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MOLECULAR IDENTIFICATION AND AN ULTRASTRUCTURE STUDY OF *HYMENOLEPIS DIMINUTA* MATURE SPERMATOZOON: NEW DATA OF PHYLOGENETIC IMPORTANCE

By

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Abstract

Integrating biological, morphological and molecular data is necessary for the phylogeny of Platyhelminthes. Besides the molecular tools, the ultrastructural description of the mature spermatozoon was found a valuable tool for this purpose. Among the studied cestodes, description of *Hymenolepis diminuta* spermatozoon has been neglected; therefore, this study was designed. Live specimens of *Hymenolepis* spp. were isolated from naturally infected rats and molecular sequencing of the internal transcribed spacer (ITS2) region of the major ribosomal RNA genes was performed and a phylogenetic tree was constructed. This analysis revealed a 99–100% identity of the isolated worm as *H. diminuta* which appeared distinctive in a subclade while *H. nana* and *H. microstoma* appeared more closely related in another subclade. After the molecular identification of the worm, an ultrastructure study was performed. This might be the first ultrastructure characterization of a parasitic Platyhelminth spermatozoon in which a molecular identification tool was utilized. The results revealed a filiform cell with a single axoneme of 9 + "1" pattern extending from the anterior to the posterior extremity, a spiraled cord-like nucleus situated posteriorly associated with a large amount of glycogen, spiraled cortical microtubules at an angle of 20-25º then they became parallel to the axoneme at the posterior extremity. For the first time, this report six crested-like bodies at the anterior extremity of *H. diminuta* mature spermatozoon. These crested bodies enabled to clarify spermatozoon orientation and provided a full schematic reconstruction of it.

Keywords: *Hymenolepis diminuta,* spermatozoon ultrastructure, molecular sequencing, ITS2, phylogenetic, crested bodies

Introduction

Hymenolepis diminuta and *H. nana* are the causative tapeworms of a worldwide neglected zoonotic disease. But, *H. nana* is the main cause of human hymenolepiasis due to man to man spread while human infections with *H. diminuta* are less reported as it requires an accidental ingestion of the cysticercoid larvae in haemocoel of an arthropod intermediate host; *Tribolium* spp. and *Tenebrio* spp. (Nkouawa *et al*, 2016). However, *H. diminuta* gains more importance good model species in modern molecular studies (Rozario and Newmark, 2015) due to its ease to culture within the laboratory conditions. Also, *H. diminuta* is a parasite of both man and rodents; thus, studying the adult parasite in a rodent model revealed the mechanisms involved in interactions and adaptation between adult tapeworm and their vertebrate host (Sulima *et al*, 2018).

Hymenolepis diminuta belongs to the family Hymenolepididae which is a diverse group of tapeworms comprising 620 species in birds and 230 in mammals. Since the molecular phylogenetic studies on the relationships within the family were primitive (Haukisalmi *et al*, 2010), these species was assigned to many genera based on their morphological traits (Czaplinski and Vaucher, 1994). This tapeworm was first discovered in the brown rat, *Rattus norvegicus*, and then several species of Eurasian field mice, *Apodemus spp*. were identified as other definitive hosts (Makarikov and Tkach, 2013). Thereafter, many cryptic *Hymenolepis* species from the same definitive rodent hosts were discovered and made the identification and taxonomy of *H. diminuta* problematic issues (Haukisalmi *et al*, 2010). Besides, solving the puzzling taxonomy, the precise discrimination of the parasites is also required for

their management and control (Pérez-Ponce de León and Poulin, 2018). In this concern, the description of ultrastructural characters of the mature spermatozoon has been found a valuable tool for the discrimination and the phylogeny within Platyhelminthes and cestodes in particular (Justine, 2001; Miquel *et al*, 2007; Levron *et al*, 2010). But, to obtain more accurate information about the phylogeny and evolution of the tapeworms, it was necessary to integrate the biological, morphological and molecular data (Waeschenbach *et al*, 2012). In this context, sequencing of the internal transcribed spacer (ITS2) region of the major ribosomal RNA genes was considered as a powerful tool in resolving taxonomic questions and in closely discriminating related genera and species (Hershkovitz and Lewis, 1996).

As to the ultrastructure of cestodes spermatozoon, several studies were published in the last few decades. These studies focused on order Cyclophyllidea and described the spermatozoon of many species (Miquel *et al*, 2015). Nonetheless, studying the ultrastructure *H. diminuta* spermatozoon was neglected and the available data were obtained by Rosario (1964); Kelsoe *et al* (1977) and Robinson and Bogitsh (1978) who did not provide a full description of *H. diminuta* mature spermatozoon.

This study aimed to concern the molecular identification of *H. diminuta* followed by an ultrastructure study to provide an updated description of its mature spermatozoon to add some required data for the taxonomic and phylogenetic purposes.

Material and methods

Live specimens of *Hymenolepis* spp. were isolated from the intestine of naturally infected rats in Menoufia Governorate, Egypt. After dissection, the recovered adult tapeworms were rinsed with a sterile physiological solution (0.9% NaCl, w/v). Samples of the isolated worms were immediately fixed in absolute ethanol and kept at -80ºC until the molecular identification by DNA sequencing technique. Other samples were fixed in cold (4°C) 2.5% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.2) for the ultrastructure study by TEM.

Molecular identification: DNA extraction: From each absolute ethanol-preserved *Hymenolepis* spp. sample, a 15mg tissue piece was excised and placed in a 1.5mL eppendorf tube containing 5% Chelex® 100 sodium form (Sigma-Aldrich, Madrid, Spain) in TE buffer (pH 8) according to the protocol described (Estoup *et al*, 1996). 2.4 U of Proteinase K (Thermo-Fisher, USA) was added. The sample was incubated at 55ºC with shaking at 30min intervals for 2hrs, boiled in a 100ºC dry bath for 20 minutes and finally stored at 4ºC until the target genes were amplified by PCR followed by the forensically informative nucleotide sequencing (FINS).

PCR amplification for ITS2 gene: PCR amplification of the internal transcribed spacer (ITS2) region of the major ribosomal RNA genes (18+28S rDNA) was carried out using universal eukaryotic primers according to the method described by White *et al*. (1990). The amplification reaction was performed in a total volume of 25µL, in which 2µL of total genomic DNA, 0.4µM of each primer (ITS2:5'-GCATCGATGAAGAA CG CAGC-3´; ITS4:5'-TCCTCCGCTTATTGA TATGC-3´), & 200ngμL^{-1} of bovine serum albumin (BSA) were used. PCR was performed using MyTaq red master mix (Bioline, London, UK) according to the manufacturer's instructions. PCR conditions included an initial denaturation at 95ºC for 5min, 35 cycles of amplification (95°C for 30s, 48°C for 30s, & 72°C for 35s), followed by a final extension step for 7 min at 72ºC. Finally, 2µL of the PCR product were electrophoresed in 2% Agarose gel, containing 0.5µg mL^{-1} of ethidium bromide. PCR samples with adequate size bands (~750 base pairs) and intensity were sent to MACROGEN Inc. (Seoul, South Korea) for the standard Sanger sequencing.

Sequences analyses: ITS2 sequence was reviewed and manually trimmed whenever necessary. The edited sequence was compared

to archived reference sequences in GenBank using BLAST algorithm (https://blast.ncbi.nlm.nih.gov). ITS2 sequences from phylogenetically related and distant cestodes were retrieved from GenBank database to assess the phylogenetic identification. These species were *H. diminuta* (accession numbers: AB494475.1, KC990409.1, KY079339.1), *H. microstoma* (AB494478.1), *H. nana* (HM536187.1), *Anoplocephala perfoliata* (AJ578153.1), *Arostrilepis macrocirrosa* (HM561423.1) and *Taenia hydatigena* (FJ886761.1). ITS2 sequence of *Hymenolepis* spp. obtained in the current study and others retrieved from GenBank were aligned using CLUSTALW integrated with MEGA 7.0.14 software program. To determine the species taxonomic relations with other members of the family Hymenolepididae, a maximum likelihood phylogenetic tree was constructed after selection of the best substitution model using Model Test algorithm appended to the MEGA7 software and applying 1,000 bootstraps as replicates.

Ultrastructure examination by TEM: From the specimens confirmed as *H. diminuta* by molecular sequencing (Roy *et al*, 2012), small pieces of mature proglottids were post fixed in 1% OsO_4 at 4ºC for 2hr followed by dehydration in an acetone series then, embedded in Spurr's epoxy resin. Semi-thin sections were prepared, stained with 1% methylene blue in borax solution. Serials of ultrathin sections were prepared for TEM, placed on copper grids, and double stained with uranyl acetate and lead citrate. Sections were examined under JEOL JEM 1230 TEM operated at 80 kV.

Results

Molecular identification: Amplification of ITS2 region resulted in a 750bp DNA sequence. After manual checking and correction, the resulting fragment of 680bp was subjected to further identification by FINS and BLAST. Comparing the obtained sequence with other cestode sequences chosen from the GenBank database showed 99-100% sequence identity with *H. diminuta* (acc. No. KY079339.1, AB494475.1). FINS exhibited that the obtained ITS2 sequence of the Menoufia sample of *H. diminuta* clus-tered with other sequences of *H. diminuta* in a single subclade. The subclade was separated from the other one containing *H. nana* & *H. microstoma* which appeared closely related and distinctive from *H. diminuta* (Fig. 1).

Ultrastructure examination by TEM: The testes of *H. diminuta* mature proglottids showed different cell types. Spermatogonia was identified by its relatively small size, condensed irregular nucleus and electron dense cytoplasm. The cells formed a syncytial rosette (Fig. 2a). The spermatocytes were recognized as large cells with large nuclei and lucent cytoplasm (Fig. 2b). Cells were inside the testicular lobules enclosed with a cytoplasmic membrane forming a lattice-like of microtubular array (Fig. 2c). Spermatocytes undergoing division were with many nuclear fragments and developed spermatozoa attached to the cytoplasmic membrane (Fig. 2a). After release of a huge number of spermatozoa; spermatocytes resi-dual bodies were detected in testes (Fig. 2d).

Multiple cross and longitudinal sections of male reproductive organs of *H. diminuta* were examined. Interpretation showed that the mature spermatozoon was a filiform cell with tapering extremities without mitochondria. Each spermatozoon has a single axoneme, a spiraled nucleus and twisted cortical microtubules (Fig. 3). Anterior part cylindrical and thin while the transitional and posterior part was broader with cytoplasmic extensions giving blade-like shape in cross-sections. Each spermatozoon body possesses five regions with distinctive characters.

Region I (Fig. 4, 6a, 7) Anterior of spermatozoon with a diameter of 0.1-0.2µm, cytoplasm with a single axoneme of 9+"1" pattern of Trepaxonematan Platyhelminthes (Ehlers, 1984). Axoneme has a central core unit surrounded with nine doublet microtubules without a periaxonemal sheath (Fig. 2b). There were 6 electron-dense thin cords, less than 6nm in thickness of crested-like

bodies running with cortical microtubules for a short distance. Crested bodies gave the anterior spermatozoon extremity a rosette shape in its cut sections (Fig.4).

Region II (Fig. 5, 6, 7) Polymorphic with rounded, ovoid, comma-shaped and blade-like in cut sections, with diameter of 0.2-0.4 µm. Cytoplasm was lucent with fine electron-dense granules with an axoneme and spiraled cortical microtubules at an angle of about 20-25º with long axis of spermatozoon.

Region III (Fig. 6, 7) Average diameter 0.3-0.5µm. with a condensed nucleus. As the nucleus was twisted around the axoneme, they alternated their places; so, in some cut sections axoneme was eccentric and central nucleus and the reverse was in other sections. Nucleus was compact, rounded or kidney-shaped in cross sections while crescent or annular nuclei were seldom seen. Cortical microtubules were spiraled and the cytoplasm was lucent with fine electron-dense granules.

Region IV (Fig. 6, 7) Cytoplasm with electron-dense rich in glycogen, an axoneme with rounded or kidney-shaped nucleus and spiraled cortical microtubules. This was the broadest region with a diameter of 0.3µm in the distal part and 0.7µm in proximal part.

Region V (Fig. 5, 7) Posterior part of spermatozoon with an average diameter of 0.1–0.2µm. It was similar to region I in structure but without crested bodies and characterized by electron-dense cytoplasm, axoneme extended to posterior spermatozoon extremity and the cortical microtubules run parallel to the axoneme at the posterior spermatozoon extremity (Fig. 3b).

Table 1: Ultrastructure characters of mature spermatozoa of Hymenolepididae

Genus species	AC	Cb	Cm	G	N		Pse	References
					pattern	Shape		
Retinometra serrata	+	6	25º	+	Straight	Kidney	Dense cone	Bâ and Marchand (1993)
Hymenolepis diminuta	-	6[a]	20-25º[a]	+	Spiraled	*Kidney*	Ax[a]	Rosario (1964); Kelsoe *et al*. (1977); Robinson and Bogitsh (1978); current one[a]
Hymenolepis(vampirolepis) microstoma	?	6	20-30º	+	Spiraled	Crescent	Ax	Bâ and Marchand (1998)
Hymenolepis straminea	+	8	30º	+	Spiraled	Crescent	G	Bâ and Marchand (1996)
Hymenolepis nana	-	12	15º	+	Spiraled	Crescent	G	Bâ and Marchand (1992)

Ac apical cone, *Cb* crested body, *Cm* angle of cortical microtubules, *G* electron-dense granules, *N* nucleus, *Pse* posterior spermatozoon extremity. -/+ presence/absence? Unknown data, [a] indicates data in present study

Discussion

In the present study, phylogenetic analysis revealed that the identified Menoufia isolate clustered with other sequences of *H. diminuta* in a single subclade. This subclade was separated from the other one containing *H. nana* and *H. microstoma* which was closely related to each other and distinctive from *H. diminuta.* This result agreed with Sharma *et al*. (2016) who reported the same relationships by sequencing of nuclear rDNA ITS2 gene.

The ultrastructure examination of *H. diminuta* testes revealed different cell types sharing in the process of spermiogenesis such as spermatogonia and spermatocytes. These cells were observed with the same characters described by Kelsoe *et al*. (1977). The spermatogonia formed a syncytial rosette. The spermatocytes were inside the testicular lobules surrounded by a cytoplasmic membrane which contains a lattice-like of microtubular array (micro-tubular organelle). Willms *et al*. (2003) described this organelle in an ultrastructure study on spermatogonia and spermatocytes lobules of *Taenia solium* and they suggested that these organelles were the possible site for the synthesis of the cortical microtubules of the developing spermatozoa. Similar to the observations by Kelsoe *et al*. (1977), in the present study, the large lucent nuclei of spermatocytes divided and gave numerous nuclear fragments of condensed chromatin with the development and the release of a huge number of spermatozoa leaving behind residual bodies in the testes.

The present study showed that the mature spermatozoon of *H. diminuta* is a filiform cell with tapering extremities, a single axoneme, a spiraled nucleus, twisted cortical microtubules and lacks mitochondria; thus, it followed the type V pattern according to Levorn *et al*. (2010) who categorized the spermatozoa of eucestodes into seven types according to their ultrastructure criteria such as the number of axonemes, the presence or absence of the apical cone, the crested-like bodies, periaxonemal sheath and parallel or spiralled cortical microtubules and nucleus.

In accordance with Robinson and Bogitsh (1978) the apical cone was never observed in *H. diminuta* mature spermatozoon in this study while it was reported in *Retinometra serrata* and *Hymenolepis straminea* (Bâ and Marchand, 1993; 1996). The cortical microtubules are spiralled in all the Cyclophyllidean spermatozoa and the angle of these microtubules with the long axis of the spermatozoon varies among the Hymenolepididae (Justine, 1991). In the present study, for the first time, the cortical microtubules angle of 20-25º and six crested-like bodies were reported in *H. diminuta* spermatozoon.

In accordance with Kelsoe *et al*. (1977) and Robinson and Bogitsh (1978), the present study revealed polymorphism of the cut sections in the different regions of the spermatozoon with the appearance of rounded, ovoid, comma and blade-like shaped sections with a rounded, ovoid or kidney-shaped nucleus.

The granular electron-dense material in the cytoplasm of spermatozoa was described in Hymenolepididae with a variable distribution in between species. For instance, in *H. nana*, *H. microstoma* (Bâ and Marchand, 1992, 1998) and *H. diminuta* in the current study, these granules exist in all the gamete regions except at the anterior extremity with crested-like bodies. While in *R. serrata* and *H. straminea* these granules are distributed all over the spermatozoa except the anterior and posterior extremities (Bâ and Marchand, 1993; 1996). Another feature of *H. diminuta* mature spermatozoon was the lucent appearance of the anterior spermatozoon part including regions I & II and the dark cytoplasm of the posterior part including regions IV & V which could be attributed to a large amount of cytoplasmic glycogen in posterior part. Robinson and Bogitsh (1978) found glycogen filling the area around the nucleus and the axoneme in the posterior part of *H. diminuta* spermatozoon. They assumed that glycogen acted as the source of energy required for spermatozoon motility in the absence of mitochondria.

In the present study, a close phylogenetic relationship between *H. nana* and *H. microstoma* was found while *H. diminuta* was in a distinctive clade (Fig. 1). Interestingly, on integrating these molecular results with the morphological features of Hymenolepididae spermatozoa, the closely related species within a single subclade showed morphological variations regarding the number of crested bodies, the apical cone, the angle of the cortical microtubules and the posterior spermatozoon extremity. On the other hand, the shape and pattern of the nucleus was of the same description within each subclade.

Regarding the *H. diminuta* spermatozoon orientation, the crested-like body (or bodies) in the cestode mature spermatozoon was (were) considered as a marker of the gamete anterior extremity. Thus, the extremity with crested bodies of *H. diminuta* spermatozoon corresponded to anterior end, and the lacking of crested-like bodies was the posterior end (Baˆ *et al*, 1991). In the current study, the cut sections with narrowest diameters were those with the crested bodies and those with the widest diameters were nucleated. Thus, the anterior portion of spermatozoon was thin. But, Kelsoe *et al*. (1977) and Robinson and Bogitsh (1978) reported that the mature spermatozoon anterior portion was broad and flattened and contained the nucleus, while most of the spermatozoon length was posterior to nuclear-axonemal complex with little cytoplasm and they assumed that the orientation of tapeworm sperm-

atozoon was similar to the mammalian sperm with anterior nucleus (Justine, 1998).

However, the orientation of *H. diminuta* spermatozoon appeared as a matter of controversy, it was fully resolved by the accumulation of many studies. For instance, Justine and Mattei (1982) studied the movement of the spermatozoa of a Digenea species and found that the axonemes moving at the anterior part and the nuclei were posteriorly situated. During the fertilization process of a Digenea species, the nucleus was the last part of the spermatozoon to enter the oocyte (Justine and Mattei, 1984). In a study on two Digenea species, visualization of tubulin by immunocytochemistry and nucleus by fluorescent labeling showed that the nucleus migration in the distal portion during spermatid elongation (Iomini *et al*, 1997; Iomini and Justine, 1997). Justine (1998) clarified the posterior position of the nucleus in the spermatozoa of the parasitic Platyhelminthes in general.

Conclusion

This might be the first study to perform a morphological ultrastructure characterization of the parasitic Platyhelminth spermatozoa after species identification by the molecular sequencing tool. The isolated worm identification by the molecular sequencing undoubtedly proved to be the *Hymenolepis diminuta*.

The ultra-structure study gave the specific description of six crested-like bodies at the anterior extremity of *H. diminuta* mature spermatozoon which enabled to clarify the spermatozoon orientation and provided a full schematic reconstruction of the spermatozoon for the first time. Also, the filiform spermatozoon was characterized with its spiraled cord-like nucleus situated posteriorly and associated with a large amount of glycogen, spiraled cortical microtubules at an angle of 20-25º in the entire length of the spermatozoon and then became parallel to the axoneme at the post-erior extremity. The molecular integration and the morphological features revealed the variations of some points such as the crested bodies and the angle of cortical microtubules within closely related species but, the shape and pattern of the nucleus varied among the genera.

Exyensive research work is ongoing to add more information with an emphasis on the Hymenolepididae and will be published in due time later elsewhere.

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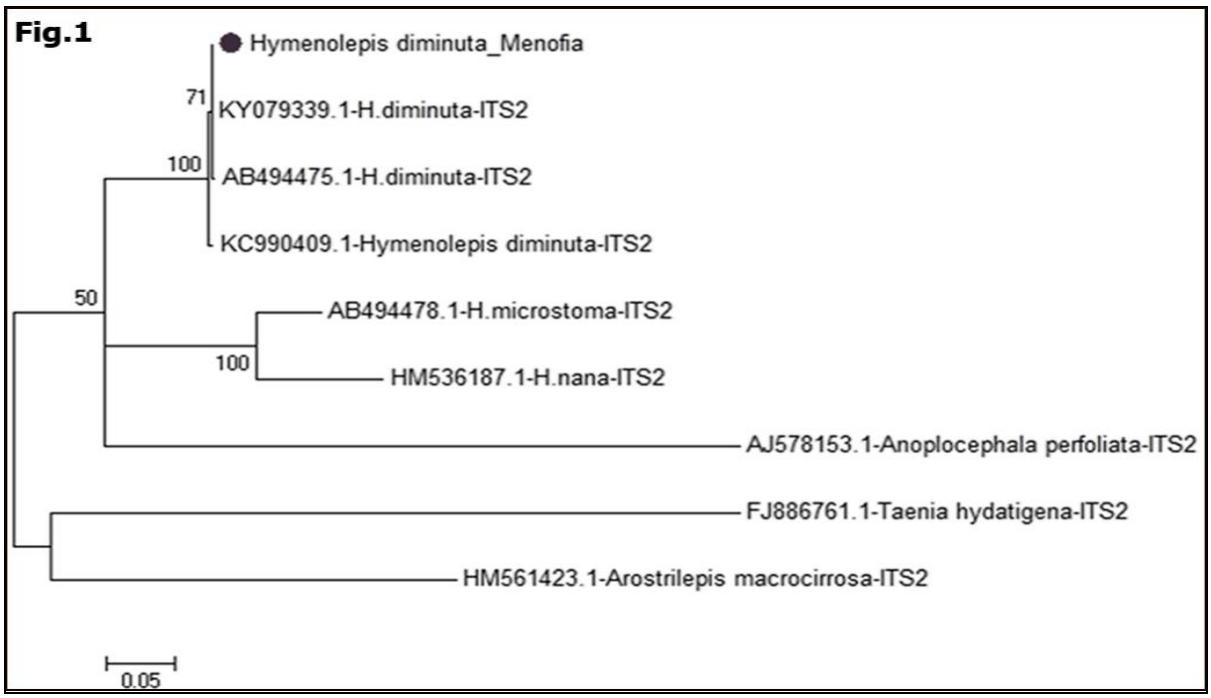
Explanation of figures

Fig.1: Midpoint-rooted phylogenetic tree of *Hymenolepis* spp. including Menoufia isolate. Tree made by maximum likelihood method using ITS2 region of major ribosomal RNA genes (18+28S rDNA). Values of main nodes bootstrap percentages after 1000 replicates. Scale bars represented estimated number of substitutions per nucleotide site.
Fig. 2: Micrographs of testes (T) of mature *H. diminuta* proglottids showing a) spermatid cluster (Spr) undergoing spermiogenesis and showing nuclear fragments (Nf), a sperm in a cytoplasmic pocket and attached to membrane (arrow) and numerous spermatozoa (Sp) transverse sections at different levels. At right side, five spermatogonia with large and condensed nuclei (N), 6000x. b, c) mature spermatocyte lobules surrounded with membranous envelopes (Env, arrow) with a microtubular organelle (Mto), glycogen granules (Gly) and numerous spermatozoa, 12000x. d) Residual body (Rb) in testes (T) with cut sections of mature spermatozoa (Sp), 12000x.
Fig. 3: a) L.S. of mature *H. diminuta* spermatozoa showing central axoneme (Ax), spiraled cuticular microtubules (Cm) and nuclei (N), 12000x. b) posterior sperm extremity (Pse) in cortical microtubules (Cm) run parallel to the axoneme (Ax), the electron dense granules (G) disappear near tip of spermatozoon, 50000x. Square represents structure of taxoneme of nine doublets centrioles (D) with a central core (9 +"1") in a transverse section, 150000x.
Fig. 4: a) T.S. in seminal receptacle showing numerous cut sections of *H. diminuta* spermatozoa at different levels. b) Magnification of red rectangle in a), each cut section at region I (red circle) shows an axoneme (Ax) surrounded with cortical microtubules and 6 small crested-like bodies (Cb) giving anterior sperm extremity a rosette shape, 12000x.
Fig. 5: Micrographs of *H. diminuta* spermatozoa transverse sections at regions II and V. a) Sections at region II in spermatozoa become flattened (blade like) with central axoneme (Ax) and spiraled cortical microtubules (Cm), 20000x. a, b) Transverse sections at region V with a characteristic electron dense cytoplasm. b, c) Circular, oval and comma shaped transverse sections at region II with a characteristic lucent cytoplasm, 30000x.
Fig. 6: Micrographs of *H. diminuta* spermatozoa transverse sections at different regions. a) Represents cut sections without crested bodies at region I, comma shaped section at region II and sections at region IV each with an eccentric axoneme (Ax), nucleus (N) which may be rounded or kidney shaped, spiraled cortical microtubules (Cm) and cytoplasm rich in glycogen (Gly), 30000x. b, c) Cut sections at region IV, (magnification of (b) 20000x and (c) 40000x). d) Cut sections at region II with spiraled cortical microtubules (Cm), eccentric axoneme (Ax) and electron dense granules, 60000x (G). e) Cut sections at region III with spiraled cortical microtubules (Cm), axoneme (Ax) and nucleus (N), 30000x.
Fig.7: A schematic reconstruction of *Hymenolepis diminuta* mature spermatozoon.



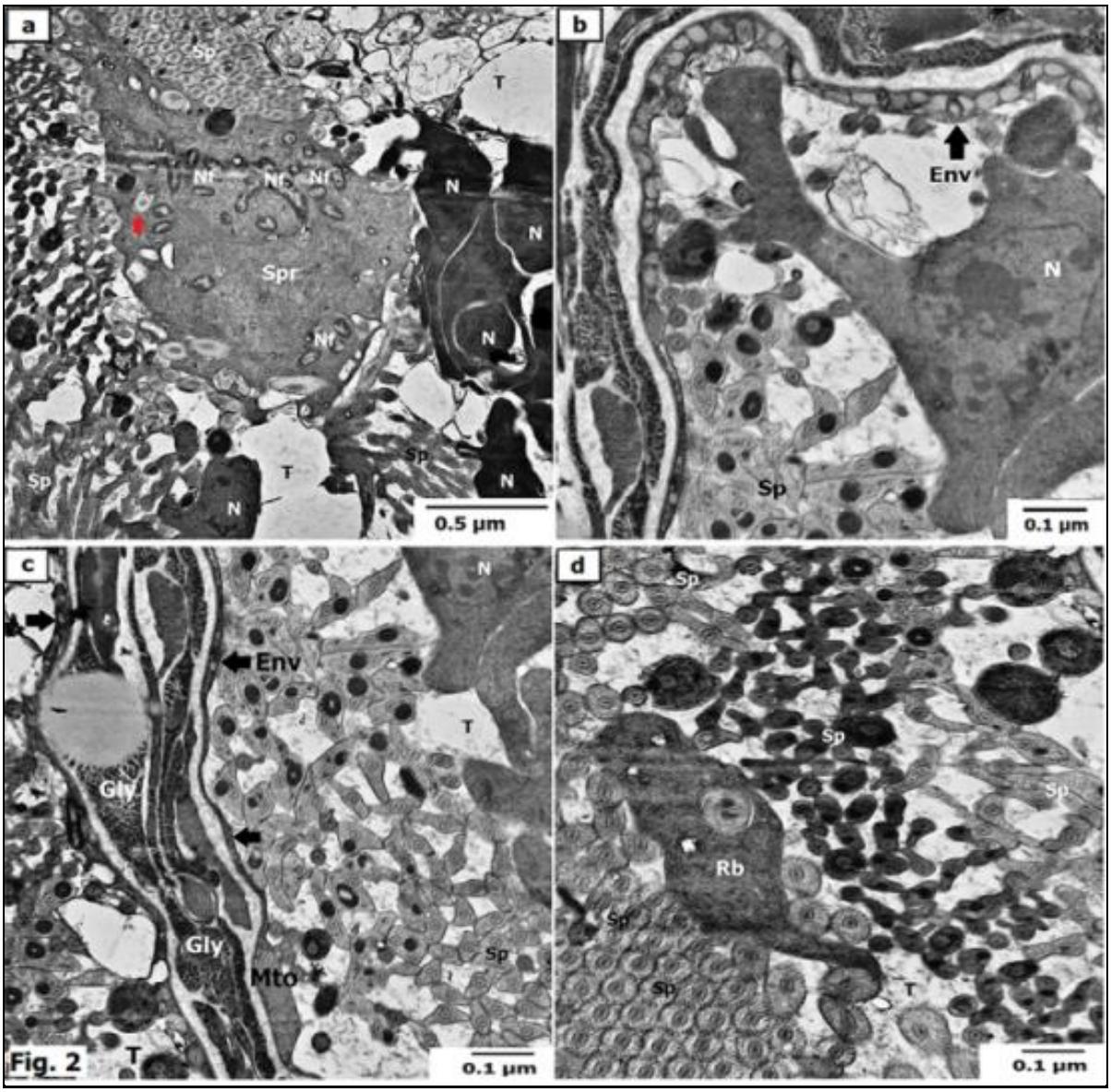

a
Sp
T
Nf
Nf
Nf
N
N
Spr
Nf
N
Sp
T
Sp
N
N
0.5 µm
b
Env
N
Sp
0.1 µm
c
N
Env
T
Gly
Gly
Mto
Sp
T
0.1 µm
d
Sp
Sp
Sp
Rb
Sp
Sp
T
0.1 µm

Fig. 2

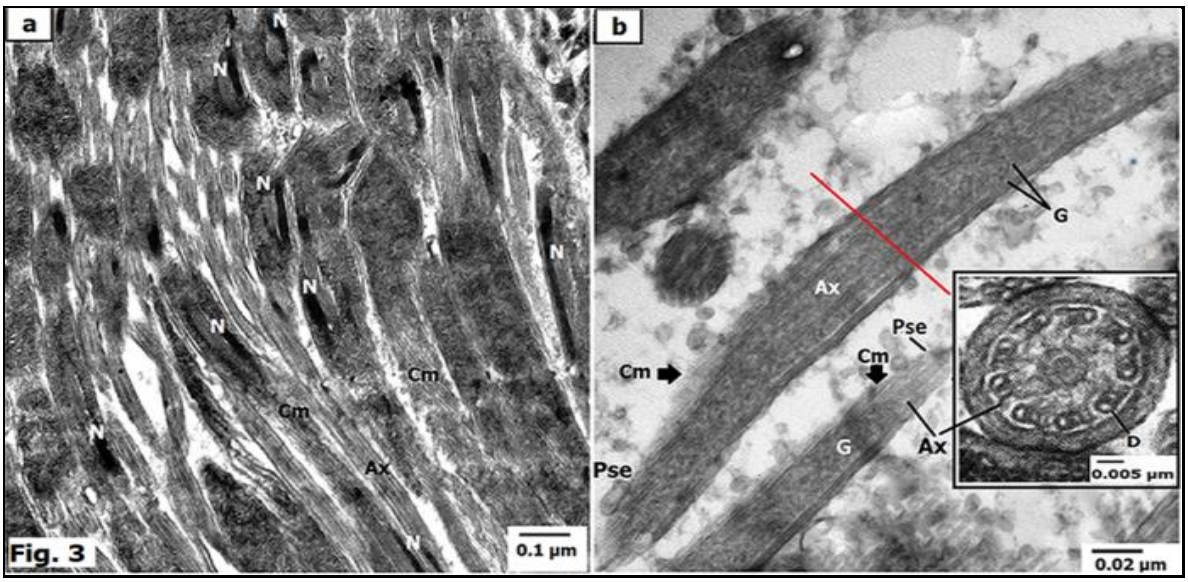
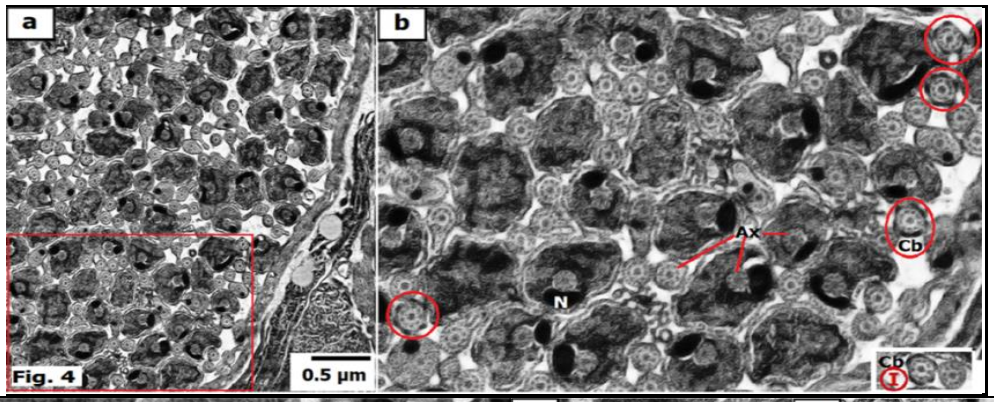
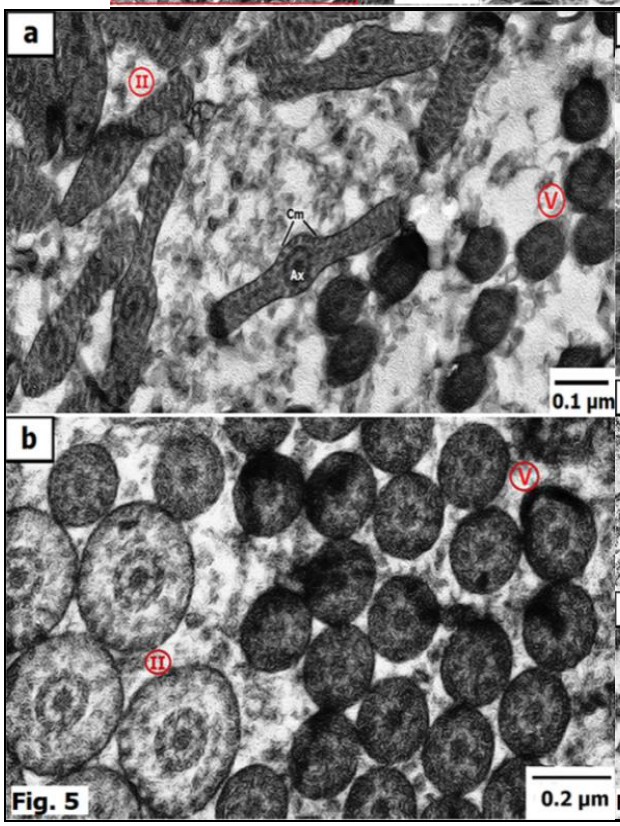
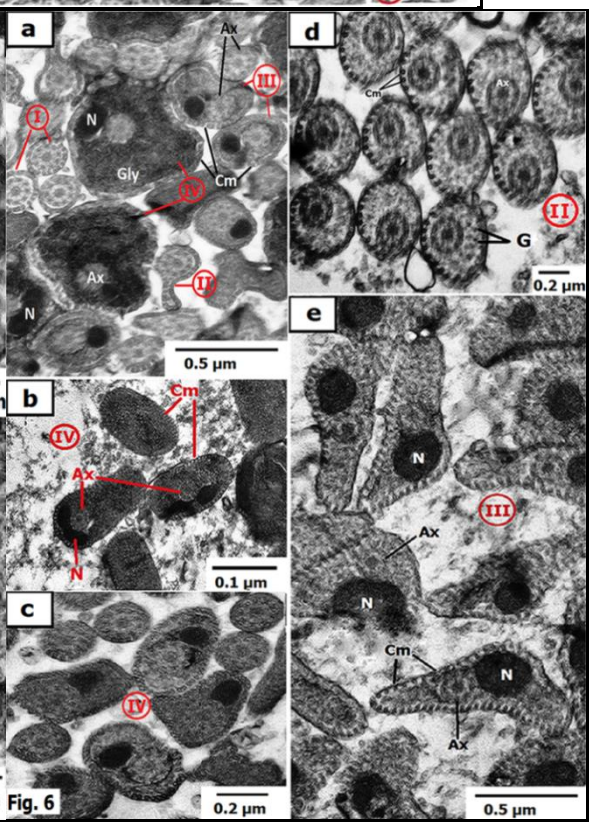
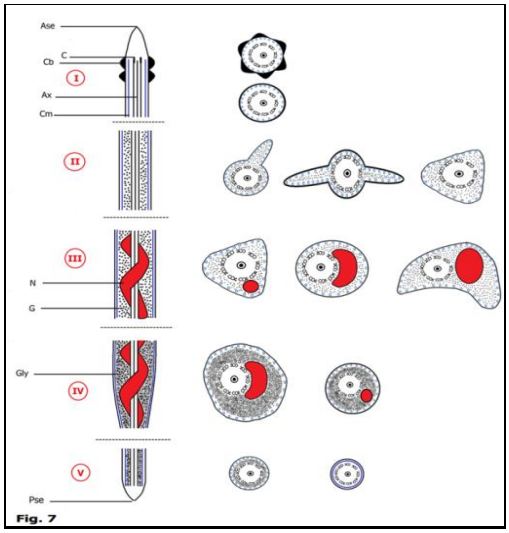

a
N
N
N
N
N
N
N
Cm
Cm
N
Ax
N
0.1 µm
b
G
Ax
Pse
Cm
Cm
G
Ax
Pse
D
0.005 µm
0.02 µm

Fig. 3


a
b
Ax
Cb
N
0.5 µm
Fig. 4


a
II
V
Cm
Ax
0.1 µm
b
V
II
0.2 µm
Fig. 5


a
Ax
III
I
N
Gly
IV
Cm
II
N
0.5 µm
b
Cm
IV
Ax
N
0.1 µm
c
IV
0.2 µm
Fig. 6
d
Cm
Ax
II
G
0.2 µm
e
N
III
Ax
N
Cm
N
Ax
0.5 µm


Ase
Cb
C
I
Ax
Cm
II
III
N
G
Gly
IV
V
Pse
Fig. 7