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Apolocystis proventus sp. nov. (Apicomplexa: Monocystinae) a New Species of Aseptate Gregarine from Egyptian Earthworms: *Pheretima californica* and *Pheretima elongata* (Annelida: Oligochaeta)

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Abstract. A new acephaline gregarine is described from the earthworms *Pheretima californica* and *Pheretima elongata*. The gregarine was either embedded in the pharyngeal glandular tissue or found free in the coelomic fluid around the pharyngeal region in front of the crop. Adult trophozoites measured 48–65 µm in diameter and are mostly active with a wavy pellicle. Heterogeneity in the endoplasm of active trophozoites was observed. Gametocysts measured 56–81 µm in diameter, with a characteristic thick cyst wall. Navicular sporocysts measured 5.8 ± 0.2 × 3.5 ± 0.4 µm, with small truncate plugs.

Key words: Gregarine, *Pheretima*, Coelom, *Apolocystis*, Pharyngeal glands, Apicomplexa and Monocystinae.

INTRODUCTION

Monocystids belonging to the eugregarines are parasitic protozoa frequently infecting the seminal vesicles and coelomic cavities of many oligochaetes. Most published papers, which described those parasites, are taxonomic in nature or check lists of monocystid species along with their related hosts (Ruston 1959, Marek 1967, Segun 1971a, b, Levine 1977 and 1988, Pizl 1989a, b and Frolov 1991). All species belonging to genus *Monocystis*, characterized by round trophozoites with no polarity, were included into the genus *Apolocystis* by Cognett de Martiis (1923). Several species of *Apolocystis* were described (Bahatia and Setna 1926, Phillips and Mackinnon 1946, Ramadan 1969, Levine 1977, Segun 1978, Pradhan and Dasgupta 1983, Armendáriz and Gullo 2002, Bandyopadhyay *et al.* 2004, Bhowmik *et al.* 2012). In this paper, a new species of *Apolocystis* is described with comparative comments on closely related species.

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MATERIALS AND METHODS

The host worms

Earthworms were collected from Maghagha region (Menia Governorate) and the Zoological Gardens (Giza Governorate), Egypt. Specimens collected were stored into soil-filled plastic containers and transferred live to the laboratory of invertebrates, depart-

ment of zoology, faculty of science, Ain shams university. Some earthworms were dissected, and the seminal vesicles were carefully removed and placed on a clean grease-free slide with a drop of 0.8% NaCl solution. A thin film of seminal fluid was pipette onto the slides and saved with glass cover slips. Wet mounts were examined for living protozoans under a compound microscope. The coelomic fluid of the examined worms was withdrawn by a micropipette and then placed on clean slides with a drop of 0.8% NaCl solution and covered with glass cover slips for examination. After initial examination of living protozoans, slides were semidried and fixed for 20 minutes in Schaudin's fluid (saturated aqueous solution of mercuric chloride and glacial acetic acid). Slides were then treated in ethyl alcohol for removal of excess of mercuric chloride.

Histological preparations

For histological examination, sectioned tissues were prepared by fixing body segments of infected worms in Bouin's fluid for 24 hours. Specimens were then transferred to a mixture of 70% ethyl alcohol and lithium carbonate, then dehydrated through an ascending series of alcohols and cleared in terpineol for 3 days. Specimens were embedded in parablast wax and sectioned (5 µm thickness) on clean slides. Slides were then dewaxed in xylene, then rehydrated through a descending series of alcohols to distilled water. A subset of slides were transferred to Mallory I solution (1% Acid fuchsin) for 15 seconds, rinsed in distilled water to differentiate red color of fuchsin for 10 seconds, treated with Phosphomolybdic acid (1%) for 1–5 minutes, then stained with Mallory II solution (equal parts of 1% Aniline blue and 4% Orange G) for 2 minutes. Differentiation was done with 90% ethyl. Slides were then washed in distilled water, dehydrated in an ascending series of alcohols, cleared in xylene, and mounted in Canada balsam. The other subset of slides were stained in haematoxylin for 30 minutes, transferred to a tap water (1 minute), and counter stained in eosin. Slides were dehydrated in ethyl alcohol, cleared and mounted. Photomicrographs were taken by a Kodak digital camera (model 1450Z) attached to a compound microscope.

RESULTS

Apolocystis proventus sp. nov.

Phylum: Apicomplexa (Levine 1977)
Order: Eugregarinida (Leger 1900)
Family: Monocystidae (Butschli 1882)
Subfamily: Monocystinae Grassè, 1953 (= Monocystidae Bhatia, 1930)

Genus: *Apolocystis* (Cognetti de martiis 1923)

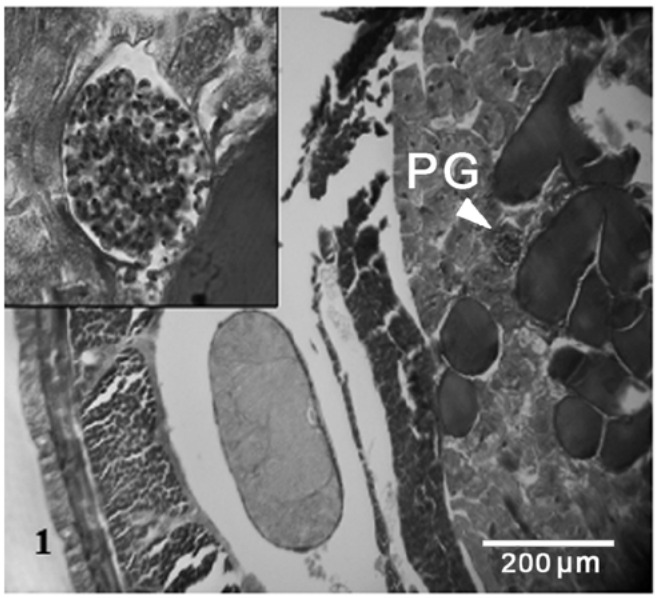
Description: sub-spherical, oval, parasite is embedded into the pharyngeal glandular tissue or free in the coelomic fluid around the pharyngeal region in front of the crop region. Adult trophozoites have no polar differentiation (48–65 µm in diameter). Most trophozoites are active with wavy pellicle. Heterogeneity (irregular distribution of paraglycogen granules) in the endoplasm of active trophozoites is observed. Gametocysts measure 56–81 µm in diameter, with a characteristic thick cyst wall. Navicular sporocysts measure 5.8 µm ± 0.2 × 3.5 µm ± 0.4, with small truncate plugs.

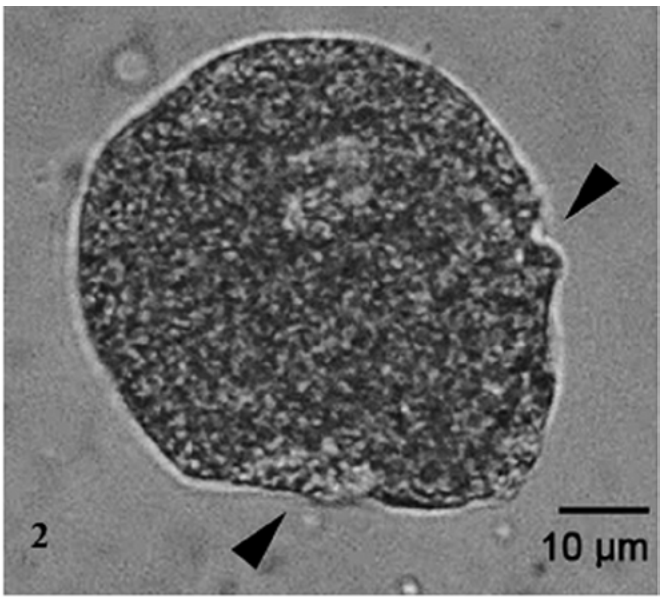
Remarks: The parasite was found embedded in the pharyngeal glands, (Fig. 1) free in the coelom, and in front of the crop region of *Pheretima californica* and *P. elongata*. Growing trophozoites were mainly round or oval and had no apparent polarity. The pellicle exhibited active short contractility waves which took many forms (Fig. 2). The endoplasm of the growing trophozoites exhibited more heterogeneity than adults. Many growing trophozoites had endoplasmic areas without paraglycogen granules. In fresh preparations, the endoplasm of some adult trophozoites exhibited an irregular darker area (more aggregation of paraglycogen granules) around the nucleus with narrow radiating extensions (Fig. 3). Paraglycogen granules were oval to elongate in shape with rounded ends and ranged from 0.3 µm to 1.6 µm in length. The nucleus was deformed (irregular in shape) in most trophozoites due to the pressure exerted by the active internal streaming of the endoplasm. The nucleus was round, eccentric, and measured about 8–12 µm in diameter (Fig. 4). Karyosomes were relatively large, eccentric and about 2.4–5.7 µm in diameter (Fig. 4). The adult trophozoites had no polar differentiation and measured about 48–65 µm in diameter (Table 1).

Gametocysts had sizes ranged from 56 to 81 µm in diameter (Fig. 5). The cyst wall is characterized by its considerable thickness. However, the morphological change of the parasite inside the cyst was hard to observe during progressive stages. In sectioned material, the cyst wall was deeply stained with Haematoxylin

▸▸

Figs 1–6. *Apolocystis proventus* a new greagarine from Egyptian earthworms. **1** – Section of the host worm showing a parasite in gametocyst stage (arrowhead) embedded in pharyngeal glands (PG), Mallory stain. Note-Insert showing a magnified gametocyst. **2** – An active growing trophozoite showing the waves of contractility (arrowheads), fresh preparation. **3** – A growing trophozoite showing an irregular darker area (more aggregation of granules) around the nucleus (N) with narrow radiating extensions (white arrowhead), fresh preparation. **4** – Adult trophozoite showing the nucleus (N) and karyosome (K), fresh preparation. **5** – A mature gametocyst filled with sporocysts (S), fresh preparation. **6** – A section of immature gametocyst showing a thick gametocyst wall (G W), Haematoxylin and eosin stains.


PG
1
200 µm


2
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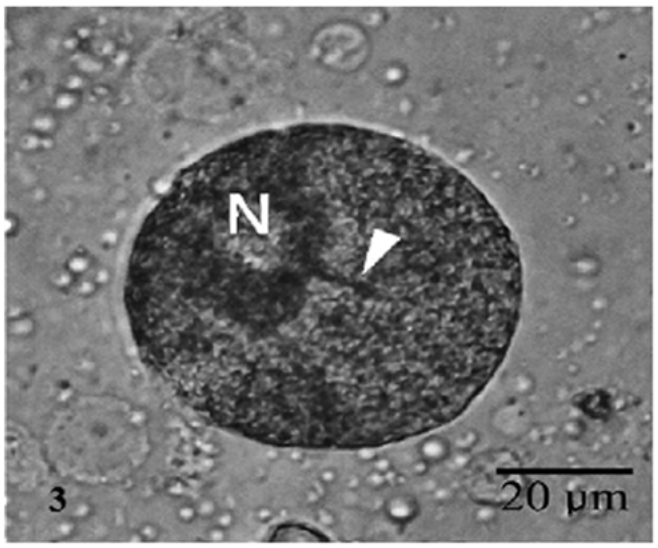
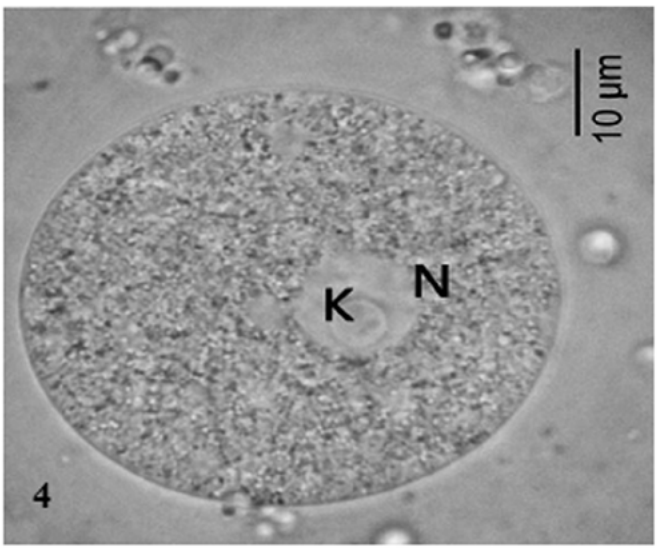
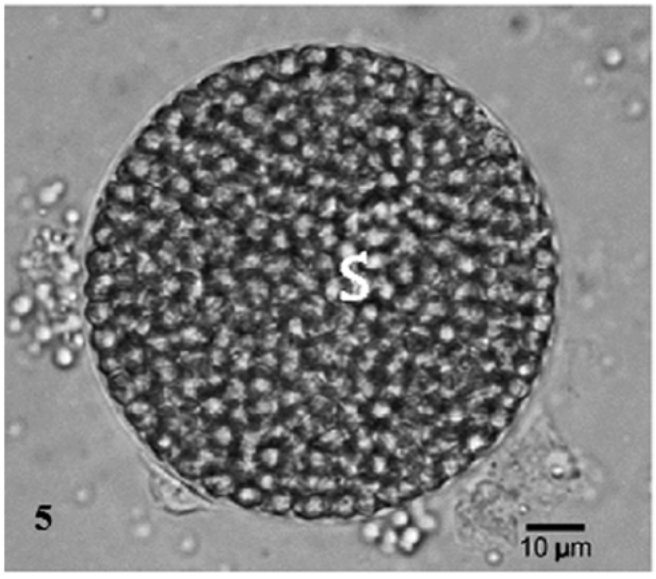

N
3
20 µm


10 µm
K
N
4


S
5
10 µm

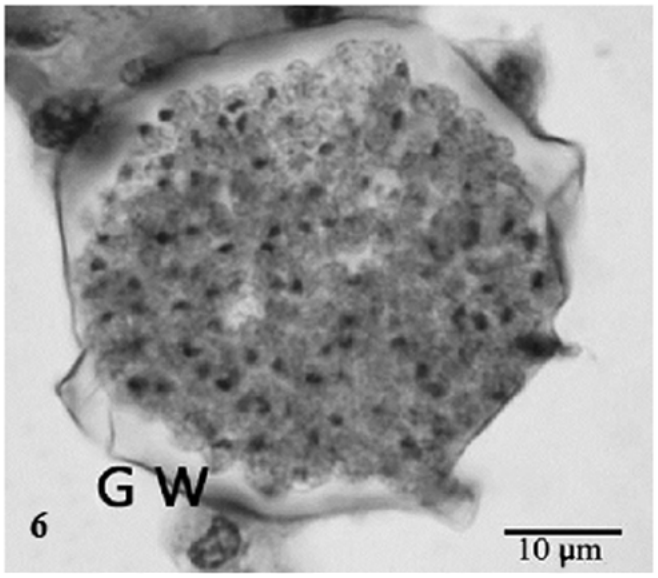

G W
6
10 µm

Table 1. Average size (μm) of the observed *Apolocystis proventus* stages in coelom of *Pheretima californica* and *P. elongata*.

Stage	Average (μm)	Range (μm)	Standard deviation (± μm)
Trophozoite			
– Cell	57.7	48–65	6.3
– Nucleus	10.3	8–12	1.7
– Karyosome	4	2.4–5.7	1.6
– Granule	0.9	0.3–1.6	0.6
Gametocyst			
– Diameter	73	56–81	5.9
Sporocyst			
– Length	5.8	5.6–6	0.2
– Width	3.5	3.1–3.9	0.4

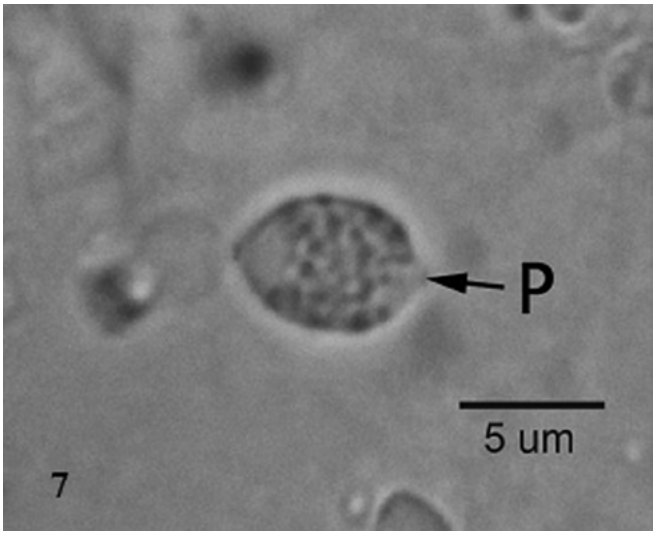


Fig. 7. A single sporocyst, note the two small flat plugs (P), fresh preparation.

and eosin (Fig. 6). Sporocysts are navicular, with small truncate plugs at both ends, and measured 5.8 ± 0.2 μm in length and 3.5 ± 0.4 μm in width (Fig. 7).

DISCUSSION

The parasite described herein belongs to the genus *Apolocystis*. The spheroidal form and lack of polarity in its trophozoites are evident characteristics. One species, *A. aggregata* was described and named by Ramadan (1969), from two Egyptian earthworms *Pheretima californica* and *Pheretima hawayana* (Table 2). Apart from the difference in trophozoite size, and larger nuclei of *A. aggregata*, and the gregarine describe herein, a special character was exhibited by *A. aggregata*. Young trophozoites displayed the tendency to aggregate in groups consisting of 5 to 20 individuals. Moreover, the size and shape of the sporocysts in *A. aggregata* differed from these of the present species. *Apolocystis aggregata* sporocysts are navicular with pointed ends and measured 10 μm × 3.5 μm, while in the present species the sporocysts are navicular with small truncated ends and measured 5.8 μm (±0.6 μm) × 3.5 μm (±0.4 μm).

An *Apolocystis* sp. was reported by Ramadan (1969) in an Egyptian oligochaete species; only trophozoites were observed. This parasite was exhibiting a striking feature; as the endoplasm of trophozoite takes the form of small vacuoles surrounded by moderately coarse granules, while the vacuoles themselves are occupied by much finer granules. This description may be comparable with some previously described aspects related to the endoplasm of the present parasite.

In the present parasite, the endoplasm of the growing trophozoite exhibited more heterogeneity than that of the adult trophozoite. The growing trophozoites had areas of their endoplasm devoid of paraglycogen granules; with progressive growth of the trophozoite and more production of paraglycogen granules, the degree of heterogeneity in the endoplasm decreased. Generally, trophozoites related to Ramadan's work were much larger in its measurements (about double) than those of the present gregarine. (Table 2)

Within the range size of trophozoites of the present parasite, were those of *A. beaufortii* (Cognetti de Martiis 1918) (50 μm); *A. chotonagpurensis* Bandyopadhyaye *et al.*, 2004 (42 to 83 μm); *A. minima* (Boisson 1957) Frolov 1991 (60 μm); and *A. minuta* Troisi, 1933 (40 to 46 μm). *A. beaufortii* was collected from the seminal vesicles of *Pheretima beaufortii*. It was characterized by the formation of two morphologically distinguishable types of gametes (anisogamy). *Apolocystis chotonagpurensis* was collected from the seminal vesicles of *Amynthasrobusta* (earthworm). It was characterized by very fine rows of cytoplasmic processes and large nucleus if compared with the diameter of our present trophozoite. *Apolocystis minima* was collected from the seminal vesicles of *P. posthuma*. It was characterized by anisogamy, a central karyosome, and larger paraglycogen granules (up to 3 μm) compared to those of the present parasite (0.3 to 1.6 μm); it also has much longer sporocysts (twice the length of those in the

Table 2. Comparison among three *Apolocystis* species infecting some earthworms in Egypt.

Parasite	Host and Localities	Measurements					Reference
		Trophozoite	Nucleus	Karyosome	Gametocysts	Sporocysts	
Apolocystis aggregata	*Pheretima californica* and *Ph. hawayana* Zoological and Orman botanical Garden	60 µm–100 µm (76.5 µm) Young troph. in groups	13 µm–22.5 µm Mainly eccentric	3 µm–5 µm	130 µm–180 µm (175 µm)	Navicular, pointed ends 10 µm × 3.5 µm	Ramadan 1969
***Apolocystis* sp.**	Unidentified species of oligichaetes Abou-Rawash	90 µm–115 µm	32 µm–35 µm Mostly eccentric	7 µm	–	–	Ramadan 1969
***Apolocystis proventus* n. sp.**	*Pheretima californica* and *Ph. elongata* Zoological Garden and Maghaga region (Menia Governorate)	Round: 48 µm–65 µm 57.7 µm (±6.3 µm)	8 µm–12 µm Mainly eccentric	2.4 µm–5.7 µm Eccentric	Round: 56 µm–81 µm 73 µm (±5.9)	Navicular, very small truncated ends 5.8 µm (±0.2) × 3.5 µm (±0.4)	Present work

present study; 10.5 and 5.8 µm, respectively). *Apolocystis minuta* was collected from the seminal vesicles of *Lumbricus terrestris, L. castaneus and L. rubellus*. It was characterized by being the smallest species belonging to genus *Apolocystis*, with 3 sizes of sporocysts and had very large paraglycogen granules (up to 5 to 7 µm) (Table 3).

Apart from the difference in morphometric parameters of trophozoites, there were other monocystid gregarines belonging to genus *Apolocystis* which were recorded in the coelom of various species of oligochaetes and exhibited more or less behavioral similarities with the present parasite. It was recorded that in four species of *Apolocystis*, some stages of their life cycle were involved into certain tissues of their host worms; *A. laverensis* Rees, 1963, *A. michaelseni* Hesse, 1909, *A. janovy* Armendariz and Gullo, 2002 and *A. herculea* Bosanquet, 1894.

Young trophozoites of *A. lavernensis* develope in the ovisac and exhibited high activity in their movements. Growing and mature trophozoites, (the syzygy stages and gametocysts) were free in the coleomic cavities of the genital segments. And mature trophozoites had no observable activity in their movement. All life cycle stages of *A. michaelseni* were attached to the peritoneal epithelium of the body wall and the splanchnic epithelial cells belonging to post-clitellar segments, This attachment was attributed by the help of what Hesse (1909) described as many thin membranes formed by the host worm itself. Moreover, some trophozoites could be occasionally found free and might be attached to the nephredia. This species was characterized by the syzygy stage which involved 2 to 3 gamonts, up to 16 sporocysts in the gametocysts, and the sporocysts with swollen equatorial planes (Table 3).

Young trophozoites of *A. janovyi* are intercellular parasites of the oesophageal epithelium, while all remaining stages of the life cycle are attached to the parietal epithelium and the visceral peritoneum of the post-clitellar segments up to the posterior end of the host. Generally, measurements of different stages of *A. janovyi*, were triple in size compared to those of the corresponding stages of *A. proventus*. Many authors described and reported *A. herculea* (Bosanquet 1894, Hesse 1909, Ress 1963, Segun 1978 and Pizl 1990). Bosanquet (1894) reported that the infection was restricted to the posterior region of the host worm. Young trophozoites and gametocysts were embedded in the splanchnic epithelial layer. The growing and mature trophozoites were mainly free in the coelomic cavity, or to a less extent, attached to nephredia, the coelomic epithelium of the intestine and of the body wall by what Bosanquet described as double membranes. Hesse (1909) agreed with what was described by Bosanquet (1894) except that the gametocysts were phagocytized by host amoebocytes and found in groups. Segun (1978) found

Table 3. Comparison among some species of *Apolocystis* infecting some earthworms in world.

Parasite	Host (Site of infection)	Trophozoite	Nucleus	Karyosome	Paraglycogen granules	Sporocyst	Gametocyst	Reference
A. pertusa	*Allolobophora chlorotica* (Seminal vesicle)	160 µm Young in amoebocyte	31 µm vacuoles around nucleus	7 µm eccentric vesicular	3 µm	12 µm long oval spore, round ends	Cyst 250 × 250	Loubatieres (1955)
A. beaufortii	*Pheretima (Parapheretima) beaufortii* (Seminal vesicle)	50 µm	–	–	–	Biconical with short flat button	– anisogamy	Cognetti de Martiis 1923
A. chotonagpurensis	*Amynthas robusta* (Seminal vesicle)	42–83 µm (57 ± 19)	12–17 × 8–16 µm	–	–	6.5–7 × 3.5–4 µm	71–96 × 46–83 µm	Bandyopadhyay, Roychoudhuri and Biswas 2004
A. minima	*Pheretima posthuma* (Seminal vesicle)	60 µm	8 µm eccentric	a central karyosome	3 µm	10.5 long With flat ends	100 um anisogamy	Boisson 1957 (Frolov 1991)
A. minuta	*Lumbricus terrestris*, *L. castaneus* and *L. rubellus* (Seminal vesicle)	40 µm to 46 µm	10 µm	–	5–7 µm	3 types of sporocysts 11 × 5.5	68–74 × 55–65 µm	Troisi 1933
A. megagranulata	*Dendrobaenarubida* (Lumbricidae) (Perivisceral coelom of genital segments)	84–450 µm	30–90 µm	9–36 µm eccentric	Spherical, 6–14 µm	27–31 × 12–16 µm	330–540 µm In coelom	Segun 1971
A. proventus	*Pheretima californica* and *P. elongata* (Coelomic cavities front of crop region)	Round, 48–65 µm 57.7 µm (±6.3µm)	8–12 µm Mainly eccentric	2.4–5.7 µm Eccentric	0.6–1.6 µm	5.8 (±0.2) × 3.5 µm (±0.4) Navicular, very small truncated ends	Round: 56–81 µm 73 µm (±5.9)	The present work
A. laverensis	*Allolopophora terrestris* (Young in ovisac, troph. free in the coleomic cavities of the genital segments)	500 µm	60 µm	30 µm	11 µm	–	–	Rees 1963
A. michaelseni	*Pheretima hawayana* (Coelom behind clitellum region, occasionally in nephredia)	225–295 µm Thin layer of host's amoebocytes, in small groups attached to the body wall of host, rarely free.	Voluminous spherical chromatic granules near the nuclear membrane and irregular small ones toward the center of nucleus.	Spherical central	–	9 × 15 µm, navicular, with remarkable swollen region in equatorial plane.	235–300 × 170–220 µm, with 16 spores as maximum.	Hesse 1909

A. janovy	*Microscolex dubius*. (Young troph. intercellular in oesophageal epithelium, other stages in coelomic cavity along worm, spores in seminal vesicles)	158.2 (±63.578) × 128.4 (±51.7) Thin mesothelial layer around most stages attached them to the parietal as well as to the visceral peritoneum, rarely free.	37.4 (±11.5) × 32.8 (±8.413), eccentric	17 (±7.7), vesicular	–	2.7–6.8 × 5–15 µm	148.8 (±41.2) × 262 (±44.3) syncytial wall	Armendariz 2002
A. herculea	*Lumbricus terrestris* *Lumbricus rubellus* *Octocalsium lacteum* (Posterior region of the host worm)	4000 × 3000 µm Young troph. embedded into the splanchnic epithelial layer, growing and mature trophozoites mainly free in the coelomic cavity or attached to epithelium of the intestine	70–80 µm	6 µm	7–8 µm	32 × 12 µm	1200 × 400 µm	Bosanquet 1894

A. herculea in the coelomic spaces of earthworm *Libyodrilus violaceus* extending from the genital segments to the posterior segments. He added that all different stages in the life cycle were attached with chloragogen cells (splanchnic epithelial layer) of the intestinal wall in the form of groups, and each group was surrounded by very thin transparent membranes. Segun (1971b) described a new species of gregarine, *A. megagranulata* from the perivisceral coelom of genital segments in *Dendrobaenarubida* sp. This parasite was different from the present parasite by the large size of its paraglycogen granules (spherical: 6 to 14 µm), and immobility of their trophozoites. The present parasite is the smallest Egyptian species of acephaline gregarine belonging to genus *Apolocystis* (57.7 ± 6 µm). It also represents the first smaller acephaline gregarine, belonging to genus *Apolocystis*, infecting the coelomic cavities of oligochaetes around the world. Universally, it seems to be the third species concerning the size of its trophozoites after *A. minuta* Troisi, 1933; (40 µm to 46 µm) and *A. beaufortii* Cognetti de Martiis, 1918 (Cognetti de Martiis, 1923); (50 µm). It is evident from the above detailed discussion that the gregarine of *P. californica* and *P. elongata* is a new species of *Apolocystis*, well characterized by its small size, heterogeneity of the endoplasm, obvious thickness of its gametocyst's wall and the restricted area of infection in the coelom around the pharyngeal region before the crop (proventriculus). The authors give it the name, *Apolocystis proventus* sp. nov. (Table 3).

Taxonomic summary

Type material: *Apolocystis proventus* sp. nov.

Type host: *Pheretima californica* and *Pheretima elongata*.

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

Site of infection: Pharyngeal glands and peripharyngeal coelom.

Type locality: Zoological gardens, Giza governorate (Lattitude and Longitude: 30°01′27.3″N 31°12′49.3″E) and Maghagha region, Menia governorate (Lattitude and Longitude: 28°39′00″N 30°50′24″E), Egypt.

Prevalence: *Pheretima californica,* 6 worm infected out of 43. (13.9%)

P. elongata, 7 worm infected out of 23 (30.4%).

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

Etymology: The new species has been named after the site of infection (restricted to ceolomic cavity front of the crop (= proventriculus) of the host worm).

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