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# Diversity of *Sarcocystis* spp shed by opossums in Brazil inferred with phylogenetic analysis of DNA coding ITS1, cytochrome B, and surface antigens

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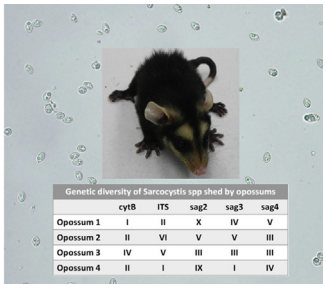
## HIGHLIGHTS

- New alleles of *sag*2, *sag*3, and *sag*4 of *Sarcocystis* shed by opossums.
- Phylogeny of *Sarcocystis* shed by opossums based on Cytochrome B and ITS1.
- Differences between *Sarcocystis* shed by opossums in Brazil and reference strains of *S. falcatula* and *S. neurona*.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Although few species of *Sarcocystis* are known to use marsupials of the genus *Didelphis* as definitive host, an extensive diversity of alleles of surface antigen genes (*sag*2, *sag*3, and *sag*4) has been described in samples of didelphid opossums in Brazil. In this work, we studied 25 samples of *Sarcocystis* derived from gastrointestinal tract of opossums of the genus *Didelphis* by accessing the variability of *sag*2, *sag*3, *sag*4, gene encoding cytochrome b (*cyt*B) and first internal transcribed spacer (ITS1). Reference samples of *Sarcocystis neurona* (SN138) and *Sarcocystis falcatula* (SF1) maintained in cell culture were also analyzed. We found four allele variants of *cytB*, seven allele variants of ITS1, 10 allele variants of *sag*2, 13 allele variants of *sag*3, and 6 allele variants of *sag*4. None of the sporocyst-derived sequences obtained from Brazilian opossums revealed 100% identity to SN138 at *cytB* gene, nor to SN138 or SF1 at ITS1 locus. In addition, none of the *sag* alleles were found identical to either SF1 or SN138 homologous sequences, and a high number of new *sag* allele types were found other than those previously described in Brazil. Out of ten *sag*2 alleles, four are novel, while eight out of 13 *sag*3 alleles are novel and one out of six *sag*4 alleles is novel. Further studies are needed to clarify if such a vast repertoire of allele variants of *Sarcocystis* is the consequence of re-assortments driven by sexual exchange, in order to form individuals with highly

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diverse characteristics, such as pathogenicity, host spectrum, among others or if it only represents allele variants of different species with different biological traits.



## 1. Introduction

*Sarcocystis falcatula* cause severe disease to a vast diversity of avian species among Passeriformes, Psittaciformes, Columbiformes, Strigiformes and Falconiformes and they are commonly associated to outbreaks of acute pulmonary sarcocystosis in zoos (Smith et al., 1990; Hillyer et al., 1991; Clubb and Frenkel, 1992; Page et al., 1992; Dubey et al., 2001b; Wünschmann et al., 2009, 2010). *Sarcocystis neurona* is the causative agent of equine protozoal myeloencephalitis (EPM), a disease associated to incoordination resulting in decreased proprioception and muscle weakness (Dubey et al., 2001a). The close relationship between *S. neurona* with *S. falcatula* has been demonstrated by sequence analysis of the 18S ribosomal RNA gene (Dame et al., 1995; Fenger et al., 1995). Despite their similarity, they can be distinguished by their biological traits (Marsh et al., 1997a, 1997b; Dubey and Lindsay, 1998).

*S. neurona* and *S. falcatula* use the marsupial of the genus *Didelphis* as definitive host (Dubey et al., 1989). These marsupials also participate in the life cycle, as definitive hosts, of other *Sarcocystis* species, such as *Sarcocystis speeri* (Dubey and Lindsay, 1998, 1999) and *Sarcocystis lindsayi* (Dubey et al., 2001b; Stabenow et al., 2012).

Surface antigens of Apicomplexan parasites are often immunodominant and have been widely studied because of their value for diagnostic purposes (Howe et al., 1998; Atkinson et al., 2000). Mitochondrial genes are suitable for phylogenetic studies because they are of maternal transmission in metazoans (Escalante et al., 1998) and they are not affected by multiple nucleotide substitutions driven by adaptive selection, since the majority of the substitutions found in this loci are synonymous (Meyer, 1994). The internal transcribed spacer (ITS) has been used for differentiation of species or strains in studies of population genetics of several organisms (Gasser and Newton, 2000; Blouin, 2002). The high evolutionary rate of ITS locus makes it appropriate to estimate phylogenetic relationships at lower taxonomic levels, such as genus and species (van Herwerden et al., 1999; Alvarez and Wendel, 2003; Prasad et al., 2007).

Extensive variability was found in surface antigens genes (*sags*) of *Sarcocystis* spp. shed by opossums in Brazil. Such large number of allele variants found for each antigen gene (*sag*2, *sag*3, and *sag*4) in unpredictable combinations within each sample, pointed to the possibility of allele exchange through sexual recombination (Monteiro et al., 2013) forming highly divergent individuals in terms of antigenic properties. Studies on DNA markers less subjected to selection pressure, in conjunction with analysis of the diversity of *sags*, would contribute for a better understanding of the phylogenetic relationships between *Sarcocystis* shed by opossums in Brazil (Monteiro et al., 2013). For this purpose, we studied phylogenetic relationships among *Sarcocystis* spp. isolates from opossums assessing molecular markers with different evolutionary rates than the *sags* coding loci, as is the case of genetic sequences of Cytochrome B (*cytB*) in the mitochondrial genome and sequences of the first internal transcribed spacer (ITS1) of the parasite.

## 2. Materials and methods

### 2.1. Samples of parasites

Twenty-seven samples of *Sarcocystis* spp. were used. Twenty-five samples of sporocysts of *Sarcocystis* spp. were recovered from anatomical parts of the small intestines of opossums of the genus *Didelphis* spp. sampled in Brazil. The intestinal scraping technique (Dubey et al., 1989) was performed in order to recover sporocysts. DNA extraction of the recovered sporocysts was done as previously described (Monteiro et al., 2013).

The Technical Division of Veterinary Medicine and Management of Wild Fauna (DEPAVE-3, from the initials in Portuguese) collected all the animals used in this study. DEPAVE-3 is a screening center for wild animals (Wild Animal Screening Center, acronymed to CETAS, from the initials in Portuguese) and belongs to the municipality of the city of São Paulo, state of São Paulo, Brazil. CETAS is any entity authorized by the Brazilian Institute of Environment (IBAMA, from the initials in Portuguese, an official institute that belongs to the Ministry of the Environment of the federal government of Brazil), that aims to receive, identify, mark, screen, evaluate, retrieve, rehabilitate wild animals brought by seizure, redemptions or animals voluntarily brought by residents from the municipality of São Paulo, State of São Paulo, Brasil.

Isolates of *S. neurona* strain 138 (SN138) and *S. falcatula* strain SF1 (SF1) were employed, and both have been kept in CV-1 cells. SN138 was obtained from intestines of an opossum (*Didelphis virginiana*) fed skeletal muscles of a raccoon (*Procyon lotor*) that had been fed sporocysts, isolated in Ohio, United States (Lindsay et al., 2004). SF1 was obtained from tissues of budgerigars (*Melopsittacus undulatus*) previously inoculated with sporocysts from intestines of opossum (*D. virginiana*), isolated in California, United States (Marsh et al., 1997b). Isolates SN138 and SF1 were kindly supplied by Dr. David S. Lindsay.

### 2.2. PCR and sequence analysis

DNA of the samples was PCR amplified after two rounds of amplification in nested, heminested or double PCR format. The first round of amplification was performed according to the manufacturer recommendations of platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The second round of amplification was performed exactly as the first round, except for the primer and target, which was the product from the first reaction. A negative control was included in each PCR run and the primers and the annealing temperature used for each primer pair are informed in Table 1.

The ITS1 primer targeting *Sarcocystis* spp. was designed from multiple alignments between ITS1 sequences of *S. falcatula* and *S. neurona*. The *cytB* primer targeting *Sarcocystis* spp. was designed from genomic sequences of *S. neurona* and *S. falcatula*. The *sag* primers used for *Sarcocystis* were those described elsewhere (Monteiro et al., 2013). Internal primers were designed in this study to be used in nested or heminested format, which were based on genetic sequences described by Monteiro et al. (2013).

The PCR products were observed through electrophoresis on a 2% agarose gel. Positive samples had their DNA excised from the gel