Advanced Studies on Monogenean Diseases of *Argyrosomus Regius*, *Dicentrarchus Labrax* and *Epinephelus Aeneus* in Port Said Governorate

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Abstract

The present study was designed to investigate the parasitic monogeneans affecting *Argyrosomus regius, Dicentrarchus labrax* and *Epinephelus aeneus* that collected seasonally from different areas of Mediterranean sea region (Port Said province). The monogenean parasites were identified as Diplectanium sp. and recovered from the gills of *Argyrosomus regius*, Diplectanium sp. in *Dicentrarchus labrax* and *Benedenia epinepheli* and *Pseudorhabdosynochus epinepheli* were isolated from *Epinephelus aeneus*. The total prevalence of monogenean infestations among the examined fishes was 45%. It was 66, 36 and 33% in *Argyrosomus regius, Epinephelus aeneus* and *Dicentrarchus labrax* respectively. The spring displayed the highest seasonal prevalence of monogenetic trematode infestations among the examined fishes as 58.67%. Molecular and histopathological studies were investigated.

Introduction

Gill parasites are not uncommon on wild fish. Many of these species were been recognized to have the potential to affect hosts fecundity, survival and growth ***(Johnson et al., 1996).*** Severe inflammation caused by the organs of attachment such as hooks and suckers in addition to sever tissue damage and rendering fish susceptible to secondary infection by viruses, fungi and bacteria **(*Dezfuli et al., 2003).*** The damage to the tissue of gills may decrease the ability of fish to maintain normal oxygen intake by impeding water flow ***(Ojha and Hughes, 2001).*** Parasites may be important in formative the health status of the fishes ***(Ferguson, 1989).*** Therefore, the present study was directed towards further understanding of *Argyrosomus regius, Dicentrarchus labrax* and *Epinephelus aeneus* in Mediterranean sea region (Port Said province). The aim of present study was to report the clinical picture,

total and seasonal prevalence of the monogenean parasitic ***infestations affecting each fish. Beside, molecular and histopathological*** studies were studied.

Materials and methods:-

Fish:

A total number of 300 alive fish (*100 Argyrosomus regius*, 100 *Dicentrarchus labrax* and 100 *Epinephelus aeneus*) of different body weights were collected randomly from Mediterranean sea in Port Said. The collected alive fishes were taken in large tanks filled with water from the water of the same sources to the lab. of Fish Diseases and Management Department, Faculty of Veterinary Medicine.

Clinical picture:

First, the examined fish's body weight were recorded and subjected to clinical examination (live fishes or freshly dead ones). Fish specimens were examined grossly for determination of external parasites and any clinical abnormalities. Postmortem investigation was performed on all fishes according to ***Amlacker (1970)***.

Parasitological examination:

1. Macroscopic examination:

Macroscopic examination was done by naked eyes and hand lens for detection of any abnormalities in fish gills.

2. Microscopic examinations:

The gill arches were removed to slides and proceed to cut away the cartilaginous arch using needles to separate gill filament, few drop of physiological saline were added on the slides to ensure a uniform distribution of the filaments under the entire cover slip ***(Lucky, 1977).*** Gill mucus was examined by transfer to slides then a drop of distilled water was added and examined under microscope to detect the parasites.

3- Smear preparations, permanent slides and staining:

The collected worms were washed with physiological saline several times to be free from debris, mucus and left in the refrigerator for complete relaxation. Then, they were gently compressed between cover and glass slide. The worms were fixed in 10% buffered neutral formalin for 12-24 hours, then the worms were washed several times by distilled water to remove the excess of the fixative, stained overnight in alum carmine, washed in tap water then distained in 1% acid alcohol, dehydrated in ascending grades of ethyl alcohol (30, 50, 70, 80, 90, 100%). Finally, clove oil was used to clear the specimens and xylene to remove the oil and mounted in Canada balsam ***(Kruse and Pritchard, 1982),*** left to dry in horizontal position.

4- Detection of Diplectanid monogenea using PCR:-

A. Preparation of the parasites :

The collected Diplectanid monogeneans were stored in alcohol 70% at -20°C until they analysed. The confirmation of

identified monogeneans was made by genetic analysis after DNA extraction and amplification a region comprising partial ssrDNA and entire internal transcribed spacer1 (ITS1).

B. DNA extraction and amplification:

Before the extraction of DNA, each parasite was kept in Eppendorf tube (0.5 ml). The parasites then plunged in an amount of 500 µl TE9 (500 mM Tris-HCl, 200 mM EDTA, and 10 mM NaCl, pH 9.0) for a period of 2–3 h. Next, the parasites were set in 20 µl lysis buffer (0.45% NP-40, 0.45% Tween-20, 1 mM EDTA, 10 mM Tris-HCl and 20 µg/ml proteinase K), and incubated at 65°C for 60 min, and then incubated at 95°C for 15 min. for proteinase K inactivation. The region including partial ssrDNA, and the whole internal transcribed spacer 1 (ITS1) was amplified using primers S1 (5'-ATTCCGATAACGAACGAGACT-3') and IR8 (5'-GCTAGCTGCGTTCTTCATCGA-3') according to ***Šimková et al. (2003)***. For each reaction , a total volume of 50 µl (9 µl of lysate, 1 × buffer (TakaRa), 1.5 mM of MgCl2, 0.8 µM of each PCR primer, 200 µM of each dNTP, and 2.5 U of Ex Taq polymerase (TakaRa) in a thermocycler (MJ Research). The optimum conditions were initial denaturation at 95°C for 4 min., followed by denaturation (35 cycles of 1 min at 92°C), annealing (1 min at 53°C) and extension (1.5 min at 72°C). The final extension was performed at 72°C for 10 min.

5- Histopathological examination:

Tissue specimens from the infested gills were taken, fixed immediately in 10% neutral buffered formalin, dehydrated, blocked in paraffin wax, sectioned at 5 – 7 microns and stained with H & E according to ***Carleton (1976).***

Results

Clinical picture:

The clinical picture of the infested fishes (*Argyrosomus regius*, *Dicentrarchus labrax* and *Epinephelus aeneus*) were represented as excessive mucus production, distress, surface swimming, emaciation, sluggish movement, and rubbing the body against hard objects. Fish gathered at the top of the water (surface breathing) with gulping the atmospheric air. Opercula were bulging. Gills of *D. labrax* showed a marbling (mosaic) appearance (areas of congestion and paleness). Gills of some fishes appeared anemic. Gills showed petechial hemorrhages, areas of thickened mucus, gill tips were sticked with necrosis and grayish coloration.

Results of parasitological examination:

1) Diplectanium sp. monogenean flukes were recovered from the gills of *A. regius*. The morphological approaches as some studies on Gyrodactylidae. The presence of compartmental, sclerotized, male copulatory organ of bulb shaped

(MCO) was a characteristic for family Diplectanidae. The morphological identification of this species is based on the vagina, shape and size of the MCO, dorsal and ventral bars, squamodisc, the marginal hooklets , dorsal and ventral hamuli and the squamodisc's rows of elements number. According to the morphological and parasitological characters, such monogenean can be classified and related to family Diplectanidae (Plate1).

2) Diplectanium sp. monogenean flukes collected from the gills of *D. labrax.* The presence of compartmental, sclerotized, male copulatory organ of bulb shaped (MCO) was a characteristic for family *Diplectanidae*. The morphological identification of this species is based on the vagina, shape and size of the MCO, dorsal and ventral bars, squamodisc, the marginal hooklets , dorsal and ventral hamuli and the squamodisc's rows of elements number. based on the morphological and parasitological characters, such monogenea can be classified and related to family Diplectanidae (Plate2).

3) *Benedenia epinepheli* (Yamaguti 1937). A monogenean fluke collected from the gills of *E. aeneus.* It is dorso-ventrally flattened, elongated in body shape, two pairs of eyes were present, posterior pair is larger than the anterior one. The anterior end has one pair of suckers. The enlarged posterior end armed with disc like opisthaptor with hooks (Plate3).

4) *Pseudorhabdosynochus epinepheli* Yamaguti,1938. Like most monogeneans, it is flat, The head is located interiorly with head glands and four oculi, the body is elongated with a posterior haptor. Digestive system characterized by muscular pharynx anteriorly, and two lateral intestinal branches (or caeca), the anus is absent. The haptor which present in the posterior part of the body, it used for the attachment to the host. The haptor includes two lateral (dorsal) bars, sclerotized elements (ventral bar), fourteen hooklets and two dorsal and two ventral hooks. The reproductive system consists of a single testis and a single ovary. The testis is posterior to the ovary (or germarium) and loops around the right intestinal caecum. Male copulatory organ is sclerosis, or "quadriloculate organ", which is bean shape contain 4 internal chambers (Plate 4).

5) Unidentified monogenean found between gill filaments of *A. regius* (Plate 5).

Prevalence of monogenean infestations among the examined fishes:

Table (1) The total monogenean infestation among all examined fishes was 45%. It was 66, 36 and 33% in *A. regius, E. aeneus* and *D. labrax* respectively.

Table (2) showed that the seasonal prevalence of monogenetic trematode infestations among all

examined fishes was 58.67% in spring, 56% in winter, 37.33% in summer and 28% in autumn. The seasonal prevalence of monogenetic trematodes infestations among *A. regius*, *D. labrax* and *E.aeneus* which were 56, 8 and 20% in autumn, 84, 56 and 28% in winter, 76, 64 and 36% in spring and 48, 4 and 60% in summer respectively .

Result of identification of family Diplectanidae using PCR:

The ITS-1 amplicons size were (~650 bp), and no detected size variation on agarose gels (Photo. 1), while (Lane 3, 4 and 5) among any of the family Diplectanidae samples representing different host species *A.regius*, *D. labrax* and *E. aeneus.*

Histopathological examination of the infested fishes:

Plate (6) showing the gills of *D. labrax* infested with monogeneasis showing congestion of blood vessels, hyperplasia of lamellar epithelial cells, severe mucinous degeneration and massive leucocytic infiltration. Gill lamellae of *E. aeneus* infested with monogeneasis showing interstitial edema, congestion, degeneration, lamellar epithelium necrosis and adhesion of lamellae. Lamellar epithelium necrosis and vacuolar degeneration was also observed.

Table (1): *Prevalence of monogenean infestations among the examined fishes:*

Fish species	No. of examined fish	No. of infested fish	%
Argyrosomus regius	**100**	**66**	**66**
Dicentrarchus labrax	**100**	**33**	**33**
Epinephelus aeneus	**100**	**36**	**36**
Total	**300**	**135**	**45**

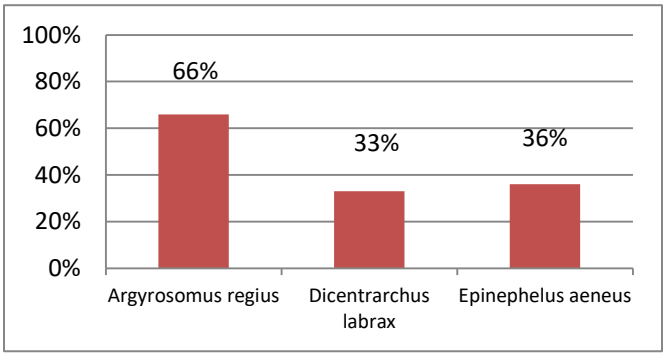
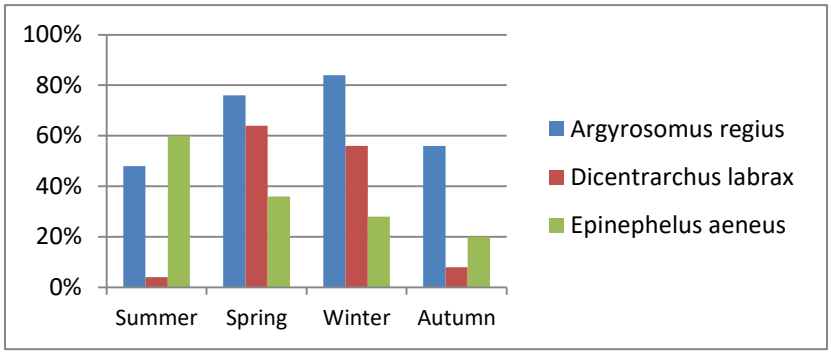


Table (2): Seasonal prevalence of monogenetic trematode infestations among the examined fishes:

Season	Autumn		Winter		Spring		Summer		Total(n=300)	
	No. infested	%	No. infested	%	No. infested	%	No. infested	%	Total no. of infested	%
Argyrosomus regius(n = 25)	14	56	21	84	19	76	12	48	66	66
Dicentrarchus labrax (n = 25)	2	8	14	56	16	64	1	4	33	33
Epinephelus aeneus(n = 25)	5	20	7	28	9	36	15	60	36	36
Total (n = 75)	21	28	42	56	44	58.67	28	37.33	135	45



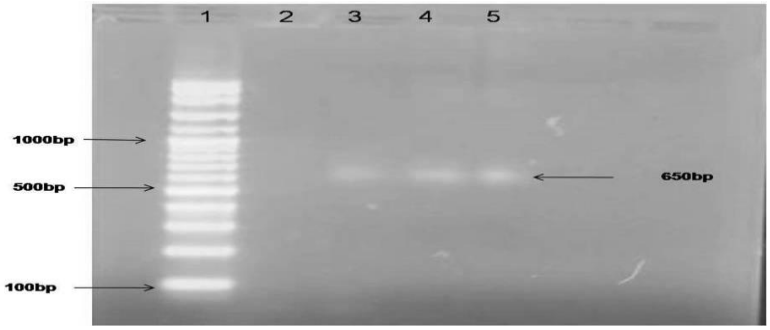


Photo. 1): A representative gel displaying the ssrDNA analysis of ITS-1 region from individual adult specimens of Diplectanum spp. lane 3, 4 and5 at 650 bp. Lane 1 represents the 100 bp DNA ladder as a marker (bp).

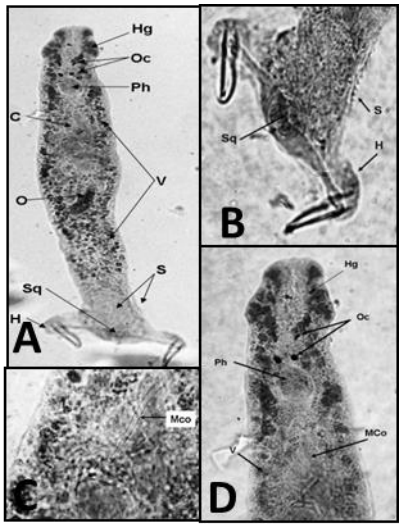


Plate (1): A. Diplectanum sp. : Hg: Head glands; Oc: Oculi; Ph:Pharynx; C: Copulatory organ; O: Ovary; T: Testes; V: Vetillaria; H: Haptor; Sq: Squamodiscs; S: Spine, B. Anterior part : Hg: Head glands; Oc: Oculi; Ph:Pharynx; Mco: Male copulatory organ; V: Vetillaria, C. Mco: Male copulatory organ, D. Posterior part: S: spine; H: Haptor; Sq: Squamodiscs

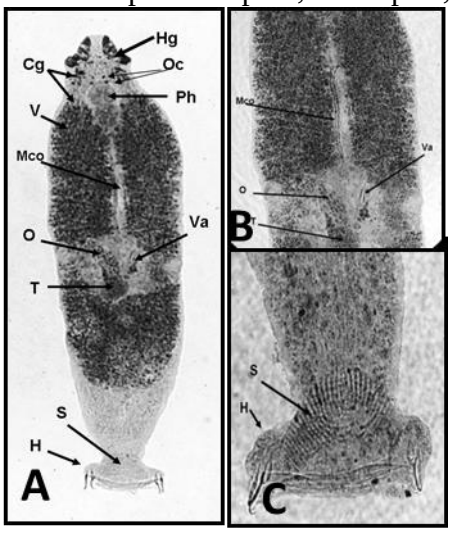


Plate (2): A. *Diplectanum sp*. : Hg: Head glands; Oc: Oculi; Ph:Pharynx; Mco: male copulatory organ; Va: Vagina; O: Ovary; T: Testes; V: Vetillaria; H: Haptor; S: Squamodiscs, **B.** Copulatory organs: ; Mco: Male copulatory organ; Va: Vagina; O: Ovary; T: Testes, **c.** Posterior part : H: Haptor; S: Squamodiscs

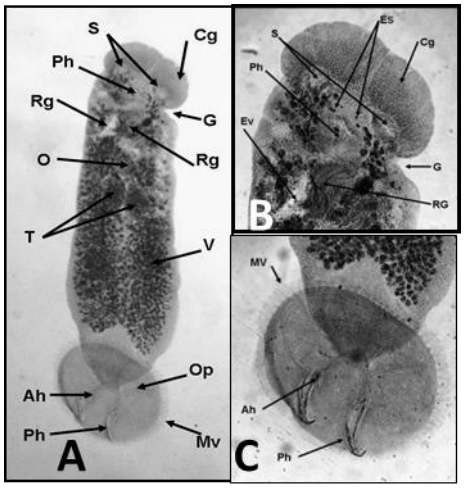


Plate (3): A. Whole adult monogenea *Benedenia epinepheli* : Cg: Cephalic gland; S: Sucker; Ph: Pharynx; G: genital pore; Ev: Excretory vessel; Rg: region of genitalia; O;: Ovary; T: Testes; V: vetelline glands; Ah: Anterior hook; Op: Opisthaptor; Ph; Posterior hook; Mv: marginal valve, **B.** Anterior end : Es: Eye spots; Ev: Excretory vessel, **C:** Opisthaptor: Mv: marginal valve; Ah: Anterior hook; PH: Posterior hook.

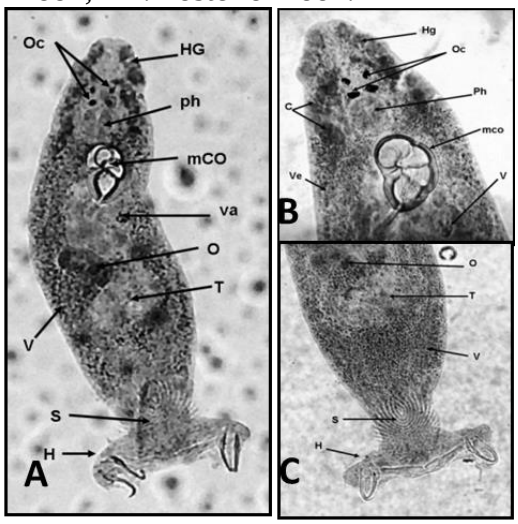


Plate (4): A. *Pseudorhabdosynochus epinephe* (D.hargisi), HG: Head glands; Oc: Oculi; Ph:Pharynx; mco: male copulatory organ; va: vagina; O: ovary; T: testes; V: vetillaria; S: squamodiscs; H: haptor, **B.** Anterior part, HG: Head glands; Oc: Oculi; Ph:Pharynx; C: cephalic glands; mco: male copulatory organ; V: vagina; Ve: vetillaria. C. Posterior part, O: ovary; T: testes; V: vetillaria; S: squamodiscs; H: haptor.

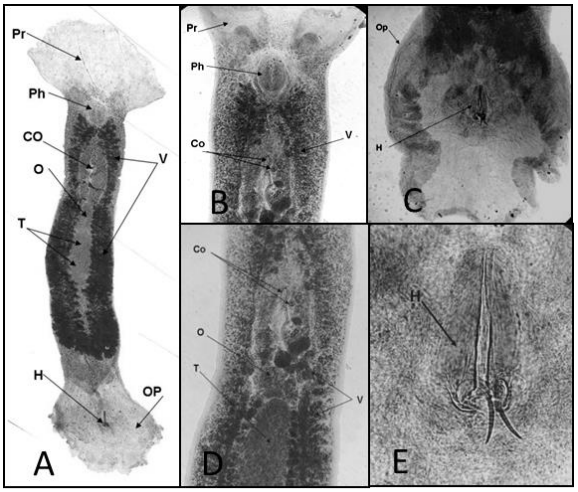
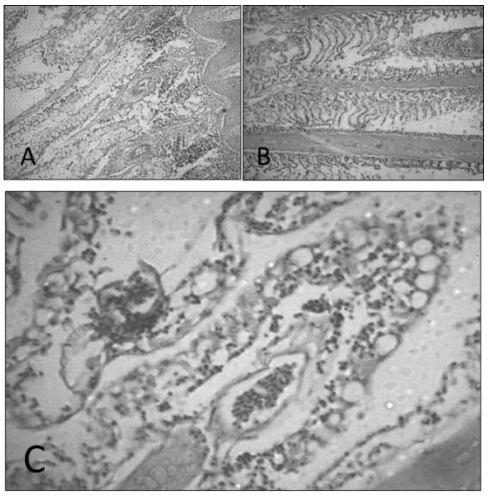


Plate (5): **A.** unidentified monogenia: Pr: Prehaptor; Ph: Pharynx; Co: copulatory organ; O: ovary; T: Testes; H: hooks; OP: Opethohaptor; V: Vitellaria, **B.** Pr: Prehaptor; Ph: Pharynx; Co: copulatory organ; V: Vitellaria, **C.** H: hooks; OP: Opethohaptor, **D.** Co: copulatory organ; O: ovary; T: Testes; V: Vitellaria. **E.** H: hooks



(Plate. 6): **A.** Gills of *D. labrax* infested with monogeneasis showing congestion of blood vessels, hyperplasia of lamellar epithelial cells, severe mucinous degeneration and massive leucocytic infiltration. **B.** Gill lamellae of *E. aeneus* infested with monogeneasis showing interstitial edema, congestion, degeneration, necrosis of lamellar epithelium and adhesion of lamellae. **C.** Gills of *A. regius* infested with monogeneasis showing vacuolar degeneration and necrosis of lamellar epithelium

Discussion

The present study deals with most of different monogenean parasitic diseases among naturally infested *Argyrosomus regius*, seabass (*Dicentrarchus labrax*) and white grouper (*Epinephelus aeneus*) which were caught from different sites of Mediterranean Sea in relation to the seasonal prevalence.

The main clinical picture observed in infested *A. regius*, *D. labrax* and *E. aeneus* with monogenean infestations were surface swimming, distress, excessive mucus production, emaciation, sluggish movement and rubbing the body against sides of aquaria and hard objects to get rid of the irritation resulted by the parasites. Surface breathing (fish gathered at water surface with gulping the air oxygen. Opercula were bulging. These results were in agreement with ***Osman (2005) and Mohamed et al. (2015).*** These findings may be attributed to the low respired oxygen because of the destructed gill epithelium due to fixation, attachment, feeding activity, and locomotion of monogenea and the resulted massive destruction of respiratory epithelial cell ***(Eissa, 2002).***

Regarding the postmortem examination of the infested *D. labrax*, it was revealed excessive mucous secretions with marbling appearance of gills, the gill tips were necrotic and sticked with grayish coloration. Excessive mucous secretion may be a defense mechanism against the infestation and to minimize the irritation. These signs agreed with those reported by ***Maather El-Lamie (2007).***

Based on the parasitological examination, the isolated monogeneans were identified as family Diplectanid, *Benedenia epinepheli* Yamaguti,1937 and *Pseudorhabdosynochus epinepheli* Yamaguti,1938 according to the description of ***Beverley-Burton and Suriano (1981), Kritsky and Beverley-Burton (1986), Bu et al. (1999), Santos et al. (2000), Jithendran et al. (2005) and Jean-Lou (2009)***

Diplectanium sp. was collected from the gills of *D. labrax* and this was in agreement with ***Banu and Zafer (2012)*** who isolated *Diplectanum aequans* from *D. labrax* and ***Ola Abu Samak and Ashraf (2008)*** who isolated *D. aequans* and *D. laubieri* from *D. labrax.*

Regarding monogenean infestations among *D. Labrax*, the total prevalence was (33%). This result was lower than that reported by ***Oktener et al. (2009)*** and ***Banu and Zafer (2012)*** which were 100% among sea bass (*D. Labrax).* Furthermore, ***González-Lanza et al. (1991)*** detected prevalence as 80.64% among sea bass (*D. Labrax)*; while, ***Dezfuli et al. (2007)*** reported the prevalence as 73.6% among sea bass (*D. Labrax*).

Regarding to the monogenean infestations among white grouper

(*E. aeneus)*, the total prevalence was (36%). This result was nearly similar to that obtained by ***Bayoumy and Hanadi Baghdadi (2013)*** who isolated Diplectanidae from *Epinephelus tauvina* and found prevalence as 33.3%. The difference may be due to the different localities from which fish samples were obtained.

Regarding to the seasonal prevalence of monogenetic trematodes, the highest was in spring 58.67% followed by winter 56% then summer 37.33% and autumn 28%. *A.. regius* showed the highest rate in winter 84% followed by spring 76% then autumn 56% and the lowest in summer 48%. This result disagree with ***Ternengo et al. (2010),*** who recorded that, the maximum prevalence of *Sciaenocotyle pancerii* among *A. regius* was in November and December. In *D. Labrax*, the peak was obtained in spring 64% followed by winter 56% and autumn 8% then summer 4%. This result disagree with ***Ola Abu Samak and Ashraf (2008)*** who recorded *D. aequans* among *D. labrax* and founded that the highest season was winter 49.9% and the lowest was in summer 9.9% and with ***González-Lanza et al. (1991)*** who recorded that the maximal infection levels of *Diplectanum aequans* and *D. laubieri* among *D. labrax* occurring in winter. In *E. aeneus* the highest season was summer 60% followed by spring 36% and winter 28% and the lowest was autumn 20%**.**

Regarding the identification of Diplectanid monogenean parasite using PCR, the nomenclature and taxonomy of Diplectanid species has since become controversial and confusing ***(Beverley-Burton and Suriano, 1981 and Wu et al., 2005).*** Therefore, the molecular methods established here may provide useful tools for future identifcations of specimens from a wide range of fish species and geographical origins. In this study, molecular identification of *Dipletanum* spp. using PCR analysis of the ssrDNA (ITS1) (size 650 bp) are a common molecular identification. The ITS-1 amplicons of size (~650 bp) were agree with ***Li et al. (2005)*** who found it (from 180 bp to 780 bp). They used ITS-1 amplicons size for species-specific PCR RFLP analysis of the D1- D3 domains analysis for Diplectanid species.

Although morphometric keys are available for the identification of adult specimens of Diplectanid spp., no such keys are available for the identification of larval stages. Hence, the PCR analysis approaches may provide useful tools for the accurate identification of different species of *Diplectanum* (irrespective of developmental stage), providing a foundation for investigating their ecology (e.g., host-parasite relationships and host preference) and population genetic

structures and for controlling the diseases they cause.

In the present study, histopathological changes due to monogenean infestations of gills of the examined fishes were congestion of blood vessels, hyperplasia of lamellar epithelial cells, degeneration, necrosis of lamellar epithelium and adhesion of lamellae, vacuolar degeneration. This result was in agreement with that obtained by ***Paperna (1980), Badawy (1994), Osman (2005) and Maather El-Lamie (2007).***

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