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***Apolocystis nephredii* (Apicomplexa: Monocystinae) A New Aseptate Gregarine Species from Nephredia of *Limnodrilus* sp. (Annelida: Oligochaeta) from Egypt**

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ABSTRACT

An aseptate gregarine, *Apolocystis nephredii*, sp. nov. was described as a new species from the nephredia of earthworm, *Limnodrilus* sp. Life cycle stages of the parasite are present inside the nephredia. Trophozoites are pear, kidney or oval in shape ranging between 81 and 124 μm long and between 75 and 85 μm broad, with an average of $92.7 (\pm 24) \times 79 (\pm 3.7) \mu\text{m}$. Gametocysts are ellipsoidal and range between 200 and 209 μm long and between 112 and 132 μm broad, with an average of $205.9 (\pm 3.8) \times 125 (\pm 4) \mu\text{m}$. Sporocysts are navicular with round ends and measure $17 - 19.2 \times 8.1 - 9 \mu\text{m}$, with an average of $18.5 (\pm 0.6) \times 8.5 (\pm 0.2) \mu\text{m}$.

INTRODUCTION

Aseptate gregarine fauna has been noted in many areas of the world including Egypt. Gregarine fauna of Egypt needs more investigations to explore the new species. About 400 species of acephaline gregarines were described in order Eugregarinida (Levine 1977). Trophozoite of aseptate gregarine characterized by a single cytoplasmic part.

Genus *Apolocystis* was documented by Cognetti de Martti (1923) to gather all species of *Monocystis* contain spherical trophozoites with no polarity. Many studies identified several species of *Apolocystis* from various regions of the world (Bhatia 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 and 2012; Ramadan *et al.* 2014 and 2015). In this paper, taxonomic description of a new species of *Apolocystis* is given and its phases of life cycle described.

MATERIALS AND METHODS

Host Worms:

Three hundred and twenty-four worms collected from both Maghagha, and Abo-Rawash regions, Menia and Giza Governorates respectively, Egypt. Worms were put into freshwater-filled plastic containers and then transferred alive to the laboratory of invertebrates, Department of Zoology, Faculty of Science, Ain Shams University. Worms were kept alive for days in small pools of dechlorinated water, Water was changed daily.

Some worms squeezed between two clean slides and slightly compressed to observe the parasite inside nephredia. Other worms were cut into small pieces in Petri dishes filled with saline solution (0.8 % NaCl), to free the parasite stages. Carefully, living parasites removed and placed on a clean grease-free slide with a drop of saline solution to be examined under a compound microscope then photographed. After the initial examination of living protozoans, slides were semi-dried and fixed for 20 minutes in Schaudin's fluid (Mercuric Chloride and Methanol). Slides were then put in ethyl alcohol for removal of excess of mercuric chloride. One set of specimens were prepared for staining by Heidenhain's haematoxylin and Haematoxylin and eosin as described in Ramadan *et al.*, (2014).

Histological Preparations:

Sections (5µm) were prepared by taking small pieces of infected worms that fixed in Bouin's fluid for 24 hours, finally, section were stained by Haematoxylin and eosin and Giemsa's stain, dehydrated, cleared and mounted. Photomicrographs were taken by a Kodak digital camera (model 1450Z) attached to the compound microscope.

RESULTS AND DISCUSSION

Apolocystis nephredii sp. nov. (Figs.1-3)

Phylum: Apicomplexa, Levine 1977

Order: Eugregarinida, Leger 1900

Family: Monocystidae, Butschli 1882

Subfamily: Monocystinae, Bhatia 1930

Genus: *Apolocystis*, Cognetti de martiis 1923

This parasite was detected in the nephredia of *Limnodrilus* sp. (Fig. 1A). From 324 collected worms, 17 (5.2 %) were found infected with *Apolocystis nephredii*. All 17 worms only infected with this parasite. The intensity of infection was higher in the nephredia of the middle segments and getting lighter toward the posterior segments. Syzygy and gametocyst stages were the most observed stages in the nephredia.

The young trophozoite was typically round in shape (Fig. 1B). The nucleus of the

early stages was relatively large, rounded, centrally located (Fig. 1B) and usually occupied a considerable space inside the young trophozoite. In sectioned material stained with haematoxylin and eosin, darker red chromatin granules appear in nuclear space (Fig. 1B). A single rounded dense centric karyosome was also observed (Figs. 1B, 1C & 1D). Many chromatin vacuoles were observed in the periphery of karyosome, these vacuoles were more or less equal in size but their distribution inside the karyosome took either a regular or irregular modes (Figs. 1C, 1D & 3A).

The adult trophozoite was pear- (Fig. 1E), kidney- or oval-shaped (Figs. 1F & 3B), surrounded by the remnant parts of the nephredial wall. The dimensions of adult trophozoites ranged between 75 – 85 x 81-124 µm, with an average of 79 (±3.7) x 92.7 (±24) µm. Moreover, the endosarc was filled with round to oval paraglycogen granules, ranging between 1 and 1.3 µm, with an average of 1.1 (±0.1) µm. The nucleus in most adult trophozoites, tended to be eccentrically situated within the parasite's body, the nucleus ranged between 19 and 24 µm, with an average of 22.4 (±2.1) µm. In fresh preparations, most of the nuclear space between the nuclear membrane and the karyosome was clear and homogenous. The internal surface of the nuclear membrane had a discontinuous layer of large dark dot-like chromatin material, which was more or less regularly distributed under the circumference of the nuclear membrane (Figs. 1E & 3C). The average dimensions of the recently formed syszygy stages were 190 (±13.7) x 117 (±14.4) µm. The mature gametocysts were mainly oval filled with numerous mature sporocysts (Figs. 2B & 3D). Generally, the gametocysts measured 200 - 219 x 112 - 132 µm, with an average of 208.9 (±9.8) x 125.1 (±4) µm.

Sporocysts were navicular with a thickened flat region at the apical end measuring about 2 µm (Figs. 2C & 3E). Generally, the sporocysts measured 17 - 19.2

x 8.1 - 9 μm , with an average of 18.5 (± 0.6) x 8.5 (± 0.2) μm .

Ramadan (1969) described some acephaline gregarines isolated from different species of *Pheretima*, *P. californica*, *P. hawayana* and *P. elongata*, which are belonging to order Opisthopora, family Megascolecidae and two species of *Alma*, belonging to family Glossoscolecidae in Egypt. The host of this parasite under consideration, genus *Limnodrilus*, was belonging to family Tubificidae, so the gregarines parasitizing genus *Alma* could be comparable with our parasite recorded in genus *Limnodrilus*.

Ramadan (1969) reported two species of *Apolocystis*, *A. almanili* and *A. centrospora* from the seminal vesicles of two *Alma* species.

According to Table 1, the measurements of the trophozoites and their nuclei belonging to *A. almanili* were slightly larger than those of corresponding stages in the parasite under investigation. Ramadan (1969) described the paraglycogen granules as following: "The preparations stained with iron-alum haematoxylin the endoplasmic granules take a faint green color, which is a characteristic feature of this parasite" this feature was not observed in the endoplasm of the parasite under question.

Table 1: Comparison between the species of *Apolocystis* infecting *Alma* sp. and *Limnodrilus* sp. in Egypt.

Parasite	Host and Localities	Measurements					Reference
		Trophozoite	Nucleus	Karyosome	Gametocysts	Sporocysts	
<i>Apolocystis almanili</i>	<i>Alma nilotica</i> Nile River banks	90 μm - 160 μm (134.5 μm)	25 μm - 35 μm No fixed position	8 μm -10 μm	130 μm - 170 μm	(3) navicular types: a) Flattened ends 15-20 μm x 7.5 μm b) Pointed ends 12 μm x 5 μm c) Flattened ends 10-15 μm x 4-6 μm	Ramadan 1969
<i>Apolocystis centrospora</i>	Unidentified Species of <i>Alma</i> sp. Abou-Rawash	190 μm -250 μm	25 μm -40 μm Eccentric	8 μm -10 μm	Round: 230 μm - 390 μm Oval: 250 - 280 μm x 200 μm	Broad navicular, blunt ends 10-11 μm x 5 μm Spores fill center of the cyst only	Ramadan 1969
<i>Apolocystis nephridii</i> n.sp.	<i>Limnodrilus</i> Nile River banks of Maghaga (Menia)	Pear, kidney, oval shape: 75-85 μm x 81-124 μm 7 $^{\circ}$ (± 3.7) x 92.7(± 24)	19 μm -24 μm 22.4 μm (± 2.1) Eccentric	8.4 μm -11.2 μm 9.9 μm (± 1)	Oval 200-209 x 112-132 20 $^{\circ}$.9(± 7.8) x 125(± 5)	rounded end 17-19.2 μm x 8.1-9 μm 18.5(± 0.6) x 8.5(± 0.2)	Present work

In fresh state, the nucleus of *A. nephredii*, exhibited a discontinuous layer of dot-like chromatin more or less regularly distributed under the nuclear membrane, while in stained materials there is an irregular chromatin layer under the nuclear membrane with thin strands of chromatin spreading toward the karyosome until fused around the karyosome. In the description of *A. almanili*, Ramadan (1969) also stated that the nucleoplasm contains scattered fine chromatin granules except at the small area around the karyosome that is clear, unstained and devoid of any granulation.

The size of the gametocyst in *A. almanili* was markedly smaller than that of the present parasite. Besides, Ramadan reported a high degree of shrinkage of the gamonts after encystment leading to a wide space left between gamonts and the cyst wall, this space may reach about 50 μm . This aspect was not observed in early gametocyst of the current parasite.

In *Apolocystis almanili*, Ramadan described three types of navicular sporocysts. In the parasite under question, only one type of sporocysts was reported with thickened flat ends measured 17-19.2 x 8.1-9 μm , with an average of 18.5 (± 0.6) x

8.5 (± 0.2) μm which is closely related to the first type of Ramadan's gregarine.

Apolocystis centrospora differs from the present Egyptian monocystid in some aspects; *A. centrospora* had markedly smaller sporocysts. Besides, they, as described by Ramadan (1969), were filling the centers of their gametocysts only. Ramadan (1969) reported two forms of gametocysts of *A. centrospora*; a round form and an ovoid form. The present parasite resembled the last form but was slightly smaller in size than that of corresponding

ovoid form of Ramadan's work. Finally, the size of the trophozoites of *A. centrospora* was twice in size, that of corresponding stages in the present parasite. (Table 1).

Among the previously known species of *Apolocystis*, Table 2 shows species fall in the range of size of the species under consideration. In *A. beaufortii*, the gamonts produce two morphologically distinguishable types of gametes (anisogametes). The sporocysts are biconical with short flat plugs and are markedly smaller than those of the parasite of *Limnodrilus* sp.

Table 2: Comparison between the species of *Apolocystis* fall in the range of size of the species under consideration.

Parasite	Organ, Host	Trophozoite	Nucleus	Gametocysts	Sporocysts	Reference
<i>Apolocystis beaufortii</i>	seminal vesicles of <i>Pheretima</i> (<i>Parapheretima</i>) <i>beaufortii</i>	up to 55 μm	----	-----	with short flat plugs 6 x 2.5 μm	Cognetti de Martiis, 1923
<i>A. chotonagpurensis</i>	seminal vesicles of <i>Amyntas robusta</i>	up to 83 μm	---	71-96 μm (77 \pm 9) x 46-83 μm (62 \pm 15)	6.5-7 μm (6 \pm 1) x 3.5-4 μm (3 \pm 0.1)	Bandyopadhyay, Roychoudhuri & Biswas, 2004
<i>A. dudichi</i>	seminal vesicles of <i>Dendrobaena platyura</i> and <i>Fitzingeria platyura</i>	80-150 μm x 70-120 μm	---	120 μm x 100 μm	10-11 μm x 4.5-5.5 μm	Bereczky, 1967
<i>A. granulata</i>	seminal vesicles of <i>Allolobophora chlorotica</i> and <i>Allolobophora gigas</i>	110 μm	50 μm	----	with round ends and reach 20 μm in long	Tuzet & Loubatieres, 1946
<i>A. saigonensis</i>	seminal vesicles of <i>Pheretima peguana</i>	up to 80 μm	---	120 μm	with pointed ends 9 μm x 4 μm	Frolov, 1991
<i>A. minima</i>	seminal vesicles of <i>Pheretima postuma</i>	up to 60 μm	8 μm	100 μm	10.5 long	Frolov, 1991
<i>A. monokaryoseminiferus</i>	seminal vesicles of <i>Amyntas robusta</i>	61-115 μm	----	-----	-----	Pradhan and Dasgupta (1983)
<i>Apolocystis nephridii</i> n.sp.	<i>Limnodrilus</i> Banks of Nile Maghaga (Menia)	Pear, kidney, oval shape: 75-85 μm x 81-124 μm 71 (\pm 13.7) x 92.7 (\pm 24)	19 μm -24 μm 22.4 μm (\pm 2.1) Eccentric	Oval 200-209 x 112-132 208.9 (\pm 9.8) x 125 (\pm 1)	rounded end 17-19.2 μm x 8.1-9 μm 18.5 (\pm 0.6) x 8.5 (\pm 0.2)	Present work

In *A. chotonagpurensis*, trophozoites characterized by very fine rows of cytoplasmic ridges, the gametocysts are ellipsoidal and mainly smaller, in addition, the sporocysts are shorter and narrower than those of the parasite of *Limnodrilus* sp. in the present work.

A. dudichi, has smaller gametocysts and sporocysts. Pizl (1989) described trophozoite of *A. dudichi* with paraglycogen granules which may reach 2 μm long by 1 μm wide.

A. granulata, the trophozoites have larger nuclei that may reach 50 μm . Bereczky (1967) recorded the presence of alveoli into which paraglycogen granules are located that cannot be observed in present parasite.

In *A. saigonensis*, the gametocysts are smaller in size than those of our parasite. The sporocysts are smaller with pointed ends.

Trophozoites of *A. minima*, have small nucleus (8 μm), the paraglycogen granules reach 3.2 μm in diameter. Boisson (1957) revealed to the presence of anisogametes in the life cycle of *A. minima*. The gametocysts and sporocysts are smaller in size.

Pradhan and Dasgupta (1983) described *A. monokaryoseminiferus* that collected from the seminal vesicles of *Amyntas robusta*, its trophozoites measured from 61-115 μm , the other stages of the life cycle were not described.

Only one species, belonging to *Apolocystis* which was mainly recorded in the coelom and occasionally in nephredia, *Apolocystis michaelsoni* Hesse, 1909 (Cognetti de Martiis 1923). The trophozoites

are spherical or ovoid (up to 225 or 295 x 230 μm), have dark opaque appearance resulting from their content of "voluminous" granules, and their cytoplasm shows certain staining irregularities. The gametocysts of two gamonts are ellipsoidal (235-300 x 170-220 μm) and abundant, cysts containing one or three individuals. The mature gametocyst contains up to 16 sporocysts. The sporocysts of *A. michaelsoni* are strongly swollen in the middle and are slightly smaller [15 x 9 μm] than those of the parasite of *Limnodrilus* sp. [17-19.2 x 8.1-9 μm] in the present work.

The present species of *Apolocystis* is considered, universally, the second record of a gregarine, belonging to family Monocystidae (after) which is parasitizing, *Limnodrilus* sp. (Family: Tubificidae) and locally, the first record in Egypt. It should be stated that, according to Frolov (1991), Janiszewska (1968) was the first one who described first monocystid gregarine, *Zygocystis limnodrili*, from the vesicula seminalis of *Limnodrilus hoffmeisteri*. However, the present species is the unique species of *Apolocystis* which complete all life stages within a specific organ other than seminal vesicles, namely; nephredia.

Thus, it is clear from the above discussion that the gregarine recorded from *Limnodrilus* sp. is the first species of *Apolocystis* which infects this genus of the

host worm. However, the present authors recommend giving a name to this new species of *Apolocystis* as *A. nephredii* n. sp.

TAXONOMIC SUMMARY

Description: Trophozoites are pear, kidney or oval in shape, with an average of 7^a (± 3.7) x 92.7 (± 24) μm . The rounded nucleus has a single karyosome and measures 19 to 24 μm in diameter, with an average of 22.4 (± 2.1) μm . Gametocysts are ellipsoidal, with an average of 205.9 (± 3.8) x 125 (± 4) μm . Sporocysts are navicular with flat thickened ends with an average of 18.5 (± 0.6) x 8.5 (± 0.2) μm .

Type hosts: *Limnodrilus* sp.

Site of infection: Nephredia

Type localities: Maghaga region (Menia Governorate), Egypt, 28°39'00.8"N 30°50'35.1"E and Abo-Rawash region (Giza Governorate), Egypt. & 30°02'45.6"N 31°05'34"E

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

Type material: Holotype 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 localization within the host.

Legend of Figures

Figure 1:

- A:** A photomicrograph of the middle region of an infected worm showing the host's nephredia (arrowhead) highly infected with different stages of the parasite. Fresh preparation.
- B:** A photomicrograph of a section of young trophozoite showing chromatin-accumulation (arrowhead) under the nuclear membrane (Nm.) and central karyosome (K.). Haematoxylin and eosin.
- C:** A photomicrograph of a section of a young trophozoite showing the chromatin vacuoles (arrowhead) inside the karyosome in one complete peripheral ring. Giemsa's stain.
- D:** A photomicrograph of a section of trophozoite showing the irregularly accumulated chromatin vacuoles (arrowhead) in the peripheral region of the karyosome. Giemsa's stain.
- E:** A photomicrograph of a trophozoite (T) inside intentionally ruptured nephredium (Nf.), Note: chromatin granules (arrowhead) under nuclear membrane. Fresh preparation.
- F:** A photomicrograph of a fully developed trophozoite, Haematoxylin and eosin.

Figure 2:

- A:** A photomicrograph of a complete syzygy of trophozoites showing the hemispherical shape of gamonts. Heidenhain's haematoxylin stain.
- B:** A photomicrograph of a gametocyst. Fresh preparation.
- C:** A photomicrograph of mature sporocysts showing thickened flat regions in the apical ends (arrowhead) of the sporocyst. Fresh preparation.

Figure 3:

Drawing of different stages of *Apolocystis nephredii* sp. nov.

- A:** Enlarged nucleus of young trophozoite showing the vacuoles of different sizes inside karyosome
- B:** Adult trophozoite showing its general shape.
- C:** Nuclear details in an adult trophozoite showing a continuous layer of chromatin (arrowhead) under nuclear membrane (Nm).
- D:** A gametocyst crowded by navicular sporocysts.
- E:** A single sporocyst showing thickened flat region at the apical end (arrowhead).

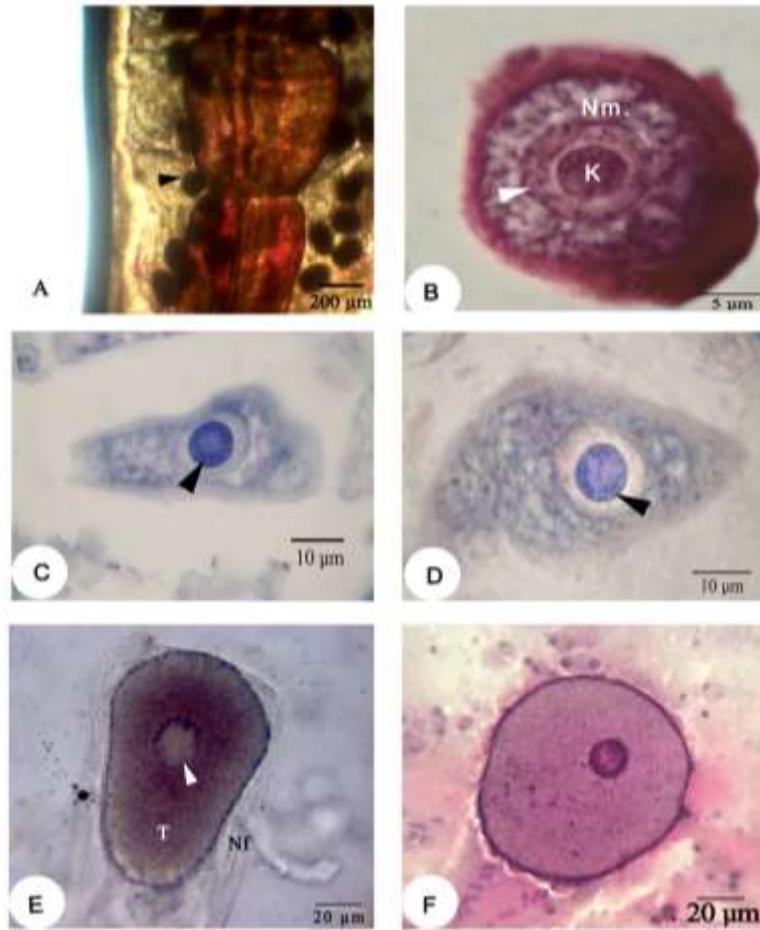


Figure (1)

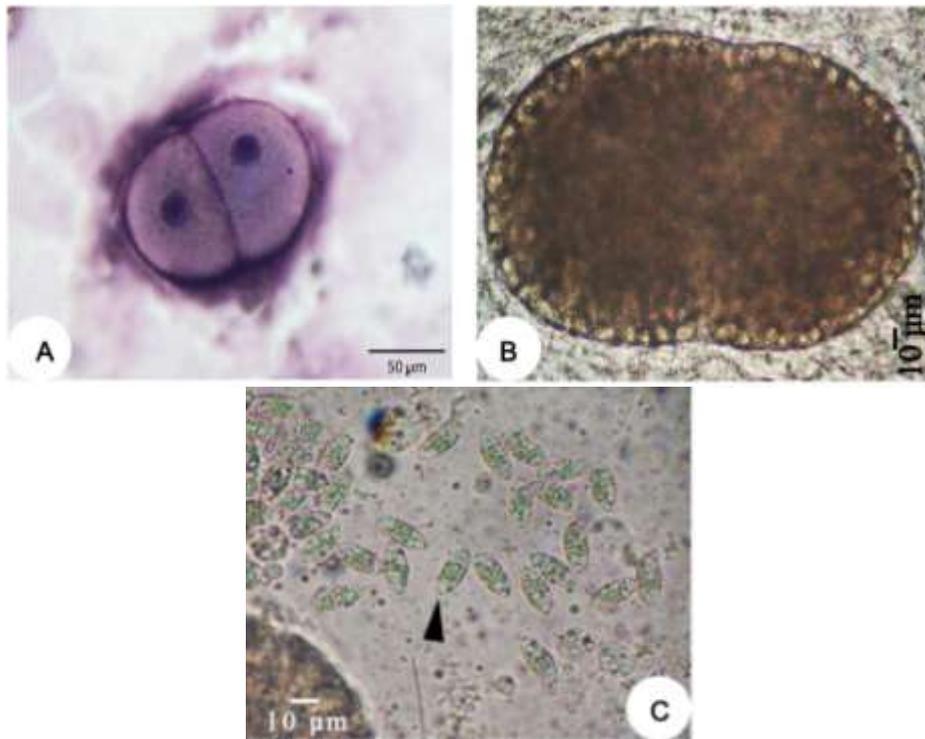


Figure (2)

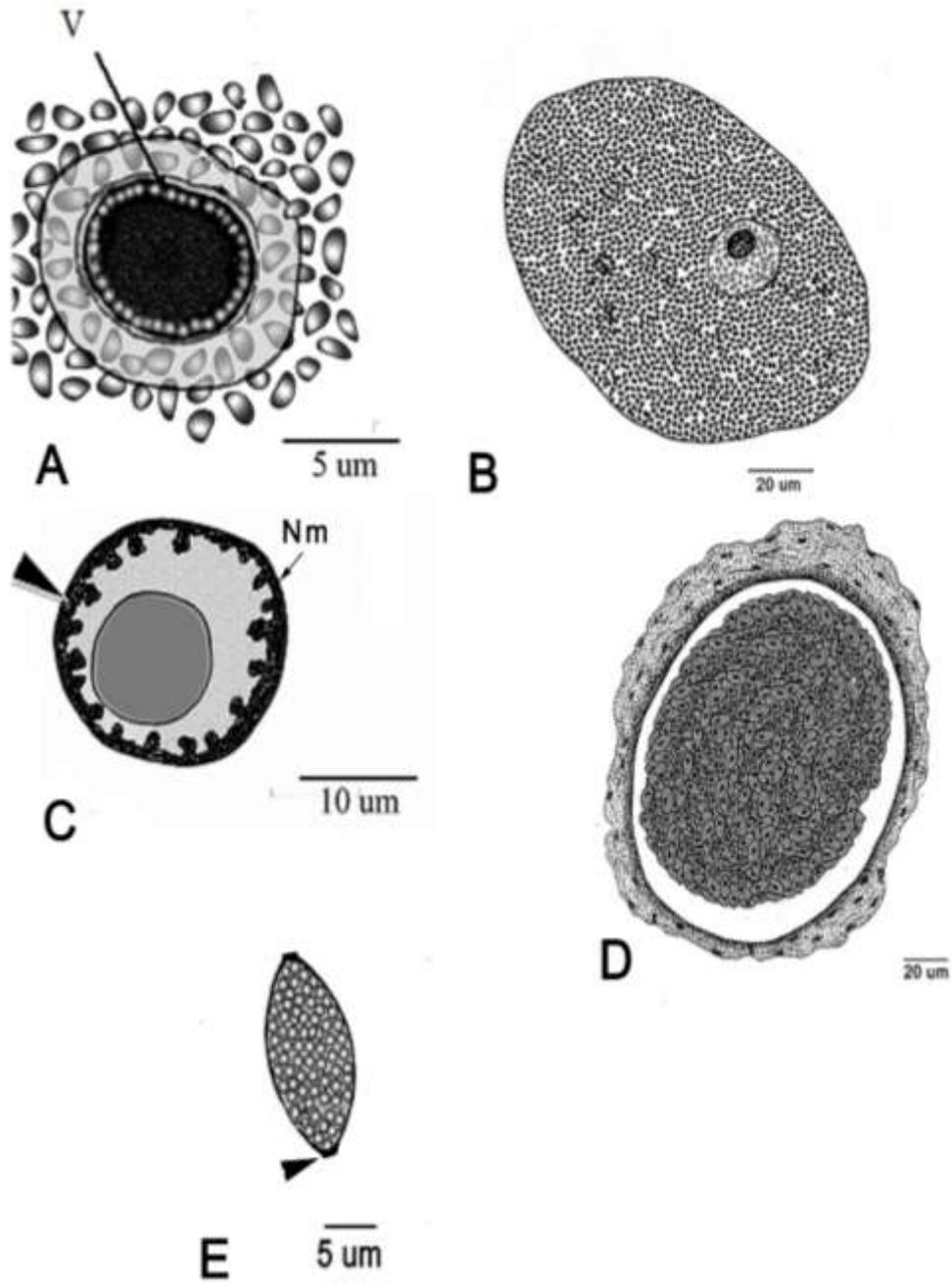


Figure (3)

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ARABIC SUMMERY

أبولوسيتس نفيدي (معقدات القمة: مونوسيتيني) نوع جديد من الجريجاريينات عديمات الرؤوس من نوع الليمنودرلس (الحلقيات: قليات الأشواك) من مصر

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تم وصف نوع جديد من طفيليات الجريجاريينات عديمة الرؤوس - أبولوسيتس نفيدي - من نفيدي دودة الأرض من نوع الليمنودرلس. ومراحل دورة حياة الطفيلي تشمل الأطوار المغذية التي تتراوح أطوالها بين ٨١ و ١٢٤ ميكرون وعرضها بين ٧٥ و ٨٥ ميكرون، بمتوسط $(92.7 \pm 24) \times$ (± 3.7) 79 ميكرون. وأكياس الأمشاج التي تشبه القطع الناقص تتراوح أطوالها بين ٢٠٠ و ٢٠٩ ميكرون وعرضها بين ١١٢ و ١٣٢ ميكرون بمتوسط قدره $205.9 (\pm 3.8) \times 125 (\pm 4)$ ميكرون. أما أكياس الأبواغ فهي زورقية الشكل ولها نهايات دائرية ويتراوح قياسها بين ١٧ - ١٩,٢ ميكرون $\times 8,1 - 9$ ميكرون بمتوسط قدره $18,5 (\pm 0,6) \times 8,5 (\pm 0,2)$ ميكرون.