Parasitology International 66 (2017) 106–111

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Isolation, molecular characterization, and *in vitro* schizogonic development of *Sarcocystis* sp. ex *Accipiter cooperii* from a naturally infected Cooper's hawk (*Accipiter cooperii*)

David S. Lindsay [a,*], Shiv K. Verma [b], David Scott [c], Jitender P. Dubey [b], Alexa R. von Dohlen [d]

[a] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Faculty of Health Sciences, Virginia Tech, Blacksburg, VA 24061, USA
[b] United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705-2350, USA
[c] Carolina Raptor Center, 6000 Sample Road, Huntersville, NC 28078, USA
[d] Department of Natural Sciences and Mathematics, Johnson C. Smith University, Charlotte, NC, 28216, USA

ARTICLE INFO

Article history:
Received 6 October 2016
Received in revised form 17 November 2016
Accepted 5 December 2016
Available online xxxx

Keywords:
Cooper's hawk
Accipiter cooperii
Cell culture
Sarcocystis sp. ex *A. cooperii*
Sarcocystis columbae
Sporocysts
Raptor

ABSTRACT

Raptors serve as the definitive host for several *Sarcocystis* species. The complete life cycles of only a few of these *Sarcocystis* species that use birds of prey as definitive hosts have been described. In the present study, *Sarcocystis* species sporocysts were obtained from the intestine of a Cooper's hawk (*Accipiter cooperii*) and were used to infect cell cultures of African green monkey kidney cells to isolate a continuous culture and describe asexual stages of the parasite. Two clones of the parasite were obtained by limiting dilution. Asexual stages were used to obtain DNA for molecular classification and identification. PCR amplification and sequencing were done at three nuclear ribosomal DNA loci; *18S rRNA*, *28S rRNA*, and *ITS-1*, and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) locus. Examination of clonal isolates of the parasite indicated a single species related to *S. columbae* (termed *Sarcocystis* sp. ex *Accipiter cooperii*) was present in the Cooper's hawk. Our results document for the first time *Sarcocystis* sp. ex *A. cooperii* occurs naturally in an unknown intermediate host in North America and that Cooper's hawks (*A. cooperii*) are a natural definitive host.

1. Introduction

Sarcocystis infection is common in the skeletal muscles [1] and intestines of raptors in the United States [2]. However, little is known about the species diversity of these parasites and the types of intermediate hosts used in their life cycles [3]. They have the potential to be the source of fatal neurological disease in other avian species [4–9], and raptors themselves can also suffer from fatal neurological disease caused by *Sarcocystis* species [10–13].

We are interested in understanding the diversity of *Sarcocystis* species that use birds of prey as intermediate and definitive hosts. The present study describes *in vitro* isolation of a *Sarcocystis* species from the intestinal contents of a naturally infected Cooper's hawk (*Accipiter cooperii*) and analysis of DNA isolated from merozoites obtained from cell cultures. Hawks of the genus *Accipiter* are definitive hosts for *S. accipitris*, *S. calchasi*, *S. columbae*, and *Sarcocystis* sp. ex *A. nisus* from Europe [14–16] that use birds as intermediate hosts. Disease cause by *S. calchasi* has recently been observed in several avian intermediate hosts in the United States [7–9] but the definitive host has not yet been identified. Our hypothesis was that Copper's hawks would serve as definitive host for 1 of these 4 *Sarcocystis* species in North America.

2. Materials and methods

2.1. Source, isolation and excystation of sporocysts

A male Cooper's hawk (#18677) was admitted March 21, 2015 from Pickens, South Carolina at the Carolina Raptor Center, Huntersville, North Carolina, USA for treatment. The patient was not able to stand due to spinal trauma. The bird was euthanized because it could not be rehabilitated and released. A necropsy was conducted by one of us (DS) and a fracture was observed at T2–T3. The intestinal tract was removed, placed in a plastic bag, and refrigerated at 4 °C until brought by motor vehicle to the Zoonotic Protozoal Diseases Laboratory (ZPDL), Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia, USA by ARD. The intestinal tract was opened using scissors and smears were made from 3 different regions and examined using light and UV microscopy [17]. After the identification of *Sarcocystis* oocysts and sporocysts, intestinal mucosal was scrapped off using a glass slide and

* Corresponding author at: Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061, USA.
E-mail address: lindsayd@vt.edu (D.S. Lindsay).

http://dx.doi.org/10.1016/j.parint.2016.12.002
1383-5769/

placed in a sterile 50 ml screw cap disposable test tube. Ten milliliters of commercial bleach was added to the test tube and it was vigorously shaken by hand for 15 to 30 s. After the bleach had been in contact with the intestinal scrapings for 10 min, the 50 ml tube was filled with Hanks's balanced salt solution (HBSS) and it was centrifuged at 1500 RPM for 10 min. The pellet was resuspended in 15 ml HBSS in a sterile 15 ml screw cap disposable test tube and it was centrifuged for 10 min. This was repeated 2 additional times and the pellet resuspended in 2 ml HBSS and stored at 4 °C until used. Sporocysts were measured using a calibrated ocular micrometer.

Sporocysts in 0.5 ml of 37 °C HBSS were combined with 0.5 ml of 37 °C excystation solution containing 1.5% (w/v) sodium taurocholic acid and 0.5% trypsin (w/v) (Sigma Chemical Co., St. Louis, MO, USA) in HBSS in a 15 ml sterile test tube. Sterile 2 mm glass beads were added up to about 0.5 ml in the test tube. The test tube was vortexed for about 10 s then the mixture was incubated at 37 °C in a water bath for 30 min. The excystation solution was washed off by repeated centrifugation in HBSS and the pellet used to inoculate a 25 cm^2 cell culture flask containing African green monkey kidney (CV-1) cells (ATCC CCL-70, Manassas, VA, USA).

2.2. Cell culture, infections, and examinations

2.2.1. Cell culture

We examined development in African green monkey kidney (CV-1) cells (ATCC CCL-70, Manassas, VA, USA). Host cells were grown in 25 cm^2 cell culture flasks or on 22 mm^2 glass coverslips in 6 welled cell culture plates in RPMI 1640 cell culture medium (Mediatech, Inc., Manassas, VA, USA) containing 100 IU penicillin/ml, 100 μg/ml streptomycin/ml and 10% (v/v) fetal bovine serum (FBS). Cells were maintained in the same medium except the concentration of FBS was 2%. Living cell cultures were examined in 25 cm^2 flasks using an inverted microscope equipped with phase-contrast optics (Zeiss Invertoskope) daily or every other day until the end of the study.

2.2.2. Infections and light microscopy

Once merozoites were observed in cell culture additional flasks of CV-1 cells were infected to serve as a source of merozoites for DNA and to infect additional flasks and coverslips to study development. Merozoites were harvested from infected CV-1 cells by removing the medium and replacing it with HBSS. The CV-1 cells were then removed from the plastic growth surface by use of a cell scraper. This merozoites containing cell mixture was then filtered through a sterile 3 μm filter to remove cellular debris. The number of merozoites in the filtrate was determined using a hemocytometer.

Coverslips containing CV-1 cells were each inoculated with 2×10^5 merozoites in 3,000 μl of HBSS. The inoculum was removed 24 h post-inoculation (PI) and replaced with maintenance medium. One or two cover slips were removed at 1, 2, 3, 4, 5, and 6 days PI and processed for light microscopic examination after staining with Giemsa. The 22-mm^2 coverslips were fixed in 10% (v/v) buffered formalin solution for 10 min then post-fixed in 100% methanol for 10 min, air dried, stained with Giemsa for 1 h, and mounted on glass slides using Permount™ (Fisher Scientific Company, Fair Lawn, NJ, USA) for microscopic examination. Slides were observed and photographed using an Olympus BH60 microscope (Olympus America Inc., Center Valley, PA, USA) equipped with epifluorescent, UV, and differential interference contrast (DIC) optics. Developmental stages were measured using a calibrated ocular micrometer.

2.2.3. Parasite cloning

Clones were obtained by inoculating CV-1 cell monolayers grown in 96 welled flat-bottomed cell culture plates. Merozoites were collected and diluted to a concentration of 1 merozoite per ml and CV-1 cell monolayers were inoculated with 100 μl of merozoites containing maintenance medium. After 9 days PI cultures were examined and wells containing merozoites were collected using a sterile pipette tip and transferred to CV-1 cells grown in 25 cm^2 culture flasks. Clones were expanded by growth in CV-1 cells and merozoites collected and used for DNA analysis (see below).

2.2.4. Transmission electron microscopy

For transmission electron microscopy (TEM) a cell culture was scraped off a 25 cm^2 flask and pelleted by centrifugation. The supernatant was removed and the pellet fixed in 3% (v/v) glutaraldehyde in phosphate buffer (PBS, pH 7.4). The infected culture was post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanols, passed through 2 changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA, USA). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10CA TEM operating at 60 kV. Digital images were captured using an AMT camera system (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

2.2.5. DNA extractions and PCR amplification

Genomic DNA from cell culture merozoites/schizonts was extracted using DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer instructions. DNA quantification and quality were determined by Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

PCR amplification and sequencing were done at three regions of nuclear ribosomal DNA unit; *18S rRNA*, *28S rRNA*, and *ITS-1*, and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) locus. 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 [18]. In addition, the complete *ITS-1* region and the partial sequence of *cox1* locus were also amplified using primer pairs SU1F/5.8SR2, and SF1/SR5 respectively [18]. The 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, CA, USA). 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 from *S. neurona* isolate) 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 by using Gel Logic 212 Imaging Systems (Eastman Kodak Company, Rochester, NY, USA).

2.2.6. DNA sequencing and phylogenetic analysis

The PCR amplicons of *18S rRNA*, *28S rRNA*, *ITS-1*, and *cox1* were excised from the gel, and purified using QIAquick Gel Extraction (Qiagen, Inc., Valencia, CA, USA) 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 amplification to obtain both reads. The resulting sequences were imported, read, edited manually if necessary, and analyzed using the software Geneious version 9.0.4 (Biomatters Ltd. Auckland, NZ). New sequences were compared with other sequences deposited in NCBI GenBank by BLASTn analysis to detect intra-species and interspecies variation on these DNA regions.

A phylogenetic tree based on *ITS-1* sequences was constructed using new sequences obtained from cell culture merozoites/schizonts of *Sarcocystis* species from Cooper's hawk. Input sequences were the *ITS-1* regions of different *Sarcocystis* species and related taxon retrieved from NCBI GenBank. One hundred bootstrap replicates of the phylogenetic relationships among these sequences were reconstructed under the criterion of maximum likelihood using PhyML as implemented in Geneious version 9.0.4 [19]. The HKY85 model of nucleotide substitution was used, estimating the transition/transversion ratio and the

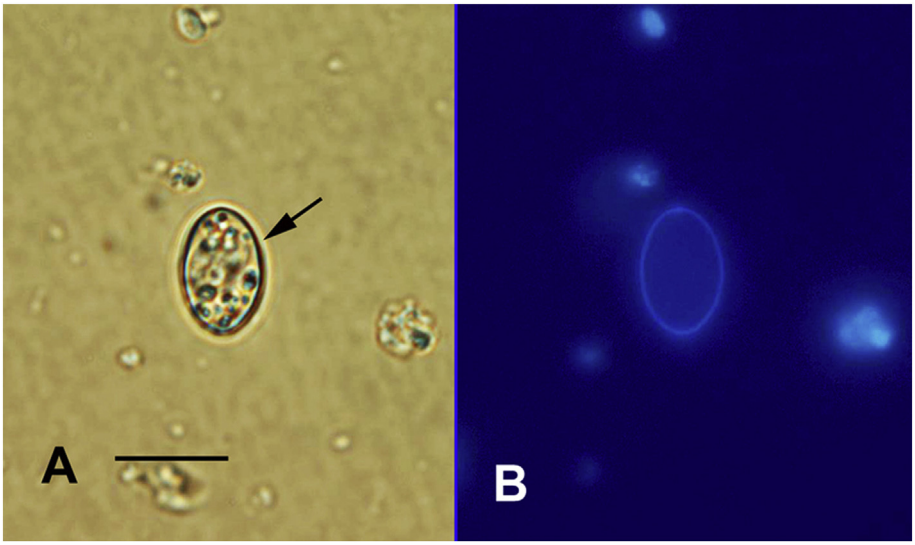

Fig. 1. Sporocyst of *Sarcocystis* sp. ex *A. cooperii* from a Cooper's hawk. A. Bright-field microscopy demonstrating a sporocyst (arrow) with a dispersed granular residuum. B. Same sporocyst as in panel A demonstrating autofluorescence using UV light. Bar = 10 μm.

gamma distribution parameter to model rate variation among sites. No sites were presumed to be invariant. The complete deletion method was used for ambiguous/missing data, and topologies were searched using the nearest neighbor interchange method. The final dataset incorporated 47 sequences, and *Besnoitia bennetti* as an out group.

The species of *Sarcocystis* was further confirmed by PCR amplification using previously published semi-nested primer pairs; SCa1/SCa2/SNCa3, SCo1/SCo2/SNCo3, and SNi1/SNi2/SNNi3 that amplifies specifically an *ITS-1* region of *S. calchasi* (136 bp), *S. columbae* (129 bp) and *Sarcocystis* sp. ex *Accipiter nisus* (124 bp) [20]. Briefly, the primary PCR amplifications were performed in 50 μl total reaction volume containing 3 μl of template DNA, 20 pmol of each primer pairs; SCa1/SCa2, SCo1/SCo2 and SNi1/SNi2, and 1 × Taq PCR Master Mix Kit (Qiagen, Inc., Valencia, CA, USA) in a thermal cycler. The thermal cycler conditions were set at initial denaturation at 95 °C for 5 min; 35 cycles of amplification (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s) and final extension at 72 °C for 10 min. Semi-nested PCR was carried out using primer pairs: SCa1/SNCa3, SCo1/SNCo3 and SNi1/SNNi3, and 2 μl of 1:100 diluted product of primary PCR as a template in 50 μl total reaction volume. Same thermal cycler conditions were used in both PCR reactions. The amplified PCR products were run on 2.5% (w/v) agarose gel with ethidium bromide stain and visualized by using Gel Logic 212 Imaging Systems.

3. Results

3.1. Isolation and excystation of sporocysts

Oocysts and sporocysts of *Sarcocystis* species were observed in all locations of the intestine that were examined. Fifteen sporocysts measured 12.9 by 7.9 μm (range = 10.0 to 15.0 by 7.5 to 10.0 μm) contained a granular sporocyst residuum and fluoresced blue using UV light (Fig. 1). Approximately 4×10^5 sporocysts were isolated from the intestine. Sporocysts but no sporozoites were observed in the preparation exposed to excysting solution prior to its use to inoculate cell cultures.

3.2. Cell culture, experimental infections, cloning, light and transmission electron microscopy

Developmental stages consisting of a few extracellular merozoites were first observed 33 days PI and intracellular schizonts were seen 34 PI. Subcultures of merozoites in cell culture fluid were done 40 days PI and the parasite has been maintained by subculture since that time.

Intracellular merozoites, and other developmental stages were observed next to the host cell (Figs. 2, 3). The merozoites increased in size to become schizonts (Fig. 2) and developed by endopolyogeny to produce numerous merozoites (Fig. 3). Mature schizonts were seen 4 days PI. Merozoites often stayed in the same host cell and many developing schizonts could be observed in what appeared to be the same host cell. Early schizonts looked like merozoites that had enlarged and the distinction between merozoites and developing schizonts was subjective. Non-staining vacuoles were often present in the cytoplasm of developing schizonts. As they enlarged schizonts usually were more sausage shaped (Fig. 2) but some still looked like large merozoites (Fig. 3). Blebs developed on the surface of schizonts and this indicated merozoites formation was about to occur. Some of these schizonts developed constrictions and eventually produced merozoites that were in a rosette pattern developing off a residuum (Fig. 3). Several schizonts producing merozoites in a rosette pattern in the same cell could be observed. Merozoites eventually separated from the rosette and became dispersed in the host cell cytoplasm. Fifteen merozoites were 5.4 by 1.9 μm (range = 5 to 6 by 1 to 2 μm). Extracellular living merozoites were actively motile.

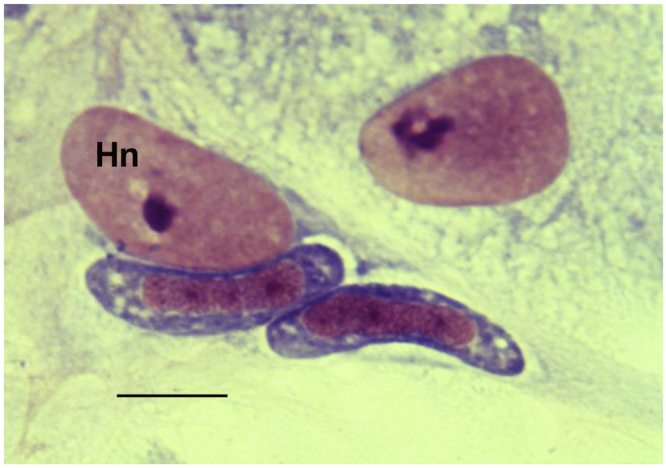

Fig. 2. Two developing sausage shaped schizonts of *Sarcocystis* sp. ex *A. cooperii* in CV-1 cells. Note that development is next to the host cell nucleus (Hn). Bar = 10 μm.

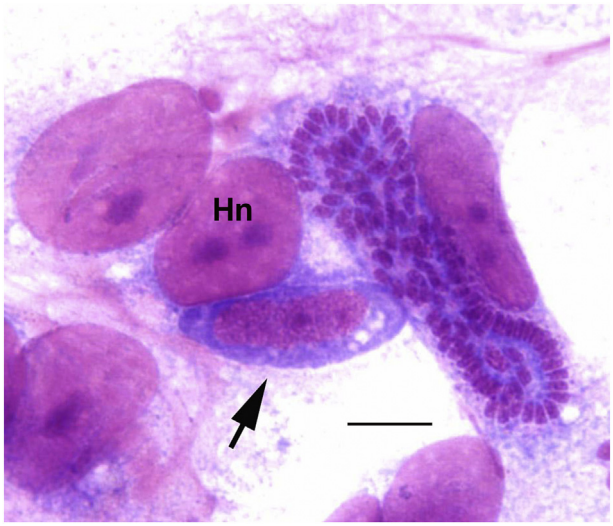

Fig. 3. Mature schizont of *Sarcocystis* sp. ex *A. cooperii* containing merozoites and an immature schizont (arrow) developing next to the host cell nucleus (Hn). Bar = 10 μm.

We obtained 2 clones named CHC-1 and CHC-2 and isolated DNA from merozoites of these clones. The DNA of the 2 clones was compared to the DNA from merozoites of the original isolate by PCR amplification and DNA sequencing using species specific *ITS-1* primers of *S. calchasi*, *S. columbae,* and *Sarcocystis* sp. ex *Accipiter nisus* [20].

Transmission electron microscopy demonstrated that the parasites developed directly in the host cell cytoplasm, that development was by endopolyogeny, that merozoites lacked rhoptries, and that they contained all organelles typical of *Sarcocystis* merozoites (Fig. 4).

3.3. PCR

PCR analysis with schizonts/merozoites DNA as the template yielded amplicons of the expected size for the *18S rRNA*, *28S rRNA*, *ITS-1*, and *cox1* loci. PCR-DNA sequencing of amplicons resulted the unambiguous sequences of three nuclear DNA regions; *18S rRNA* (1325 bp), *28S rRNA* (752 bp), and *ITS-1* (1000 bp), and the mitochondrial DNA locus; *cox1* (900 bp). These sequences were submitted to NCBI GenBank with accession numbers KY348753 (*18S rRNA*), KY348754 (*28S rRNA*), KY348755 (*ITS-1*), and KY348756 (*cox1*) and designated as originating from *Sarcocystis* sp. ex *Accipiter cooperii*.

Analysis of *ITS-1* sequences obtained from the DNA of cell cultured merozoites of *Sarcocystis* sp. ex *Accipiter cooperii* confirmed its membership among genus *Sarcocystis* and indicated an especially close relationship to another parasite in this genus that employs birds as their hosts (Fig. 5); *S. columbae*, *S. corvusi*, *Sarcocystis* sp. ex *Phalacrocorax carbo*, *Sarcocystis* sp. ex *Columba livia*, *Sarcocystis calchasi*, and *Sarcocystis wobeseri*. *ITS-1* sequences that show higher divergence, shared the highest sequence identity (96.0%) of *Sarcocystis* sp. ex *A. cooperii* with *S. columbae* (GU253885, HM125052), followed by 94.0% identity with *S. corvusi* isolate kuos1 (JN256119), *Sarcocystis* sp. ex *P. carbo* isolate Dkorm1 (JQ733513), and 84.0% *Sarcocystis* sp. ex *C. livia* (FJ232948), *S. calchasi* strain A1259 (KC733715), *S. wobeseri* isolate kir01 (HM159421), and *Sarcocystis* sp. ex *A. nisus* (GU253886). In the phylogenetic tree based on *ITS-1* sequences; *Sarcocystis* sp. ex *A. cooperii* clustered consistently in a clade together with *S. columbae* (HM125052), and with less bootstrap support with *S. corvusi* (JN256119), and *Sarcocystis* sp. ex *P. carbo* (JQ733513) (Fig. 5); all three employs birds as their hosts [3,15,21,22].

The *18S rRNA* sequences are commonly used to show phylogenetic relationship and diagnosis of *Sarcocystis* species [3]. The *18S rRNA* sequence (KY348753) of *Sarcocystis* sp. ex *A. cooperii* shared 100% identity with sequences of *Sarcocystis* sp. ex *P. carbo* isolate Dkorm (JQ733511), *Sarcocystis* sp. ex *C. livia* (GQ245670), and *Sarcocystis* sp. cyst type I ex *Anser albifrons* isolate 09 (EU502869), followed by 99.0% identity with other sequences; *S. corvusi* isolate kuos1 (JN256117), *S. columbae* isolate lith (HM125054), *S. turdusi* isolate M 41 (JF975681), *S. lari* isolate balnkr1 (JQ733508), and many other species of *Sarcocystis*.

The partial *28S rRNA* sequence of *Sarcocystis* sp. ex *A. cooperii* (KY348754) shared 100% identity with sequences of *S. corvusi* isolate kuos1 (JN256118), *Sarcocystis* sp. ex *P. carbo* isolate Dkorm1 (JQ733512), and *S. columbae* (HM125053, GU253887).

The partial *cox1* sequence of *Sarcocystis* sp. ex *A. cooperii* (KY348756) shared 99% identity with *S. lutrae* (KM657808, KF601326), *S. arctica* (KF601318-KF601321), and *S. speeri* (KT207461) and 98% identity with *S. neurona* strain BR2012 (KF854272).

The semi-nested PCR resulted in amplification of 129 bp long single bands by the *S. columbae* specific primer pairs. There was no PCR amplification product visible in the gel by both the *S. calchasi*, and the *Sarcocystis* sp. *ex A. nisus* specific primers. PCR-DNA sequencing of DNA from both clones; CHC-1, CHC-2 and the original isolate showed identical results, and confirmed *S. columbae* infection in Cooper's hawk [20]. Complete *ITS-1* sequences (KY348755) obtained by PCR-DNA sequencing of DNA from original cell cultured merozoites and from both clones; CHC-1, CHC-2 of *Sarcocystis* sp. ex *A. cooperii* resulted unambiguous 100% identical sequences, and confirmed *S. columbae*-like infection in this Cooper's hawk.

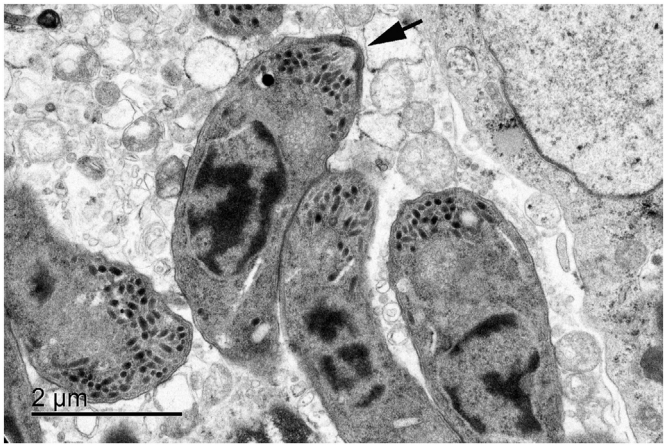

Fig. 4. Transmission electron micrograph of merozoites of *Sarcocystis* sp. ex *A. cooperii*. Note the conoid (arrow) and absence of rhoptries in the merozoites.

4. Discussion

The measurements of sporocysts in our study were 12.9 × 7.9 μm and the sporocysts of *S. calchasi* were 11.9 × 7.9 μm. Sporocysts measuring 11.9 × 8.3 μm were reported from sparrow hawks (*A. nisus*) after feeding meat from wood pigeon (*Columba palumbus*) containing sarcocysts of *S. columbae* and or *Sarcocystis* sp. ex *A. nisus* [15]. The structure of the oocyst and sporocysts traditionally used to determine species of other coccidia has limited taxonomic value in *Sarcocystis* because, except for minor variations in size, all *Sarcocystis* sporocysts and oocysts are structurally similar [3]. Additionally, the sporocysts of numerous species of *Sarcocystis* in a given host may overlap in dimensions. For example, dogs are definitive hosts for 22 species and cats are host for 12 species, and sporocysts of most canine species are about 15 × 10 μm and those of feline species are about 12 × 10 μm [3]. PCR-DNA sequencing of DNA from original cell cultured merozoites and from both clones; CHC-1, CHC-2 of *Sarcocystis* sp. ex *A. cooperii* resulted unambiguous 100% identical sequences (1000 bp that covered partial *18S rRNA gene*, complete *ITS-1* sequence; and partial *5.8S rRNA gene*), and confirmed *S. columbae*-like single species infection in our isolate from a Cooper's hawk. Additional transmission studies and the examination of sarcocyst

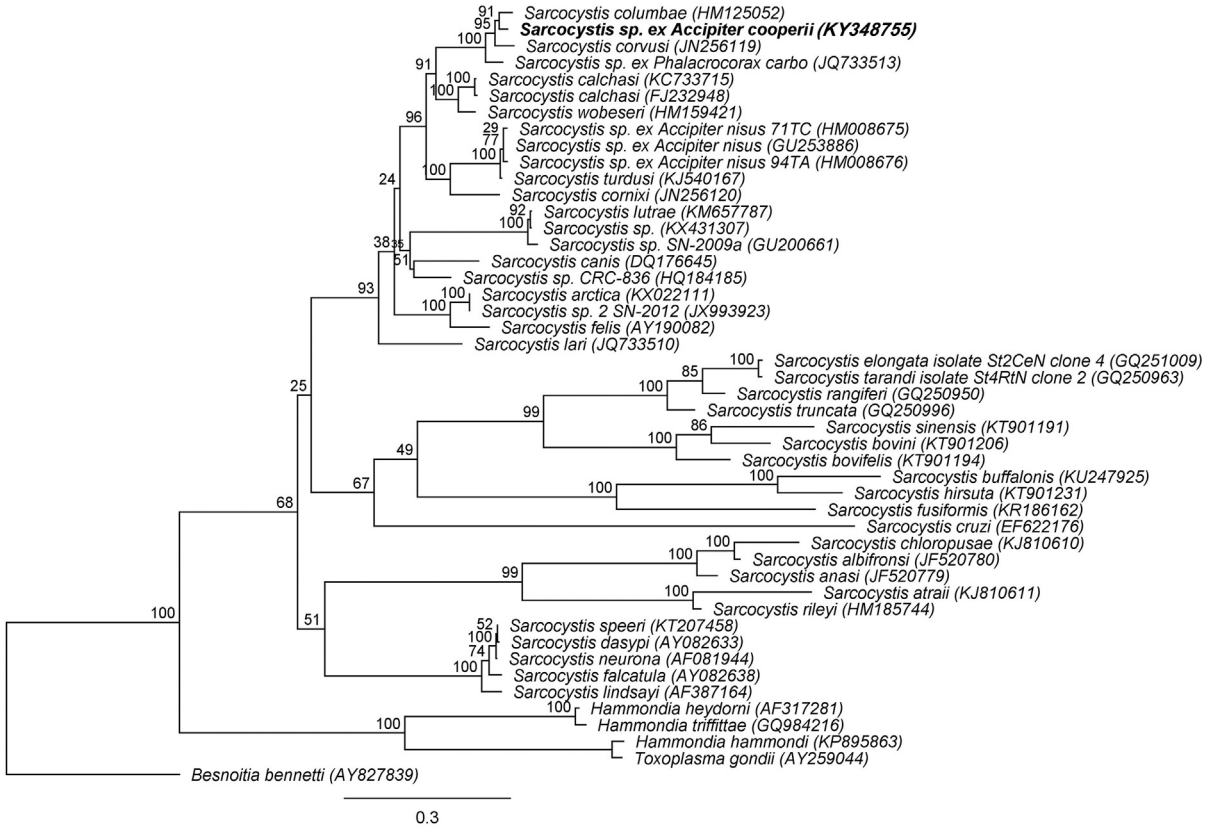

Fig. 5. Phylogenetic tree based on *ITS-1* sequences. Input sequences were the *ITS-1* regions of various species of *Sarcocystis*, and related taxon retrieved from NCBI GenBank, and new *ITS-1* sequence obtained from *Sarcocystis* sp. ex *A. cooperii* from a Cooper's hawk. Accession numbers of sequences were given in parenthesis following the species name. One hundred bootstrap replicates of the phylogenetic relationships among these sequences were reconstructed under the criterion of maximum likelihood using PhyML as implemented in Geneious version 9.0.4. *Sarcocystis* sp. ex *Accipiter cooperii* showed close relationship with those that employ birds as their hosts.

structure using transmission electron microscopy is needed before the parasite we isolated in this study is confirmed as *S. columbae* or demonstrated to be some other species of *Sarcocystis* that use *Accipiter* species as definitive hosts.

The sausage shaped appearance of schizonts in this study is different from the shape of schizonts reported for *S. falcatula* or *S. neurona* that have been grown in cell culture [23]. These species have been reported from cases of encephalitis in raptors in the United States [3]. Other than the occurrence of sausage shaped schizonts of *Sarcocystis* sp. ex *A. cooperii* development was similar to *S. falcatula* and *S. neurona* by light and TEM. Olias et al. [16] described the schizonts and sarcocysts of *S. calchasi* from domestic pigeons (*Columba livia f. domestica*) and determined that Northern goshawks (*A. gentilis*) were suitable definitive hosts. Olias et al. [15] described *S. columbae* from sarcocysts present in muscles of the wood pigeon (*C. palumbus*) but did not describe the schizont stages.

Yabsley et al. [2] found a prevalence of 66.8% (159 of 238) *Sarcocystis* oocysts/sporocysts in 4 species of hawks from Georgia. They found that 86 of 102 (84.3%) Cooper's hawks (*A. cooperii*), 17 of 37 (45.9%) Sharp-shinned hawks (*A. striatus*), 39 of 57 (68.4%) Red-tailed hawks (*Buteo jamaicensis*), and 17 of 42 (40.5%) Red-shouldered hawks (*B. lineatus*) were positive [2]. They examined 10 isolates using molecular methods and concluded that more than one species was present in the 4 hawk species examined [2] but this was before the reports of *S. calchasi* and *S. columbae* so no comparisons could be attempted. Additional studies need to be conducted on sporocysts isolated from *Accipiter* species to determine the genetic diversity of *Sarcocystis* species occurring in these birds in the United States.

In summary, we document that Cooper's hawks are a definitive host for *Sarcocystis* sp. ex *A. cooperii* a species closely related to *S. columbae* in the USA and describe schizogony of the parasite in cell culture.

Acknowledgements

This work was supported by grant # 1505407 from the National Science Foundation Historically Black Colleges and Universities Undergraduate Program to ACR and an IRC grant from the Virginia-Maryland College of Veterinary Medicine to DSL.

References

[1] D.S. Lindsay, B.L. Blagburn, Prevalence of encysted apicomplexans in muscles of raptors, Vet. Parasitol. 80 (1999) 341–344.
[2] M.J. Yabsley, A.E. Ellis, D.E. Stallknecht, E.W. Howerth, Characterization of *Sarcocystis* from four species of hawks from Georgia, USA, J. Parasitol. 95 (2009) 256–259.
[3] J.P. Dubey, R. Calero-Bernal, B.M. Rosenthal, C.A. Speer, R. Fayer, Sarcocystosis of Animals and Humans, CRC Press, 2015.
[4] M.B. Teglas, S.E. Little, K.S. Latimer, J.P. Dubey, *Sarcocystis*-associated encephalitis and myocarditis in a wild Turkey (*Meleagris gallopavo*), J. Parasitol. 84 (1998) 661–663.
[5] M.G. Spalding, C.A. Yowell, D.S. Lindsay, E.C. Greiner, J.B. Dame, *Sarcocystis* meningoencephalitis in a northern gannet (*Morus bassanus*), J Wildlife Dis 38 (2002) 432–437.
[6] P. Olias, A.D. Gruber, A.O. Heydorn, A. Kohls, H.M. Hafez, M. Lierz, Unusual biphasic disease in domestic pigeons (*Columba livia* f. *domestica*) following experimental infection with *Sarcocystis calchasi*, Avian Dis. 54 (2010) 1032–1037.
[7] J.G. Trupkiewicz, R. Calero-Bernal, S.K. Verma, et al., Acute, fatal *Sarcocystis calchasi*-associated hepatitis in Roller pigeons (*Columba livia* f. *dom.*) at Philadelphia Zoo, Vet. Parasitol. 216 (2016) 52–58.
[8] A. Wünschmann, A.G. Armien, L. Reed, A.D. Gruber, P. Olias, *Sarcocystis calchasi*-associated neurologic disease in a domestic pigeon in North America, Transbound. Emerg. Dis. 58 (2011) 526–530.
[9] G. Rimoldi, B. Speer, J.F. Wellehan Jr., et al., An outbreak of *Sarcocystis calchasi* encephalitis in multiple psittacine species within an enclosed zoological aviary, J. Vet. Diagn. Investig. 25 (2013) 775–781.
[10] R.F. Aguilar, D.P. Shaw, J.P. Dubey, P. Redig, *Sarcocystis*-associated encephalitis in an immature northern goshawk (*Accipiter gentilis atricapillus*), J Zoo Wildlife Med 22 (1991) 466–469.
[11] E.J. Olson, A. Wünschmann, J.P. Dubey, *Sarcocystis*-associated meningoencephalitis in a bald eagle (*Haliaeetus leucocephalus*), J. Vet. Diagn. Investig. 19 (2007) 564–568.

[12] A. Wunschmann, D. Rejmanek, P.A. Conrad, et al., Natural fatal *Sarcocystis falcatula* infections in free-ranging eagles in North America, J. Vet. Diagn. Investig. 22 (2010) 282–289.
[13] A. Wünschmann, D. Rejmanek, L. Cruz-Martinez, B.C. Barr, *Sarcocystis falcatula*-associated encephalitis in a free-ranging great horned owl (*Bubo virginianus*), J. Vet. Diagn. Investig. 21 (2009) 283–287.
[14] Z. Cerna, Z. Kvasnovska, Life-cycle involving bird-bird relation in *Sarcocystis*-coccidia with the description of *Sarcocystis accipitris* sp. n, Folia Parasitol. 33 (1986) 305–309.
[15] P. Olias, L. Olias, M. Lierz, H. Mehlhorn, A.D. Gruber, *Sarcocystis calchasi* is distinct to *Sarcocystis columbae* sp. nov. from the wood pigion (*Columba palumbus*) amd *Sarcocystis* sp. from the sparrowhawk (*Accipiter nisus*), Vet. Parasitol. 171 (2010) 7–14.
[16] P. Olias, A.D. Gruber, H.M. Hafez, A.O. Heydorn, H. Mehlhorn, M. Lierz, Sarcocystis calchasi sp. nov. of the domestic pigeon (*Columba livia* f. *domestica*) and the Northern goshawk (*Accipiter gentilis*): light and electron microscopical characteristics, Parasitol. Res. 106 (2010) 577–585.
[17] H.D.A. Lindquist, J.W. Bennett, J.D. Hester, M.W. Ware, J.P. Dubey, W.V. Everson, Autofluorescence of *Toxoplasma gondii* and related coccidian oocysts, J. Parasitol. 89 (2003) 865–867.
[18] B. Gjerde, T.D. Josefsen, Molecular characterisation of *Sarcocystis lutrae* n. sp. and *Toxoplasma gondii* from the musculature of two Eurasian otters (*Lutra lutra*) in Norway, Parasitol. Res. 114 (2015) 873–886.
[19] S. Guindon, J.F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, O. Gascuel, New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0, Syst. Biol. 59 (2010) 307–321.
[20] P. Olias, L. Olias, J. Krücken, M. Lierz, A.D. Gruber, High prevalence of *Sarcocystis calchasi* sporocysts in European *accipiter* hawks, Vet. Parasitol. 175 (2011) 230–236.
[21] P. Prakas, L. Kutkiene, D. Butkauskas, A. Sruoga, M. Zalakevicius, Molecular and morphological investigations of *Sarcocystis corvusi* sp. nov. from the jackdaw (*Corvus monedula*), Parasitol. Res. 112 (2013) 1163–1167.
[22] S.L. Mayr, K. Maier, J. Muller, D. Enderlein, A.D. Gruber, M. Lierz, Accipiter hawks (Accipitridae) confirmed as definitive hosts of *Sarcocystis turdusi*, *Sarcocystis cornixi* and *Sarcocystis* sp. ex *Phalacrocorax carbo*, Parasitol. Res. 115 (2016) 3041–3047.
[23] D.S. Lindsay, J.P. Dubey, K.M. Horton, D.D. Bowman, Development of *Sarcocystis falcatula* in cell cultures demonstrates that it is different from *Sarcocystis neurona*, Parasitology 118 (1999) 227–233.