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Identification of Caligus parasites infesting *Morone Labrax* and its impact on fish health status and pathological alteration

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ABSTRACT

Objective: The present study was aimed to investigate sea lice infestation including morphometric, molecular identification, disease prevalence and pathological tissue alterations in wild Sea bass *(Morone Labrax)* naturally collected from Ezbet Elborg, Damietta province, Egypt from October 2018 to end of August 2019.
Design: Descriptive study
Fish: 120 wild seabass *(Morone Labrax)*
Procedures: The collected fish from Ezbet Elborg area, Damietta province, Egypt were clinically and parasitological investigated for the prevalence of sea lice parasite with molecular identification of the collected parasite samples with study of the pathological alteration of the affected tissue.
Results: The study revealed that 48 fish (40%) of the examined fish were infested with *Caligus clemensi,* while the higher prevalence recorded during summer season (57%) followed spring (33%), autumn (10%) and no recording during winter. The molecular identification using 18-S rDNA was done with phylogenetic analysis compared with the previously recorded caligus species on database confirming the same results of parasitology. Histopathological examination of gills, buccal cavity revealed that the gill arch contained congested blood vessels beside edema, hemorrhage and inflammatory cells infiltration due to parasitic infestation.
Conclusions and clinical relevance: Caligus infestation in wild sea bass caused by *Caligus clemensi* lead to mortalities due to pathological tissue alterations in gills and buccal cavity.

Keywords: Wild *Morone labrax*, *Caligus clemensi*, molecular identification, histopathological examination

1.INTRODUCTION

Fish are considered one of the most important sources of animal proteins all over the world. Marine fish are preferable than freshwater fish as the former are rich in phosphorus and iodine which are essential for cell metabolism. Parasitic copepods are common crustacean parasites infesting fresh, cultured and wild marine fish [1] Members of Caligidae are the most commonly reported species infesting marine fish known as sea lice, comprises 28 genera and more than 400 species [2]. Genus caligus is the most species within caligidae, according to their mouthparts, the caligids have tubular mouth, their mandibles are flat, long blades with distal end carrying arrow of teeth on one margin [3].

The sea bass is euryhaline (0–40 ppt salinity) and eurythermal (2–32 °C) and is often found foraging in estuaries and lagoons from spring to fall, especially at the juvenile stage. During winter, juvenile and adult sea bass migrate from the coastline to deeper waters, where the temperature is more stable, as they prefer temperatures above 9–10 °C [4]. Sea lice is commonly used to refer to several species of marine ectoparasitic copepods of the family Caligidae (order Copepoda: suborder Siphonostomatoida), prevalence of it increases with higher temperatures [5]. Moreover, sea temperature affects the occurrence and life cycle of caligids [6], the feeding behavior of larval stages may be planktotrophic or lecithotrophic [7], the planktons need sun light for photosynthesis and napulius needs planktons to survive so the caligus spp is believed to live with sufficient light. Caligid parasites present in the environment on wild fish populations will most probably infect the new caged hosts and re-infect wild fish populations. Members of Caligidae are the most commonly reported species infesting marine fish known as sea lice, comprises 28 genera and more but the main difference between the Caligus species are the relative sizes of the four component body parts, particularly the shape and size of the genital complex, length and segmentation of abdomen [8].

Parasitic infestations represent the majority of the known infectious diseases affecting fish, they cause mortality, deformity, weight loss and different clinical abnormalities among the affected fish [9]. Parasitic copepods feed on host mucous, tissues, and blood. In addition, their attachment and feeding activities are responsible for any primary disease that develops [10]. In disease situations, death may be caused by the development of secondary infections exacerbated by stress and the formation of open wounds, osmoregulatory failure, and in the case of the gills, respiratory impairment [11-12-13]. In many cases, increased fibroplasia and spongiosis is noticed within dermal collagenous connective tissue [14]. Parasites of the gills can cause irritation, leading to hyperplasia and increased mucous production, which result in decreased respiration and ion exchanging capabilities [15-16].

2.Materials and Methods

2.1. Sample Collection

A total number of 120 wild seabass *(Morone Labrax)* fish suffering parasitic infestation with recorded mortality were collected during October 2018 to end of August 2019 from naturalfish collection areas in Ezbet Elborg area (wild fish), Damietta province, Egypt. The collected fish were transported to parasitology laboratory, Animal Health Research Institute, Mansoura branches, Egypt in polyethylene bags. The lengths and body weights of examined fish were ranged from 30-60 cm and 200-440g respectively. The collected fish were examined clinically for postmortem lesions according to [17].

2.2. Parasitological examination

The caligus parasites were collected using a fine brush and special needle. Placed into petri-dish containing normal saline and washed several times to get rid of mucus preserved and cleared in lactophenol and mounted by polyvol then, left to dry according to [18-19]

2.3. Molecular identification

2.3.1. DNA extraction, amplification and sequencing of 18srRNA gene

DNA extraction for the isolated copepods was conducted according to the protocol of [20], where the concentration of DNA and purity was determined using Quawell nano-spectrophotometer (Q5000 UV-VIS) (USA). The integrity of DNA was checked using 2% agarose gel electrophoresis. For amplification of 18srRNA gene of isolated copepods, an alignment of sequences of *Lepeophtheirus* species as follow: *(JX896389.1 Lepeophtheirus frecuens, JX896386.1 L. chilensi, AF208263.1 L. salmonis, JX896402.1 L. yanezi, JX896400.1 L. zbigniewi, DQ123831.1 L. hospitalis, DQ123830.1 L. parvicruris, KR048780.1 L. parviventris voucher) and caligus species as followed: (MF077737.1 caligus curutus, EF088406 C. centrodonti, EF088410.1 C. gurnardi, KC569364.1 C.fugu, DQ123833.1 C.clemensi, EF088409.1 C. elongates, KR048777.1, C. puctatus, EF088405.1 C. belones, EF088412.1 C. quadratus, KP681600.1 C. rogercresseyi)*, where a primer sequence for caligadae was manually designed after the alignment; CalF: CAATGATCGAAGATCGAGGTAGTG, CalR: TCTTTGGTTTCCCGGAAGCT. PCR cycling condition was conducted with 2x master mix provided from EmeraldAmp MAX premix (Takara, Japan) and 20 pmol primers with initial denaturation of 95 °C/5 min that was followed by 40 cycles of denaturation (95 °C/50 sec), elongation (72 °C/45sec) and annealing (56 °C/30sec) and the reaction was terminated with final elongation (72° C/9min). After the termination of PCR, gel electrophoresis was performed using 40 l of PCR product on 1.5% agarose gel electrophoresis for visualization of PCR product, where the expected amplicon size of PCR product was standardized against 100bp DNA ladder (Gene ruler, ThermoFisher Scientific). The visualized PCR product was excised using sterilized clean scalpel and transferred to sterilized clean epindorf tube for gel extraction protocol using GeneJET gel extraction kit (ThermoFisher Scientific). After gel purification, sequencing for PCR product was determined by ligating the PCR product with cloning vector (pTZ57R/T) (ThermoFisher Scientific). Clone containing DNA insert was sequenced using forward primer M13/pUCF: GTAAAACGACGGCCAGT and reverse primer M13/pUCR: CCAGTATCGACAAAGGAC. Sequencing was performed using automated sequencer (ABI 377) (Applied biosystem) according to manufacturer instructions.

2.4. Histopathological examination

The affected fish tissue with by caligus parasites including gills and oral cavity were dissected out and fixed in 10% phosphate buffered formalin, then dehydrated in ascending grades of alcohol and cleaned in xylol, then embedded in paraffin wax, cut into thin 5um sections and floated on warm water. The sections were left from the water path on microscope slides, coated with Myers albumin, allowed to dry thoroughly and stained by hematoxylin eosin according to [21]

3.RESULTS

3.1. Clinical examination

The clinical signs in the naturally infested wild fishes (*Morone labrax*) revealed congestion of gills, erosions on hard palate with presence of caligus parasites with no pathognomonic internal clinical abnormalities. Presence of both male and female adult stages of *Caligus clemensi* on hard palate and gills of the infected fish (figure 1)

3.2. Prevalence of Caligus clemensi in seabass (Morone labrax) fish

Table (1) showing the seasonal prevalence *of Caligus clemensi* parasite in wild seabass from October 2018 to end of August 2019 in Ezbet Elborg, Damietta province, Egypt. The study revealed that 48 fish (40%) of the examined fish were infested

with *Caligus clemensi,* while the higher prevalence recorded during summer season (57%) followed spring (33%), autumn (10%) and no recording during winter.

Table 1. Seasonal prevalence of sea lice *Caligus clemensi* parasite among wild seabass (*Morone labrax).*

Season	Examined	Infected fish with *Caligus clemensi*	% of infected
Autumn	30	3	10%
Winter	5	0	0
Spring	15	5	33%
Summer	70	40	57%
Total	120	48	40%

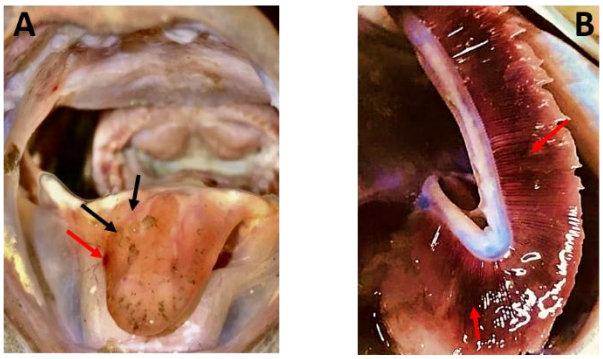
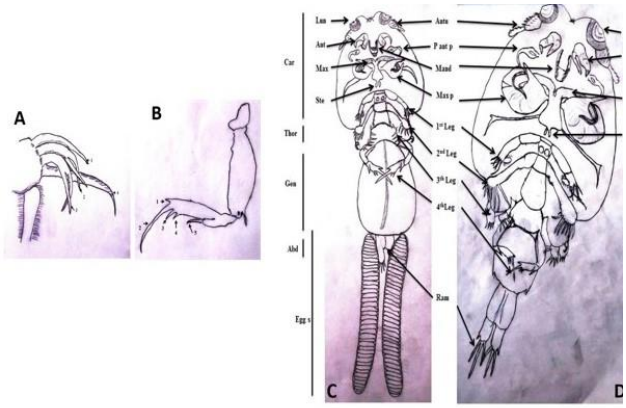


Figure 1. Macroscopic detectionshowing Presence of both male and female adult stages of caligus clemensi on hardpalate of the infected Morone labrax (black arrow) causing erosions and congestion on tongue (red arrow) (A). Congestion of gills Sea bass (Morone labrax) infested with Caligus clemensi parasite causing erosions of gills (red arrow) (B)

Figure 2. Microscopical identification of adult male caligus clemensi (x40).

Figure 3. Microscopical identification of adult female caligus clemens(x40).



Car	Carapace or cephalothorax segment	**Thor**	Thoracic segment
Gen	Genital segment	**Abd**	Abdominal segment
Egg s	Egg sacs	**Lun**	Lunules
Antu	Antennules	**Ant**	Antenna
P ant p	Post antennal precess	**Mand**	Mandibles
Max	Maxillae	**Max p**	Maxillipeds
Ste	Sternal furca	**Ram**	Ramii
1st leg	1st pair of legs	**2nd leg**	2nd pair of legs
3rd leg	3rd pair of legs	**4th leg**	4th pair of legs

Figure 4. Line drawing of male and female.

3.3. Morphological description of parasites

Caligus clemensi Parker & Margolis, 1964 Kingdom: Animalia Class: Crustacea Subfamily: Caliginae, Family: Caligidae Number of parasitological examined parasites: 25

Caligus clemensi either male (Fig 2) or female (Fig3) are identified by 1st and 4th legs. The distal margin of 1st pair of leg are charectrized by presence of setae 1 and 4 undivided and unarmed, setae 2 and 3 bifid (fig 4 A) while 4th pair of legs consists of 3 segments bearing bristilles and 5 setae, 4 seta found in the destal end of the last segment, one in the next. (fig 4 B). On the other hand, male and female were differ in some morphological findings (Figures 4 C and D respectivly). Females was ovate carapace but not longer than remaining body. Frontal plate was broad with anterior grouve. Free thorathic segment was very short and small approximately. Genital segment was oval with curved lateral adges and exceed in length the half carapace. Abdomen was short and somewhat enlarged towards posterior end. Caudal rami were one short and three long with alittle bent and each had bristiles. Two long egg sacs about the same length of body were present. Body ranged from 10- 14mm long (fig 4 C). While male had very broad carapace with enlargement towards posterior end. Free thotacic segment was short. Genital segment was less broad with rounded lateral adges. Abdomen consistes of one segment. were one short and three long with alittle bent and each had bristiles.. Body ranged from 12- 24 mm long (fig 4 D).

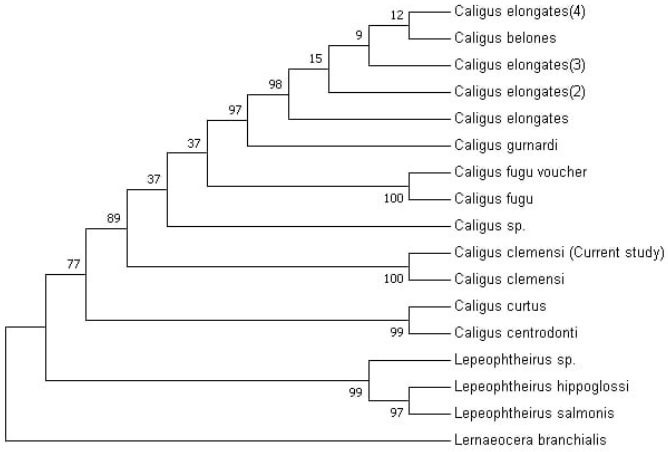


Figure 5. Phylogenetic tree of the 18S rRNA gene obtained *from C. clemenis.*

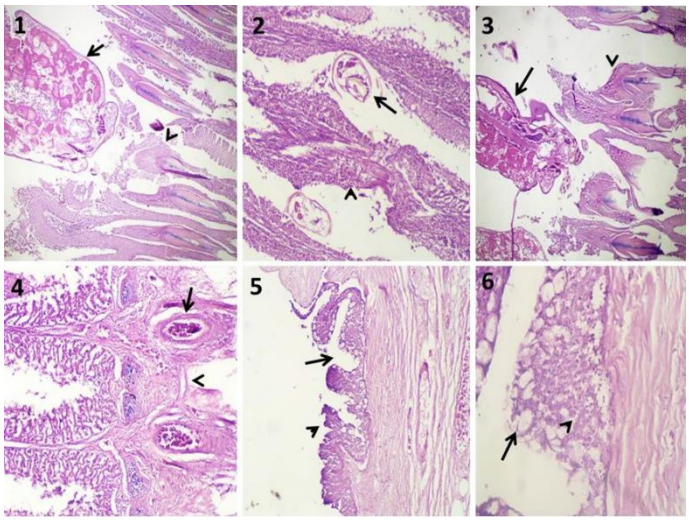
3.4. Molecular identification

The sequencing result of *C. clemensi* produced 1135 bp and had been deposited on genbank with accession number of (MT151385) that was checked with similarity index on genbank and gave 99.74% similarity index with C. clemensi isolated from chum salmon in Canada. As shown in figure 5, phylogenetic analysis showed the presence of two clades; Clade A that contained all caligids species according to Blast result and Clade B that contained all Lepeophtheirus species. Lernaeocera branchialis is assigned as an outgroup. Clade A was divided into two sub-clades; Clade AI and Clade AII. Clade AI was consisted of All caligids species except *C. curtus* and *C. centrodonti* (Clade AII). The current *C. clemensi* showed high similarity index and close relationship between *C. clemenis* isolated for chum salmon and formed a specific sub sub-clade with the other *C.clemensi* species.

3.5. Histopatholothological finding

Parasites are stacked on gill filaments with partial or complete sloughing of their lamellar epithelium which results in denuded filaments (fig.6-1). Sometimes hyperplastic secondary lamellar epithelium in the adjacent area which led to fusion of the filaments were encounted thickened filaments by numerous inflammatory cells and edema beside dilated capillaries were observed. Fragments from the parasite were detected between primary filaments with ulceration and distortion of the adjacent filaments (fig.6-2). Sometimes, heavy parasitic infestation with atrophy, distortion and blunting of the gill filaments could be seen (fig.6-3). The base of primary filaments was covered by sheath from hyperplastic lamellar epithelium. The gill arch contained congested blood vessels beside edema, hemorrhage and inflammatory cells infiltration mainly eosinophil, granular cells in their necrotic muscles (Fig.6-4). Oral cavity showed erosion or ulceration of their lining epithelium beside hyperplastic mucus secreting cells beside numerous inflammatory cells infiltration mainly lymphocytes (fig.6-5 and 6-6).

Figure 6. Photomicrograph from gills and oral cavity of Morons Labarax section



stained with H&E. 1: Gills showing parasite (arrow) sticked on gill filaments with sloughing of the adjacent lamellar epithelium (arrow head) x100. 2: Gills showing fragments of parasitic elements (arrow) with ulceration of primary filaments (arrow head) x100. 3: Gills showing numerous parasites (arrow) with atrophy and distortion of the adjacent filaments x100. 4: Gill arch showing congested blood vessels (arrow) with necrosis, odema and leukocytes in the muscles (arrow head) x100. 5: Oral mucosa showing erosion and ulceration (arrow) of their epithelium x100. 6: High power of the pervious figure to show numerous mucus secreting cells (arrow) and inflammatory cells in the mucosa (arrow head) x400.

4. DISCUSSION

Damietta province is one of the most important area for production of fish in Egypt, caligus is considered important pathogen infesting marine fish. The aim of work is the determination of prevailing Caligus parasite disease affecting *Morone Labrax* fish and their impact on the fish health as well as the study the molecular of parasite. The main clinical signs observed in infested *Morone labrax* with crustacean infestations were excessive mucus production, erosion and ulceration. These signs are as a result of the attachment by means of second pair of the antennae which were inserted into the host epidermal tissue which caused the low respired oxygen of destructed gill epithelium of the parasites. These results are in agreement with those reported by [8-22]. Focal hemorrhage, abrasions on hard palate. These may be attributed to the parasites penetration of hard palate for fed and facilitate the invasion of the opportunistic microorganisms. These results are in agreement with that reported by [23-24]. Emaciation was recorded in M. labrax may be due to crustacean infestation which reduce fish appetite and became off food, this agreed with [25-22]. *Morone labrax* was infested with Caligus parasite with a percent of 40%, the result agreed with that given by [26] from Suez Canal area, while higher than recorded from Port Said province (19%) by [27] and lower than reported by [24]

who reported (47%) from different areas of Suez Canal (Ismailia province). The difference in prevalence of caliguse infestation may be reported due to feeding of fish, light, temperature and degree of water pollution with different locality. This study showed the higher prevalence during summer (57%) followed by spring (33%), autumn (10%) and no recording during winter, the present findings nearly agreed with previous results reported by [28-29-16] in which they recorded the summer season as the highest infection rate and no recording during the winter season, the variation between seasons may be attributed to seabass were investigated from spring to summer [30], the change of tempreature and golbal warming that affected prevalence of sea lice with changed tempreture [5].

Seabass infested with different species of caligus, in our study caligus clemenis is recorded while recorded by [31] from adult Pacific salmon in Canada, also [10] said that caligus clemenis was the major species that able to create problem to salmonid culturing industry in few countres within American.

Caligus clemenis was parasitological identified by 1st and 4th legs. The distal margin of 1st pair of leg are charectrized by presence of setae 1 and 4 undivided and unarmed, setae 2 and 3 bifid, while 4th pair of legs consists of 3 segments bearing bristilles and 5 setae, 4 seta found in the destal end of the last segment, confirmed by [32-33-34], male and female were differ in females was ovate carapace but not longer than remaining body, while male had very broad carapace with enlargement towards posterior end, this agreed with [35].

The present study showed histopathological lesions mainly in gills and oral cavity. Gills lesions were characterized by complete sloughing of their lamellar epithelium and necrosis of the underlying musculature with numerous inflammatory cells. In advanced stages hyperplastic secondary lamellar epithelium and edema beside dilated capillaries. Similar histopathological pictures were recorded by [16- 36]. These lesions may be attributed to the parasites attachment and feeding activity causing massive destruction of respiratory epithelial cells [9].[37]reported that degenerative and necrotic changes in the epithelial cell with hyperplasia of fin filaments associated with chronic inflammatory cells infiltration .. The changes recorded in the oral cavity in the present study came in parallel with that recorded by [38-39]. The increase of phagocytic cells and mucous cell hyperplasia in response to fish parasitizes as defense mechanism for elimination of parasites. This result was compatible with that recorded by [16].

Conflict of Interest

No Conflict of Interest

Animal ethics permission committee

This study was followed the guidelines of Committee of Animal Welfare and Research Ethics, Project management and planning code number 13429 Ser.19, Animal Health Research Institute (AHRI), Agriculture Research center (ARC), P.O. Box 246 Dokki, 12618 – Giza, Egypt.

Authors' Contribution

All authors are equally contributed

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