Journal of Parasitology 2021 107(4) 562–565

Published 20 July 2021

Contents and archives available through www.bioone.org or www.jstor.org

Journal of Parasitology

journal homepage: www.journalofparasitology.org

DOI: 10.1645/20-63

GAMOGONY OF *SARCOCYSTIS STRIXI* IN MAMMALIAN CELL CULTURES

David S. Lindsay[1], S. K. Verma[2,3], J. P. Dubey[2], David Scott[4], and Alexa Rosypal von Dohlen[5]

[1] Department of Biomedical Sciences and Pathobiology, Virginia Maryland College of Veterinary Medicine, Faculty of Health Sciences, 205 Duck Pond Drive, Virginia Tech, Blacksburg, Virginia 24061.

[2] United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal Parasitic Diseases Laboratory, 10300 Baltimore Avenue, Bldg. 1040, Beltsville, Maryland 20705.

[3] Present address: Public Health and Environmental Laboratories, New Jersey Department of Health, Ewing, New Jersey 08628.

[4] Carolina Raptor Center, 6000 Sample Road, Huntersville, North Carolina 28078.

[5] Department of Natural Sciences and Mathematics, Johnson C. Smith University, 100 Beatties Ford Rd., Charlotte, North Carolina 28216.

Correspondence should be sent to David S. Lindsay at: *lindsayd@vt.edu*

KEY WORDS

Sarcocystis strixi
Barred Owl
Strix varia
Sarcocysts
Bradyzoites
Macrogamonts
Microgamonts
Oocysts
Cell Cultures

ABSTRACT

We are interested in the disease ecology of *Sarcocystis* species that infect birds of prey as definitive and intermediate hosts. The present study was done to test our hypothesis that a laboratory model can be developed for sarcocystis infection in mammals using gamma interferon gene knockout (KO) mice as a source of *Sarcocystis strixi* bradyzoites and mammalian cell cultures as a source of sporulated *S. strixi* oocysts. Sporocysts of *S. strixi* from a naturally infected barred owl (*Strix varia*) were fed to KO mice to produce sarcocysts, and the enclosed bradyzoites were obtained by acid-pepsin digestion of abdominal and thigh muscles. Bradyzoites, metrocytes, and an unusual spherical stage were seen in digest before the inoculation of host cells. The spherical stages stained dark with Giemsa stain, but no nucleus was observed, and they were seen free and associated with the concave portion of some bradyzoites. Examination of infected cell cultures demonstrated that macrogamonts and microgamonts were present at 24 hr post-inoculation. Since sporulated oocysts were not observed, we had to reject our current hypothesis.

Sarcocystis species use 2 hosts in their life cycle (Dubey et al., 2016). An intermediate host is infected after consuming sporulated oocysts/sporocysts. Sporozoites undergo schizogony in endothelial cells of the vascular system, and eventually merozoites enter heart, skeletal muscle, or, rarely, neurological tissue and develop by endodyogeny producing bradyzoites. Bradyzoites are infective asexual stages found in mature sarcocysts in the intermediate host. After tissues containing sarcocysts have been ingested, bradyzoites are released into the intestinal tract. In an appropriate definitive host, bradyzoites penetrate the intestinal epithelium and forgo asexual development and develop sexually in host cells in the lamina propria producing gamonts (sexual stages). Microgamonts produce microgametes that fertilize macrogamonts. Fertilized macrogamonts develop into oocysts that sporulate in vivo and contain 2 sporocysts each with 4 sporozoites. These sporulated oocysts make their way out of the lamina propria, often rupturing the thin oocyst wall, and are excreted in the feces as sporulated oocysts and free sporocysts (Dubey et al., 2016).

Surprisingly, few studies have examined the in vitro development of bradyzoites (syn., merozoites, endozoites, cystozoites) of *Sarcocystis* in cell culture. This is even though the early in vitro studies by Fayer (1970, 1972) stimulated others to investigate the potential of *Sarcocystis* species as having a 2-host life cycle. Bradyzoites obtained from sarcocysts from naturally infected grackles (*Quiscalus quiscula*) (presumably *Sarcocystis falcatula*-like) entered avian and mammalian cells and transformed into ellipsoidal or spherical stages resembling gamonts of intestinal coccidia (Fayer, 1970, 1972; Fayer and Thompson, 1975). These light microscopical observations were later confirmed using transmission electron microscopy (Vetterling et al., 1973; Mehlhorn and Heydorn, 1979). Dubremetz et al. (1975) examined the development of bradyzoites of *Sarcocystis tenella* from sheep in cell cultures. Becker et al. (1979) reported on the development of bradyzoites of 4 *Sarcocystis* species in cell cultures using light and transmission electron microscopy. Mehlhorn and Heydorn (1979) and Becker et al. (1979) demonstrated that bradyzoites of *Sarcocystis suihominis* developed to microgamonts and macrogamonts within 12 hr of inoculation of human cell cultures. They reported that at 22 hr a process similar to sporulation was observed in some oocysts, although no sporulated oocysts were observed, and they attributed this lack of sporulation to degeneration of infected host cells. Similar findings were reported by Becker et al. (1979) for *Sarcocystis muris* from mice, a *Sarcocystis* species from goats, and *Sarcocystis ovifelis* from sheep.

We have previously demonstrated that sporocysts of *Sarcocystis strixi* from barred owls (*Strix varia*) produced sarcocysts in the muscles of gamma interferon gene knockout (GKO) mice but not outbred immune-competent mice (Verma et al., 2017). Attempts to infect African green monkey kidney (CV-1) cells (ATCC CCL-70, Manassas, Virginia) with sporozoites obtained from the same batch of oocysts/sporocysts from the same barred owl used to orally inoculate mice were not successful (Verma et al., 2017). The present study was done to determine if bradyzoites from *S. strixi* collected from infected GKO mice would infect mammalian cells and differentiate into sexual stages and become sporulated oocysts containing sporocysts with sporozoites. If successful, this would let us test our hypothesis that a laboratory model can be developed for *S. strixi* using GKO mice as a source of bradyzoites and cell cultures as a source of sporulated oocysts.

Cultures of CV-1 cells, human pigmented retinal epithelial cell (HRE, ATCC CRL-2302, Manassas, Virginia), and bovine monocytes (BM; a gift from John Dame, University of Florida, Gainesville, Florida) were grown in 25 cm^2 cell culture flasks or on 22 mm^2 coverslips in 6-welled tissue culture plates in RPMI 1640 cell culture medium (Mediatech, Inc., Manassas, Virginia) containing 100 IU penicillin/ml, 100 μg/ml streptomycin/ml, and 10% (v/v) fetal bovine serum (FBS) (growth media) (Lindsay et al., 2017). Bradyzoite infected cell cultures were maintained in the same medium except the concentration of FBS was lowered from 10% to 2% (maintenance media).

Bradyzoites were collected from the abdominal and hind leg muscles containing sarcocysts of *S. strixi* from a GKO mouse infected 206 days previously and necropsied at the U.S. Department of Agriculture, Agricultural Research Service, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, that was sent by overnight carrier to the Zoonotic Protozoal Diseases Laboratory, Center for One Health Research, Virginia Tech, Blacksburg, Virginia. The isolate of *S. strixi* used to infect GKO mice was the same as used previously (Verma et al., 2017). Muscle tissue was processed using sterile HBSS and dissecting tools. The muscle was minced with a scalpel blade, and then 5 ml of sample was transferred to a 50 ml sterile screw cap centrifuge tube. A 5 ml aliquot of the acid-pepsin digest was added, and the tube was mixed every 30 sec for a total of 3 min. The liquid was collected into a 15 ml sterile screw cap centrifuge tube, and RPMI growth medium was added until 15 ml was present in the tube. The tube was inverted to mix and the top 13 ml collected and placed into a 15 ml sterile screw cap centrifuge tube after letting large particles settle to the bottom for 45 sec. Two milliliters of RPMI growth medium was then added to the tube, and it was capped and centrifuged for 10 min at 800 *g*. After the first centrifugation to pellet the bradyzoites, the entire supernatant was collected and discarded. The pellet was resuspended in growth medium, and this process was repeated 2 times to reduce the acidity of the solution. The bradyzoites were counted in a hemocytometer, and 5×10^5 were inoculated into each of 2 flasks of CV-1 cells, 2 flasks of HRE cells, and 2 flasks of BM cells. Lastly, 1×10^4 bradyzoites were inoculated onto each of 8 coverslips containing monolayers of CV-1 cells.

A portion of the digest that was used to inoculate cell cultures was placed on a glass slide, air-dried, fixed in 100% methanol, and stained with Giemsa stain. Additionally, bradyzoites were collected from surplus muscle not used on day 1 of the study. Surplus muscle tissue was stored for 24 hr, collected using the same process as described above, and 1×10^4 bradyzoites were inoculated onto 3 coverslips containing monolayers of HRE cells. After 2 hr, the inoculum containing growth media was removed and replaced with maintenance media.

Living cell cultures were examined using an inverted microscope equipped with phase-contrast optics. Coverslips were removed and processed for light microscopic examination after staining with Giemsa various days post-inoculation (PI) (see below). The 22 mm^2 coverslips were fixed in 10% (v/v) buffered formalin solution for 10 min, post-fixed in 100% methanol, air-dried, and stained with Giemsa stain for 1 hr. Stained coverslips were mounted on glass slides using Permount™ (Fisher Scientific Company, Fair Lawn, New Jersey) for microscopic examination. Coverslips of infected CV-1 cells were processed for examination at 6 hr PI (n = 2), 16 hr PI (n = 2), 24 hr PI (n = 1), 30 hr PI (n = 1), 2 days PI (n = 1), and 3 days PI (n = 1), while coverslips of HRE cells were examined 16 hr PI (n = 1), 40 hr PI (n = 1), and 4 days PI (n = 1). On selected days PI, cells on 25 cm^2 flasks were scraped using a cell scraper, and the cell pelleted by centrifugation. The cell pellet was resuspended in 0.5 ml phosphate-buffered saline (7.2 pH), and a drop was placed on a glass slide, the slide was inverted to spread out the cell mixture. then it was air-dried and stained with Giemsa for bright field microscopy. Slides were observed and photographed using an Olympus BH60 microscope (Olympus America Inc., Center Valley, Pennsylvania) equipped and differential interference contrast (DIC) optics.

Stages in the digest consisted of bradyzoites, metrocytes, and smaller spherical dark staining bodies (Fig. 1A, B). Bradyzoites were elongate to mainly crescent-shaped. The nucleus of most bradyzoites was not distinctly stained with Giemsa, and several dark staining bodies were scattered in the cytoplasm of the anterior one-half to two-thirds of bradyzoites. Some bradyzoites were weakly motile, flexing or spinning at the apical end. Metrocytes were 6.1 by 3.0 μm (5.0 to 8.0 by 2.0 to 4.0 μm; n = 10) and 3.0 by 2.7 μm (3.0 to 3.0 by 2.0 to 3.0 μm; n = 7) and varied in size depending on the stage of development. Some presumably early developmental stage metrocytes were spherical, while more developed metrocytes were elongate to elliptical. The nucleus of metrocytes was dark staining and occupied almost the entire width of the metrocyte. The cytoplasm of metrocytes was usually free of dark staining bodies. Most metrocytes had a singular spherical nucleus, but occasionally a metrocyte that was binucleate or had a horseshoe shaped nucleus was seen in Giemsa stained preparations (Fig. 1B). Spherical stages that were observed were often associated with the concave portion of bradyzoites. Cell penetration was not observed in living cultures.

Bradyzoites penetrated CV-1 cells and rounded up to produce uninucleate gamonts by 6 to 16 hr PI that were 7.8 by 6.4 μm (5.0 to 10.0 by 4.0 to 8.0 μm, n = 9) and contained a centrally located nucleus (Fig. 2A). Microgamonts and macrogamonts were observed at 24 hr in CV-1 cell cultures (Fig. 2B, C). Macrogamonts were larger than gamonts measuring 10.3 by 8.8 μm (9.0 to 11.0 by 7.0 to 11.0 μm; n = 12) and contained a single nucleus and had many non-staining vacuoles in the cytoplasm (Fig. 2B). Immature microgamonts were 8.9 by 8.0 μm (8.0 to 10.0 by 7.0 to 9.0 μm; n = 10) and contained a lobed nucleus. Mature microgamonts with microgametes were not observed during the study.

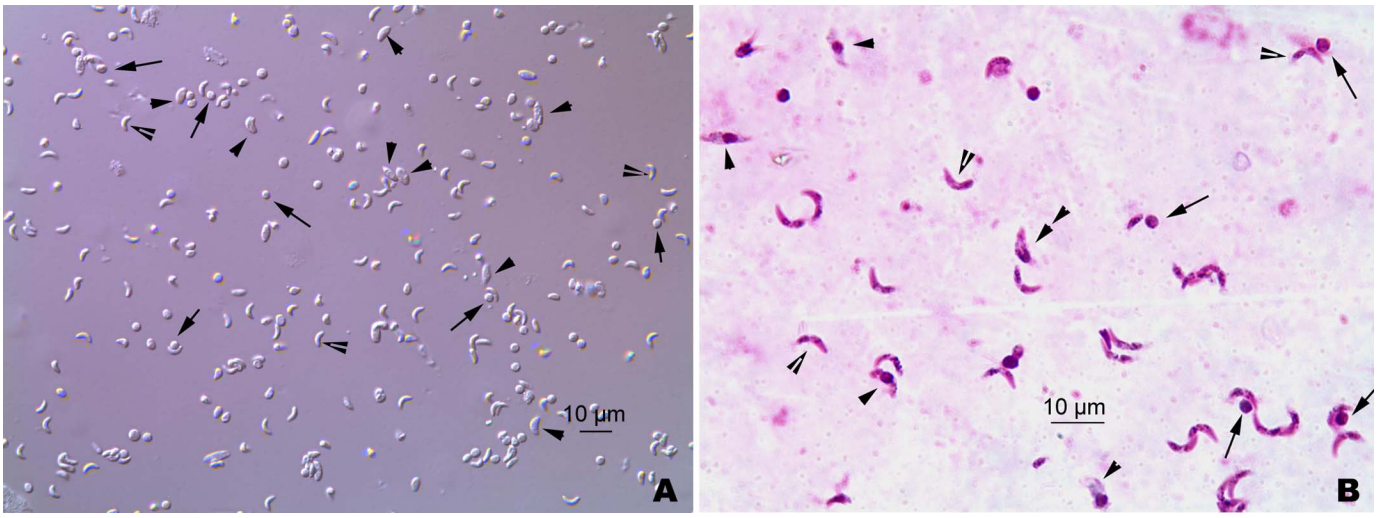

Figure 1. Stages of *Sarcocystis strixi* after treatment with acid-pepsin digest solution. (**A**) Non-fixed sample of bradyzoites (empty arrowheads), metrocytes (arrowheads), and small spherical stages (arrows). Interference contrast microscopy. (**B**) Fixed and stained sample of bradyzoites (empty arrowheads), metrocytes (arrowheads), and small spherical stages (arrows). Note lack of dark staining granules in metrocytes and the presence of a horseshoe shaped nucleus in the metrocyte with the double arrowhead label, Giemsa stain. Scale bars =10 µm. Color version available online.

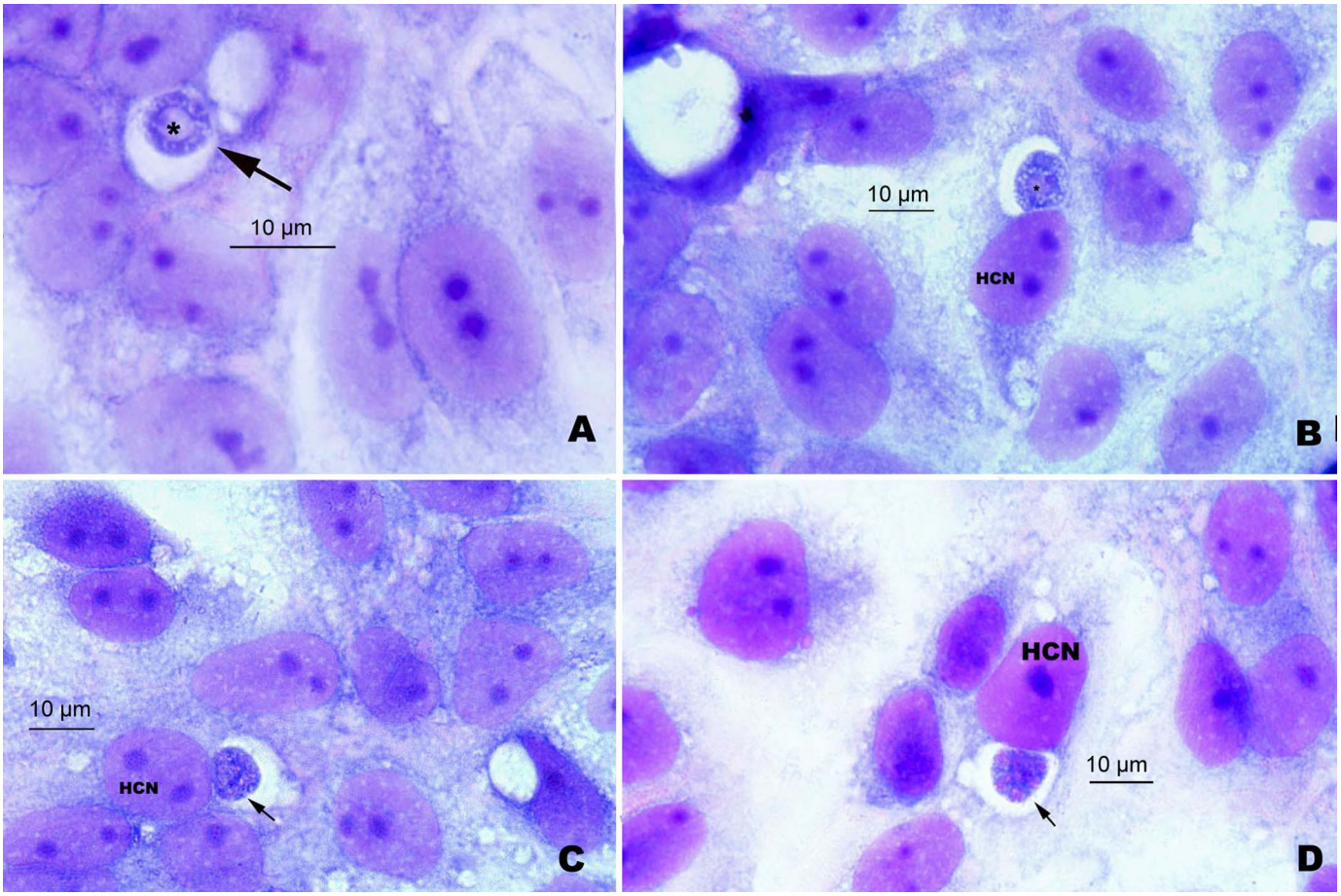

Figure 2. Stages of *Sarcocystis strixi* in cell cultures of CV-1 cells stained with Giemsa stain. (**A**) Gamont stage (arrow) at 16 hr. Note the nucleus (*) and few white granules in cytoplasm. (**B**) Macrogamont at 30 hr with a single nucleus (*) and several white granules of probable wall-forming bodies in the gamont cytoplasm. It is located near the host cell nucleus (HCN). (**C**) Microgamont (arrow) with a nucleus that is elongating and developing lobes. It is located against the host cell nucleus. (**D**) Microgamont at 30 hr post-inoculation that has a lobed nucleus nuclei that is producing several nuclei. The microgamont is located next to the host cell nucleus (HCN). Scale bars = 10 µm. Color version available online.

Oocysts were not identified, and no evidence of division of macrogamont into sporoblasts was seen during the study. We must therefore reject our hypothesis that a laboratory model can be developed for *S. strixi* using GKO mice as a source of bradyzoites and cell cultures as a source of sporulated oocysts.

We did not directly observe active cell penetration by bradyzoites of *S. strixi* in this study. The motility of living *S. strixi* bradyzoites was weak, and very few infected host cells were present. This may have been due to the use of acid-pepsin solution in the present study. Fayer (1970) used mechanical grinding of host muscle from grackles to obtain bradyzoites for cell culture studies, and others obtained bradyzoites by trypsinization of muscle tissues for cell culture studies on the development of bradyzoite to gamonts in host cells (Becker et al., 1979; Mehlhorn and Heydorn, 1979). Trypsinized bradyzoites were used to demonstrate the events that occur during host cell penetration by bradyzoites of *S. muris*, and it was determined that 2 types of parasitophorous vacuoles occurred (Entzeroth, 1984, 1985; Entzeroth and Chobotar, 1989). One was associated with bradyzoites initially penetrating host cells, and the second type of parasitophorous vacuole began to develop within 30–45 min of the bradyzoites becoming intracellular as they left the first vacuole and began producing a new vacuole in which bradyzoites underwent stage conversion into gamont stages. Metrocytes are less resistant to acid-pepsin solution than bradyzoites (Verma et al., 2018), and their presence in digests along with the small spherical stages is interesting and deserves further research.

The rapid transformation of *S. strixi* bradyzoites into gamonts is similar to previous studies (Fayer, 1970, 1972; Vetterling et al., 1973; Fayer and Thompson, 1975; Dubremetz et al., 1975; Becker et al., 1979). We observed only immature microgamonts that had a single lobed nucleus. No mirogametes were produced based on the lack of finding free microgametes in the parasitophorous vacuole of developing microgamonts. We did not observe sporulated oocysts of *S. strixi*; we only observed developing macrogamonts and microgamonts.

This work was supported in part by grant number 1505407 from the National Science Foundation Historically Black Colleges and Universities Undergraduate Program to A.R.V.D. and an Internal Research Competition (IRC) grant from the Virginia-Maryland College of Veterinary Medicine to D.S.L. This research was supported in part by an appointment to the Agricultural Research Service (ARS) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract number DE-SC0014664. All opinions expressed in this paper are the authors' and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE. The USDA is an equal opportunity provider and employer.

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