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Research paper

Serologic cross-reactivity between *Sarcocystis neurona* and *Sarcocystis falcatula*-like in experimentally infected Mongolian gerbils

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ABSTRACT

Sarcocystis neurona is the major cause of the equine protozoal myeloencephalitis (EPM) in the Americas and has opossums of the genus *Didelphis* as definitive hosts. Most isolates of *Sarcocystis* sp. shed by opossums in Brazil differ genetically from the known species of *Sarcocystis*. These Brazilian isolates behave similarly as *Sarcocystis falcatula*, which causes sarcocystosis in birds, and for this reason, have been classified as *Sarcocystis falcatula*-like. Genes coding for the immunodominant surface antigens SAG2, SAG3 and SAG4 of *S. falcatula*-like are similar to those from *S. neurona*. It is unknown the *Sarcocystis* species that causes EPM in Brazil, as *S. neurona* has never been genetically confirmed in Brazilian horses. All cases associated with EPM in Brazil were diagnosed by immunological tests, which are not specific for *S. neurona* infection. It is possible that *S. falcatula*-like may infect horses in Brazil. The aims of the current study were to test the susceptibility of gerbils (*Meriones unguiculatus*) to experimental infections with *S. neurona* and *S. falcatula*-like, and to investigate potential serologic cross-reactivity to these parasites by immunofluorescent antibody test (IFAT) and Western blot (WB). A total of 27 gerbils, distributed in five experimental groups (G1-G5), were employed in this work (G1: 4 negative controls; G2: 6 infected with *S. neurona* merozoites, G3: 6 infected with *S. falcatula*-like merozoites; G4 and G5 (5 and 6, respectively, infected with different doses of sporocysts). None of the 17 animals that seroconverted for the parasites in IFAT presented any visualized organism or *Sarcocystis* DNA in the examined tissues. No serologic cross-reactivity was observed using IFAT. However, sera from animals infected with *S. falcatula*-like and *S. neurona* presented the same pattern of antigenic recognition when *S. neurona* merozoites were used as antigen in WB, including reactivity to proteins of 30 and 16 kDa, regarded as specific markers for *S. neurona*-infected animals. Gerbils did not sustain infection by these parasites, although produced antibodies after inoculation. These results are suggestive that other animal species that are exposed to *S. falcatula*-like, including horses, may present serologic cross-reactivity to *S. neurona* in WB. IFAT was demonstrated to be more specific that WB for the detection of antibodies to *S. falcatula*-like and *S. neurona* in the experimental conditions of this study.

1. Introduction

Sarcocystis neurona is a coccidian parasite associated with neurologic abnormalities in a variety of animal species and regarded as the major cause of the equine protozoal myeloencephalitis (EPM) in the Americas (Reed et al., 2016). Parasite sporocysts/oocysts are shed in the feces of *Didelphis virginiana* (North and Central Americas) (Fenger et al., 1995) and *Didelphis albiventris* (South America) (Dubey et al., 2001a). *Sarcocystis falcatula* is another important pathogen, which is shed in the feces of *D. virginiana*, *D. albiventris* and *Didelphis marsupialis* and causes pulmonary sarcocystosis in exotic birds in the Americas (Smith et al., 1987). There is no evidence that *S. falcatula* from North America (SF1 strain) is able to infect horses (Cutler et al., 1999).

In recent years, most *Sarcocystis* sp. isolates obtained from *Didelphis* spp. in Brazil were genetically distinct from the known species of *Sarcocystis* that use opossums as definitive hosts; as they caused infection in budgerigars, they have been classified as *Sarcocystis falcatula*-like (Cesar et al., 2018; Gondim et al., 2017; Valadas et al., 2016). A common characteristic of the Brazilian isolates is the high genetic diversity of the genes coding for some surface antigens (SAG2, SAG3 and

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Table 1
Inoculation of Mongolian gerbils (*Meriones unguiculatus*) with *Sarcocystis neurona* and *Sarcocystis falcatula*-like.

Group (number of animals)	Inoculum	Parasite stage	Volume	Number of parasites	Administration route
01 (4)	PBS	–	0.5 m L	–	Subcutaneous
02 (6)	*S. neurona*	Merozoite	0.5 m L	1×10^4	Subcutaneous
03 (6)	*S. falcatula*-like	Merozoite	0.5 m L	1×10^4	Subcutaneous
04 (5)	*S. falcatula*-like	Sporocyst	0.8 m L	1×10^3	Oral
05 (6)	*S. falcatula*-like	Sporocyst	0.8 m L	1×10^5	Oral

SAG4). Due to the similarities of these surface antigens with those found in *S. neurona* (Cesar et al., 2018; Gondim et al., 2017; Valadas et al., 2016), the authors of the current study hypothesized that cross-reactivity may occur in the most frequently employed immunological tests, including ELISA, IFAT, immunoblot and immunohistochemistry. It is possible that *S. falcatula*-like may infect horses in Brazil, as *S. neurona* has never been genetically confirmed in Brazilian horses. In previous serological studies conducted in Brazil using *S. neurona* antigens (Gennari et al., 2016; Hoane et al., 2006; Meneses et al., 2014), it is unknown whether positive reactions represented exposure of animals to *S. neurona* or to other *Sarcocystis* spp. shed by opossums, including *S. falcatula*-like.

In the absence of animal models that are susceptible to both *S. neurona* and *S. falcatula*-like, Mongolian gerbils (*Meriones unguiculatus*) were selected for the current study, as they have origin in Asia and did not inhabit the same geographical regions where the definitive hosts (opossums) of these parasites are resident. The aims of this work were to test the susceptibility of gerbils (*Meriones unguiculatus*) to experimental infections with *S. neurona* and *S. falcatula*-like, and to investigate potential serologic cross-reactivity to these parasites by immunofluorescent antibody tests (IFAT) and Western blot (WB).

2. Material and methods

2.1. Sporocysts, merozoites and antigen production

Sporocysts of *S. falcatula*-like (Sarco-BA1 strain) were obtained by intestinal scraping of an opossum (*Didelphis aurita* or *D. marsupialis,* morphologically indistinguishable species) and presented molecular markers (ITS1: GenBank accession number MK803362; SAG2: accession number MK809526; SAG3: accession number MK821087, and SAG4: accession number MK809527) compatible with *S. falcatula*-like, as described elsewhere (Gondim et al., 2019). Sporocysts were stored at 4 °C for five months in antibiotic/antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B) (Gibco-Invitrogen, Carlsbad, CA, USA). Some days before bioassay, 1×10^6 esporocysts of Sarco-BA1 were ruptured with glass beads (425–600 µm) to release the sporozoites (Gondim et al., 2015) and inoculated into VERO cells to verify their infectivity. After 14 days, it was possible to observe free merozoites in the supernatant, indicating the maintenance of the infectivity of the sporocysts.

Merozoites of *S. neurona* (SN-138 strain) (Lindsay et al., 2004) were maintained in Vero cells supplemented with RPMI-1650 + L-glutamin (Invitrogen/ Gibco®, Carlsbad, USA), 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B) (Gibco®, Carlsbad, USA) and 5% of inactivate bovine serum (Invitrogen/ Gibco®, Auckland, NZ), at 37 °C in a humidified incubator containing 5% CO_2. *S. falcatula*-like merozoites were cultured in the same conditions as described above, but instead of Vero cells, the parasites were grown in a permanent chicken cell line (UMNSAH/DF-1) (Foster and Foster, 1997).

Merozoites from both parasite species were removed from cell monolayers using a cell scrapper, purified in Sephadex® G-25 columns and washed four times in PBS by centrifugation (1500 *g* for 5 min). The numbers of purified merozoites for each serologic test are detailed in the specific sections.

2.2. Mongolian gerbils

A total of 27 female gerbils (*Meriones unguiculatus*), with ages between four and six weeks, were obtained from the Federal University of Alagoas, Brazil. The animals were maintained in the animal facility of the Veterinary Hospital at Federal University of Bahia. The cages, water, rodent commercial food and bed were autoclaved at 121 °C for 15 min before use. The use of animals was approved by the Ethical Committee of the School of Veterinary Medicine from Federal University of Bahia (License number: 58/2018).

2.3. Experimental infections

The 27 gerbils were distributed in five experimental groups (G1-G5) as follows: G1: four negative controls; G2: six infected with *S. neurona* merozoites, G3: six infected with *S. falcatula*-like merozoites; G4 and G5 (5 and 6 gerbils, respectively, infected with different doses of sporocysts). The dose and way of infection are shown in Table 1.

Gerbils from groups 4 and 5 were inoculated by gavage under inhalation anesthesia with isoflurane. All animals were observed daily for appearance of any clinical sign until the day of euthanasia. At 21 days after inoculation (DAI), 14 animals were euthanized using inhalation with isoflurane, followed by cardiac puncture blood collection and cervical dislocation. The remaining 13 gerbils had blood samples collected from the retro-orbital plexus at 21 DAI (after local anesthesia with proxymetacaine) and were euthanized at 60 DAI, as described above.

2.4. Immunofluorescent antibody tests

Merozoites from *S. neurona* (SN138 strain) and *S. falcatula*-like (Sarco-BA1 strain) were used to coat IFAT slides. A number of 3×10^3 merozoites were added to each well of 5 mm of diameter. A 1:25 cutoff was employed for animal sera (Gondim et al., 2017), which were incubated at 37 °C for 30 min in a humid chamber. The slides were washed for 10 min in a FA buffer (26.9 mM Na_2CO_3; 100 m $NaHCO_3$; 70.6 mM NaCl; pH = 9.0), 10 min in PBS, and dried at 37 °C. A FITC conjugate anti-mouse IgG (Sigma-Aldrich®, St Louis, USA), which cross-reacts with gerbil IgG (Sager et al., 2006) was used as secondary antibody at 1:50 dilution and incubated for 30 min in a dark and humid chamber. The slides were washed in FA e PBS, as described above, dried at 37 °C and mounted with buffered glycerin for observation by immunofluorescence microscope. IFAT was performed using sera from gerbils pre-inoculation and at 21 and 60 DAI. Positive controls consisted of sera from gerbils inoculated with merozoites of each parasite (*S. neurona* or *S. falcatula*-like). Serum from a gerbil inoculated with PBS was used as negative control.

2.5. Western blot

The term Western blot (WB) is used in this section and throughout the manuscript to encompass both WB and immunoblot. Purified merozoites from *S. neurona* (4×10^7) and from *S. falcatula*-like (2×10^7) were pelleted by centrifugation and used for antigen preparation. Antigens from the two parasite species were used under reduced and non-reduced forms, because no data were available in literature about

Table 2
Sera from Mongolian gerbils (*Meriones unguiculatus*) experimentally infected with *Sarcocystis neurona* and *Sarcocystis falcatula*-like and tested by immunofluorescent antibody test (IFAT) using merozoites from each parasite as antigens.

Group	Animal	IFAT at 21 DAI		IFAT at 60 DAI	
		S. neurona	*S. falcatula*-like	*S. neurona*	*S. falcatula*-like
G1	1	neg	neg	x	x
	2	neg	neg	x	x
	3	neg	neg	neg	neg
	4	neg	neg	neg	neg
G2	1	1:200	neg	x	x
	2	1:100	neg	x	x
	3	1:200	neg	x	x
	4	1:400	neg	1:100	neg
	5	1:400	neg	1:50	neg
	6	1:50	neg	1:100	neg
G3	1	neg	neg	x	x
	2	neg	1:100	x	x
	3	neg	1:25	x	x
	4	neg	neg	neg	neg
	5	neg	neg	neg	1:25
	6	neg	neg	neg	1:50
G4	1	neg	neg	x	x
	2	neg	neg	x	x
	3	neg	neg	x	x
	4	neg	neg	neg	1:25
	5	neg	neg	neg	neg
G5	1	neg	neg	x	x
	2	neg	1:100	x	x
	3	neg	neg	x	x
	4	neg	neg	neg	1:25
	5	neg	neg	neg	1:50
	6	neg	neg	neg	1:25

G1 = negative control; G2: inoculated with *S. neurona* merozoites, G3: inoculated with *S. falcatula*-like merozoites; G4 and G5 (inoculated with different doses of sporocysts); DAI = days after inoculation; x = serum not available due to euthanasia at 21 days after inoculation; neg = negative result.

antigen preparation for WB for *S. falcatula*-like. The parasites were mixed with a reducing sample buffer (1% 2-mercaptoethanol, 2% SDS, 7% glycerol, 48 mM TrisHCl, pH 6.8) or a non-reducing one (2% SDS, 7% glycerol, 48 mM TrisHCl, pH 6.8), heated at 97 °C for 10 min, and centrifuged for 13,000*g* for 10 min at 4 °C.

Western blot was performed similarly as reported by Gondim et al. (2016). Animal sera were diluted 1:10 (Rossano et al., 2000) and anti-mouse IgG conjugate with peroxidase was used as secondary antibody. The reactions were revealed using diaminobenzidine (DAB) peroxidase tablets. Sera from 14/27 gerbils were tested at 21 DAI and sera from 13/27 animals were examined at 60 DAI.

2.6. Histopathology

Samples from liver, kidneys, lungs, heart, spleen, hindlimb muscle, brain, diaphragm, tongue and esophagus were collected from four *S. falcatula*-like inoculated animals. Tissues were fixed in 10% buffered formalin and processed for conventional hematoxilin-eosin staining. Three slides from each tissue were examined for parasite stages in their tissues.

2.7. Molecular analysis of Sarcocystis sp. in animal tissues

The same repertoire of animal tissues employed in the histopathological analysis was collected immediately after euthanasia. Pools of the same tissues from each experimental group, totalizing ten pools per group (total of 50 pools), and five negative controls (ultrapure water) from 21 and 60 DAI (total of 100 pools of tissues and 10 negative controls) were used for DNA extraction by means of a commercial DNA extraction kit (Easy-DNA, Invitrogen®, Carlsbad, USA).

PCR was conducted with the primer pair JNB25/JD396 (Tanhauser et al., 1999), which amplifies DNA from *Sarcocystis* spp. shed by *Didelphis* spp. Each reaction was performed to a final of 25 µL, 1 µL (50 pmol) of each primer, 12.5 µL of a commercial Master Mix (Promega, USA), 9.5 µL of ultrapure water and 1 µL of DNA, according to the same conditions proposed by Tanhauser et al. (1999). Positive controls consisted of extracted DNA from cultured *S. neurona* (SN138 strain) and *S. falcatula*-like (Sarco-BA1 strain) merozoites.

2.8. In vitro isolation of the parasites using animal tissues

Tissue pools (liver, kidneys, lungs, heart, spleen, hindlimb muscle, brain, diaphragm, tongue and esophagus) from each experimental group were grinded using mortar and pestle and mixed with PBS containing 2% antibiotic/antimycotic (200 units/mL of penicillin, 200 µg/mL of streptomycin, and 0.5 µg/mL of amphotericin B) (Gibco-Invitrogen, Carlsbad, CA, USA). The homogenate was filtered in sterile gauze, transferred to 50-ml centrifuge tubes and centrifuged at 4 °C, 1200 *g*, for 10 min. The sediment was resuspended in 2 ml of PBS with 2% antibiotic/antimycotic and inoculated in two culture flasks (25 cm^2), one containing Vero cells and the other DF-1 cells at the same culture conditions described in the section 2.1. The medium was replaced 12 h after inoculation and changed every 48 or 72 h.

3. Results

3.1. Immunofluorescent antibody test

All six gerbils that were inoculated with *S. neurona* merozoites (G2) seroconverted at 21 DAI when tested by IFAT using *S. neurona* merozoites as antigen; 3/6 animals were kept until 60 DAI and remained seropositive. The six animals from this group (G2) tested negative by IFAT when merozoites of *S. falcatula*-like were employed as antigen. In contrast to *S. neurona*-inoculated gerbils, only two of the six animals inoculated with *S. falcatula*-like merozoites (G3) seroconverted at 21 DAI when tested by IFAT with *S. falcatula*-like merozoites as antigen. Three gerbils from this group (G3) were maintained until 60 DAI and 2/3 animals seroconverted at this time when tested with *S. falcatula*-like antigen (Table 2).

Among the 11 gerbils that were inoculated with different doses of *S. falcatula*-like sporocysts (G4 and G5), 5/11 animals seroconverted; 4/5 animals that seroconverted belonged to the group that received a higher dose of sporocysts (G5) (Table 2).

None of the negative control animals tested positive for any parasite using IFAT. No serologic cross-reactivity was observed between animals infected with *S. neurona* or *S. falcatula*-like by IFAT at serum dilution of 1:25, although apical reactions were noted.

3.2. Western blot

Sera from gerbils post-inoculation with *S. neurona* and with *S. falcatula*-like were tested by WB using merozoite antigens from both parasites in reduced and non-reduced forms. Serological cross-reactivity was evident and characterized by similar patterns of antigen recognition.

3.2.1. Non-reduced antigens from both parasites

Using non-reduced antigen from *S. falcatula*-like, sera from gerbils at 21 DAI with *S. neurona* (2 animals), *S. falcatula*-like merozoites (3 animals) and *S. falcatula*-like (higher dose of sporocysts, 3 animals) reacted to a protein of approximately 20 kDa (Fig. 1A). Part of the animals from these three groups, including two inoculated with *S. neurona*, also recognized a protein of 12.5 kDa from *S. falcatula*-like antigen. Using *S. neurona* merozoites as non-reduced antigen in WB, the recognition of proteins by sera at 21 DAI from three gerbils inoculated with *S. falcatula*-like (10^5 sporocysts) was identical to reactions by sera

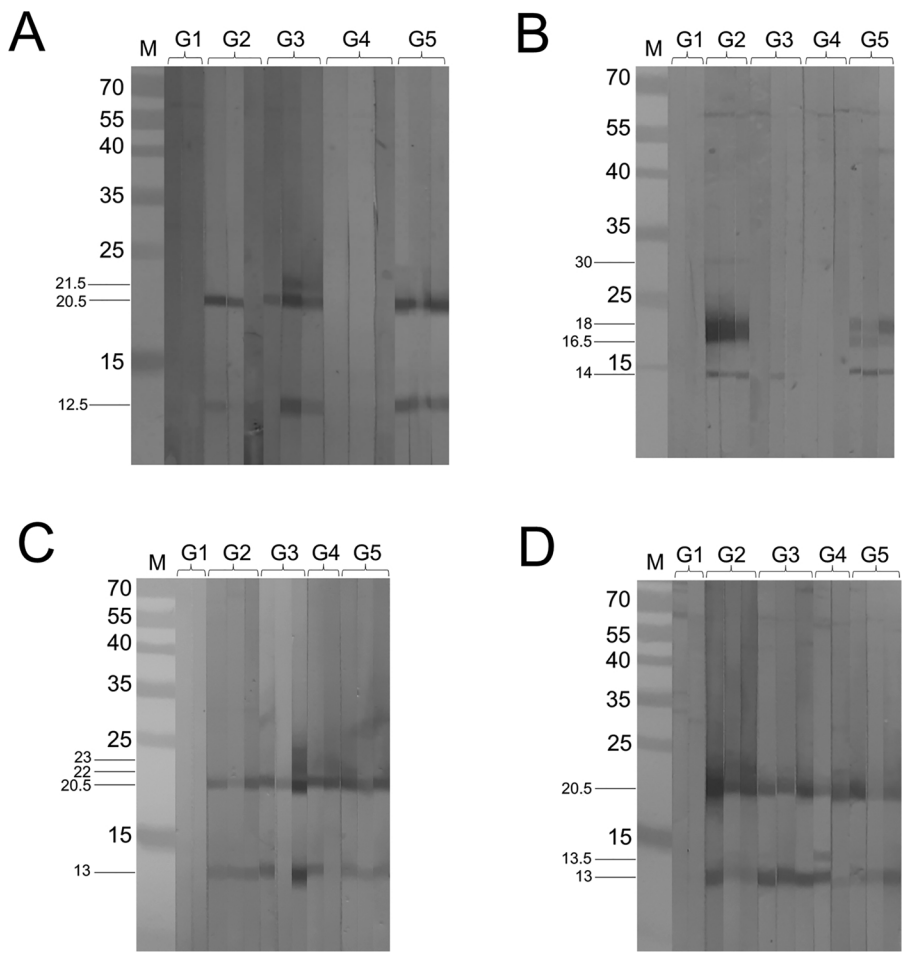

Fig. 1. Sera from Mongolian gerbils at 21 (A and B) and 60 (C and D) days post inoculation with *Sarcocystis neurona* and *Sarcocystis falcatula*-like, and tested by Western blot using non-reduced antigens from *S. falcatula*-like (A and C) and *S. neurona* (B and D).
M: molecular weight marker (kDa); G1: negative control; G2: inoculated with 10^4 *S. neurona* merozoites; G3: 10^4 *S. falcatula*-like merozoites; G4: 10^3 *S. falcatula*-like sporocysts; G5: 10^5 *S. falcatula*-like sporocysts.

from three animals inoculated with *S. neurona* (Fig. 1B).

Examining sera at 60 DAI using *S. falcatula*-like non-reducing antigen, all sera labeled a protein band of 20.5 kDa, except sera from the negative controls, whereas a protein of 13 kDa was recognized by part of the animals from all parasite-inoculated groups (Fig. 1C). When tested with *S. neurona* antigens, all sera at 60 DAI from animals inoculated either with *S. neurona* or *S. falcatula*-like, recognized proteins of 20.5 and 13 kDa. (Fig. 1D).

3.2.2. Reduced antigens from both parasites

Reduced forms of antigens from both parasites (*S. neurona* and *S. falcatula*-like) were also employed for sera from all inoculated groups of gerbils at 21 DAI. The recognition pattern of the membranes containing *S. falcatula*-like antigen was weaker than that with *S. neurona* (Fig. 2A). When tested with *S. neurona* reduced antigen, seven inoculated animals recognized proteins of 15 to 15.5 kDa, and four animals also reacted to bands of 13 KDa (Fig. 2B). Two tested animals from G2 and 2/3 animals in G4 reacted to a protein of 30 KDa (Fig. 2B).

When using sera at 60 DAI, the recognition pattern in WB for *S. falcatula*-like antigen was also weaker than that for *S. neurona* antigen, but the 30 kDa protein was recognized by all three animals inoculated with *S. neurona* merozoites, and the 13 kDa was recognized by two of them (similar to that observed at 21 DAI) (Fig. 2C). Using *S. neurona* as antigen, sera from six animals recognized a protein of 15.5 kDa, whereas six sera reacted to a 13 kDa antigen (Fig. 2D).

3.3. Histopatholy, molecular detection and in vitro isolation attempts

No parasite stages of *Sarcocystis* sp. were observed were observed in the examined tissue sections stained by H&E. All tissue samples analyzed by PCR were also negative for *Sarcocystis* sp. Merozoites or schizonts of the parasites were not detected in cell culture flasks maintained until 45 DAI in Vero or DF-1 cells.

4. Discussion

In the current study, serological responses of experimentally infected animals tested by WB and IFAT were compared for the first time using a Brazilian strain of *S. falcatula*-like (Sarco-BA1) and a North-American strain of *S. neurona* (SN138). Antigens from the two parasites were used in both reduced and non-reduced forms, as *S. falcatula*-like antigens have never been used in WB. Due to the similarity of the genes coding for the surface antigens SAG2, SAG3, and SAG4 from these two parasites (Cesar et al., 2018; Gondim et al., 2017; Valadas et al., 2016), we hypothesized that serological cross-reactivity might occur in sera from the inoculated animals.

Serological diagnosis of equine protozoal myeloencephalitis (EPM) in North America was originally based on serum reactivity to low molecular weight proteins of 22.5, 13 and 10.5 kDa by WB using reduced antigens (Granstrom et al., 1993). An improved WB was developed to increase the specificity of the test by blocking the *S. neurona* reduced antigen using bovine sera reactive to *Sarcocystis cruzi* (Rossano et al.,

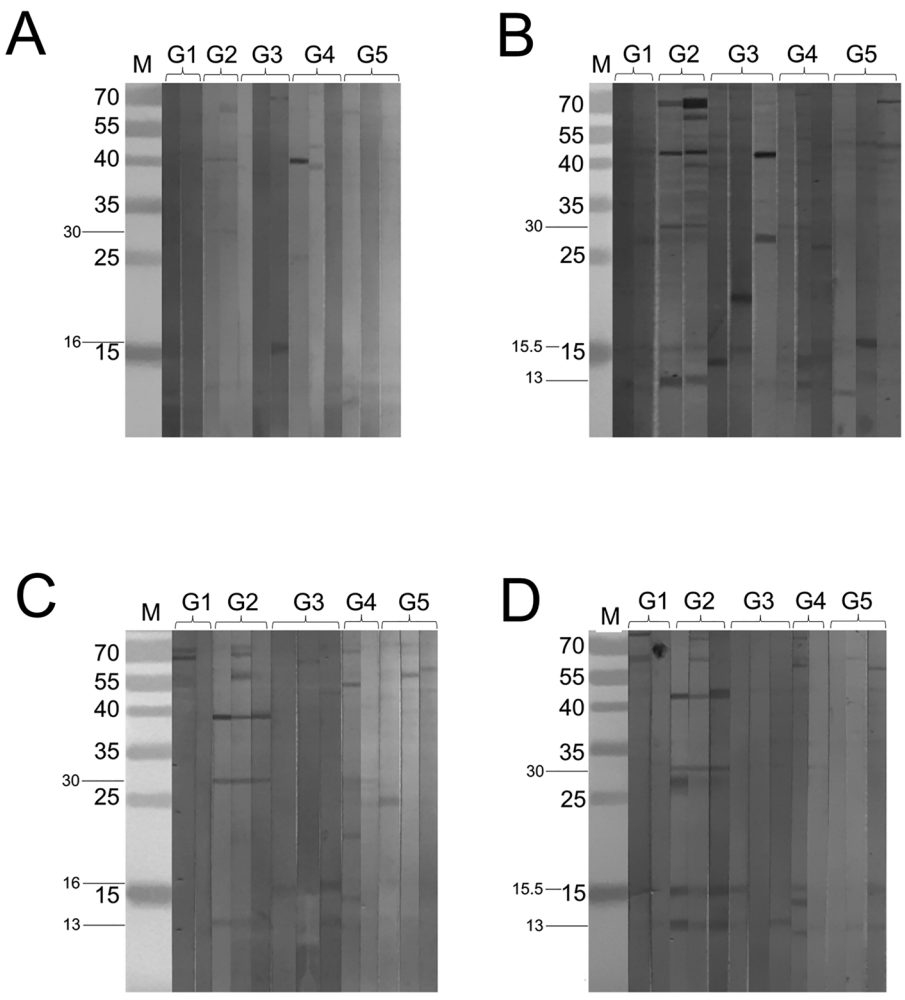

Fig. 2. Sera from Mongolian gerbils at 21 (A and B) and 60 (C and D) days post inoculation with *Sarcocystis neurona* and *Sarcocystis falcatula*-like, and tested by Western blot using reduced antigens from *S. falcatula*-like (A and C) and *S. neurona* (B and D).
M: molecular weight marker (kDa); G1: negative control; G2: inoculated with 10^4 *S. neurona* merozoites; G3: 10^4 *S. falcatula*-like merozoites; G4: 10^3 *S. falcatula*-like sporocysts; G5: 10^5 *S. falcatula*-like sporocysts.
Note: serum from the third gerbil from group 2 in A and B was insufficient for Western blot analysis.

2000); this approach would avoid potential cross-reactivity of equine sera to other *Sarcocystis* spp. The authors pointed that proteins of 16 kDa and 30 kDa are immunodominant antigens for *S. neurona*-infected horses (Rossano et al., 2000).

In the current work, Mongolian gerbils that were experimentally infected with merozoites and sporocysts of *S. falcatula*-like reacted against antigens of *S. neurona*, as well as to antigens of *S. falcatula*-like in WB. The authors used antigens from both parasites in reduced and non-reduced forms, as no information was available on the best antigen preparation for WB using *S. falcatula*-like antigen.

Using reduced antigens of *S. neurona*, a protein of 13 kDa was recognized by sera from four animals at 21 DAI, which received *S. neurona* merozoites or *S. falcatula*-like sporocysts as inocula. This same molecular weight protein (13 kDa) was also recognized by six animals tested at 60 DAI. Sera from six gerbils (two inoculated with *S. neurona* and four inoculated with *S. falcatula*-like) at 60 DAI labeled a protein of 15.5 kDa, which may probably correspond to the 16 kDa protein described by Rossano et al. (2000). Although the 30 kDa protein was recognized by sera from animals 21 DAI with *S. neurona* (G2) and *S. falcatula*-like (G4) in *S. neurona* reduced antigen, at 60 DAI only sera from gerbils inoculated with *S. neurona* recognized the 30 kDa protein in both *S. neurona* or *S. falcatula*-like reduced antigens. The proteins of 16 kDa and 30 kDa, that are considered immunodominant for horses infected with *S. neurona*, are also recognized by gerbil sera inoculated with *S. falcatula*-like.

In previous studies, the robustness of the 16 and 30 KDa as specific markers has been weakened. Rossano et al. (2000) reported that an equine serum from India, where the definitive host of *S. neurona* does not exist, reacted to proteins of 16 and 30 KDa in WB. In another study, it was demonstrated that a 29 KDa protein of *S. neurona* (SnSAG1) was 30% homologous to a protein derived from *Sarcocystis muris* produced *in vitro* (Ellison et al., 2002).

It has been recently assumed that there is no gold standard serological test for the diagnosis of EPM (Reed et al., 2016). Among the tests routinely used for detecting antibodies to *S. neurona*, ELISA based on recombinant proteins (SAG2, SAG3 and SAG4), as well as IFAT on serum and spinal fluid, have been regarded as useful tests (Reed et al., 2016). In the current study, IFAT using sera from *S. neurona* inoculated gerbils showed 100% of sensitivity and specificity. No serologic cross-reactivity was observed for gerbil sera inoculated with *S. neurona* and *S. falcatula*-like and tested with antigens from both parasites. Despite the small number of tested animals, the serological differences observed by IFAT between *S. neurona* and *S. falcatula*-like inoculated animals suggest that the repertoire of immunodominant antigens for each parasite is probably higher than expected. The lack of cross-reactivity using IFAT is probably due to existence of additional specific antigens besides those originally described by WB.

Proteins shared between *S. neurona* and *S. falcatula*-like were clearly observed in WB using both reduced and non-reduced antigens. The high similarity between surface antigens (SAG2, SAG3 and SAG4) from *S. neurona* and *S. falcatula*-like (Cesar et al., 2018; Gondim et al., 2017) may partially explain the serological cross-reactivity observed in the present work. It is possible that some specific membrane proteins of merozoites remain intact when tested by IFAT and lose their antigenicity during antigen solubilization for WB. This may explain the higher specificity of IFAT compared to WB in the current work. In

previous studies using sera from horses that were naturally infected with *S. neurona*, IFAT was shown to be superior to WB (Duarte et al., 2003, 2004).

In Brazil, high seropositivities for *S. neurona* were reported for horses. In a study performed in São Paulo, 35% of 101 horses were reactive to the parasite by WB (Dubey et al., 1999). Using an ELISA based on SnSAG4, 69.6% of 961 horses from different regions in Brazil were seropositive for SnSAG4 protein (Hoane et al., 2006). Isolation of *S. neurona* from a Brazilian opossum was reported almost two decades ago (Dubey et al., 2001), however, the molecular tests employed at that time were not specific for *S. neurona*. Surprisingly, *S. neurona* has never been confirmed again by molecular methods. It is unkown weather *S. neurona* exists in Brazil. Studies conducted in recent years showed that most *Sarcocystis* spp. isolates derived from Brazilian opossums are *S. falcatula*-like organisms (Cesar et al., 2018; Gondim et al., 2017). Interestingly, *S. neurona* has never been genetically confirmed in the country, besides the study reported in 2001 (Dubey et al., 2001a,b).

In the present work, despite seconversion in gerbils inoculated either with *S. neurona* or *S. falcatula*-like, infection was not confirmed by examining gerbil tissues for *in vitro* isolation of the parasites, as well as by histological and molecular examinations. Susceptibility of animals to *S. neurona* infection could not be properly assessed because the parasite strain (SN-138) employed in the study has been propagated for years in cell culture and might have lost part of its infectivity *in vivo*. Loss of infectivity of *S. neurona* merozoites has been shown to occur after 100 passages of the parasite in cell culture (Dubey et al., 2001b).

In conclusion, serological cross-reactivity was demonstrated in the present study using sera from gerbils experimentally inoculated with a Brazilian strain of *S. falcatula*-like, which recognized antigens derived from *S. neurona* merozoites in WB. However, serological cross-reactivity did not occur when the same sera were examined by IFAT at a 1:25 cutoff. No evidence of disease was observed in gerbils inoculated with any of the two parasites. Seroconversion in animals inoculated with sporocysts is suggestive of infection, although no parasite stages were detected in tissues from inoculated animals. In the conditions of the present study, IFAT was more specific than WB to differentiate exposure to *S. neurona* and *S. falcatula*-like. It is crucial to test whether horses seroconvert or become infected after exposure to *S. falcatula*-like sporocysts. It also imperative to investigate the potential of *S. falcatula*-like to cause disease in horses.

Ethical statement

The use of Mongolian gerbils was approved by the Ethical Committee for Animal Experimentation of the School of Veterinary Medicine, at the Federal University of Bahia (Protocol 58/2018).

Declaration of Competing Interest

None.

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