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ORIGINAL PAPER

Sarcocystis acanthocolubri sp. n. infecting three lizard species of the genus *Acanthodactylus* and the problem of host specificity. Light and electron microscopic study

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Abstract In the present investigation, macroscopic sarcocysts of *Sarcocystis acanthocolubri* were observed in muscles of 42 (4.3%) out of 975 *Acanthodactylus* sp. lizards collected from different geographical areas in Egypt. The infection rate was 6.4% in *Acanthodactylus boskianus*, 2.1% in *Acanthodactylus sculentus*, and 5% in *Acanthodactylus paradalis*. The highest infection rate was recorded in the lizards captured from Baltem (10% in *A. boskianus* and 8% in *A. paradalis*). The infection rate was usually higher in females (7.4%) than in males (3.8%). Moreover, the highest infection rate was recorded in summer (7.53%), autumn (3.57%), and spring (3.11%), and the lowest was recorded in winter (0.91%). Also, old animals had higher infection rates (10.8%) than young ones (0–2.7%). Macrocysts measured 0.95×10.12 mm. Both macroscopic and microscopic sarcocysts were enclosed only by a primary cyst wall, which had many finger-like, stalkless, and non-branched protrusions giving it a striated appearance. The primary cyst wall measured 3.9 μm. A dark granulated ground substance was found directly underneath the protrusions and is extended interiorly dividing the cyst cavity into many compartments containing the parasites (metrocytes and merozoites). Metrocytes were found directly under the ground substance and usually multiply asexually by endodyogeny producing two merozoites from each metrocyte. Both metrocytes and merozoites had the apical complex structures characteristic to the genus *Sarcocystis*. Transmission experiments with three snake species indicated that the snake *Spalerosophis diadema* is the proper final host belonging to the family Colubridae. The prepatent period was 16 days, while the patent period was 35 days. The results obtained from the present investigation revealed that this is a new species which was named *Sarcocystis acanthocolubri*.

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Introduction

Reptiles have long been considered as targets of many parasitic coccidian protozoa especially those belonging to phylum Apicomplexa. The cyst-building coccidian is the most common group representing these parasites (Mehlhorn 2008). Since the elucidation of the coccidian nature and life cycle of *Sarcocystis* (Rommel et al. 1972), many reports had appeared describing many new species from different vertebrates until now (Mehlhorn and Heydorn 1978; Dubey and Odening 2001; Al-Hoot et al. 2005; Abdel-Ghaffar et al. 1990, 2009).

On the other hand, wild animals specially reptiles had been rarely investigated, and only few reports appeared in this field (Mehlhorn and Matuschka 1986; Abdel-Ghaffar et al. 1990, 2009; Abdel-Aziz 2003; Abdel-Ghaffar and Al-

Johany 2002; Šlapeta et al. 2001, 2002, 2003). Moreover, information about Egyptian reptile fauna and the infecting parasites are scarce and fragmented. Hence, the present study aimed to study the rate and prevalence of the *Sarcocystis* infection in the lizards of the genus *Acanthodactylus* and the role of such hosts in the obligatory heteroxenous prey–predator life cycle of this genus in three snake species of the family Colubridae through experimental infection of uninfected snakes.

Materials and methods

Three species of lizards from the family Lacertidae: *Acanthodactylus boskianus*, *Acanthodactylus sculentus*, and *Acanthodactylus paradalis* were collected from Giza, Cairo, Baltem, Rashid, Marsa-Matrouh, and Beni-suef and were identified according to Saleh (1997), as well as three different species of snakes belonging to the family Colubridae (*Spalerosophis diadema*, *Psammophis sibilans*, and *Malpolon monspessulana*). The snakes were captured from the same localities and habitat of the lizards, brought to the laboratory, where they were identified according to Saleh (1997), and maintained singly in glass cages with sand at room temperature (27 C±2). Lizards were fed regularly with insect larvae, and snakes received laboratory-reared, coccidia-free mice.

To detect the incidence of natural *Sarcocystis* infection in the lizards, muscles from tails were examined by naked eye for macroscopic forms of sarcocysts. At the same time, microscopic cysts were examined by help of cryosections. Uninfected lizards were set free in the field; meanwhile, small pieces of heavily infected muscles were immediately fixed and processed for light and electron microscopy as described before (Abdel-Ghaffar et al. 2009). Fecal samples from lizards and snakes were examined daily for coccidian oocysts (Long et al. 1976), for at least 1 month prior to experimental infection.

For experimental transmission, sarcocysts from naturally infected lizards were fed to 36 coccidia-free snakes (*S. diadema*, *P. sibilans*, and *M. monspessulana)*. As controls, three non-infected snakes of each species were kept under the same conditions. These control animals never showed any coccidian oocysts or sporocysts in their fecal samples during the period of the experiment. To study developmental stages of the parasite in the snake (*S. diadema*), two experimentally infected snakes were killed each at 2, 4, 6, 8, 10, 12, and 15 days post infection (p.i.). The small intestine of these snakes was divided into five equal parts. The mucosa and submucosa of these parts were removed by scraping, fixed in glutaraldehyde (3% buffered in 0.1 M sodium cacodylate). As well, small pieces of the infected muscles from lizards were processed for electron microscopy as described by Abdel-Ghaffar et al. (2009). Ultrathin sections were cut on a Reichert ultracut, stained with saturated alcoholic uranyl acetate and alkaline lead citrate, and finally examined with Philips 400 TEM at 25 kV at Ain Shams University in Cairo. For light microscopy, samples were fixed in 10% buffered formalin (pH 7.3), processed in a series of alcohols, and stained with hematoxylin and eosin. All data are given in micrometers as results of 30 or 50 measurements.

Results

Prevalence of natural infection

All lizard species examined were proven to be infected only with sarcocysts and showed no shedding of any coccidian oocysts. Out of 975 *Acanthodactylus* lizards collected from March 2006 to November 2009, only 42 (4.3%) lizards were infected with sarcocysts. These cysts were either macroscopic or microscopic. The infection rate was 6.4% in *A. boskianus*, 2.1% in *A. sculentus*, and 5% in *A. paradalis*. The highest infection rates recorded were 10% in *A. boskianus* and 8% in *A. paradalis* captured from Baltem. The present results also showed that the infection rate is usually higher in females (7.4%) than in males (3.8%). At the same time, old lizards harbored higher numbers of cysts (10.8%) than young ones (0–2.7%). Moreover, the infection rate was higher in summer (7.53%) and spring (3.11%), while the lowest values were recorded in winter (0.91%).

Mature sarcocysts (Figs. 1 and 2) are surrounded by a thick, striated cyst wall formed by upfoldings of the primary cyst wall (Figs. 3, 4). These tissue cysts measured 0.4–1.5×0.6–18 mm with a mean of 0.95×10.12 mm (Figs. 1 and 2). However, microscopic cysts measured 61–249 μm. The primary cyst wall has many protrusions that introduced the striated appearance, whereas the dark granulated ground substance is found directly beneath the protrusions and extended interiorly dividing the cyst cavity into many chamber-like compartments separated from each other by septae. The chambers contained the parasites (metrocytes and merozoites) (Figs. 2 and 4).

Electron microscopic studies of the materials examined from the different *Acanthodactylus* spp. (naturally or experimentally infected) proved that all sarcocysts observed belong to the same species having only a single primary cyst wall, while a secondary cyst wall was never observed. The primary cyst wall measured 2–5.8 μm (n=50) with a mean of 3.9 μm and had finger-like, stalkless, and non-branched protrusions. A characteristic row of successive knob-like electron-dense, rather thick elevations are found between the invaginations (Fig. 4). The protrusions

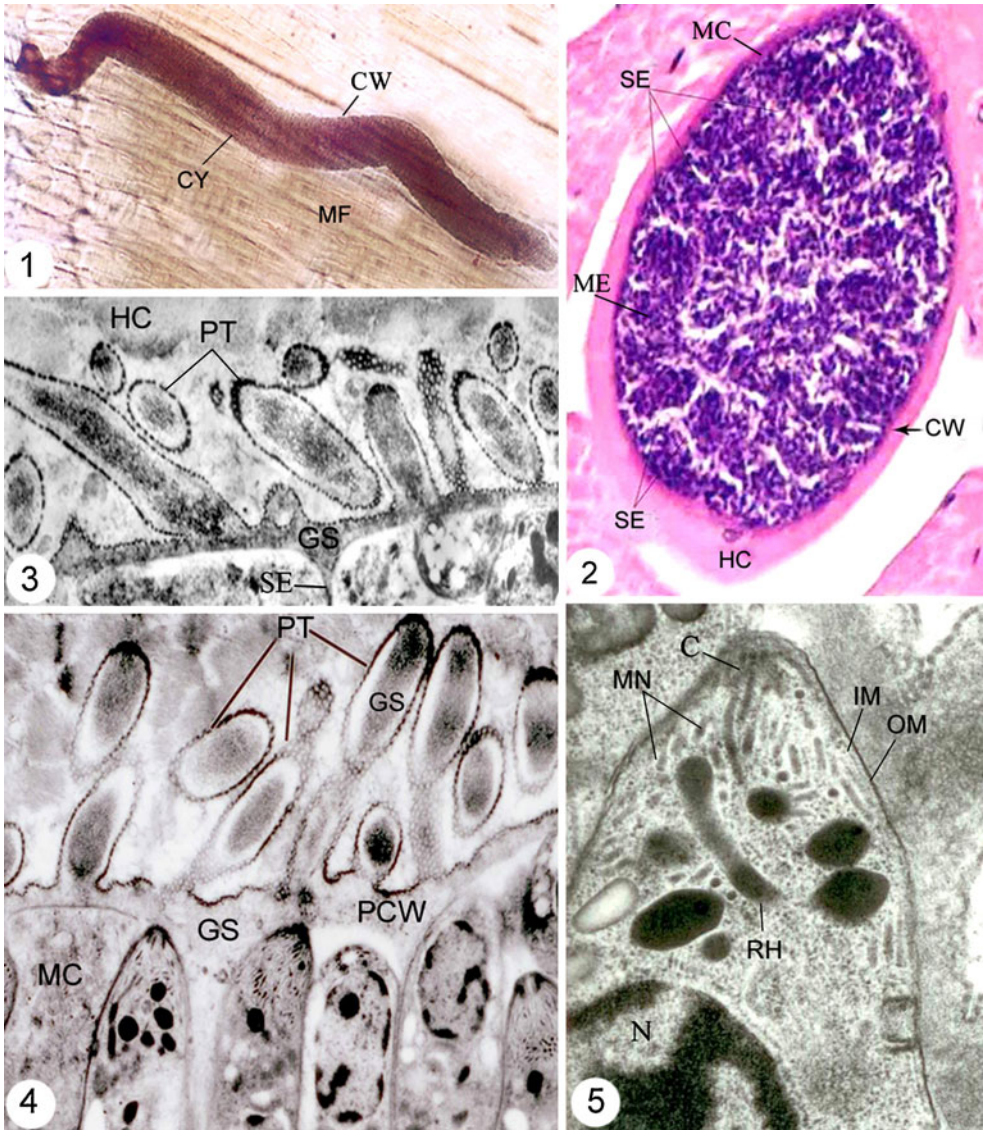

Fig. 1 Photomicrograph of a fresh preparation of infected muscles of *Acanthodactylus* lizards showing sarcocysts (*CY*), cyst wall (*CW*), and muscle fibers (*MF*). ×400

Fig. 2 Transverse sections of cysts, showing the primary cyst wall (*CW*), septae (*SE*), merozoites (*ME*), metrocytes (*MC*), and host cells (*HC*). ×1,200

Fig. 3 Transmission electron micrograph showing the fine structure of the 1ry cyst wall as protrusions (*PT*) and ground substance (*GS*). ×21,000

Fig. 4 Transmission electron micrograph showing metrocytes (*MC*) and the primary cyst wall structure with protrusions (*PT*), ground substance (*GS*). ×25,000

Fig. 5 Transmission electron micrograph showing a magnified apical pole of a cyst merozoite showing anterior polar ring (AP), rhoptries (*RH*), micronemes (*MN*), conoid (*C*), outer membrane (*OM*), and inner membrane (*IM*). ×35,000

contained neither filaments nor tubular elements. These protrusions measured 1.6–37 μm (n=50) with a mean of 2.65 μm in length and 0.8–2 μm (n=50) with a mean of 1.4 μm in cross sections (Figs. 3 and 4).

In some sections, protrusions were filled with numerous mitochondria of the host cells (Fig. 4). Just underneath the primary cyst wall, a relatively thick, granulated, and homogenous ground substance of 0.37 μm (n=50) was found. It extended inside the cyst as septae dividing its cavity into several compartments containing the parasites (Figs. 2–4). The few globular metrocytes occupied the peripheral region of the cyst, while the major banana-shaped cyst merozoites were concentrated in the central region of the cyst (Figs. 3, 4, and 6). The fine structural characteristics of both metrocytes and merozoites (Figs. 5 and 6) were similar to those described for many other *Sarcocystis* species (see Abdel-Ghaffar et al. 2009) and also to other members of the Apicomplexa.

Endodyogeny was the only mode of multiplication seen in sarcocysts of the present investigation, and successive events of this process were reported (Figs. 6, 7, 8, 9). This division started by the appearance of two nuclear cones and

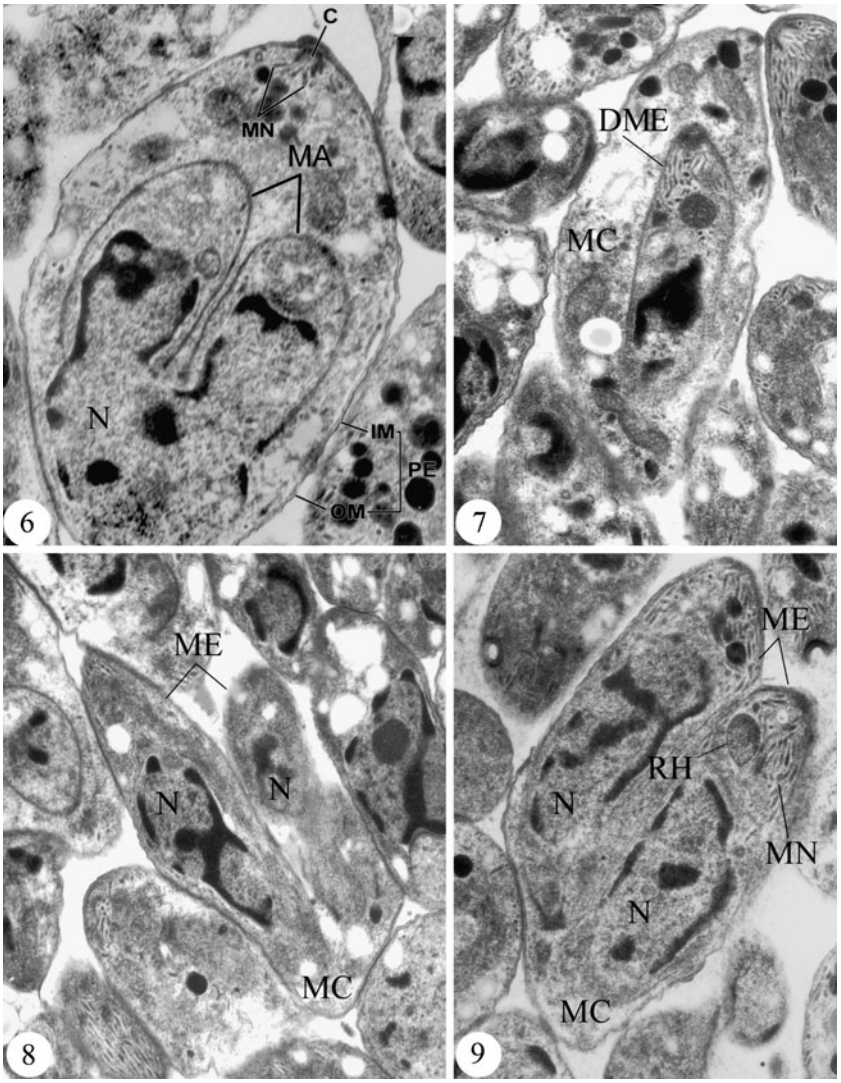

Fig. 6 Transmission electron micrograph showing a magnified part of a metrocyte (*MC*) with two merozoite anlagen (*MA*) and two nuclei (*N*). Note that the metrocyte (*MC*) had a conoid (*C*), a two-membrane pellicle (*PE*), and micronemes (*MN*). ×22,500

Fig. 7 Transmission electron micrograph showing events of endodyogeny within metrocyte (*MC*), only a developing merozoite (*DME*) was seen in this stage. ×22,000

Fig. 8 Transmission electron micrograph showing events of endodyogeny within metrocyte (*MC*), nearly two mature merozoites (*ME*) were observed. ×22,000

Fig. 9 Transmission electron micrograph showing two completely formed merozoites within metrocyte (*MC*) with the typical apical complex structures were seen. ×22,000

two merozoite anlagen inside the mother cell (metrocyte) which mostly show most characteristics of the apicomplexan motile stages (Fig. 6). The formation of two daughter cyst merozoites from the two longitudinal protuberances was clearly demonstrated (Figs. 7, 8). Endopolygeny of metrocytes was not observed in the present study.

In the present study, 36 snakes of three different species were fed by sarcocysts from infected muscles from the three *Acanthodactylus* spp. After ingestion (infection), only the specimens of the snake *S. diadema* excreted sporocysts in feces after 16 days and thus were proven to be the valid final hosts. No oocysts or sporocysts were shed from the other snake two species or from control snakes. However, all gamogonic and sporogenic stages were observed in the small intestine of the experimentally inoculated *S. diadema*.

Gamogony Small oval gamonts of 6.04×6.40 μm (n=30) were seen in the intestinal sections of experimentally infected snakes after 24 h p.i. (Fig. 10). These gamonts developed up to a size 6.60×6.90 μm (n=30) until the 3rd day p.i. and then began differentiation into micro- and macrogamonts (Figs. 11 and 12). Macrogametes are characterized by their large median prominent nuclei and by their wall-forming bodies situated at the periphery (Fig. 4). These macrogamonts finally measured 7.2×6.4 μm (n=30). Microgamonts were seen at the same time

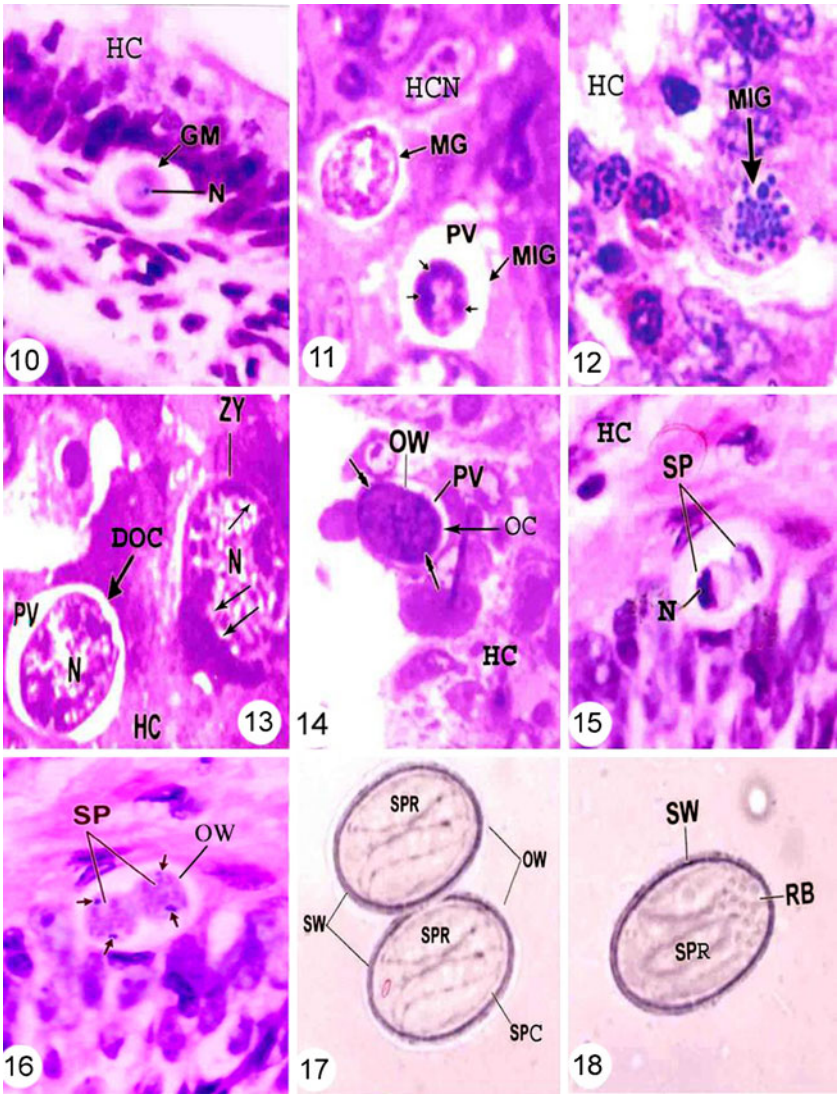

Fig. 10 Photomicrograph showing developing gamont (*GM*) in host cells (*HC*) from the intestine of experimentally infected *S. diadema* stained with hematoxylin and eosin. ×650

Fig. 11 Photomicrograph showing developing macrogamete (*MG*) and microgamont (*MIG*) with nuclei, each within parasitophorous vacuole (*PV*). ×750

Fig. 12 Photomicrograph showing microgamont (*MIG*) with many nuclei1. ×900

Fig. 13 Photomicrograph showing developing oocysts (*DOC*) after fertilization; note the fusion of the wall-forming bodies to form the oocyst wall. ×1,100

Fig. 14 Photomicrograph showing an oocyst (*OC*) with its oocyst wall (*OW*) within parasitophorous vacuole (*PV*) and host cell (*HC*). ×900

Fig. 15 Photomicrograph showing a dividing oocyst with two sporoblasts (*SP*). ×850

Fig. 16 Photomicrograph showing a dividing oocyst with its oocyst wall (*OW*) and two sporoblasts (*SP*), each containing four nuclei (*arrows*). ×850

Fig. 17 Photomicrograph of a fresh preparation of sporulated oocysts with oocyst wall (*OW*) and two sporocysts (*SPC*), each containing four sporozoites (*SPR*). ×850

Fig. 18 Photomicrograph of a magnified sporocyst, showing the sporocyst wall (*SW*), sporozoites (*SPR*), and the sporocyst residual body (*RB*). ×1,050

containing 30–40 nuclei measuring 6.1×5.4 μm (n=30) (Figs. 11 and 12).

Sporogony On the 5th day p.i., only zygotes were found in the lamina propria below the epithelial cells of the small intestine of the experimentally infected *S. diadema* snakes. These stages measured 8×9 μm (n=30), contained a vacuolated cytoplasm, and were termed small oocysts or unsporulated oocysts, measuring 11.2×7.8 μm (Figs. 13 and 14). During the 10th to 12th days p.i., the nucleus of the oocyst divided once, being followed by a cytoplasmic division producing two sporoblasts (Figs. 15 and 16). At the 14th day p.i., the nucleus of each sporoblast divided twice producing four nuclei (Fig. 16). Then, at the 15th day p.i., the sporoblast cytoplasm divided producing four sporozoites (Fig. 17). At this stage, each oocyst contained

two sporocysts, each with four sporozoites. These sporocysts were usually shed at the 16th day p.i., since the thin oocyst wall broke in feces (Fig. 18). Thus, the prepatent period was determined as 16 days.

Such sporulated oocysts and sporocysts were shed with the feces by the experimentally infected snakes for 35 days. These sporocysts measured 15.20×7.70 μm (n=30), and each contained four sporozoites. The sporocysts were enclosed by a double-layered wall consisting of an outer thin layer and an inner thick one. The number of sporocysts shed was increased gradually reaching the maximum at the 24th day, while the minimum number was shed on the 35th day. Thus, the patent period was 35 days.

Taxonomic summary

Type host: The lizards *A. boskianus*, *A. sculentus*, and *A. paradalis* are intermediate hosts. Final host: the snake *S. diadema*.
Site of infection: Skeletal muscles of the tail, fore-limb, hind-limb, and subcutaneous tissue; in snakes: lamina propria of intestine.
Type locality: Different geographical localities in Egypt; prevalence: 4.4%.
Prevalence: 42 (4.3%) out of 975 *Acanthodactylus* spp. were infected with sarcocysts.
Etymology: The specific name originates from the combination of the genus names of the lizards and the family name of the snake (*S. acanthocolubri*).

- Size of tissue cyst: 0.95×10.12 mm (macroscopic), 155×100 μm
- Occurrence of septae inside the cyst: yes
- Occurrence of protrusions of the cyst wall: yes, long without inner filaments or microtubules
- Size of metrocytes: 6–9 μm
- Size of cyst merozoites: 4–7 μm
- Size of oocysts: to be added
- Size of sporocysts: 15.20×7.70 μm

Discussion

While *Sarcocystis* has been described in a wide range of mammals and birds, it appears to be uncommon in ectothermia, and investigations concerning reptiles are scarce (Levine and Tadros 1980; Matuschka 1987; Paperna 2002). On the other hand, reports on Egyptian reptiles are very few (Abdel-Ghaffar et al. 1990; Sakran 2000; Abdel-Aziz 2003).

The percentage of natural infection of *Sarcocystis* varied widely among different animals (Abdel-Ghaffar et al. 1990, 2009; Fukuyo et al. 2002; Al-Hoot et al. 2005; Dubey and Morales 2006). In the present study, the natural prevalence was 6.4% in *A. boskianus*, 2.3% in *A. sculentus*, and 6% in *A. paradalis*, which are similar to results reported by some authors (Sakran 2000; Abdel-Ghaffar and Al-Johany 2002). However, numbers found in the present study are lower than those reported by Abdel-Ghaffar et al. (1990). Nevertheless, the differences reported in the prevalence of infection could be attributed to climatic and natural factors covering the whole ecosystem during different months and seasons of the year as well as the biotic relationships demonstrated in this system as food chains and webs. The maximum values of prevalence were recorded in summer among females, while the lowest values were recorded in winter among males which agreed with (Sakran and Ahmed 2000; Elsheikha et al. 2004).

Sarcocysts were usually found within skeletal muscles of the hind- and fore-limbs, tails, and the subcutaneous tissue and were never seen in the tongue, cardiac muscles, or brain of infected lizards. This is in agreement with many reports on reptiles (Abdel-Ghaffar et al. 1990, 1994, 2009). However, reports on mammals are usually in contradiction to the presented results herein (Sakran 2000; Modry et al. 2000, Al-Hoot et al. 2005; Elsheikha et al. 2006).

Sarcocysts of *S. acanthocolubri* were filiform measuring 0.95×0.12 μm which is closely similar to those recorded before (Abdel-Aziz 2003; Sakran and Ahmed 2000; Abdel-Ghaffar and Al-Johany 2002). These sarcocysts usually occur within a typical coccidian parasitophorous vacuole initially limited by a single unit membrane that finally developed into the primary cyst wall (Sakran and Ahmed 2000; Abdel-Aziz 2003; Elsheikha et al. 2006; Abdel-Ghaffar et al. 2009).

An identification of the genus *Sarcocystis* is mainly dependent on the morphology and fine structure of the primary cyst wall (Mehlhorn 2008; Dubey et al. 2006; Wouda et al. 2006; Kutkiene et al. 2006). Thus, the comparison of species differences should be made only from mature cysts (Bashtar et al. 1988; Abdel-Ghaffar et al. 1990, 1994, 2009; Paperna and Finkelman 1996; Modry et al. 2000; Mehlhorn, 2008).

During the last three decades, the basic accepted criteria for identification and diagnosis of sarcosporidia have changed many times. Some authors suggested the ultrastructure of the developmental stages and host specificity as proven in experimental transmissions (Mehlhorn and Heydorn 1978; Dubey et al. 1989; Dubey and Odening 2001); others claimed that the knowledge of the final host is desirable but not imperative (Odening 1998). The present study described the whole developmental stages of *S. acanthocolubri* in both the intermediate reptile (lizards of the genus *Acanthodactylus*) and the final host (the snake *S. diadema*) from the family Colubridae.

In the present study, thick-walled sarcocysts with striated appearance were reported being situated in muscles of the infected *Acanthodactylus* lizards. These closely packed finger-like protrusions are stalkless, nonbranching, and measured 2.55×0.81 μm. Similar results were recorded in *Sarcocystis chalcidicolubris* (Matuschka 1987) and *Sarcocystis gongyli* (Sakran 1993). Along the whole surface of these protrusions, numerous knob-like elevations represented the thickened portions of the primary cyst wall besides a number of slight invaginations. These invaginations may represent absorptive areas since they form canal-like structures through which food materials might pass from the host to the parasites within the cyst. The site of these invaginations varied among the different *Sarcocystis* species (Mehlhorn 2008; Abdel-Ghaffar et al. 2009).

Endodyogeny is the only mode of multiplication of the cyst merozoites and metrocytes described in the present investigation. However, polyendodyogeny is described for the schizonts by many observers (Matuschka and Mehlhorn 1984; Häfner and Frank 1986; Dubey et al. 1989; Sakran and Ahmed 2000; Abdel-Ghaffar et al. 2009).

Considering the cyst parasites described in the present work, both metrocytes and merozoites have the characteristic architecture of the specimens of the *Sarcocystis* species (rhoptries, micronemes, dense bodies, apicoplast, conoid, polar ring, pellicle, micropore, etc.). The cyst merozoites appeared variable in size and sometimes showed an abundance of reserve materials (amylopectin and lipid droplets). These findings were reported in *Sarcocystis turcicii*, too (Abdel-Ghaffar et al. 2009), and they suggested that the cyst merozoites may develop from different generations within the cyst.

The life cycle of the *Sarcocystis* species occurs in three different successive phases throughout two hosts, an intermediate host, within which merogony and cyst formation take place, and a final host, within which gamogony and sporogony occur (Mehlhorn and Matuschka 1986; Abdel-Ghaffar et al. 1990; Sakran and Ahmed 2000; Šlapeta et al. 2001, 2003; Svobodova et al. 2004).

In the present study, experimental transmission succeeded only in the snake *S. diadema* as final host when uninfected snakes were inoculated orally by infected muscles from *Acanthodactylus* lizards of the three species under investigation demonstrating that the prepatent period is 16 days, and the patent period covers about 35 days. These results indicate that these two periods are specific for *S. acanthocolubri* and are quite different from the finding of 14 and 60 days for *S. gongyli* in the same final host (Abdel-Ghaffar et al. 1990) and differ from other *Sarcocystis* species infecting lizards and using snakes as final hosts (Matuschka 1987). The gamogonic and sporogonic stages were similar to those described for other sarcosporidians and thus cannot be used for differentiation or identification (Mehlhorn and Matuschka 1986). Nevertheless, the failure to demonstrate a *Sarcocystis* infection in the other experimentally infected snakes (*P. sibilans* and *M. monspessulana*) of the family Colubridae proved that *S. acanthocolubri* is highly host specific for the final host as are all other *Sarcocystis* species (Mehlhorn and Heydorn 1978). Whereas, a broad spectrum of rodents and/or lizards may act as intermediate hosts in the life cycle (Häfner and Matuschka 1984).

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