



## Digenean *Transversotrema haasi* (Witenberg, 1944) infection in cultured *Dicentrarchus labrax*, and in vitro anthelmintic assays

**Amr Fadel\***

Laboratory of Fish Diseases, Aquaculture Division, National Institute of Oceanography and Fisheries, Egypt.

**\*Corresponding author**

Amr Fadel  
Laboratory of Fish Diseases, National Institute of Oceanography and Fisheries, Alexandria, Egypt. ElAnfoshy, Qyiet Bay Castle, Alexandria, Egypt.  
NIOF, Zipcode: 21556  
Telephone NIOF: +203 4807138 & 4807140  
Mobile no: 202-01008580037  
Af.morsy@niof.sci.eg  
fadelniof@gmail.com  
&orcid.org/0000-0002-8177

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**Abstract**

A little knowledge still available for fish digenean, although some of them have pathogenic and economic importance. Fish morbidity and mortalities were reported in two fish farms culturing *D. labrax* fingerling with the primarily isolated parasites belonged to fish digenean trematodes. Therefore, this study was directed mainly to investigate the main causative digenean parasites. Additionally, the ecological water parameters were reported, and the anthelmintic efficacy using levamisole was evaluated. The prevalent digenean parasite was identified Digenean, *Transversotrema haasi*, an extra-intestinal digenean that was mainly isolated from gill and skin. The infected *D. labrax* exhibited abnormal external, and internal lesions indicative of deteriorating health status. The recorded infection rates were 48.67 and 38.67%, parasitic intensities, 6.14 and 2.4 and mortalities rates 28 and 19.33% in Manzala, and Wadi-mariot respectively. The recorded ecological parameters were ranged from 14.22 - 29.7 °C temperature, 7.49 - 8.36 pH, and 13.54 - 34.36 ppt for salinity. Besides, the in vitro therapeutic baths with levamisole were dose-dependent, with the highest anthelmintic efficacy at higher doses effective against digenean *T. haasi*. Higher anthelmintic efficacy was obtained at 70 µg/l levamisole concentration, the lowest mean viability 0.33, the highest parasitic mortality rate 81.81% occurred at the lowest exposure time. Although the anthelmintic efficacy of the levamisole bath has been proven, other friendly environmental agents of plant origin should be taken under consideration to avoiding potential environmental and human hazards.

**Keywords:**

Digenean; Outbreak; *D. labrax*; levamisole; Assays.

## INTRODUCTION

Various significant challenges against aquaculture development and

productivity are out there, that requiring the more understanding of frequent problems related to management and diseases problems (Islam et al., 2020, Abdel-aziz et al., 2020).

Marine fishes are receiving widely commercial importance; however, it faces many obstacles including infectious diseases, that constrain the expansion of the aquaculture industry worldwide (Bessat and Fadel, 2018; Fadel et al., 2020; Aly et al., 2021). Parasitic disease outbreaks have become a clear threat, stems directly from its pathological effect, and indirectly through its impacts on fecundity, productivity, and economic situation (Przybyla et al., 2014; Eichner et al., 2018).

Parasitic trematodes of monogenean and digenean orders are considered serious pathological problems facing the aquaculture sector (Alvarez-Pellitero, et al., 2004). They affect fish of various habitats and environments including freshwater, marine, and brackish (Buchmann and Bresciani, 2006). Different species of fish trematodes have been isolated locally, and worldwide. *Diplomonorchis sphaerovarium* was isolated from *S. testudineus* (Fernandes et al., 2004), *D. Hopkins*, and *D. catarinensis* from *M. furnieri* (Amato, 1982), *D. leiostomi* (Kohn et al., 1982), and *Boridia grossidens* (Fernandes et al., 1985). Other species were also identified that include *T. praeterita*, *P. panamense*, *D. sphaerovarium*, *M. crassulata*, *D.*

*sphaerovarium*, and *T. patialense* (Erik 2002; Morsy et al., 2013; Abou Zaid et al., 2018). In Egypt, *Derogenes varicus* was identified from *M. surmuletus* (Amel et al., 2009; Abou Zaid et al., 2018), Hemiurata suborder species (Al-Zubaidy and Mhaisen, 2011), *A. secundus*, *C. serranis*, and *P. parupnai* were isolated from *M. labrax* (Heba and Walaa, 2012).

Fish infected with trematodes exhibited signs that include scratching, excess mucus, lethargy, anoxia, and loss of appetite (Eissa et al., 2017; Aly et al., 2020). In severe infection cases, opacity, ulcers, hemorrhages, in addition to histopathological alterations of focal hyperplasia, lamellar fusion, hemorrhages, and inflammatory infiltration also appeared in the infected gill tissues (GonzálezLanza et al., 1991; Woo 2006).

To improve the efficiency of parasite treatment, coordinated treatment measures are recommended which have been successfully applied in controlling parasites (Bumgarner et al., 1986; Costello 2004; Kelly et al., 2010). Nevertheless, the lack of understanding of the effective treatment protocols has been implicated in reduced management efficiency (Bravo et al., 2014). Moreover, the extensive misusing and overdosing of chemotherapeutic agents

resulted in the emergency of antimicrobial resistance phenomena in environment, human, and animal products (Cabello, 2006).

Although the pathogenic importance of some fish digenean, a little knowledge still available regarding its identification, biological, and ecological related features, as well the efficient treatment aspects. Fish morbidity and mortalities were reported in two fish farms culturing *D. labrax* fingerlings in two locations in Egypt. This study was directed mainly to investigate the main causative digenean parasites in Damietta Governorate and Alexandria Governorate. Moreover, the ecological water parameters were reported, and the anthelmintic efficacy using levamisole was evaluated.

## MATERIALS AND METHODS

### 1. Fish sampling

A total of 300 European Sea bass *Dicentrarchus labrax* samples cultured in earthen ponds, with an average weight of  $74.15 \pm 9.24$  g. and length  $17.54 \pm 2.04$  cm. was collected throughout June- December 2020. The first Farm A is located in Damietta, Manzala,  $31^{\circ} 24' 32''$  N,  $31^{\circ} 52' 19''$ , and the second Farm B is located Alexandria Governorate, Wadi-maiot  $30^{\circ} 50' 56''$  N,  $29^{\circ} 36' 42''$  E. Fish samples were then kept in ice boxes and transported quickly to

the Laboratory of Fish Diseases, National Institute of Oceanography and Fisheries, Alexandria. The fish sampling procedures, handling, and examination were conducted according to the addressed legislation of animal welfare guidelines and humane treatment (Ecpakfp 2006 and Branson, 2008).

### 2. Water basic analysis

Representative water samples from the same ponds were collected in plastic bottles and kept in insulated coolers then transported directly to the laboratory. The physical and chemical basic parameters of water quality parameters; water temperature, oxygen, pH, and salinity were measured using a specific Electronic probe, and the testing photometer (APHA 2005).

### 3. Parasitological examination

Gill and skin biopsies and scraping were prepared by dissection of the gill arch, covered with coverslips placed on a slide, submerged in water, and examined freshly under a light microscope and dissecting microscope (Optika). The isolated trematodes were carefully collected fixed in buffered formal saline 3%, and preserved in 1:4 alcohol 70% and 5% glycerin respectively. The fixed trematodes were then stained with Semichon's aceto-carmin and dehydrated in graded concentrations of

alcohol, cleared in clove oil and xylene, and were finally mounted in Canada balsam (Manter, 1961; Cribb et al., 1992; Pirarat et al., 2011). The prevalence rate of the isolated parasites was calculated (Reiczigel and Rózsa, 2005). The mean intensity of infection was represented as a number of parasites per microscopical examination field (Silan and Maillard, 1989).

#### 4. Clinical and Post Mortem (PM) investigation

Throughout the study period and the experimental conditions, the fish were checked visually for behavior abnormalities, external clinical signs such as erosions, ulcers, and the abnormal discoloration of the skin, and gills. Also, the internal post-mortem changes, and the mortalities and morbidities were reported according to Schmidt (1992) Woo (2006), Mahmoud, et al., (2014).

#### 5. In vitro assays of anthelmintic efficacy of levamisole against Digenean, *T. haasi*

##### 5.1. Preparation of levamisole stock solutions

The initial concentration of the commercial levamisole hydrochloride 10 % suspension was diluted in fresh seawater to the required concentrations (Székely & Molnár 1987; Butcher 1993). The tested dilution concentrations of chemotherapeutic

substances were 20, 40, 50, 60, and 70,  $\mu\text{g}/\text{L}$  for 1 h.

##### 5.2. Experimental conduction

A total of 60 infected *D. labrax* fingerlings were collected from Farm A, Manzala. They were anesthetized by TMS (MS-222), before the experimental conduction, gills and skins swabs were collected inspected, and checked for digenean presence, prevalences, and other infectious agents (Bush et al., 1997).

Then the fish were divided into seven groups; G1 negative control, G2 positive control, G3, G4, G5, G6, and G7 exposed to 20, 40, 50, 60, and 70  $\mu\text{g}/\text{L}$  of levamisole, respectively, for 1 hour. For each fish group; 5 ml of the corresponding prepared concentrations of levamisole were poured, then the branchial arch and skin scrapings of the naturally parasitized *D. labrax* was placed and immersed. After submerging, the branchial arches of the different concentrations of anthelmintics were observed at 15-min intervals to detect parasite viability, activity, and mortality. The anthelmintic efficacy was recorded based upon the time taken till 100% of digenean were dead within an hour time span (Zhang et al., 2014; Soares et al., 2017).

##### 6. Statistical analysis

Statistical analysis was made with the computer program **Statistical Package for Social Sciences) SPSS**. Differences among groups were performed with a chi-squared

## RESULTS

### The prevalence of *Transversotrema*

#### *haasi* in *D. labrax*

Higher infection rates, parasitic intensities, and mortalities of 48.67, 6.14, and 28 were recorded in Farm A, Manzala than Farm B, Wadi-mariot of 38.67%, 2.4, and 19.33% respectively (**Table 1**).

### The parasitological examination

The isolated parasites were identified as *Transversotrema haasi* (Witenberg, 1944a), belonged to, digenetic trematodes, Digenea, Transversotrematidae, Platyhelminthes. This is according to the distinct morphological features as the following: the body of the digenetic trematode is elongated, flattened, transversal lancet with eye-spots, small ventral sucker, absence of oral sucker, lobed testis, and the presence of only a single ovary. The identified developmental stages were mostly adult and often with the nearby smaller stages, and mainly recovered from the skin and gill (**Figure 1**).

test using the SAS package (SAS 2004). Levels of significance were set at ( $p < 0.05$ ) as indicated by an asterisk (\*) in figures and tables.

### The clinical signs and post-mortem examinations

The infected *D. labrax* showed abnormal swimming, debilitation, decreased appetite, and sluggish behavior. Besides, skin darkness, gills paleness, excess mucous secretion, congestion, and hemorrhage may also be exhibited. The internal organs often appeared anemic, with pale liver, and spleen, and congested kidney (**Figure 2**).

### Physiochemical water parameters

The recorded water quality parameters were significantly differed ( $p \leq 0.05$ ) among the two locations throughout the study period. Overall higher levels were recorded of water temperature 26.16 °C, salinity 30.34 ppt, ammonia 0.46, pH 8.02, and lower oxygen tension 5.37 in Farm A, Manzala. On the other hand, decreased levels in the Farm B, Wadi-mariot were reported of temperature 24.85 °C, salinity 14.51 ppt, ammonia 0.13, pH 7.45, and higher oxygen tension 7.61 (**Table 2**).

**Table 1:** The prevalence of *T. haasi* in examined *D. labrax* in the farms under study (Farm A, Manzala, and Farm B, Wadi-mariot).

Location	No. of examined fishes	No. of the infected fish	Prevalence (%)	Parasitic intensity	Cumulative mortalities (%)
Farm A	150	58	38.67	2.4	19.33
Farm B	150	73 *	48.67*	6.14*	28*

\*: Averages in the same column having different superscripts were significantly different ( $P \leq 0.05$ ).



**Figure 1:** (A&B): showing non-stained digenean, *Transversotrema haasi* skin scraping from *D. labrax* (X10), (B) carmine stained *T. haasi* (X 200). The scale bar is 500  $\mu$ M



**Figure 2:** *D. labrax* fingerlings infected with *Transversotrema haasi*, showing debilitation, erosions, and anemic discoloration of the internal organs. The scale bar is 1 cm.

**Table 2:** The physiochemical parameters of the collected water samples in Farm A, Manzala, and Farm B, Wadi-mariot.

	Temperature (°C)	Salinity (ppt)	Ammonia (ppt)	pH	Oxygen (mg l <sup>-1</sup> )
Farm A	26.16	30.34	0.46	8.02	5.37
Farm B	24.85*	14.51*	0.13*	7.45*	7.61*

\*: Averages in the same column having different superscripts were significantly different ( $P \leq 0.05$ ).

### The anthelmintic efficacy of levamisole against *T. haasi*.

Levamisole application exhibited an exponential efficiency that correlated with its increasing dosage rates. Generally, the higher doses led to the subsequent decreased parasitic viability and increased parasitic mortality rates. Thus, in a lower concentration of 20 µg/L levamisole and the highest exposure time, the decreased anthelmintic efficacy and higher parasitic

viability were reported. Meanwhile, from 40 µg/L levamisole elevated efficacy, decreased parasitic viabilities, higher parasitic mortalities, and lower exposure time which were the best in 60 µg/L, and 70 µg/L levamisole. Accordingly, at 70 µg/L levamisole concentration, the lowest mean viability 0.33, the highest parasitic mortality rate 81.81% were reported (**Table 3**).

**Table 3:** The effect of different concentrations of levamisole on the viability and mortality of *T. Hassi* in the infected *D. labrax*.

Exposure time	G2 <sup>†</sup> + Control		G3 20 µg/l		G4 40 µg/l		G5 50 µg/l		G6 60 µg/l		G7 70 µg/l	
	Viable <sup>‡</sup>	Dead <sup>§</sup>	Viable	Dead								
0 hr	9.67	0	8.67	7.6	10.33	3.2	10.33	9.68	10.67	15.62	11	18.18
15 min.	9.67	0	8.33	12	7	32.26	5	51.61	6	37.5	5	39.39
30 min.	9.67	0	8	27	5.67	45.16	4.67	58.06	3.33	46.88	4	48.48
45 min.	9.67	0	8	27	5.33	48.39	3.33	67.74	2	75	0.33	81.81
1 hr.	9.67	0	7.33	30.77	5	54.84	1	87.10	0.33	90.63	0	0

<sup>†</sup>: Mean skin and gill scrapings of 10 infected *D. labrax*, <sup>‡</sup>: Mean parasitic intensities of *T. Hassi*, <sup>§</sup>: Mean parasitic mortality (%).

## DISCUSSION

Infectious parasites are of great pathogenic, biological, ecological, and economic importance for marine habitats (Cribb et al., 2002; Carvallho et al., 2014; Abou Zaid et al., 2018). Numerous parasitic trematodes species were reported in a wide range of fresh, marine, and brackish water fish in Egypt, and worldwide, but the knowledge of their pathogenic effects still little and questionable (Fernandes et al., 2004; Amel et al., 2009; Al-Zubaidy and Mhaisen, 2011; Heba and Walaa, 2012; Morsy et al., 2013; Abou Zaid et al., 2018). Digenetic trematodes have indirect life cycles and their outbreaks need other hosts that include crustaceans, snails, and birds (Scholtz and Salgado, 2000). Under optimum favorable rearing, ecological, stress conditions, the life cycle of digenetic trematodes, and their potential parasitic infection was initiated (Woo, 2006; Noga, 2010).

Morphologically, the prevalent digenetic parasite was identified as *Transversotrema haasi* (Witenberg, 1944a). The taxonomical features have belonged to Digenea, Transversotrematidae, Platyhelminthes. The isolated parasite was extra-intestinal digenetic, mainly isolated from gill and skin, and the developmental

stages were mostly adult with the near smaller stages. Their adult and developmental stage are mostly pathogenic for fish (Smith, 1972; Woo, 2006). The prevalence of *T. haasi* in the examined *D. labrax* recorded higher infection rates (48.67, and 38.67%), parasitic intensities (6.14, and 2.4), and (28, and 19.33%) mortalities rates in Farm A, Manzala, and Farm B, Wadi-mariot respectively. The dissemination of digenetic infection from one geographical region to another could be allowed, translocated, and altered through human intervention, piscivorous bird trematodes snails carried with cultured fish stock (Mitchell et al., 2002).

Clinically, the infected *D. labrax* exhibited bad health status, abnormal behavior, and decreased growth performances. Besides, the skin exhibited darkness, excess mucous secretion, and gills paleness congestion, hemorrhagic, and oedematous may also be exhibited. These serious abnormal external and internal postmortem changes indicated the harm, inflammatory, and irritation reaction of the skin-inhabiting *Transversotrema* spp.

*Transversotrematidae* is an ectoparasitic inhabit under the scales of fish. They have leaf-like, transversely elongated with their oral sucker is used for attachment to the host

skin (Angel, 1969; Mills, 1979; Woo, 2006; Abou Zaid et al., 2018). Moreover, the metabolic function and nutrition of digeneans depend on the plasma of blood capillaries, ingestion of blood cells, and in particular, transversotrematidae, their feeding habits were digestion of the host epidermis (Lester, 1980; Woo, 2006).

The ecological parameters recorded throughout the study period could consider other predisposing factors for outbreaks of digenean infections. Throughout different seasons, we recorded water temperature ranges from 14.22 - 29.7 °C, 7.49 - 8.36, and salinity 13.54 ppt- 34.36 ppt. Accordingly, the highest prevalence specifically in Farm A indicates the availability of the ecological, environmental, and biological factors that favor the growth of the primary and the definitive host that assist the survival and spread of trematodes in different localities (Syobodova and Kolarova, 2004). For example, the snail is a major intermediate host, its growth needs water temperature over 17 ° C (Noga, 2010).

Given, the ecological stresses have negative influences on fish homeostasis and could inhibit the fish resistance to infectious pathogens (Small and Bilodeau, 2005). Particularly in Manzala farm, a higher infection rate of 48.67% was reported,

where, higher water temperature 26.16 °C, salinity 30.34 ppt, ammonia 0.46, pH 8.02, and lower oxygen tension 5.37 were recorded. The higher water temperature and ammonia levels cause decreased oxygen consumption, and subsequent delayed innate immunity to equilibrate these higher levels (Meyers et al., 1994; Noga, 2010). Moreover, the higher ammonia values in the second Farm A, could reflected the occurrence of the poor sanitary conditions, and microbial load (Santhosh and Singh, 2007). Further, the recorded water parameters were suitable for the growth of trematode and their propagation between their definitive and intermediate hosts (Syobodova and Kolarova, 2004; Noga, 2010).

Importantly, the integrated principles of pest management advocate the limitation of pesticide usage to avoid the development of resistance and minimize environmental residues (Brooks, 2009; Burrige et al., 2010). Most chemical and husbandry practices were used to maintain the parasitic infestation levels at their lowest permits following each country's authorities (Helgesen et al., 2015). However, these managing tasks are greatly complicated by the limited licensed anthelmintic drugs (Morales-Serna

et al., 2018; Zuskova et al., 2018). In our study, we reported the efficient lowest concentration of levamisole at which efficient control was achieved in the form of decreased parasitic load and increased fish health status. It was observed that the increasing concentration dose of levamisole, was corresponding to increased anthelmintic efficacy of the therapeutic agent. Thus, the higher anthelmintic efficacy was obtained at 70 µg/L levamisole concentration, wherein, the lowest mean viability 0.33, the highest parasitic mortality rate 81.81% has been recorded. Higher concentrations of 100 and 125 mg/L levamisole were used by **Alves et al., (2018)**, which cause parasites inactivity and mortality just after 20 min exposure time, and 88.2% efficacy. Levamisole can block the neuromuscular junctions and stimulated both sympathetic and parasympathetic ganglia causing detachment of the parasites from the host gills (**Martins et al., 2017**). Similarly, **Santamarina et al., (1991)** reported the efficacy of 100 mg/L of levamisole, but at 3-hour baths against *Gyrodactylus* sp. In *O. Mykiss*, a lower concentration of 20–50 mg/L levamisole in 10–18 min baths was effective against *U. vistulensis* in *Silurus glanis* (**Szekely and Molnar, 1990**).

## CONCLUSION

The pathological importance of the digenean *Transversotrema hassi* was indicated in the fish morbidities, mortalities, and clinical lesions. Also, the digenean infection was greatly influenced by the associated ecological factors that favor the growth and outbreaks between hosts. Besides, in vitro therapeutic baths with levamisole were dose and exposure time-dependent, with the highest anthelmintic efficacy was recorded at higher doses. Nevertheless, other friendly environmental natural agents of plant origin should be taken under consideration to avoid environmental and human hazards.

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## الملخص العربي

### انتشار الإصابة بتريماتودا ترانسفيرسوتريما هازى فيأسماك القاروص الأوروبي المستزرع وتقييم معلمي للمكافحة الطفيلية

Amr Fadel

معمل امراض الاسماك- شعبة تربية الاحياء المائية- المعهد القومي لعلوم البحار والمصايد- مصر

على الرغم من الأهمية المرضية لبعض أنواع ديدان التريمتودا التي تصيب الأسماك إلا أنه مازال القليل المعروف عن الصفات المرضية والبيولوجية لها. وقد سجلنا إصابة طفيلية لأصبعيات الأسماك القاروص الأوروبي وأوضح الفحص الطفيلي الأولي أحد أنواع تريماودا الدايجينيا. وعليه، فقد ركزت تلك الدراسة على معرفة نسبة الحدوث الطفيلي، التأثير الإكلينيكي، وكذلك التصنيف الباراسيتولوجي لديدان الدايجينيا وما يرتبط بها من العوامل الأيكولوجية بموقعين مختلفين في المنزلة بمحافظة دمياط، ووادى مريوط بالإسكندرية. والأهم وسيلة مكافحته، وذلك بتقييم الكفاءة العلاجية لأحد المواد المعمول بها دولياً وهي الليفاميزول تحت تركيزات مختلفة 20، 40، 50، 60، 70 ملليجرام/لتر. ثم اختبار مقاومتها للطفيل معملياً في أطباق تحوى مسحات خيشومية وجلدية من الأسماك المصابة مغموسة في المحلول وتحديد الأعلى كفاءة علاجية.

وقد أوضحت النتائج أن الأسماك تحوى طفيل تريمتودا يصنف إلى نوع ترانسفيرسوتريما هازى *Transversotrema haasi*. وهو نوع من التريمتودا غير المعوية يتطفل على الجلد والخياشيم ويؤدى إلى حدوث أعراض سلوكية وإكلينيكية غير طبيعية من هيجان في الجلد والخياشيم، التهابات، وإفرازات كثيفة للمخاط، ضعف الإقبال على الغذاء، ضعف النمو، وتدهور الوضع الصحي للأسماك. وكانت نسبة الإصابة تتراوح بين 48.67 و38.67%، الكثافة الطفيلية 6.14 و2.4، ونسبة النفوق في الأسماك 28 و19.33% في كلا من المنزلة ووادى مريوط على الترتيب. وسجلت معايير جودة المياه أعلاها في موقع المنزلة والتي تراوحت خلال فترة الدراسة من درجة الحرارة 29.7-14.22 درجة سيليزية، الأس الهيدروجيني 8.36-7.49، ودرجة الملوحة 34.36-13.54 جزء في الألف. وقد وجد أن كفاءة الليفاميزول العلاجية تتزايد بزيادة التركيز الذى يصل إلى أحسن كفاءة عند 70 ملليجرام / لتر، وفيه أقل كثافة طفيلية 0.33، وأعلى نسبة نفوق للطفيل 81.81%. وعلى الرغم من كفاءته العلاجية، إلا أنه من الضروري البحث عن الوسائل العلاجية الصديقة للبيئة من المواد الطبيعية لتجنب مخاطر المقاومة الطفيلية، وأثرها على البيئة والإنسان.