1 | | Gondim et al. 2019 |
| | COX1-885R25 | GAAATATGCACGAGTATCTACCTCT | 1 | | Gondim et al. 2019 |
| | COX1-275F22 | TGTACCCACGAATTAATGCAGT | 2 | 590 | Gondim et al. 2019 |
| | COX1-844R21 | GTGTGCCCATACTAGAGAACC | 2 | | Gondim et al. 2019 |
| ITS1 | JS4 | CGAAATGGGAAGTTTGAAC | 1 | | Slapeta et al. 2002 |
| | CT2c | CTGCAATTCACATTCGC | 1 | | Soares et al. 2011 |
| | JS4b | AGTCGTAACAAGGTTTCCGTAGG | 2 | ~ 1100 | Soares et al. 2011 |
| | CT2b | TTGCGCGAGCCAAGACATC | 2 | | Soares et al. 2011 |
| SAG2 | SAG2-F1 | CAACAATTGCGTGCACACAA | 1 | | Valadas et al. 2016 |
| | SAG2-R1 | ACAACACTGTGAGAGATGCGA | 1 | | Monteiro et al. 2013 |
| | SAG2-F2 | GGTCAGAGCTTTGTGCTGAA | 2 | 338 | Monteiro et al. 2013 |
| | SAG2-459R21 | CACATTGCAAGCASGACACCA | 2 | | Gondim et al. 2019 |
| SAG3 | SAG3-F1 | CTCGCAGTTGCCTGCCTTG | 1 | 511 | Monteiro et al. 2013 |
| | SAG3-053F19 | GATCCACCTGTYGCAACTT | 2 | | This study |
| | SAG3-589R21 | TGGTCCTGTAGCAGTAACACA | 1 + 2 | | Valadas et al. 2016 |
| SAG4 | SAG4-F2 | CCGAGGTACAGTTCAAGGCG | 1 | | Monteiro et al. 2013 |
| | SAG4-R1 | CGACGACGATACCCAATGCC | 1 | | Monteiro et al. 2013 |
| | SAG4-541F21 | GGCAACGCCGCAGCMCTGCAA | 2 | 282 | Valadas et al. 2016 |
| | SAG4-803R20 | CAATGCCGAMGCGGTACGAG | 2 | | Gondim et al. 2019 |

[a] (1) Primers used only in the first round of amplification, (2) primers used only in the second round of amplification, and (1 + 2) primers used in the first and second round of amplification

[b] Predicted size of the nested PCR product

in Codon Code Aligner, version 4.2.1. The final nucleotide sequences from each locus were analyzed using Blast search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS1 and SAG sequences were aligned with homologous sequences available in GenBank using the program Clustal W in BioEdit Sequence Alignment Editor v. 7.0.5.3 (Hall 1999). ITS1 phylogenetic tree was inferred with maximum likelihood method, computing evolutionary distance using K2P + G method. The ITS1 phylogeny was inferred with sequences of *S. neurona*, *S. speeri*, *S. falcatula*, and related organisms retrieved from GenBank after Blast search tool using *S. neurona* from a cat as query. ITS1 phylogeny was reconstructed using sequences with more than 90% coverage containing at most one degenerate site by using MEGA 7 (Kumar et al. 2016). The software PopART (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015) was used to infer evolutionary relationships and Integer NJ networks inference method among isolates of *S. neurona* and *S. falcatula* based on SAG loci. The genetic sequences were deposited in GenBank under the following accession numbers: SAG2 MN175964, SAG3 MN175965, SAG4 MN175966, CO1 MN175967, ITS1 MN172273, and 18S MN169125.

## Results

Immunohistochemistry anti-*S. neurona* was strongly positive (Fig. 1d), and immunohistochemistry anti-*T. gondii* and anti-*N. caninum* were negative. Brain tissue samples were positive by all nested PCRs and the results of the multilocus analysis revealed the presence of *S. neurona*, which was called as *S. neurona*-07-2015-RS-BR. The complete nucleotide sequence of the nPCR-18S amplicon of *S. neurona*-07-2015-RS-BR (783 nucleotides, primers excluded) disclosed 100% identity and 100% coverage to *S. speeri* detected in opossums from Argentina (KT207459), and 99.87% identity and 100% coverage to *S. falcatula* detected in fatal *Sarcocystis* infection in the Rainbow Lorikeets (*Trichoglossus moluccanus*) (isolate Lorikeet #205850, MH626537) (Verma et al. 2018). A G-A substitution differs *S. neurona*-07-2015-RS-BR and *S. falcatula* isolate Lorikeet #205850 at nucleotide position 30 (taking MH626537 as reference). Only two single nucleotide polymorphisms differ *S. falcatula* isolate Lorikeet #205850 from *S. speeri* among 1618 paired nucleotides (G-A and C-A substitutions at nucleotide positions 30 and 1563, respectively, taking MH626537 as reference). nPCR-COX1

products of *S. neurona*-07-2015-RS-BR (547 nucleotides, primers excluded) differs from both *S. falcatula* (MH665257) and *S. speeri* (KT207461) at two nucleotide positions (A-T and C-A substitutions at nucleotide positions 347 and 497, respectively, taking KT207461 as reference). Curiously, *S. falcatula* MH665257 and *S. speeri* KT207461 disclosed 100% identity and 100% coverage among 1002 paired nucleotides. No gap was included in the alignments of 18S and COX1 from *S. neurona*-07-2015-RS-BR, *S. falcatula* isolate #205850, and *S. speeri*.

The almost complete nPCR-ITS1 amplicon was sequenced from S. *neurona*-07-2015-RS-BR (992 nucleotides). From the Blast search, the ITS1 sequence of *S. neurona*-07-2015-RS-BR is most similar to *S. speeri* detected in opossums in Argentina (KT207458), showing 97.89% identity, including 10 gaps. From the sequences annotated as *S. falcatula*, the most similar to *S. neurona*-07-2015-RS-BR is *S. falcatula* isolate Lorikeet #205850 (MH626538). These sequences share 97.08% nucleotide identity, including 16 gaps. ITS1 phylogenetic tree including *S. falcatula*, *S. neurona*, *S. speeri*, and *S. falcatula* related sequences can be found in Fig. 2. Curiously, the topology of the ITS1 tree shows that *S. neurona*-07-2015-RS-BR is clearly divergent from *S. neurona*/*S. speeri*. The monophyly of *S. neurona*/*S. speeri*/*S. neurona*-07-2015-RS-BR clade is supported with low bootstrap value (< 70, not shown).

Evolutionary relationships among *S. neurona* and *S. falcatula* inferred with SAG loci revealed that *S. neurona*-07-2015-RS-BR clustered closer to *S. neurona* (Fig. 3). In addition to *S. neurona*, the networks in Fig. 3 included *S. falcatula* obtained from budgerigars that were experimentally infected with opossum's sporocysts (Gondim et al. 2017, Cesar et al. 2018) and *S. falcatula* directly detected in intermediate hosts (Konradt et al. 2017, Acosta et al. 2018). At the three SAG loci, *S. neurona*-07-2015-RS-BR was identical to *Sarcocystis* sp. genotype II derived from opossum's sporocysts in the state of Rio Grande do Sul, Brazil, described by Monteiro et al. (2013).

## Discussion

In the present study, the diagnosis of *S. neurona*-associated meningoencephalitis in a domestic cat was established through the association of the pathological, immunohistochemical, and molecular findings. Data regarding CNS disease caused by *S. neurona* in domestic cats are scarce. In North America, a recent case report described encephalomyelitis in an 8-year-old cat associated with *S. neurona*-like infection. The ITS1 sequence showed the most similarity with *S. neurona* and *S. dasypi* (Zitzer et al. 2017). The experimental infection of domestic cats by *S. neurona*, with subsequent formation of cysts in their muscular tissue, has been reported (Dubey et al., 2001b), which suggests that cats may act as one of the intermediate hosts of *S. neurona*, at least in experimental conditions. Studies conducted in Michigan (Rossano et al. 2002), Missouri (Turay et al. 2002), and Ohio (Stanek et al. 2003) revealed antibodies to *S. neurona* in feral cats, indicating possible exposure and natural infection by *S. neurona* in domestic felines with access to the outdoors in general, similarly to what was observed in the present case. However, in some of the mentioned studies, antibody cross-reactivity against other closely *Sarcocystis* species could not be completely rule out (Rossano et al. 2002).

Rapid reproduction after oral infection of the intermediate hosts is likely responsible for the clinical manifestations (Wünschmann et al. 2009). Possibly, the use of corticosteroids, FeLV infection, and chemotherapy had great importance in the development of the clinical condition in the present case. Similarly, other immunosuppressive factors such as lesions, surgery, and stressful events have been associated with *S. neurona* infection in horses (Saville et al. 2001, Cooley et al. 2007). It has been suggested that the use of corticosteroids influences the proliferation of the protozoan in the CNS (Cooley et al. 2007), which may explain the aggravation of the clinical condition.

Microscopic lesions observed in the inspected organs were restricted to the brain and cerebellum and were characterized by meningoencephalitis associated with intralesional parasitic structures compatible with *S. neurona*, confirmed by immunohistochemistry and PCR. Inflammatory lesions were marked in the present case; however, unlike horses, in which *S. neurona* is rarely observed (Dubey et al. 2015), numerous parasites were noted within areas of inflammation. *Toxoplasma gondii* and *N. caninum*, two coccidians candidates that could be also involved in neurological infections, were ruled out because nPCR-ITS1 are capable of detecting both sarocystinae and toxoplasmatinae and genetic sequences of the latter was not found. In addition, immunohistochemistry anti-*N. caninum* and anti-*T. gondii* were negative.

To our knowledge, this is the first report on molecular characterization of *S. neurona* detected in a case of neurologic disease affecting an animal in Brazil. *Sarcocystis neurona* and *S. falcatula* are closely related organisms exclusively transmitted within the American continents that use opossums of the genus *Didelphis* as definitive hosts. Despite morphological and molecular similarities, *S. neurona* infects mammals predominantly, while *S. falcatula* exclusively infects birds. Two evolutionarily distant strains have been well recognized among isolates of *S. falcatula* in the Americas (Valadas et al. 2016, Gondim et al. 2017, Cesar et al. 2018), and to our knowledge, a similar situation has not yet been detected in *S. neurona*, which appeared to be formed by only one genetic lineage. However, from the results presented here, it is probable that this diversity also occurs within *S. neurona*. The characterization of the ITS1 locus of the *S. neurona*-07-

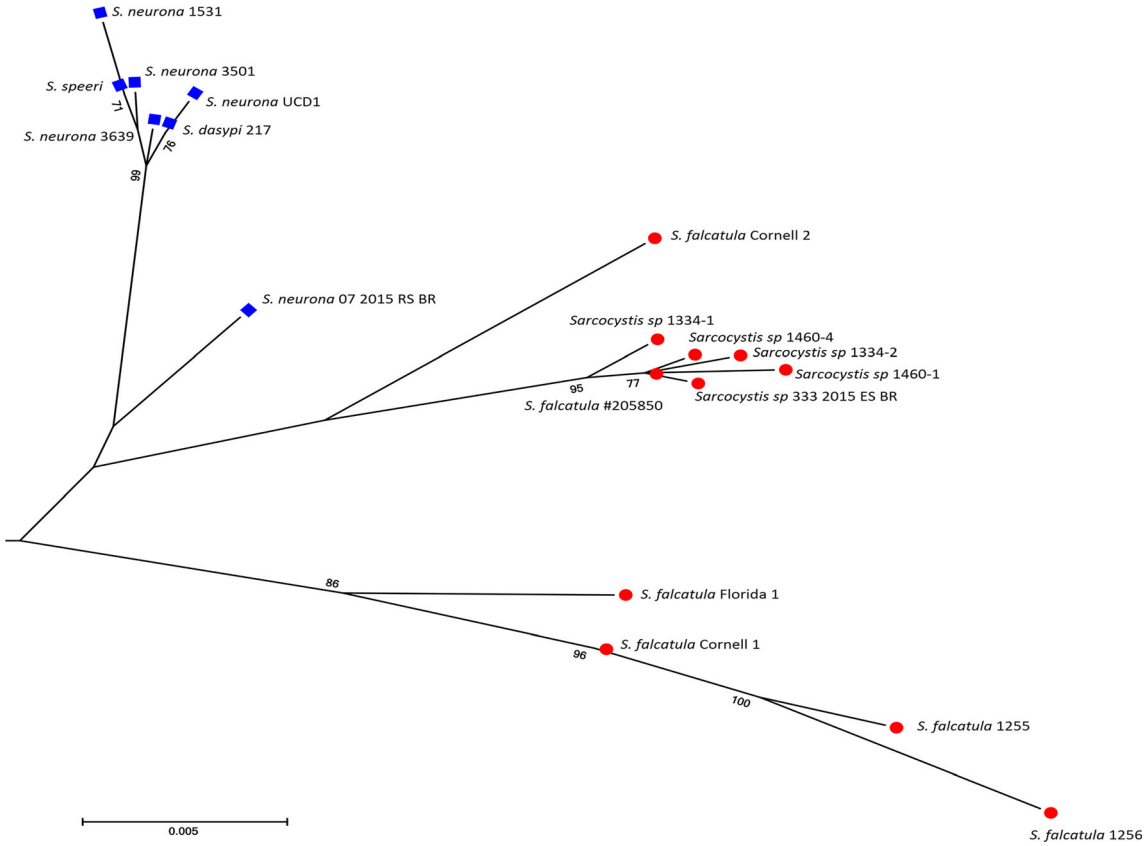


**Fig. 2** Evolutionary relationships among *S. neurona*, *S. speeri*, and *S. falcatula* inferred with ITS1. The tree is rooted with *S. lindsayi*. Bootstraps values less than 70 are not shown. *S. neurona*/*S. speeri* in blue, *S. falcatula* in red. The list of taxa presented in the tree is depicted in the supplementary table

2015-RS-BR isolate revealed an ITS1 lineage, which is considerably distant from other organisms classified as *S. neurona* (including *S. speeri*). By ITS1-based analysis, *S. speeri*, found in sporocysts of opossums in Argentina, is almost identical to *S. neurona* isolates found in several other hosts in North America and the clade that congregates all species of *S. neurona* and *S. speeri* with high statistical support excludes *S. neurona*-07-2015-RS-BR isolate.

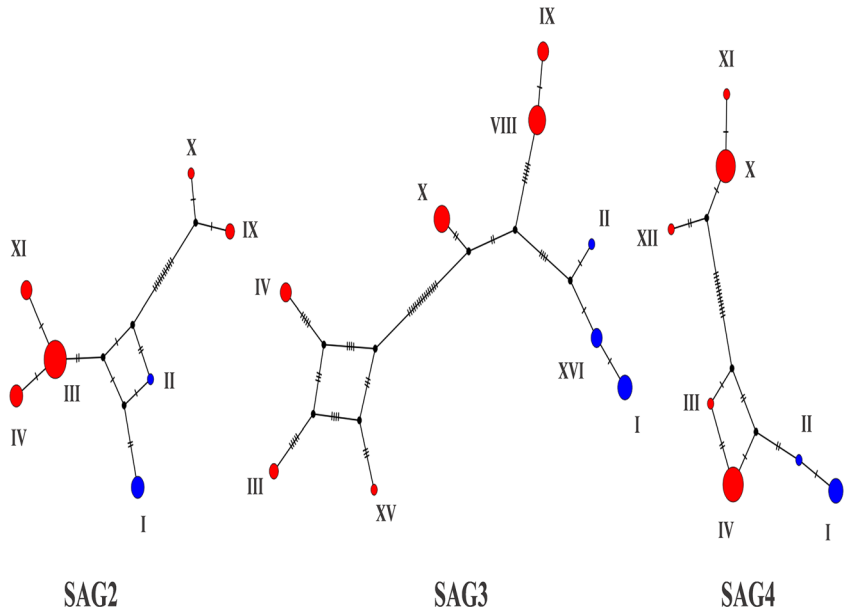


**Fig. 3** Phylogenetic networks on SAG2, SAG3, and SAG4 genotypes from *Sarcocystis* spp. *Sarcocystis neurona*-07-2015-RS-BR is represented by genotype #II in all networks. *S. neurona* in blue, *S. falcatula* in red. The size of the circles is proportional to the number of samples. Mutations are shown in hatch marks. The list of taxa presented in the networks is depicted in the supplementary table

Although COX1-based analysis does not distinguish *S. speeri* and *S. falcatula*, both species differ from the *S. neurona*-07-2015-RS-BR isolate by two nucleotide substitutions. It is worth noting that two nucleotide substitutions (considering the segment homologous to nPCR-COX1 fragment) differentiate *S. halieti* (MH138308) from *S. turdusi* (KT588511), *S. lari* (MF596283), and *S. lutrae* (MG273670), which are all recognized as distinct species (data not shown).

Herein, the divergence of isolate *S. neurona*-07-2015-RS-BR from *S. neurona* was further investigated with the SAG loci and the isolate 07-2015-RS-BR reveals, although not identical, very closely related to other samples of *S. neurona*, which allows concluding that the sarcocystid isolate found in the cat investigated may in fact be classified as *S. neurona*.

From the analysis of SAG2, SAG3, and SAG4, *S. neurona*-07-2015-RS-BR was identical to *Sarcocystis* sp. identified in opossum sporocysts in the state of Rio Grande do Sul, Brazil (Monteiro et al. 2013), indicating that variants of *S. neurona* different from those occurring elsewhere in the Americas are being transmitted in Brazil. Studies including more samples of *S. neurona* would be required to test this hypothesis, as well as to assess the impact of this diversity.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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