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Sarcocystis atheridis sp. nov., a new sarcosporidian coccidium from Nitsche's bush viper, *Atheris nitschei* Tornier, 1902, from Uganda

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Abstract Transmission experiments were performed to elucidate the life cycle of a *Sarcocystis* sp. found in a Nitsche's bush viper, *Atheris nitschei nitschei* (Serpentes: Viperidae), from Uganda. Sporocysts measuring 10.4 (10.0–11.0) × 8.0 (7.0–8.5) μm were given to laboratory mice (Crl: CD1), laboratory rats (Wistar H), and Barbary striped mice, *Lemniscomys barbarus*. Sarcocysts developed in the skeletal muscles of laboratory mice and *L. barbarus*. No sarcocyst was observed in laboratory rats. Merogony was observed in the liver of *L. barbarus* at 7 and 12 days postinfection. Mature sarcocysts in mice reached a length of 30 mm and did not exceed 0.9 mm in diameter at 121 DPI. The primary sarcocyst wall was 0.6–0.8 μm thick and displayed small osmiophilic knob-like protrusions that were up to 150 nm long and 90 nm wide. Two types of asexual multiplication, endodyogony and endopolygony, were found within sarcocysts. Our results indicate that the newly found *Sarcocystis* represents a new species.

Introduction

Sarcosporidia represent a highly diversified group of coccidian parasites of snakes. Members of the genus *Sarcocystis* using reptiles as definitive hosts and rodents or reptiles as intermediate hosts have been studied in the last two decades (Matuschka 1987; Paperna and Finkelman 1996a, b). Since the pioneer work of Rzepczyk (1974), when a full python-rat life cycle for *Sarcocystis* sp. from the carpet python *Morelia spilotes variegata* was described, 12 further species possessing a snake-rodent life cycle have been discovered and named, including *S. idahoensis*, *S. dirumpens*, *S. hoarensis*, *S. muriviperae*, *S. crotali*, *S. gerbilliechis*, *S. clethrionomyelaphis*, *S. montanensis*, *S. zamani*, *S. villivillosi*, *S. singaporensis*, and *S. murinotechis* (Beaver and Maleckar 1981; Bledsoe 1980; Dubey 1983; Enzeroth et al. 1985; Häfner and Matuschka 1984; Jäkel 1995; Matuschka 1986; Matuschka et al. 1987; Munday and Mason 1980; Upton et al. 1992; Zaman and Colley 1975).

The present paper reports the results of a study of the life cycle of a new species of *Sarcocystis* with a rodent-snake life cycle, which originated from an arboreal viperid snake, *Atheris nitschei*, occurring around the Great Lakes in Central Africa. It feeds mainly on rodents, although feeding on amphibians, reptiles, and birds has also been reported (Pitman 1974). Based on diet composition, infection experiments with rodents were performed to identify suitable intermediate hosts.

Materials and methods

Infectious material

Seven (four adult and three semiadult) specimens of Nitsche's bush viper, *Atheris nitschei nitschei*, were collected during a parasitological and herpetological expedition to Uganda in August 1996 in the vicinity of the town Kilembe (0°14′N, 30°3′E). Snakes were kept and examined as described previously (Modrý et al. 1997). Fecal samples were routinely screened for parasites using flotation in modified Sheater's sugar solution (s.g. 1.30). Isolated coccidian oocysts and sporocysts were examined and photographed using Nomarski interference contrast (NIC) optics. A total of 30 sporocysts were measured using bright-field microscopy (100× objective) with a calibrated ocular micrometer. All measurements are given in micrometers and are usually expressed as mean values followed by ranges in parentheses. Sporocysts were stored at a temperature of 4–5 °C in 2.5% aqueous potassium dichromate solution. Prior to inoculation the sporocysts were counted using a hemocytometer.

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Infection and maintenance of experimental animals

For determination of the intermediate host the following rodents were used in a primary set of transmission experiments. The origin of animals and the numbers of inoculated and control animals are listed in parentheses, respectively: Crl: CD-1(ICR)BR mice (Anlab Brno, 13, 1); Barbary striped mice, *Lemniscomys barbarus* (captive-born, 5, 1); and Wistar Han laboratory rats (VúFB Konárovice, 9, 1). Rodents were caged in plastic cages with wooden bedding and were fed on standard laboratory mouse pellets. Prior to use, oocysts were disinfected in 50% commercial bleach (SAVO, Bochemie, Czech Republic) for 5 min and washed three times by centrifugation in sterile phosphate-buffered solution (PBS). All experimental rodents were inoculated perorally (p. o.) with 10^2–10^6 sporulated sporocysts in a single 0.5-ml volume (for detailed data on inoculated animals, see Table 1). The clinical status of all rodents was monitored daily. Individual animals were euthanized by overdosing of barbiturates and then necropsied at different days postinfection (see Table 1).

In the definitive-host study, one adult, coccidia-free *A. n. nitschei* and one subadult captive-born *Vipera ammodytes* were used. Snakes were fed solely on laboratory mice. Feces were monitored using the flotation technique, with negative results being noted for 4 months prior to the experimental infection. Both experimental snakes were force-fed with the musculature of a laboratory mouse that had been euthanized at 61 days postinfection (DPI); sarcocysts were macroscopically visible in the musculature.

Sporocysts isolated from the feces of experimentally infected *A. nitschei* were used for back-transmission to mice and *L. barbarus*. In all, 5 Crl: CD-1(ICR)BR mice (Anlab Brno) and 2 *L. barbarus* were infected with 10^4 sporocysts and were kept and examined as described above.

Light microscopy and histology

At necropsy the following tissue samples for light microscopy were collected and fixed in 10% buffered formalin: from naturally infected *A. nitschei*, eight equidistantly spaced sections of the intestine, stomach, liver, lung, kidneys, and heart; and from rodents, the esophagus, stomach, duodenum, jejunum, ileum, cecum, rectum, lung, liver, kidney, spleen, mediastinal lymphatic nodes, abdominal wall, tongue, heart, diaphragm, muscles of the abdominal wall, brachial muscles (m. triceps brachii), and left quadriceps femoris and masseter muscles. Fixed tissues were processed for light microscopy using standard methods. Paraffin sections were stained with hematoxylin and eosin (H&E) and Giemsa. Smears of cystozoite homogenate (121 DPI) were air-dried, fixed with methanol, and stained with Giemsa.

Electron microscopy

Muscles containing sarcocysts were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C and postfixed in 1% osmium tetroxide in the same buffer. Specimens were washed three times in the same buffer, dehydrated in graded alcohols, and embedded in Durcupan. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 1010 transmission electron microscope.

Results

Stages found in the definitive host

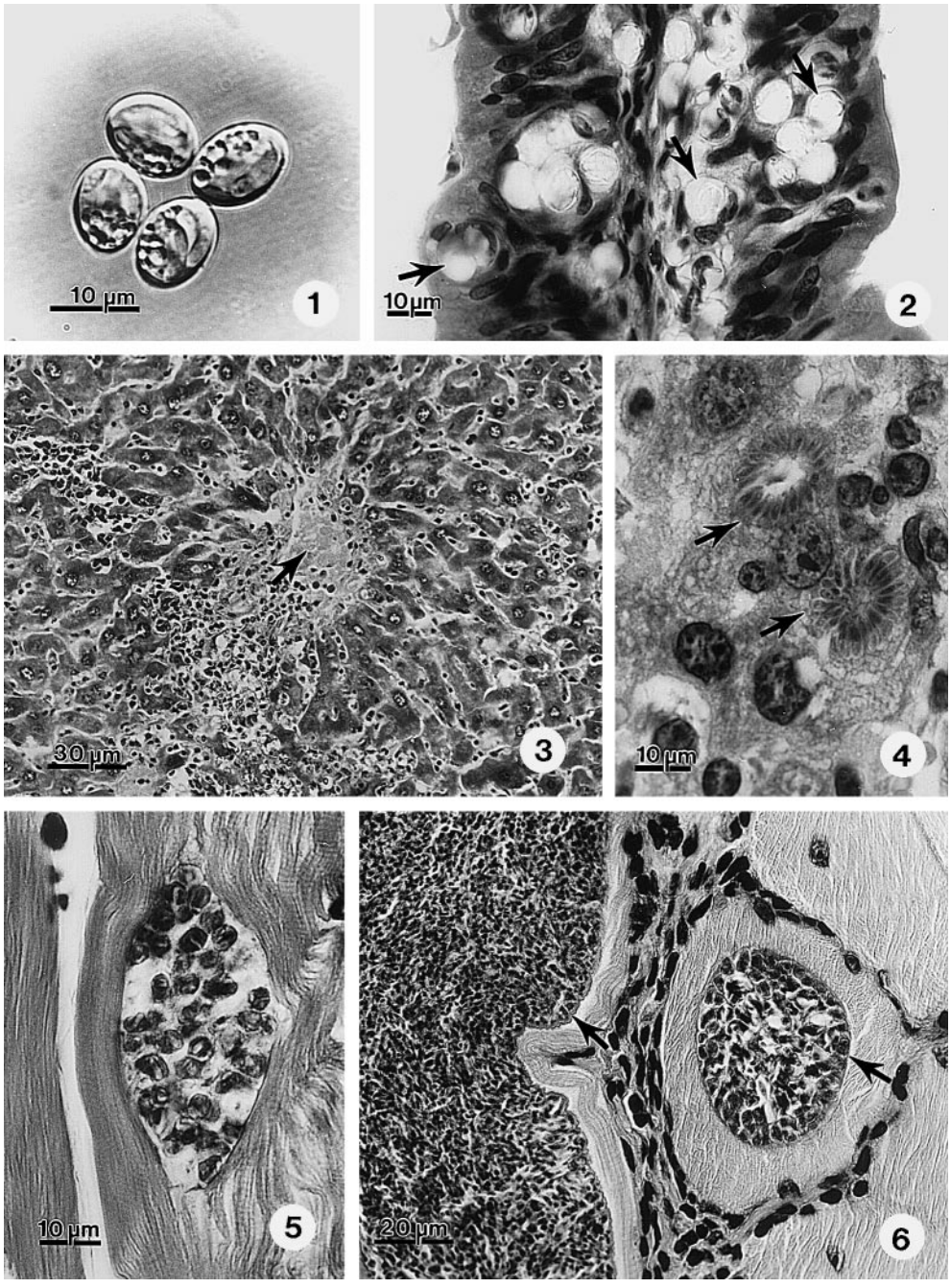
Repeated coprological examination of seven *Atheris nitschei* revealed six specimens (86%) to be passing numerous sporocysts of *Sarcocystis* sp. in their feces. Sporulated oocysts possessed a thin wall that surrounded two sporocysts. Most oocysts had ruptured when excreted in the feces, and individual sporocysts were seen. Oocysts and sporocysts isolated from all naturally infected *A. nitschei* were identical in size and morphology. Sporocysts were tetrazoic and ellipsoid, measuring 10.4 (10.0–11.0) × 8.0 (7.0–8.5) μm, with a shape index (length/width) of 1.3 (1.24–1.43). Stieda and substieda bodies were absent, and a sporocyst residue was present that consisted of numerous small granules measuring 0.75–1.15 μm in diameter. The sporocyst wall was single-layered, smooth, and colorless (Figs. 1, 11).

Histological examination of tissue samples from the intestine of a naturally infected female *A. nitschei* revealed numerous fully developed bisporocystic oocysts between enterocytes and in the lamina propria in all parts of the small intestine, with heavier occurrence being noted in its middle part. Oocysts were found mainly individually or in small, scanty groups (Fig. 2). Stages of merogony or gamogony were not observed. The intestinal mucosa was not altered significantly, and infected snakes did not show any clinical sign of health alteration due to sarcosporidian infection. The snakes excreted sarcosporidian oocysts/sporocysts for at least 17 months.

Table 1 Results of sarcocyst development in rodents after inoculation of the latter with sporocysts of *Sarcocystis atheridis* sp. nov. isolated from naturally infected *Atheris nitschei*

Rodent species	Sporocyst dose	Animals inoculated/ sarcocyst-positive (*n*)	Examination schedule (DPI)
Primary inoculation:			
Crl: CD-1(ICR)BR mice	10^2	2/0	61, 63
	10^3	2/1	61, 63
	10^4	3/3	61, 63, 121
	10^5	3/2	61, 61, 93
	10^6	3/2	19, 61, 63
Wistar Han rats	5×10^2	3/0	9, 9, 30
	5×10^3	3/0	30, 62, 62
	5×10^4	3/0	30, 62, 62
Lemniscomys barbarus	10^5	2/2[a]	12[a], 12[a]
	10^4	3/3[a]	7[a], 38, 61
Back-transmission:			
Crl: CD-1(ICR)BR mice	10^4	5/4	53, 53, 65, 65, 65
L. barbarus	10^4	2/2	53, 65

[a] Based on the finding of merogony stages in hepatocytes



Figs. 1–5 Development of *Sarcocystis atheridis* sp. nov. **Fig. 1** Nomarski interference contrast (NIC) photographs of sporocysts from feces of naturally infected *Atheris nitschei*. **Fig. 2** Histological section of an intestinal villus of a viper's small intestine with clusters of oocysts (*arrows*). H&E. **Fig. 3** Centrolobular coagulative necrosis (*arrow*) surrounded and infiltrated by inflammatory cells in the histological section of the liver from *Lemniscomys barbarus* at 12 DPI. H&E. **Fig. 4** Rosette-like meronts in hepatocytes of *L. barbarus* at 7 DPI. H&E. **Fig. 5** Longitudinal histological section of a sarcocyst containing metrocytes at 19 DPI. H&E

Experimental infection of intermediate hosts

The susceptibility to infection, the intensity of infection, and the prevalence of sarcocysts in infected rodents varied. Whereas all *Lemniscomys barbarus* were infected, 50–100% prevalence was observed in laboratory mice. All laboratory rats remained uninfected. Similarly, all control animals were sarcocyst-negative (Table 1).

Of all inoculated rodents, only *L. barbarus* developed clinical signs of acute sarcosporidiosis. In *L. barbarus* given 10^5 sporocysts, acute hepatitis resulting in death developed at 12 DPI. Clinical signs that became obvious at 10 DPI included anorexia and weakness. The most apparent macroscopic pathological changes involved hepatomegaly, splenomegaly, and petechial hemorrhages on the serosal surface of the liver. Histopathological changes at 7 and 12 DPI were characterized by hemorrhages and numerous centrolobular necroses surrounded by and infiltrated with inflammatory cells (Fig. 3). Hepatocytes were infected with mature meronts that contained merozoites arranged parallel to each other, forming rosette-like structures (Fig. 4). No clinical sign of acute sarcosporidiosis was observed in *L. barbarus* inoculated with 10^4 sporocysts or in other infected rodent species.

Sarcocysts that developed in laboratory mice and in *L. barbarus* were localized in the musculature of the tongue, in the m. triceps brachii, in the m. quadriceps femoris, in the abdominal wall musculature, in muscles of the labium superior et inferior, in the m. masseter, and, in one mouse, also in the diaphragm. The tongue and the abdominal wall were the most frequent sites occupied by sarcocysts.

The earliest stages observed in mice were young sarcocysts in the skeletal muscles of a mouse necropsied at 19 DPI. These sarcocysts contained spherical mononuclear metrocytes measuring 6–8 μm (Fig. 5). Sarcocysts found in the muscles of rodents euthanized at 61–63 DPI were macroscopically visible, reaching a length of 19 mm and a diameter of 0.4–0.9 mm, depending on the muscles parasitized. In a mouse necropsied at 121 DPI the sarcocyst attained the maximal length of 30 mm. The primary cyst wall appeared smooth and thin in native preparations and histology. No evidence of a secondary sarcocyst wall was observed. At 61–63 DPI, cysts contained rounded metrocytes as well as many elongated cystozoites (Fig. 6). The cystozoites measured 6.8 (6–8) × 1.02 (1–1.5) μm in smears. At 93–121 DPI, only the tips of the sarcocysts contained metrocytes; the rest of the cyst was filled with fully developed, elongated cystozoites. Sarcocysts surrounded by inflammatory cells were occasionally observed in muscles.

Ultrastructurally, the primary sarcocyst wall formed small osmiophilic knob-like protrusions, which were up to 150 nm long and 90 nm wide. The protrusions were located at regular distances, giving a granulated appearance to the cyst wall under the lower magnification. The ground substance was 0.6–0.8 μm thick (Fig. 10). Two types of asexual multiplication, endodyogony – producing two progeny within the parasite cell – and endopolygony – producing multinucleated stages – were found in sarcocysts (Figs. 8, 9). No ultrastructural difference was observed among sarcocysts that developed in laboratory mice or in *L. barbarus*.

Experimental infection of the definitive host

Oocysts and sporocysts, identical to the original isolate, were found at 34 DPI in the feces of experimentally infected *A. n. nitschei*. The last negative sample was collected at 15 DPI. Shedding of the sporocysts lasted for at least 6 consecutive months. Experimentally inoculated *Vipera ammodytes* remained noninfected.

Experimental back-transmission to rodents

Infection of mice and *L. barbarus* with sporocysts isolated from the aforementioned experimental *A. nitschei* led to the finding of numerous muscular sarcocysts in all inoculated *L. barbarus* and in four of five inoculated mice. The sarcocysts found were morphologically as well as ultrastructurally identical to those described above.

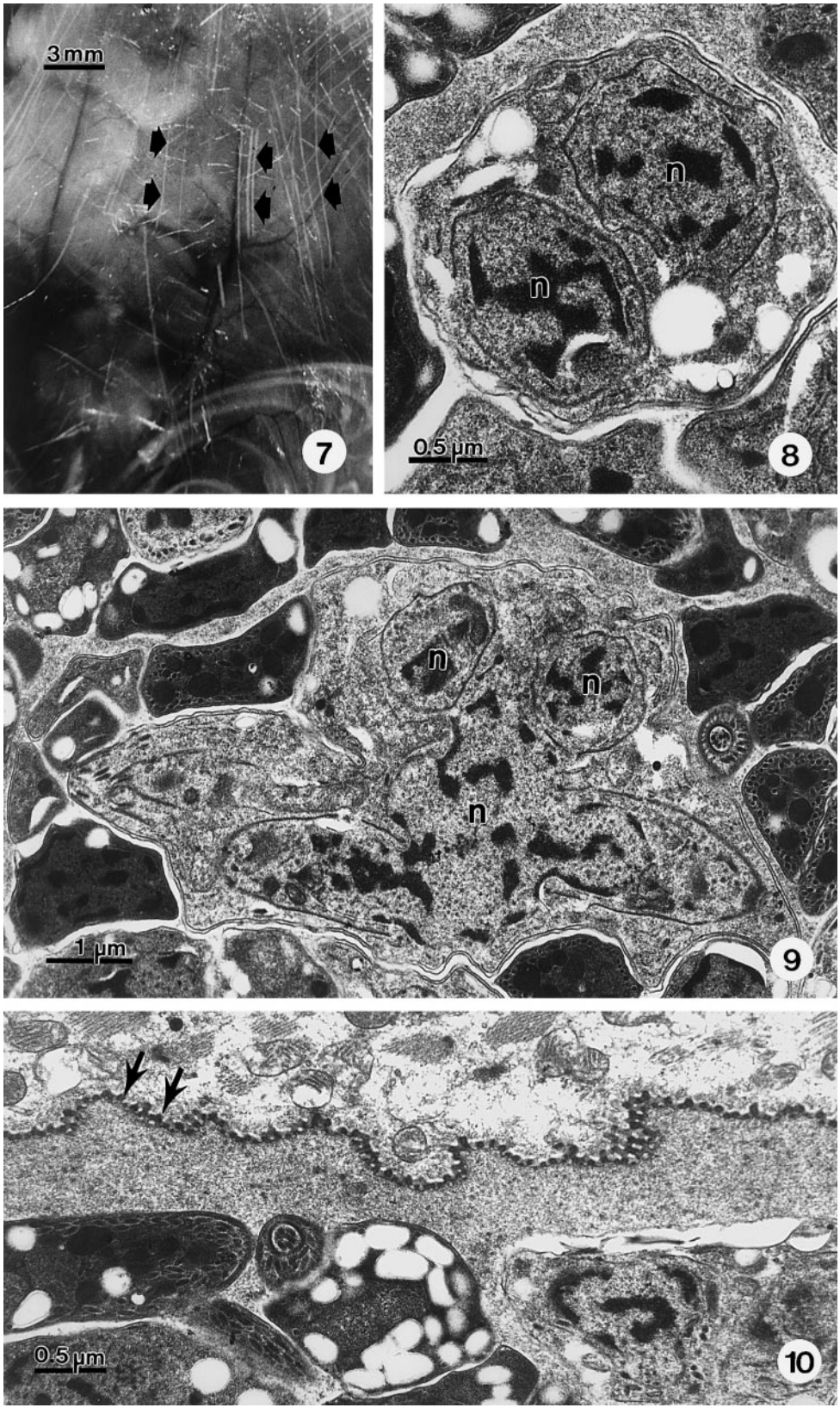
Taxonomic summary

Sarcocystis atheridis sp. nov.
Exogenous stages: Oocysts were only rarely seen; liberated sporocysts represented the majority of stages found in the feces. Sporocysts were colorless and ellipsoid and measured 10.4 (10.0–11.0) × 8.0 (7.0–8.5) μm, showing a shape index of 1.3 (1.24–1.43); sporocyst residue was composed of granules measuring 0.75–1.15 μm in diameter.
Stages in the intermediate host: Rosette-like developmental stages were observed in the liver of *L. barbarus* at 7 and 12 DPI. Sarcocysts measured at 61–63 DPI were filiform, macroscopically visible, and up to 19 mm long and 0.4–0.9 mm wide and were found in the abdominal and apendicular musculature, in the tongue, in the diaphragm, and in the skin musculature; at 121 DPI the sarcocyst attained the maximal length of 30 mm. Ultrastructurally, the primary sarcocyst wall was 0.6–0.8 μm thick and formed small electron-dense, knob-like protrusions, which were up to 150 nm long and 90 nm wide. Two types of multiplication within the sarcocyst, endodyogony and endopolygony, were observed.
Type host: *Atheris nitschei nitschei* (Serpentes: Viperidae)
Intermediate hosts (*experimental*): Laboratory mice and *L. barbarus* (*both Muridae*).
Type locality: Uganda, at the foot of the Ruwenzori Mountains, Jambo village near Kilembe (0°14′N, 30°3′E), altitude 1700 m.
Type specimens: Phototypes and histology slides have been deposited in the Parasitological Institute of the Academy of Sciences of the Czech Republic in České Budějovice (Coll. No. R 193/96). The symbiotype of the final host has been deposited in the Herpetological Collection of the Forschungsinstitut und Museum A. Koenig, Bonn (Coll. No. ZFMK 62371).
Etymology: The specific epithet *atheridis* is derived from the generic name of the type host.

Discussion

The taxonomic criteria for sarcosporidian coccidia as summarized by Dubey et al. (1989) comprise the morphology of sarcocysts, meronts, oocysts, and sporocysts; host specificity; and isoenzymes. Ultrastructural details of the primary cyst wall and host specificity are pointed out as the most valuable data. Recently, molecular biological characteristics have extended the spectrum of taxonomic criteria (Fisher and Odening 1998; Jeffries et al. 1997; Votýpka et al. 1998). The taxonomic value of oocyst/sporocyst morphology is usually considered to be low; most *Sarcocystis* species are similar in morphology and vary only slightly in size (Dubey et al. 1989; Häfner 1987; Jäckel 1995).

Sarcocystis species are usually regarded as host-specific and possess a narrow spectrum of definitive hosts (Matuschka 1987). Data reported by Lindsay et al. (1992) and Paperna and Finkelman (1996a, b) do not



Figs. 6–10 Morphology of sarcocysts of *S. atheridis* sp. nov. **Fig. 6** Longitudinal and cross (histological) sections of sarcocysts at 61 DPI. Note the thin, wrinkled cyst wall (*arrows*). TEM. **Fig. 7** Abdominal muscles of a mouse, showing large numbers of sarcocysts (*arrows*) at 63 DPI. **Fig. 8** Endodyogony in a metrocyte. Note the pair of daughter nuclei observed at 61 DPI. TEM. **Fig. 9** Endopolygony in a metrocyte. Note the separation of daughter cells with prominent nuclei (*n*) at 61 DPI. TEM. **Fig. 10** Interface between the host cell and the cyst wall, showing electron-dense knob-like protrusions (*arrows*) of the primary cyst wall at 61 DPI. TEM

match these suggestions, but as they are not based on transmission experiments, the conspecificity of the studied isolates is therefore not confirmed. All *Sarcocystis* species described from members of the subfamily Viperinae – *S. gerbilliechis* from *Echis coloratum*, *S. muriviperae* from *Vipera palestinae*, and *S. dirumpens* from

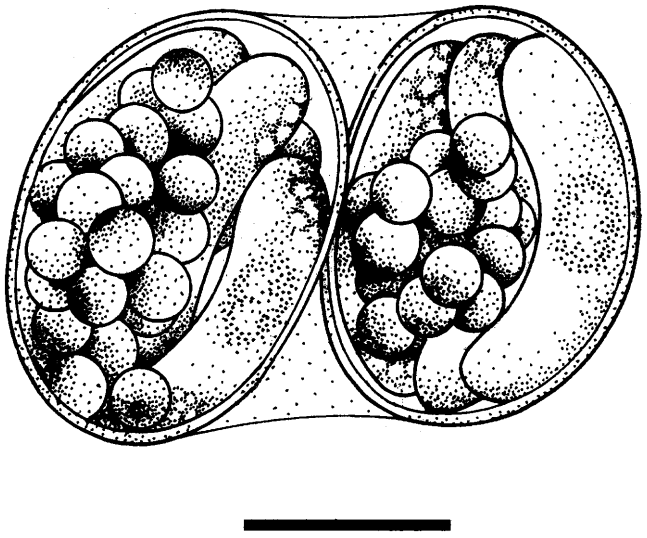


Fig. 11 Composite line drawing of the oocyst of *S. atheridis* sp. nov. *Bar* 5 μm

members of the genus *Bitis* (Häfner 1987; Paperna and Finkelman 1996a, b) – show specificity at the level of the host genus or even species. In our experiments the transmission of *S. atheridis* sp. nov. to its natural definitive host was successful, whereas the transmission to *V. ammodytes* failed. The genus *Atheris* is considered to be phylogenetically distant from other viperids (Herrmann and Joger 1995), and the inability of *S. atheridis* sp. nov. to infect *V. ammodytes* is therefore not surprising.

The sarcocyst wall of *S. atheridis* is thin and smooth; the primary cyst wall represents type I according Dubey et al. (1989). There are 13 *Sarcocystis* species with a snake-rodent life cycle, 5 of which – *S. crotali, S. montanensis*, *S. idahoensis*, *S. dirumpens*, and *S. gerbilliechis* – possess a type I primary cyst wall (Dubey et al. 1989; Jäckel 1995; Lindsay et al. 1992; Odening 1998).

S. idahoensis shows histological similarities to *S. atheridis* sp. nov.; the ultrastructure of the primary cyst wall of this species is unknown (Bledsoe 1980). Its geographical distribution, host specificity, and sarcocyst size as well as the presence of villus-like projections on the primary sarcocyst wall exclude its conspecificity with *S. atheridis* sp. nov. *S. montanensis* and *S. crotali* possess similar knob-like protrusions on the primary cyst wall (Enzeroth et al. 1985; Lindsay et al. 1992). Both of these species could be distinguished by the size of their protrusions, the thickness of the ground substance, their spectrum of intermediate hosts, and their geographical distribution as well. Additionally, a typical feature of sarcocysts of *S. crotali* is the electron-dense layer underlying the protrusions, which is absent in *S. atheridis* sp. nov.

Sarcocysts of *S. gerbilliechis* develop only in gerbils, predominantly in the esophagus; merogonic stages were found in the liver, lung, kidney, and heart (Jäckel 1995). This species possesses a smooth primary cyst wall with knob-like electron-dense protrusions similar to that of *S. atheridis* sp. nov. The localization of sarcocysts and merogonic stages as well as its strict host specificity well differentiate *S. gerbilliechis* from *S. atheridis* sp. nov. Although the ultrastructure of *S. dirumpens* shows great similarity to that of *S. atheridis* sp. nov., there are remarkable biological and morphological characteristics that clearly differentiate the two species. Whereas *S. dirumpens* is infectious for laboratory rats, the inoculation of rats with *S. atheridis* sp. nov. failed over a wide range of doses. The localization of sarcocysts of *S. atheridis* sp. nov. differs from that of *S. dirumpens*. Whereas the esophagus is the typical localization site of *S. dirumpens*, no sarcocyst was found in the esophagus of rodents infected with *S. atheridis* sp. nov. Ultrastructurally, the proportion of knob-like protrusions to ground substance represents a significant difference as well.

The prepatent period in reptilian hosts is of questionable taxonomic value because it is mostly biased by feeding and defecating habits (Matuschka 1986). More important is the time required for the development of fully sporulated oocysts in the lamina propria (Paperna and Finkelman 1996a). Consequently, the patent period lasts until the oocysts in the lamina propria have been depleted, which may take months or even years (Dubey et al. 1989). Determination of the prepatent period of the isolate of *S. atheridis* sp. nov. was based only on examination of feces, and its value is therefore limited. Daszak and Cunningham (1995) have reported clinical sarcosporidiosis in the colubrid snake of the genus *Pituophis*. Neither naturally nor experimentally infected *Atheris nitschei* exhibited clinical signs of infection.

The pathogenicity of sarcosporidian infections for rodents is variable, depending on the coccidian species, the host, and the infection dose as well. High infection doses may cause severe pathological lesions in the liver (Bledsoe 1980; Mehlhorn and Matuschka 1986) and/or in the vascular endothelial cells, lungs, kidney, and heart of suitable intermediate hosts (Jäckel 1995; Zaman and Colley 1975). The liver reaction seems to be due not only to the parasite itself but also to the host reaction to metabolites or unknown substances (Lindsay et al. 1992; Paperna and Finkelman 1996b). Histological observations have revealed rosette-like meronts morphologically corresponding to those described in other studies (Bledsoe 1980; Lindsay et al. 1992; Mehlhorn and Matuschka 1986; Paperna and Finkelman 1996b).

Although sporocysts from naturally infected *A. nitschei* were successfully transmitted to two species of rodents, the prevalence of infection in individual groups of animals varied. Whereas the infection of *Lemniscomys barbarus* with 10^5 sporocysts led to the death of experimental animals, rodents infected with the lower dose of 10^4 sporocysts showed no clinical sign of sarcosporidiosis and finally recovered, with numerous sarcocysts being seen in the musculature of 100% of the animals. The prevalence of tissue cysts in other infected rodent species was not strictly biased by the infection dose and varied between 50% and 100%. The phenomenon of ineffectiveness is often discussed in the lit-

erature (Blewaska 1981; Häfner 1987; Matuschka et al. 1987) and is probably due to the use of incorrect intermediate host species (experimental versus natural intermediate hosts), to the degeneration of sarcocysts after the infection, or to an inappropriate dose.

Endodyogony has been suggested to be the only type of asexual reproduction occuring in the tissue cysts of *Sarcocystis* spp. Häfner and Frank (1986) and Häfner (1987) first proved endopolygony of metrocytes in several rodent species. Recently, Jäckel (1995) described endopolygony as the only means of multiplication for *S. gerbilliechis*. Paperna and Finkelman (1996b) found zoites dividing by endopolygony in early sarcocysts, whereas metrocytes divided by endodyogony. Both types of multiplication were observed in sarcocysts of *S. atheridis* at 61 and 63 DPI.

Considering the aforementioned differences, it seems quite obvious that *S. atheridis*, found to be transmitted by the viperid snake *A. nitschei* to two species of murine rodents, is a hitherto unknown *Sarcocystis* species.

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