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Review Article

Sarcocystis neurona and related *Sarcocystis* spp. shed by opossums (*Didelphis* spp.) in South America

Sarcocystis neurona e *Sarcocystis* spp. relacionados, excretados por gambás (*Didelphis* spp.) na América do Sul

Luís Fernando Pita Gondim[1]*; Rodrigo Martins Soares[2]; Gastón Moré[3,4]; Rogério Fernando de Jesus[1]; Horwald Alexander Bedoya Llano[5]

[1] Departamento de Anatomia, Patologia e Clínicas, Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia – UFBA, Salvador, BA, Brasil
[2] Departamento de Medicina Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo – USP, São Paulo, SP, Brasil
[3] Consejo Nacional de Investigaciones Científicas y Técnicas – CONICET, Buenos Aires, Argentina
[4] Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina
[5] Grupo de Investigación – GINVER, Facultad de Medicina Veterinaria, Corporación Universitaria Remington, Medellín, Colombia

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Abstract

Protozoan parasites of the genus *Sarcocystis* are obligatory heteroxenous cyst-forming coccidia that infect a wide variety of animals and encompass approximately 200 described species. At least four *Sarcocystis* spp. (*S. falcatula*, *S. neurona*, *S. lindsayi* and *S. speeri*) use opossums (*Didelphis* spp.) as definitive hosts, and two of them, *S. neurona* and *S. falcatula*, are known to cause disease in horses and birds, respectively. Opossums are restricted to the Americas, but their distribution in the Americas is heterogeneous. Five *Didelphis* spp. are distributed in South America (*D. aurita*, *D. albiventris*, *D. marsupialis*, *D. imperfecta* and *D. pernigra*) whereas just one opossum species (*D. virginiana*) is found in North America. Studies conducted in the last decades show that *Sarcocystis* spp., derived from South American *Didelphis* spp., have biological and genetic differences in relation to *Sarcocystis* spp. shed by the North American opossum *D. virginiana*. The aim of this review was to address the peculiar scenario of *Sarcocystis* species shed by South American opossums, with a special focus on diagnosis, epidemiology, and animal infections, as well as the genetic characteristics of these parasites.

Keywords: *Sarcocystis neurona, Sarcocystis falcatula*, marsupial, Sarcocystidae.

Resumo

Parasitos protozoários do gênero *Sarcocystis* são coccídios heteroxenos formadores de cistos, que infectam variadas espécies animais e compreendem cerca de 200 espécies descritas. Pelo menos quatro *Sarcocystis* spp. (*S. falcatula*, *S. neurona*, *S. lindsayi* e *S. speeri*) utilizam gambás (*Didelphis* spp.) como hospedeiros definitivos; e duas delas, *S. neurona* and *S. falcatula* são conhecidas por causarem doença em equinos e aves, respectivamente. Gambás didelfídeos são restritos ao continente americano, contudo são distribuídos de forma heterogênea nas Américas. Cinco *Didelphis* spp. são distribuídos na América do Sul (*D. aurita*, *D. albiventris*, *D. marsupialis*, *D. imperfecta* e *D. pernigra*), enquanto somente uma espécie (*D. virginiana*) é encontrada na América do Norte. Trabalhos conduzidos, nas últimas décadas, mostram que *Sarcocystis* spp. derivados de *Didelphis* spp. sul-americanos possuem diferenças biológicas e genéticas, quando comparados a *Sarcocystis* spp. excretados pelo gambá norte-americano *D. virginiana*. O objetivo desta revisão é discutir a situação peculiar das espécies de *Sarcocystis* na América do Sul com um foco especial em diagnóstico, epidemiologia e infecções animais, assim como nas características genéticas desses parasitos.

Palavras-chave: *Sarcocystis neurona*, *Sarcocystis falcatula*, marsupial, Sarcocystidae.

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*Corresponding author: Luís Fernando Pita Gondim. E-mail: pita@ufba.br

Introduction

Sarcocystis spp. are obligatory cyst-forming Apicomplexan parasites that infect a broad spectrum of animal hosts (Levine, 1986). Despite the large variety of described *Sarcocystis* spp., with more than 200 named species, complete life cycles are known for less than a quarter of them (Dubey et al., 2016). Among the *Sarcocystis* spp. infecting mammalian and avian hosts, two, that use opossums (*Didelphis* spp.) as definitive hosts, *Sarcocystis falcatula* and *S. neurona*, may cause serious disease in birds (Smith et al., 1987a, b) and horses (Dubey et al., 1991), respectively.

In recent years, numerous studies have shown that *Sarcocystis* spp. shed by South American opossums differ, in several aspects, from those found in North America (Acosta et al., 2018; Cesar et al., 2018; Gondim et al., 2019; Gondim et al., 2017; Valadas et al., 2016). Moreover, other species have been described and referred to, such as *S. speeri* (Dubey & Lindsay, 1999), *S. lindsayi* (Dubey et al., 2001d) and *S. falcatula*-like (Dubey et al., 2000b). The differences and diversity of *Sarcocystis* spp. in South America may be partly related to the existence of five *Didelphis* spp. that may act as definitive hosts in this region (*Didelphis aurita*, *D. albiventris*, *D. marsupialis*, *D. imperfecta* and *D. pernigra*), contrasting with North America, where only one species of opossum is found (*D. virginiana*) (Cerqueira, 1985; Lemos & Cerqueira, 2002). The diversity of South American fauna acting as intermediate hosts for *Sarcocystis* spp. is also higher than that of North America, which thus allows a potentially elevated degree of genetic recombination in South American opossums.

The aim of the current study was to review the published knowledge on *Sarcocystis* spp. from South American opossums as definitive hosts, with emphasis on diagnosis, epidemiology and genetic characteristics of these parasites. Special focus is given to studies about *S. neurona* and *S. falcatula*.

Material and Methods

Peer-reviewed papers on *S. falcatula, S. falcatula*-like, *S. lindsayi, S. neurona* and *S. speeri* involving South American animals were retrieved using the following databases: PubMed (2020, 2021), Scopus (2020, 2021), Web of Science (2020, 2021) and SciELO (2020, 2021), as well as recent textbooks. Meeting abstracts and conference proceedings were not included as references. Additional papers, not restricted to South American studies on *Sarcocystis* spp. derived from opossums, were added to help in relation to basic information on the parasites and *Didelphis* spp..

Sarcocystis spp. using South American opossums as definitive hosts

The most common and most widely distributed South American opossum is *D. albiventris,* especially in Argentina and Brazil (Cerqueira, 1985). The first studies conducted to identify *Sarcocystis* spp. sporocysts in Argentinian and Brazilian opossums were developed in collaboration with Dr. J.P. Dubey between 1998 and 2001 (Dubey et al., 2001d; Dubey et al., 1999b). Species identification in these studies was performed based on opossums' derived sporocyst infectivity to avian species, particularly budgerigars (*Melopsittacus undulatus*), and to immunodeficient mice, mostly gamma-interferon gene knockout mice (KO). *Sarcocystis falcatula* and *S. lindsayi* are infective for birds while *S. neurona* and *S. speeri* are infective for mice (Dubey et al., 2016).

Among all *Sarcocystis* spp., *S. neurona* is probably the most studied species, because it causes neurological disease in horses and in some marine mammals. *Sarcocystis* spp. identified in opossums from Brazil and Argentina are summarized in Table 1. Most studies have aimed to isolate *S. neurona* from *D. albiventris*, but its frequency in South American opossums appears to be lower than expected (Dubey et al., 2016). Moreover, studies conducted in Argentinian *D. albiventris* detected sporocysts from *S. falcatula* in four samples (Dubey et al., 1999b) and *S. speeri* in two animals (Dubey et al., 2000e). Further characterization of two *S. falcatula* isolates from Argentina resulted in fatal infections for budgerigars (Dubey et al., 2000c). On the other hand, *S. speeri* induced non-fatal infections in KO mice and was transmitted via mouse tissues to *D. virginiana* (Dubey et al., 2000d). Similarly, the first studies conducted in Brazil identified *S. falcatula*-like in one specimen of *D. albiventris* (Dubey et al., 2000b), and later in one of *D. marsupialis* and eight of *D. albiventris* (Dubey et al., 2001c). *Sarcocystis neurona* was identified in two out of eight *D. albiventris* samples (Dubey et al., 2001b) and *S. lindsayi* from one of *D. albiventris* (Dubey et al., 2001d). The opossum *D. marsupialis* from Brazil was identified as another definitive host for *S. speeri* (Dubey et al., 2000a).

Recently, several DNA samples derived from *Sarcocystis* spp. sporocysts, and cultured *Sarcocystis* spp., obtained from Brazilian opossums, were identified as *S. falcatula*-like due to their genetic characteristics and/or experimental infectivity to budgerigars (Gondim et al., 2017; Monteiro et al., 2013; Valadas et al., 2016). In summary, only two *S. neurona* isolates have been obtained from opossums in South America, both in *D. albiventris* from Brazil (Dubey et al., 2001b).

Table 1. Identification of sporocysts and oocysts shed by opossums (*Didelphis* spp.) in Argentina and Brazil.

Country	State / province	Host	No. positive/no. tested (% pos)	Recovery of sporocysts	Bioassay	Molecular biology	Identified species	Reference
Argentina	Buenos Aires	*D. albiventris*	4/4	Intestinal scraping	Budgerigars (+)	ND	*S. falcatula*	(Dubey et al., 1999b)
					ɣ -IFN KO mice (-)			
	Buenos Aires	*D. albiventris*	2/2 [A]	Intestinal scraping	ɣ -IFN KO mice (+)	ND	*S. speeri*	(Dubey et al., 2000e)
					nude Swiss mice (-)			
Brazil	São Paulo	*D. albiventris*	1/3 (33.3%)	Intestinal scraping	Budgerigars (+)	ITS + RFLP (HinfI / DraI), *locus* 25/396	*S. falcatula*-like	(Dubey et al., 2000b)
					ɣ-IFN KO mice (-)	Large subunit ribosomal, ITS1	Posteriorly identified as *S. lindsayi*	(Dubey et al., 2001b)
	São Paulo	*D. marsupialis*	1/NI	Intestinal scraping	Budgerigars (-)	ND	*S. speeri*	(Dubey et al., 2000a)
					ɣ-IFN KO mice (+)			
					Nude mice (+)			
	São Paulo	*D. marsupialis* (1) *D. albiventris* (8)	9/NI	Intestinal scraping	Budgerigars (+)	ITS + RFLP (HinfI / DraI), *locus* 25/396, large subunit ribosomal, ITS1	*S. falcatula*-like	(Dubey et al., 2001c)
	São Paulo	*D. albiventris*	8/NI	Intestinal scraping	ɣ-IFN KO mice (+)	ITS + RFLP (HinfI / DraI), *locus* 25/396	*S. neurona,* *S. speeri*	(Dubey et al., 2001b)
	Rio de Janeiro	*D. aurita*	3/5 (60%)	Intestinal scraping	Budgerigars (+)	ND	*S. lindsayi*-like	(Stabenow et al., 2008)
	São Paulo	*D. aurita* (66 dead)	6/66 (9.1%) *D. aurita* (dead)	Intestinal scraping, fecal flotation (live animals)	ND	ND	*Sarcocystis* sp.	(Casagrande et al., 2009)
		D. albiventris (32 dead)	0/32 (0%) *D. albiventris* (dead)					
		D. aurita (28 alive)	2/28 (7.1%) *D. aurita* (alive)					
		D. albiventris (05 alive)	0/5 (0%) *D. albiventris* (alive)					
	Rio de Janeiro	*D. aurita*	3/9 (33.3%)	Intestinal scraping	Budgerigars (+)	ND	*S. lindsayi*	(da Silva Stabenow et al., 2012)
	Rio Grande do Sul	*D. albiventris,* *D. aurita*	27/NI	Intestinal scraping, fecal flotation	ND	SAG2, SAG3, SAG4	*S. falcatula*-like	(Monteiro et al., 2013)

References: ND = not done; NI = not informed; [A] = Both opossum samples were previously identified as having *S. falcatula* sporocysts (Dubey et al., 1999b)

Table 1. Continued...

Country	State / province	Host	No. positive/no. tested (% pos)	Recovery of sporocysts	Bioassay	Molecular biology	Identified species	Reference
	São Paulo	*Didelphis* sp.	25/NI	Intestinal scraping	ND	SAG2, SAG3, SAG4, ITS1, CytB		(Valadas et al., 2016)
	Bahia	*D. albiventris* (16)	0/39	Intestinal scraping	Budgerigars (+)	SAG2, SAG3, SAG4, ITS1, CytB, *locus* 25/396	*S. falcatula*-like	(Gondim et al., 2017)
		D. aurita/ *marsupialis* (23)	9 positive samples from independent study of São Paulo state		ɣ-IFN KO mice (-)			
	Rio de Janeiro	*D. aurita*	8/13 (61.5%)	Intestinal scraping, fecal flotation	Budgerigars (+)	ITS + RFLP (HinfI / DraI), ITS	*S. falcatula*-like	(Gallo et al., 2018)
					Nude mice (-)		Maybe *S. lindsayi* in one sample	
	São Paulo	*D. albiventris* (1)	12/NI	Intestinal scraping	Budgerigars (+)	SAG2, SAG3, SAG4,	*S. falcatula*-like	(Cesar et al., 2018)
		D. aurita/ *marsupialis* (11)				ITS1, CytB, 18S		
	Bahia	*D. aurita*/ *marsupialis*	1/1	Intestinal scraping	Budgerigars (+)	SAG2, SAG3, SAG4, ITS1, 18S, Cox1, *locus* 25/396	*S. falcatula*-like	(Gondim et al., 2019)

References: ND = not done; NI = not informed; [A] = Both opossum samples were previously identified as having *S. falcatula* sporocysts (Dubey et al., 1999b)

Genetic characteristics of *Sarcocystis* spp. shed by South American opossums

Overview on molecular identification of *Sarcocystis* spp.

The taxonomy of the genus *Sarcocystis* is based on the morphological characteristics of the sarcocysts, life cycle characteristics (host specificity) and molecular data (Dubey et al., 2016; Lindsay et al., 1995).

However, the morphology of mature sarcocysts, the structures typically found in the natural intermediate hosts of the parasite, such as size, shape, and cyst wall structure, may be shared by several closely related species (Gjerde, 2013). Moreover, *Sarcocystis* species might be inaccurately described because the structural characteristics of sarcocysts may vary according to the method of fixation, the degree of development (age) of the sarcocyst and the type of tissue in which it is found, among other factors (Dubey et al., 2016).

Since each species of *Sarcocystis* may have a particular spectrum of hosts, knowledge on infectivity to intermediate, definitive and eventual aberrant hosts, might offer information of great value for identification of the parasite (Butcher et al., 2002; Cutler et al., 1999; Dubey & Lindsay, 1998). Full knowledge of the host specificity of the parasite is often difficult to obtain, since it depends on experimental infections or on detection of the parasite in naturally infected animals, in wild or domestic fauna.

Thus, molecular data, especially those based on phylogenies, have become mandatory for identification of species of the genus *Sarcocystis* (Gjerde, 2014; Gjerde et al., 2017; Morrison et al., 2004). Molecular data have made it possible to discriminate between species that had previously been considered synonymous, by means of morphological analysis or expansion of the knowledge of their host spectrum. Moreover, *S. falcatula* and *S. neurona* were determined to definitely be distinct species by means of molecular differentiation (Marsh et al., 1999). As additional example, molecular analysis on a sarcocyst in muscle tissue, identified herring gull (*Larus argentatus,*

order Charadriiformes) as a new intermediate host for *S. wobeseri*; this *Sarcocystis* species had exclusively been found in the order Anseriformes (geese and ducks) (Prakas et al., 2011).

Molecular analyses on viruses and bacteria have become significantly enhanced through next-generation sequencing (NGS)-based technologies, in which complete genomes are evaluated within a few hours. On the other hand, technologies based on NGS are less commonly used for routine investigation of molecular diversity in eukaryotes, due to the large size and complexity of their genomes, along with the high costs involved (Maljkovic Berry et al., 2020). Thus, traditional approaches based on the Sanger sequencing method, directed to complete or partial gene segments, have been widely used to access the diversity of eukaryotic organisms, including protozoa of the genus *Sarcocystis*.

The nuclear genome sequences encoding ribosomal RNA products and the genes encoding mitochondrial products in the genome of this organelle are the molecular markers most used for identifying *Sarcocystis* species (Gjerde, 2013, 2016). The gene encoding the small ribosomal unit (18S rDNA) has been identified for most *Sarcocystis* spp. (Dubey et al., 2016). The 18S rDNA gene alternates highly conserved and highly variable domains (Morrison et al., 2004; Morrison & Ellis, 1997; Ogedengbe et al., 2016), which makes it possible to design of universal primers that are complementary to conserved regions (helices) that flank segments of high variability (loops). Therefore, this favors detection and identification of unknown species belonging to the genus.

Although 18S rDNA is widely used for molecular identification of *Sarcocystis* spp., closely related species are occasionally almost identical at this locus, as is the case of *S. falcatula* and *S. neurona*. These species had erroneously been regarded as synonymous because of minimal differences at this locus (Dame et al., 1995; Fenger et al., 1995). In fact, 18S rDNA gene is not sufficiently variable to differentiate between certain *Sarcocystis* species that use birds as intermediate hosts (Olias et al., 2010; Prakas et al., 2013). However, because 18S rDNA is considered to be a universal marker for molecular identification, it is highly recommended that new descriptions of species of the genus *Sarcocystis* should include nucleotide sequences of this gene. Thus, 18S sequences of new *Sarcocystis* spp. will enable phylogenetic comparisons and reconstructions with homologous sequences that are available in public-access databases (Dubey et al., 2016; Morrison et al., 2004).

With phylogenetic resolution slightly superior to that of 18S rDNA, the gene encoding the large ribosomal unit (28S rDNA) (Mugridge et al., 1999) has also been used to identify *Sarcocystis*, although much less frequently. While a search at GenBank using the terms [18S + *Sarcocystis*] brings 2166 items, the same search with the terms [28S + *Sarcocystis*] brings 231 items (NCBI, 2020).

The mitochondrial gene encoding cytochrome c oxidase subunit I (COI) is a molecular marker that has shown good phylogenetic resolution for discrimination of living organisms among different taxa (Pentinsaari et al., 2016), including those among the *Sarcocystis* genus. The COI genetic sequences have been successfully used to discriminate among *Sarcocystis* spp., using ruminants as intermediate hosts (Gjerde, 2013). However, the differences identified at this locus are minimal among *Sarcocystis* spp. that use birds or carnivorous mammals as intermediate hosts (Gjerde et al., 2017).

As mentioned above, the genes encoding ribosomal RNA and the genes encoding mitochondrial products may not be variable enough to discriminate between certain *Sarcocystis* spp. In these cases, markers with higher evolutionary rates, such as the first and second internal transcribed spacers (ITS1 and ITS2, respectively), can be used (Marsh et al., 1999). The ITS1 and ITS2 sequences are located between coding sequences of the ribosomal units 18S and 5.8S and between 5.8S and 28S, respectively (Hillis & Dixon, 1991). Public-access databases have a much larger number of ITS1 sequences than ITS2 for *Sarcocystis* spp., and thus the first locus has been used most to discriminate between species (Watthanakaiwan et al., 2017). As in other ribosomal loci, the advantages of using ITS1 and ITS2 are the universality and sensitivity of the assays that aim towards these loci, since the ribosomal loci are present in several copies within the eukaryote genome (Alvarez & Wendel, 2003). However, because ITS1 and ITS2 have much higher evolutionary rates than 18S rDNA, COI and 28S rDNA, phylogenetic studies using these markers need to only contain very similar organisms. ITS sequences from distant organisms cannot be unambiguously aligned because many insertions and deletions are present, which greatly reduces the reliability of the phylogenetic alignment (Prakas et al., 2013). For this reason, ITS-based phylogenies for the genus *Sarcocystis* should not be rooted with organisms of other genera. The ITS1-based phylogeny of species of Toxoplasmatinae could not be rooted because the sequences could not be aligned against the outgroup (Ellis et al., 1999). ITS1 sequences of *Sarcocystis* spp. typically range from 600 to 1000, while ITS1 of Toxoplasmatinae species encompass about 500 nucleotides in length.

From the above, it is clear that more than one gene is needed to produce a species phylogeny for the *Sarcocystis* genus, in order to obtain consistent identification.

Genetic characterization of *Sarcocystis* spp. excreted by didelphid opossums in Brazil

Marsupials of the genus *Didelphis*, which are exclusive to the Americas, are definitive hosts of at least four morphologically and very similar *Sarcocystis* spp.: *S. neurona*, *S. falcatula*, *S. lindsayi* and *S. speeri*. In addition to the morphological similarity, these four species are closely related from a phylogenetic point of view, although they can be differentiated using molecular methods.

Due to the high similarity between *S. falcatula* and *S. neurona* at 18S rDNA, it was initially suggested that these species were synonymous (Dame et al., 1995). However, through comparing ITS1 sequences, Marsh et al. (1999) demonstrated that *S. falcatula* and *S. neurona* were, in fact, organisms with significant divergence and could be unequivocally differentiated through this marker. They also identified considerable diversity of ITS1 among samples of *S. falcatula*, thus indicating that this species should constitute a heterogeneous population.

Sarcocystis speeri and *S. neurona* are infectious species for mammals and have high molecular similarity. Although a few morphological differences between these two species have been identified, these agents are practically identical at molecular level (Dubey et al., 2015b). Complete ITS1 sequences from these two agents have up to 99.7% identity (only 3 SNPs difference), which strongly suggests that they are synonymous species.

Sarcocystis lindsayi and *S. falcatula* are species that are infectious for birds and, contrary to what is observed between *S. neurona* and *S. speeri*, have much more extensive molecular differences. At ITS1, *S. lindsayi* has 93.3% and 92.6% identity with *S. falcatula* and *S. speeri*, respectively, although at 28S, these three species are almost identical (Dubey et al., 2001d). The only isolate of *S. lindsayi* that has been molecularly identified was from budgerigars (*Melopsittacus undulates*) experimentally infected with *Didelphis albiventris* sporocysts from Brazil (reference).

Sarcocystis spp. excreted by opossums of the genus *Didelphis* have intraspecific diversity. Tanhauser et al. (1999) and Marsh et al. (1999) demonstrated that some North American isolates of *Sarcocystis* spp. derived from opossum sporocysts were distinct from both *S. neurona* and *S. falcatula*. Through sequencing ITS1, these isolates were found to be 96.0-96.8% and 95.5-96.4% similar to *S. neurona* and *S. falcatula*, respectively (Tanhauser et al., 1999). Although at that time *S. lindsayi* had not yet been described, it is now well known that these isolates are even more divergent from *S. lindsayi*. Later on, isolates that were equally divergent at ITS1 from both *S. neurona* and *S. falcatula* were detected in Brazil. These isolates had proved infectious for budgerigars, and they were named *Sarcocystis falcatula*-like organisms (Dubey et al., 2001c). Since then, the *S. falcatula*-like ITS1 genotype has been systematically found in isolates from Brazil, both in budgerigars experimentally infected with opossum sporocysts (Cesar et al., 2018; Gondim et al., 2017) and in organs of naturally infected wild birds (Acosta et al., 2018; Konradt et al., 2017).

The *S. falcatula* ITS1 alleles described in the studies by Marsh et al. (1999) and Tanhauser et al. (1999) have never been detected in *Sarcocystis* spp. from South America, but *S. falcatula*-like ITS1 alleles were recently described in the United States in naturally infected rainbow lorikeets (Verma et al., 2018). Further studies are needed to test the hypothesis that this diversity is due to heterogeneity of ITS1 copies. It is well known that the ribosomal locus is present in more than one copy in the apicomplexan nuclear genome (Morrison & Ellis, 1997; Mugridge et al., 2000). Nevertheless, no sequence data with a mixture of sequences compatible with the simultaneous occurrence of the two alleles have yet been described. The allelic diversity of *S. falcatula*-like ITS1 has been described, but not at the nucleotide positions that consistently differentiate between *S. falcatula* and *S. falcatula*-like alleles (Marsh et al., 1999).

PCR and restriction endonuclease digestion (PCR-RFLP) of the locus JNB 33/54 were formerly used in the differential diagnosis of *S. neurona* and *S. falcatula* (Tanhauser et al., 1999). The endonuclease *Dra*I is able to cut the 1100 bp JNB 33/54 PCR products of *S. neurona* isolates, but not from *S. falcatula*. Conversely, *Hinf*I does not cut the 1100 bp product of *S. neurona*, but cuts those from *S. falcatula*. However, PCR-RPLP of the locus JNB 33/54 of some isolates of opossum derived *Sarcocystis* sp. were cut by both endonucleases and such isolates have been identified as *S. falcatula*-like. Using this molecular method, Gallo et al. (2018) demonstrated for the first time that *S. falcatula*-like could be shed by the *Didelphis aurita*.

There are only two reports on molecular descriptions of *S. neurona* in intermediate hosts in Brazil. These include a naturally infected cat with meningoencephalitis (Hammerschmitt et al., 2020) and naturally infected horses with EPM (Henker et al., 2020). Both studies were conducted in the state of Rio Grande do Sul, Brazil. The molecular identification of the parasite in these studies revealed ITS1 alleles with less than 98% similarity to the

homologous sequences of *S. neurona* and *S. speeri* that had been described up to that time. Moreover, phylogenetic reconstructions have shown that the parasites were related to *S. neurona* (Hammerschmitt et al., 2020; Henker et al., 2020). The isolate from the cat (Hammerschmitt et al., 2020) and isolates of *S. falcatula* and *S. neurona* were also compared at genes encoding surface antigens and the results corroborated the information obtained using ITS1. Interestingly, this feline sample was revealed to be identical, at ITS1 and surface antigen genes, to sporocysts of *Sarcocystis* spp. detected in didelphid opossums in Brazil (Valadas et al., 2016).

Evidence of *S. neurona* infection in cats was also reported by Lucio et al. (2021). In this study, the authors described the natural occurrence of *S. neurona* muscular sarcocysts in cats without *Sarcocystis*-associated disease. However, in spite of the fact that sarcocysts were unequivocally identified in skeletal muscle of cats, the molecular identification of the parasites was performed by using genetic fragment with insufficient discriminatory power to differentiate species within the genus *Sarcocystis*.

The results from Hammerschmitt et al. (2020) and Henker et al. (2020) suggest that more than one strain of *S. neurona* also occurs in the Americas. While the *S. neurona* genotype detected in cats and horses in Brazil has not yet been detected elsewhere, alleles relating to the other *S. neurona* strains have been detected across the America, in *D. virginiana* (Dubey & Lindsay, 1998) and *D. albiventris* (Dubey et al., 2001b), as well as in many intermediate hosts (Dubey et al., 2015a).

After identifying the molecular diversity of *Sarcocystis* spp. detected in 50 samples of intestinal scraps from didelphid opossums in Brazil, Valadas et al. (2016) found ITS1 sequences from *S. falcatula*-like, but not from *S. falcatula*. In addition to *S. falcatula*-like, they identified four alleles of ITS1, among which one was identical to the ITS1 of *S. neurona* detected in a cat by Hammerschmitt et al. (2020) in Brazil. The other three alleles were phylogenetically related, but distant from the four known species of opossum derived *Sarcocystis*. These three alleles are possibly from a still-unknown species of *Sarcocystis*.

The intraspecific diversity of *Sarcocystis* spp. shed by didelphid opossums has been identified through multilocus analyses. Multilocus characterizations of *S. falcatula*-like were performed after analyses on three loci encoding surface antigens: SAG2, SAG3 and SAG4. From SAG-based multilocus analyses on isolates obtained from budgerigars experimentally infected with opossum sporocysts (Cesar et al., 2018; Gondim et al., 2017) and from naturally infected birds (Acosta et al., 2018; Konradt et al., 2017), four SAG2 alleles, five SAG3 alleles and four SAG4 alleles have been identified in *S. falcatula*-like. Interestingly, each SAG-based phylogeny demonstrates the occurrence of two families (clades) of alleles with significant inter-group diversity (Figure 1). These alleles seem to randomly recombine, but the biological implications of the SAG-admixture, such as pathogenicity or infectivity to the host, are yet to be clarified.

The diversity of *S. falcatula*-like organisms revealed through multilocus analysis contrasts with the results from similar investigations carried out using samples of *S. neurona* detected in different mammals in North America (Barbosa et al., 2015; Rejmanek et al., 2010; Wendte et al., 2010). All the samples of *S. neurona* investigated by these authors were identical at ITS1 and were almost identical at the SAG2, SAG3 and SAG4 alleles. Rejmanek et al. (2010) found no variability in SAG2 and only one polymorphic site in SAG4, between isolates from different mammal species such as horses, didelphids, felines and sea otters. Among marine mammals, the variability of *S. neurona* in SAG3 and SAG4 (Barbosa et al., 2015; Wendte et al., 2010) is much less than that found among *S. falcatula*-like detected in parakeets experimentally infected with sporocysts from *Didelphis* spp. (Cesar et al., 2018; Gondim et al., 2017). While only seven polymorphic sites in SAG3 and one in SAG4 (in about 1000 nucleotides analyzed) discriminated 37 isolates of *S. neurona* sampled by Barbosa et al. (2015), up to 30 polymorphic sites were detected in SAG3 and 17 in SAG4 (in about 300-400 nucleotides analyzed) among different isolates of *S. falcatula*-like obtained from experimentally infected parakeets.

Didelphid opossums originated in South America and the genus possibly expanded its range to North America before the rise of the Panamanian land bridge was completed by 3.1–2.7 Ma and the Great American Biotic Interchange took place (Dias & Perini, 2018). A new separation between these continental masses occurred after the construction of the Panama Canal at the end of 19th century. Dias & Perini (2018) also suggested that, after a northern South American origin for the genus, climatic-driven modifications in Neotropical environments during the late Pliocene and early Pleistocene favored the dispersion to new ecosystems triggering the emergence of new lineages. Two highly similar lineages of *S. falcatula* and two highly similar lineages of *S. neurona* may have recently emerged. Molecular clock tests would help to date when the differentiation between these strains occurred and whether such a tiny divergence would be related in time to the migratory movements of marsupial species across the Americas.

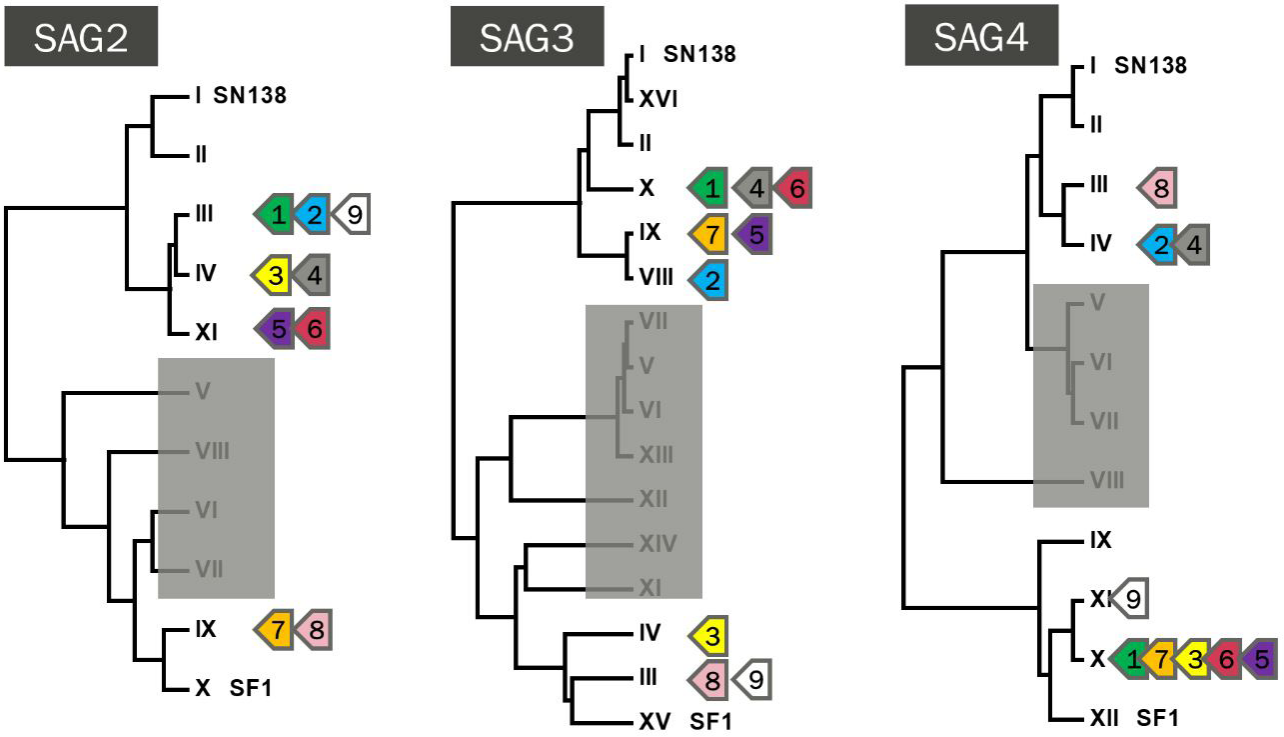

Figure 1. Published in Gondim et al. (2019). Dendrograms on *SAG2*, *SAG3* and *SAG4* genotypes from *Sarcocystis* spp. that use Brazilian *Didelphis* spp. as definitive hosts, as proposed by Monteiro et al. (2013) and Valadas et al. (2016). Arrows 1 to 7 correspond to *Sarcocystis* spp. genotypes derived from budgerigars that were experimentally infected with *Didelphis* spp. sporocysts (Cesar et al., 2018; Gondim et al., 2017). Arrow 8 identifies a genotype of *S. falcatula* isolated from a naturally infected bare-faced ibis (*Phimosus infuscatus*) (Konradt et al., 2017). Arrow 9 represents a *S. falcatula*-like isolate from Bahia (Gondim et al., 2019) and those observed in Magellanic penguins (Acosta et al., 2018). SN138 (Lindsay et al., 2004) and SF1 (Marsh et al., 1997) are reference strains of *Sarcocystis neurona* and *Sarcocystis falcatula*, respectively. Alleles in shaded boxes correspond to genotypes identified in opossum-derived sporocysts that have not been associated with *S. falcatula* so far.

Studies on population genetics or experimental infections are needed in order to test the hypothesis that the two molecular types of *S. neurona* and the two molecular types of *S. falcatula* are in fact distinct species. Likewise, further studies are needed to assess the epidemiological or biological relevance of the extensive intraspecific diversity detected in *S. falcatula*-like organisms.

Confusion on classification between *Didelphis aurita* and *D. marsupialis*

Six species of Didelphidae opossums have been identified in the Americas. Whereas only one species (*D. virginiana*) is found in North America, five *Didelphis* spp. exist in South America, which have been grossly divided into white-eared opossums (*D. albiventris*, *D. pernigra* and *D. imperfecta*) and black-eared opossums (*D. aurita* and *D. marsupialis*) (Cerqueira, 1985; Lemos & Cerqueira, 2002). Combination of morphological and molecular studies on *Didelphis* spp. has significantly aided in differentiation and characterization of each separate species (Cervantes et al., 2010; Sousa et al., 2012).

Some published reports on *Sarcocystis* spp. derived from South American opossums seem to show confusion regarding the classification of *D. aurita* and *D. marsupialis*, which are morphologically similar, but have different geographical distributions. In a study conducted in southeastern Brazil, *S. speeri* sporocysts were reported from intestinal scraps of *D. marsupialis* that inhabited the vicinities of the city of São Paulo (Dubey et al., 2000a). Sporocysts derived from an opossum referred to as *D. marsupialis* (Dubey et al., 2000a; Dubey et al., 2001b) were used in a second study (Dubey et al., 2001c) in which *S. falcatula*-like in *D. marsupialis* from São Paulo was reported. It is noteworthy that *D. marsupialis* does not exist in the state of São Paulo: this species has only been described in the northern and central regions of the country (Cerqueira, 1985; Lemos & Cerqueira, 2002). The findings of

Sarcocystis spp. in *D. marsupialis* reported in the state of São Paulo were probably related to *D. aurita*, which is the only black-eared opossum in this state.

Due to morphological similarities between *D. aurita* and *D. marsupialis*, some authors have referred to black-eared opossums as *D. aurita*/D. *marsupialis*, given that precise differentiation between these species requires molecular analysis, which has poor availability (Gondim et al., 2017).

Diagnosis of Equine Protozoal Myeloencephalitis (EPM) and *S. neurona* in horses

Equine protozoal myeloencephalitis (EPM) was first diagnosed in Brazil several decades ago. In one of the first published studies, 77 thoroughbred horses in the state of São Paulo, including animals with neurological disease, abortion and without clinical signs, were examined for *T. gondii* antibodies. Based on the serological results, the authors assumed that clinical signs were attributable to toxoplasmosis (Macruz et al., 1975). Another study reported the presence of protozoal organisms in the spinal cord of a 10-year-old horse (Lombardo de Barros et al., 1986); however, at that time, the causative agent was not elucidated. A few years later, two horses were diagnosed with neurological disease caused by *Sarcocystis* sp. infection, given that mature schizonts and merozoites were associated with the lesions (Masri et al., 1992); one of the horses was born and reared in Brazil, while the other was born in Argentina and reared in Brazil. Protozoal organisms were labelled in CNS tissues by means of immunohistochemistry (IHC) using a polyclonal serum against *S. cruzi* as the primary antibody. However, the authors assumed that *S. neurona* was the causative agent of the lesions (Masri et al., 1992), since *S. neurona* had been isolated and classified from a horse with EPM in the United States (Dubey et al., 1991).

In a retrospective study conducted in Minas Gerais, formalin-fixed fragments from the central nervous system (CNS) of 187 horses and 16 mules that had been placed in storage between 1942 and 2005 were screened for lesions (Paixão et al., 2007). Inflammation was observed in 54 samples that were processed for immunohistochemical analysis using a primary antibody against *S. neurona* that was provided by a laboratory in the USA (Paixão et al., 2007). Severe multifocal nonsuppurative encephalitis was observed in a seven-year-old thoroughbred horse that had been admitted to the Animal Hospital in 2004 and was treated with corticosteroid (dexamethasone), DMSO, vitamin B1 and fluid therapy. The clinical condition of the horse progressed to paralysis of the limbs and the animal was euthanized (Paixão et al., 2007).

In Argentina, many anecdotal results circulate among horse breeders and veterinarians. However, there has only been one recent confirmed EPM case report, on a 12-year-old mare from Buenos Aires Province that presented asynchronous walking and bilateral stringhalt. It was decided to euthanize the animal because of severe clinical progression and lack of response to treatment. Microscopic lesions were observed in brain and spinal cord samples and IHC analysis revealed the presence of *S. neurona* meronts and free merozoites. PCR-RFLP analysis showed a specific *S. neurona* restriction pattern in brain samples (Moré et al., 2019).

In a retrospective study in Brazil, formalin-fixed and paraffin-embedded tissues from 38 horses that presented myelitis, encephalitis and/or meningitis were examined for EPM (Henker et al., 2020). Thirteen of the horses tested were diagnosed as having EPM based on the following criteria: mononuclear perivascular cuffing, inflammatory infiltrate of eosinophils and multinucleated giant cells. Immunostaining using a polyclonal serum for *S. neurona* was positive in 11 horses, and partial nucleotide sequences of ITS1 from 6 horses presented the best match with *S. neurona* (Henker et al., 2020).

So far, no *in vitro* isolation of *S. neurona* has been obtained from affected horses in South America. In a study conducted on Brazilian opossums (*D. albiventris*), *Sarcocystis* sporocysts were detected and shipped to the US and processed by means of mouse bioassay. The parasite isolated in cell culture from murine tissues was classified as *S. neurona* (Dubey et al., 2001b). No subsequent studies on this *S. neurona* isolate have been published.

Serological studies on South American horses

Indirect or serological diagnostic techniques are an important tool for identifying exposure to *S. neurona* in horses, as well as for aiding in EPM monitoring and clinical intervention (Dubey et al., 2016). The Western blot or Immunoblot test (IB), using a full extract of merozoite antigen, was the first assay developed for qualitative detection of antibodies against *S. neurona* in horses (Granstrom et al., 1993). Antibodies against *S. neurona* were attached to immune dominant antigens (IDA) both in serum and CSF (cerebral spinal fluid) samples. However, the results were only semi-quantitative (i.e. negative, weak-positive and positive). This test has been extensively used

in North America, but because it is laborious and requires significant expertise for accurate interpretation, several other assays for identifying and quantifying antibodies against *S. neurona* have subsequently been developed (Dubey et al., 2015a). Among these assays, the immunofluorescent antibody test (IFAT) and various enzyme-linked immunosorbent assays (ELISAs) are widely used for EPM diagnosis and seroepidemiological studies in South America (Table 2). In the absence of cultured *S. neurona* isolates derived from South American animals, serological studies on horses have been conducted with merozoites from North American strains or proteins (recombinant or crude) of the parasite (Table 2).

An IFAT for *S. neurona* was developed and consisted of whole-cell culture-derived merozoites (Duarte et al., 2003). It allowed detection of antibodies to *S. neurona* surface antigens, but some of these antigens are probably shared among different species of the genus *Sarcocystis* (Dubey et al., 2016). Therefore, use of IFAT requires validation and proper definition of cutoff titers in order to avoid false-positive results due to antibodies against *Sarcocystis* spp. other than *S. neurona* (Dubey et al., 2016).

Several ELISAs have been developed using *S. neurona* merozoite surface antigens (SnSAGs), mainly expressed as recombinant proteins (Dubey et al., 2015a). The largest serological study performed on South American horses was based on SnSAG4 ELISA (Hoane et al., 2006). However, due to the possibility of false-positive results from using a single antigen protein and through variation of SnSAGs among isolates, an improved SnSAG ELISA was developed, based on three recombinant surface antigens (Reed et al., 2013).

The majority of seroepidemiological studies performed on South American equids have been conducted in Brazil, and have been carried out in several states, distributed in the five main regions of the country (North, Northeast, Center-West, Southeast and South) (Table 2).

Combining all the published seroepidemiological surveys conducted in South America, more than 5000 horses have been tested for *S. neurona* antibodies by means of different techniques. The most frequently used test in these studies was IFAT, followed by ELISAs and, at a lower proportion, by IB (Table 2). Samples tested using IFAT have shown a range of seropositivity, from 2.8 to 84.1%, with most studies showing values around 40% (Table 2). IFAT has been conducted with cutoffs between 1:20 and 1:80. Even in some studies using low cutoffs (1:25 or 1:50), the frequencies of antibodies in horses were below 50% (Antonello et al., 2015; Cazarotto et al., 2016; Koch et al., 2019; Portella et al., 2017; Spohr et al., 2018). It is noteworthy that serological studies conducted using SnSAG4 ELISA reported higher antibody frequencies (higher than 60% in most regions) than studies conducted using IFAT (Table 2). On the other hand, the few populational studies conducted using IB showed moderate positivity ranging from 26.1 to 35.6% (Dubey et al., 1999a; Moré et al., 2014). As previously mentioned, the studies conducted by means of *S. neurona* ELISA and IFAT presented the possibility of detection of some false-positive results, due to horse antibodies against other *Sarcocystis* spp. In addition, the studies using combinations of tests or IB as confirmatory after a positive IFAT or ELISA result showed a lower number of "true positive" samples (Borges et al., 2017; Valença et al., 2019). A study performed in Argentina using IB revealed a twofold seropositivity rate (odds ratio 2.27) in horses with neurological signs, compared with horses without clinical signs (39.2% versus 22.1%, respectively), thus suggesting that *S. neurona* might be implicated in the occurrence of neurological disorders (Moré et al., 2014). In the same study, 71% of the samples showed reactivity to protein bands with a relative motility of 30 kDa (which includes the antigens SnSAG1 and SnSAG4). This result is comparable what was obtained using SnSAG4 ELISA in Brazil and could represent the presence of antibodies against other *Sarcocystis* spp., rather than against *S. neurona*.

Serological studies performed in Brazil with precolostral foal serum samples have suggested that there is potential for vertical transmission of *Sarcocystis* spp. in horses (Antonello et al., 2016; Pivoto et al., 2014). However, Antonello et al. (2016) found that 7.4% (14/189) of their foal samples were seropositive according to IFAT at low antibody titers, and the same samples were negative using IB. Pivoto et al. (2014) detected that 6.6% (12/181) of their samples were positive according to an ELISA using total lysate antigen (TLA) from *S. neurona*. Altogether, these results are controversial, since some seropositive foals were born from seronegative mares, and the specificity of the detected antibodies is doubtful. Identification of *Sarcocystis* spp. in tissues from aborted or newborn foals could help to confirm the vertical transmission hypothesis.

Other equids from Brazil have also been tested for antibodies against *S. neurona.* In a study using 47 mule samples processed using IFAT (SN37R at 1:25 dilution), seropositivity of 17% was recorded (Borges et al., 2017). In the same study, from 500 horse serum samples, 112 tested positive through IFAT, and only 33 were confirmed through rSnSAG2/4/3 *S. neurona* ELISA (Table 2). A subset of 28 samples (from the 33 ELISA positive samples) were tested using IB, and a reaction against *S. neurona* IDA was only detected in 15 samples (Borges et al., 2017). These

Table 2. Serological studies on *Sarcocystis neurona* in South American horses

Country	State/Province	No. tested	No. positive (%)	Test	Antigen /strain	Cut-off	Reference
Argentina	Chaco	76	27 (35.5)	Immunoblot	TLA/SN3	-	(Dubey et al., 1999c)
	Buenos Aires (531),Cordoba (51), Santa Fe (11), La Pampa (8), Corrientes (5), Santiago del Estero (4), San Juan (4), Neuquen (1), Entre Rios (1), unknown (24)	640	167 (26.1)	Immunoblot	TLA/SN3	-	(Moré et al., 2014)
Brazil	Alagoas	427	12 (2.8)	IFAT and immunoblot	Merozoite/ SN37R TLA/SN3	1:80 -	(Valença et al., 2019)
	Bahia	9	7 (77.8)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Goiás	15	9 (60)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Mato Grosso	28	15 (53.6)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Mato Grosso	500	104 (20.8)	IFAT and	Merozoite/ SN37R	1:25	(Borges et al., 2017)
			33 (6) [a]	rSnSAG ELISA	rSnSAG2/4/3	10%	
	Mato Grosso do Sul	11	9 (81.8)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Minas Gerais	10	9 (90)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Minas Gerais	506	117 (23.09)	IFAT	Merozoite/SN37R	1:80	(Ribeiro et al., 2016)
	Paraná	146	88 (60.3)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Paraná	100	42 (42)	IFAT	Merozoite/ SN37R	1:50	(Koch et al., 2019)
	Rio Grande do Sul	2	1 (50)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Rio Grande do Sul	189	57 (33.86)	IFAT	Merozoite/ SN37R	1:50	(Antonello et al., 2015)
	Rio Grande do Sul	195 (M and PF)	M 159 (84.1)	IFAT	Merozoite/ SN37R	M= 1:50	(Antonello et al., 2016)
			PF 14 (7.4)			PF= 1:25	
	Rio Grande do Sul	181 (M and PF)	M 61 (33.7)	ELISA	TLA/SN37R	EI >1.2	(Pivoto et al., 2014)
			PF 12 (6.6)				
	Rio Grande do Sul and Paraná	197	71 (36)	IFAT	Merozoite/ SN37R	1:50	(Portella et al., 2017)

References: TLA= total lysate antigen. ND= not described. EI= ELISA index. M= mares. PF: pre-colostral foals. [a] = confirmed by ELISA. * = absorbance units.

Table 2. Continued...

Country	State/Province	No. tested	No. positive (%)	Test	Antigen /strain	Cut-off	Reference
	Rondônia	192	162 (84.4)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Roraima	303	141 (43.2)	IFAT	Merozoite/SN37R	1:25	(Spohr et al., 2018)
	Roraima	213	86 (40.4)	IFAT	Merozoite/SN-138	1:80	(Gomes et al., 2019)
	Santa Catarina	24	11 (45.8)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	Santa Catarina	174	72 (41.37)	IFAT	Merozoite/SN37R	1:50	(Cazarotto et al., 2016)
	São Paulo	101	36 (35.6)	Immunoblot	TLA/SN3	-	(Dubey et al., 1999a)
	São Paulo	513	348 (67.8)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
	São Paulo	116	27 (23.8)	IFAT	Merozoite/SN-138	1:80	(Oliveira et al., 2017)
	Unknown	11	10 (90.9)	SnSAG4 ELISA	SnSAG4/SN3	25%	(Hoane et al., 2006)
Colombia	Córdoba	73	48 (65.7)	ELISA/ND	ELISA/ND	100*	(Calderón et al. 2014)
	Bogotá	1	1	Immunoblot	TLA/SN3	-	(Medina & Oliver, 2003)

References: TLA= total lysate antigen. ND= not described. EI= ELISA index. M= mares. PF: pre-colostral foals. [a] = confirmed by ELISA. * = absorbance units.

results showed that there was a lack of or poor agreement among the serological methods and suggested that cross-reactivity of equid serum samples against *Sarcocystis* spp. was potentially occurring.

A seroepidemiological study performed on serum samples from 329 donkeys (*Equus asinus*) detected antibody frequencies of 3% using IFAT and 21% using a direct agglutination test (SAT), at 1:40 and 1:50 cutoffs, respectively. In both tests, the antigens consisted of merozoites from a North American strain of *S. neurona* (SN3) (Gennari et al., 2016). The correlation between the two tests used was poor (kappa = 0.051), and the potential for cross-reaction with antibodies generated by other *Sarcocystis* spp. in the donkeys tested could not be ruled out (Gennari et al., 2016).

Recently, a serological comparative study was conducted on 409 horse samples from Brazil, which was performed using IFAT (starting dilution of 1:20) with antigens derived from a Brazilian strain of *S. falcatula*-like (Sarco-BA1) and from a North American strain of *S. neurona* (SN138). Out of all the samples, 10.5% and 17.1% were reactive to *S. falcatula*-like and *S. neurona* antigens, respectively. The poor agreement observed between the two IFATs (k = 0.364) indicated that the horses were exposed to more than one *Sarcocystis* species. Some IFAT-positive samples were also tested using IB (using the same isolates as antigens) and showed cross-reactivity to proteins in the range of 16 and 30 kDa (Borges-Silva et al., 2020).

The relatively higher frequency of isolation of *S. falcatula*-like in Brazil, and the detection of horse serum samples reacting to antigens derived from North American *S. neurona* isolates, open the question about the real "specificity" of serological results targeted to *S. neurona* antibodies in South America. It is highly probable that South American horses are exposed to *S. falcatula*-like, as well as to *S. neurona*, *S. lindsayi* and *S. speeri*, which are also shed by opossums. In addition, horses are intermediate hosts (harboring muscle cysts) of *Sarcocystis bertrami* (syn. *Sarcocystis fayeri*), which uses canids as definitive hosts (Zeng et al., 2018). Combination of serological methods could help to increase specificity, as also would combining these with direct detection methods for *Sarcocystis* spp.

in horse tissues. The potential implication of other *Sarcocystis* spp. shed by South American opossums in horse neurological disorders and EPM remains uncertain.

Sarcocystis neurona infection or exposure in domestic carnivores and in wildlife

Few serological investigations have been performed to detect antibodies against *S. neurona* in domestic carnivores in South America. This is probably explained, in part, by the lack of clinical reports of *S. neurona* infection in South American dogs and cats, except for one recent report of clinical *S. neurona* infection in a Brazilian cat (Hammerschmitt et al., 2020). Poor access to serological tests for *S. neurona* in most South American countries is another factor that may hamper serological investigations of the parasite in these countries.

Two studies using canine serum samples were conducted in the Brazilian states of Paraná (Koch et al., 2019) and Bahia (Oliveira et al., 2020). In both studies, low frequencies of seropositivity for *S. neurona* were found using IFAT, with 7/100 (7%) and 12/353 (3.4%) positive animals, respectively, with antibody titers reaching 100 (Oliveira et al., 2020) and 500 (Koch et al., 2019), thus indicating low exposure of these animals to the pathogen (Table 3). No association with neurological signs was observed by Koch et al. (2019), who used 35 animals with neurological signs and 65 asymptomatic animals ($p = 0.69$). In the study conducted in Bahia, none of the animals selected for the study presented any clinical signs (Oliveira et al., 2020).

In three serological studies performed on cats in Brazil, low frequencies of antibodies against *S. neurona* were observed in the animals examined. In the first study, 502 cats were tested using SAT (*S. neurona* agglutination test), and all of them were seronegative (Dubey et al., 2002). It is worth mentioning that all the cats tested were from urban areas and they probably had lower exposure to *S. neurona* sporocysts than cats from rural areas. Subsequently, two studies using IFAT found that 4% (Meneses et al., 2014) and 7% (Koch et al., 2019) of the cats

Table 3. Serological studies on *Sarcocystis neurona* in South American domestic carnivores and wildlife.

	Country	State/region	Species	No. tested	No. positive (%)	Serology	Cut-off	Reference
Domestic Carnivores	Brazil	São Paulo	Cat	502	0 (0%)	SAT	1:50	(Dubey et al., 2002)
		Bahia	Cat	272	11 (4.04%)	IFAT	1:25	(Meneses et al., 2014)
		Paraná	Dog	100	7 (7%)	IFAT	1:50	(Koch et al., 2019)
			Cat	100	5 (5%)	IFAT	1:50	
		Bahia	Dog	353	12 (3.39)	IFAT	1:25	(Oliveira et al., 2020)
Wildlife	Brazil	São Paulo	Capybara (*Hydrochoerus hydrochaeris*)	63	2 (3.17%)	IFAT	1:25	(Valadas et al., 2010)
		Mato Grosso	Jaguar (*Panthera onca*)	11	8 (72.7%)	IFAT	1:25	(Onuma et al., 2014)
		Paraná	Red-tailed Amazon parrot *(Amazona brasiliensis)*	51	0 (0%)	IFAT	1:5	(Sato et al., 2020)
	Peru	Ica	South American fur seal (*Arctocephalus australis*)	29	0 (0%)	IFAT	1:40	(Jankowski et al., 2015)

References: SAT: *Sarcocystis* agglutination test; IFAT: Indirect Fluorescent Antibody Test.

were seropositive. Although seropositivity was similar in these two studies, Koch et al. (2019) obtained a maximum titer of 100, while Meneses et al. (2014) observed 800 as the maximum titer (Table 3). As in dogs, no association with neurological signs was reported for the seropositive cats.

In a recent study, a 1.7-year-old domestic cat, which presented anorexia, dyspnea, pleural effusion, positive test for feline leukemia virus (FeLV) and a presumptive diagnosis of mediastinal lymphoma, was treated with vincristine, prednisolone and cyclophosphamide (Hammerschmitt et al., 2020). Twenty-one days after the first clinical presentation, the cat also showed neurological signs, that progressed to death. Histopathological examination revealed meningoencephalitis in the brain and cerebellum, associated with parasites resembling *S. neurona*. The parasites observed were labelled immunohistochemically using polyclonal serum against *S. neurona*. PCR was performed using DNA extracted from the feline brain. Nucleotide sequencing of ITS1 and SAG loci, as shown in detail in section 4.1, showed that the parasite detected, which was regarded as *S. neurona*, was genetically different from strains isolated in North America (Hammerschmitt et al., 2020).

Studies on *S. neurona* in South American wildlife are also scarce. Little is known about cross-reactivity among *Sarcocystis* spp. excreted by *Didelphis* spp., or among other species of *Sarcocystis* (de Jesus et al., 2019). The pathogenicity to wildlife species of *Sarcocystis* spp. derived from opossums is also largely unknown (Onuma et al., 2014; Valadas et al., 2010). Few intermediate hosts of *S. neurona* have been confirmed in North America (Dubey et al., 2015a), which makes investigations on wild species less common. Serological agglutination tests on a related parasite, *Toxoplasma gondii*, are frequently used for wildlife species (Kornacka et al., 2016), but the *S. neurona* direct agglutination test (SAT) has only rarely been used in serological investigations. Large quantities of parasites are required as antigens for agglutination tests: in the case of *S. neurona*, multiplication of merozoites in cell cultures is poor in comparison with related parasites (Ellison et al., 2001), which may hamper preparation of antigen for SAT. Most seroepidemiological surveys for *S. neurona* in wildlife have been carried out using IFAT with specific or cross-reactive secondary antibodies (Jankowski et al., 2015; Onuma et al., 2014; Sato et al., 2020).

In Peru, antibodies against *S. neurona* were investigated in South American fur seals (*Arctocephalus australis*) from a marine protected area, as part of a health evaluation on this animal species (Jankowski et al., 2015). Despite mortality caused by *S. neurona* in sea otters (*Enhydra lutris nereis*) in the North Pacific (Miller et al., 2010), susceptibility or exposure of South American fur seals to the pathogen was not confirmed in any of the 29 animals tested (Jankowski et al., 2015). In Brazil, two apparently healthy adult capybaras (*Hydrochoerus hydrochaeris*) (2/63) tested positive through IFAT for *S. neurona* antibodies in the state of São Paulo (Valadas et al., 2010). Also in Brazil, antibodies reactive to *S. neurona* were detected in 8/11 jaguars in the state of Mato Grosso (Onuma et al., 2014). Despite the small sample size, the finding of high antibody titers reactive to *S. neurona* in these felids is suggestive of environmental contamination of the parasite sporocysts in the region studied (Onuma et al., 2014).

In North America, there has been a single report of *S. neurona* infection in an avian species. *S. neurona* tissue cysts were detected in brown-headed cow birds (*Molothrus ater*) and merozoites of the cultured parasite were infective to opossums (*D. virginiana*) and to gamma-interferon gene knockout mice (Mansfield et al., 2008). Limited investigations have been conducted on *S. neurona* in South American birds. In the state of Paraná, southern Brazil, 51 red-tailed Amazon parrots (*Amazona brasiliensis*) were tested for the parasite using IFAT and none of them were seropositive for *S. neurona* (Sato et al., 2020). So far, there have not been any confirmed cases of infection by *S. neurona* in South American birds.

Sarcocystis falcatula, *S. falcatula*-like and sarcocystosis in birds

Birds become infected with *S. falcatula* through ingesting food or water contaminated with feces from infected opossums. Another route of infection consists of consumption of paratenic hosts such as flies and cockroaches that are carrying sporocysts (Clubb & Frenkel, 1992).

Reports of *S. falcatula* or related species in birds in South America are scarce and are often associated with Old World species. The first reported outbreak of acute pulmonary sarcocystosis in birds occurred in a zoological collection in Belo Horizonte, Brazil (Ecco et al., 2008). Eight psittacines belonging to three different species and one pigeon were found dead and had exhibited no previous clinical signs. At necropsy, pulmonary congestion and edema were the most common findings. Immunohistochemical analysis confirmed the presence of mature schizonts and merozoites, diagnosed as *S. falcatula*, in the capillaries of the lungs, heart, liver and spleen of the birds. Another outbreak in a zoo in Brazil was documented a year later (Godoy et al., 2009). A total of 47 psittacines housed in a bird park in Foz do Iguaçu, state of Paraná, died within a 15-month period as a result of *Sarcocystis* sp. infection.

Using histopathology, immunohistochemistry, electron microscopy and bioassay, *S. falcatula* was indicated as the cause of death. Although fatalities have only affected a few New World psittacine birds, mortality among Old World species has been found to be higher. The latter were more susceptible to the pulmonary form of sarcocystosis.

Respiratory alterations are the predominant abnormalities associated with *S. falcatula* and *S. falcatula*-like infection in captive or free-living birds (Dubey et al., 2001a; Hillyer et al., 1991; Suedmeyer et al., 2001; Verma et al., 2018; Villar et al., 2008; Wünschmann et al., 2010). However, a few cases of these protozoa causing encephalitis in wild birds have also been described (Siegal-Willott et al., 2005; Wünschmann et al., 2009). In Brazil, a young free-ranging bare-faced ibis (*Phimosus infuscatus*) was clinically examined because of wing paralysis and mild motor incoordination. The bird died four days after admission to a veterinary center and at necropsy presented multifocal to coalescing soft yellowish areas in the brain. The results from histopathological and molecular analyses revealed necrotizing meningoencephalitis in cerebellum and brainstem caused by *S. falcatula* (Konradt et al., 2017). This was the first record of necrotizing meningoencephalitis in a native South American bird caused by *S. falcatula*.

There is limited knowledge on the occurrence of *S. falcatula* or related species in naturally infected birds without clinical disease. Molecular evidence of a species closely related to *S. falcatula* was detected in the carcasses of 16 Magellanic penguins (*Spheniscus magellanicus*) that were rescued on the coast of Brazil (Acosta et al., 2018). Given that parasite DNA sequences were obtained in muscle tissue samples, these authors suggested that the genetic material originated from tissue cysts, which are structures characteristic of the chronic phase of infection, and hence, it was unlikely that the parasite was the cause of death.

Conclusions and future directions

The great majority of the studies examined, on *Sarcocystis* spp. shed by South American opossums, were restricted to Brazil and Argentina. In most of these studies, *S. falcatula* or *S. falcatula*-like species were identified in *D. aurita* and *D. albiventris*. In a few reports, it seems that *D. aurita* was mistakenly identified as *D. marsupialis*, because this latter species does not inhabit the geographical area where it was classified.

Up to the time of conclusion of the current review, no viable isolate of *S. neurona* had been obtained in South America. For this reason, serological tests for *S. neurona* in South American animals have been made using North American strains of the parasite. There is no gold-standard serological test for EPM caused by *S. neurona* in South America due to the lack of a serum panel of truly *S. neurona*-infected horses.

Viable *S. falcatula*-like merozoites have been obtained in Brazil and used in serological tests and in experimental infections. Brazilian horses have been found to be reactive to *S. falcatula*-like antigen; however, it is unknown whether horses are naturally exposed to *S. falcatula*-like or whether the seropositivity in horses resulted from cross-reactivity with other *Sarcocystis* spp.

The scenario of *Sarcocystis* spp. shed by opossums in South America is very peculiar, in comparison with North America. Therefore, future investigations should be conducted in several directions, as follows: 1) the role of *D. albiventris*, *D. aurita* and other *Didelphis* spp. (*D. marsupialis*, *D. imperfecta* and *D. pernigra*) as definitive hosts of *S. neurona*, *S. falcatula* and related species should be investigated; 2) intermediate hosts of *Sarcocystis* spp. derived from South American opossums should be identified; 3) viable South American isolates of *S. neurona* and related *Sarcocystis* spp. should be obtained for diagnosis and molecular studies; 4) it is crucial to investigate whether South American horses are susceptible to other *Sarcocystis* spp. derived from opossums, besides *S. neurona*; and 5) the serodiagnosis of EPM should be developed using South American isolates of *Sarcocystis* sp.

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