Molecular characterization and development of *Sarcocystis speeri* sarcocysts in gamma interferon gene knockout mice

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SUMMARY

The North American opossum (*Didelphis virginiana*) is the definitive host for at least three named species of *Sarcocystis: Sarcocystis falcatula, Sarcocystis neurona* and *Sarcocystis speeri*. The South American opossums (*Didelphis albiventris, Didelphis marsupialis* and *Didelphis aurita*) are definitive hosts for *S. falcatula* and *S. lindsayi*. The sporocysts of these *Sarcocystis* species are similar morphologically. They are also not easily distinguished genetically because of the difficulties of DNA extraction from sporocysts and availability of distinguishing genetic markers. Some of these species can be distinguished by bioassay; *S. neurona* and *S. speeri* are infective to gamma interferon gene knockout (KO) mice, but not to budgerigars (*Melopsittacus undulatus*); whereas *S. falcatula* and *S. lindsayi* are infective to budgerigars but not to KO mice. The natural intermediate host of *S. speeri* is unknown. In the present study, development of sarcocysts of *S. speeri* in the KO mice is described. Sarcocysts were first seen at 12 days post-inoculation (p.i.), and they became macroscopic (up to 4 mm long) by 25 days p.i. The structure of the sarcocyst wall did not change from the time bradyzoites had formed at 50–220 days p.i. Sarcocysts contained unique villar protrusions, 'type 38'. The polymerase chain reaction amplifications and sequences analysis of three nuclear loci (*18S rRNA, 28S rRNA* and *ITS1*) and two mitochondrial loci (*cox1* and *cytb*) of *S. speeri* isolate from an Argentinean opossum (*D. albiventris*) confirmed its membership among species of *Sarcocystis* and indicated an especially close relationship to another parasite in this genus that employs opossums as its definitive host, *S. neurona*. These results should be useful in finding natural intermediate host of *S. speeri*.

Key words: *Sarcocystis speeri*, *Sarcocystis falcatula*, *Sarcocystis neurona*, Opossum (*Didelphis virginiana*), Diagnosis.

INTRODUCTION

The North American opossum (*Didelphis virginiana*) is the definitive host for at least three named species of *Sarcocystis: Sarcocystis falcatula* (Box and Duszynski, 1978; Box *et al*. 1984), *Sarcocystis neurona* (Dubey *et al*. 1991; Fenger *et al*. 1997; Dubey and Lindsay, 1998, 1999) and *Sarcocystis speeri* (Dubey *et al*. 1998; Dubey and Lindsay, 1999). It appears that there may be additional undescribed species of *Sarcocystis* in *D. virginiana* feces (Tanhauser *et al*. 1999; Dubey *et al*. 2015). The South American opossums (*Didelphis albiventris, Didelphis marsupialis* and *Didelphis aurita*) act as the definitive host for *S. falcatula* and *Sarcocystis lindsayi* (Dubey *et al*. 1999, 2000*a*, *b*; 2001*b*; Stabenow *et al*. 2008, 2012). Additionally, *Didelphis albiventris* is a definitive host for *S. neurona* (Dubey *et al*. 2001*a*). The sporocysts of these *Sarcocystis* species are similar morphologically. They are also not easily distinguished genetically, in part because of the non-availability of distinguishing genetic markers (Dame *et al*. 1995; Tanhauser *et al*. 1999).

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Infectivity to immunodeficient mice and budgerigars (*Melopsittacus undulatus*) has been one important means to distinguish among species of *Sarcocystis* in opossum feces (Dubey, 2000). *Sarcocystis neurona* and *S. speeri* are infective to immunodeficient mice but not budgerigars, whereas *S. falcatula* and *S. lindsayi* are infectious for budgerigars, but not mice (Marsh *et al*. 1997; Dubey *et al*. 1998; Dubey and Lindsay, 1999). However, there is an unconfirmed report of cowbirds (*Molothrus ater*) as an intermediate host of *S. neurona* (Mansfield *et al*. 2008). Both *S. neurona* and *S. speeri* can induce encephalitis in mice associated with schizonts and merozoites (Dubey *et al*. 1998; Dubey and Lindsay, 1999), but *S. neurona* does not form sarcocysts in mice (Dubey *et al*. 2001*b*). *Sarcocystis speeri*, however, can form sarcocysts in interferon gene knockout (KO) and nude mice, but full details of sarcocyst development and details of bradyzoite structure are not known (Table 1). Natural intermediate hosts for *S. speeri*, also remain unknown.

Sarcocystis speeri has not been characterized molecularly because of the possibility of several species of *Sarcocystis* sporocysts in opossum feces. Unlike *S. neurona, S. speeri* was cultivated *in vitro* only once (Dubey *et al*. 2000*d*). This culture was obtained from an Argentinian opossum, *D.*

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Table 1. Transmission electron microscopic examination of sarcocysts of *Sarcocystis speeri*

			TEM of sarcocysts		
Opossum (no.)	Origin	Day p.i	Cyst wall	Bradyzoites	References
Didelphis virginiana (# 8026)	Florida, USA	50, 54	Described	Not described	Dubey *et al*. (1998); Dubey and Lindsay (1999)
Didelphis marsupialis	Sao Paulo, Brazil	53	Illustrated, not described	Not described	Dubey *et al*. (2000*a*)
Didelphis albiventris (# 8157)	Chascomus, Argentina	64	Illustrated, not described	Not described	Dubey *et al*. (2000*e*)
Didelphis albiventris[a]	Chascomus, Argentina	85	Illustrated, not described	Not described	Dubey *et al*. (2000*c*)
Didelphis virginiana (# 9062)	Florida, USA	See text	Described	Described	Present study
Didelphis albiventris (# 9068)	Argentina	See text			

[a] Transmitted to *Didelphis virginiana* via KO mice from *Didelphis albiventris*.

albiventris. *Sarcocystis neurona*, unlike *S. speeri* does not form sarcocysts in any strain of mice.

Here we report the development of *S. speeri* sarcocysts in KO mice. We also provide molecular characterization of *S. speeri* from culture derived merozoites with the objective that findings may be helpful in the discovery of its natural intermediate host.

MATERIALS AND METHODS

For the present study, *Sarcocystis* sporocysts were obtained from the intestinal scrapings of two adult opossums # 9062 and # 9068 (Table 1) using procedures described by Dubey (2000). Opossum # 9062 was *D. virginiana* from Metro Zoo, Florida and received at the Animal Parasitic Diseases Laboratory (APDL) on 12 April 1999. It had a mixed infection of *S. falcatula* and *S. speeri* sporocysts in feces, based on bioassay in mice and budgerigars (Dubey, 2000). Sporocysts from opossum # 9062 were fed to KO mice and sporocysts had been stored at 4 °C for 30 days before inoculation of mice.

Sporocysts from opossum # 9068, *D. albiventris* were received at APDL from Argentina on 21 April 1999, and information on this specimen is reported here for the first time. Sporocysts from opossum # 9068 were inoculated orally into 18 KO mice (BALB/c-Ifngtm1Ts) that were euthanized 1, 2, 4 (2 mice), 6, 8, 9, 11 (2 mice), 14, 17, 22, 25, 46, 52, 59, 104 and 140 days post-inoculation (p.i.). The sporocysts had been stored at 4 °C for 28–70 days before oral inoculation into KO mice in 1999. Sporocysts from opossum # 9062 were inoculated orally into 16 KO mice, and the mice were euthanized on days 1, 3, 5, 7, 9, 11, 13, 16 (2 mice), 34, 35, 37, 38, 39 (3 mice); the sporocysts had been stored in refrigerator from 1 to 135 days. The KO mice were obtained from Jackson Laboratories as described by Dubey and Lindsay (1998).

The number of *S. speeri* sporocysts in feces of the opossums was unknown; both opossums had also *S. falcatula* sporocysts as revealed by bioassay in budgerigars (data not shown). For estimation of *S. speeri* sporocysts in opossum # 9068, 10-fold serial dilutions were inoculated orally into groups of two KO mice. These inoculated mice were observed for 83 days. Bradyzoites were collected by pepsin digestion of infected muscles as described (Dubey *et al*. 1989).

Mice that were killed or died were necropsied. Portions of muscles (heart, tongue, leg) were fixed in 10% buffered neutral formalin and processed for histology. Paraffin-embedded sections were cut at 5 μm and examined after staining with haematoxylin and eosin (H and E).

For immunohistochemical (IHC) staining, paraffin sections were reacted with antibodies to *S. speeri* bradyzoites as described by Dubey and Lindsay (1999). For transmission electron microscopy (TEM), skeletal muscle from a KO mouse euthanized 220 day p.i. (Table 1) from a formalin-fixed specimen, embedded in paraffin, was processed for TEM as described (Dubey *et al*. 2015). Briefly, for TEM, ultra-thin sections (60–90 nm) were stained with uranyl acetate and lead citrate then examined in a JEOL JEM 1400 electron microscope.

The cryo-preserved culture of merozoites/schizonts derived from the liver of a nude mouse # 4309 infected with *S. speeri* from the third opossum (# 8157); collected from Argentina were used for molecular characterization. This culture was free of *S. neurona* and *S. falcatula* as evidenced by lack of infectivity to budgerigars and KO mice (Dubey *et al*. 2000*d*).

Culture-derived merozoites/schizonts of *S. speeri* isolate from opossums # 8157 were sequenced at three nuclear DNA regions; *18S* rRNA, *28S* rRNA, and *ITS1*, and two mitochondrial DNA regions; *cox1* and *cytb*. DNA was extracted using

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DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. DNA quantification and quality were determined by Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The complete regions of *18S* rRNA and *28S* rRNA were amplified using overlapping fragments and primer pairs; ERIB1/S2r, S5f/S4r, S3f/Primer Bsarc and KL1/LS2R, LS1F/KL3, respectively as described previously (Gjerde and Josefsen, 2015). In addition, the complete *ITS1* region and the partial sequence of *cox1* and *cytb* loci were also amplified using primer pairs SU1F/5·8SR2, SF1/SR5 and CybF1/CybR1, respectively (Gjerde and Josefsen, 2015). The polymerase chain reaction (PCR) amplifications were performed in 50 μL total reaction volume containing 10 pmol of each primer and 1× Taq PCR Master Mix Kit (Qiagen, Inc., Valencia). The thermal cycler (Veriti® Thermal Cycler, Applied Biosystems, Foster City, CA, USA) conditions were set at initial denaturation at 95 °C for 10 min; 40 cycles of amplification (95 °C for 45 s, 52–56 °C for 45 s and 72 °C for 1 min) and final extension at 72 °C for 10 min. Both, the positive (DNA of *Sarcocystis felis*) and the negative (H_2O) controls were included in all the batches, respectively. The amplified PCR products were run on 2·5% (w/v) agarose gel with ethidium bromide stain and visualized using Gel Logic 212 Imaging Systems (Eastman Kodak Company, Rochester, NY, USA).

The single PCR amplicons of *18S rRNA, 28S rRNA, ITS1, cox1* and *cytb* were excised from the gel and purified using QIAquick Gel Extraction (Qiagen, Inc., Valencia) according to the manufacturer's recommendation. The purified PCR products were sent to Macrogen Corporation (Rockville, MD, USA) for direct sequencing using the same primer pair used in PCR amplification to obtain both reads. The resulting sequences were imported, read, edited manually if necessary, and analysed using the software Geneious version 8.0·4 (Biomatters Ltd. Auckland, NZ). The sequences obtained were aligned against each other and various *Sarcocystis* spp. sequences published in NCBI database to detect interspecies variation on these DNA regions, respectively.

Phylogenetic trees were estimated by the Neighbour-Joining algorithm applied to Tamura-Nei genetic distances, as implemented by Geneious version 8.0·4. A phylogenetic tree based on *18S rRNA* sequences was constructed using sequences of the *S. speeri* isolate (opossum # 8157) and previously published sequences of various *Sarcocystis* spp. using software Geneious version 8.0·4. Input sequences were the *18S rRNA* regions of various *Sarcocystis* species and related taxon retrieved from GenBank. The second phylogenetic tree was constructed based on *ITS1* sequence obtained from *S. speeri* isolate (opossum # 8157). Input sequences were the *ITS1* regions of different *Sarcocystis* species and related taxon retrieved from NCBI GenBank. Trees were tested by selecting bootstrap method with the value of 1000 replicates.

RESULTS

Individual metrocytes were first seen in skeletal muscle of the mouse killed on 12 days p.i. (Fig. 1A). The presence of a prominent parasitophorous vacuole helped in the recognition of these early metrocytes (Fig. 1A and B). In H and E stained sections metrocytes were stained faintly, but stained strongly with anti-bradyzoite *S. speeri* rabbit antibodies (Fig. 1A, C and F). In a Giemsa-stained smear of infected mouse killed day 25 p.i., sarcocysts were serpentined, up to 4 mm long and up to 40 μm wide; metrocytes were often rectangular to ovoid and 10 μm long (Fig. 1D and E). Spike-like villar protrusions on the sarcocyst wall began to appear on day 34 p.i. and were clearly visible on day 39 p.i. (Fig. 1G). Few bradyzoites had formed by 46 days p.i., but sarcocysts at day 50 p.i. contained predominantly metrocytes.

Each of two KO mice fed a 10^{-4} dilution of the sporocyst inoculums from opossum # 9068 was infective to KO mice; numerous bradyzoites were recovered in pepsin digests of muscles of mice 83 days p.i. Bradyzoites were not found in muscle digests of two KO mice killed 83 days after oral inoculation with 10^{-6} dilution of sporocysts; 10^{-5} dilution was not tested. Thus, at least 1000 infective sporocysts were in the inocula used to study development of sarcocysts.

Sarcocysts from KO mice killed 104, 140 and 220 days p.i. were examined ultra-structurally. The sarcocyst wall at 220 days p.i. (Fig. 2) was identical to sarcocysts illustrated previously from KO mice killed 50 – 85 days p.i. (Table 1). The sarcocyst wall from the tip of the villar protrusions to the base of bradyzoites was up to 2·5 μm thick, depending on the length of villar protrusions, the ground substance (gs) was approximately 1 μm thick (Fig. 2A). The villar protrusions were steeple-shaped surmounted by a spire, type 38 (Dubey *et al.* 2015). The longitudinally cut bradyzoites ($n = 7$) in TEM sections measured 7·5–9·5 × 2·0–3·2 μm in size (Fig. 3). They contained a conoid, rhoptries, micronemes, amylopectin granules and a nucleus. Micronemes were numerous, often arranged haphazardly, and located in the 1–2 μm conoidal part of the bradyzoite; they were approximately 0·4 μm long and slender. Rhoptries were few; no more than two were identified with certainty because their contents appeared similar to those of

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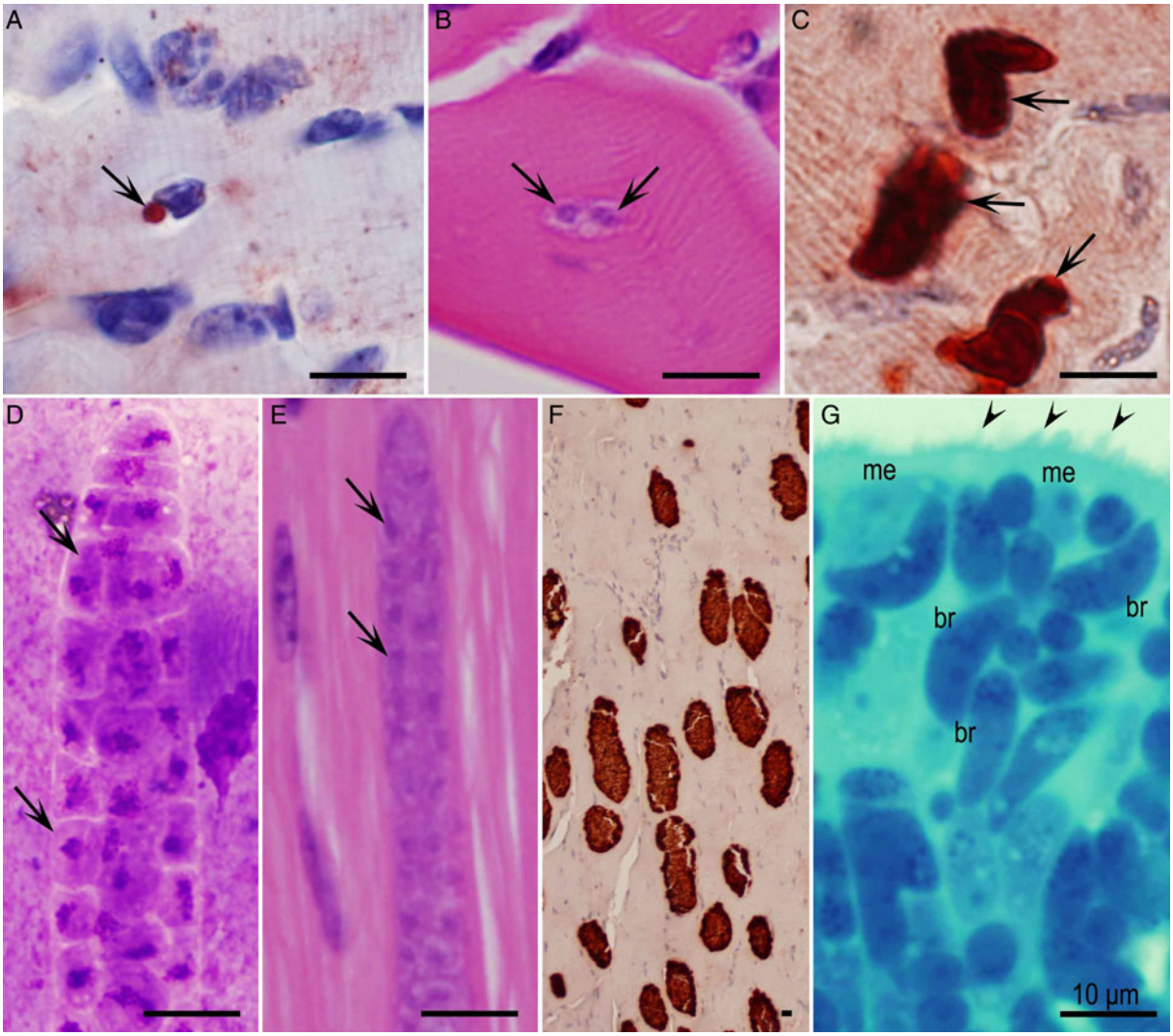

Fig. 1. *Sarcocystis speeri* sarcocysts in skeletal muscles of KO mice fed sporocysts. D –smear stained with Giemsa, the rest are tissue sections. A, C and F –IHC staining with anti–*S. speeri* antibodies, B and E stained with haematoxylin and eosin, G stained with Toluidine Blue, (A) Myocyte containing 1 metrocyte inside a vacuole (arrow), day 14 p.i. (B) A myocyte with two metrocytes (arrows), each in a separate vacuole. day 14 p.i. (C) Three immature sarcocysts. Day 17 p.i. (D, E) Immature sacrocysts with metrocytes (arrows). Day 25 p.i. The metrocytes in smear are much bigger in size than metrocytes in sections. (F) Numerous sarcocysts. Day 52 p.i. (G) Mature sarcocyst. Note villar protrusions (arrowheads) on sarcocyst wall, few metrocytes (me) and longitudinally cut bradyzoites (br). Day 222 p.i. Bar applies to all parts.

dense granules. There was one convoluted mitochondrion. Amylopectin granules were numerous and distributed in most of the bradyzoite length, except the region containing micronemes (Fig. 3). The nucleus was up to 3 μm long and located terminally/sub-terminally. The non-conoidal end was round to conical in shape.

The *18S rRNA* (in three fragments), *28S rRNA* (in two fragments), *ITS1*, *cox1* and *cytb* loci were amplified by PCR using DNA of *S. speeri* isolate from opossum # 8157. DNA sequencing of PCR amplicons resulted in unambiguous sequences of three nuclear DNA regions; *18S rRNA* (1751 bp), *28S rRNA* (1522 bp) and *ITS1* (1190 bp), and two mitochondrial DNA loci; *cox1* (1057 bp) and *cytb* (741 bp). These sequences were submitted to GenBank with accession numbers; KT207459 (*18S rRNA*), KT207460 (*28S rRNA*), KT207458 (*ITS1*), KT207461 (*cox1*) and KT207462 (*cytb*).

Phylogenetic analysis based on both the *18S rRNA* and the *ITS1* sequences obtained from *S. speeri* isolate from opossum # 8157 confirmed its membership among species of *Sarcocystis* and indicated an especially close relationship to another parasite in this genus that employs opossums as its definitive host, *S. neurona* (Figs 4 and 5). The *28S rRNA*, *cox1* and *cytb* sequences of *S. speeri* shared the highest identity with sequences of *S. neurona* (AF092927) 99·0%, *Sarcocystis lutrae* (KM657808) 99·0% and *Toxoplasma gondii* strain RH (JX473253) 100%, respectively.

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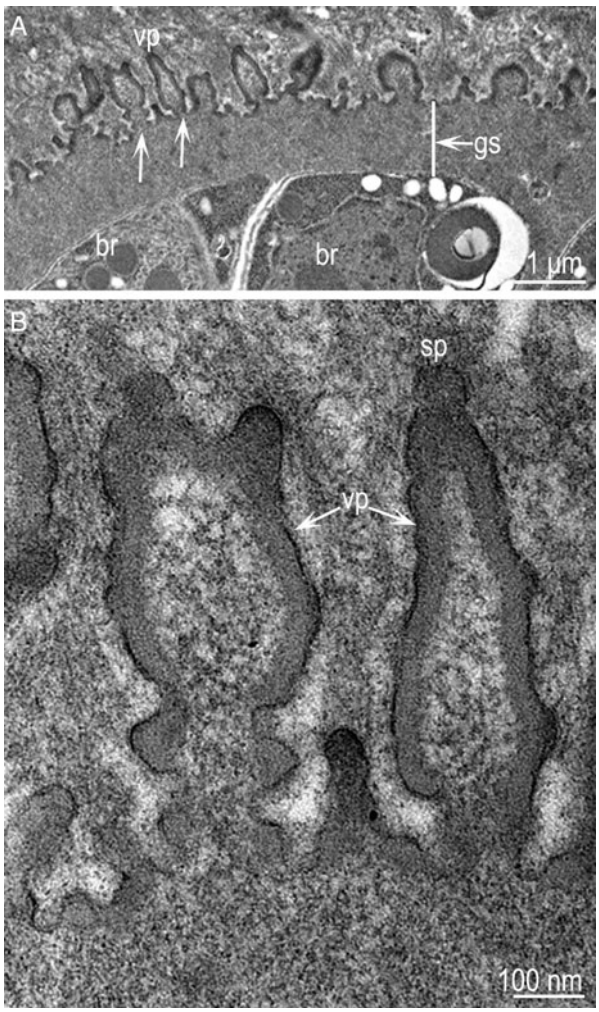

Fig. 2. TEM of the sarcocyst wall of *Sarcocystis speeri* in KO mouse, 222 days p.i. (A) Note steeple-shaped surmounted by a spire villar protrusions (vp, arrows), a thick ground substance (gs) without granules, and bradyzoites (br). (B) Higher magnification of villar protrusions marked with arrows in Fig. 2A. Note spire (sp) and absence of microtubules in vp. Abbreviation: TEM, transmission electron microscopic.

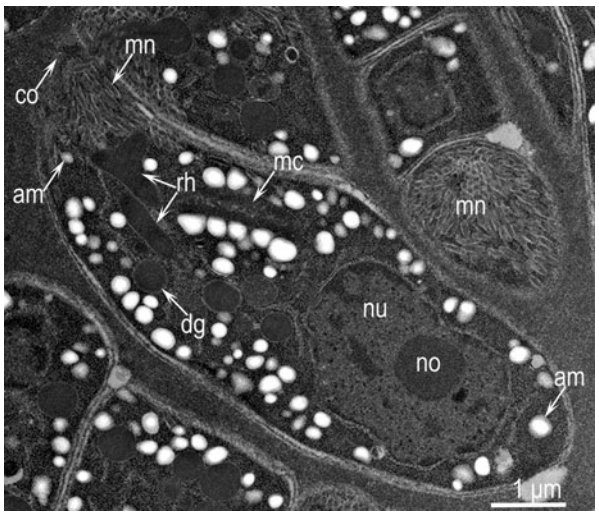

Fig. 3. TEM of a longitudinally cut bradyzoite from the sarcocyst in Fig. 2A. Note conoid (co), micronemes (mn), amylopectin granules (am), 2 rhoptries (rh), a mitochondrion (mc), dense granules (dg) and a nucleus (nu) with a prominent nucleolus (no). Abbreviation: TEM, transmission electron microscopic.

A nearly complete locus of *18S rRNA* sequences (1751 bp) was obtained from *S. speeri* isolate after PCR amplification and sequencing of three overlapping fragments. The *18S rRNA* sequences obtained from *S. speeri* isolate shared the highest identity with sequences of *S. neurona* strain SN5 (U07812). *Sarcocystis speeri* and *S. neurona* (U07812) differed at 5 of 1751 aligned sites.

The *ITS1* sequence obtained from *S. speeri* isolate also shared the highest identity with sequences of *S. neurona* (AF252407). Ten of 1190 nucleotide positions were recorded having variations between these two sequences (99·4% identity).Four of 10 SNPs are confirmed between *S. speeri* (KT207458) and *S. neurona* (AF252407) *ITS1* sequences. The other six nucleotides differences between these two sequences indicating uncertainty or a potential of two different nucleotides at particular sites because *S. speeri* sequence chromatograms in both, the forward and the reverse reads recorded double peaks at these positions.

The *28S rRNA* sequences of *S. speeri* and *S. neurona* (AF092927) shared the highest identity (99·0%). *Sarcocystis speeri* sequences chromatograms in both, the forward and the reverse reads recorded double peaks (C and G) at position 482; the C base was predominant and selected base call. *28S rRNA* of *S. speeri* also shared the higher sequence identity with other species; *S. calchasi* (FJ232949), *S. arctica* (KF601312), *Sarcocystis* (*Frenkelia*) *glareoli* (AF044251), *S. lutrae* (KM657772) and *S. rileyi* (KJ396585).

The partial *cox1* sequence (1057 bp) was obtained from *S. speeri* isolate after PCR amplification and sequencing. The *cox1* sequences obtained from *S. speeri* isolate shared 99% identity with *S. lutrae* (KM657808), 98% identity with *S. arctica* (KF601321), 97% identity with *S. rileyi* (KJ396582) and 83% identity with *S. hardangeri* (KC209630).

The partial *cytb* sequences (741 bp) obtained from *S. speeri* isolate shared 100% identity with sequences of *T. gondii* strain RH (JX473253), corresponding to nucleotide positions 13–754. It did not match with any available *Sarcocystis* spp. *cytb* sequences. Identical results have been reported in the previous studies that applied same primers; CybF1/CybR1 for amplification of *S. lutrae* (Gjerde and Josefsen, 2015). It was possible that the target region of these primers was more conserved portion of the gene. Limited sequences data of *cytb* gene from *Sarcocystis* spp. are available to compare.

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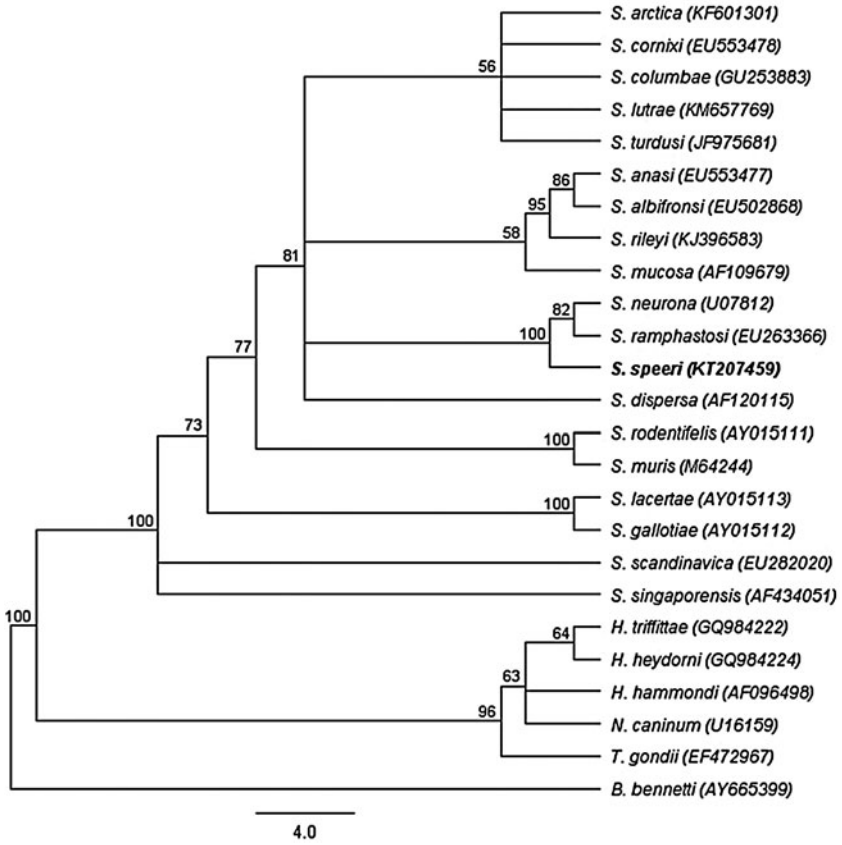

Fig. 4. Phylogenetic tree based on *18S rRNA* sequences. Input sequences were the *18S rRNA* regions of various species retrieved from GenBank, and 1751 bp long sequence obtained from *S. speeri* isolate. Accession number of gene sequences was given in parenthesis following the species name. Tree was built by selecting the Tamura-Nei genetic distance model and Neighbour-Joining tree methods (Geneious version 8.0·4). Tree was tested by selecting bootstrap method with value of 1000 replicates. *Sarcocystis speeri* was inferred to share an especially close relationship to *S. neurona*, another species employing opossums as its definitive host and *S. ramphastosi* an avian species of *Sarcocystis* with unknown definite host.

DISCUSSION

The sarcocyst seen in the present study were structurally similar to those observed previously as described in Table 1. In the present study, additional details on development of the cyst wall and structures of bradyzoites are provided. Sarcocysts were first seen at 12 days p.i., and they became macroscopic by 25 days p.i. The structure of the sarcocyst wall did not change from the time bradyzoites had formed at 50 – 220 days p.i.

To enhance the genetic characterization of this specimen, we sequenced three nuclear (*18S rRNA, 28S rRNA* and *ITS1*), and two mitochondrial loci; *cox1* and *cytb* of *S. speeri* isolate were sequenced and compared with homologues in other species of *Sarcocystis*. Phylogenetic inferences based on sequences of *18S* and *ITS1* portions of *rRNA* confirmed a close relationship of *S. speeri* to other species of *Sarcocystis*, particularly those which also employ the opossums as their definitive hosts, i.e. *S. neurona*. In the phylogenetic tree based on *18S rRNA* sequences, *S. speeri* grouped closely with *S. neurona* and *S. ramphastosi*. *Sarcocystis ramphastosi* is an avian species of *Sarcocystis* with unknown definite host (Dubey *et al*. 2004). In the phylogenetic tree based on *ITS1* sequences; *S. speeri* clustered in a clade together with *S. neurona, S. falcatula, S. dasypi* and *S. lindsayi*; all four employs opossums as the definitive host.

The *28S rRNA* sequences of *S. speeri* shared the highest identity with sequences of *S. neurona* (AF092927). The *cox1* sequences of *S. speeri* isolate shared the highest identity with sequences of *S. lutrae* (KM657808). These two sequences can be differentiated by 10 SNPs. The use of *cox1* gene as a genetic marker for *Sarcocystis* species discrimination has been proposed recently, so only limited few sequences are now available for comparative use (Gjerde, 2013).

These data sufficed to conclude, however, that *S. speeri* is very closely related other species of *Sarcocystis* that employ opossums as their definitive hosts. There were few SNPs that differentiated this isolate of *S. speeri* exemplars of *S. neurona*. *Sarcocystis speeri*, complementing certain phenotypic characteristics (i.e. development of sarcocysts in KO mice within 12 days that become macroscopic within 25 days bearing a 'type 38' wall structure that does

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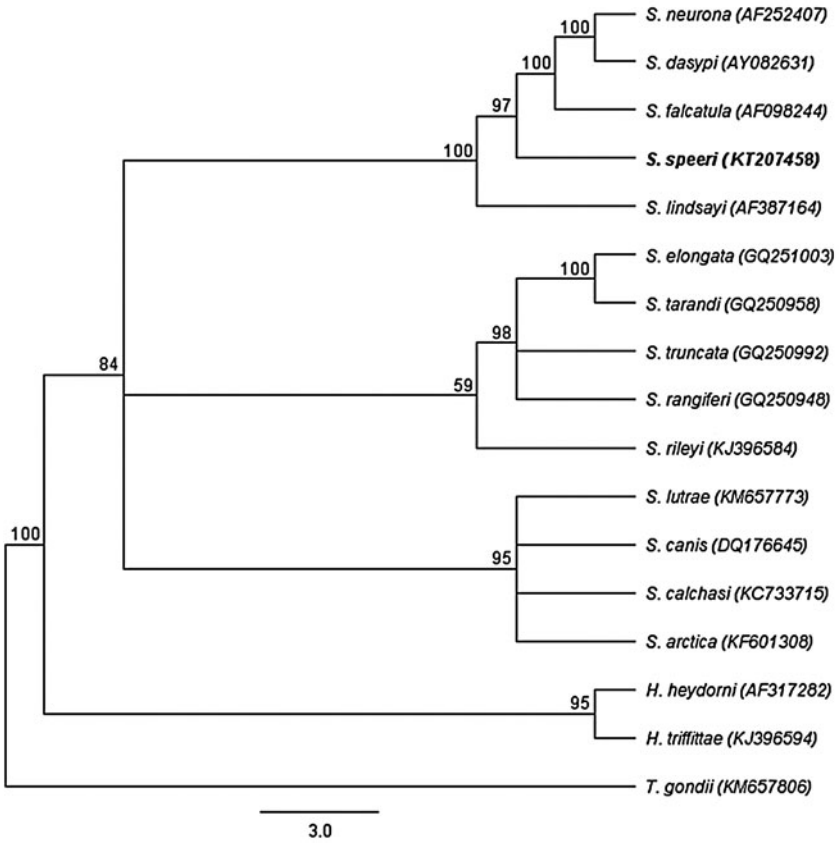

Fig. 5. Phylogenetic tree based on *ITS1* sequences. Input sequences were the *ITS1* regions of various species retrieved from GenBank, and 1190 bp long sequences obtained from *Sarcocystis speeri* isolate. Accession number of gene sequences was given in parenthesis following the species name. Tree was built by selecting the Tamura–Nei genetic distance model and Neighbour-Joining tree methods (Geneious version 8.0·4). Tree was tested by selecting bootstrap method with value of 1000 replicates. *Sarcocystis speeri* clustered together with *Sarcocystis* species that employs opossums as a definitive host.

not change with age) that set them apart from other known species such as *S. falcatula* and *S. neurona*.

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