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Molecular Identification of *Enterogyrus* sp. Parasite (Dactylogyridae: Ancyrocephalinae) and its Impact on the Health Status of the Red tilapia (*Oreochromis* sp.) in Egypt

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ABSTRACT

Monogenean parasites are flatworms commonly found in fish and lower aquatic invertebrates. The present study was performed to genetically analyze one parasitic species of genera monogeneans belonging to the family Dactylogyridae, based on its morphological structures isolated from the red tilapia fish (*Oreochromis* sp.) in Egypt. Hematological and biochemical parameters were measured in the infested fish. Histopathological examinations of different organs of infested fish were also performed. A total of 126 freshwater fish with an average weight and length of 2.57±0.25 g. and 5.26±0.2 cm, respectively were examined. Fish were randomly collected alive or freshly dead from March to December 2020 from a fish farm in Suez Governorate, Egypt. Naturally, infested fish revealed pathognomonic clinical signs such as excess mucus secretions, opening of the mouth, and in some cases, exophthalmia. An empty, shrinking stomach with pale colorations of the internal organs was depicted during a postmortem examination. The total infestation recorded was 41.27%. The isolated intestinal monogeneans from the red tilapia were *Enterogyrus* sp. according to primary morphological characterizations. Molecular diagnosis (PCR), phylogenetic and sequencing were used to confirm infestation with parasites. Highly significant differences were detected between infested and non-infested samples ($P \leq 0.05$). In addition, histopathological sections of the liver, spleen, and intestine recorded abnormalities occurring due to infestations.

1-INTRODUCTION

Red tilapia (*Oreochromis* sp.) is a freshwater fish with significant economic and development potential. Tilapias are the third most successful aquaculture species in the world after shrimp and salmon. There are many reasons for using red tilapia in aquaculture, such as being easy to cultivate and breed, with a relatively fast growth rate and the ability to resist some environmental conditions **(Koniyo *et al.*, 2020)**.

The class Monogenea is a large group of external or internal parasitic worms that have a direct life cycle with only one final host, commonly fishes and lower invertebrates. These parasites feed on mucus or the epithelial cells of the gills and skin externally, also the intestinal walls or the blood internally. In addition, it may be isolated from

both captive and wild fishes, causing excessive mortalities. After hatching this parasite, larvae search for a host, especially when stress conditions are present such as pollution or overcrowded populations **(Chaudhary *et al.*, 2013)**. The larva is usually a small ciliated microorganism (oncomiracidium) swimming to infect another host **(Mhaisen *et al.*, 2015)**. Species belonging to the genus *Enterogyrus* **(Paperna, 1963)** Dactylogyridae: Ancyrocephalinae, inhabits the digestive system of its fish hosts **(Zhang *et al.*, 2019b)**. Presently, this genus contains twelve known species **(Madanireand Avenant, 2014)**; they could hardly be attached to the intestinal wall by its terminal ends of anchors, causing severe wounds and chronic morbidity to the host intestinal cell wall **(Madanire & Avenant, 2015)**. Their migrations from the external parts of hosts such as gills or skin to the intestinal cavity, where light and oxygen are unavailable and aligned with high acidity, are neither physiologically nor genetically explained **(Zhang*et al.*, 2019a).**

For their rareness, the molecular data on *Enterogyrus* spp. within the nucleotide database needs further study. Few records from Egypt describe *Enterogyrus* sp. **(Khidr, 1990)**. Previous studies on *Enterogyrus* sp. were carried out using the morphology and size of the attachment organs **(Madanireand Avenant, 2014)**. Only three sequences for *Enterogyrus* sp. were recorded in GenBank **(Mendlová *et al.*, 2010;Yurie *et al.*, 2017),** hence future research is required to fill the missing data.

As a result, this study was conducted to evaluate the morphological and genetic characteristics of the *Enterogyrus* sp. infestation and its impact on immunological, biochemical, and histopathological changes in the red tilapia (*Oreochromis* sp.).

2. MATERIALS AND METHODS

2.1. Ethics statement

All the experimental protocols including animals were carried out according to the guidelines of the Ethics of Animal Use in the Research Committee (EAURC), Faculty of Science, Suez Canal University, Egypt, with an approval number (REC 9/10/2022).

2.2. Samples collection

Using nets, 126 red tilapia individuals were collected from a fish farm in Suez City, Egypt from March to December 2020 (50 and 70 mm). Fish were collected live or freshly dead and transferred to the fish pathology lab at the National Institute of Oceanography and Fisheries (NIOF) in Suez City, Egypt, to complete parasitic examinations.

2.3. Clinical signs and Postmortem examinations

Fish samples were examined for clinical signs and postmortem (P.M.) lesions using the methods of **Noga (2010)**.

2.4. Parasitological examinations

Fish samples were examined under a light microscope before decomposition. A single sample was put in a petri dish to complete parasitic examinations **(Amlacher, 1970)**. Parasites fixation and preservation were done according to **Hoffman (2019)**. Parasite

identification was done using the major taxonomic accounts of **Gussev (1985)**, **(Yamaguti, 1985)** and **Pugachev *et al.* (2009)**. Prevalence (P%) was calculated according to the following equation **(Mgbemena *et al.*, 2020)**.

$$\text{Parasite prevalence (P \%)} = \frac{\text{No. of infected fish}}{\text{Total no. of examined fish}} \times 100$$

For molecular identification, samples of monogeneans were carefully collected from the intestine by a needle and forceps, then preserved in 95% ethanol for later processing **(Koskova *et al.*, 2010)**.

2.5. Hematological and biochemical examinations

Blood samples were collected from the caudal vein and divided into two aliquots for hematological evaluations; the first aliquot was transferred into EDTA-labeled blood tubes, for measuring total leukocyte counts (TLC), differential leukocyte counts (DLC), total red blood cells (RBCs), hemoglobin content (Hb), hematocrit test (HCT), mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), and platelet counts according to **Tran-Duy *et al.* (2008)**. The second aliquot was collected in dry, clean tubes (without anticoagulants), and serum was collected for further biochemical tests to determine the liver enzymes, alanine amino-transferase (ALT) and aspartate amino-transferase (AST). In addition, total protein (TP), albumen (ALB), globulin (GL), creatinine (CR), urea (U) and uric acid (UA) levels were measured stepping the procedures of **Blaxhall and Daisley (1973)**.

2.6. Histopathological examination

Samples from the liver, spleen, and intestine were preserved in 10% neutral formalin for 24 hours before being transferred to 70% ethyl alcohol for complete preservation. The complete processing was done according to **Drury and Wallington (1967)** and **Bancroft (1996)**.

2.7. DNA extraction and amplification

After collecting the parasites from the tilapia intestine, the preserved specimens' ethanol was then evaporated in a vacuum centrifuge. The DNA sample was extracted according to the manufacture procedure of Gene JET Genomic DNA Purification Kit (Thermo Scientific, Cat. No. K0721). The LSU region was amplified using primers "C1 (5′-ACCCGCTGAATTTAAGCAT-3′)" and "D2 (5′TGGTCCGTGTTT-CAAGAC-3′)" **(Hassouna *et al.*, 1984;Chisholm *et al.*, 2001)** using the protocol of **Mendlová *et al.* (2010)**; the PCR products were then electrophoresed on a 1%agarose gel, and purified by the manufacture procedure of "Gene JET PCR Purification Kit (Thermo Scientific, Cat. No. K0701)".

2.8. DNA Sequencing

Sequencing was performed using the same primers used in the initial amplification, on "ABI 3730XL DNA" Sequencer at Macrogen sequencing services (Macrogen, Seol, South Korea), using the standard Sanger method.

2.9. Phylogenetic Analysis

The partial LSU rDNA gene sequences were initially aligned and compared to another sequences presented in the "GenBank"for a number of related species (*Pseudempleurosoma*, *Paradiplectanotrema*, *Dactylogyrus*, and *Euryhaliotrema* genera were used as out groups**.** All sequences were aligned using the Blast online tool. The top twenty similar sequences obtained from GenBank were also analyzed (Table 1). MEGA version X was used to analyze aligned sequences using Maximum Likelihood (ML), Maximum Parsimony (MP) and Minimum Evolution (ME). Prior to analysis, an evolutionary model for ML and ME was selected by MEGA version X using the Bayesian information criterion. Support for inferred cluster was obtained through non-parametric bootstrap with 2000 replicates.

2.10. Statistical analysis

The SPSS 20 biostatistics program was used to compare means with independent samples. T-test was used to get the significant values ($P \leq 0.05$) **(Dytham, 2011)**.

3. RESULTS

3.1 Physical parameters and infestations

126 red tilapia individuals (*Oreochromis* sp.) were examined; the average body mass and total length were 2.57±0.25g and 5.26±0.2cm, respectively. The average body mass of infested males and females was 2.61±0.44 and 3.36±0.96 g. respectively, with a total length of 5.59±0.25 and 6±0.79 cm, respectively.

3.2 Clinical signs and postmortem findings

Excess mucus secretions, mouth opening, and exophthalmia were addressed in all infested fish. While, postmortem examinations showed the presence of an empty shrinking stomach with internal organs of pale coloration.

3.3 Parasitological findings

The prevalence of infestations with monogenean parasites in all examined fish was 41.27% (52 out of 126 fish). The most infested fish were males (44 fish) at 84.62 %, while the lower percentage was for females (8 fish), recording a percentage of 15.38%. The morphological examination of the parasite showed that it was *Enterogyrus* sp. belonging to the monogenean group and found in the intestinal cavity. Internal examinations of the intestinal content showed severe infestation of the monogenean *Enterogyrus* sp., either attached to the intestinal wall or free moving inside the intestine.

3.4 Morphological findings

The numbering and main morphological characters of *Enterogyrus* sp. were detected by a light microscope (Optica B-150), which showed the main structures of the

parasite, such as the four eye spots, clearly appearing in the fresh mound slide; clear gut tube in the middle part of the body, and an anchor (hook) in the posterior part which helps in attaching to the host cells, especially inside the intestine (Fig. 1).

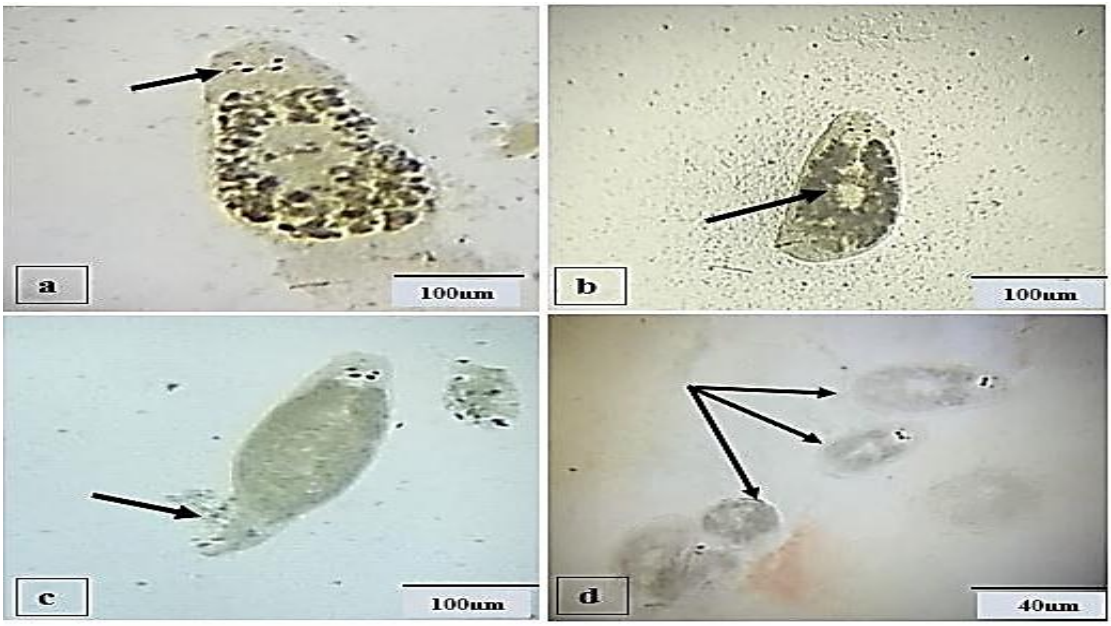

Fig. 1. Different captures of wet mounts to life monogenean parasite *Enterogyrus* sp. inside the intestine of red tilapia, **(a)** *Enterogyrus* sp. showing the four eye spots (black arrow)(100x); **(b)** *Enterogyrus* sp. showing the main internal structures with clear digestive system (black arrow) (100x); **(c)** *Enterogyrus* sp. freshly mount showing the posterior marginal hocks (black arrow) (100x), and **(d)** Heavy infestation with *Enterogyrus* sp. Parasite in the intestine detected in a freely movement (black arrows)(40x).

3.5 Hematological analyses

There were highly significant differences ($P \leq 0.05$) between the infested and non-infested groups in the results of hemoglobin (Hb), red blood cells (RBCs), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), with a significant increase in non- infested group, compared to the infested one. The mean corpuscular hemoglobin concentration (M.C.H.C) results showed a significant difference ($P \leq 0.05$) between the two groups, while the infested group showed a significant increase compared to the other one. The total leukocyte count (TLC) revealed a highly significant difference ($P \leq 0.05$) in the two groups, and the non-infested one increased significantly more than the other one. Other results of differential leukocyte count showed no significant difference ($P > 0.05$) between the two groups, except for the results of monocytes, which significantly increased in the non-infested group. Platelet count (PL) showed a highly significant difference ($P \leq 0.05$) between the two groups, and a significant increase in the non-infested group than the other one (Table 1).

Table 1. Hematological analyses between infested and non-infested groups

Parameter	Infested	Not infested	*P*-Value *
Hb (g/dl)	1.3±0.12	5.6±0.26	0.000*
RBCs (mm^3)	0.26±0.06	1.27±0.12	0.002*
HCT %	3.07±0.26	18±1.15	0.000*
MCV (μm^3)	107.27±1.3	164.67±1.76	0.000*
MCH g/dl	34.13±1.16	49.17±1.17	0.001*
MCHC g/dl	31.77±0.86	27.17±1.17	0.034*
TLC (µl)	5.67±1.3	72±3.61	0.000*
Neutrophils %	51±0.58	52±0.58	0.288
Lymphocytes %	36±1.15	38±1.15	0.288
Monocytes %	9±0.58	11±0.58	0.070
Eosinophils %	1±0	2±0	-
PL (µL)	86±1.15	38±1.15	0.000*

*Indicates significant difference between values of the two groups ($P \leq 0.05$). Hb: hemoglobin, RBCs: red blood cells, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, TLC: total leukocyte count, PL: platelets.

3.6 Biochemical parameters

There was a significant difference ($P \leq 0.05$) between the infested and non-infested groups in the results of blood TP and ALB, with a clear and significant increase in the non-infested one. Moreover, there was a significant difference ($P \leq 0.05$) between the two groups in the results of blood (CR and U), with a significant increase in the infested group than the other one. The results of blood GL and UA revealed a non-significant difference ($P > 0.05$) between the two groups. While, blood ALT and AST values showed a highly significant increase ($P \leq 0.05$) between the two groups and a significant increase in the result of the infested group, compared to the other one (Table 2).

Table 2. Biochemical parameters for infested and non-infested groups

Parameters	Infested	Not infested	*P*- Value*
TP (g/dl)	2.47±0.2	4.47±0.2	0.002*
ALB (g/dl)	1.47±0.15	3.5±0.17	0.001*
GL (g/dl)	1.37±0.15	0.8±0.17	0.066
CR (g/dl)	0.77±0.18	0.27±0.03	0.050*
U (g/dl)	15.33±0.88	10.33±0.88	0.016*
UA (g/dl)	5.4±0.26	5±0.29	0.365
ALT (U/L)	58±1.73	27.67±1.45	0.000*
AST (U/L)	52.67±1.76	26±1.15	0.000*

*Indicates significant difference between values of the two groups ($P \leq 0.05$). TP: total protein, ALB: albumin, GL: globulin, CR: creatinine, U: urea, UA: uric acid, ALT: alanine amino-transferase, AST: aspartate amino-transferase.

3.7 Histopathological results

Liver tissue revealed normal tissue structure with no pathological changes in the non-infested sample (Fig. 2a), while the infested one showed areas of necrosis with drop-out hepatocytes (black arrows) with chronic congestion and hemorrhage (Fig. 2b). Spleen tissue showed normal splenic tissue in the non-infested sample (Fig. 2c), while the infested one revealed chronic congestion with lymphoid hyperplasia (Fig. 2d). Intestinal tissue in non-infested samples showed normal mucous secreting epithelium lining villi with no pathological changes (Fig. 2e), while in infested samplesrevealed spotty infiltration by inflammatory cells not involving the entire mucosa or sub-mucosal thickness with epithelial cell erosion (Fig. 2f).

3.8 DNA findings and phylogenetic results

According to morphological data, the results were insufficientto identify thespecies, consequently, molecular analysis was performed to complete identification.The genetic sequences obtained were closely related to the sequence of *Enterogyrus sp*.2 AS-2010 (HQ010031.1)and*Enterogyrus malmbergi*(MN152976.1) that showed 90.94–% identities with*Enterogyrus* sp. 2 AS-2010 , 90.88 % identity withand*Enterogyrus malmbergi*.This was confirmed by the phylogenetic reconstruction (Fig.3)

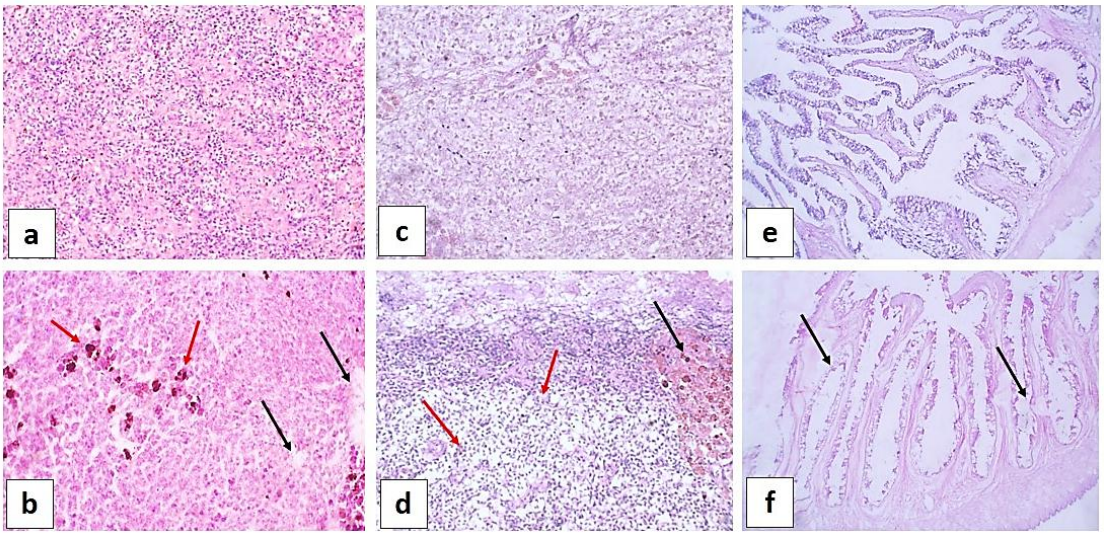

Fig. 2: Histopathological sections of liver, spleen and intestine of red tilapia infested with *Enterogyrus* sp. **(a)**Uniform liver tissue (H&E, 40x). **(b)** Liver of infested fish showed area of drop out hepatocytes; lytic necrosis and evidence of chronic congestion and hemorrhage in the form of hemosiderin-laden macrophages (red arrow) (H&E, 40x). **(c)**Uniform splenic tissue (H&E, 40x). **(d)**Spleen of infested fish showed dilated sinusoids in red pulp (red arrows) and focus of hemorrhage (black arrow) (H&E, 40x).**(e)**Uniform intestinal villi with regular epithelial lining (H&E, 10x). **(f)** Intestinal tissue of infested fish revealed villi retained their regular morphology, nut with areas of epithelial erosions (black arrows). No inflammatory infiltrate could be seen. (H&E, 10x).

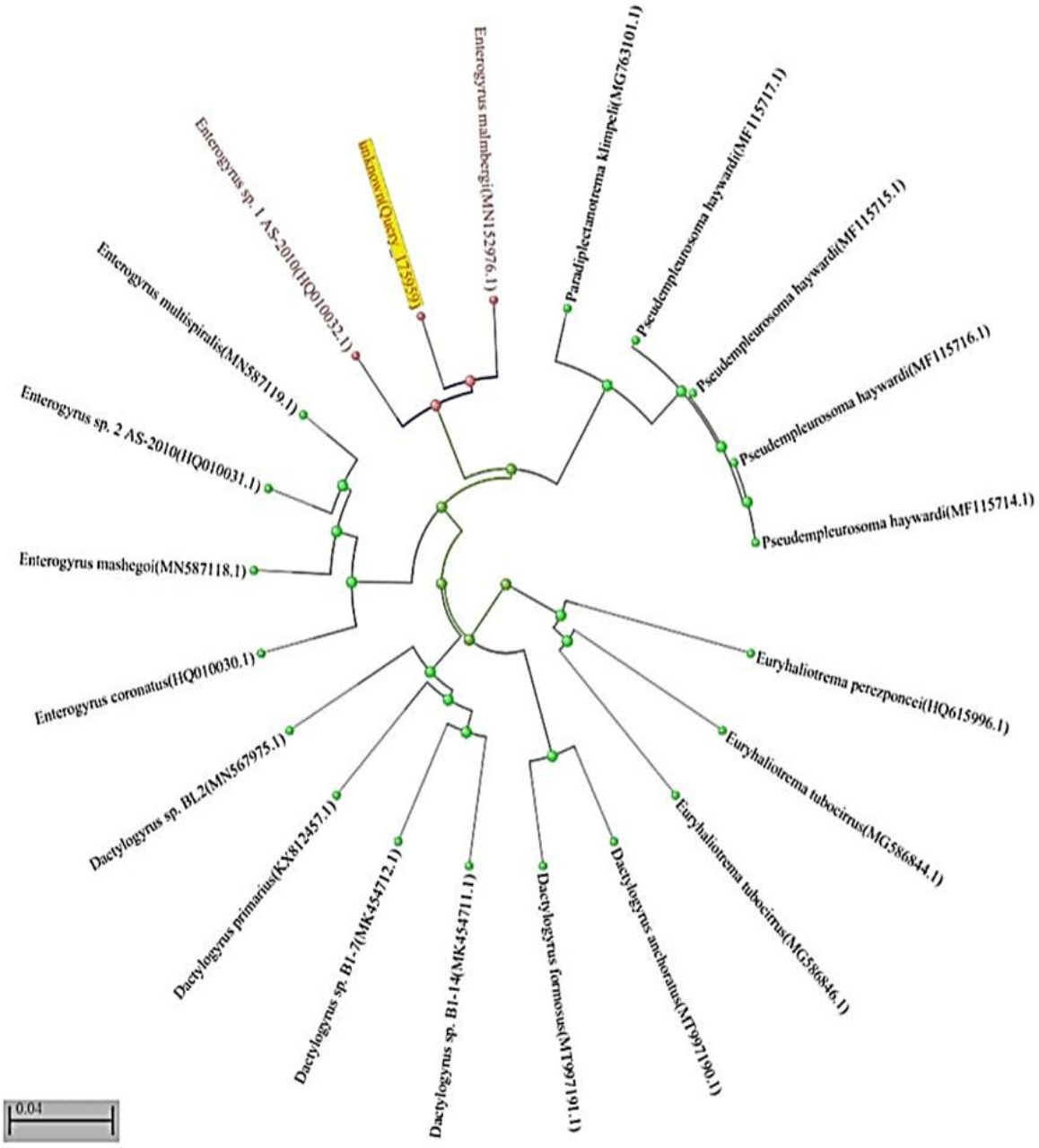

Fig. 3: Phylogenetic tree obtained with Maximum Likelihood analysis. Bootstrap value was 2000 iterations. Species newly sequenced for this study is in Red (Query). The highlighted clusters represent the most similar 20 sequences/species with our submitted query. The Species belonging to *Enterogyrus*, genera. The GenBank accession number for each sequence ID precedes its scientific name.

4. DISCUSSION

The current study established for detection the parasitic infestation inred tilapia (*Oreochromis* sp.) with endoparasitic monogenean (*Enterogyrus* sp.) by basic morphological characters, genetic identification, and the phylogenetic tree. Phylogenetic relationships between monogenean species have been inferred usingmorphological characters **(Pouyaud *et al.*, 2006).**There is little genetic information about *Enterogyrus* sp., so the results completed the previous studies about this species. The clinical signs and the harmful effect detected on the infested fish were explained by the presence of the parasite, which has been observed embedding between the villi of the intestine, causing peritonitis and local damage to the intestinal mucosa due to proteolytic enzymes that are released from some adult worms, degrading the intestinal tissues of the host.**(Luus-Powell*et al.*, 2020b).**

Only three species of *Enterogyrus* sp. are isolated from Western Africa, Egypt, and South Africa **(Eid and Negm, 1987; Khidr, 1990; Pariselle and Euzet, 2009; Madanire-Moyo and Avenant-Oldewage, 2014)**.The adaptation from natural external parasitism to internal or mesoparasitic life makes them interesting and needs more studies to understand the host speciation, which may aid in understanding more about adaptation mechanisms.**(Luus-Powell*et al.*, 2020a).**Host specificity may be resulting from various factors as, phylogenetic, physiological and ecological aspects**(Desdevises *et al.*, 2002; Randhawa and Burt, 2008)**.The majority of parasites represent some degree of host specificity**(Bush and Clayton, 2006)**. Monogenean were selected as a model for studying the manner and processes connected with the evolution of parasite specialization, resulting in host specificity **(Šimková *et al.*, 2002)**.*Enterogyrus* sp. might not be highly host-specific due to its presence in more than one host species, which makes it more interesting to study genetically**(Paperna, 1963; Bilong *et al.*, 1991).**

The total prevalence of Enterogyrosis recorded in this study was 41.27% which was greater than what was recorded by **Eissa *et al.* (2011)** as 13.3% and **Madanire-Moyo and Avenant-Oldewage (2014)** who stated that *Enterogyrus* sp. may be found not high prevalence per host. These variations may be due to different factors affecting disease distribution, such as the nature of fish feeding, fish age, and size, because worms are considered stomach flukes, which require a mature fish with a well-developed digestive system with thicker walls for adaptation and fixation. The parasitological findings in this study were regarded similar to the results described by **Khidr (1990)** and **Eissa *et al.* (2011)** who explained morphologically another intestinal monogenean species (*Enterogyrus cichlidarum*).

Evaluation of blood cells could be useful for the measurement of physiological changes in parasitized fish, and measure the level of damage in the host and predictions for the diseases **(Tavares-Dias and Moraes, 2007).Stosik (2001),** stated that the mechanisms of specific immunity in fishes are not well developed and didn't had an important role such in animals, birds or mammals. Thus, fishes have non-specific immune system plays an important role in the defense against pathogenic

microorganisms. In this study, hematological parameters recorded a significant variations between infested and non-infested groups. There weresignificant decrease in values of total and differential leukocytes count in the infested group than non-infested one, resembling that observed by **Shah *et al.* (2009)**with helminthic infestations in*Schizothorax* spp. and *Cyprinus* spp.habituate the lake Anchar, Kashmir. This study revealed also significantly increase in lymphocytes and eosinophils count in non-infested group than infested one, which may be agreed with what mentioned by **Sexena (1993),** who found similar results in *Heteroponeutus fossilis* (The Asian stinging catfish) infected with cestodes(*Lucknowia indica*). Opposite results recorded by **Ranzani *et al.* (1997)** and **Azevedo *et al.* (2006)** who found that no changes in leukocytes count in *Mugil platanus* parasitized by monogenean, copepods, trypanosomes, and in *Oreochromis niloticus* parasitized by *Trichodina* sp., *Lamproglena* sp., and monogenean respectively.Red blood cells and Hb counts showed significantly decrease in fish infested with Enterogyrosis than non-infested one, leading to anemia as mentioned by**Martins *et al.* (2004).** There were a significant increasein the platelets count in infested group than the other one, agreed with results reported by **Şahan *et al.* (2007)**for European eels. The decreasing of hematological parameters and increasing of the platelets count were due to response and efficiency of cellular immune system toward monogenean infection which is considered as one of defense mechanisms against parasites**(Shah *et al.*, 2009).** Another different observations by **Tavares *et al.* (1999)**, who found no changes in RBC, Hb, MCV, MCH, and MCHC in *P. mesopotamicus* infected by *Argulus* sp. and **Soberon *et al.* (2014)**, who stated that no significant alterations in hematological parameters of *Colossoma macropomum* naturally parasitized by *Anacanthorus spathulatus* (Monogenea: Dactylogyridae) in fish farm in the Peruvian Amazon.

Decreasing of serum total protein and albumin levels in the infested group compared to the non-infested one may be due to stress occurred by monogenean infestation, which result due to loss of appetite in the infested fish and explains the decrease in total protein concentration in many diseased fish**(Patriche *et al.*, 2011)**. Blood proteins acting as buffer to keep hydrogen ion concentration and osmotic pressure in balance **(De Lisle, 1971)**. So, the concentration of total protein decreased in many diseased fishes due to decreasing the capacity of synthesis, reduced absorption or protein loss **(Yang and Chen, 2003)**. That was disagree with what stated by **Nnabuchi, *et al.* (2015a)** who found significantly increased ($P> 0.05$) in the same parameters due to protozoan, cestodes and nematodes infestations of some fish species in Nigeria. AST and ALT liver enzymes, Urea, and other biochemical parameters were significantly higher in the infested group than in the non-infested one, another opposite results were observed by **Osman *et al.* (2009)**who found that Trichodinea affected *Clarias gariepinus* recorded higher levels of serum ALT, AST enzymes, creatinine and Urea values, and **Abou Zaid (2011)** stated that serum ALT, AST, creatinine and urea values were increased in *O. niloticus* and *C. gariepinus*

infected with external parasites as monogenean group. Activity of ALT and AST in the serum of the infested fishes with parasites were increased due toits effect on the parenchyma's tissues and skeletal musculature, which increasing the permeability and cell organelles integrity **(Adamu and Iloba, 2008). Nnabuchi*et al.* (2015b),** found that urea and creatinine levels were increased due to parasitic infestation of catfishes infested with protozoa, cestodes and nematodes,which seems to corroborate with that observed herein.**Adamu and Kori (2011),** stated that, creatinine take the pathway from the muscles after breaking down,reaches to the blood where it is considered as a waste product. It is removed by filtration through the renal tubules of the kidney and excreted as urine which is waste product metabolized in the liver and excreted by the kidney, So the increasing in creatinine levels in infested fish may regarding to alteration of the muscles structure resulting from parasites**(Egbu, 2017).**

Histopathological changes in liver, spleen and intestine of infested group showed acute to chronic signs of inflammations, necrosis and fibrosis that may be a simple way to detect the effect of parasites on the host, in most cases, a normal balance has been achieved between the host cells and the parasites, even when histopathology is an evidencefor parasitism effect **(Feist and Longshaw, 2008)**.

The use of molecular analysis proved to be more effective in detecting this species. The genetic sequences obtained from the Egypt specimens were found to be related to the sequence of *Enterogyrus malmbergi* from China, which was obtained from the stomach of *Oreochromis niloticus***(Zhang *et al.*, 2019a),**as confirmed by the phylogenetic reconstruction. All studies on *Enterogyrus*sp. depended on molecular data are required to accurate determination of Enterogyrids to species level.

5. CONCLUSION

This studyrevealed the molecular identification based on some morphological characters of the parasitic species *Enterogyrus* sp. following the group of monogenean flukes. The parasitic infestations showed significant variations in hematological and biochemical parameters in the infested group than in the non-infested one. That means the presence of parasitic infestation leads to mechanical immune defense from the fish body. Also, histopathological sections for the main immunological organs such as liver and spleen revealed pathological changes as a result of responses toward infestation. Intestinal cells where the parasite habituated showed also inflammations and depletions.

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