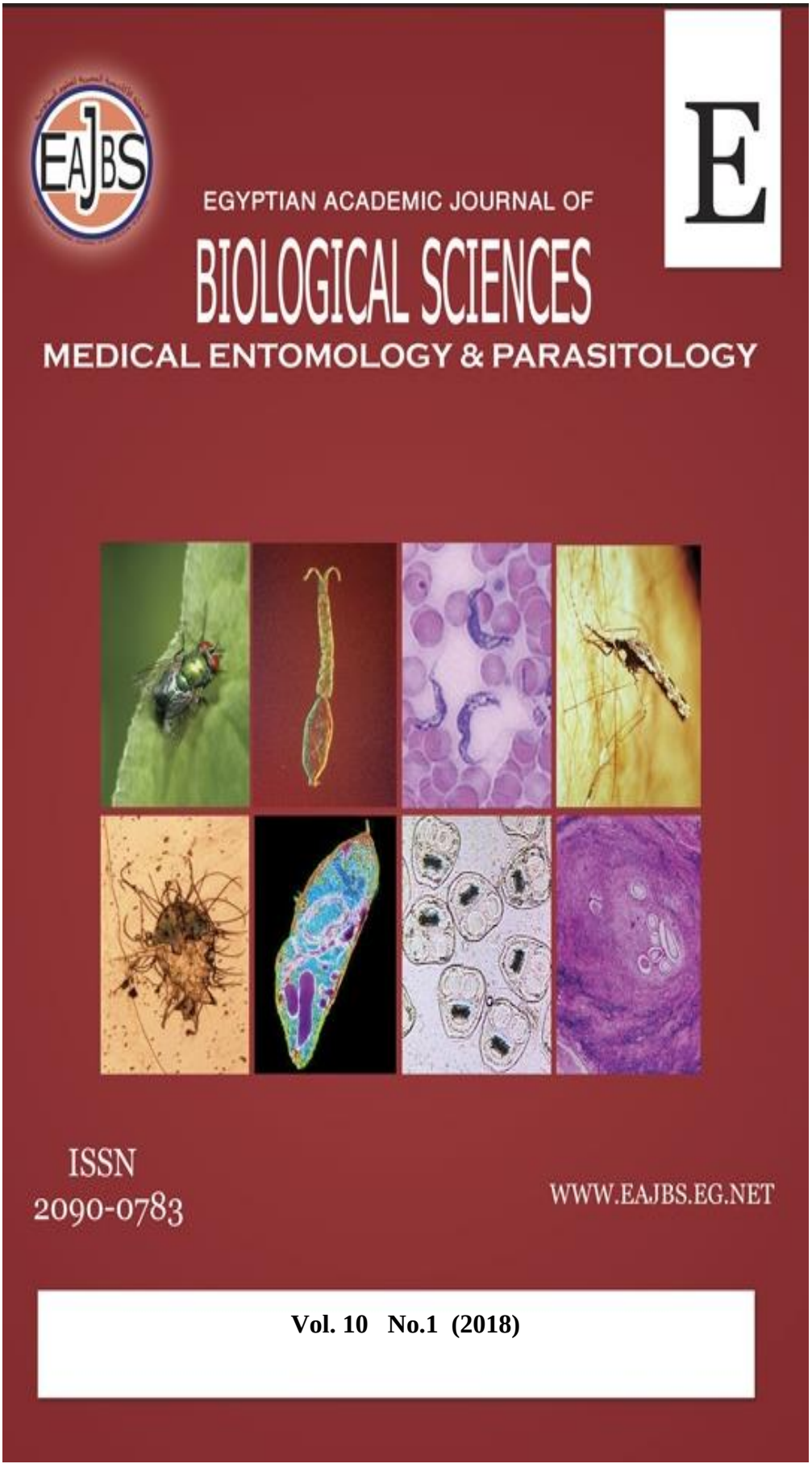
EAJBS
EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
E
MEDICAL ENTOMOLOGY & PARASITOLOGY
ISSN
2090-0783
WWW.EAJBS.EG.NET
Vol. 10 No.1 (2018)

Egypt. Acad. J. Biolog. Sci., 10 (1):105 – 115 (2018)

Egyptian Academic Journal of Biological Sciences
E. Medical Entom. & Parasitology

ISSN: 2090 – 0783
www.eajbse.journals.ekb.eg

Morphological and Electrophoretic Differentiation of Two Clinostomatid Metacercariae Infecting *Oreochromis niloticus* from the River Nile at El-Minia District, Egypt

Manal Ahmed, Sahar El-Ganainy and Shaban H. Abd El-Aziz*
Department of Zoology and Entomology, Faculty of Science, Minia University, Minia 61519, Egypt.
E-mail: shaban.abdallah@mu.edu.eg

ARTICLE INFO

Article History
Received:1/3/2018
Accepted:5/5/2018

Keywords:
Euclinostomum ardeolae; *Clinostomum phalacrocoracis*; *Oreochromis niloticus*; Electrophoresis.

ABSTRACT

Morphological characteristics and electrophoretic protein patterns were considered in the present study to distinguish between two types of clinostomatid metacercariae from *Oreochromis niloticus* fish caught from the River Nile at El-Minia district, Egypt. Fifty-two specimens of randomly collected 120 examined fish were infected with both *Euclinostomum ardeolae* and *Clinostomum phalacrocoracis* metacercariae or either. *Euclinostomum ardeolae* metacercariae were embedded in *Oreochromis niloticus* tissues of the kidney as round to oval greyish black cysts giving the area around faint black color with a 15% prevalence rate of infection. Cyst was of variable size (3-4 mm) and thin completely tight wall. Metacercaria is aspinose pyriform in shaped, long 6.5 wide 2.3 mm, of blunt anterior end but the posterior one is nearly rounded. *Clinostomum phalacrocoracis* yellow to orange in color cysts were detected in the pharyngeal region and branchial chambers and the prevalence rate of infection was 39.16%. They varied in size (4-5.2 mm) with a thin transparent membrane full of yellowish fluid. The body of metacercaria was elongated, tongue-shaped, and slightly wider in gonadic region, long 16.4 wide 4.01mm. Electrophoretic protein profiles were resolved using one dimensional SDS-PAGE stained with Coomassie Brilliant Blue R-250 stain. Encysted and excysted *Euclinostomum ardeolae* metacercariae showed bands of 61 kDa, 29 kDa, 28 kDa, 15 kDa and 14 kDa, while *Clinostomum phalacrocoracis* encysted and excysted metacercariae revealed bands of 61 kDa, 54 kDa, 32 kDa, 31 kDa, 20 kDa, 17 kDa, 15 kDa and 14 kDa. Conclusively, biochemical findings are in concordance with morphological characterization confirming both metacercariae to be of two distant species.

INTRODUCTION

Some internal parasitic diseases affecting *Oreochromis niloticus* (formerly *Tilapia nilotica*) fish such as yellow grub disease caused by clinostomatid species can be transmitted to human causing Halzoun like disease leading to laryngo-pharyngitis, while others like *Prohemistomum vivax* were rarely recorded to infect human, but may cause death (Nasr, 1941; Williams and Jones, 1976).

Piscivorous birds (final host) in Europe, Asia, Africa, and America were reported to be infected with species of Euclinostomum Travassos, 1928 (Synonyms *Tumaclinostomum* van der Kuyp,1953; *Metaclinostomum* Pande and Baugh, 1970) in the buccal cavity and oesophagus (Kanev *et al.,* 2002). Seventeen species of *Euclinostomum* have been described by Purivirojkul and Sumontha (2013), some of which showed intermediate host specificity at the genus level such as *E. clarias* metacercaria found in *Clarias* species fishes (Onuoha, 2010), while others like *E. heterostomum* has a wide range of freshwater fish hosts (Abd Al Aal *et al.,* 2008; Echi *et al.,* 2009; Vankara *et al.,* 2011). Metacercariae of *Euclinostomum* species may be located in various organs of fish host: buccal cavity, skin, eyes, coelomic cavity, liver, spleen, kidney, muscles, pharyngeal wall, gill cavity, and external surface of the alimentary canal (Arthur and Ahmed, 2002; Arthur and Te, 2006; Britz *et al.,* 2017; Echi *et al.,* 2009; El-ganiny, 1995; Purivirojkul and Sumontha, 2013). Furthermore, more than one species of *Euclinostomum* metacercariae may be found in the same fish such as *E. gastrocaecum*, *E. heterocaecum*, *E. nephrostomum* and *E. indicum* from *Channa marulius* (Bilqees, 1972). Besides the usual morphological description used in metacercariae characterization, recently, a combination with molecular phylogenetic analysis was reported for *E. heterostomum* metacercaria (Caffara *et al.,* 2016).

In Egypt, clinostomatid metacercariae seem to be very common parasites of *Tilapia* sp. (Abd Al Aal *et al.,* 2008; Abou-Eisha *et al.,* 2008; Eissa *et al.,* 2011; El-ganiny, 1995; Taher, 2009). Among the 14 *Clinostomum* species considered valid so far (Caffara *et al.,* 2011; Gustinelli *et al.,* 2010; Sereno-Uribe *et al.,* 2013), only a few are supported by the complete morphological description and most of them were reported only at the adult stage, while the metacercarial phase hosted by fish or amphibians, known as ‘‘yellow grub’’, is often poorly described. Dzikowski *et al.* (2004) confirmed the presence of *C. complanatum* by molecular analysis, but molecular data are available so far only for a limited number of *Clinostomum* species. Recently, analyses of mitochondrial and/or ribosomal DNA have confirmed the validity of five *Clinostomum* species (Caffara *et al.,* 2011; Dzikowski *et al.,* 2004; Gustinelli *et al.,* 2010; Sereno-Uribe *et al.,* 2013). Moreover, the coupling of morphological description with molecular analysis carried out on ITS rDNA and COI mtDNA sequences was reported for the metacercarial stage of *Clinostomum phalacrocoracis* (Caffara *et al.,* 2014).

Molecular analyses, multilocus enzyme electrophoresis (MLEE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are used as confirmation methods for morphological description. The latter is a cheap, reliable, and still an important tool in the study of parasites and other pathogens (Tibayrenc, 2009). Strains of *Leishmania*, *Plasmodium* spp., and their subspecies were analyzed using one and two-dimensional gel electrophoresis (Armijos *et al.,* 1990; Gongora *et al.,* 2003; Heidrich *et al.,* 1982). Moreover, SDS-PAGE had been used to determine the metacercariae total body protein band profiles of different *Diplostomum* spp. (Faulkner, 1989), and encysted metacercariae from *Oreochromis niloticus* (Mousa *et al.,* 2000).

This study aims to verify the morphological differentiation of clinostomatid metacercariae infected *Orechromis niloticus* caught from the River Nile in El-Minia district, Egypt, using one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

MATERIALS AND METHODS

Samples Collection:

A total number of 120 specimens of *Oreochromis niloticus* (25-100 gm BW)

were collected during the period from October, 2015 to April, 2016 from the River Nile in El-Minia district, Egypt. The gills, gill chambers, and muscles of the freshly caught fish were examined for macroscopic clinostomatid metacercariae cysts. Then, fishes were dissected and examined where the cysts were removed from infected specimens, and some were manually excysted. Collected metacercariae were divided according to their morphological characters into two kinds, where whole mounts were fixed in hot F.A. A. and stained with Carmine stains (Kurse and Pritchard, 1982). Metacercariae were measured using eyepiece micrometer of dissecting microscope, photomicrographed and identified according to the keys given by Yamaguti (1958) and Ukoli (1966).

Preparation of Metacercariae Soluble Proteins:

Some encysted and excysted clinostomatid metacercariae collected from *Oreochromis niloticus* fishes were kept immediately at -80 °C. On preparation, metacercariae (100 mg) were further tissue-hardened with liquid nitrogen and homogenized for 15 minutes in 1 ml ice-cold distilled water. Then, homogenates were sonicated for five minutes and centrifuged at 20,000 rpm for 45 minutes at 4 °C. Supernatants were harvested, aliquoted, and kept at -80 °C (Mousa *et al.*, 2000).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Twenty micrograms of encysted and excysted clinostomatid metacercariae supernatants were dissolved in loading sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromphenol blue; pH 6.8, 1% 2-mercaptoethanol), boiled for 2 minutes (Abd El- Aziz et al., 2007) and total proteins were resolved using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). Gels were stained overnight in Coomassie Brilliant Blue R-250 stain (0.25% Coomassie Brilliant Blue R-250, 40% methanol, 10% glacial acetic acid, 50% distilled water). Excess of stain was removed by destain solution (40% methanol, 10% glacial acetic acid, 50% distilled water) (Mousa et al., 2000). Different band patterns were visualized against a pre-stained standard (Nippongenetics, Japan) and molecular weights were determined using GelAnalyzer 2010 Software.

RESULTS

Out of 120 examined *Oreochromis niloticus* fish specimens, 52 were infected with one or two kinds of macroscopic encysted clinostomatid metacercariae (Trematoda: Clinostomatidae) with an overall prevalence rate of infection of 43.33% (Table 1). Identification of clinostomatid metacercariae attained from naturally infected *Oreochromis niloticus* fish in the present work was based on the keys of Yamaguti (1958) and Ukoli (1966). They were assigned as metacercariae of two species belonging to two subfamilies of family Clinostomatidae Lühe 1901 emend Dollfus 1932:

Table 1: Clinostomatid metacercariae prevalence rate and intensity of infection in *Oreochromis niloticus* fishes collected from the River Nile in El-Minia district, Egypt.

Metacercaria	No. of examined fishes	No. of infected fishes	% of infection	Intensity per fish
Euclinostomum ardeolae	120	18	15.00	1-4
Clinostomum phalacrocoracis	120	47	39.17	2-15

1-Subfamily: Euclinostomatinae Yamaguti 1958:

Genus: *Euclinostomum* Travassos 1928:

Metacercariae of *Euclinostomum ardeolae* El-Naffar and Khalifa 1981:

Encysted metacercariae were embedded in the *Oreochromis niloticus* tissues of the kidney. They appeared as round to oval greyish black visible cysts and gave the area around faint black color. Cysts were of variable size (3-4 mm) with a thin completely tight wall, where no free space inside around the larva. The prevalence rate of infection was 15 % and the intensity of infection varied from 1-4 metacercarial cysts per infected fish (Fig. 1).

External Features (Description of metacercaria, in mm, based on six specimens):

Excysted metacercaria is aspinose pyriform in shaped, long 6.5 wide 2.3. The anterior end is blunt but the posterior one is nearly rounded. Oral sucker, long 0.27 wide 0.23, slightly oval in shaped sub terminal surrounded by will developed oral collar. Acetabulum more or less rounded, long 1.18 wide 1.16. Oesophagus very short and opens into a well-developed pharyngeal organ which opens by a valve-like pattern. Intestinal bifurcation is close to the anterior end of the body just behind the pharyngeal organ. They are smooth, simple till the posterior margin of the acetabulum. Intestinal diverticula about 9-12 on each side, this number may be equal but frequently differs. Precursors of genitalia are at the fourth-fifth of the body. The anterior testis is a horse-shoe in shaped, long 0.62 wide 0.19, and enclosing the genital atrium between its arms, the posterior testis is tripartite in shaped, long 0.47 wide 0.29, and it lies just behind the ovary. Rounded small ovary, long 0.17 wide 0.15, situated between two testes on the right side. The future uterus is seen with its ascending and descending loops opening at the genital atrium in the concavity of the anterior testis. The ootype is easily seen and it is situated in the intertesticular space near the posterior margin of the ovary. The vitellaria are not discernible. The excretory aperture is rounded to oval in shape and lies near the posterior body extremity (Fig. 2).

2- Subfamily: Clinostomatinae, Pralt 1902

Genus: *Clinostomum* Leidy 1856

Metacercariae of *Clinostomum phalacrocoracis* Dubois 1931:

Encysted metacercariae had a thin transparent membrane and contained a yellowish fluid when excysted. The prevalence rate of infection was 39.16 %. They were yellow to orange and varied in size from 4-5.2 mm. They were detected in pharyngeal region and branchial chambers. The intensity of infection ranged from 2-15 metacercarial cysts per fish (Fig. 1).

External Features (Description of metacercaria, in mm, based on six specimens):

The body of excysted metacercaria is elongated, tongue-shaped, and slightly wider in the gonadic region, long 16.4 wide 4.01. Oral sucker, long 0.73 wide 0.65, smaller than acetabulum, long 1.5 wide 1.4, surrounded by the weakly developed oral collar. Pharynx is evident, long 0.58 wide 0.53. Intestine bifurcates immediately posterior to pharynx. Intestinal caeca run laterally to the ventral sucker and immature genital complex, with small diverticula more evident posteriorly to the ventral sucker. Testes arranged in tandem between the middle and posterior third of the body. Anterior testis, long 1.46 wide 1.72, in the posterior part of middle third of the body, fan-shaped, consists of four to eight blunt lobes, some of which are sub-lobed. Posterior testis, long 1.44 wide 1.40, in the anterior part of the posterior third of the body, fan-shaped with anterior margin concave and with two major lateral lobes and one posterior lobe, each of which is sub-lobed. Cirrus sac, long 1.22 wide 0.36, bean-shaped, in dextral intertesticular space, anterior to the ovary, with a genital pore opening laterally at the posterior margin of anterior testis between right and posterior lobe. Ovary, long 0.34 wide 0.26, irregular,

round, smaller than cirrus sac, located in dextral intertesticular space. Uterus runs straight from ventral sucker to anterior testis. Uteroduct runs around the left margin of the anterior testis and opens into the uterine sac. Metraterm, straight and overlapping the right half of the anterior testis, connects the uterus to the genital atrium. Vitellarium is not evident. Tegumental surface covered by very thin papillae (Fig. 2).

Electrophoretically, protein profiles derived from the two types of metacercariae revealed several band patterns of different molecular weights. Both metacercariae recorded one common band of 61 kDa, but differed in other protein band patterns. Excluding too faint bands, four bands were identified for *Euclinostomum ardeolae* and 7 bands for *Clinostomum phalacrocoracis* metacercariae of molecular weights more than 61 kDa. Low molecular weight bands of 32 kDa and lower were the obvious discriminatory band patterns between the two types of metacercariae (Fig. 3 & Table 2).

Euclinostomum ardeolae and *Clinostomum phalacrocoracis* encysted metacercariae differed in their protein band patterns except for the 61 kDa and 14 kDa bands. Also, excysted metacercariae protein band patterns differed in all except for the band of 61 kDa. Protein profile of both encysted and excysted *Euclinostomum ardeolae* metacercariae showed bands of 61 kDa and 14 kDa, but encysted metacercaria recorded bands of 29 kDa and 15 kDa, while excysted metacercaria recorded a band of 28 kDa. On the other hand, the protein band patterns of both encysted and excysted *Clinostomum phalacrocoracis* metacercariae revealed 61 kDa and 17 kDa bands. Exclusively, *Clinostomum phalacrocoracis* encysted metacercaria showed 32 kDa, 20 kDa and 14 kDa bands, while excysted metacercariae showed 54 kDa, 31 kDa and 15 kDa bands (Fig. 3 & Table 2).

Table 2: Electrophoretic band patterns and their molecular weight of clinostomatid metacercariae infected *Oreochromis niloticus* fishes collected from the River Nile in El-Minia district, Egypt.

Metacercariae	**Encysted**									**Excysted**							
	Euclinostomum ardeolae				*Clinostomum phalacrocoracis*					*Euclinostomum ardeolae*			*Clinostomum phalacrocoracis*				
Band No.	1	2	3	4	1	2	3	4	5	1	2	3	1	2	3	4	5
MW (kDa)	61	29	15	14	61	32	20	17	14	61	28	14	61	54	31	17	15

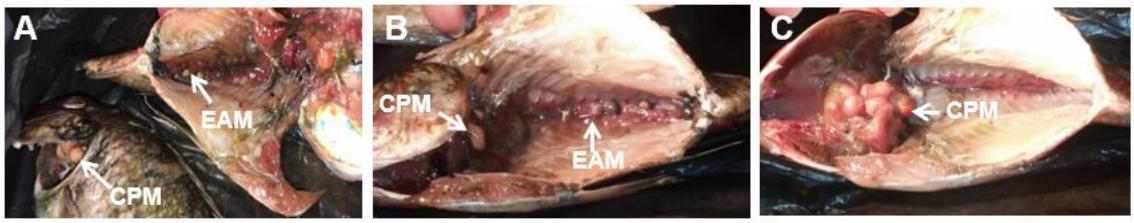

Fig. 1 *Euclinostomum ardeolae* (EAM) and *Clinostomum phalacrocoracis* (CPM) encysted metacercariae. A, branchial chamber and kidney double infection; B, pharyngeal region and kidney double infection; and C, pharyngeal region single infection

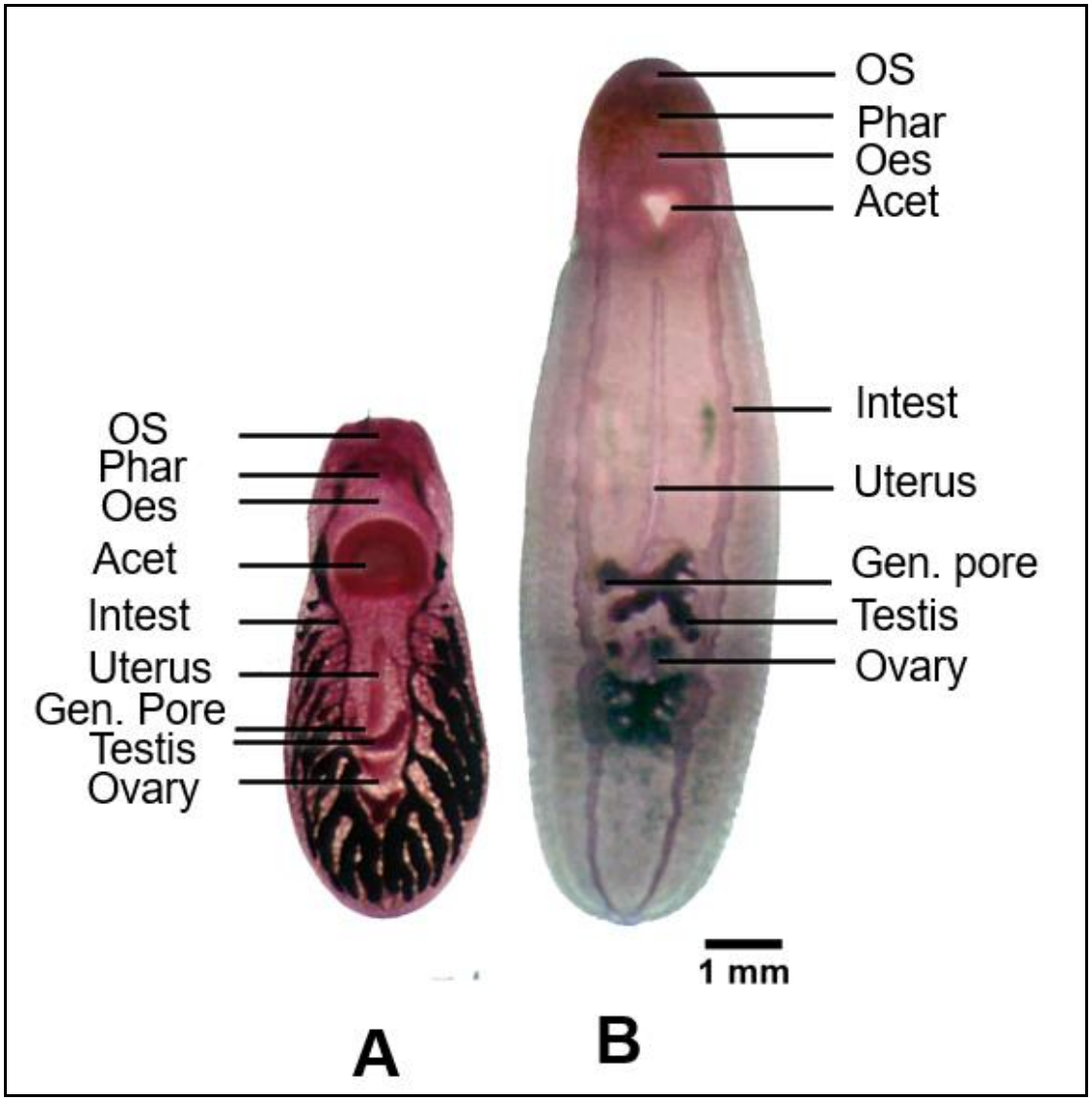

Fig. 2 *Euclinostomum ardeolae,* A, and *Clinostomum phalacrocoracis* excysted metacercariae, B. OS, oral sucker; Phar, pharynx; Oes, oesophagus; Acet, acetabulum; Intest, intestine; Gen. pore, genital pore

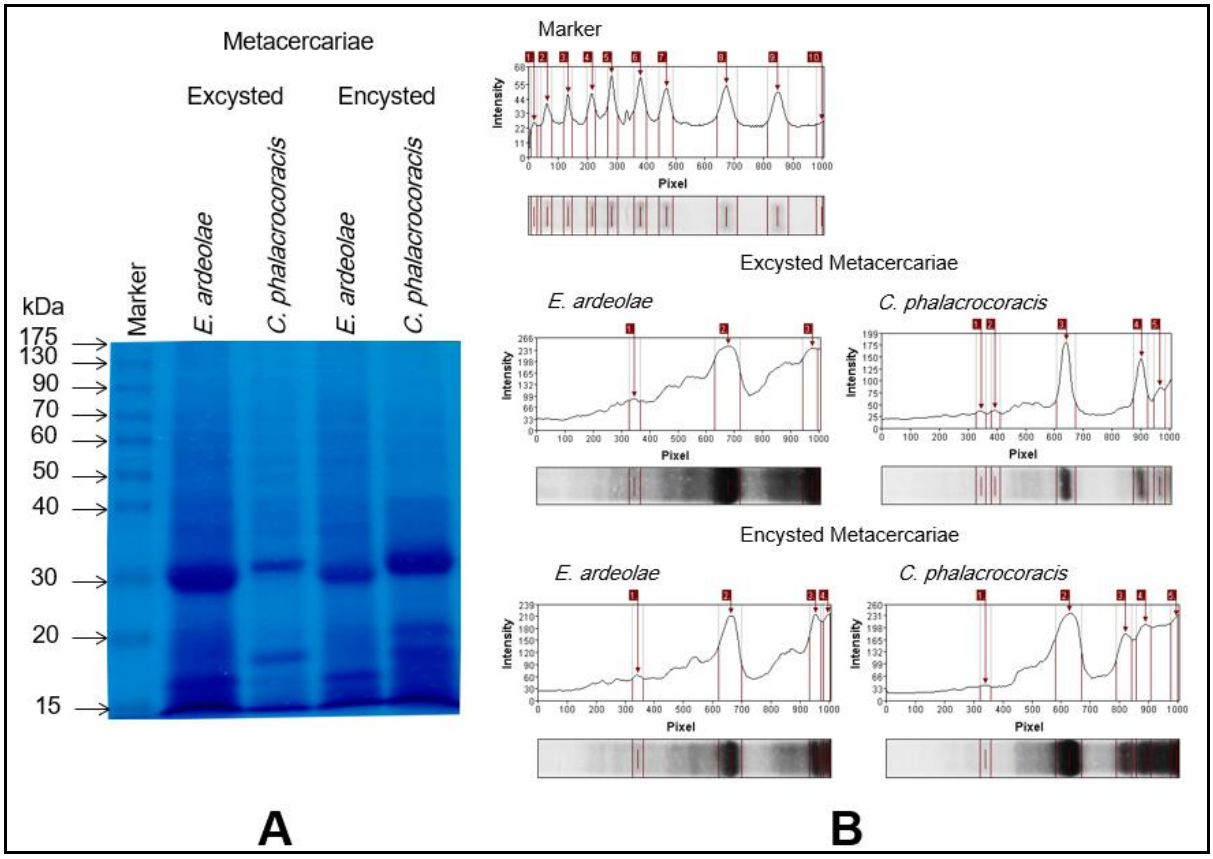

Fig. 3 Protein profiles of *Euclinostomum ardeolae* and *Clinostomum phalacrocoracis* metacercariae resolved by SDS- PAGE, A, and band patterns analyses, B

DISCUSSION

Euclinostomum species are not cosmopolitan parasites such as *E. africanum* reported in Africa (Canaris and Gardner, 2003), *E. ardeolae* in Egypt (El-Naffar and Khalifa, 1981; Taher, 2009), *E. indicum* in India (Agarwal, 1958) and *E. robustum* in Pakistan (Perveen *et al.*, 2011). The metacercariae under the present discussion show identical similarity with specimens described by El-Naffar and Khalifa (1981), El-Shahawi (1983) and El-ganiny (1995) in their general body form, shape and position of testes, and arrangement of intestinal caeca diverticula. Therefore, it is identified as *Euclinostomum ardeolae*.

Dubois (1930) originally described the adult stage of *Clinostomum phalacrocoracis* in *Phalacrocorax levaillanti* L. from Angola. Later, Ukoli (1966) reported this species in African darter (*Anhinga rufa rufa*) in Ghana, Peirce and Din (1970) in *Pelecanus* spp. from Uganda, and Tendeiro *et al.* (1974) in *P. onocrotalus* from Mozambique. Kabunda and Sommerville (1984) described metacercariae of *Clinostomum* sp. in tilapias (*Oreochromis* spp.) captured in Zaire showing morphological similarities to *C. phalacrocoracis* and *C. giganticum*. Accordingly, it could be stated that the present study second type specimens are *C. phalacrocoracis* with morphological characters consistent with the metacercaria described by Ebraheem (1992), El-ganiny (1995), Kabunda and Sommerville (1984) and Caffara et al. (2014), except the anterior testis, which is fan-shaped, not saddle-shaped, as reported by Kabunda and Sommerville (1984). This may be related to differences in metacercarial maturity, as stated by Ukoli (1966), who reported less digitation in the testes of younger specimens. Kabunda and Sommerville (1984), also, noted a remarkable similarity between their metacercaria and *C. giganticum* described by Agarwal (1959) in *Ophiocephalus punctatus*, but the two species are different in the

position of the genital pore. The genital pore is at the right posterior margin of the anterior testis between the lobes in *C. phalacrocoracis*, while it is at the level of the equator of the anterior testis in *C. giganticum*. Concerning the other species described by Caffara *et al.* (2014), at the metacercarial stage, the morphological characters observed in the present *C. phalacrocoracis* differ from *C. cutaneum*, *C. tilapiae*, and from *C. complanatum* in the position of the genital pore and the shape of the testes.

Protozoan parasites electrophoretic protein profiles analysis took great consideration to discriminate between taxa and sub-taxa and proved to be a useful tool in the identification of unknown species and sub-species (Armijos *et al.*, 1990; Fernandez-Boo *et al.*, 2014; Gongora *et al.*, 2003). In helminths, sodium dodecyl sulphate-polyacrylamide gel electrophoresis had been used to determine the metacercariae total body protein profiles of different *Diplostomum* spp., where four fish species were investigated: roach (*Rutilus rutilus*), gywniad (*Coregonus laveratus*), ruff (*Gymnocephalus cernua*) and perch (*Perca fluviatilis*). The four species of *Diplostomum* metacercariae were distinguished by three different bands of molecular weight, 55.5 kDa, 53.5 kDa and 52 kDa. *D. coregonus* metacercariae, from *C. laveratus*, showed reduced protein concentrations between bands from 32 kDa to 40 kDa (Faulkner, 1989). Also, metacercariae of *Prohemistomum vivax*, *Pygidiopsis genata*, *Procerovum warium* and *Haplorchis pumilio* from *Oreochromis niloticus*, where protein profile molecular weights range from 13 kDa to 112 kDa (Mousa *et al.*, 2000).

In the present study, protein profiles between 61 kDa and 32 kDa bands are shown in parallel for both metacercariae, but the band of 54 kDa appeared only in *Clinostomum phalacrocoracis* excysted metacercariae. *Clinostomum phalacrocoracis* are generally distinguished by 1-2 kDa added between the 32 kDa and 14 kDa faced prominent bands, but the band of 20 kDa is recorded only in *Clinostomum phalacrocoracis* encysted metacercariae. Consequently, the overall metacercariae band patterns analysis emphasizes the dissimilarity between *Euclinostomum ardeolae* and *Clinostomum phalacrocoracis*.

In conclusion, biochemical findings are in concordance with a morphological characterization of both *Euclinostomum ardeolae* and *Clinostomum phalacrocoracis* metacercariae confirming them to be metacercariae of two distant species.

Acknowledgments

The authors wish to thank Dr. Doaa Moustafa, Department of Botany and Microbiology, Faculty of Science, Minia University, for helping us to carry out this study.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest: The authors declare that they have no conflict of interest.

REFERENCES

Abd Al Aal, Z., Amer, O. H., Badawy, A. I. I. and El-Ashram, A. M. M. (2008) Digenetic trematodes the little egret, *Egretta garzetta*, and possibility of transmission to *Oreochromis niloticus* at El-abbassa fish farms, Egypt *8th International Symposium on Tilapia in Aquaculture*, pp. 1351- .1364.

Abd El- Aziz, S. H., Endo, Y., Miyamaori, H., Takino, T. and Sato, H. (2007) Cleavage of growth differentiation factor 15 (GDF15) by membrane type 1- matrix metalloproteinase abrogates GDF15- mediated suppression of tumor cell growth. *Cancer Science*, 98 (9), 1330- 1335.

Abou-Eisha, A. M., Saleh, R. E., Fadel, H. M., Youssef, E. M. and Helmy, Y. A. (2008) Role of freshwater fishes in the epidemiology of some zoonotic trematodes in Ismailia province. *Suez Canal Veterinary Medical Journl*, 13, 653-677.

Agarwal, S. M. (1958) Further observations on *Euclinostomum indicum* Bhalerao,

1942. *Indian Journal of Helminthology*, 10, 31-39.

Agarwal, S. M. (1959) Studies on the morphology, systematics and life history of *Clinostomum giganticum* n. sp. (Trematoda: Clinostomatidea). *Indian Journal of Helminthology*, 11, 75–115.

Armijos, R. X., Chico, M. E., Cruz, M. E., Guderian, R. H., Kreutzer, R. D., Berman, J. D., Rogers, M. D. and Grogl, M. (1990) Human cutaneous leishmaniasis in Ecuador: identification of parasites by enzyme electrophoresis. *American Journal of Tropical Medical Hygiene* 42, 424-428.

Arthur, J. R. and Ahmed, A. (2002) Checklist of the parasites of fishes of Bangladesh. *FAO Fisheries Technical Paper. No. 369/1. Rome, FAO*, 77.

Arthur, J. R. and Te, B. Q. (2006) Checklist of the parasites of fishes of Vietnam. *FAO Fisheries Technical Paper. No. 369/2. Rome, FAO*, 133.

Bilqees, F. M. (1972) Freshwater fish trematodes of Pakistan III. Metacercarial forms of *Euclinostomum Travassos*, 1928 from Ophiocephalus spp. *Acta Parasitologica Polonica*, 20, 55-62.

Britz, J., Saayman, J. E. and van As, J. G. (2017) Anatomy of the metacercaria and adult of *Euclinostomum heterostomum* (Rudolphi, 1809) (Trematoda: Clinostomatidae). *South African Journal of Zoology*, 19, 91-96.

Caffara, M., Davidovich, N., Falk, R., Smirnov, M., Ofek, T., Cummings, D., Gustinelli, A. and Fioravanti, M. L. (2014) Redescription of *Clinostomum phalacrocoracis* metacercariae(Digenea: Clinostomidae) in cichlids from Lake Kinneret, Israel. *Parasite* 21, 32.

Caffara, M., Locke, S. A., Cristanini, C., Davidovich, N., Markovich, M. P. and Fioravanti, M. L. (2016) A Combined Morphometric and Molecular Approach to Identifying Metacercariae of *Euclinostomum heterostomum* (Digenea). *Journal of Parasitology*, 102, 239–248.

Caffara, M., Locke, S. A., Gustinelli, A., Marcogliese, D. J. and Fioravanti, M. L. (2011) Morphological and molecular differentiation of *Clinostomum complanatum* and *Clinostomum marginatum* (Digenea: Clinostomidae) metacercariae and adults. *Journal of Parasitology*, 97, 884–891.

Canaris, A. G. and Gardner, S. L. (2003) Bibliography of Helminth Species Described from African Vertebrates 1800 - 1967. *Faculty Publications from the Harold W. Manter Laboratory of Parasitology*, 3, 1-30.

Dubois, G. (1930) Trematoda. Mate´riaux de la mission scientifique Suisse en Angola. *Bulletin de la Socie´te´ Neucha^teloise des Sciences Naturelles*, 54, 61–72.

Dzikowski, R., Levy, M. G., Poore, M. F., Flowers, J. R. and Paperna, I. (2004) *Clinostomum complanatum* and *Clinostomum marginatum* (Rudolphi, 1819) (Digenea: Clinostomidae) are separate species based on differences in ribosomal DNA. *Journal of Parasitology*, 90, 413–414.

Ebraheem, M. E. (1992) Studies on some of the parasites in some Nile fishes in Sohag Governorate, Assiut University, Egypt.

Echi, P. C., Eyo, J. E. and Okafor, F. C. (2009) Co-parasitism and morphometrics of three clinostomatids (digenea: clinostomatidae) in sarotherodon melanotheron from a tropical freshwater lake. *Animal Research International*, 6, 982-986.

Eissa, I. A. M., Gado, M. S., Laila, A. M., Zaki, M. S. and Noor El-Deen, A. (2011) Field Studies Encysted Metacercariae infested Natural Male Tilapias and Monosex Tilapias in

Kafr El-Sheikh Governorate. *Fish Farms Life Science Journal*, 8, 7-12.

El-ganiny, S. S. E. (1995) Studies on the parasites of Nile fishes at EL-Minia Governorate, Minia University, Egypt.

El-Naffar, M. K. and Khalifa, R. M. (1981) *Euclinostomum ardeolae* Sp. Nov.(Trematoda: Clinostomatidae). *Journal of the Egyptian Society of Parasitology,* 11, 175-181.

El-Shahawi, G. A. Z. (1983) Studies on some fishes as intermediate hosts of helminth parasites of some birds and mammals, Minia University, Egypt.

Faulkner, M. (1989) The application of sodium dodecyl sulphate-polyacrylamide gel electrophoresis to the taxonomic identification of the total body protein band profiles of Diplostomum spp. metacercariae (Digenea), parasites of fish eyes. *Electrophoresis*, 10, 260-264.

Fernandez-Boo, S., Chicano-Galvez, E., Alhama, J., Barea, J. L., Villalba, A. and Cao, A. (2014) Comparison of protein expression profiles between three Perkinsus spp., protozoan parasites of molluscs, through 2D electrophoresis and mass spectrometry. *Journal of Invertebrate Pathology*, 118, 47-58.

Gongora, R., Acestor, N., Quadroni, M., Fasel, N., Saravia, N. G. and Walker, J. (2003) Mapping the proteome of Leishmania Viannia parasites using two-dimensional polyacrylamide gel electrophoresis and associated technologies.*Biomedica,* 23, 153-160.

Gustinelli, A., Caffara, M., Florio, D., Otachi, E. O., Wathuta, E. M. and Fioravanti, M. L. (2010) First description of the adult stage of *Clinostomum cutaneum* Paperna, 1964 (Digenea: Clinostomidae) from grey herons *Ardea cinerea* L. and a redescription of the metacercaria from the Nile tilapia *Oreochromis niloticus niloticus* (L.) in Kenya. *Systematic Parasitology,* 76, 39–51.

Heidrich, H. G., Mrema, J. E., Vander Jagt, D. L., Reyes, P. and Rieckmann, K. H. (1982) Isolation of intracellular parasites (Plasmodium falciparum) from culture using free-flow electrophoresis: separation of the free parasites according to stages. *Journal of Parasitol*ogy, 68, 443-450.

Kabunda, M. Y. and Sommerville, C. (1984) Parasitic worm causing the rejection of tilapia (*Oreochromis* species) in Zaire. *British Veterinary Journal*, 140, 263–268.

Kanev, I., Radev, V. and Fried, B. (2002) Family Clinostomidae LÜhe, 1901. In Gibson, D. I., Jones, A. & Bray, R. A. (eds) *Keys to the Trematode* CAB internationa, New York, USA. pp. 113-120.

Kurse, G. O. W. and Pritchard, M. H. (1982) *The collection and preservation of animal parasites*. Nebraska University Press, USA.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head Bacteriophage T4. *Nature*, 227, 680-685.

Mousa, W. M., Mahdy, O. A. and Kandil, O. M. (2000) Electrophoretic analysis to confirm the identification of some kinds of encysted metacercaiae from *Oreochromis niloticus*. *Assiut Veterinary Medical Journal*, 43, 199-209.

Nasr, M. (1941) The occurance of *Prohemistomum vivax* infection in man,with redescription of the parasite. *Laboratory and Medical Progress*, 2, 135-149.

Onuoha, E. O. (2010) Liver pathology of the african catfish, *Clarias gariepinus* infected with *Euclinostomum clarias* metacercariae in Anambra river basin, Nigeria. *Animal Research International*, 7, 1151-1155.

Peirce, M. A. and Din, N. A. (1970) Two new hosts for *Clinostomum phalacrocoracis* Dubois, 1930 (Trematoda), from Uganda. *Journal of Parasitology*, 56, 489.

````
````

Perveen, F., Shaikh, G. S., Bilqees, F. M. and Khatoon, N. (2011) A new metacercarial form *Euclinostomum robustum* (Trematoda: Clinostomidae: Euclinostominae) from a fresh water fish of Sindh, Pakistan. *Proceedings of Parasitology,* 52, 35-41.

Purivirojkul, W. and Sumontha, M. (2013) *Euclinostomum heterostomum* (Rudolphi, 1809) Metacercarial Infection in Three Osphronemid Fish Species Walailak. *Journal of Science and Technology,* 10, 97-102.

Sereno-Uribe, A. L., Pinacho-Pinacho, C. D., Garcı´a-Varela, M. and Pe´rez-Ponce de Leo´n, G. (2013) Using mitochondrial and ribosomal DNA sequences to test the taxonomic validity of *Clinostomum complanatum* Rudolphi, 1814 in fish-eating birds and freshwater fishes in Mexico, with the description of a new species. *Parasitology Research*, 112, 2855–2870.

Taher, G. A. (2009) Some studies on metacercarial infection in *Oreochromis niloticus* in assiut governorate and their role in transmission of some trematodes to dogs. *Assiut University Bulletin of Environmental. Researches,* 12, 63-79.

Tendeiro, J., Travassos Santos Dias, J. A. and Fazendeiro do Carmo Martins, M. I. (1974) Sobre dois clinostomideos (Trematoda, Digenea) parasitas do pelicano, *Pelecanus onocrotalus* L. com descricao de uma especie nova *Revista de Cie^ncias Veterina´rias. Universidade de Lourenc¸o Marques, Mozambique (Ser A)* 7, 45–71.

Tibayrenc, M. (2009) Multilocus enzyme electrophoresis for parasites and other pathogens. *Methods in Molecular Biology*, 551, 13-25.

Ukoli, F. M. A. (1966) On *Clinostomum tilapiae* n. sp., and *C. phalacrocoracis* Dubois, 1931 from Ghana, and a discussion of the systematics of the Genus *Clinostomum* Leidy, 1856. *Journal of Helminthology*, 40, 187–214.

Vankara, A. P., Mani, G. and Vijayalakshmi, C. (2011) A report on various digenetic metacercariae from the freshwater fishes of River Godavari, Rajahmundry. *Journal Parasitic Diseases*, 35, 177-185.

Williams, H. H. and Jones, A. (1976) Marine helminthes and human health. *Commonwealth Institute of Helminthology Miscellaneous Publication* 3, 47.

Yamaguti, S. (1958) *Systema Helminthum*. Inter Science Publishers Inc. , New York, USA.