

Apolocystis perienteron sp. nov. (Apicomplexa: Monocystinae) a new aseptate gregarine from *Pheretima californica* (Annelida: Oligochaeta) from Egypt

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Summary

An acephaline gregarine *Apolocystis perienteron* sp. nov. is described from the coelomic cavity and intestinal coelomic epithelium of earthworms *Pheretima californica*. Young trophozoites resided inside the coelomic epithelium cells of the intestine. Adult trophozoites (48–72.5 µm in diameter) were found extracellular within coelomic cavity, or attached to either somatic or splanchnic epithelium. Gametocysts were 76–150 µm in minor diameter. Navicular sporocysts (17×10 µm) demonstrated slightly projected flat plugs. The most conspicuous features differentiating the new species from the congeners are encapsulations by the host amoebocytes throughout the gregarine lifespan, and tight attachment of the parasite to the epithelial layer of the host intestine (enteron).

Key words: *Apolocystis*, coelom, gregarines, *Pheretima*, systematics

Introduction

Over 400 species of aseptate gregarines were described in the order Eugregarinida (Levine, 1977). Early divergence within Apicomplexa suggested by SSU rDNA-based phylogenies, and presence of numerous presumably plesiomorphic characters, make aseptate gregarines a significant group for understanding the directions of evolution of apicomplexan parasites (Théodoridès, 1984; Vivier and Desportes, 1990; Cox, 1994). The genus *Monocystis* Stein, 1848 was revised and split into four

genera (*Monocystis*, *Nematocystis*, *Rhynchocystis* and *Pleurocystis*) by Hesse (1909). The genus *Apolocystis* erected by Cognetti de Martiis (1923) included all species of *Monocystis* with spherical trophozoites with no polarity. Species of *Apolocystis* were described from all over the world (Bhatia and Setna, 1926; Phillips and MacKinnon, 1946; Ramadan, 1969; Levine, 1977; Segun, 1978; Pradhan and Dasgupta, 1983; Gullo and Armendáriz, 2002; Bandyopadhyay et al., 2004; Bhowmik et al., 2012). In this work, a new species, *A. perienteron*, is described and compared to the closely related *Apolocystis* spp.

Material and methods

The host. Earthworms were collected in zoological gardens, Giza governorate, Egypt. Worms were then put into soil-filled plastic containers and transferred alive to the Laboratory of Invertebrates, Department of Zoology, Faculty of Science, Ain Shams University. Some worms were dissected and the seminal vesicles were carefully removed and placed on clean grease-free slides with drops of 0.8% NaCl solution. The seminal fluid was smeared on slides and covered with glass cover slips. Slides were examined for living protozoans under a compound microscope (Carl Zeiss Jena, Laboval 4, Germany) at 100×, 400× and 1000×. The coelomic fluid of the worms was withdrawn by a micropipette, placed on clean slides with drops of 0.8% NaCl solution, covered by glass cover slips and examined.

After the initial examination of living protozoans, slides were semidried and fixed for 20 min in Schaudin's fluid (saturated aqueous solution of mercuric chloride and glacial acetic acid, El Nasr Pharmaceutical chemicals company, Egypt). Slides were then stored in 70% ethyl alcohol for removal of excess of mercuric chloride.

Histology. Body segments of infected worms were fixed in Bouin's fluid for 24 hours. Specimens were transferred into a mixture of 70% ethyl alcohol and lithium carbonate for removal of yellow colour of Bouin's fluid, passed through an ascending alcohol series, cleared in terpineol for 3 days, and embedded in parablax wax. Five-micrometer sections were cut with Erma microtome. Sections were placed on clean slides and dewaxed using xylene. The slides were then passed through descending series of alcohol and finally were placed in distilled water. Some slides were transferred to 3% iron alum solution for 3 h and stained with Heidenhain's haematoxylin for 20 min. Differentiation was performed using 1% iron alum solution. The slides were washed thoroughly in distilled water, dehydrated, cleared in xylene and mounted in Canada balsam. Some slides with sections were stained with haematoxylin (Merck, Germany) for 30 min. These slides were transferred to tap water for 1 min, dipped in eosin stain (E), dehydrated, cleared and mounted. Photomicrographs were taken by Kodak digital camera (model 1450Z) attached to the compound microscope.

Electron microscopy. Infected parts of worms were washed in 0.8% NaCl and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 24 h. The specimens were then washed in phosphate buffer for 2 h, and postfixed in 1% OsO₄

followed by washing twice in distilled water. Samples were dehydrated, embedded in Epon 812-Araldite 502 resin (Fluka Chemical Company, USA). Semithin sections (1 µm) and ultrathin sections (50-100 nm) were cut by the ultramicrotome (Leica Ultracut EMUC7). Semithin sections were stained with toluidine blue. Thin sections were placed on copper grids and stained with uranyl acetate and lead citrate (Electron Microscopy Sciences, USA). Sections were examined by JEOL's electron microscope (JSM-6300) at the Regional Center of Fungi, El Azhar University, Cairo.

Results

Two *Pheretima californica* worms out of 28 collected (7%) were infected. Young trophozoites were located intracellularly in the coelomic epithelium of the intestine (Figs 1A, 3A). Developing and mature trophozoites (Figs 1B, 3B) and gametocysts (Figs 1G, 3C) were found in coelomic cavities in the middle portion of the infected worm. Histological sections showed that most stages of the parasite were surrounded by host amoebocytes (Fig. 1C). These amoebocytes were attached to the epithelial cells (Fig. 1D). Examination by electron microscope indicated that the parasite-attached amoebocytes were linked to the epithelial cells by branched processes protruded from amoebocytes (Fig. 2A). A trophozoite, surrounded by amoebocytes, in turn, exhibited epicytic extensions (Fig. 2B). These extensions followed the perimeter of the trophozoite. In some trophozoites chromatophilic material accumulated below the cell membrane; in others it was scattered through the cytoplasm and around the nucleus (Fig. 1E). The mature trophozoites (Fig. 3B) measured 48-72.5 µm in diameter and did not demonstrate polar differentiation (Table 1).

A nucleus of mature trophozoites was 9-18.5 µm in diameter (Table 1); its position within the cell was not fixed (Figs 1C, 1D). The nucleus contained an eccentrically located spherical endosome 4-7 µm in diameter (Table 1). The nucleoplasm was granulated (Fig. 2D) and the nuclear membrane was wavy with nuclear pores (Fig. 2C). The paraglycogen granules, 0.6 to 1.7 µm in diameter (Table 1), were round to oval (Fig. 2C) and demonstrated a strong reaction to Periodic Acid-Schiff (PAS).

Gametocysts were observed in the coelomic cavity of the host worm. Gametocysts measured 76-150 µm in the minor diameter and 114-165 µm in the major diameter (Table 1). Sporocysts were navicular, with projecting flat plugs at both ends, 10±0.4 µm wide and 16.8±0.7 µm long (Table 1) (Figs 1F, 3D).

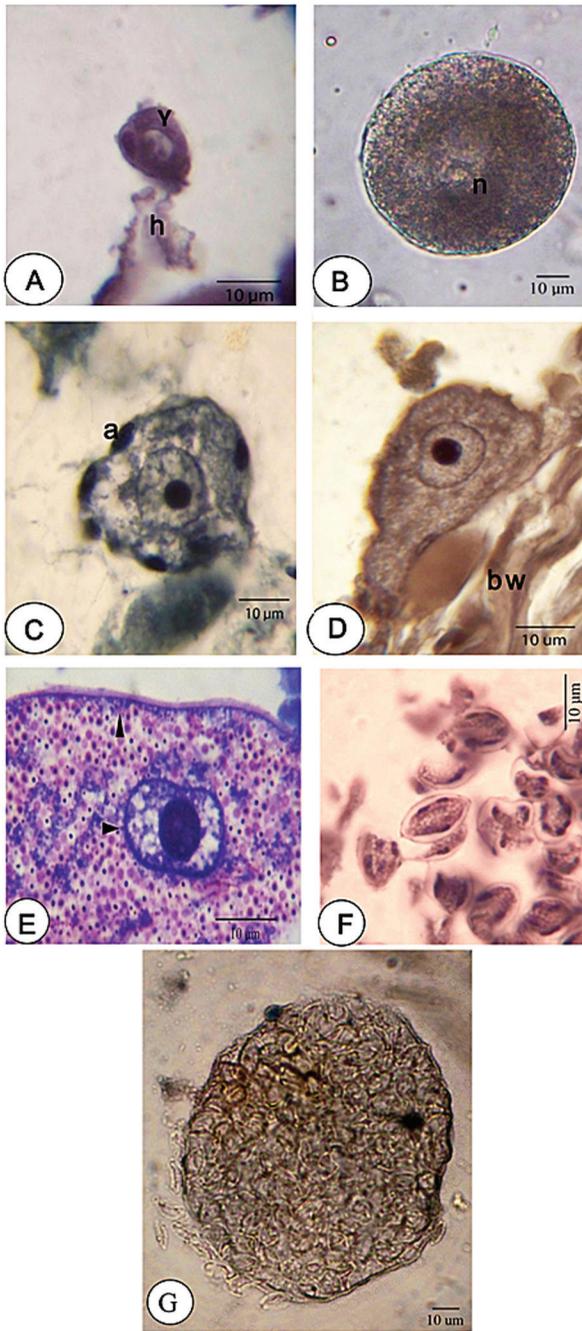


Fig. 1. *Apolocystis perienteron* sp. nov., light microscopy. A – Young trophozoite (y) stained with Heidenhain's haematoxylin (HX); B – mature trophozoite in a fresh smear; C – section through immature trophozoite, surrounded by ameobocytes, HX; D – section through trophozoite attached to coelomic epithelium underlying the oligochaete body wall, HX; E – trophozoite with chromatophilic material (arrowheads) scattered all over the cell cytoplasm, and concentrated in vicinity of the plasma membrane and nuclear envelope; a semithin section stained with toluidine blue; F – navicular sporocysts with projecting flat plugs at both the ends, HX; G – gametocyst filled with sporocysts, HX. *Abbreviations:* a – ameobocytes, bw – body wall, h – host cell, n – nucleus.

small groups of 5-20 individuals, which is not the case for the described species; (iii) the size of *A. aggregata* gametocysts is larger, than the ones of the novel species (Table 2); (iv) *A. aggregata* spores are larger, and possess pointed ends, while the new species exhibited small blunt ends. In addition, in *A. aggregata*, the amoebocyte layer was formed only around young stages (Ramadan, 1969); while in the studied species such layer surrounded all stages until the formation of gametocyst around two gamonts.

The new species has certain similarities to other species of *Apolocystis*, which partially or completely perform their life cycles in the host coelomic cavity, like: *A. catenata* (Mulsow, 1911); *A. michaelseni* (Hesse, 1909) and *A. janovyi* (Gullo and Armendariz, 2002). Trophozoites and gametocysts of *A. catenata* are huge, measuring 425 and 500 μm in diameter, respectively, while their sporocysts are smaller than those of our parasite (Table 3). The trophozoites and gametocysts of *A. michaelseni* were also markedly larger (225-295 μm and 235-300 $\mu\text{m} \times 170$ -220 μm respectively). Besides, the number of sporocysts in *A. michaelseni* gametocyst was 16 at maximum (Table 3), while in the described species gametocysts were more numerous (Figs 1g, 3c). Finally, a swollen part in the equatorial plane of the sporocyst reported for *A. michaelseni* (Loubatières, 1955) was not observed in *A. perienteron*. Gullo and Armendariz (2002) reported the presence of basophilic granules of 0.62-1.8 μm in diameter in the cytoplasm and nucleoplasm of *A. janovyi* trophozoites. In trophozoites of the new species, a comparable endoplasmic chromatophilic material was observed. This chromatophilic material was located under the pellicle of trophozoites or scattered randomly throughout the cytoplasm. Besides, young trophozoites of *A. janovyi* were

Discussion

Differential diagnosis of the described species.

The acephaline gregarine described above can be assigned to the genus *Apolocystis*, due to its spherical non-polar trophozoites, and navicular sporocysts. The present species can be differentiated from *Apolocystis aggregata* (Ramadan, 1969) by following characters: (i) *A. aggregata* trophozoites are larger; (ii) young *A. aggregata* trophozoites aggregate in

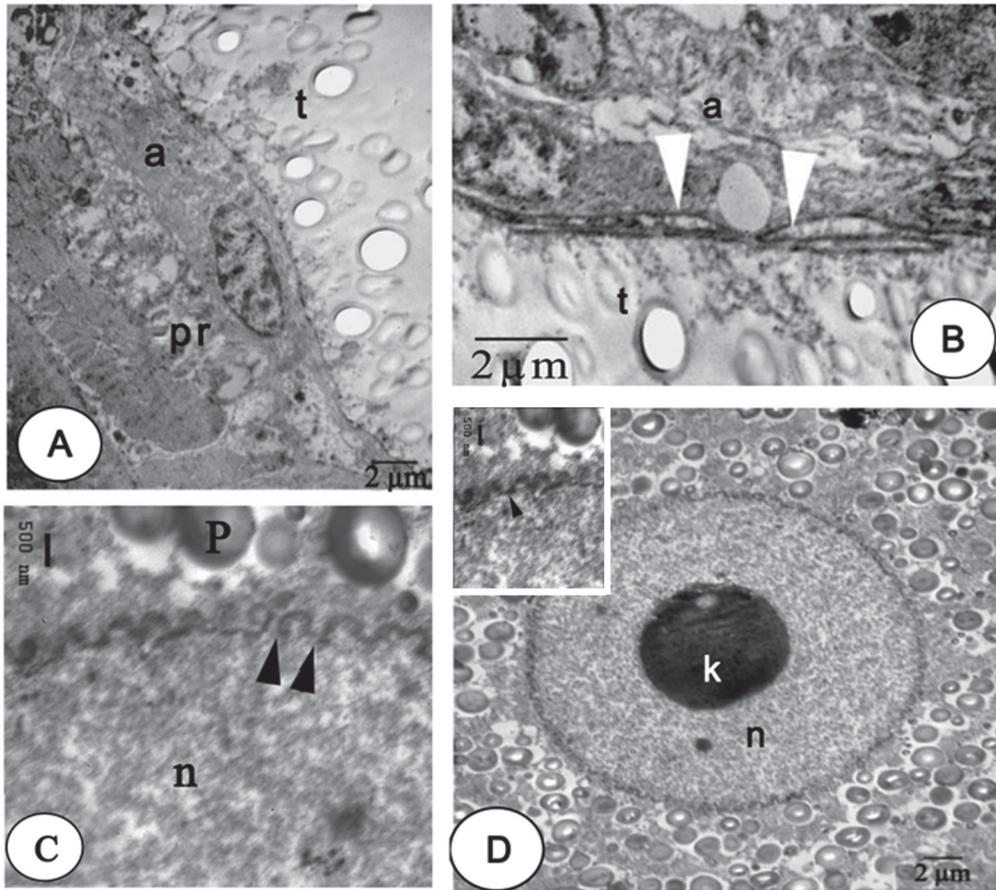


Fig. 2. *Apolocystis perienteron* sp. nov., electron microscopy. A – Amoebocyte attached to the trophozoite with branched processes; B – trophozoite with epicytic extensions (*arrowheads*) on its surface; C – nuclear membrane containing nuclear pores (*arrowheads*), note the spherical paraglycogen granules; D – trophozoite’s nucleus is surrounded by a wavy envelope (magnified in the insert at the *arrowhead*) and containing a coarsely granulated nucleoplasm, note a round condensed karyosome. *Abbreviations:* a – amoebocytes, k – karyosome, n – nucleus, p – paraglycogen granules, pr – branched processes, t – trophozoite.

Table 1. Average size of *A. perienteron* stages in *P. californica*.

| Stage | Range of diameter (μm) | Average diameter | Standard deviation |
|--------------------------|------------------------|------------------|--------------------|
| Adult trophozoite | | | |
| Cell | 48–72.5 | 59.7 | 7.5 |
| Nucleus | 9–18.5 | 13.8 | 3.5 |
| Karyosome | 4–7 | 5.6 | 1.5 |
| Granule | 0.6–1.7 | 0.9 | 0.3 |
| Gametocyst | | | |
| Major diameter | 114–165 | 134 | 20.9 |
| Minor diameter | 76–150 | 110 | 32.7 |
| Sporocyst | | | |
| Length | 16–17.5 | 16.8 | 0.7 |
| Width | 9.6–10.5 | 10 | 0.4 |

located inside intestine epithelial cells near the basal membrane (Gullo and Armendariz, 2002), while in the present study, trophozoites resided in visceral peritoneal cells in the post-clitellar region of the worm. Martinucci and Crespi (1979) described filiform extensions equipped with internal microtubules on the surface of *Apolocystis* sp. trophozoites. These extensions were similar to the above described epicytic extensions in the trophozoites of *A. perientron*. No internal microtubules were observed in the present study.

The comparative analyses presented above, suggest that the novel gregarine belongs to the genus *Apolocystis*, but differs from the previously known species of this genus, thus justifying the erection of a new species. The most conspicuous features differentiating the new species from congeners are encapsulations by amoebocytes throughout the life

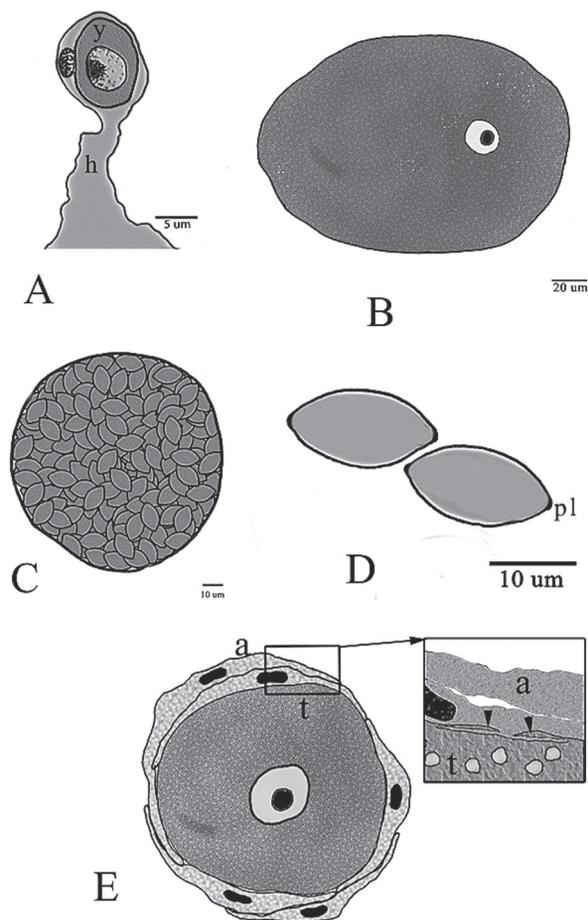


Fig. 3. *Apolocystis perienteron* sp. nov., schematic drawings. A – Young trophozoite inside the host cell; B – mature trophozoite; C – gametocyst; D – navicular sporocysts with projecting flat plugs at both ends (sporozoites not visible); E – trophozoite surrounded by amoebocytes; the enlarged portion shows the epicytic extensions (arrowheads). Abbreviations: a – amoebocytes, h – host cell, pl – flat plugs, t – trophozoite, y – young trophozoite.

cycle, and tight attachment to the epithelial layer of the intestinal region (enteron) of the host worm.

TAXONOMIC SUMMARY

Apolocystis perienteron sp. nov. (Figs 1-3, Tables 1-3)

Phylum: Apicomplexa, Levine 1977
Order: Eugregarinida, Leger 1900
Family: Monocystidae, Butschli 1882
Subfamily: Monocystinae, Bhatia 1930
Genus: *Apolocystis*, Cognetti de Martiis 1923
Type host: *Pheretima californica*

Table 2. Comparison between *A. aggregata* and *A. perienteron* sp. nov. infecting earthworms in Egypt.

| Host, stage | <i>A. aggregata</i> | <i>A. perienteron</i> sp. nov. |
|---------------------|---|--|
| Host and localities | <i>Pheretima californica</i> , <i>P. hawayana</i> ; Zoological and Orman botanical Gardens | <i>Pheretima californica</i> ; Zoological Gardens |
| Trophozoite | 60–100 μ m (76.5 μ m) Young troph. in groups | Round: 48–72.5 μ m (59.7 \pm 7.5 μ m) |
| Nucleus | 13–22.5 μ m Mainly eccentric | 9–18 μ m (13.8 \pm 3.5 μ m) No fixed position |
| Karyosome | 3–5 μ m | 4–7 μ m (5.6 \pm 1.5 μ m) |
| Gametocysts | 130–180 μ m (175 μ m) | Oval: 134.3 \pm 20.9 \times 110 \pm 32.7 μ m |
| Sporocysts | Navicular, pointed ends 10 \times 3.5 μ m | Navicular, small flat ends 16.8 \pm 0.7 \times 10 \pm 0.4 μ m |

Symbiotype: Host is deposited in the Parasitology Laboratory, Department of Zoology, Faculty of Science, University of Ain Shams, Abbassia 11566, Cairo, Egypt

Site of infection: Coelom

Type locality: Zoological Gardens (Giza governorate), Egypt. Latitude and Longitude: 30°01'27.3"N and 31°12'49.3"E.

Description: Young trophozoites are intracellular; reside in the coelomic epithelium of the intestine, sub-spherical to oval in shape. Mature trophozoites 48–72.5 μ m in diameter, attached to external surface of host intestine. Trophozoites without polar differentiation or ectoplasmic processes. Gametocysts 76–150 μ m in diameter. Navicular sporocysts 16.8 \pm 0.7 \times 10 \pm 0.4 μ m, with slightly projecting flat plugs.

Type material: Holotype and paratypes are deposited in the Parasitology Laboratory, Department of Zoology, Faculty of Science University of Ain Shams, Abbassia 11566, Cairo, Egypt.

Etymology: The new species has been named after the site of localization within the host.

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Table 3. Comparison among *A. perienteron* sp. nov., *A. michaelseni*, *A. catenata* and *A. janovyi*.

| Host, stage | <i>A. perienteron</i> sp. nov. | <i>A. michaelseni</i> | <i>A. catenata</i> | <i>A. janovyi</i> |
|----------------|---|---|---|--|
| Host | <i>Pheretima californica</i> | <i>Pheretima hawayana</i> | <i>Lumbricus terrestris</i> <i>L. rubellus</i> | <i>Microscolex dubius</i> |
| Infection site | Coelom behind clitellar region, occasionally in nephredia | Coelom behind clitellar region, occasionally in nephredia | Coelom | Young is intercellular parasite in oesophageal epithelium, other stages in coelomic cavity |
| Trophozoite | 48–72.5 (59.7±7.5) µm | 225–295 µm | 425 µm | 17– 283 (158.2±63.578) × 17–250 (128.4±51.7) µm |
| Nuclei | 9–18.5 (13.8 ±3.5) µm | – | – | 37.4 ±11.5 × 32.8±8.413 µm, eccentric |
| Karyosome | 4–7 (5.6 ±1.5) µm | – | – | 17±7.7 µm |
| Gametocysts | 110±32.7 µm × 134.2±20.9 µm, a large number of sporocysts | 235–300 µm × 170–220 µm, with 16 sporocysts as maximum | 500 µm | 116–206 × 193–369 (148.8±41.2 × 262±44.3) µm |
| Sporocysts | 10±0.4 × 16.8±0.7 µm | 9 × 15 µm | 6 × 14 µm | 2.7–6.8 × 5 -15 (4.1±0.8 × 9.9±2.2) µm |

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